The Flowering of APOMIXIS:

From Mechanisms to Genetic Engineering

Y. Savidan, J. G. Carman, and T. Dresselhaus, Editors

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CIMMYT.
International Maize and Wheat Improvement Center

European Union

IRD
Institut de recherche
pour le développement

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The International Maize and Wheat Improvement Center (CIMMYT)® (www.cimmyt.org) is an internationally funded, nonprofit, scientific research and training organization. Headquartered in Mexico, CIMMYT works with agricultural research institutions worldwide to improve the productivity, profitability, and sustainability of maize and wheat systems for poor farmers in developing countries. It is one of 16 food and environmental organizations known as the Future Harvest Centers.

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Abstract: Apomixis, the asexual reproduction of plants through seeds, has received increasing attention as technological advances have led to a rapid increase in knowledge about cellular biology, molecular genetics, and the mechanisms and pathways behind plant reproduction. The fourteen chapters of this book address a wide range of theoretical and technical issues related to apomixis, as well as its potential impact on agriculture in both the developing and developed world. The technical chapters address aspects of two complementary research paths in the ultimate quest to produce apomictic food crop plants. One path essentially seeks to either transfer the apomictic trait from a wild apomictic relative into a crop plant or mutagenize sexual genes into apomictic genes in the crop plant itself. This research is currently being conducted in important food crops such as maize, wheat, and millet, as well as forages used for livestock, and model plant species such as *Arabidopsis*. The other path is rigorously exploring apomictic and sexual mechanisms and pathways in order to provide a more complete understanding of the overall apomixis process. This could ultimately allow scientists to target and induce the interrelated processes of apomixis through natural or artificial means.

AGROVOC Descriptors: Apomixis; Genetic engineering; Asexual reproduction; Sexual reproduction; Biotechnology; Chromosome translocation; Molecular genetics; Genetic variation; Genetic control; Genomes; Tripsacum; Zea mays; progeny forms; Plant breeding; Breeding methods

Additional Keywords: CIMMYT, IRD, European Commission

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Yves Savidan, John G. Carman, and Thomas Dresselhaus
March, 2001

Foreword

As implied by the title of the book—"The Flowering of Apomixis: From Mechanisms to Genetic Engineering"—this complex and mysterious aspect of reproduction is beginning to yield its secrets to more than a century of scientific inquiry by researchers from around the world. Building on this foundation of knowledge, and by using the rapidly advancing tools and techniques of biotechnology, we are probing the intricate processes of apomixis more deeply and broadly than ever before. Consequently, our grasp of the mechanisms of both asexual and sexual reproduction has expanded tremendously in the last decade. And though timetables for research discoveries cannot be dictated, the promise of applying apomixis technology to real world needs and issues has never been brighter.

One of the most urgent applications for the technology will be feeding and raising the standard of living for the burgeoning populations of the developing world. It is fitting that in the book's opening chapter, Gary Toenniessen, Director of Food Security for the Rockefeller Foundation, succinctly sets forth the magnitude and gravity of the situation we face, and the tremendous potential apomixis holds for helping to meet those challenges. By producing crops that produce asexually through seeds, we can greatly hasten the development of new higher-yielding hybrid varieties, a keystone of past productivity gains and one that will be required to boost productivity in coming years. With costs of development coming down, seed prices to farmers may also decrease. Of particular import to small-holder farmers, apomixis will allow scientists to efficiently breed varieties specifically tailored to a multitude of niche environments, many of them situated in the most marginal agricultural areas. Finally, because apomictic seed is self-replicating, developing world farmers should be able to recycle seed without losing valuable hybrid characteristics. Furthermore, the technology could be used in such a way that farmers may be able to better fix the traits they deem desirable within their own indigenous varieties and landraces. Needless to say, however, there is work yet to be done.

In the following chapters, the authors follow two complementary paths in the ultimate quest to produce apomictic food crop plants. One path is to either transfer the apomictic trait from a wild apomictic relative into a crop plant or change sexual genes into apomictic genes in the crop plant itself. This research is currently being conducted in important

food crops such as maize, wheat, and millet, as well as forages used for livestock, and model plant species such as *Arabidopsis*. The other path is rigorously exploring apomictic and sexual mechanisms and pathways in order to provide a more complete understanding of the overall apomixis process. This should ultimately allow scientists to target and induce the interrelated processes of apomixis through natural or artificial means. The knowledge gained through research following both approaches has significantly accelerated advances in the field as a whole.

It is with great pleasure that I invite those with an interest in apomixis—students, academics, plant breeders, geneticists, and those simply with a scientifically inquisitive bent—to read and reference this book. Finally, I must commend the authors and editors for their diligence in producing this important and timely work.

Professor Timothy Reeves
Director General,
The International Maize and Wheat Improvement Center (CIMMYT)
El Batan, Mexico.
March, 2001

Chapter 1

Feeding the World in the 21st Century: Plant Breeding, Biotechnology, and the Potential Role of Apomixis

GARY H. TOENNIESSEN

A surplus of food in many of the world's wealthier countries has led to a certain complacency there about future supplies and availability. But for the vast majority of the world's people, who live in poorer developing countries faced with growing populations and increasing demand for food, concern rather than complacency is the order of the day. For the nations of the South, the task of feeding their future generations presents a critical and formidable challenge for agriculture over the next half century or longer.

Population Projections

Fortunately, there are reasons to be optimistic that an end to population growth is finally in sight, albeit at some distance (Lutz et al. 1997). The rate of world population growth peaked around 1970 and has been steadily declining since then. As societies have moved from dependence on subsistence agriculture to more intensive agriculture and more modern economies—in the process providing improved nutrition and health care and expanded educational opportunities to their girls and boys—desired family size has dropped. A family planning revolution in the developing world, under way now for more than two decades, has lowered the average number of children in a family from six to three, which is reflected in a respective decline in annual population growth from 2.5 to around 1.8 percent (United Nations 1997). Contraceptive use by women of child-bearing age in developing countries has risen from about 10 percent to more than 50 percent during the last three decades; and it is estimated that there are at least an additional 100 million women who wish to regulate their fertility, but who are not now using contraceptives. If effective family planning and reproductive health services were provided to all those wishing to use them, demographers now predict that replacement level fertility could be reached as early as 2020 and that the world's population would stabilize at 8–11 billion people near the middle of the 21st century (Bongaarts 1994; Lutz et al. 1997).

Although the task of curbing population growth will be arduous, generally speaking the agencies and institutions that provide family planning services have the technical know-how required to achieve this goal; now they are working on mobilizing the necessary financial resources and political commitments. To complement this effort, the agricultural sector must provide the basic nutrition and economic growth needed to fuel the desire for smaller families and the requisite family planning services, until the time that replacement level fertility is reached.

These encouraging population trends will, over the long term, be good for agriculture, as they imply that sometime during the next century the ever-increasing demand for greater food production should finally stabilize. The downside is that even given these positive trends, the developing world will need to produce two to three times as

much food as it does today. In many developing countries, more than half of the people are just entering or are still under reproductive age. Even if these people were to have only two children per family, a near doubling of total population is inevitable. In addition, economic growth will further increase the demand for food.

The challenge facing agriculture in the first half of the 21st century is formidable. It must provide adequate nutrition for billions more people and contribute to their economic development, thereby stoking the desire to limit family size. Furthermore, agriculture must accomplish this without jeopardizing the capacity of the natural resource base to meet the needs of future generations. Currently, agriculture does not have the technologies to double or triple food production in developing countries, and so the threat arises that farmers will irreparably damage the natural resource base in their efforts to feed growing populations—this scenario is already becoming a reality in certain locations. Meeting the food challenge will demand the discovery of new knowledge and the development of innovative technologies, which, combined with the broader adaptation and application of existing technologies, will allow greater intensification of production on a sustainable basis.

Plant Breeding

Many of the institutional structures and financial support systems needed to address the food challenge are already in place and can rightly claim an impressive record of accomplishment. International cooperation in plant breeding has been particularly successful in producing improved crop varieties that benefit the developing world. When combined with appropriate management practices, these modern varieties have substantially increased productivity and contributed significantly to food self-sufficiency and economic development in many countries of Asia and Latin America.

In Asia, farmers have for centuries used irrigation, organic fertilizer, and hand weeding on their small holdings. More recently, they have readily adopted modern varieties and, using their traditional intensive management practices together with purchased inputs, have in many locations pushed yield per hectare close to the maximum potential. Modern varieties of rice and wheat are now grown on nearly 70 percent of the area planted to these crops in Asia. Because many of these varieties have short growing seasons, farmers can obtain two or three crops per year on fertile land under irrigation. Improved varieties have also been produced for the poorer upland and seasonally flooded regions of Asia, however their performance and rates of adoption have been less dramatic. During the past 20 years, the proportion of the Asian population affected by inadequate nutrition declined from 40 to 19 percent. Nevertheless, Asia still has the greatest number of chronically undernourished people, 528 million, and the largest projected increase in population (FAO 1992; Bongaarts 1994; Lutz et al. 1997).

In Latin America, modern varieties have made an enormous impact, however, due to the highly skewed and inequitable distribution of land in the region, it is primarily the commercial farmers (who control most of the fertile land) that have adopted them. Production on the larger farms has increased significantly and consumers have benefited from lower prices. However, the majority of Latin American farmers, who work small holdings on less fertile land in the highly heterogeneous hill regions, have not gleaned the benefits offered by modern crop varieties. Developing improved varieties for them is a difficult task and only limited progress has been made. No single elite breeding line is

broadly applicable across such diverse agronomic and socio-economic conditions, and plant breeders are just beginning to provide improved varieties tailored to a few of the multitude of niche environments found in the region.

Of the major developing regions, improved varieties have had the least impact in sub-Saharan Africa; food production there has lagged behind rapid population growth. In Africa as a whole, more than 168 million people are chronically undernourished, and, alarmingly, nearly a fourfold increase in population, from 740 million in 1996 to 2.8 billion by the end of the 21st century, is now projected (FAO 1992, 1998; Bongaarts 1994). The defining characteristics of African agriculture are its complexity and heterogeneity. Most farmers have small holdings on which they grow a variety of crops, often intercropped with one another. In each of the continent's countries, soils and climate are highly diverse and variable. Economic realities limit the development of irrigation and other forms of yield enhancing and risk averting infrastructure. As in much of Latin America, no elite breeding lines are broadly applicable and improved varieties with specific characteristics need to be developed for many different types of agronomic and socioeconomic niches. Such niche breeding has been successful in a few locations and has potential for expansion, but it is a slow process when based on conventional breeding technology. Notably, while there is no such thing as low input/high output agriculture, average yields in Africa are so low (often less than 1t/ha) that a doubling or tripling of production should be possible with locally well-adapted varieties using just minimal inputs. Undoubtedly, better management practices would help boost yields (the use of nutrient and soil-enhancing crop rotations and associations looks especially promising), but over the long term, greater use of inputs, particularly fertilizer, will be necessary.

Biotechnology

Modern plant breeding, which revolutionized agriculture in the 20th century, is now on the verge of significantly extending its technological potential. New genetic monitoring and manipulation tools, in aggregate commonly referred to as biotechnology, are becoming available as a result of advances in molecular and cellular biology. As indicated in Figure 1.1, these new tools are contributing to both phases of plant

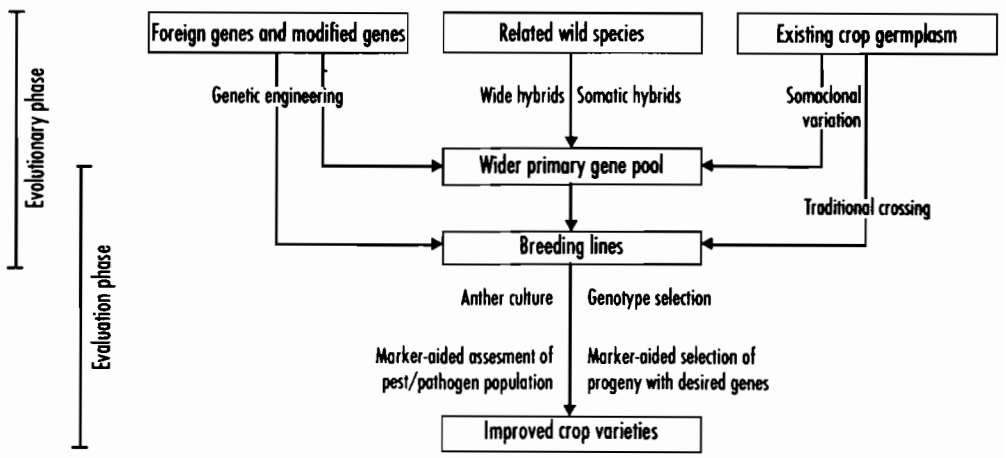


Figure 1.1 Biotechology tools for strengthening plant breeding.

breeding: the evolutionary phase, in which variable populations are produced, and the evaluation phase, in which desirable genotypes are selected.

Variability, at the heart of the evolutionary phase, traditionally has been created by hybridization and to a lesser extent by mutations. Wide hybridization through embryo rescue or somatic hybridization, somaclonal variation, and genetic engineering are biotechnology tools that can dramatically expand the range of variability available to breeders. Genetic engineering, especially, should make the process of generating desirable variability much more predictable and help obtain other goals that are beyond the reach of conventional techniques. Meanwhile, the evaluation phase will become much more efficient through the use of the following biotechnology tools: anther culture to produce doubled haploids and eliminate dominance variance; molecular maps and markers of the crop genome to tag and follow the inheritance of genes for important traits, particularly quantitative traits and those that are difficult to score; and molecular genetic maps and markers of pests and pathogens that can be used to characterize and monitor population structures and dynamics, thereby promoting more effective selection and deployment of resistant plants.

The international agricultural research system, which has been so successful at producing improved varieties for developing countries, is itself evolving and adding new institutions (see Tables 1.1 and 1.2) to take advantage of these new tools. It is drawing more on results generated by fundamental and strategic

Table 1.1 International Agricultural Research Centers

International Center		Crops	Location
CIAT	Centro Internacional de Agricultura Tropical	Cassava, field beans, rice	Colombia
CIFOR	Center for International Forestry Research	Forestry	Indonesia
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo	Maize, wheat, triticale	Mexico
CIP	Centro Internacional de la Papa	Potatoes, sweet potatoes	Peru
ICARDA	International Center for Agricultural Research in Dry Areas	Wheat, barley, lentils, chickpea	Syria
ICLARM*	International Center for Living Aquatic Resources		Philippines
ICRAF	International Center for Research in Agroforestry	Forestry, tree crops	Kenya
ICRISAT	International Crop Research Institute for the Semi-Arid Tropics	Sorghum, pearl millet, groundnut, pigeon pea	India
IFPRI*	International Food Policy Research Institute		USA
IITA	International Institute for Tropical Agriculture	Cassava, yams, cowpea, maize	Nigeria
ILRI	International Livestock Res. Institute	Forages	Kenya
IPGRI	International Plant Genetic Resources Institute		Italy
IRRI	International Rice Research Institute	Rice	Philippines
ISNAR*	International Service for National Agricultural Research		The Netherlands
IWMI*	International Water Management Institute		Sri Lanka
WARDA	West Africa Rice Development Association	Rice	Côte d'Ivoire

* ICLARM, IFPRI, ISNAR, and IWMI do not directly handle plant research programs.

research institutions and exploring new ways of gaining access to proprietary technologies. Some of the key institutions that make up this system in the era of biotechnology are noted in Figure 1.2. Their work includes

- fundamental research conducted primarily in advanced research universities and institutes that expands the knowledge base on plants, insects, and microbes, and their interactions with one another and with their environment;
- strategic research, conducted primarily at agricultural universities, national agricultural research institutes, in corporations, and increasingly at the international agricultural research centers (IARCs), which generates new and strengthens existing technologies for crop genetic improvement;
- applied research (including germplasm collection and evaluation), conducted primarily at IARCs and national crop breeding institutions, often collaboratively, which generates new breeding lines;
- adaptive research, conducted primarily at the national level, which combines elite breeding lines with traditional varieties to produce improved finished varieties that are well-suited to local needs and conditions; and
- seed multiplication and delivery of improved varieties, usually by national agencies, local farmers, non-governmental organizations (NGOs), and increasingly through market mechanisms.

The IARCs with plant research programs (see Table 1.1) have the mandate and primary

Table 1.2 Institutions facilitating the application of biotechnology to international agriculture

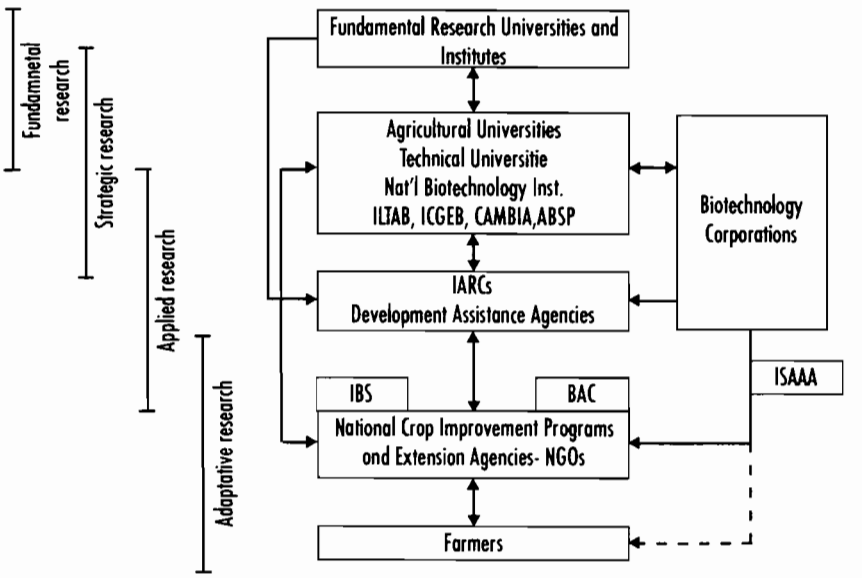
ISAAA	**International Service for Acquisition of Agri-Biotech Applications**, Ithaca, New York, USA, is a not-for-profit international organization committed to the acquisition and transfer of proprietary agricultural biotechnologies from the industrial countries for the benefit of the developing world. It assists in identifying biotechnology needs and opportunities, evaluates the availability of proprietary technologies, serves as an "honest broker" that matches needs with available technology, and when necessary mobilizes the financial resources required to implement brokered proposals.
ILTAB	**International Laboratory for Tropical Agricultural Biotechnology**, St. Louis, Missouri, USA, is a unit of the Danforth Plant Science Center. Technology is transferred first from the center, where scientists are engaged in pioneering work on the development of disease resistant plants, to five ILTAB scientists and fellows from developing countries. These scientists use the technology to produce new sources of disease resistance in tropical crops including cassava, rice, sweet potato, and yam.
CAMBIA	**Center for the Application of Molecular Biology to International Agriculture**, Canberra, Australia is a research and technology transfer organization committed to the application of biotechnology to international agriculture. It specializes in producing inexpensive biotechnology tools that can be effectively utilized in developing countries.
ICGEB	**International Center for Genetic Engineering and Biotechnology** was established by the United Nations Industrial Development Organization. It has headquarters and information gathering and dissemination facilities in Trieste, Italy, and agricultural biotechnology research and training facilities in New Delhi, India.
ABSP	**Agricultural Biotechnology for Sustainable Productivity**, East Lansing, Michigan, USA, is a project headquartered at Michigan State University and funded by USAID. It is a unique bilateral program in that it supports research at and technology transfer from public and corporate sector crop research institutions to developing countries.
IBS	**Intermediary Biotechnology Service**, The Hague, Netherlands, is a unit of the International Service for National Agricultural Research (ISNAR). It provides national agricultural research agencies with information, advice, and assistance to help strengthen their agricultural biotechnology capacities and to enable them to establish collaborative arrangements with international biotechnology programs.
BAC	**Biotechnology Advisory Center**, Stockholm, Sweden, a unit of the Stockholm Environment Institute, is an independent resource for impartial biosafety advice. It was established to help developing countries assess the possible environmental, health, and socioeconomic impacts of proposed biotechnology introductions.

responsibility for linking the components of this system together and assuring that it functions effectively. This system has the ability to take relevant scientific discoveries from the "ivory towers" of academe and, through a series of technology transfers and collaborative research projects, incorporate the new knowledge and technology into improved seeds that will be sown in fields throughout the developing world—and to do so in an amazingly short time frame. The improved cultivars and agronomic practices generated by this system have helped literally billions of people who daily consume the end products. If over the next century we are to achieve a stabilized world population fed by sustainable agriculture, this unique public sector research establishment must also be sustained, both financially and technologically.

Potential Role of Apomixis

The potential role of apomixis in boosting yields in the developing world is considerable and varies according to region. From a plant breeding perspective, Asia most needs new varieties of its staple cereal crops that have significantly higher yield potentials than today's high-yielding varieties. Africa and Latin America most need a large number of improved varieties of food crops, each well-suited for production in one or more of the many ecologically and/or socioeconomically unique niches that can be found in these continents.

Asia must more than double its cereal production over the next fifty years and do it on the same or less area than is currently in production. Accomplishing this will require



IARCs International Agricultural Research Centers (see Table 1)
ILTAB International Laboratory for Tropical Agricultural Biotechnology, La Jolla, CA, USA
ICGEB International Center for Genetic Engineering and Biotechnology, New Delhi, India and Trieste, Italia
CAMBIA Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia
ABSP Agricultural Biotechnology for Sustainable Productivity, East Lansing, MI, USA
ISAAA International Service for Acquisition of Agri-Biotech Applications, Ithaca, New York, USA
IBS Intermediary Biotechnology Service, The Hague, Netherlands
BAC Biotechnology Advisory Commission, Stockholm Environment Institute, Stockholm, Sweden

Figure 1.2 The International Agricultural Research System in the era of biotechnology.

even more double and triple annual cropping cycles and more extensive use of yield-enhancing technologies such as hybrid seed, by the overwhelming majority of farmers, including those with limited purchasing power. Hybrid seed's potential to increase production has been demonstrated by hybrid rice in China. From 1980 to 1990, China increased its rice production by roughly 32.5 million tons, or 22 percent, while decreasing the area planted to rice by roughly 2.2 million hectares, or six percent (FAO 1990). Yuan Longping, the "father of hybrid rice" in China, speculates that full exploitation of the heterosis available in rice could provide another 30–50 percent increase in yield (Yuan 1993). New hybrid lines that are suitable for other regions of Asia are slowly becoming available. Biotechnological tools (such as genetically engineered male sterility systems for elite breeding lines) and the use of molecular markers to select parental lines that combine high levels of heterosis with other desirable characteristics can accelerate this process and make the use of hybrid rice technology more broadly applicable. And, as reported later in this book, progress is being made on using apomixis as the ultimate tool for fixing heterosis in cereals, thereby making the benefits of hybrid seed available to farmers at minimal cost.

African and Latin American farmers could also benefit from hybrid seed that self-replicates through apomixis, although the application of apomixis to niche breeding could yield even more consequential results. If apomixis can be introduced into staple food crops, cultivars that perform well under local conditions could be genetically fixed early in the selection cycle. Under this scheme, variability would be generated through traditional hybridization or any other technique noted in the evolutionary phase (see Figure 1.1). The resulting population of plants would be grown and evaluated under local conditions, and the plants that performed best could be selected and quickly developed into genetically stable superior cultivars by incorporating the gene(s) for apomixis. For crops that are normally reproduced from tubers or vegetative cuttings, apomixis would enable the multiplication and dissemination of improved varieties as true seed.

In short, apomixis has the potential to make a significant contribution toward meeting food production demand throughout the developing world in the 21st century. Because of its limited profit potential, this technology will probably not be fully developed in the private sector. Therefore, if the full potential of apomixis as a breeding tool to help the poor is to be realized, the necessary research and development must be undertaken by the public sector international agricultural research system—and the results must remain freely available to public sector crop breeding programs.

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Chapter 2

Apomixis and the Management of Genetic Diversity

Julien Berthaud

Introduction

Apomixis is a mode of reproduction (asexual propagation through seeds) that exists in many plants from different botanical families (review in Asker and Jerling 1992; Carman 1997). It is most frequent in the dicots Rosaceae and Asteraceae and in the moncot Poaceae. Some of these Poaceae genera are tropical forages with wide colonizing ability, e.g., *Panicum maximum*. From its center of origin in East Africa, through human activities it has expanded to West Africa, where it can be found colonizing roadsides, and to tropical regions of the Americas and Asia.

Apomixis attracts considerable theoretical interest as it may help us better understand the sexual mode of reproduction. It is also of practical interest to breeders as a means of genetic fixation, potentially offering the capability of indefinite multiplication of heterotic genetic combinations. In the case of apomictic tropical forages (see Valle and Miles, Chap. 10), the problem faced by breeders is how to overcome apomixis to take advantage of genetic recombination in order to create new genetic combinations to be maintained through apomixis. Another challenge is to transfer apomixis into crops in which heterosis has been well documented. Research projects focused on this goal are underway for pearl millet, *Pennisetum glaucum* (Hanna et al. 1993) maize (see Savidan, Chap. 11), and wheat (Carman 1992). Rice breeders are also interested as F_1 hybrids in rice show heterosis (see Toenniessen, Chap. 1).

Some scientists have solely pursued the simplest model of apomixis, that with a complete lack of sexuality, i.e., no possibility of recombination and evolution. In this case, population genetics models show a diffusion of apomixis genes into natural populations without a need for some form of selective advantage (Pernes 1971; Marshall and Brown 1981). If this holds true, transferring apomixis to crops could ultimately decrease genetic diversity in those crops and pose a threat to the environment. From modern apomictic varieties, the apomixis gene could move to landraces and wild ancestors in their center of origin. In a recent review of apomictic risk, van Dijk and van Damme (2000) based their discussion almost entirely on this model. However, before overstating this possibility, one should know more precisely how apomixis functions, what diversity is conserved in wild populations where apomixis is the dominant mode of reproduction, and how apomixis could be transferred to landraces.

To address these issues, this chapter discusses (*i*) genetic variation observed in progeny of apomicts, (*ii*) diversity observed in wild apomictic populations, (*iii*) evolution—processes of agamic complexes, and (*iv*) the possibility of transferring apomixis from synthetic apomictic crops to landraces and wild relatives.

Progeny of Apomictic Plants

In most apomicts, the apomictic mode of reproduction is linked to pseudogamy, whereby the endosperm develops only after fertilization while the embryo develops parthenogenetically (Nogler 1984; see Crane, Chap. 3). Apomixis can be split into two logical stages, which does not necessarily imply two different genetic controls (see Sherwood, Chap. 5): (*i*) development of an embryo sac without reduction and (*ii*) parthenogenetic development of the embryo without fertilization. This results in an embryo with $2n + 0$ chromosomes that is genetically identical to the maternal plant. However, in some cases, the embryo sac is reduced while in others fertilization occurs. It is therefore possible to distinguish four categories in the progeny of apomictic plants, with respective frequencies dependent on success rates of the different stages (Table 2.1).

The four categories can be identified through the use of chromosome counts (or flow cytometry) and isozymes or molecular markers. The IRD-CIMMYT Apomixis team used isozymes to score *Tripsacum* progeny (Berthaud et al. 1993). In *Poa*, isozymes and random amplified polymorphic DNA (RAPDs) have been used (Huff and Bara 1993: Barcaccia et al. 1994). It is difficult to find detailed results of progeny analyses in the literature. Frequently, only morphological distinctions between true (maternal) and off-type progeny are reported.

Data on *Panicum maximum* (Combes 1975) are presented in Table 2.2. In the F_2 generation of a *P. infestum* x *P. maximum* cross (T19 progeny), frequencies of plants produced through sexuality and of haploid plant production were high. In one case (progeny from T19-36-5), 40% of a 177-plant progeny were off-types, including seven haploids. F_2 progeny from other crosses involving accession T19 were less variable; only four haploids ($n + 0$) were found out of 1,500 observed. In *P. maximum*, the proportion of off-types, including $2n + n$ and $n + n$ was 3%, based on a total of 2,100 progeny observed. We can therefore conclude apomixis in *P. maximum* is facultative.

For the *Parthenium* (Asteraceae) species, Guayule and Mariola, frequencies of the four categories of progeny (Table 2.3) were extracted from Powers and Rollins (1945). Haploid plants were produced at a low rate. Most plants were produced from unfertilized unreduced female gametes, however, the

Table 2.1 Genetic constitution of progeny from apomictic plants

Female meiosis	Fertilization	
	yes	no
Yes	$n+n$*	$n+0$
No	$2n+n$**	$2n+0$

* also called 8_{II} (Rutishauser 1948)
** also called 8_{III} (Rutishauser 1948)

Table 2.2 Size of four categories defined in Table 2.1 for two *Panicum maximum* clones (from Combes 1975)

Clone	Progeny size	$n+0$	$n+n$	$2n+n$	$2n+0$
256	551	0	16	6	529
H267,1*	238	4**	27	2	205

* hexaploid plant, from $2n + n$ progeny of "267"
** 3 plants with $2n = 24$ and 1 plant with $2n = 23$

Table 2.3 Size of four categories defined in Table 2.1 for three types of progeny involving two *Parthenium* species. Adapted from Powers and Rollins (1945)

Combination	Progeny size	$n+0$	$n+n$ (%)	$2n+n$ (%)	$2n+0$ (%)
P. argentatum x *P. argentatum*	342	0	14 (4)	5 (1.5)	323 (94.5)
P. argentatum x *P. incanum*	888	2	48 (5.4)	78 (8.8)	760 (85.6)
P. incanum x *P. argentatum*	567	0	76 (13.4)	66 (11.7)	425 (75.0)

categories $n + n$ and $2n + n$ appeared at significant rates. Stebbins and Kodani (1944) showed a frequency of occurrence of $2n+n$ progeny of 5.6%, ranging from 0.14% to 49%. Thus, apomixis in *Parthenium* is largely facultative.

In *Tripsacum* we found an average 2.7% ($n + n$) progeny, 8.1% ($2n + n$), and 89.2% ($2n + 0$) progeny (Table 2.4). From seeds collected in wild populations, we analyzed the occurrence of $2n + n$ progeny (it is difficult to test for $n + n$ progeny in this situation because clones are distributed in small niches of land and interpollination occurs from identical genotypes, making detection of new isozyme patterns difficult). The frequencies for three wild populations of *Tripsacum* we observed are presented in Table 2.5. According to the table, it appears that within one species, but in various populations, the rate of $2n + n$ progeny is variable and significant, being quite high in the case of population #39 "La Toma." Experiments are in progress to analyze the effect of the environment (flowering and pollination) on the stability of these parameters.

For *Dichanthium* and *Bothriochloa* (Poaceae), Harlan et al. (1964) reported rates of $2n+n$ progeny from crosses between tetraploid species. These combinations, however, are interspecific and therefore are difficult to compare with the former examples. Bashaw et al. (1992) showed that in crosses between

Table 2.4 Estimation of apomixis rate and categories of progeny from chromosome counts and isozyme analyses of *Tripsacum* populations (Berthaud et al., unpublished data)

Pop ID	Plant ID	Species tested	Size	2n = 72	90	108	n+n	2n+n	% n+n	2n+n	2n+0
24	143	DHBV	10	10	0	0	0	0	0	0	100
28	163, 164	MZ	20	18	0	2	0	2	0	10	90
29	183	MZ	12	10	0	2	0	2	0	16.7	83.3
37	282, 283, 772	DM	46	43	0	3	1	3	2.1	6.5	91.4
43	358, 361	DM	14	14	0	0	0	0	0	0	100
47	414	BV	12	12	0	0	3	0	25	0	75
48	421, 423	DM	39	38	0	1	0	0	0	5	95
52	497	DM	10	5	0	5	0	5	0	50	50
53	545	IT	19	17	0	2	2	2	10.5	10.5	79
54	588	DH	15	13	0	2	0	2	0	13.3	86.7
55	608	DH	18	12	2	4	0	6	0	33.3	66.7
59	641	DM	7	7	0	0	0	0	0	0	100
60	654, 655	DM	23	21	0	2	0	2	0	8.7	91.3
62	675	DH	14	12	0	2	0	2	0	14.3	85.7
63	689	DH	12	11	0	1	0	1	0	8.3	91.7
67	734	DH	11	11	0	0	0	0	0	0	100
71	853	BV	14	13	0	1	0	1	0	7.1	92.8
72	879	DM	19	18	0	1	0	1	0	5.3	94.7
74	898	DM	21	20	0	1	5	1	23.8	4.8	71.4
83	960	DM	8	8	0	0	0	0	0	0	100
87	990	DM	5	5	0	0	0	0	0	0	100
96	1076	JL	35	31	0	4	1	4	2.85	11.4	85.7
98	1093	DHIT	23	23	0	0	0	0	0	0	100
100	11, 201, 121	IT	40	38	0	2	0	2	0	5	95
Total			**446**				**12**	**36**	**2.7**	**8.1**	**89.2**

Abbreviations used: TBV=*T. bravum* Gray, TDH=*T.dactyloides* var. *hispidum* (Hitchc.) De Wet et Harlan, TDM=*T.dactyloides* var. *mexicanum* De Wet et Harlan, TIT=*T. intermedium* De Wet et Harlan, TJL=*T. jalapense* De Wet et Brink, TLC=*T. lanceolatum* Ruprecht ex Fournier, TLT=*T. latifolium* Hitchc., TMZ=*T. maizar* Hernandez et Randolph.

Pennisetum flaccidum and *P. mezianum*, progeny of the $2n + n$ and $n + n$ types are produced (Table 2.6).

In summary, when progenies are produced from apomictic plants, we can observe plants of the maternal type, plants with a ploidy level different from the maternal type (genome addition), and/or plants with the same ploidy level that have undergone a cycle of recombination. With the apomictic mode of reproduction, we have a system favoring changes toward higher or lower ploidy levels. Changes toward higher ploidy levels are the result of fertilization within unreduced embryo sacs. Changes toward lower ploidy levels come from parthenogenetic development of a reduced egg cell, which is the result of meiosis and recombination. When apomixis is active, sexuality is not eliminated but rather distributed over several generations. This topic is discussed in greater detail below.

Table 2.5 Variation in chromosome number for progeny from wild populations of *Tripsacum dactyloides mexicanum*. (Seeds were collected in the wild)

Population	Genotype	Progeny tested	Chromosome number 72	90	108	2n+n	2n+n (%)
38	DM38-01	78	73		5	5	6.4
39	DM39-04	172	111	4	57	61	35.5
39	DM39-15	16	9		7	7	43.8
39	DM39-16	7	4	2	1	3	42.9
39	DM39-20	17	11		6	6	35.3
39	DM39-21	10	9		1	1	10.0
39	DM39-22	12	10		2	2	16.7
39	DM39-23	12	7		5	5	41.7
40	DM40-01	56	55		1	1	1.8
40	DM40-02	208	198		10	10	4.8
40	DM40-03	17	15		2	2	11.8
Totals/averages per population							
38		78	73		5	5	6.4
39		246	161	6	79	85	34.6
40		281	268		13	13	4.6
Totals/averages, all populations							
		605				103	17.0

Diversity in Wild Apomictic Populations

Pernes (1975) described polymorphisms observed in wild populations of *Panicum maximum* in East Africa, which is the center of diversity for this species. He identified three types of populations: (*i*) monomorphic populations; (*ii*) polymorphic, with disjointed variation and distinct genotypes; and (*iii*) polymorphic, with discrete variation.

The latter was discovered in zones where sexual diploids and apomictic tetraploids were sympatric. The IRD-CIMMYT team's observations during collections of wild *Tripsacum* led to the same typology. In the case of *Tripsacum*, however, different species can coexist in the same population. Diploid populations are more frequent than in *Panicum*, and several ploidy levels in within species have been discovered in the same populations.

Three different species were found to coexist in a multispecific wild *Tripsacum* population ("La Toma" population #39) near Tequila, Jalisco, Mexico: *T. pilosum*, a diploid sexual species, and two apomictic tetraploid species, *T.bravum* and *T. dactyloides mexicanum*. Using fingerprinting, restriction fragment length polymorphisms (RFLPs) and isozymes, M. Barré et al. (personal comm.) identified most of the diploid plants. Plants belonging to the

Table 2.6 Size of categories defined in Table 2.1 for two *Pennisetum flaccidum* x *P. mezianum* crosses. From Bashaw et al. (1992)

Progeny type	Progeny size	n+0	n+n	2n+n & n+n*	2n+n	2n+0 (%)
PI315868xPI214061	2,505	-	51	20	77	2428 (96.9%)
PI220606xPI214061	3,040	-	58	72	148	2892 (95.1%)

* This hybrid category has been recognized on morphological traits. Not all the hybrids were analyzed cytologically.

two tetraploid species were distributed in clones of variable size (Table 2.7). The genetic diversity in this population was distributed among 54 different diploid plants, six triploid clones (11 plants), and 18 tetraploid clones (83 plants). We conclude that there are almost no "widespread" genotypes in these populations. Moving from one population to another, new genotypes of the same species are found. In Mexico, populations #38 and #39 ar e about 10 km apart and both contain *T. bravum* and *T. dactyloides mexicanum*. Nevertheless, their genotypes are distinct. As a rule of thumb, the probability of finding distinct genotypes within a distance of 50 to 100 m is quite high.

In population #38, we analyzed 94 asexually reproducing triploid and tetraploid plants, distributed in 24 clones, i.e., four plants per genotype on average. Ellstrand and Roose (1987) observed 5.9 plants per clone in a literature survey of studies involving asexually reproducing plants. Wild populations of dandelion (*Taraxacum sp*, Asteraceae) and *Antennaria sp.*(Asteraceae) are comparable (Lyman and Ellstrand 1984; Ford and Richards 1985; Bayer 1990).

Table 2.7 Distribution of clones in *Tripsacum* wild population "La Toma"

Type*	Chromosome no.	Size	Type*	Chromosome no.	Size
BV1	72	33	DM12	54	1
BV2	72	3	DM13	54	2
DM1	72	4	DM14	72	1
DM2	72	2	DM15	72	1
DM3	72	2	DM16	72	1
DM4	72	27	DM17	54	1
DM5	54	5	DM18	54	1
DM6	90	1	DM19	54	1
DM7	108	1	DM20	72	1
DM8	72	1	DM21	72	1
DM9	72	1	DM22	72	1
DM10	72	1	DM23	72	1

* BV = *T. bravum*; DM = *T. dactyloides mexicanum*

In summary, studies of wild populations demonstrate that apomixis does not produce the uniformity that is often simplistically suggested. Diversity is maintained in these populations. Mechanisms generating and maintaining this diversity may involve genetic exchanges between different *Tripsacum* types and genetic recombination as previously described.

Ploidy Cycles and Organization of Agamic Complexes

In agamic complexes, two pools exist: one is sexual diploid and the other is apomictic polyploid (very often triploids and tetraploids). Plants considered to be apomictic present a certain amount of sexuality, at a rate we will call "k." Authors of reviews on apomixis (Nogler 1984; Asker and Jerling 1992) conclude that facultative apomixis is the most common. Obligate apomixis, when found, occurs when k = 0, and is under the same genetic control as facultative apomixis.

In many cases, apomixis and pseudogamy (endosperm produced after fertilization by pollen) are found together. Pseudogamy is the rule for apomictic Poaceae, Rosaceae, and Ranunculaceae. In *Taraxacum* (Asteraceae), fertilization is not needed for endosperm development (Ford and Richards 1985), while in *Parthenium*, which belongs to the same family, seeds are produced only after pollination, demonstrating that fertilization is needed for endosperm development (Powers and Rollins 1945).

Taraxacum and *Parthenium* Agamic Complexes (Asteraceae)

Taraxacum sp. is present on five continents and about 2,000 species have been described. The base chromosome number is eight, and diploid and tetraploid forms exist. Diploid forms are sexual and, depending on the species, self-incompatible or self-compatible.

Polyploid forms are autonomous apomicts, either facultative or obligate. Fruits (propagules) can be obtained without pollination, after eliminating anthers and stigmas (Mogie and Ford 1988).

In *Parthenium* (Asteraceae), diploid forms with $2n = 2x = 36$ are sexual, and polyploid forms with $2n = 54, 72, 90$, or 108, are apomictic. In this genus, pseudogamy is prevalent and therefore fruits are not produced in the absence of pollen (Powers and Rollin 1945). Ploidy buildup occurs through production of $2n + n$ progeny (Powers and Rollins 1945), and production of haploids from hexaploids has been documented (Powers 1945). In this case, a cycle exists between tetraploids, hexaploids, and triploids, with a possibility of incorporating diploid forms into the cycle through their production of $2n + n$ progeny with 54 chromosomes.

Capillipedium-Dichanthium-Bothriochloa Agamic Complex (Poaceae)

The genera of *Capillipedium, Dichanthium, and Bothriochloa* are distributed over Europe, the Mediterranean region, Asia, Australia, and the New World, and have been studied in detail by Harlan, de Wet, and coworkers. De Wet (1968) described a possible evolution in the genus *Dichanthium* based on ploidy cycles involving diploids, tetraploids, and haploids. In a broader approach, de Wet and Harlan (1970) described the interrelationships between species of the three genera of this agamic complex (Figure 2.1). The most common ploidy levels are $2x, 4x, 6x$, as well as some pentaploid forms. Diploids are sexual, and polyploids are apomictic. However, forms from the New World are sexual and polyploid. Triploid forms are not mentioned. Gene flow occurs in several directions, but in some cases is limited by incompatibility barriers. Genetic exchanges between *Capillipedium* and *Dichanthium* are effective only when species of *Bothriochloa* are involved as genetic bridges. Haploid production was detected experimentally and haploid plants were found to be either sexual or sterile. Tetraploid plants can be recovered from these dihaploids through the formation of $2n + n$ progeny, with n proceeding from pollen of tetraploid plants. Rates of $2n + n$ production of up to 15% have been observed.

Panicum maximum Agamic Complex (Poaceae)

"Guineagrass" has its origin in East Africa. It has colonized West Africa as well as the tropical areas of the New World. This agamic complex includes three species: *Panicum maximum, P. trichocladum,* and *P. infestum* (Combes 1975). *Panicum maximum* is widely distributed and sexual diploid forms have been identified (Combes and Pernes 1970), though they are very rare, having only been found in three very limited areas in Tanzania (Combes and Pernes 1970; Nakajima et al. 1979). The other forms are tetraploid and facultative apomicts. Occasionally, penta- and hexaploid forms have been detected.

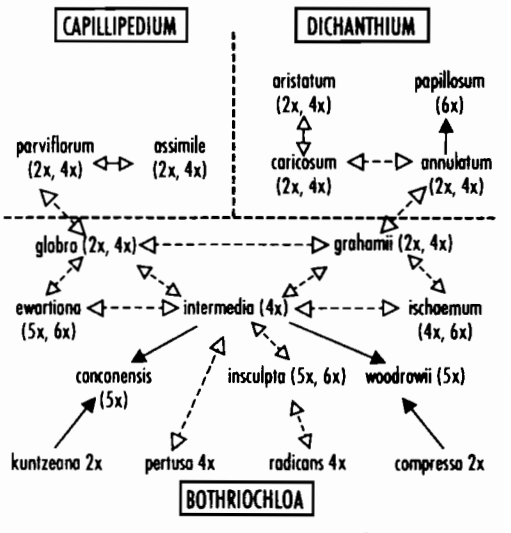

Figure 2.1 Continuous introgression and hybridization without further introgression in an apomictic complex including three genera, *Bothriochloa, Capillipedium, Dichanthium,* and 18 species. Adapted from De Wet and Harlan (1970).

Table 2.8 Distribution of clones according to ploidy level from the *P. maximum* collection established in Côte d'Ivoire (Combes 1975)

Total	2x	3x*	4x	5x**	6x**
551	19	0	506	12	13

* 3x ploidy level not found in wild populations
** 5x and 6x overrepresented in this collection (from over-collecting in these populations)

Table 2.8 shows the distribution of clones according to their ploidy level in a collection established in Côte d'Ivoire.

Triploid plants have been experimentally obtained from hexaploids ($n + 0$ progeny) as well as from diploid x tetraploid crosses. (Poly-) haploids also have been experimentally obtained from tetraploids, and the resultant plants have been either sexual or sterile (potentially apomictic as shown by embryo sac analyses). These findings led Savidan and Pernès (1982) to propose an evolutionary scheme based on ploidy cycles involving di-tetra-haploid levels as in the *Dichanthium* complex. The change from diploid to tetraploid is realized through $2n + n$ hybridization with pollen from tetraploid plants. In this system, sexuality is maintained at the diploid level. Contact between diploid and tetraploid plants allows genetic exchange between these pools (compartments) and creation of sexual tetraploid plants, allowing the release of new genetic diversity at the tetraploid level.

Paspalum Agamic Complex (Poaceae)

The center of diversity for the genus *Paspalum* is in South America. Studies conducted by Quarin (1992), Norrman et al. (1989) and collaborators at the Instituto de Botanica del Nordeste, Corrientes, Argentina (IBONE) show that many species in this genus have genetic pools at two or more ploidy levels (Table 2.9). In the pool with the lowest ploidy level, plants are sexual and self-incompatible, while in pools with higher ploidy levels, they are apomictic and self-compatible. In many cases, the two pools are at the diploid and tetraploid level (group 3 of Table 2.9). In some species, however, the sexually self-incompatible plants are tetraploid and the self-compatible apomictic plants are hexaploid or octoploid (group 6 of Table 2.9).

Some species that are sexual and self-compatible at the tetraploid or hexaploid level (groups 5 and 7 of Table 2.9) are not apomictic at higher ploidy levels. In other species, triploids are often apomictic and are found in species with sexual diploids and apomictic tetraploids.

As with previously cited agamic complexes, sexual forms are found at the lowest ploidy level and apomictic forms at the other levels. However, in this example, the relationship includes the incompatibility system. Apomictic plants are self-compatible and the corresponding sexual plants are self-incompatible. Experiments should be conducted to determine whether this also occurs in other agamic complexes.

Tripsacum Agamic Complex (Poaceae)

The *Tripsacum* genus is restricted to the New World, from 42°N to 24°S. Its center of diversity (or origin) is located in Mexico and Guatemala, and 11 of the 16 species described for the genus are found in this region. These 11 species show different ploidy levels both within and among themselves. The collection the team assembled from Mexico displayed the following distribution (unit = one ploidy level of one species in one population): diploids, 16.4%; triploids, 7.9%; tetraploids, 72%; penta- and hexaploids, 3.7%.

When compared to other agamic complexes, a high frequency of triploid plants in the *Tripsacum* complex was observed. These wild triploid plants are apomictic, produce fertile pollen, and set good seed. All of the natural polyploids we observed were apomictic (Leblanc et al. 1995; and unpublished data).

Diploids are sexual, and progeny with $2n + n$ chromosomes from apomictic plants occur at a significant frequency (Tables 2.4 and 2.5). Through this mechanism, many hexaploids were produced experimentally or detected in seeds collected from a wild population. Natural hexaploid plants in wild populations were observed at a lower frequency than in the seed progeny we analyzed.

Triploid plants can be obtained in four ways: (*i*) from $2n + n$ hybridization within diploids, (*ii*) from crosses between diploid and tetraploid plants, (*iii*) from haploidization of hexaploids ($n + 0$ progeny), or (*iv*) from asexual propagation of apomictic triploids. Evaluation of these possibilities is currently underway. In addition we have observed the presence of triploids, tetraploids, and hexaploids, and absence of diploids in some wild populations, which suggests that some triploids could have originated from haploidization of hexaploid plants. In populations containing diploids and triploids, there is a possibility of $2n + n$ hybridization, with $2n$ from the triploid female and n from a diploid male leading to the production of new tetraploid plants. We have documented such an event in seeds from one wild population. This event shows one possible route of gene exchange from the diploid to the tetraploid genetic pool. We did not discover any sexual tetraploid *Tripsacum*, but residual sexuality exists in apomicts, which permits production of $n + n$ progeny. This sexuality favors creation of new diversity at the tetraploid level by allowing crosses between apomictic plants.

Our model (Figure 2.2) suggests that in the *Tripsacum* agamic complex, sexuality fosters two stages: (*i*) a change from tetraploidy to

Table 2.9 Distribution of species of *Paspalum* according to their incompatibility system, ploidy level, and meiosis behavior (from studies at IBONE, Quarin, personal comm.)

Species	2x	3x	4x	5x	6x	8x
almum	sex, SI+	-	apo, SC*			
bertonii	sex, SI+	-	apo, SC*			
brunneum	sex, SI+	-	apo, SC*			
compressifolium	sex, SI+	-	apo, SC*		apo, SC*	
coryphaeum	sex, SI+	-	apo, SC*			
cromyorrhizon	sex, SI+	-	apo, SC*			
dedeccae	sex, SI+	-	apo, SC*			
denticulatum	sex, SI+	-	apo, SC*			
distichum	sex, SI+	-	apo, SC*		apo, SC*	
equitans	sex, SI+	-	apo, SC*			
haumanii	sex, SI+	-	apo, SC*			
hydrophilum	sex, SI+	apo, SC*	apo, SC*			
indecorum	sex, SI+	-	apo, SC*			
intermedium	sex, SI+	apo, SC*	apo, SC*			
maculosum	sex, SI+	-	apo, SC*			
modestum	sex, SI+	-	apo, SC*			
notatum	sex, SI+	apo, SC*	apo, SC*			
palustre	sex, SI+	-	apo, SC*			
procurrens	sex, SI+	-	apo, SC*			
proliferum	sex, SI+	-	apo, SC*			
quadrifarium	sex, SI+	apo, SC*	apo, SC*			
rufum	sex, SI+	-	apo, SC*			
simplex	sex, SI+	-	apo, SC*			
boscianum	-	-	sex, SC+			
dasypleurum	-	-	sex, SC+			
dilatatum	-	-	sex, SC+	apo, SC-		
regnellii	-	-	sex, SC+			
virgatum	-	-	sex, SC+			
durifolium	-	-	sex, SI+		apo, SC*	
ionathum	-	-	sex, SI+			apo, SC*
conspersum	-	-	-		sex, SC+	
inaequivalve	-	-	-		sex, SC+	
laxum	-	-	-		sex, SC+	
ramboi	-	-	-		sex, SC+	

sex = sexual mode of reproduction; apo = apomictic mode of reproduction; SC = self compatible; SI = self incompatible; + = meiosis regular; * = meiosis irregular; - = meiosis with many univalents

hexaploidy through $2n + n$ hybridization, and (*ii*) a change from hexaploidy to triploidy by meiosis and parthenogenetic development of the embryo. From triploidy to tetraploidy the pathway is as previously described ($2n + n$ hybridization) and involves diploid plants as pollinators. Complete cycles of tri-tetra-hexa-haploid plants linked to diploid plants are possible. During these cycles, recombination and fertilization events occur, helped by the parthenogenetic development of reduced embryo sacs and by fertilization of unreduced embryo sacs. Apomixis, in this case, enhances the functioning of sexuality that is distributed over several generations.

Cycles and Sexuality

In all agamic complexes, two different ploidy pools are found: a lower ploidy pool (usually diploid) with sexual forms and a higher ploidy pool (usually several ploidy levels, the most frequent being the tetraploid level) with apomictic forms. Absence of apomixis at the diploid level is thought to be due to either a lack of expression of this trait at this ploidy level or to an absence of transmission through haploid gametes (Nogler 1984; Grimanelli et al. 1998). The sexual pool is where most of the genetic recombination occurs and is therefore the pool where most of the selection on new combinations is acting.

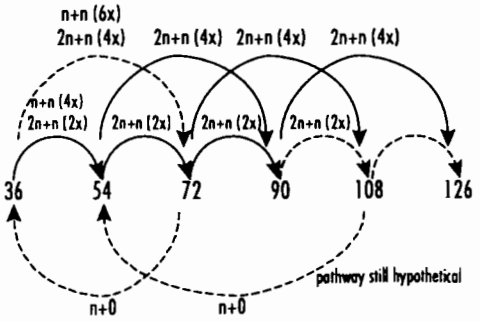

Figure 2.2 Evolution of ploidy levels in *Tripsacum* from fertilization of female gamete (n or 2n) by a male gamete (n) from 2x, 4x or 6x plants or parthenogenetic development of egg cell (n+0).

Gene flow from the diploid to the polyploid pool is realized in several ways. Diploid sexual plants, in some cases, can produce $2n$ female gametes (Harlan and de Wet 1975). If these gametes are pollinated by pollen from tetraploid plants, tetraploid progeny will be produced that will be sexual to a certain extent, providing an opportunity for a new burst of diversity to be tested at the tetraploid level. Another flow, as discussed earlier, comes from the pollination of unreduced eggs from triploid plants by normal pollen grains from diploid plants. The triploid plants can result from crosses between diploid and tetraploid plants. As can be seen, many opportunities exist for the diploid pool to contribute to the genetic diversity of the apomictic tetraploid pool. In the *Antennaria* complex, several genomes from diploid species can be accumulated in polyploid species (Bayer 1987).

In the polyploid apomictic pool, new genetic combinations may also arise through residual sexuality ($n + n$ progeny). We have also seen evidence that sexuality is distributed over several generations by creation of $2n + n$ progeny in one generation, followed by $n + 0$ progeny in the next generation. By permitting some perenniality for each stage of the sexual cycle, this wealth of genetic recombination is favored by apomixis, and it may be characteristic of the apomictic mode. More experimental data and modeling are required to isolate all of the factors involved in the genetic recombination of apomicts.

Management of Apomictic Varieties

Two types of apomictic varieties can be distinguished: forage varieties, which are already released as apomictic varieties, and apomictic varieties of crops such as maize and pearl millet, which may be released in the near future.

In the breeding of apomictic forage grasses, sexuality is involved at different steps and permits genetic recombination (Valle and Miles 1992; see Valle and Miles, Chap. 10). Released varieties are apomictic and have been distributed mainly outside their centers of diversity. In this instance, breeding activity is generating new genetic diversity.

Because projects are now underway to transfer apomixis to pearl millet, maize, wheat, and rice, we must consider the consequences of apomixis on the diversity management of landraces and that apomixis drastically reduces the recombination rate. It is important to remember that these landraces and their wild ancestors represent our current reservoir of genetic diversity. Thought should also be given to conserving the diversity of wild ancestors that grow near fields planted with apomictic varieties, which could be recipients of apomixis genes through naturally occurring gene flow.

Projects to transfer apomixis to pearl millet and maize have reached an intermediate stage: advanced generations of interspecific hybrids between apomictic forms and cultivated species have been produced that retain the apomictic trait. In the case of rice, possible sources of apomixis are yet to be identified. For wheat, F_1 and BC_1 hybrids between *Triticum* and *Elymus* have been produced (Peel et al. 1997; Savidan et al., Chap. 11). Pearl millet and maize are allogamous crops and so methods must be developed to maintain genetically adaptative processes once this new mode of reproduction is introduced. In its current design, the *Pennisetum* project considers the creation of tetraploid apomictic varieties of pearl millet (Dujardin and Hanna 1989). Upon release, the distinct ploidy levels of currently cultivated millet and the tetraploid apomictic new varieties will act as a genetic barrier between them. Dissemination of apomixis gene(s) from the tetraploid to the diploid level would involve production of triploid plants, which are usually male sterile; so dissemination through triploids should be negligible. However, in agamic complexes, apomixis seldom occurs at the diploid level. Some mechanism may suppress the expression of apomixis or impeach transmission to the diploid level. In the pearl millet program, there is no clear evidence that apomixis can be expressed at the diploid level. In contrast, a few BC_2 diploid-like hybrids in the maize-*Tripsacum* program were found to express apomixis (Leblanc et al. 1996). These plants are $2n = 28$ with $x = 10$ from maize and $x = 18$ from *Tripsacum*. Furthermore, triploid *Tripsacum* are male and female fertile. Thus, tetraploid apomictic varieties of maize will probably not restrict diffusion of apomixis gene(s) to other maize lines or its wild ancestor, teosinte. Therefore, the models of diffusion of apomixis discussed below are based on diploidy.

Apomixis fixes heterosis, thereby presenting two options for its use: (*i*) to produce apomictic F_1 hybrids through breeding programs and release them to farmers as end products; and (*ii*) to release to farmers apomictic varieties that would be used to transfer (diffuse) gene(s) to landraces, which would eventually become apomictic. In the latter case, breeding for apomixis would be a local activity. In fact, these two options are complementary and related as they pertain to the diffusion of apomixis gene(s). F_1 apomictic hybrids could be released in an area where landraces and wild relatives still exist. The transfer of the gene to these landraces and wild relatives will depend on the parameters cited above in option 2.

Transfer of Apomixis Gene(s) and Evolution of Landraces

We deduce from Sherwood (see Chap. 5), that apomixis is probably initiated by one dominant gene (see also Valle and Savidan 1996). The active *A* allele of this "apomixis gene" would be found mostly in the

heterozygous condition (*Aa*). The homozygous stage (*AA*) has been considered lethal in some cases (Nogler 1984). Nevertheless, in discussing apomixis transfer, we will consider three models: (*i*) apomixis is active as a dominant trait, either heterozygous or homozygous (*Aa* or *AA*) with the recessive homozygote (aa) being sexual; (*ii*) apomixis is active only as a heterozygote (*Aa*), with the recessive homozygote (*aa*) being sexual; and (*iii*) apomixis is only expressed as a recessive homozygote (*ss*), while *sS* and *SS* are sexual. We will also consider a residual rate of sexuality, k, in apomictic plants, with $0 < k < 1$.

Simple models of population genetics predict, in the absence of selection, the diffusion of the apomixis gene (Pernès 1971; Marshall and Brown 1981). According to the models, it is possible for the apomixis gene to transfer to landraces, such as maize or pearl millet, and to inadvertently move to wild relatives (Pernès 1971; van Dijk and van Damme 2000).

In model 1, there is one dominant allele for apomixis and three categories of genotypes at generation n: *AA* (apomictic) at a frequency of P_n, *Aa* (apomictic) at a frequency of $2Q_n$, and *aa* (sexual) at a frequency of R_n. Gametes for generation n+1 are distributed according to the following frequencies: male gametes *A* have a frequency of $P_n + Q_n$ and gametes *a* have a frequency of $Q_n + R_n$; female gametes *A* have a frequency of 0, gametes *a*, a frequency of R_n, gametes *AA*, a frequency of P_n, and gametes *Aa* a frequency of $2Q_n$.

Three genotypes will appear at generation n + 1 with the following frequencies (random mating of gametes): *AA* at a frequency of $P_{n+1} = P_n$, *Aa* at a frequency of $2Q_{n+1} = 2Q_n + R_n(P_n + Q_n)$, and *aa* at a frequency of $R_{n+1} = R_n(R_n + Q_n)$.

With $P_n + 2Q_n + R_n = 1$, we obtain $Q_n = 1/2(1 - P_n - R_n)$ and the recurrence relation:

$$R_{n+1} = 1/2R_n(1 - P_n + R_n)$$

Equilibrium is reached for $R = 1$, the population being entirely sexual, or for $R = 0$, the population being completely apomictic. This model is identical to the model proposed by Fisher (1941) for autogamy. In fact, apomictic plants self-reproduce, however they simultaneously release pollen with the dominant allele to the sexual plant forms; consequently, a portion of the progeny of sexual forms becomes apomictic.

If we take into account a rate of residual sexuality, k, the variation in frequency for *A* allele becomes $P_{n+1} + Q_{n+1} = (P_n + Q_n)(1+1/2(1-k)R_n)$ (Pernès 1971).

The change in frequency of allele *A* from generation n to generation n + 1 is a function of Rn, the frequency of the recessive allele, and a function of k. A zero value for k (obligate apomixis) maximizes the frequency of *A*, while higher values of k reduce the frequency of *A*. This variation would be zero if $k = 1$, i.e., when all plants are sexual with either the *A* or *a* allele.

In this model, we assume random mating of gametes. Transfer would be favored if an apomictic variety, homozygous for *A*, were interplanted with the variety (landrace) to be modified. In the case of maize, by detasselling and harvesting only the landrace, only heterozygous progeny would be produced. These new plants would be apomictic and genetically fixed. Their ability to evolve would rely on the rate of residual sexuality, k. A proportion k of the apomictic forms can be fertilized by pollen from other sources. Moreover, pollen from the first generation of apomictic forms can be used to pollinate the landrace. After several cycles of such backcrossing, the new variety will be identical to the landrace except that it carries the apomixis gene. Evolution in these "new" landraces will depend on the rate of residual sexuality that is retained at the end of the transfer process.

In model 2, apomixis is active in plants with the *Aa* association of alleles. The aa genotypes are sexual. If Rn is the frequency of *aa* genotypes (sexual) and Qn is the frequency of *Aa* genotypes (apomictic), frequencies in the next generation (n + 1) will be $R_{n+1} = R_n(1-1/2Q_n)$, and $Q_{n+1} = Q_n(1 + 1/2R_n)$. In this case, the apomixis allele, *A*, diffuses in the population as

$$1 + 1/2Rn > 1 \text{ and } Qn + 1 > Qn.$$

We can use this model to define conditions of equilibrium between sexual and apomictic forms if a differential fitness exists between the two forms. With a fitness of 1 + s for the *aa* and 1 for the *Aa*, the frequency changes from generation n to generation n + 1 are as follows:

$$R_{n+1} = R_n(1 - 1/2Q_n).(1 + s)/(1 + sR_n + sR_n^2)$$

$$Q_{n+1} = Q_n(1 + 1/2R_n).1/(1 + sR_n + sR_n^2).$$

In this case, equilibrium between sexual and apomictic forms will be reached for s = 1/1 + R. Initially, when apomixis starts to be established in a population, R is close to 1, and equilibrium can be reached with s values close to 0.5. The fitness advantage of the sexual forms in relation to the apomictic forms has to be at least 1.5:1 to reach the equilibrium. Once apomixis is widely established, R is lower, and equilibrium will be reached only with higher s values. In the extreme case of Q close to 1, equilibrium will be reached with s values close to 1. In this instance, sexual forms will have to produce twice as many seeds as apomictic forms to survive in the successive generations.

If model 2 applies to apomictic varieties, transfer of apomixis to landraces could be accomplished according to the process found in model 1; but the transfer will take longer (at least one more generation) because the first generation will be made from *Aa* x *aa* crosses producing *Aa* and *aa* genotypes, not from *AA* x *aa* crosses, which produce only *Aa* progeny. Conservation of diversity in the apomictic landraces will depend, as in the former model, on the rate of residual sexuality, k.

In model 3, apomixis is active only in plants that are homozygous for the recessive allele *s*. In this case, *SS* (sexual) has a frequency of Pn, *Ss* (sexual) has a frequency of 2Qn, and *ss* (apomictic) has a frequency of Rn. Using this model, it can be shown (Pernès 1971) that the frequency of *S* behaves as follows:

$$Pn + 1 + Qn + 1 = (Pn + Qn)(1 - 1/2Rn)$$

The frequency of *S* is reduced from one generation to the next, as 1 - 1/2Rn is always lower than 1.

If the genetic control of apomixis follows this model, then transfer of apomixis will require at least two generations. The pathway to transfer can be imagined as follows:

1st generation: *SS* [sexual] female x *ss* [apomictic] male = *Ss* [sexual]

2nd generation: *Ss* [sexual] female x *ss* [apomictic] male = *Ss* [sexual] + *ss* [apomictic]

3rd generation: *Ss* [sexual] + *ss* [apomictic] x *ss* [apomictic] or *Ss* [sexual] + *ss* [apomictic] = *Ss* [sexual] + *ss* [apomictic] or *SS*[sexual] + *Ss* [sexual] + *ss* [apomictic]

The apomixis gene can diffuse within the population through backcrossing between plants from the first generation and the donor variety as male parent. In order to have apomixis transferred within a reasonable timeframe, the donor must be used as the male variety of each generation. After several backcrosses, the local variety will be transformed to an apomictic variety, but it will be almost identical to the donor variety. Therefore, if apomixis is active only when recessive alleles are present, it will be difficult to transfer apomixis to landraces while at the same time maintaining the original traits of these landraces. It would require (*i*) the use of

markers to retain the *a* allele, (*ii*) the production of near isogenic lines through backcrossing with the landrace, and (*iii*) the selfing of isogenic, heterozygous (*Aa*) lines to produce *aa* apomicts.

2*n* + *n* Progeny

In *Tripsacum*, we saw an average of 10% of progeny come from 2*n* + *n* hybridization; in some samples, this rate rises to 35%. Crosses between apomictic species of *Pennisetum* also produced this type of progeny (Bashaw et al. 1992). These forms are less frequent in other species, such as *Panicum maximum*. If this trait is inherited during the transfer of apomixis, what behavior can be expected from cultivated apomictic forms?

The transfer projects now underway consider a type of apomixis linked to pseudogamy. Once apomictic varieties are produced, most probably they will be also pseudogamous. In this case, we are concerned with the ratio between embryo ploidy and endosperm ploidy, as it has been often reported that a ratio different from 2:3 (or 2:5) would introduce some developmental incompatibility at the seed level and a loss in productivity (endosperm development also depends on maternal:paternal genome ratio; see Chap. 6, 11, 12, and 13). However, for the *Tripsacum*, we observed that triploid plants produce seeds even when their pollen environment comes mostly from tetraploid plants. In this case, the ploidy ratio between embryo and endosperm is 3:8. The 2*n* + *n* progeny we detected were from normal seeds with normally developed endosperm. In *Tripsacum*, the 2:3 ratio (or 2:5) between embryo ploidy and endosperm ploidy does not appear to be necessary for seed filling. In general terms, we have two hypotheses to consider:

1. Endosperm development is deficient when the ratio of embryo ploidy to endosperm ploidy is different from 2:3 (or 2:5). In this case, ears display poorly filled kernels (with 2*n* + *n* embryo) at harvest time. There is a potential loss of production due to the presence of these 2*n* + *n* embryos, but these kernels would not be selected as seed for the next generation.
2. Endosperm development is not affected by a ratio of embryo ploidy to endosperm ploidy different from 2:3 (or 2:5). In this case, kernels with 2*n* + *n* embryos would go undetected and could be used as seed for the next generation. Apomictic plants obtained from such embryos are triploid; they may produce normal seeds but the pollen could be sterile, which could limit field production. If the pollen is still fertile, as noted with triploid *Tripsacum*, no loss in production should be detected. However, ploidy buildup will occur, and many different ploidy levels will be stored in the same variety. This ploidy buildup could raise chromosome numbers to levels far above the optimum for productivity, potentially resulting in lower production.

In nature, 2*n* + *n* progeny production is a strategy that takes advantage of genetic recombination, as these plants would give rise, after meiosis, to some haploid progeny by parthenogenetic development of reduced embryo sacs. In the case of an apomictic crop, it is a trait that should be reduced or eliminated.

Relationship between Wild Relatives and Apomictic Varieties

For the purpose of discussing the relationship between wild relatives and apomictic varieties, we will use the maize-teosinte model, however, it is our belief that it can be extrapolated to pearl millet in instances where wild relatives are still in contact with cultivated plants. Teosinte is only found in Mexico and Guatemala. Relationships between wild relatives and maize are not identical over the distribution area of teosinte. The variety

parviglumis may be found in southwest Mexico and is considered to be a very wild form, with almost no link to modern maize. In the states of Michoacan and Mexico, teosinte should be considered a weed. An incompatibility system exhibited by these weedy teosintes, which efficiently controls gene flow from maize to teosinte, has been detected and analyzed (Kermicle and Allen 1990). Moreover, as described by Wilkes (1967), teosintes generally have a flowering period that is distinct from maize. These mechanisms limit gene flow between this wild relative and maize.

If we use model 1 to explain the transfer of apomixis from apomictic plants to landraces, we can envisage the following process. The first generation hybrid between teosinte (sexual, *aa*) and apomictic maize (*AA*) would be apomictic (*Aa*), and BC_1 plants with teosinte as female would produce *Aa* (apomictic) and *aa* (sexual) progeny. At each generation, the apomictic forms are fixed but they still participate in the next generation from sexual plants through their pollen, which can transfer the apomixis allele to sexual plants. Therefore, a portion of each generation's progeny becomes apomictic. We can then deduce that the apomictic allele will diffuse into the wild population. However, the assumptions made to simplify the model may not prove accurate when applied to the relationship between cultivated plants and wild relatives.

Cultivated maize and its wild teosinte relatives are, morphologically, widely distinct. Apomictic maize x teosinte F_1 hybrids will be apomictic and will breed true. Sexual maize x teosinte F_1s are known to have a low fitness due to their intermediate morphology and adaptation, and they are easily recognized morphologically. When they grow in a field, they are not harvested. However, if the hybrid is apomictic, its pollen will transmit the *A* allele at a rate of 50%. Pollination efficiency depends on synchronization between flowering of these hybrids and the wild relatives. As a lack of synchronization between the two types of plants is anticipated, the gene flow between them should be minimal. These observations deviate considerably from the assumptions posited in the model in which apomictic plants are expected to engage in pollination in proportion to their frequency in the population. Moreover, in the long run, the apomictic intermediate forms should have a lower fitness than the sexual forms, because the latter can take advantage of more new recombinations and adapt faster to environmental changes. As noted earlier, a stable polymorphism between sexual and apomictic forms is possible when fitness values of the two forms reach a certain ratio. We have also observed that the speed of apomixis diffusion is a function of the rate of residual sexuality—a high level of residual sexuality will slow apomixis diffusion.

Promoting Genetic Diversity and the Release of Apomictic Varieties

We base our models for apomixis diffusion on the hypothesis that this mode of reproduction is under a simple genetic control. Current knowledge about the mechanisms underlying apomixis, however, is very incomplete, especially regarding the expression of an apomixis gene in a new genetic background, as would be the case with a *Tripsacum* apomixis gene transferred into a maize background. If genetic control of apomixis in landraces and new varieties involves several genes or a major gene and modifiers, the dynamics of diffusion will be more difficult to describe and transformation of current varieties to apomictic varieties would have to be carried out by professional breeders. In this instance, apomixis could be used as a genetic fixation tool and new varieties with a complex genetic structure could be created and released. Such varieties would contribute to the maintenance of diversity at the farmer's field level.

Furthermore, if apomixis is controlled by multiple genes, the probability of diffusing this trait to wild relatives is extremely low. A wild plant would need to receive several genes (probably on several different chromosomes) from the cultivated plant to become apomictic. This transfer would certainly lower its fitness to a value unacceptable for survival in the wild.

If apomixis is under a simple genetic control, diffusion of apomixis to landraces and wild relatives is possible. Apomixis reduces recombination rates and could be perceived as a danger for conservation of genetic diversity of wild relatives and landraces. In actuality, current genetic diversity is the result of a long process of domestication, which is still underway in some regions of the world, especially where wild and cultivated plants continue to exchange genes, often within a traditional agricultural system. Somewhat surprisingly, it is in regions where traditional agriculture prevails that apomixis could be the most helpful. We know that obligate apomixis is an exception and facultative apomixis is predominant (Asker 1979). If during the transfer of apomixis to crops, residual sexuality is also transmitted and expressed in the new apomictic crop, we could rely on the rate of recombination inherent in this process to generate new genetic combinations. Even at low rates, new combinations may be interesting to farmers who could select and propagate them easily. As long as apomixis is not obligate, landraces can still evolve. It may also be possible to introduce new genes from "exotic" and modern sexual varieties. Crosses will occur only in the proportion k (rate of residual sexuality). But if these new products can be detected by markers or by their hybrid vigor, following selection, they could serve as an important source of seed for the next generation. The possibility and rate of evolution of these apomictic varieties will eventually depend upon the rate of residual sexuality; therefore, it will be important to consider this parameter when transferring apomixis from wild apomixis donor plants to first apomictic varieties. This rate of residual sexuality may depend on genetic factors. Controlling these factors, in order to adapt the value of this parameter in new apomictic varieties, could be extremely useful as we seek to conserve the genetic diversity of landraces and allow for their continual evolution.

Areas of traditional agriculture are repositories for most of the genetic diversity of crops. The conservation of this diversity is threatened, however, by changes in technical practices that can suppress current gene flow and by the introduction of new modern varieties with limited genetic diversity (e.g., F_1 hybrids). Producing new varieties from local germplasm may be advantageous to farmers, and it could be more easily accomplished if apomixis is incorporated into the breeding scheme (see Toenniessen, Chap.1). In this scenario, landraces with high genetic diversity would be maintained in these farming systems, thus limiting the diffusion of varieties with low genetic diversity. This diversity would serve as a reservoir for future evolution.

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Chapter 3

Classification of Apomictic Mechanisms

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Introduction

Apomixis has traditionally been separated into asexual seed production (agamospermy) and replacement of flowers by vegetative propagules (vivipary). In practice, many researchers define apomixis as agamospermy, which in turn is divided into adventitious embryony and gametophytic apomixis. Adventitious embryony is the formation of somatic embryos from ovular tissues outside the embryo sac, although endosperm in the embryo sac usually is necessary to support their maturation, and the resulting somatic embryos sometimes compete with a zygotic embryo within the same ovule. Gametophytic apomixis is, at the least, a two-step process involving the production of a $2n$ megagametophyte, whose egg develops without fusion of egg and sperm nuclei; other aspects of fertilization can be completely normal or completely missing, depending on the type.

Adventitious embryony can be divided into nucellar and integumentary embryony, depending on where the embryos arise. According to Naumova (1993), adventitious embryos in nature always originate from single cells, termed embryocytes, which can differentiate in the nucellus or integuments, before or after fertilization of the embryo sac. As summarized by Naumova (1993), the early organization of adventitious embryos characteristically differs from that of zygotic embryos, possibly because space is constrained and the mechanical stress field in the nucellus differs from that in the embryo sac. Multiple adventitious embryos can develop asynchronously within the same ovule (Gustafsson 1946), whereupon competition and packing further affect their morphology. Polyembryony is very frequent in most cases of adventitious embryony, and fertilization of the central cell is generally necessary for seed set. The developmental interpretation of adventitious embryony is simple: the embryocytes are induced to act like zygotes. The number of ways in which this induction can occur is not known, but they all must cause repeated mitosis.

Types of Gametophytic Apomixis

In gametophytic apomixis, $2n$ embryo sacs can arise in at least nine different ways, depending on the species. The embryo and endosperm in such embryo sacs can develop in at least five ways. Therefore, at least 45 types of gametophytic apomixis are theoretically possible, but only half of these account for nearly all cases of naturally occurring gametophytic apomixis. The nine types are schematically diagrammed in Figure 3.1, which lays out their proposed developmental basis. The types are described below (references are given afterward as part of a historical perspective). Note that "strike" (nondivision or precocious degeneration of nuclei in the chalazal end of developing

embryo sacs) is not mentioned in these descriptions, but it regularly occurs in a number of angiosperm families and is expected to affect sexual and apomictic species equally.

Nine Types of Embryo-Sac Development

1) The Allium odorum-type. In the Allium odorum-type, the megasporocyte undergoes endomitosis and then proceeds through a mechanically normal development of the Allium (bisporic)-type. The endomitosis is not specific to the megasporocyte, and many of the nucellar cells also become endopolyploid. The $2n$ number of bivalents is observed at meiotic metaphase I, but all seem to involve former sister chromatids, and genetic segregation is thwarted despite high chiasma frequency. The first meiotic division yields a dyad of cells, whose chalazal member gives rise to the mature 8-nucleate embryo sac after three rounds of mitotic division. Sometimes a second round of endomitosis precedes meiosis, resulting in $4n$ bivalents and a $4n$ embryo sac. In other ovules, the megasporocyte does not undergo endomitosis, bivalents and multivalents are seen at metaphase I, and the embryo sac is $1n$ and genetically segregated.

2) The Taraxacum-type. In the Taraxacum-type, meiotic prophase I and metaphase I generally lack chiasmata. The condensed univalent chromosomes congregate and decondense, resulting in a restitution nucleus. The megasporocyte then undertakes the second meiotic division normally, and the 8-nucleate embryo sac develops through three rounds of mitosis from the chalazal member of the ensuing $2n$ megaspore dyad.

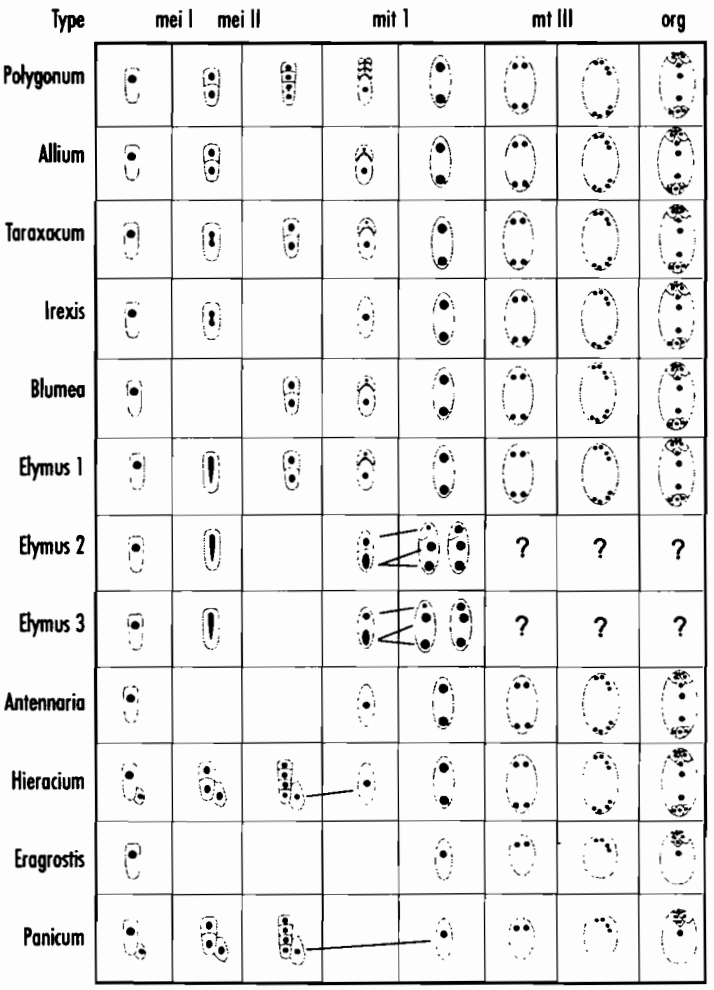

Figure 3.1 Schematic diagram of apomictic embryo sacs.
The top two lines give the sexual Polygonum- and Allium-types for comparison. All are arranged according to the concept of Crane (1978), in which late stages of differentiation are more critical to function. A mitotic checkpoint for chromosomal double-strandedness is also assumed. The final morphology of the Elymus-hemidyad and Elymus-binucleate types is inadequately understood for reliable depiction here, but extra micropylar nuclei and extra eggs are very likely, as evidenced by polyembryony in some clones of *E. rectisetus*.

3) The Ixeris-type. The Ixeris-type follows the Taraxacum-type through the formation of a meiotic restitution nucleus. The next nuclear division is not followed by formation of a cell wall, nor is the division after it. Cell walls form only as the embryo sac matures after the third round of divisions. All daughter nuclei from the restitution nucleus contribute to the mature, 8-nucleate embryo sac.

4) The Blumea-type. In the Blumea-type, a more or less mitotic division of the megasporocyte yields a dyad of $2n$ megaspores. The chalazal megaspore gives rise to the mature, 8-nucleate embryo sac. The development is identical to the Taraxacum-type after the restitution nucleus has formed. Therefore, existence of the Blumea-type must be demonstrated by the absence of the restitutional stages of the Taraxacum-type; this proof requires careful, thorough sampling and ordering of all the meiotic stages.

5) The Elymus rectisetus-type. In *Elymus rectisetus*, three interrelated developments occur that superficially resemble the Blumea-type. The megasporocyte nucleus enlarges and is deformed in all three types. The nucleus appears to be under tension, as evidenced by parallel alignment of its chromatin, and by its chalazally pointed or occasionally wasp-waisted shape. Triads and dyads with unequal nuclei indicate amitotic division in a very low percentage of megasporocytes. The first visible prophase follows the nuclear deformation episode and leads to what could be considered, more or less, a mitotic division. Here the three subtypes diverge: this division can result in a dyad of completely separated cells, a hemidyad of incompletely separated cells, or a binucleate embryo sac. All three behaviors can occur on the same individual. The chalazal-end nucleus in all three cases often undergoes a milder form of the deformation seen in the megasporocyte. The fate of hemidyads and directly binucleate embryo sacs has not been thoroughly investigated, but the chalazal member of dyads typically undergoes three rounds of mitosis and forms the mature, 8-nucleate embryo sac. The micropylar nucleus of hemidyads sometimes degenerates as if it had been completely cut off.

6) The Antennaria-type. In the Antennaria-type, there are no meiotic stages. The megasporocyte undergoes three rounds of mitosis, typically after an initial period of enlargement and vacuolation. The mature embryo sac has eight nuclei, which are arranged as in the conventional Polygonum-type.

7) The Hieracium-type. In the Hieracium-type, one to several nucellar cells enlarge, become vacuolate, and go through three rounds of mitosis, resulting ideally in a conventionally organized 8-nucleate embryo sac. The megasporocyte undergoes meiosis in some species and degenerates in others. In some cases, reduced and unreduced embryo sacs can coexist in the same nucellus. The ability to form multiple embryo sacs in the same ovule increases the frequency of polyembryony.

8) The Eragrostis-type. In the Eragrostis-type, there are no meiotic stages. The megasporocyte undergoes only two rounds of mitotic division, leading to a 4-nucleate embryo sac with an egg, two synergids, and one polar nucleus, or alternatively, to an egg, one synergid, and two polar nuclei. The original description of the type also included bipolar sacs with more than four nuclei, which at that time were interpreted as modifications of the Antennaria-type (Streetman 1963). Such sacs may instead represent facultative occurrence of the Polygonum-type.

9) The Panicum-type. In the Panicum-type, one to 25 nucellar cells enlarge, increase in vacuolation, and ideally divide twice mitotically, although some divide only once

and others not at all. The mature embryo sac has an egg, two synergids, and a polar nucleus, or else, an egg, one synergid, and two polar nuclei. The megasporocyte can undergo meiosis or degenerate, depending on the species. The potential for polyembryony is high in this type.

Most of these developments were discussed by Battaglia (1963), who also discussed various aberrations, and later by Nogler (1984) and Asker and Jerling (1992). Descriptions were first published for the Antennaria-type in 1898 (Juel 1898), the Taraxacum-type in 1904 (Juel 1904), the Hieracium-type in 1907 (Rosenberg 1907), the Ixeris-type in 1919 (Holmgren 1919; Okabe 1932), the Allium odorum-type in 1951 (Hakansson and Levan 1951, 1957), the Panicum-type in 1954 (Warmke 1954), the Eragrostis-type in 1963 (Streetman 1963; Voigt and Bashaw 1972), and the Elymus-syndrome in 1956 (Hair 1956), with significant amendments in 1987 (Crane and Carman 1987). The Blumea-type, described for *Erigeron ramosus* by Holmgren (1919), was later generically refuted (Fagerlind 1947) and finally rehabilitated for *Blumea eriantha* (Chennaveeraiah and Patil 1971).

A number of previous classifications, e.g., Asker and Jerling (1992), have recognized automixis, which is the fusion of nuclei in the same gametophyte to produce a $2n$, homozygous egg cell. Automixis has been purported in several species of *Axonopus* (Gledhill 1967), but the evidence can easily be reinterpreted as early degeneration of one of the synergids in the Polygonum-type before fertilization. Like gametophytic selfing in ferns, the mechanism would produce individuals that are completely homozygous within genomes. There is no genetic evidence for or against apomixis in the affected *Axonopus* species. Automixis has also been claimed for *Rubus caesius*, as discussed by Asker and Jerling (1992), but the case is scarcely closed. The evolution of automixis is implausible in angiosperms, because autogamy evolves easily in most groups and has the same genetic result over the course of generations.

Subsequent Steps of Development

1) Embryos. Apomictic embryo development from egg cells has been summarized in earlier research, e.g., Asker and Jerling (1992) and Battaglia (1963). There are three major types: pseudogamy, semigamy (= hemigamy), and autonomous parthenogenesis. There is no tradition for naming these last three developments for the type genera in which they occur. The developments differ in the order in which egg and primary endospermatic nuclei divide, the ability of the central-cell nuclei to divide without fertilization, the number of polar nuclei that contribute to the endosperm, and the ability of the egg cell to undergo plasmogamy.

Pseudogamy is traditionally defined as asexual seed set that requires pollination and does not involve adventitious embryos. However, a more restricted definition seems preferable: pseudogamy is seed set through fertilization of the central cell, but not the egg, in the absence of adventitious embryos.

Semigamy is seed set with full fertilization of the central cell and only plasmogamy in the egg, where the sperm nucleus can be walled off, or divide one to several times, or contribute equally to chimeric or twinned embryos. The last of these behaviors is not found routinely in recurrent apomicts, but is typical of the *Se* mutant in cotton (Turcotte and Feaster 1969), in which the sectors can be easily visualized with leaf-color mutations. In *Se* cotton, complex chimeras with maternal haploid, paternal haploid, and hybrid sectors are frequent; they probably arise from coalescence of one pair of adjacent spindle poles upon

simultaneous division of the proximate egg and sperm nuclei in the zygote (Turcotte and Feaster 1973, 1974).

Autonomous parthenogenesis is the formation of embryo and endosperm without fertilization of either the egg or the central cell; such apomicts require no pollination for seed set and frequently are male-sterile. Autonomous endosperm formation has also been reported in a case of adventitious embryony, namely *Euphorbia dulcis* (Gustafsson 1946).

2) Endosperm and embryo development. Pseudogamy and autonomous parthenogenesis can be subdivided on the basis of their capacity to form embryos in the absence of endosperm. The apomictic egg divides autonomously in *Potentilla* (pseudogamy) and *Wikstroemia* (autonomous parthenogenesis) (Gustafsson 1946). The egg divides after the first endosperm nucleus in *Themeda* (pseudogamy) and *Crepis* (autonomous parthenogenesis), although the potential for its eventual division in the absence of endosperm has not been critically evaluated in most cases. In semigamy, the unfertilized egg degenerates without dividing (Crane 1978), contrary to an earlier report of eventual division (Coe 1953). There is also variation in the fusion of the polar nuclei with each other in apomicts. In autonomous parthenogenesis, the polar nuclei can fuse before dividing (e.g., in *Taraxacum*, *Elatostema*, and most *Alchemilla* species), facultatively fuse (e.g., in *Crepis*), or divide without fusing (e.g., in *Antennaria*) (Gustafsson 1946). In pseudogamy, the polar nuclei may (*Ranunculus auricomus*; Nogler 1972) or may not (*Amelanchier*; Campbell et al. 1987) fuse with each other when the central cell is fertilized. Both polar nuclei contribute to the endosperm in semigamous *Cooperia* and in the *Se* mutant of cotton (Crane 1978; G. L. Hodnett, unpublished).

Alternative Classifications

The standard classification of apomictic embryo sacs (e.g., Asker and Jerling 1992) has followed Edman (1931) and Gustafsson (1946) in recognizing embryo sacs that arise from diploid megaspores (diplospory) and embryo sacs that do not arise from megaspores (apospory). Under this scheme, the Hieracium- and Panicum-types are aposporous, and the remaining seven types are diplosporous (Note: Types that were undescribed at the time of these old papers (*Eragrostis*, *Panicum*, *Elymus*, and *Allium odorum*) have been assigned on the basis of the classifying paper's underlying concepts). Fagerlind (1940, 1944) defined diplospory to be the production of diploid megaspores from meiosis, in effect restricting it to the Taraxacum-, Ixeris-, and Allium odorum-types. He termed purely mitotic developments apospory, which could be generative (*Antennaria*, *Eragrostis*) or somatic (*Hieracium*, *Panicum*). The Blumea- and dyad-forming Elymus-types fell into a halfway category, semiapospory. Battaglia (1963) also recognized apospory for purely mitotic developments and differentiated gonial and somatic subtypes. However, he grouped all modifications of meiosis under aneuspory. Thus he considered the Allium odorum-, Taraxacum-, Ixeris-, Blumea-, and dyad-forming Elymus-types to be fundamentally similar in that they involved modified meiosis. Consequently, one issue that has emerged is the definition of a megaspore: is it more fundamentally the product of a megasporocyte or the product of female meiosis? Many other terms have been defined and redefined, leading to terminological and conceptual confusion. For example, Johri et al. (1992) speak of "apospory of the Taraxacum-type" in *Balanophora globosa*. The major problem has been an absence of hard evidence as to what genetic changes are necessary to establish apomixis and what the relevant gene products do. Furthermore, the traditional

Edman terminology reflects an outlook in which the site of gene action is more important than the type of gene action, and this outlook has probably inhibited comprehension of apomictic development. Until the arrival of suitable optics (especially Nomarski differential interference contrast) and clearing media for the rapid, accurate analysis of statistically meaningful numbers of intact ovules, it was difficult enough to accurately describe apomictic development, such as the maximum extent of prophase I chromosome contraction or the relative sequence of division and apparently restitutional stages.

Developmental Interpretation

The computer program is a good, although imperfect, paradigm for understanding apomictic developments. In many computer programs, statements are executed in a definite order, and calls to a later statement prevent execution of intervening statements. Because control passes to the later statement, the computer program is a direct counterpart to heterochronic developmental changes, in which development abruptly jumps to a later stage, and to homeotic developmental changes, in which development in one cell type or organ abruptly switches to a pathway found in another cell type or organ. As outlined below, most aspects of apomixis can be speculatively rationalized in this way, however, some cannot. Some developments require parallel operation of steps from different programs, as would occur in a computer with multiple processors running in parallel.

Programmatic behavior is consistent with a cascade of sequential inductions of gene expression, in which each gene activity is induced only by some condition unique to the preceding step in the development. Until better evidence becomes available about the molecular function of apomictic genes, the interpretation of apomictic development must remain speculative, based on the timing and position of divisions, restitution, chromosome pairing, and so on.

Meiotic Development of Megagametophytes

The basic and most frequent embryo-sac development in flowering plants is the Polygonum-type. Its developmental program can be represented by a sufficiently detailed list of events: differentiation of the megasporocyte from parietal or other nucellar cells, commitment to meiosis I, premeiotic DNA synthesis, premeiotic G_2 phase, leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase I, anaphase I, telophase I, meiotic interphase, prophase II, metaphase II, anaphase II, telophase II, tetrad stage, germination of the surviving megaspore, first embryo-sac mitosis, binucleate embryo sac, second embryo-sac mitosis, tetranucleate embryo sac, third embryo-sac mitosis, and cellularization. One reasonable, but unproven assumption is that DNA synthesis requires a checkpoint for chromosomal single-strandedness, such that already double-stranded chromosomes are not re-replicated. Such a checkpoint would suffice to prevent DNA synthesis during meiotic interphase and allow the second meiotic division to be bypassed without affecting the ploidy of the mature embryo sac. Another assumption, supported by the pattern of numerical abnormalities observed and not observed in mature embryo sacs of *Cooperia drummondii* (Crane 1978), and consistent with the developmental sisterhood of synergids (Cass et al. 1986), is that embryo sacs differentiate through progressive capacitation of individual nuclei at each interphase, rather than simultaneously upon cellularization. At the micropylar end in the tetranucleate stage, one nucleus is already committed to differentiating synergids, and the other one is committed to

differentiating the egg and micropylar-end polar nucleus. The egg cannot form if the third mitotic division does not occur; its precursor defaults to being a polar nucleus. Thus meiosis II and the first embryo-sac mitosis can be skipped without affecting female fertility or offspring ploidy, as in the Adoxa type of sexual embryo sac, however the last divisions are critical for female fertility. The details of the numerical abnormalities observed in *Cooperia*, and their implications for the evolution of sexual embryo sacs, are reported elsewhere. Following are the author's interpretations of the nine apomictic types of embryo sacs.

Ameiotic Developments of Mgagametophytes

Endomitosis in the Allium odorum-type would result from relaxation of the chromosomal double-strandedness checkpoint postulated above for DNA synthesis. Endomitosis can occur in a wide variety of plant cells, including, most relevantly, the anther tapetum (Crane et al. 1993), which is homologous to cells in the nucellus. Megasporocyte endomitosis does not affect the morphological course of meiosis; it can precede any of the sexual types of embryo-sac development, and it is probably underreported. One consideration is how the chromosomes behave after chromatid separation and secondary replication in the premeiotic G_2 nucleus. If they scarcely move, the former sister chromatids would lie in close proximity as pairing begins in leptotene, and multivalents rarely, if ever, would form.

The Taraxacum-type results from induction of the meiotic interphase during the prophase or metaphase of meiosis I. The contracted chromosomes are thus induced to decondense and await the second meiotic division. Another possible explanation for the Taraxacum-type is induction of an Elymus-style "waiting state" (see under *Elymus* below) during prophase I, with recovery in time to undertake meiosis II, although this induction would not necessarily result in a restitution nucleus. Direct induction of meiotic interphase would likely accelerate megasporogenesis relative to sexual ovules, whereas entering a "waiting state" would cause a significant delay.

Induction of meiotic interphase also explains the Ixeris-type, but in this case the induction is superimposed on a bisporic or tetrasporic type of reduced embryo sac, e.g., the Fritillaria-type in *Rudbeckia* (Battaglia 1946, 1963). Assuming a checkpoint for chromosomal double-strandedness, bisporic and tetrasporic types lack the second meiotic division, and thus have their own induction of megaspore germination after the first meiotic division (bisporic types) or during the first meiotic division (tetrasporic types). Accordingly, the restitution nucleus awaits the first embryo-sac mitosis rather than meiosis II in the Ixeris-type, and there is no cross-wall to separate $2n$ "megaspores."

In the Blumea-type, meiotic interphase is induced in the premeiotic megasporocyte. Its genetic basis could be the same as in the Taraxacum-type, and the two types could coexist in the same species or individual if the timing of induction varied. Proving the existence of the Blumea-type is intrinsically difficult because it must be shown that the restitutional stage of the Taraxacum-type is absent. Therefore, measuring the frequencies of these two types in a polymorphic individual might not be feasible with microscopic techniques that kill the ovule before it is examined. The outcome of superimposing the Blumea-type on a bisporic or tetrasporic sexual type remains unknown. It might superficially conform to the Antennaria-type.

The Elymus-type appears to result from incomplete differentiation of the megasporocyte from the nucellus. Not only is

its callosic wall deficient, but the pattern of vacuolation and the occasional presence of an oxalate crystal in the megasporocyte conform to typical behaviors of the adjacent nucellar cells. The extent to which this similarity causes the wall to be deficient is not clear, nor is the degree to which the deficiency prevents a necessary physiological isolation for full differentiation of the megasporocyte. The cytoskeleton of the megasporocyte is disturbed, as evidenced by frequently oblique division planes and the deformation of the nucleus. Perhaps the meiotic spindle apparatus is not completely suppressed, and so interacts with the preapomeiotic nucleus. But this does not explain the second deformation in the chalazal $2n$ megaspore. Another feature of the Elymus-type is the need for parallel control of development. The megasporocyte enters a "waiting" state and variably emerges in time to undertake meiosis II, the first embryo-sac mitosis, or an intermediate condition, respectively accounting for dyads, directly binucleate sacs, or hemidyads. During the "wait," a master "clock" continues to run, albeit abnormally slowly in comparison to the Polygonum-type in related sexual genotypes; this clock allows recovery from the wait.

The Antennaria-type can be explained in two ways: by induction of megaspore germination in the megasporocyte or as a much belated recovery from an Elymus-style waiting condition. The frequently abnormal enlargement and vacuolation of the megasporocyte are consistent with the latter explanation, but intraspecific polymorphism for the Antennaria- and Hieracium-types in *Potentilla* and *Poa* is consistent with the former explanation. Details of gene expression in germinating Polygonum-type megaspores should provide clues as to which examples of the Antennaria-type result from megaspore germination. It should be noted that the tetrasporic sexual types also involve induction of megaspore germination during meiosis I, and that having tetrasporic sacs does not seem to predispose individual taxa to evolve the Antennaria-type or vice versa. Thus different, separately inducible aspects of megaspore germination might impact meiosis in different ways, or the event that calls up megaspore germination might fundamentally differ between the Antennaria- and tetrasporic sexual types.

The Hieracium-type results from induction of megaspore germination in the affected nucellar cells. The ability of an induced embryo sac to mature is related to its position, the time of its induction, and the number of competing sacs. In theory, Hieracium-type sacs can coexist with the Polygonum-type, resulting in polyembryony and facultative sexuality. One would expect apomicts with the Hieracium-type to accumulate mutations that debilitate the *Polygonum* development and permit a single, unreduced sac to mature.

The Eragrostis- and Panicum-types share induction of the second embryo-sac mitosis (or its preceding interphase), respectively, in the megasporocyte or in one to many nucellar cells. The single nucleus is induced to behave like the micropylar-end nucleus at the binucleate stage in the Polygonum-type. These types are known only from related subfamilies of grasses and possibly share the same genetic basis, modified only for site of induction. From a developmental viewpoint, they are related to the sexual Oenothera-type, in which a megaspore behaves as if it were a binucleate embryo sac.

Subsequent Steps of Development

In a broad sense, apomictic egg cells are induced to act like zygotes, with the possible exception of semigamy. The question about semigamy is whether it functions exclusively in the embryo sac; anecdotal examples of

haploids from normal cotton plants pollinated with semigamous pollen have been reported (Turcotte and Feaster 1963). In the absence of such rare haploids, one is tempted to presume that semigamy results from induction of a subset of zygotic behavior that prevents karyogamy but not plasmogamy. If semigamy were found to function in both eggs and sperms, one would instead suspect a general defect in fertilization capacity.

The variation from autonomous to pollination-dependent embryo induction in pseudogamy suggests that embryos can be induced in more than one way. Perhaps one way triggers full embryonic development, while another merely primes the egg (or sexual zygote) to respond to a stimulus from the dividing endosperm. In the latter case, the apomictic egg of an unfertilized ovule would degenerate without ever dividing, whereas in the former case, the apomictic egg would eventually divide, even if it does not regularly do so before the primary endosperm nucleus divides or before unfertilized embryo sacs degenerate. Savidan (1989) has proposed that because pseudogamous *Panicum maximum* undergoes such early induction and maturation of the embryo sac, the egg cell completes its cell wall before pollination. While the formation of the egg wall is progressive in grasses (Cass et al. 1986), it is not clear that old eggs inevitably would complete their wall. In vitro fusion of egg and sperm protoplasts of maize leads to rapid cell wall formation (Breton et al. 1995, and references cited therein), suggesting that induction of zygotic behavior is responsible for the physical barrier to fertilization of pseudogamous eggs.

Autonomous parthenogenesis would seem to require separate inductions of endosperm division and either the "triggered" (*Wikstroemia*) or merely the "primed" (*Crepis*) condition. Yet separate inductions would imply that the genes for each could be separated and that genotypes lacking the autonomous endosperm induction would be fully pseudogamous. This is possible in genera where instances of pseudogamy and autonomous parthenogenesis are known, e.g., *Poa* and *Crataegus*, but it is inconsistent with unreduced autonomous parthenogenesis in the absence of pseudogamy in apomictic Cichoreae (*Taraxacum*, *Chondrilla*, *Crepis*, and *Ixeris*).

Genomic imprinting of gametic nuclei is another consideration in the developmental interpretation of apomictic embryogenesis. In the Polygonum-type, the endosperm has a 2:1 maternal:paternal genome ratio; deviations from this ratio frequently cause endosperm abortion in interploidy crosses. In semigamous *Cooperia*, the reduced sperm nucleus fuses with both unreduced polar nuclei (Crane 1978), resulting in a 4:1 maternal:paternal ratio in the endosperm, which functions adequately for seed germination in this genus. In pseudogamous *Ranunculus*, both sperm nuclei can fertilize the central cell, resulting in a 4:2 maternal:paternal ratio and normal endosperm function (Nogler 1972). In pseudogamous *Crataegus*, the polar nuclei do not fuse with each other, and a sperm fuses with only one polar nucleus, resulting in the normal 2:1 maternal:paternal ratio (Campbell et al. 1987). The situation in autonomous parthenogenesis is not clear, because the number and biochemical effect of imprinted loci is not obvious. Imprinting of a tubulin locus (Lund et al. 1995) and the *dzr1* locus (Chaudhuri and Messin 1994) has been documented in developing maize endosperm, and it is possible that the number and sex-specificity of imprinted loci vary greatly among plant species. What matters here is the number of loci that orchestrate the imprinting pattern. Since the evolution of apomixis is easier if fewer loci control it, one might expect

one or a few key loci to affect this pattern. Ehlenfeldt and Hanneman (1988) analyzed the crossing behavior of diploid hybrids between two diploid, sexual *Solanum* species that differ in endosperm balance number, and they concluded that three unlinked loci controlled it. Obviously, comparable evidence is needed for the immediate relatives of autonomously parthenogenetic apomicts.

Outlook

The speculative nature of the preceding discussion emphasizes how little is experimentally known about meiosis, megasporogenesis, gametogenesis, and fertilization in sexual plants, and how little of the diversity of apomictic behaviors has been studied with modern methods of genetics, cell biology, and molecular biology. Most apomicts are polyploid, perennial herbs with long generation times, and the effort to maintain suitably large, uncontaminated populations of advanced generation hybrids has repeatedly defeated even rudimentary attempts to understand the inheritance of apomixis. The pattern of genomic affinity is not known for most pseudogamous or semigamous apomicts; recombination between the centromere and the causative loci can further change the expected segregation. The general problem is that more than one equally best-fitting model can be found for the same segregation data, especially when multiple allelism and complex dosage requirements are considered and advanced generation selfs and backcrosses are not available. Since the number of developmental inductions is constrained by the number and interaction of causative loci, the difficulty in completely understanding the genetics of apomixis has hamstrung the more direct developmental approaches. This is particularly true for the relationship among imprintable loci, endosperm function, and embryo induction in autonomous apomicts. Endosperm imprinting relationships might well become the biggest obstacle to the utilization of apomixis in sexual crop species.

At the level of cell and molecular biology, critical questions remain regarding the sequential and parallel nature of clocks that govern the relative and absolute duration of events in the Polygonum-type, and thus the number of control points and interconnections that suffice to push the whole program along. Diagnostic mRNA or protein suites have not yet been recognized for the steps in the Polygonum-type and applied to recognize parallel steps in apomictic developments. The promoters, reading frame sequences, and action of genes at control points remain a mystery. Thus the basis of one of the main assumptions in the preceding developmental interpretations, namely that the induction of late steps cancels intervening steps within the Polygonum-type, has yet to be justified.

Natural apomicts are difficult experimental subjects, but they provide embryological behaviors that might be even more difficult to screen from mutagenized populations of *Arabidopsis thaliana* or annual crop species, especially if the appropriate mutations require gains in function. Apomictic studies might contribute significantly to our future understanding of sexual plant reproduction and to the successful utilization of apomixis in currently sexual crops.

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Appendix: Methods to Clear Angiosperm Ovules

Apomictic development can be studied with any of the tools used to study other plant anatomical problems. Particularly useful tools include thick and thin sections, clearings, flow cytometry, and Feulgen microspectrophotometry. Clearings have played a prominent role in recent studies because they allow characterization of large, reproductively heterogeneous samples. Sections and clearings allow the positions and divisions of nuclei and protoplasts to be observed directly; sequences of nuclear divisions and movements can be inferred by observing the set of still images. In this respect, both are inferior to cinematography of living embryo sacs in ovules suspended in silicone oil (Erdelska et al. 1971, 1979), but the latter technique has not been widely applied because of the usually insufficient transparency of the nucellus and integuments. Flow cytometry provides information on the DNA content (ploidy) of embryonic and endospermatic nuclei and thus on the frequency of apomixis and the role of the sperm nuclei in apomictic endosperm development. The older technique of Feulgen microspectrophotometry provides this same information for species or developmental stages in which there are too few nuclei for flow cytometry or for which positive identification of all nuclei is desired. Except for cinematography, these methods require fixation of the specimen to stop all divisions and preserve the cells of interest in a sufficiently "lifelike" condition. The type of fixation limits the quality of the resulting data. The researcher's objectives (both scholarly and microscopic) determine the image quality that is needed and therefore the time and labor that are required; screening a segregating F_2 population for the Panicum-type does not require ultrastructural preservation or freedom from plasmolysis.

Clearings usually require less effort than embedding and sectioning, but the overall quality is limited by the amount of visual information that can be accrued in a single optical section (plane of focus) before the ovule as a whole is rendered opaque. Objects can be seen because they differ from their surroundings in color or refractive index; the latter causes light to bend at the surface of the object. Clearing media are designed to closely, but not perfectly, match the refractive index of cell walls or organelles; an object becomes invisible when immersed in a medium of equal refractive index. Variation in refractive index among cellular components assures that some aspect of the specimen will always be visible when the refractive index of the medium is close to that of the bulk specimen. Furthermore, many structures, such as cell walls, starch grains, and oxalate crystals, vary internally in refractive index as the light path moves relative to their crystal axes. This property is termed birefringence, and it can be a severe nuisance in examining cleared ovules.

Specimen thickness, refractive index, and information content (organelle or nuclear frequency per unit thickness) determine the optimal refractive index of a clearing medium. Single cells can be successfully examined in water, and a movie has been made of nuclear movements and divisions within viable ovules of *Jasione montana* (Campanulaceae) immersed in silicone oil (Erdelska et al. 1971). "Normal" ovules and grass ovaries require a considerably closer matching of refractive index, i.e., greater loss of visual information from individual planes of focus, for successful visualization of their interiors. Information content reflects organellar preservation and thus depends on the type and duration of fixation. The organellar refractive index appears to respond to hydration, and thus the optimum refractive index differs between hydrous and anhydrous

media. There may also be a statistical correlation of optimum refractive index to DNA content per chromosome, with large genomes tending to require a higher n_D. For example, *Zephyranthes* can be cleared in methyl salicylate (n_D = 1.537), but *Nothoscordum* (which has bigger chromosomes) requires a higher n_D, as provided by 2:1 benzyl benzoate: dibutyl phthalate (n_D = 1.542). Possibly this correlates more directly to G-C content, pitch of the double helix, or degree of cytosine-methylation in heterochromatin, than to total DNA content; the smaller genomes simply carry less heterochromatin.

Table 3.1 presents refractive indices of various published and candidate clearing media as measured with an Abbe refractometer at ca. 20°C. Most ovule-clearing studies have used one of two classes of media: modified lactophenol (Herr's 4-1/2 and BB 4-1/2, Herr 1971 and 1974) and aromatic esters (Crane 1978; Young et al. 1979; Crane and Carman 1987). The latter have been combined successfully with staining in hemalum (Stelly et al. 1984) or azure dyes (G. L. Hodnett, personal comm.) for brightfield observation. The Herr media do not fully dehydrate the ovule because of the 15% water in commercial lactic acid, and organelles and cell walls therein seem to clear optimally around n_D 1.51, whereas the optimum in aromatic esters is around n_D 1.53 or 1.54. A third class, nearly saturated sugar solutions, has been used to document callose deposition (Peel et al. 1997), with lesser success in displaying nuclear and cellular locations. A fourth class, salts in glycerol or sugar solutions, remains to be tested, but appropriate refractive indices have been obtained for high-quality clearing (Table 3.1).

Both of Herr's 4-1/2 media contain chloral hydrate, a federally controlled substance that sometimes presents legal problems for institutions that possess it. They also contain eugenol, which turns yellow upon exposure to light, and phenol, which likewise discolors and is a chemical contact hazard. On the other hand, excellent photographs have been obtained with Herr's media in appropriate species, and their refractive index probably can be adjusted upward without

Table 3.1 Refractive index (n_D) of common and potential clearing media

Substance	n_D
20% sucrose	1.363
40% sucrose	1.399
60% sucrose	1.441
70% sucrose (saturated)	1.465
saturated sucrose in 50% glycerol	1.468
80% fructose (saturated)	1.483
80% fructose in 10M K acetate, 10 mM KOH	1.494
80% fructose in saturated (ca. 8.6 M) KI, 10 mM KOH	1.517
95% glycerol saturated with KI, 1 mM KOH	1.506
saturated $CaCl_2$ in H_2O	1.469
saturated $CaCl_2$ in glycerol (glassy solid at room temperature)	1.534
saturated $ZnCl_2$ in H_2O	1.550
saturated $ZnCl_2$ in 80% (v:v) glycerol	1.532
1:1:4 (v:v:w) water, glycerol, $ZnCl_2$	1.518
$FeCl_3 \cdot 6H_2O$ liquefied with a trace of glycerol	1.560
50% polyvinylpyrrolidone (MW 10000)	1.429
75% polyvinylpyrrolidone (MW 10000)	1.487
saturated naphthalene in glycerol	1.472
Herr's 4 1/2 medium	1.503
Herr's BB 4 1/2 medium	1.510
methyl salicylate	1.537
dibutyl phthalate (DBP)	1.492
benzyl benzoate (BB)	1.569
BB:DBP mixes, v:v:	
50:50	1.530
55:45	1.533
60:40	1.537
65:35	1.541
70:30	1.545
75:25	1.549
80:20	1.552
saturated KI + 2.5 mg/ml tris base in 50% DMSO	1.475
saturated KI + 2.5 mg/ml tris base in 70% DMSO	1.496
saturated KI + 2.5 mg/ml tris base in 80% DMSO	1.508
saturated KI + 2.5 mg/ml tris base in 90% DMSO	1.519
saturated KI + 2.5 mg/ml tris base in DMSO	1.525

affecting their general properties by partially substituting isoeugenol (n_D = 1.574; Windholz et al. 1983) for eugenol (n_D = 1.541; Windholz et al. 1983) in the recipes given below.

Aromatic oils require complete or nearly complete dehydration of the ovule, which typically lengthens the clearing procedure and leads to shrinkage and possibly distortion. The ovules normally become hard and brittle, which is good on the microscope slide but not on the dissecting stage. While the oils that have been used are only mildly toxic, this is not uniformly true, and some oils (polynuclear aromatic hydrocarbons) must be rejected as components of clearing media because of their carcinogenicity.

Both the lactophenol and aromatic-oil clearing agents offer limited opportunities for cytochemistry. Most of the established cytochemical procedures are based on reactions in water and may behave abnormally in less polar solvents. Opaque reaction products can obscure unstained regions behind them, and accessibility to reactants is always more difficult in intact ovules than in sections thereof. Reaction products can be mobilized or lost upon dehydration and infiltration with clearing agents. Nevertheless, there is interest in distinguishing various types of cell walls (especially callose) and cellular inclusions (e.g., starch grains) in cleared ovules, and a medium that can be used to this end is introduced here for further investigation.

Several inorganic salts, including those listed in Table 3.1, dissolve to a considerable degree in DMSO, glycerol, concentrated sugar solutions, or polyethylene glycol. None of them has proven completely satisfactory as an alternative to the aromatic oils or 4-1/2 media, but they might permit additional cytochemistry to be performed on whole mounts. Some media with KI equal the 4-1/2 media in refractive index (Table 3.1), but do not clear the tissue well. This is attributed to iodination of the macromolecules in the cell, which raises its refractive index. The problem with $CaCl_2$ is the high viscosity of its solutions in polyols. Its hygroscopicity also causes the refractive index of the preparation to vary with the relative humidity in the room. Ferric and zinc chlorides are extremely corrosive to metals and human flesh, and they destroy nuclei relatively rapidly. Potassium thiocyanate gives a high refractive index in solution, but is chaotropic (destroys nuclei again) and hazardous to the mental health of the slide user (Handbook of Chemistry and Physics). Finally, the problem with DMSO for embryological clearings is its denaturant action on DNA and proteins, resulting in poor quality of nuclei or no nuclei at all. This action is the basis of a very effective recipe (provided below) for destructively clearing leaves for vascular and epidermal study.

The following protocols are mostly based on fixation in organic solvents that kill the cell quickly and preserve the chromosomes well. The choices include FAA (37% aqueous formaldehyde, acetic acid, and 50% or 70% ethanol, 1:1:18 by volume); FPA (the same solution with propionic acid substituted for acetic acid), 3:1 ethanol:acetic acid;, Carnoy's solution (6:3:1 ethanol:chloroform:acetic acid, by volume); and 2:1 acetone:acetic acid. Craf and Allen-Bouin-type fixatives can also be used, but require longer dehydrations. Formaldehyde and glutaraldehyde, alone or in combination, also require long dehydrations and typically leave the ovules yellow. They also contribute to autofluorescence in the detection of callose with aniline blue. The less polar fixatives generally cause severe plasmolysis of embryo sacs with two or more nuclei, but they also extract chlorophyll and carotenoid pigments more completely from ovary walls. Acetone/acetic acid is particularly effective at this and often leaves tissues snow-white.

The following recipes include examples in which the fixative and clearing agent are immiscible, requiring an intermediary dehydrating agent, and examples in which the fixative and clearing agent mix freely. In both cases, one must pay close attention to the solubility of air in each liquid in the procedure. Air appears to be much less soluble in aromatic oils and concentrated salt solutions than in ethanol or acetone. Over-rapid infiltration typically results in exsolution of bubbles or films of air that do not disappear with time. Affected ovules generally float and remain opaque during the infiltration series. If such bubbles appear, one must backtrack, possibly all the way to the fixative, and start again. Adding extra steps at closer gradations in concentration eliminates bubbling more effectively than does lengthening individual steps.

The dehydration and infiltration steps can be advantageously recombined in many of the following recipes. When the clearing agent and fixative are miscible, a general principle is to progress through a graded series of mixtures of the two, including enough steps to avoid exsolution, as discussed above. A good starting point is 10 gradations from fixative to the microscope slide. Users should feel free to experiment, especially when adapting a recipe to novel species or stages. The requirements for dissection must also be considered; 70% ethanol is much less noxious and corrosive than FAA, 2:1 acetone: acetic acid, or salt solutions in dimethyl sulfoxide (DMSO). In many cases, one does not have time to dissect out ovules before fixation or does not want to lose small, nearly invisible ovules when solutions are changed.

Microscopy can make or break any clearing procedure. Confocal laser scanning microscopy has been used impressively on thin orchid ovules (Fredrikson 1990), but its usefulness on thicker specimens remains untested, and it is not widely accessible because of its expense. Nomarski interference-contrast has been applied successfully to apomictic amaryllids (Crane 1978) and panicoid grasses (Young et al. 1979). Both of these methods create a very shallow depth of focus that helps to eliminate information from outside the focal plane. Hoffman modulation-contrast is a less expensive alternative to interference-contrast. Phase-contrast provides "optical staining" that can help distinguish nuclei from similarly sized vacuoles, but it requires a closer match between the sample and the medium in refractive index. Stain-cleared specimens (and many unstained specimens) can be examined satisfactorily under brightfield optics once the medium is properly matched to the specimen in refractive index. Brightfield is also insensitive to birefringent cell walls or crystals in the specimen, which greatly degrade an interference-contrast or phase-contrast image.

Specimen orientation is frequently critical to easy and correct interpretation of cleared embryo sacs. Ovaries of pooid grasses typically are laterally compressed and present a nearly end-on view of the embryo sac when they are allowed to lie flat. Standing them on edge in a sawtooth cut in index card or aluminum foil yields the desired sagittal optical section. Stelly et al. (1984) observed that squashing cleared ovules was generally counterproductive; the image got worse instead of better as more visual information was crammed into nearly the same plane. Thus clearings are often examined with "Raj" slides (Herr 1974), with which the cover slip is supported by underlying adjacent cover slips that are conveniently held in place with "Superglue" (G. Hodnett, personal comm.).

Recipes:

Clearing unstained specimens in Herr's chloral-lactophenol media (Herr 1971, 1974):

1. Fix specimens in FAA-50 or FPA-50 (these are variants of FAA and FPA prepared with 50% ethanol).
2. Transfer the specimens to 70% ethanol, in which they can be stored.
3. (Optionally) Dehydrate the specimens through 95% and 100% ethanol.
4. Transfer the specimens to the clearing agent. Two media are available: the 4-1/2 medium, which is 2:2:2:2:1 chloral hydrate, clove oil (primarily eugenol), lactic acid, phenol, and xylene by weight, and the BB-4-1/2 medium, which is 2:2:2:2:1:1 chloral hydrate, clove oil, lactic acid, phenol, xylene, and benzyl benzoate. The latter has a higher refractive index (Table 3.1).

Comments: Pretreating the specimens with lactic acid frequently improves the cleared image. In C. Crane's experience, these media turn brown within a few days, especially if they are exposed to strong light, since the phenol and eugenol are readily oxidized. The cleared ovules are fragile and can be macerated gently (Herr 1971) to isolate megasporocytes and so on.

Making permanent preparations after clearing in Herr's 4-1/2 media (Herr 1982):

1. Clear specimens 24 hr beforehand in 4-1/2 or BB-4-1/2 media as in the preceding protocol.
2. Prepare a fresh 5:3:13 (v:v:v) mixture of vinylcyclohexene dioxide, diglycidyl ether of polypropylene glycol, and nonenyl succinic anhydride. These are the ingredients of Spurr's low viscosity resin. Prepare an infiltration series of 3:1, 1:1, and 1:3 (v:v) clearing medium to Spurr's resin, and pass the specimens through the series with 15 min per stage. Then give the specimens 15 min in the pure resin. Herr measures his volumes as drops from a Pasteur pipette, e.g. 10, 6, and 26 drops of the resin ingredients.
3. Add the cure accelerator, dimethylaminoethanol, at a concentration of ca. 2 _l per ml of the Spurr's resin, i.e., 2 mm in the tip of a Pasteur pipette added to 42 drops of the resin. Mount the specimen and resin on a Raj slide; orient the specimen by sliding the cover slip on its supports. When orientation is satisfactory, cure the slide 24 hr at 60° C. The preparation will turn yellow with too much cure accelerator and fail to solidify without enough.
4. Herr also cites protocols for permanently mounting 4-1/2-cleared specimens in Permount, Piccolyte, and Euparal, all of which cause specimens to shrink. Only the Spurr's protocol gives the appearance of unplasmolyzed cells.

Clearing unstained specimens in aromatic oils:

1. Fix specimens in 2:1 acetone: acetic acid (v:v) (AnA) long enough to decolorize tissue, making a change to fresh fixative after 2–3 hr for pigmented specimens. A 24 hr fixation is good. Do not crowd the specimens in the fixative, as pigment extraction and dehydration will be compromised. See step 5 for alternative fixatives and dehydration schedules.
2. Prepare a 2:1 (v:v) mixture of benzyl benzoate and dibutyl phthalate (BBDP). Make the following solutions in sufficient volume to circulate the specimens freely: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 AnA:BBDP (v:v). Pass the specimens through this ascending series at room temperature, swirling them thoroughly at the beginning of each step. On the first try, use 1.5 hr or longer per step to avoid exsolution of air bubbles. There is no harm in letting a step run overnight or over a weekend, but the vial should be

tightly capped. It is probably possible to clear small ovules (e.g., of *Solanum*) after an hour of AnA fixation and 15 min in each step of this series, but it is risky if the gas-permeability of the specimens is not previously known.

3. Place the specimens in the optimal ratio of BB to DBP for final clearing. On the first attempt, prepare a series of mixtures from 40:60 to 75:25 (v:v) BB:DBP, e.g., 40:60, 45:55, 50:50, and so on, and clear a few specimens in each. Examine them for optimal resolution of nuclei and cell walls. If nuclei obscure the interior of the specimen, the refractive index of the medium is too low and a higher BB:DBP ratio is needed. If the cell walls are strongly visible and the nuclei are invisible, lower the refractive index with a lower BB:DBP ratio. The cleared specimens are good for at least 20 years if they are stored in the dark under refrigeration. Unsealed slides of BB:DBP are good for several months at room temperature and can be replenished with the clearing solution indefinitely, so long as the ovules have not dried out.
4. The procedure also works well with methyl salicylate, which can be mixed with the AnA fixative in all the same ratios for the same infiltration intervals.
5. Crane and Carman (1987) fixed wheat grass spikes in Carnoy's 6:3:1 ethanol, chloroform, acetic acid, and stored them in 70% ethanol. Florets were sorted by pollen meiotic stage in 70% ethanol. Batches of sorted florets were passed through 95% ethanol; 2:1 95% ethanol: benzyl benzoate; 1:2 95% ethanol: benzyl benzoate; and finally 2:1 benzyl benzoate:dibutyl phthalate. Unfortunately, this infiltration schedule exsolves air in the ovules of many dicot species. Crane (1978) fixed excised *Cooperia* ovules in FPA-50, dehydrated them through 70, 85, 95, and 100% ethanol, and infiltrated them in methyl salicylate in three steps. The first step entailed adding one-half volume of methyl salicylate to the ethanol in the ovule vial and mixing it in completely. In the second step, half of the liquid volume in the vial was removed and replaced with methyl salicylate. The third step used pure methyl salicylate. Young et al. (1979) followed a similar schedule, emphasizing complete dehydration. After FAA fixation (95% ethanol, water, 37% formaldehyde, and glacial acetic acid, 40:14:3:3 by volume), pistils were excised and passed through 50%, 70%, and 85% ethanol, three changes of absolute ethanol, 1:1 absolute ethanol: methyl salicylate, 1:3 absolute ethanol: methyl salicylate, and two changes of pure methyl salicylate, with 30 min per step. Groups of pistils were also cleared after being wrapped in a small envelope of Kimwipe tissue paper, with 2 hr per step. It should be noted that complete dehydration is not necessary for complete infiltration with methyl salicylate, which is miscible with ethanol solutions containing up to about 10% water. Perhaps removing the last traces of water from the starch grains and cell walls brought their refractive indices closer to that of nuclei.

Stain-clearing with Mayer's hemalum and methyl salicylate (Stelly et al. 1984):

1. Fix the specimen in FAA or CRAF V (Berlyn and Miksche 1976) for at least 24 hr.
2. Hydrate FAA-fixed material through 50% and 25% ethanol to water; wash CRAF-fixed material in water. In either case, the last water rinse is for 2–24 hr. Stelly et al. used tap water that was alkaline and calcareous. It might be possible to duplicate this action in distilled water by adding a small amount of calcium bicarbonate.

3. Stain the specimen for one to two days in Sass's modification of Mayer's hemalum (Sass 1958).
4. Differentially destain the specimen in 0.5% to 2.0% acetic acid for one or two days, until satisfactory stain intensity remains. Then stop the destaining with alkaline tap water or 0.1% $NaHCO_3$ for 2 to 24 hours.
5. Dehydrate the specimen through an ethanol series, e.g., at least 15 min each in 25%, 50%, 70%, 95%, 100%, and 100% ethanol, followed by 2–8 hr in a final change of 100% ethanol.
6. Infiltrate through 2:1 and 1:2 100% ethanol:methyl salicylate for at least 15 min each, and then change to pure methyl salicylate. Stain-cleared ovules are conveniently dissected from sliced potato ovaries at this stage.

Comments: The stain is best aged at least one week before use, and staining is better after long hydration, staining, and destaining steps. The procedure should be amenable to any of the other aromatic-oil procedures, and possibly to the high-salt media below for callose detection, but these remain to be tested. One would expect total stain extraction in acidic (e.g., Herr's 4-1/2) media.

Stain-clearing with azure C and methyl salicylate (Hodnett et al. 1997):

1. Fix the specimen (here excised cotton ovules) in FAA-50 for at least 24 hr, or in 2.5% glutaraldehyde buffered to pH 7.0 with 0.01 M phosphate buffer. The remaining steps are given on the assumption of glutaraldehyde fixation; see step 6 for the necessary modifications for FAA-50 fixation.
2. Perform any necessary dissection in 0.01 M phosphate buffer, e.g., removal of the integuments from the cotton ovules.
3. Stain the specimen with 0.05% azure C in 0.01 M phosphate buffer at pH 7, at least overnight. Azure A, azure B, methylene blue, and toluidine blue can also be used successfully, alone or in mixtures, at approximately the same total concentration and duration. Giemsa stain should be avoided because it often precipitates red gels from its eosin component.
4. Transfer the specimens to 0.01 M phosphate buffer, pH 7, and then dehydrate them through an ethanol series (25%, 50%, 75%, 95%, each for 2–3 hr). The aqueous ethanol solutions should be buffered to pH 7 with 0.01 M phosphate. Dehydration can be interrupted overnight in a small volume of 75% or 95% ethanol, so as to minimize extraction of stain from the specimen.
5. Infiltrate the specimen through 2:1, 1:1, and 1:2 (v:v) solutions of 95% ethanol and methyl salicylate. Then transfer the specimens to methyl salicylate for mounting and examination. It should be possible to substitute other optimized aromatic oils directly for the methyl salicylate, and to use a succession of 3:1, 1:1, and 1:3 mixtures for more uniform infiltration rates. Each step should take 2–3 hr.
6. After FAA-50 fixation, transfer the specimens to 50% ethanol for any necessary dissection, and stain them in 0.05% azure C in 50% ethanol at least overnight. Any of the other stains listed in step 3 can also be used. Proceed to 50% ethanol and complete the rest of step 4. Staining is more intense in phosphate buffer than in ethanolic solutions, and excessive extraction of the stain can be prevented during dehydration by including stain in the 25% and 50% ethanol stages of step 4. If the stain is too intense, increase the duration of each stage in the dehydration, rehydrating the specimen if necessary. A trace of blue stain is surprisingly effective in rendering gametic nuclei visible under brightfield microscopy.

7. The nuclei can be stained in DAPI (3 g/ml in McIlvaine's buffer), dehydrated, and cleared in aromatic oils. Epifluorescence microscopy of such preparations with ultraviolet excitation can yield stunning photographs, but the necessary control of destaining and the requisite photographic skill are much higher than for azure dyes.

Clearing in salty glycerol (Crane, unpublished data):

1. Fix specimens in FAA-50 or 2:1 (v:v) acetone:acetic acid (AnA), as in the general aromatic-oils protocol.
2. Bring the specimens to 70% ethanol.
3. Infiltrate the specimens with 3:1:4 (v:v:w) glycerol: polyethylene glycol (MW ca. 200): $CaCl_2$ _ $2H_2O$. A series of 6–10 half-hour gradations, e.g., 7:1, 6:2, 5:3, 4:4, 3:5, 2:6, 1:7, of 70% ethanol to glycerol solution is reasonable. The necessary number of gradations is determined by specimen size and permeability to gases. If air is exsolved, use more steps in the infiltration series. Although it is tempting to heat the clearing agent at the latter stages of the infiltration in order to reduce its viscosity, doing so risks denaturing callose and obliterating its aniline-blue response.
4. Mount the specimen on a slide, apply a cover slip, and seal it with rubber cement or viscous silicone oil. The latter is messy but will probably provide a more permanent seal. Unsealed slides eventually would take up water from the air.
5. Alternative clearing agents for this recipe can be found in Table 3.1. Dimethyl sulfoxide dissolves many salts, but it denatures proteins over time and cannot reliably give sharp nuclei. One can add tris-hydroxyaminomethane (tris base; 2.5 mg/ml) to these media to raise the pH for attempting the aniline blue reaction for callose. However, this reaction is known to fail in DMSO, because the callose is denatured and partially dissolves. Likewise, the iodine test for starch fails for media with more than 50% DMSO; the helical structure of the starch is presumably disrupted upon solvation with DMSO.

Rapid clearing of leaf tissue (Crane and Y. Ma, unpublished data)

1. Cut the specimen into ca. 1 cm squares. **IN A WORKING FUME HOOD,** immerse a few of these in 50 ml of a 2:1 (v:v) mixture of DMSO and chloroform and heat on a hot plate until it begins to boil. Move the beaker on and off the hot plate to keep the solution just below boiling (ca. 70°C) until the solution has become green. Change to fresh 2:1 DMSO: chloroform and continue. Repeat until leaf pigments are no longer extracted into the liquid. Use tongs to handle the beaker, and wear impermeable gloves. **THIS FIXATIVE IS CARCINOGENIC!!!**
2. Infiltrate the squares with 3:1 or 7:3 (v:v) benzyl benzoate:dibutyl phthalate. The optimum mixture depends on the thickness of the specimen and its secondary cell walls. Use at least 2 ml of fluid per square. One possible schedule is 30 min each in 3:1, 2:2, 1:3 mixtures of the fixative with the clearing agent. Finally change to the pure clearing agent, wait 30 min, and change again to the pure clearing solution. This will reduce the amount of chloroform liberated around the user when the slide is mounted.
3. Mount the cleared square in the clearing medium on a slide, apply a cover slip, and examine it with phase-contrast or differential interference contrast optics. Keep track of the abaxial and adaxial surfaces of the specimen.

4. The method works also for meristems, floral primordia, and other general subjects where one wants to see only the cell walls. Oxalate crystals and opaline silica survive the procedure intact. If the epidermis separates from the mesophyll, use less heat next time during fixation.

Acknowledgments

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Chapter 4

Ultrastructural Analysis of Apomictic Development

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Introduction

Since the first applications of electron microscopy to the observation of plant tissues, numerous studies have focused on the ultrastructural characteristics of sexual reproduction in flowering plants (Jensen 1965; Jensen and Fischer 1968). In many species, a wealth of information has been presented on the morphology of the female gametophyte (megagametophyte or embryo sac) before and after pollen tube arrival (Russell 1985; Mogensen 1988; Russell 1992; Huang and Russell 1992). Valuable contributions to the understanding of double fertilization have also emerged from studies that combine light and electron microscopy with technological advances on the in vitro isolation and fusion of gametes (Kranz et al. 1991; Faure et al. 1994). Nevertheless, the fundamental mysteries involving cell to cell signals and interactions during megagametogenesis and fertilization in sexual and apomictic species are still unsolved. These enigmas include the nature of the movement of sperm cells within the egg apparatus, the specific recognition of male and female gametes, the molecular nature of plasma membrane adherence, and the association, fusion, and activation of parental nuclei (Gerassimova-Navashina 1957; Russell 1992).

Despite the increasing scientific interest in apomixis (Asker and Jerling 1992; Vielle-Calzada et al. 1996a), few studies have characterized the fine structure of megagametophytes involved in apomictic reproduction. Cytological studies based on light microscopy have identified the fundamental differences existing between the general organization of sexual and apomictic female gametophyes in different genera (Bashaw and Holt 1958; Voigt and Bashaw 1972; Philipson 1978; Campbell et al. 1987; Crane and Carman 1987; Burson et al. 1990). The fine structure of nucellar and integumentary embryony has been investigated in some detail for a limited number of species (Naumova and Willemse 1982; Wilms et al. 1983; Naumova 1993), and ultrastructural studies of diplospory have been initiated in only a few apomicitic species of *Poa* (Naumova et al. 1999). In addition, a few ultrastructural characterizations of megagametophyte development and fertilization have been conducted in some aposporous members of the *Poaceae* (Chapman and Busri 1994; Naumova and Willemse 1995; Vielle et al. 1995).

The occurrence of apomixis in species that have conserved the ability to reproduce sexually provides unique opportunities for comparative ultrastructural studies. The formation of sexual and apomictic megagametophytes can simultaneously occur in the same genotype or in different genotypes of the same population. Ultrastructural comparisons of megagametogenesis and early fertilization events in sexual and apomictic

ovules of the same species can be used to obtain mechanistic information on specific cellular differences that distinguish these developmental processes. Although the number of species in which ultrastructural studies have been conducted remains limited, recent electron microscopy studies in some aposporous grasses have provided new information on megasporogenesis, aposporous initiation, the organization of the differentiated megagametophyte, and the autonomous division of unreduced egg cells.

Nucellar and Integumentary Embryony

During integumentary or nucellar embryony (also called adventive embryony), asexual embryos are formed from inner integumentary or nucellar cells that are differentiating in tissues external to the meiotically derived megagametophyte. Nucellar or integumentary cells that give rise to adventive embryos are called embryocytes. A comprehensive review of adventitive embryony provides significant ultrastructural information about this process (Naumova 1993) by summarizing light and electron microscopy observations and reviewing embryological information on representatives of more than 250 species of flowering plants.

The first morphological evidence of embryocyte differentiation is usually observed after the initiation of megagametogenesis. As the nucellar cells divide, they invade the central cell of the sexually functional embryo sac. The formation of viable seed from nucellar embryos usually requires fertilization of the polar nuclei and subsequent development of the endosperm; however, autonomous endosperm formation sporadically occurs in some species. Nucellar embryos are usually initiated independently of pollination. Polyembryony, the formation of several embryos in a single ovule, is characteristic of adventive embryony. Several ultrastructural investigations have improved our understanding of embryocyte differentiation and early adventive embryogenesis (Naumova 1978; Naumova and Willemse 1982; Wilms et al. 1983).

Embryocytes are generally characterized by a dense cytoplasm, an irregularly shaped nucleus, and a larger volume than most of the nucellar cells present in the ovule. The abundance of polysomes, free ribosomes, mitochondria, and plastids suggests high physiological activity in embryocytes. Their cell walls are significantly thickened and lacking plasmodesmata. In the genus *Sarcococca*, the number of plasmodesmata gradually decreases during embryocyte differentiation (Naumova and Willemse 1982). Using light microscopy, Koltunow et al (1995) found that the nucellar initials form a thick cell wall in *Citrus sinensis*. They also found a localized degeneration of the nucellar tissue at the chalazal pole, a region where nucellar adventive initials are confined. In *Sarcoccoca*, the general organization of the embryocyte cytoplasm is modified during consecutive stages of mitosis, undergoing events similar to those characterizing the dedifferentiation of pre-meiotic megaspore mother cells (MMC) in sexual species (Dickinson and Potter 1978). In *Euonymus macroptera*, integumentary embryo formation occurs in tenuinucellate ovules. Asexual embryos originate from two or three cell layers enveloping the micropylar region of the sexually functional megagametophyte (Figure 4.1a,b,c; Naumova 1990). Integumentary embryos coexisting in a single ovule usually develop asynchronously (Figure 4.1d,e). Plasmodesmata are not present in the embryocyte cell wall or in the integumentary wall that is in direct contact with the central cell (Figure 4.1f,g). The transversal cell wall that separates sister integumentary cells varies in thickness (Figure 4.1h,j).

The ultrastructure of both cells contained in a two-cellular adventive embryo is extremely similar: their external cell wall remains thick and lacks plasmodesmata. Young adventive embryos also lack a distinct polarity, and no regular cell divisions can be discerned; a suspensor is frequently missing. Nucellar embryos do not arise synchronously, and their early development can be severely delayed. Whereas some embryos can appear to be composed of only a few cells, others in the same ovule may already be undergoing organogenesis. Adventive embryos are able to penetrate into the adjacent central cell of a sexually derived megagametophyte.

Diplospory

In diplosporous species, the megagametophyte is formed from an aberrant meiotic cycle that prevents reduction and recombination. An ultrastructural charac-terization of diplospory has only been initiated in two apomicts: *Poa palustris* and *Poa nemoralis* (Naumova et al. 1999). In these two species, diplospory is characterized by a complete omission of meiosis; the unreduced megasporocyte develops into an embryo sac after gradual vacuolation and three consecutive mitotic divisions. In the young developing ovule, the archesporial cell progressively differentiates into a diplosporous precursor or diplosporous-embryo sac megaspore mother cell (DMC). Subsequently, after three mitotic divisions, the DMC develops into an 8-nucleate embryo sac of the Antennaria-type.

In contrast to nucellar cells present within the developing ovule, the archesporial cell of both species is characterized by a large nucleus with decreased chromatin contraction in regions having close contact with the surface of the nucleolus. Numerous poorly differentiated mitochondria and plastids are uniformly distributed in the enlarged cell. Interestingly, isolated enclaves of cytoplasm containing multiple membranes are often observed, whereas the endoplasmic reticulum (ER) is poorly developed. Compared to the DMC, vacuoles are also abundant but small and located mainly at the micropylar and chalazal poles of the cell.

Significant cellular elongation marks the transition from archesporial cell to DMC. Whereas the archespore is 160 µm on average, the DMC is about 350–380 µm long, with little or no changes in width. This increase in size is associated with a reduction in chromatin condensation and nuclear volume. In the DMC, the nucleus is positioned at the micropylar end of the cell and becomes elongated and irregular in shape. There is a substantial increase in the population of ribosomes, polysomes, dictyosomes. At this stage, the vacuoles fuse into two large ones located at the micropylar and chalazal poles, and there is an obvious increase in the thickness of the cell wall. The transition from DMC to a noncellularized embryo sac is also characterized by a gradual increase in cell length to about 500 µm. The nucleus becomes irregular and lobbed, with numerous protuberances in the nuclear envelope. Several nucleoli differing in size and shape can be simultaneously observed. There is also a marked increase in the population of mitochondria and plastids that often form clusters heterogeneously distributed throughout the cytoplasm. Contacts between the external membrane of the nuclear envelope and granular ER cisternae are often observed. Additionally, series of concentric membranous structures were also observed in the cytoplasm. The cell wall became slightly thick during the change of the DMC into a one nucleate embryo sac. Nucellar cells adjacent to the developing diplosporous embryo sac showed signs of degeneration, with an electron-opaque cytoplasm and highly condensed nuclei.

Apospory

Apospory is a form of apomixis in which sporophytic cells in the ovule give rise to unreduced female gametophytes (Gustafsson 1947). The autonomous division of aposporous egg cells generates viable embryos without fertilization; however, the majority of aposporous species are pseudogamous and require fertilization of the polar nuclei for endosperm development (Nogler 1984).

An ultrastructural characterization of aposporous megagametophytes has been conducted in *Panicum* and *Pennisetum*. In these two grass genera, the mechanism of aposporous female gametophyte formation is very similar. Early during megasporogenesis, the orientation of the ovule changes within the ovary, and the integuments progressively enclose the nucellus. The ovule becomes anatropous as it rotates toward the base of the pistil, leaving the micropyle facing away from the style. In *Panicum maximum* and *Pennisetum ciliare* (syn = *Cenchrus ciliaris* L.), sexual megagametophyte development is monosporic; a single meiotically derived megaspore gives rise to the embryo sac. The functional megaspore enlarges, and its nucleus divides mitotically three times to form the megagametophyte. Both species develop a sexual megagametophyte of the *Polygonum* type: two synergids, the egg cell, a binucleate central cell whose nuclei fuse prior to fertilization, and three antipodals that proliferate to give a cluster of cells at the chalazal pole. After fertilization of both the egg cell and the central cell, the ovule develops into a seed.

Whereas sexual ovules only develop a meiotically derived megagametophyte, in aposporous plants one or several nucellar cells acquire a reproductive fate and are able to pursue growth and differentiation while megasporogenesis proceeds. The spatial and temporal patterns of megaspore mother cell (MMC) differentiation and meiosis are the same during sexual and aposporous development. In obligately apomictic genotypes, all meiotically derived megaspores die and reduced megagametophytes are not formed. Active nucellar cells undergo two mitotic divisions. After cellularization, 4-nucleated female gametophytes differentiate into an egg apparatus and a central cell containing usually one or two polar nuclei. In both species, the egg cell is able to divide parthenogenetically and give rise to viable embryos. Fertilization of the polar nucleus (or nuclei) is necessary for endosperm formation and seed set. In facultative plants, both developmental processes are viable; sexual and aposporous female gametophytes can coexist within the same ovule, or in different ovules of the same inflorescence.

Differentiation of Aposporous Initials

Aposporous development requires the differentiation of nucellar cells into unreduced organized female gametophytes. In most aposporous species, the differentiation of the MMC appears to take place in a subepidermal layer of the nucellus, and follows morphological characteristics that have been previously described in many sexually reproducing angiosperms (Huang and Russell 1992). In *Panicum maximum*, the MMC is characterized by a centrally located nucleus containing a conspicuous nucleolus and dense cytoplasm. In contrast, the nucellar cells surrounding the MMC have thinner cell walls and numerous plasmodesmata that share ultrastructural similarities with young meristematic cells. In aposporous genotypes, meiotically derived dyads and linear tetrads often degenerate during megasporogenesis. Aposporous initials usually differentiate adjacent to degenerating megaspores or one-nucleated sexual embryo sacs. Chalazally-located nucellar cells assume vacuolized spherical shapes and increase in volume

(Figure 4.2a,b,c). Their cytoplasm contains numerous plastids and mitochondria, and plasmodemata are scarce or appear to be filled with a cellulose-like matrix. The degeneration of meiotically derived megaspores and early stages of aposporous initiation has recently been investigated in *Brachiaria brizantha*. In this grass, aposporous initials appear to contain dedifferentiated organelles reminiscent of organellar populations found in pre-meiotic MMCs (A.C. Guerra de Araujo and V. Carneiro, personal communication).

Aposporous Megagametogenesis

Several aposporous embryo sacs can develop in a single ovule (Figure 4.2d). Aposporous female gametophytes show a different orientation with respect to the micropylar-chalazal axis, and usually differentiate heterochronically with respect to each other. The transition from aposporous initial to one-nucleated embryo sac is characterized by an increase in cell size (Figure 4.3a). The first signs of cellular polarity are the consequence of vesicular fusion; two large vacuoles are formed at opposite sides of a centrally located nucleus. Cell wall thickness also increases, and no plasmodesmata can be discerned (Figure 4.2d,e). In *Pennisetum ciliare*, the nucleus migrates to the periphery of the cell before dividing (Figure 4.3b). The first mitotic division is perpendicular to the long axis of the cell and usually close to the micropylar region of the one-nucleated embryo sac (Figure 4.3c). The second mitotic division is synchronous in both sister nuclei, giving rise to a four-nucleated type of embryo sac that lacks antipodals at the chalazal pole (Figure 4.3c,d).

The Cellularized Aposporous Megagametophyte

The second mitotic division is followed by the cellularization of individual nuclei. Little is known about the processes that regulate cellularization and differentiation of aposporous megagametophytes. In *Pennisetum ciliare*, as in most reported aposporous species, female gametophytes usually contain two synergids, an egg cell, and a single polar nucleus in the central cell; however, a variable number of female gametophytes (up to 20% in certain genotypes) may be composed of a single synergid, an egg cell, and two polar nuclei. On rare occasions, embryo sacs containing three polar nuclei and no synergids have been observed. This variable organization appears to be associated with the localization of nuclei prior to cellularization, and suggests that positional information plays a role in gametophytic cell specification.

In all aposporous grasses ultrastructurally examined to date, the egg apparatus differentiates with morphological characteristics similar to those found in sexually functional synergids and egg cells. These cells are attached at the micropylar apex, but the triangular organization of the egg apparatus is not necessarily conserved, as the unreduced egg cell may appear in a more lateral position with respect to one of the synergids. Except for the presence of the filiform apparatus, few differences are found in the ultrastructural constitution of the synergids and the egg cell in unpollinated pistils of *Panicum maximum*. The three cells appear vacuolated, with a centrally located nucleus, and organelles preferentially located in the micropylar pole (Figure 4.2f,g,h). Naumova and Willemse (1995) characterized the ultrastructure of aposporous embryo sacs, but only observed events prior to pollination. In unpollinated pistils, the egg cell and synergids were similar to the egg apparatus of an embryo sac of the Polygonum-type typically found in the grasses. The egg cell wall showed variable thickness, and the chalazal plasma membrane surface of the egg, central cell, and synergids were in direct contact (Figure 4.2f,g,h). Chapman and Busri (1994) described the ultrastructure of mature

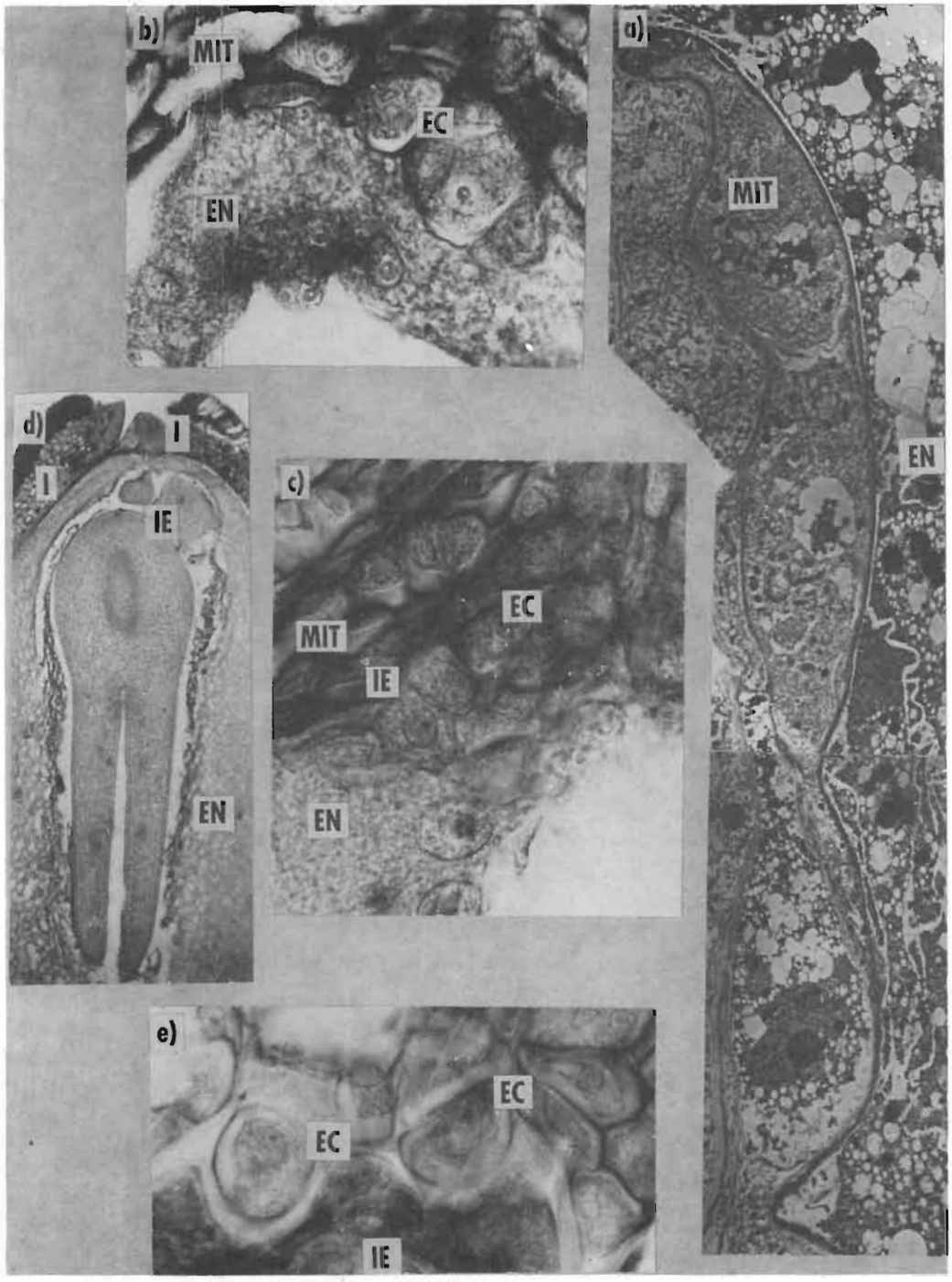

Figure 4.1 Integumentary embryony in *Euonymus macroptera*.
(a) Junction between meristematic integumentary tissue and endosperm, x 5.000; (b) and (c) Micropylar region of ovules containing meristematic integumentary tissue embryocytes and endosperm (b), x 570, and integumentary embryo (c), x 850; (d) Seed with three integumentary embryos at different developmental stages, x 130; (e) Initial cells of integumentary embryo at micropylar region, x 850; (f) Integumentary cell adjacent to the endosperm, x 8.500; (g) Cell wall junction between an integumentary cell and endosperm, x 42.500; (h) to (j) Cell walls tangential to the meristematic integumentary cells; (h), x 34.000; (i), x 10.200; (j), x 42.500.
Abbreviations on following page.

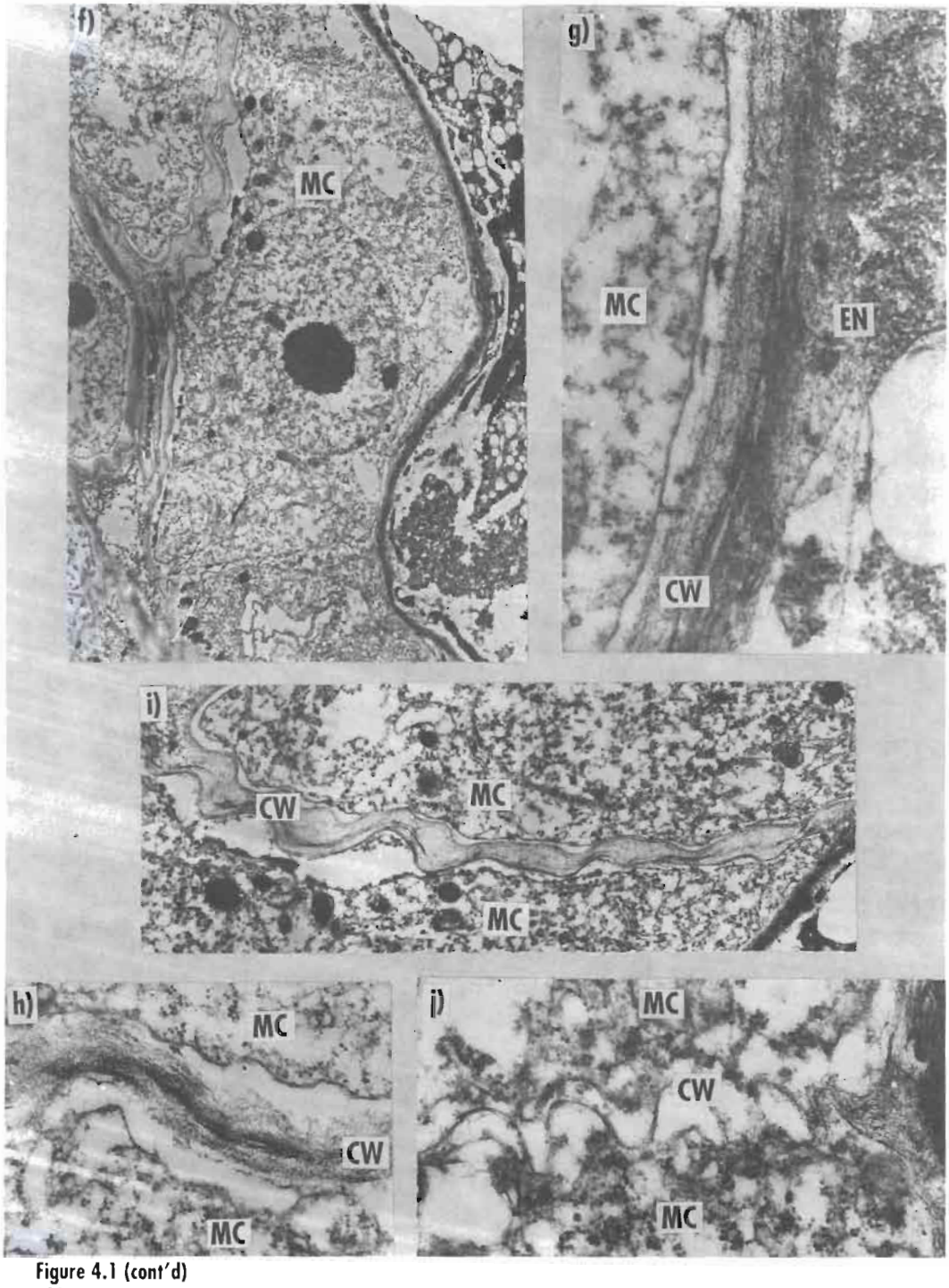

Figure 4.1 (cont'd)
Abbreviations

AE - aposporous embryo sac; CC - central cell; E - egg cell; EC - initial cell of nucellar or integumentary embryo = embryocyte; ES - embryo sac; I - inner integument; IA - initial cell of aposporous embryo sac; IE - integumentary embryo; M - micropyle; MC - meristematic cell; MIT - meristematic inner integument tissue; EN - endosperm; PN - polar nuclei; S - synergid; T - tetrade of megaspores; Z - zygote; ZE - zygotic embryo; SE - synergid embryo; FA - filiform apparatus; CW - cell wall; C - cuticle; M - mitochondria; N - nucleus; P - plastid; PD - plasmodesmata; V - vacuole.

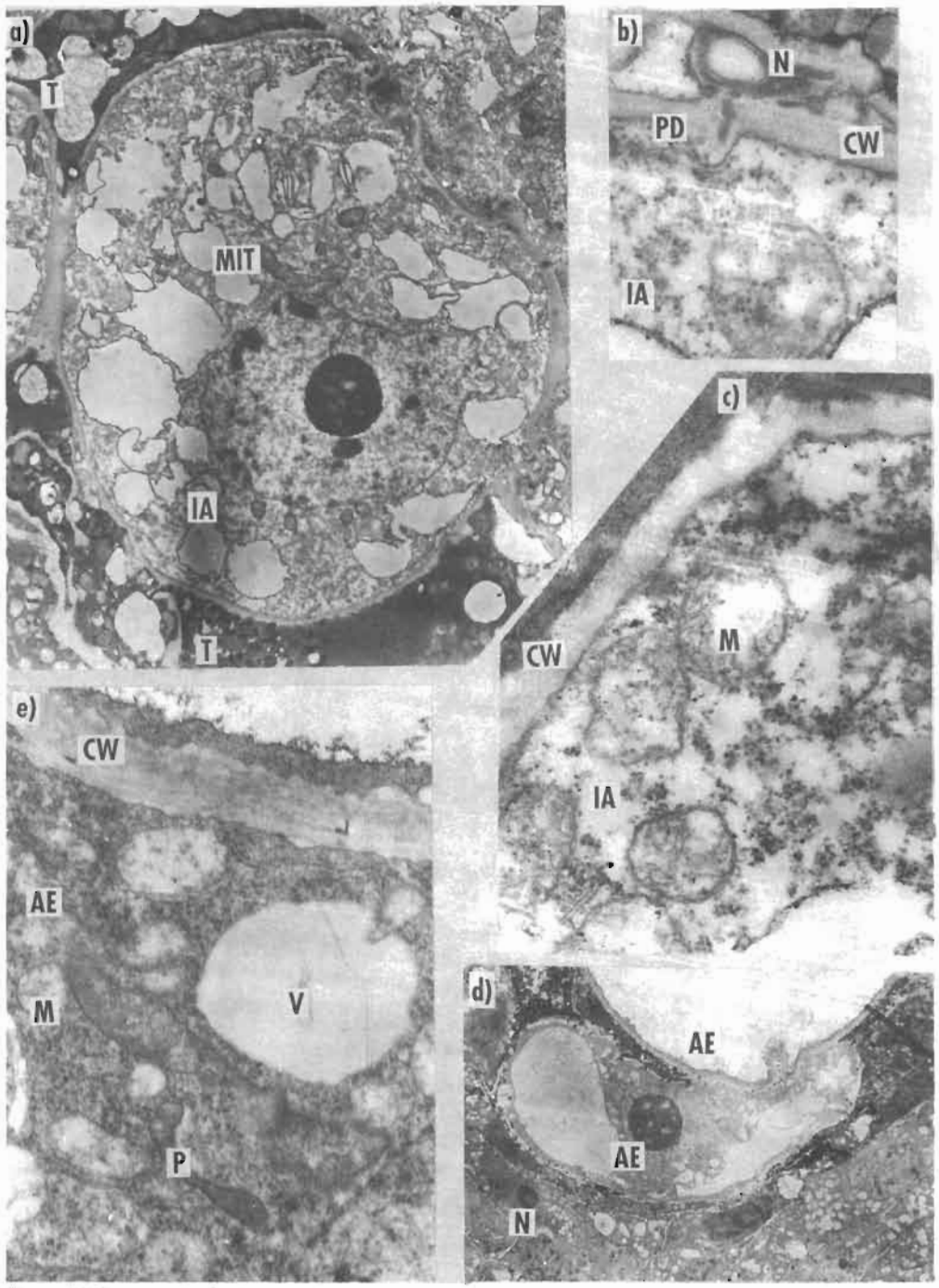

Figure 4.2 Apospory in *Panicum maximum*.
(a) Initial cell of aposporous embryo sac close to the degenerating tetrad, x 6.800; (b) and (c) Cell wall of an aposporous initial, remnants of plasmodesmata are observed in (b), x 5.000; (c), x 42.500; (d) Two aposporous embryo sacs in close contact to each other: one of them is of the Panicum-type, with no antipodals at the chalaza, a second one is at the one-nucleated stage, x 1.700; (e) One-nucleated aposporous embryo sac with no plasmodesmata, x 20.400; (f) Egg cell and synergid of aposporous embryo sac, x 4.250; (g) Plasma membranes between egg cell and central cell of an aposporous embryo sac, x 34.000; (h) Plasmodesmata in the cell wall separating the aposporous egg cell and a synergid, x 34.000; (i) Cell wall in basal part of an aposporous synergid, x 34.000.

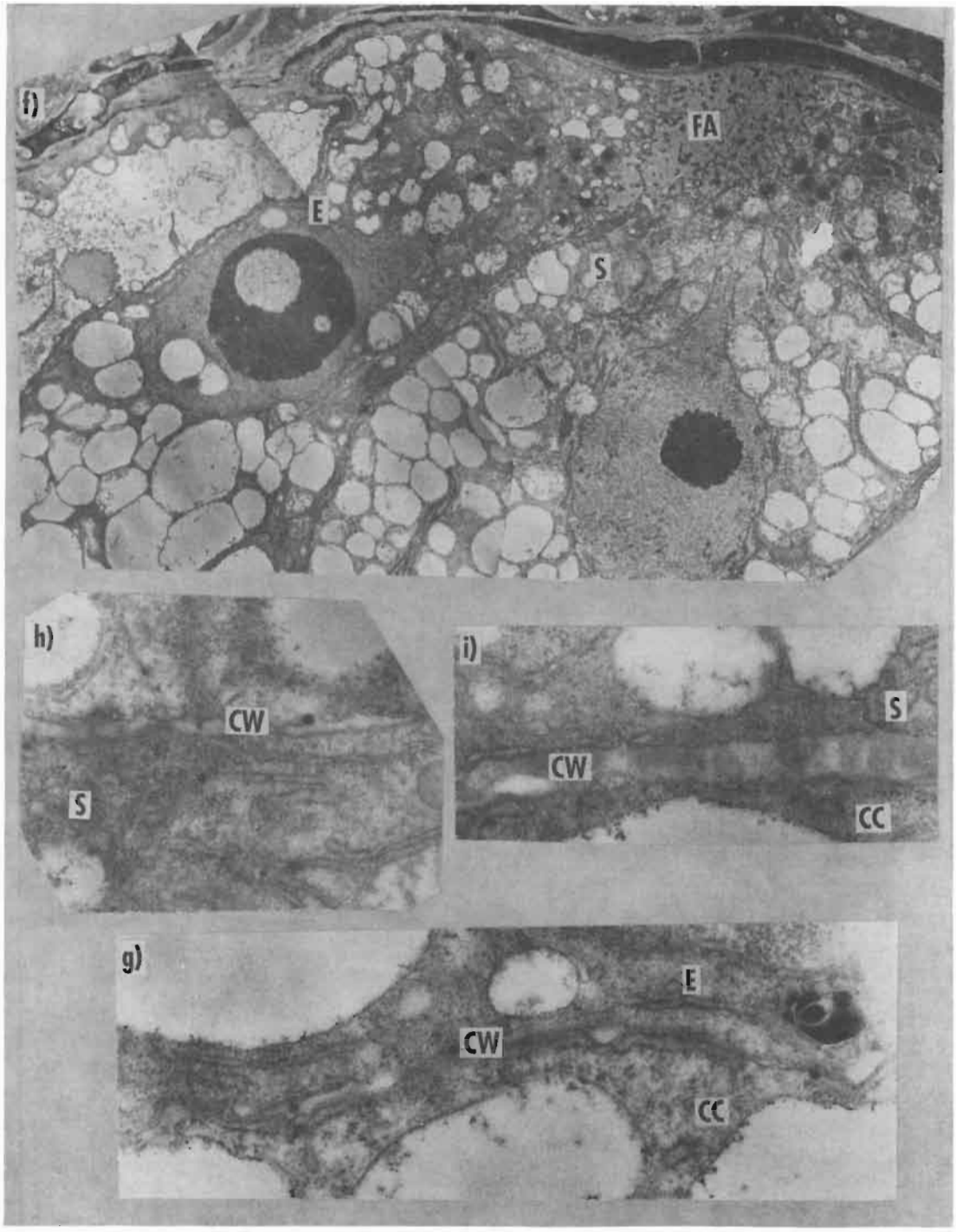

Figure 4.2 (cont'd)

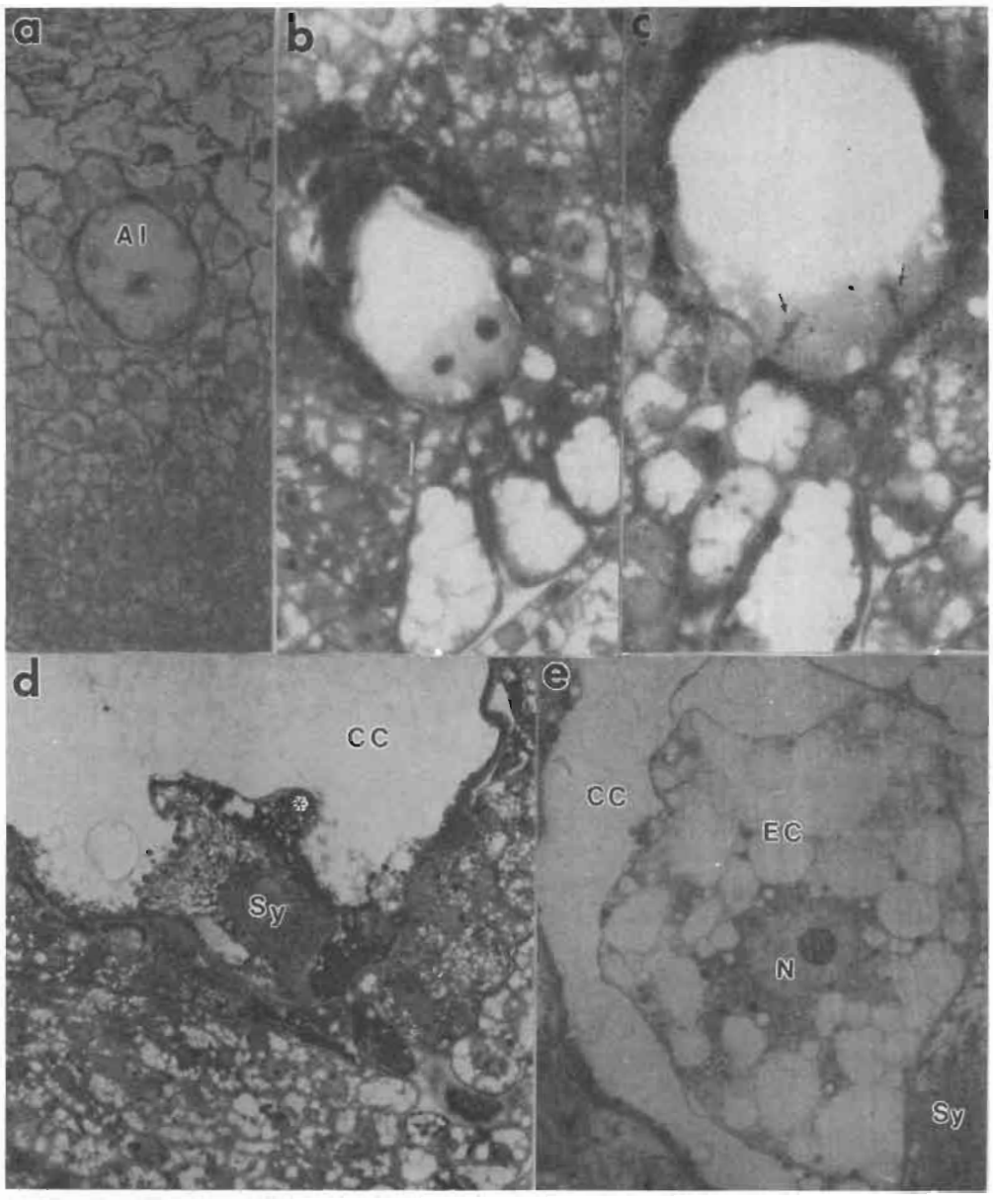

Figure 4.3 Aposporous megagametophyte development in *Pennistum ciliare*.
(a) A single aposporous initial (AI) differentiating in the nucellus (Nuc); (b) Two-nucleated aposporous embryo sac — in contrast to sexual megagametogenesis, both nuclei are located at the micropylar pale; (c) Twa-nucleated aposporous embryo sac undergoing synchronous mitosis. Arrows show chromosomes in late metaphase; (d) Precociaus degeneration of ane synergid in an aposporous embryo sac prior to pollinatian; (e) The aposporous egg cell in an aposporous embryo sac prior to pollination.

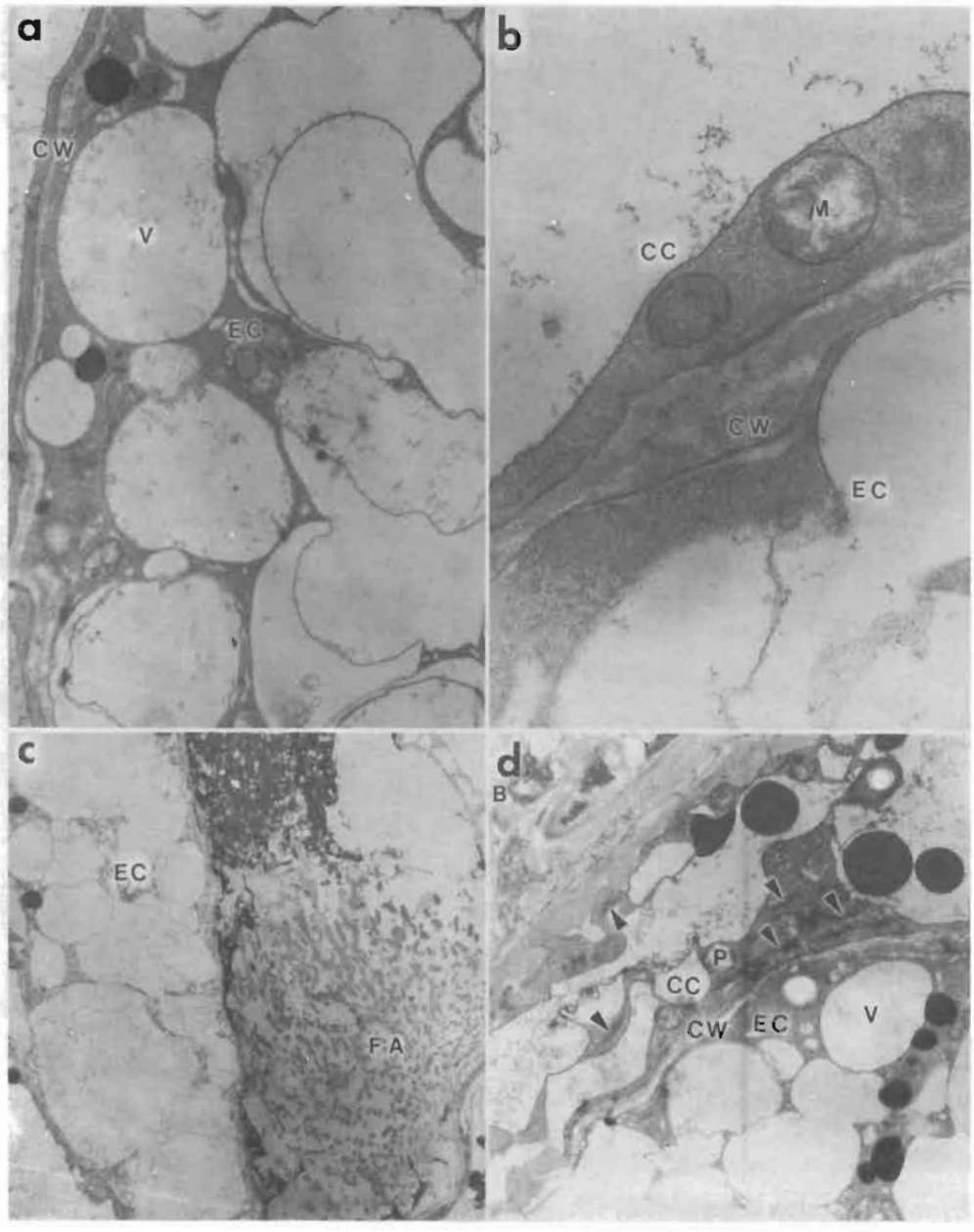

Figure 4.4 Organization of the mature aposporous egg apparatus in *Pennisetum ciliare*.
(a) The egg cell three hours after pollination. The chalazal end is completely covered by a cell wall (CW),, vacuole (V), egg cell (EC); (b) Detail of the cell wall (CW) separating the chalazal region of the egg cell (EC) from the central cell (CC) cytoplasm; M, mitochondria; (c) Micropylar region of the aposporous egg apparatus, filiform apparatus (FA), egg cell (EC); (d) Numerous Golgi (arrowheads) are present in the apical pocket, a region located between the central cell (CC) wall and the egg apparatus, egg cell (EC), plastid (P), vacuole (V), and cell wall (CW).

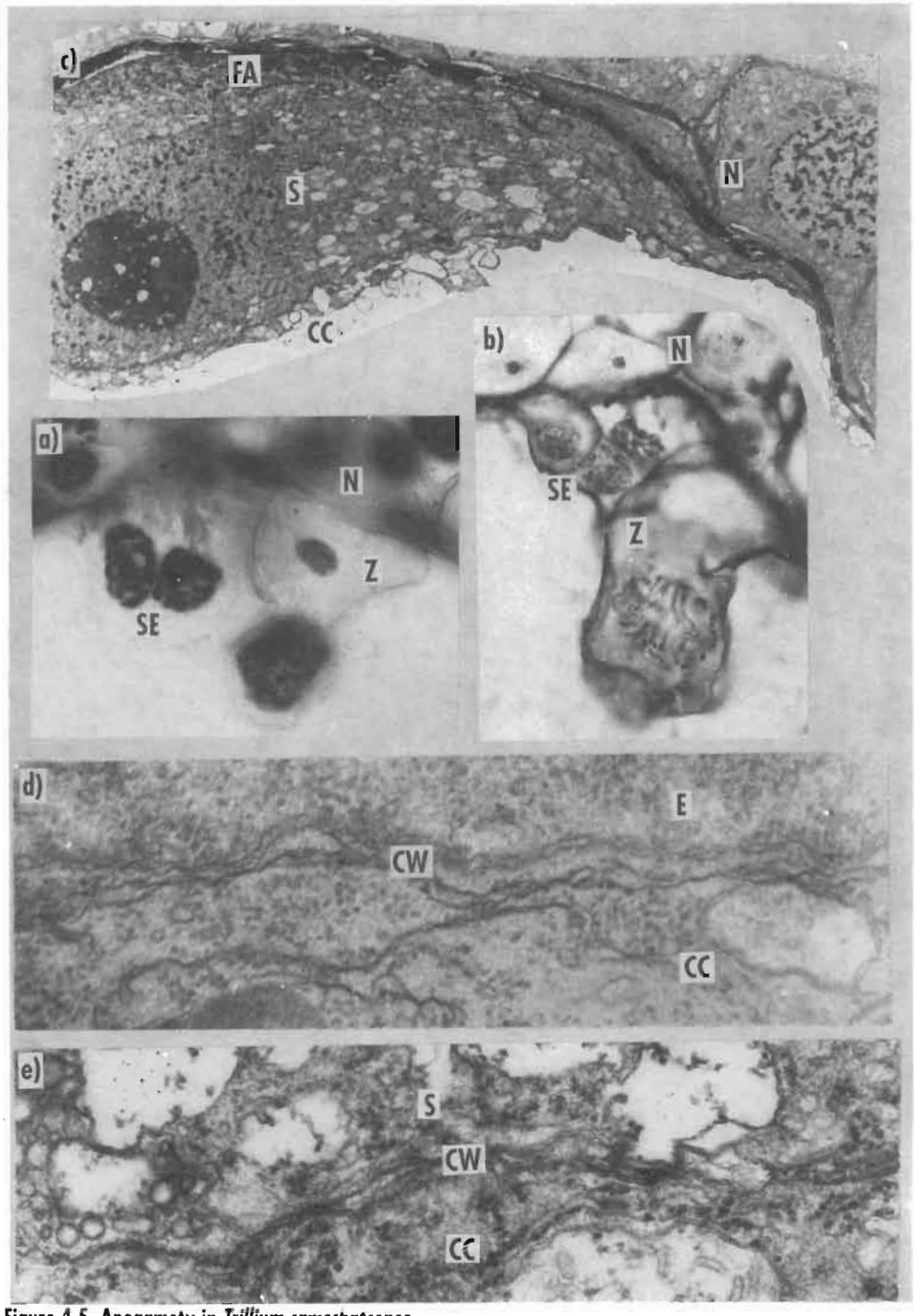

Figure 4.5. Apogamety in *Trillium camschatcense*.
(a) A 2-celled embryo derived from a synergid adjacent to a zygotic embryo, x 850; (b) Embryo sac with 3-celled synergid embryo and a zygotic embryo, x 850; (c) Fine structure of a synergid, x 6.000; (d) Cell wall separating the egg cell from the central cell, x 72.000; (e) cell walls between synergid and central cell of the mature embryo sac, x 68.000; (f) zygotic embryo, terminal port, x 1.600; (g) outer cell wall of zygotic embryo, x 24.000; (h) 2-cellular synergid embryo, x 1.500; (i) cell wall and cytoplasm of the synergid embryo, x 12.00.

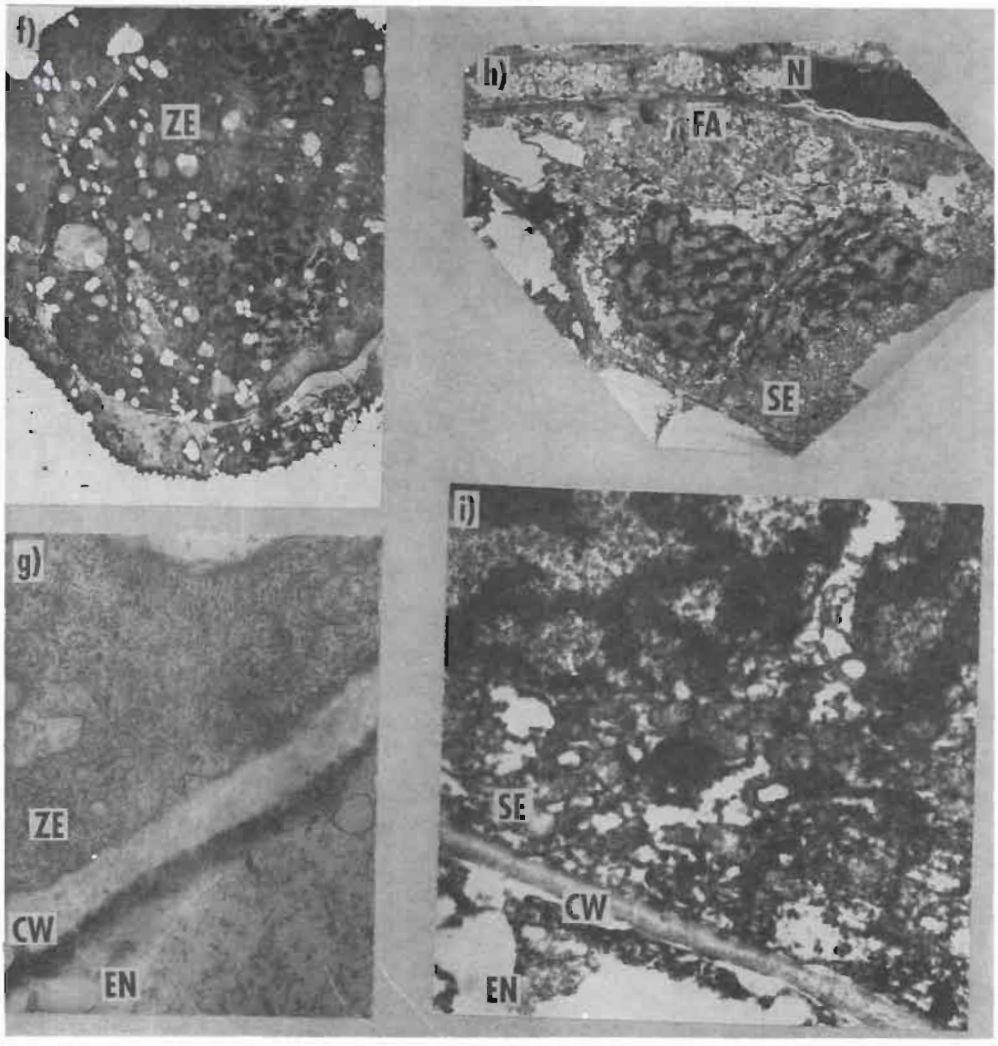

Figure 4.5 (cont'd)

aposporous embryo sacs in the progeny of a *Pennisetum glaucum* (sexual) x *Pennisetum squamulatum* (obligate apomict) interspecific cross. In facultative apomictic genotypes, they compared sexual and aposporous embryo sacs in the same ovule. Particular attention was given to the distribution of internal cell wall ingrowths (transfer walls) in the central cell. Many wall projections were observed in the micropylar region, but few ingrowths were found in the chalazal region of both sexual and apomictic megagametophytes. Plasmodesmata were present in the cell wall that separates the central cell from the antipodals, but appeared to be absent at the chalazal pole of aposporous embryo sacs.

Parthenogenesis and Fertilization

In *Pennisetum ciliare*, most genotypes reproduce by obligate apospory. Breeding efforts are based on the identification of rare genotypes that have completely lost the ability to differentiate aposporous megagametophytes and only form sexually functional, reduced (haploid) embryo sacs of the Polygonum-type. The comparison of the egg apparatus in sexual and aposporous megagametophytes of *P. ciliare* offers an opportunity to analyze the cellular dynamics of fertilization in apomictic plants. Such a comparison is particularly valuable if conducted where independent genotypes of apomictic and sexual germplasm are available within a population segregating for method of reproduction.

In P. *ciliare*, fertilization occurs 3–4 hours after pollination. The examination of the egg apparatus of buffelgrass at several time intervals after pollination has provided some information on the structural and functional features that distinguish sexual and apomictic development (Vielle et al. 1995). Compared to the synergids of the sexually derived egg apparatus, the degenerative process in one or both aposporous synergids appears to be accelerated. Prior to pollination, the cytoplasm of both cells contains small vacuoles, and few organelles can be identified. In general, the cells are similar in appearance to the sexual synergids during the first two hours following pollination, but before pollen tube arrival.

During the first two hours following pollination, the cytoplasm of one of the synergids becomes electron-dense, the plasma membrane appears disrupted, organelles cannot be identified, and the nucleus is irregularly shaped and pressed to the plasma membrane. Increased amounts of vesicular traffic are observed. Later, the cytoplasm of the second synergid also shows signs of degeneration. Two to three hours after pollination, the degenerated synergid has entirely collapsed and its remnants appear as an electron-dense fringe closely associated with the egg cell. The time of initiation of the degenerative process is variable in both sexual and aposporous embryo sacs, and in some cases, highly degenerated synergids are present in unpollinated ovules during or just after embryo-sac cellularization.

Significant differences are also observed between the aposporous and the sexual egg cell. After cellularization but before pollination, the aposporous egg cell is characterized by a conspicuous centrally located nucleus that contains a single nucleolus (Figure 4.3e). The nucleus of the sexually derived egg cell is generally centrally located, but has a smaller nucleolus. The chalazal vacuole present in the sexually derived egg cell is replaced by several smaller vacuoles that restrict the cytoplasm to a region located around the nucleus. In contrast to the sexual egg cell, the cytoplasm contains abundant mitochondria and polysomes, but few Golgi bodies and endoplasmic reticulum (ER) can be observed. At the micropylar region, both sexual and aposporous eggs share common cell walls with both synergids, but these walls disappear in the middle portions

of the cell. The plasma membranes of the egg and synergids are in direct contact at the chalazal pole.

Three to four hours after pollination, striking changes are detected in the ultrastructure of the aposporous egg cell. A cell wall without a middle lamella has covered the chalazal region of the egg plasma membrane, separating the cell from the degenerated cytoplasm of a collapsed synergid (Figure 4.4a,b). No cell wall covers the chalazal end of the sexual egg cell, even after pollen tube discharge. The aposporous egg cell cytoplasm appears vacuolated and contains numerous undifferentiated plastids preferentially organized in clusters at the periphery of the nuclear membrane. A thin layer of central cell cytoplasm is associated with the external surface of this *de novo* formed cell wall (Figure 4.4c,d). The central cell cytoplasm contains a large number of Golgi cisternae and mitochondria, particularly in the so-called apical pocket, a region of the central cell formed by the proximity of the egg apparatus to the central cell wall (Figure 4.4d). The unreduced polar nucleus usually contains more than one nucleolus. In some rare occasions, a multicellular embryo can be present before pollen tube arrival into the micropyle.

Apogamety

Apogamety designates the formation of embryos from a cell of the megagametophyte other than the egg. Even if this phenomenon has rarely been reported in sexual and apomictic species (Asker and Jerling 1992), it implies the autonomous activation of reproductive cells and can be considered a nonrecurrent form of apomixis.

The fine structure of synergids undergoing autonomous activation has been described in *Trillium camschatcense* Ker. Gawl., a species that is endemic to the extreme eastern region of the former USSR. Synergid embryos develop in more than 80% of the embryo sacs investigated in this species (Naumova 1978, 1990). Light microscopy studies have shown that the egg cell and the synergids undergo limited differentiation. They are characterized by a centrally located nucleus and no central vacuole. The three cells appear similar in size and shape, but the presence of a conspicuous filiform apparatus is characteristic of the synergids. Differences between their cytoplasmic constitution can only be discerned at the ultrastructural level. In contrast to the egg cell, synergids are rich in endoplasmic reticulum and Golgi cisterneae. Plastids, mitochondria, and polysomes are abundant in the egg cell. Before pollen tube arrival into one of the synergids, the three cells have an incomplete cell wall at their chalazal pole (Figure 4.5a,b).

Following fertilization of the egg and central cell, drastic ultrastructural changes occur in the persistent synergid. An increase in the nuclear and nucleolar size is followed by a complete reorganization of the organellar population, which becomes similar to the one present in the egg cell before sperm cell delivery. Mitochondria with numerous cristae and plastids dense in stroma are abundant. Whereas the sexually derived embryo is already surrounded by a thick cell wall devoid of plasmodesmata, the cell wall of the 2-cellular embryo derived from autonomous synergid activation thickens (Figure 4.5c,d) and becomes progressively isolated through the loss of all plasmodesmata. In summary, the ultrastructural transformations taking place in embryogenic synergids of *T. camschatcense* are comparable to the changes occurring in the egg cell during the gametophytic to sporophytic transition of sexual flowering plants.

Discussion

Comparisons between sexual and apomictic megagametophytes represent an ideal system to start dissecting the cellular and molecular differences that distinguish sexuality from apomixis. Sexual reproduction is strictly dependent on the production and fusion of haploid male and female gametes. In contrast, apomictic reproduction is dependent on the formation of unreduced female gametophytes, the autonomous activation of the egg cell, and the eventual fertilization of the polar nucleus (or nuclei). Regardless of the reproductive method, the megagametophyte forms entirely within the nucellar tissue and is dependent on the sporophyte for its development and function. Ultrastructural analysis of apomictic development has provided new, but limited, information on the developmental particularities of adventive embryony and gametophytic apomixis. So far, results have mainly described specific aspects of differentiated embryocytes and aposporous initials, and the fine structure of cellularized megagametophytic cells before and after parthenogenetic activation.

The similarity of ultrastructural characteristics shared by adventive embryocytes and aposporous initials deserves special attention. The presence of a thick cell wall appears to be prevalent around embryocytes and aposporous initials. An extensive survey of the gametophytic-sporophytic junction in land plants reveals that the two generations of the sexual angiosperm life cycle are almost invariably separated by thickened cell walls lacking plasmodesmata (Ligrone et al. 1993; Bell 1995). The presence of a conspicuous boundary surrounding the megagametophyte of some mosses and ferns of the early Devonian (Remy et al. 1993) suggests that gametophytic cell isolation may have a crucial function of fundamental importance for the evolution of the angiosperms. Some also suggest that a combination of hydrated polysaccharides and callose in the cell wall of meiotically derived megaspores may act as a molecular filter that impedes the transport of low molecular weight peptides and/or nucleic acids expressed in nucellar cells, since their presence may represent a threat to the initiation of gametophytic development (Knox and Heslop-Harrison 1970). In sexual plants, the presence of callose surrounding degenerating megaspores (but not viable ones) suggests that callose may be suppressing the nonfunctional megaspores by isolation. In lower plants (mosses and ferns), selective permeability of thick wall boundaries allows only the transport of minerals and simple sugars (Bell 1988, 1992); plasmodesmata appear to be completely absent. Callose deposition was detected in adventive embryocytes in young *Citrus* seeds (Wakana and Uemoto 1987). Carman et al. (1991) compared the distribution of callose in sexual and apomictic megaspores of *Elymus recticetus* and found that, contrary to normal meiosis, the cell wall of diplosporous MMCs invariably lacks callose. These results have been confirmed in several diplosporous grass genera (Leblanc et al. 1995; Peel et al. 1997; see also Leblanc and Mazzucato, Chap. 9). The drastic cytoplasmic transformations occurring in young embryocytes and aposporous initials may depend on a developmental program that is only initiated in the absence of informative molecules originating in the nucellus. A recent hypothesis postulated by Carman (1997) suggests that the role of isolation during megasporogenesis may depend on expression of duplicate genes, especially in polyploids in which the time and duration of specific cytological events could be the cause of anomalies during megasporogenesis. Needless to say, the role of cell wall thickening and callose deposition during megasporo-genesis, diplospory, aposporous initiation, or nucellar embryony remains an unsolved problem.

The examination of the apomictic megagametophyte in some aposporous grasses illustrates many ultrastructural characteristics that are conserved in the angiosperms. Ultrastructural comparisons to sexually functional megagametophytes do not always distinguish facultative apomictic genotypes (with sexual and aposporous embryo sacs in the same ovule or inflorescence) from obligate apomictic ones (aposporous embryo sacs exclusively present). In addition, the distinction between the ultrastructural observations of unpollinated or pollinated pistils has rarely been reported. In *Panicum maximum* (Naumova and Willemse 1995) and *Pennisetum* interspecific progeny (Chapman and Busri 1994) the general organization of the egg apparatus appears to be very similar to that of corresponding sexual megagametophytes.

The ultrastructural comparison of the sexual and aposporous egg apparatus in *Pennisetum ciliare* raises questions regarding the role of synergids and the nature of the signal that triggers egg cell parthenogenesis. The severe signs of synergid degeneration present in some unpollinated pistils indicate that a certain flexibility characterizes the timing and duration of megagametophytic development in sexual and apomictic ovules, and suggest that the fate of the degenerating synergid might be independent of pollen germination or pollen tube growth. The spatial association between the egg cell and the degenerating synergid is particularly close and suggests a possible involvement of the degenerative events in the activation or repression of the egg cell. In the sexually derived egg cell, the low frequency of endoplasmic reticulum, Golgi bodies, and polysomes, together with the small number of cristae present in the mitochondrial population, suggests that the cell is in a rather quiescent physiological state, presumably prolonged until pollen tube arrival into the micropylar vicinity. A quiescent egg cell prior to fertilization has also been described in several sexual species (Diboll 1968; Schultz and Jensen 1968). In contrast, the large amount of ribosomes and polysomes and large number of cristae in mitochondria suggest that the mature aposporous egg cell is in a highly active metabolic state even before pollination.

The changes that occur in the egg cell several hours after pollination are likely to be the result of an important reorganization of the cytoplasm that occurs during cell wall formation. The chalazal portion of the egg cell wall is synthesized *de novo* and does not depend on cell division or the formation of a cell plate. The completion of the egg cell wall lacking plasmodesmata is presumably the result of a previous activation process; however, a role for cell wall completion in the induction of egg cell division can not be discarded. Some researchers suggest that a cell wall might impede fusion of the sperm and egg plasma membranes (Savidan 1982; Asker and Jerling 1992). The ultrastructural observations in *Pennisetum ciliare* represent the first direct evidence demonstrating the presence of a complete egg cell wall before pollen tube arrival (Vielle et al. 1995). The abundance of Golgi bodies in the apical pocket suggests that the central cell may be responsible for the synthesis and transport of polysaccharides necessary for the formation of this unique portion of the egg cell wall. Whereas it is unlikely that a chalazally-located wall is the only factor preventing egg cell fertilization, it is worth mentioning that fertilization of apomictic egg cells occurs at variable frequencies in the grasses, and that precocious pollination increases the frequency of this phenomenon (Martinez et al. 1994). The presence of multicellular embryos in unpollinated pistils suggests that autonomous egg cell activation may also be independent

of pollination (Naumova et al. 1992; Naumova and Matzk 1998). In the case of apogametic development, synergid activation appears to depend on cytological modifications that mirror events occurring in fertilized egg cells.

Future Trends

The number of studies dealing with ultrastructural analyses of apomictic development is extremely limited. Even if some insights have been gained on the fine structure of adventive embryocytes and aposporous initials, the characterization of the mechanistic events that distinguish apomictic initiation from sexual megasporogenesis is far from complete. Additional descriptions of early megasporogenesis in members of dicotyledonous apomicts would be very valuable. Low-magnification electron microscopy could provide a better understanding of the sporophytic-gametophytic junction, and of the general distribution of aposporous initials differentiating in the nucellus. Whereas the deposition of callose has provided an excellent cytological marker to distinguish normal meiosis from diplosporous differentiation (Carman et al. 1991), a detailed ultrastructural analysis of diplospory and all its variants is urgently needed. A description of apomeiosis at the ultrastructural level could provide unique insights about the specifc frequency of chiasma formation for different species; this type of analysis has been extensively used to characterize mutants affecting female meiosis in maize (Golubovskaya 1979; Golubovskaya et al. 1992).

Electron microscopy investigations can now be combined with immunocytochemical approaches that take advantage of a wide range of monoclonal antibodies raised against specific components of the plant cell surface. The use of monoclonal antibodies represents a valuable alternative to the scarce cDNA probes and gene expression studies that relate to regulatory genes involved in megagametophyte development (Vielle-Calzada et al. 1996b; see also Chapter 12). Of particular interest are probes that identify specific glycoproteins involved in events such as positional sensing and cell determination (Knox 1992; Pennell 1992). The establishment of a reproductive lineage can be associated with changes in the distribution of glycoprotein epitopes present at the outer face of the plant cell plasma membrane (Pennell and Roberts 1990; Pennell et al. 1991). These and other discoveries (Knox et al. 1990) suggest that cell surface arabinogalactan proteins (AGPs) participate in the local control of reproductive transitions, from a sporophytic to a gametophytic development during gametogenesis, and from a gametophytic to a sporophytic condition after fertilization. In this regard, plasma membrane AGPs bear comparison with components of animal cell glycocalyx and suggest a functional similarity between plant and animal cell surfaces (Pennell 1992). These particular patterns of AGP distribution seem to be the consequence of a reproductive cellular commitment associated with gametophytic gene expression. The investigation of these proteins in apomictic ovules could provide valuable information on the mechanisms that regulate megagametophyte development in the angiosperms. In *Pennisetum ciliare* for example, AGP epitopes recognized by JIM13, a monoclonal antibody implicated in embryogenic cell specification (Pennell and Roberts 1990), are abundantly localized in the plasma membrane of sexual and aposporous synergids. This localization is conserved in aposporous megagametophytes aberrantly positioned with respect to the micropylar-chalazal axis, suggesting that the sporophytic tissue is unlikely to play a fundamental role on the specification of the egg apparatus (J-P Vielle-Calzada and K. VandenBosch, unpublished results).

The comparison of early fertilization events in *Pennisetum ciliare* has identified ultrastructural differences related to the control of egg cell parthenogenesis, an essential event in the apomictic life cycle that is absent from sexual reproduction; however, the dynamics of pollen tube arrival and sperm cell delivery have not been investigated. Further ultrastructural studies will be necessary to determine if sperm cell delivery and movement are equivalent in sexual and aposporous female gametophytes. The nature of the signal that activates the aposporous egg cell and the fate of the sperm cells after pollen tube delivery remain to be elucidated. Levels of calcium and other elements have never been measured in synergids of apomictic embryo sacs (Chaubal and Reger 1992). Additional studies should include three-dimensional reconstruction of egg cells during the first hours following pollination and quantitative information on the pattern of synergid degeneration and on the specific fate of sperm cells within the megagametophyte. Attempts to follow the movement of fluorochromatically-stained sperm cells in sexual embryo sacs have been limited, and the reliability of such studies using conventional clearing techniques is questionable; however, in vitro analysis of sperm nuclei movement within isolated embryo sacs has been accomplished (Faure et al. 1994). Isolation and fusion of gametes can now be used to compare the interaction of sperm cells with sexual or aposporous egg cells, in order to determine if cell to cell interactions impede the fusion of sperm nuclei with an apomictically-derived egg cell. Finally, some very enlightening findings may be obtained in the near future by following on the pioneering work of Olga Erdelská (Slovakian Academy of Sciences, Bratislava). Recent investigations in *Torenia fournieri* have shown that, at least in certain species in which the megagametophyte can be readily dissected or is partially uncovered by the integuments, some unique discoveries can be made by observing the process of double fertilization in vivo (Higashiyama et al. 2000). Microinjection and cell ablation technology may soon be combined with microcinematography to investigate sexual and apomictic megagametophyte development in vivo.

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Chapter 5

Genetic Analysis of Apomixis

Robert T. Sherwood

Introduction

Geneticists interested in analyzing the inheritance of apomixis face challenging problems. Apomixis overrides certain processes essential to the analysis of inheritance, i.e., it usurps meiotic megasporogenesis and megagametogenesis. Obligately apomictic plants cannot serve as maternal parents in hybridization. However, since apomixis normally does not prevent meiotic pollen formation, apomicts can be used as male parents in crosses with sexual or facultatively apomictic female parents. When facultative apomicts function as maternal parents, three types of progeny may be formed (see Berthaud, Chap.2), and each type must be distinguished when testing genetic models, i.e., normal B_{II} hybrids from fertilization of reduced embryo sacs, apomictic progeny from parthenogenetic embryogenesis of unreduced eggs, and B_{III} progeny from fertilization of unreduced eggs by reduced pollen yielding nonmaternal types at an increased ploidy level. Sexual reproduction is unknown in some apomictic species, accordingly, sexual plants must be found or created before genetic manipulation is possible. Sexual members of agamic complexes usually are at a different ploidy level than the apomicts. The polyploid, highly heterozygous nature of most apomictic plants complicates genetic analysis (Stebbins 1950; Nogler 1984a).

Early reports indicated that apomixis is heritable, but did not point to specific genes or genetic systems (Gustaffson 1946–47; Nogler 1984a). Successful hybridization was difficult, and methods for classifying progeny were tedious and unreliable. Discovery of the cytologically distinctive Panicum-type of apospory in the 1950s fostered the beginning of creditable inheritance studies. Pistil clearing techniques were introduced in the 1970s (see Crane, Chap. 3) that permitted the classification of large numbers of progeny. More recently, the application of molecular technology to characterizing, locating, and isolating apomixis sequences has augmented our understanding of the regulation of apomixis (see Grimanelli et al., Chap. 6). Presently, it appears that the expression of aposporous apomixis requires a dominantly acting master gene or linkage unit; roles have been indicated for dosage, additivity, recessive lethality, and modifying genes. The limited data available for diplosporous taxa indicate that diplospory also may be regulated by a dominant linkat with modifiers. Applied diligently, the methods suited to hybridization and classification of apomictic plants described below can lead eventually to a resolution of these difficult problems.

Methods

This section discusses methods for selecting parents, characterizing parents and progeny, and making crosses of apomictic species. Chapters 2, 6, 9, and 10 should also be consulted. Chromosomal constitution, reproductive behavior, and phenotype of both parents must be completely known.

Chromosome Number

Chromosome counts in progeny are essential when the parents are at different ploidy levels, whenever aneuploidy is suspected, and for detecting B_{III} hybrids. When facultative apomicts are used as the maternal parent, the nonmaternal progeny, or a sample thereof, should be examined for their chromosome numbers to determine whether they are B_{II} or B_{III} hybrids. Counts are made from anaphase figures of root tips using standard techniques (Sherwood et al. 1980; Dujardin and Hanna 1989; Hignight et al. 1991). Some $2n + 1$ aneuploid plants have a tendency to eliminate one chromosome in root tips, making detection of aneuploidy difficult (Nogler 1989).

Flow cytometry is useful in determining approximate ploidy levels (den Nijs 1990; Huff and Bara 1993; Naumova et al. 1993; Mazzucato et al. 1994; Leblanc et al. 1995a; Leblanc and Mazzucato, Chap.9). Ploidy level of individual reproductive nuclei can be determined with image cytometry of Feulgen stained sections (Sherwood 1995) or photocytometry of DAPI-stained isolated embryo sacs (Naumova et al. 1993). Cell cycle stages must be accounted for when applying flow cytometry or photocytometry.

Chromosome pairing and disjunction usually can be determined by examining microsporogenesis (Hignight et al. 1991; Burson 1992). Pollen viability is determined using I_2KI stain, fluorescein diacetate stain, or germination tests (Dujardin and Hanna 1989; Hill et al. 1989). Female fertility is estimated by determining percentage of seed set per 100 florets in open or self-pollinated inflorescences (Hignight et al. 1991).

Progeny Testing

Progeny testing originally was practiced as whole plant morphological comparison of progeny with the maternal parent. Broadly speaking, comparative analysis of any trait including cytological and molecular markers constitutes progeny testing. Identity of offspring with the maternal type in all respects indicates possible apomictic origin (Bashaw 1980). However, maternal appearance can also stem from matrocliny and from self-fertilization of highly homozygous parental material, as found in advanced generations of naturally inbred plants (Asker 1980).

Embryo-Sac Cytology

It is necessary to recognize all stages of normal meiotic megasporogenesis and megagametogenesis, as well as the stages of apomeiotic megasporogenesis and gametogenesis. Luckily, the number of different types found among species for which inheritance studies are feasible is small compared with the total range displayed by angiosperms. For about 90% of genera known to reproduce both sexually and apomictically, normal meiotic reproduction is based exclusively on formation of the Polygonum-type of embryo sac. About 10% of the genera commonly have bisporic or tetrasporic sexual embryo sac development (Carman 1997). Fortunately, most apomictic species exhibit only one type of apomictic sac from among the four types described below (Nogler 1984a).

The distinction between meiotic (= sexual) and apomeiotic (= apomictic) events becomes cytologically discernable after differentiation of the megaspore mother cell (MMC) in all ovules (see Leblanc and Mazzucato, Chap. 9). In the normal monosporic Polygonum-type meiosis, walls of megasporocytes and megaspores (tetrad cells) become invested with callose. It is a simple matter to visualize this cage-like indicator of meiotic activity using fluorescence microscopy of intact, aniline blue treated pistils (see Leblanc and Mazzucato, Chap. 9). The fully differentiated Polygonum-type sac is based on an 8-nucleate scheme, with an egg apparatus, polar nuclei, and antipodal cells.

In mitotic diplospory and restitutional diplospory, the MMC is diverted into functioning as an unreduced, apomictic embryo sac initial. Its size and shape may vary from those of reduced megasporocytes and provide fleeting evidence for diplospory that is tedious to acquire. Illustrations are given by Gustafsson (1946-1947), Hair (1956), Battaglia (1963), Rutishauser (1969), Nogler (1984a), and Crane and Carman (1987). A much more convenient test for diplospory is based on the absence of callose deposition in pistils during diplosporous megasporogenesis (Carman et al. 1991; Leblanc et al. 1995c).

Monopolar aposporous embryo sac (= Panicum-type embryo sac) formation follows a 4-nucleate scheme. Panicum-type sacs are known only in the Panicoideae and Arundinaceae (Brown and Emery 1958, Nogler 1984a). Illustrations are given in Fisher et al. (1954), Snyder et al. (1955), Bashaw and Holt (1956), and Bashaw (1962). Each embryo sac develops from a somatic cell (usually a nucellar cell) about the time that meiosis begins in the MMC. Meiotic and apomeiotic features, including callose deposition around tetrads, occur in the same ovule. The mature Panicum-type sacs lack antipodals and usually can be distinguished from sexual sacs by this feature. Occasionally, large antipodals of sexual sacs can be mistaken for multiple aposporous sacs. Aposporous activity usually crowds out sexual sac formation. However, some pistils of facultative plants eventually may contain a fully differentiated sexual sac, or even sacs of both types (Sherwood et al. 1980). Bipolar aposporous sacs and diplosporous sacs form antipodals and at maturity closely resemble the sexual sacs (Nogler 1984a).

Sectioning or Clearing Pistils to Classify Reproductive Type

The reproductive mode can be classified by examining diagnostic stages of megasporogenesis and/or megagametogenesis in cleared or sectioned pistils. In monopolar (Panicum-type) aposporous species, the pistils can be examined at any stage after embryo sac enlargement has begun. In other species, stages of megasporogenesis are examined. To accurately determine whether embryo sacs of a plant are exclusively or predominantly sexual or apomictic, at least 20–100 pistils from the plant should be viewed. A few pistils will be unclassifiable.

Sectioning paraffin embedded pistils is technically demanding and time-consuming. Early studies were based on sectioning and only small samples of ovules were examined, consequently, facultativeness was not readily detected. However, sectioning remains useful for classifying certain species (Burson 1992). Spikelets of *Eragrostis* have multiple florets with a range of developmental stages. Careful sectioning of a spikelet permits observation of developmental sequences and assists in interpretation in facultative apomicts in which diplospory affects timing of meiotic events relative to those of normal sexual development.

Clearing methods are much faster and easier than sectioning. Nuclei and walls of reproductive cells can be visualized in their proper relations in intact pistils or ovules. Several clearants and protocols are available (Herr 1971, 1982; Crane 1978; Young et al. 1979; Crane and Carman 1987; Nogler 1990, Savidan 1990a; Crane, appendix of Chap. 3; Leblanc and Mazzucato, Chap. 9). Users must determine which clearant provides the best results for their material; they should also be familiar with sectioned material of the species.

Markers

Aside from their effects on embryo-sac formation, parthenogenesis, and conservation of the maternal genotype, apomixis genes have no known effect on plant characteristics—nor have transcripts or other direct products of the

genes been located. No conventional morphological, agronomic, or physiological traits are specifically associated with apomixis. The lack of linkage information is hardly unexpected given the obstacles to traditional mapping in species that have the barrier to crossing imposed by apomixis and for the most part are alloploids with indistinguishable chromosomes, irregular chromosome duplication, and secondary economic status.

Conventional, unlinked, monogenically inherited traits have been used as markers to distinguish maternal from hybrid progeny. If the maternal parent is homozygous for a recessive trait, and the pollen parent is homozygous dominant, uniformity of progeny for the maternal marker suggests maternal inheritance. Homozygous or heterozygous dominant markers in the pollen parent have been employed to reveal hybrid F_1s (Hanna et al. 1970; Hanna and Powell 1973; Dujardin and Hanna 1989; Hignight et al. 1991).

Isozyme polymorphism has been used to characterize variability in apomictic parents and progeny (Marshall and Downes 1977; Hacker 1988; Cruz et al. 1989; Roy and Rieseberg 1989; Bayer et al. 1990; Kojima et al. 1991; Poverene and Voigt 1995; Gustine et al. 1996; Berthaud, Chap. 2; Leblanc and Mazzucato, Chap. 9).

Several molecular markers that apparently are linked with apomixis genes have been found (Leblanc and Mazzucato, Chap. 9; Grimanelli et al., Chap. 6). Ozias-Akins et al. (1993, 1998) and Lubbers et al. (1994) described a random amplified polymorphic DNA (RAPD) marker and a sequence-tagged site (STS) marker tightly linked with apomixis in *Pennisetum* species. Gustine et al. (1997) described two additional linked RAPD markers in *P. ciliare* and derived a preliminary linkage map of three markers with the apospory locus. Leblanc et al. (1995b) prepared three restriction fragment length probes (RFLP) from a maize-*Tripsacum dactyloides* F_1 population that cosegregated with diplospory. They also were linked on the long arm of chromosome 6 of maize.

Biological Tests for Parthenogenesis

Matzk (1991a) devised an auxin test for detecting parthenogenetic capacity. Unpollinated plants are treated with DIC; 2,4-D; 2,4,5-T; or CPAA. Parthenogenetic individuals form grains with a mature embryo but no endosperm. Results are positive for parthenogenetic mutants of nonapomictic species (barley, wheat) and for apomictic plants of apomictic species. The test can be used to screen for parthenogenetic plants in sexual species and to detect sexual plants in apomictic populations. It has proven useful in characterizing *Poa pratensis* lines that vary in degree of facultative apomixis (Matzk 1991b; Mazzucato et al. 1996).

Naumova et al. (1993) described a cytological test for quantitative analysis of parthenogenesis in *Poa pratensis*. Embryo sacs were isolated mechanically and examined for spontaneous embryogenesis.

An ovule culture medium facilitated identification of apomixis in diplosporous *Allium tuberosum* (Kojima and Kawaguchi 1989). Up to 80% of apomictic embryos, but no sexual embryos, showed development on the medium.

Combined Cytological, Progeny, Biological, and Marker Testing

When used alone, none of the progeny testing methods discussed above can unequivocally establish the reproductive status of every plant. Two or more approaches applied together are more informative (Naumova et al. 1993; Mazzucato et al. 1996).

Whole plant progeny testing views the end product of seed formation and is the ultimate test of whether apomixis is functional. Cytological examination of ovules during

megasporogenesis and megagametogenesis tests whether the plant has a capacity for apomictic embryo-sac formation and how strong that capacity may be relative to the sexual alternative, but it does not indicate whether the unreduced sacs will form functional seeds. Cytological and whole plant methods must be used in tandem to characterize parents (Savidan 1992).

Comparisons of methods of classification generally show good agreement. Cytology and whole plant progeny testing give similar results for most lines of *Eragrostis curvula* (Voigt and Burson 1983) and *Panicum maximum* (Nakajima and Mochizuki 1983). In *Brachiaria*, Miles and Valle (1991) found correspondence between the two methods in classifying 54 F_1s as sexual and 37 F_1s as apomictic, however, 10 plants that appeared sexual in progeny tests were facultatively apomictic in embryo-sac analysis, i.e., progeny testing underestimated the genetic potential for apomixis. Cytological analyses generally reveal higher sexual potential than is indicated by whole plant progeny testing (Savidan 1982a; Mazzucato et al. 1996).

When apomixis is essentially obligate, progeny tests are considered as reliable as cytological analyses (Savidan 1982a). In facultative lines with high levels of apomeiosis, progeny testing in conjunction with a determination of chromosome levels of off-type progeny may be efficient in detecting sexuality (Sherwood et al. 1980; Savidan 1982a). Progeny tests are unreliable in detecting low levels of apomixis in a predominantly sexual line (Savidan 1982a; Voigt and Burson 1992). Heterogeneity within a progeny cannot be considered proof of the absence of apomixis (Yudin 1994).

Early identification of nonmaternal plants in progeny of facultatively apomictic *Poa pratensis* has been facilitated by isozyme and RAPD markers (Huff and Bara 1993; Mazzucato et al. 1995). Estimates of the degree of apomixis or parthenogenesis in *P. pratensis* were higher with progeny testing than with embryo sac analysis (Mazzucato et al. 1996). The auxin test gave similar or higher estimates than embryo-sac analysis. It is necessary to examine a large sample of ovules or seed (upwards of 100 individuals) from each potential parent to detect any tendencies toward facultativeness.

Controlled Pollination

Accidental self- or cross-pollination can dramatically influence genetic inferences, especially when wide ratios are being tested. Unintentional self-fertilization will skew ratios in favor of the phenotype of the maternal parent. Markers to recognize hybrid or selfed progeny should be used when available.

Several approaches have been used to reduce unwanted fertilization in apomixis research:

1) Protogyny. If the inflorescence exserts receptive stigmas before anthers are exserted and dehisce, stigmas can be pollinated before the maternal floret sheds pollen (Voigt and Bashaw 1972; Hanna and Powell 1973; Voigt and Burson 1983; Bashaw et al. 1992; Valle and Miles, Chap. 10). Extraneous pollen is excluded by covering the head with a paper or glassine bag prior to stigma exsertion and continuing until seeds are set.

2) Suppressed anther dehiscence. Dehiscence of exserted anthers can be suppressed by maintaining high humidity. Humidity chambers (Taliaferro and Bashaw 1966), glassine bags (Hanna et al. 1973), and glass bottles lined with moist filter paper (Sherwood et al. 1994) have been used for that purpose.

3) Hand emasculation. Emasculation prior to opening of flowers, followed by bagging, has been practiced for *Potentilla* (Asker 1970a) and *Ranunculus* (Nogler 1984b). Valle et al. (1991) showed that emasculation and bagging of

Brachiaria did not totally prevent selfing. Techniques have been published for emasculating small flowered grasses (Burson 1985, 1992; Richardson 1958).

4) Male gametocide. Young inflorescences of *Pennisetum ciliare* were sprayed with a male gametocide (Bashaw and Hignigh 1990; Hignight et al. 1991).

5) Self incompatibility. Self incompatibility of sexual female lines has been used to advantage in *Taraxacum* (Richards 1970), *Paspalum notatum* (Burton and Forbes 1960), and *Hieracium* (Gadella 1987). Dujardin and Hanna (1989) used male sterile pearl millet (*Pennisetum glaucum*) as a female parent. Self-incompatibility is incomplete in guineagrass (*Panicum maximum*); the degree of cross fertilization depends upon the procedure practiced for isolation (Savidan et al. 1989; Savidan 1990b). Because genotypes can vary in degree of spontaneous selfing, it may be necessary to use control tests to establish reliability of each female parent (Matzk 1989; Valle et al. 1991).

Reciprocal Crossing

Reciprocal crossing detects nuclear and cytoplasmic maternal effects (including matrocliny). The apomixis gene(s) has such a powerful maternal effect that geneticists have been discouraged from using this technique, but apomixis need not be a deterrent to its use. It is feasible to use one or two facultative parents in the crossing scheme (Savidan 1981). Nogler (1984b) conducted reciprocal backcrosses with a sexual diploid genotype as the male or female in pairings with facultative lines. Jassem (1990) used reciprocals in beets.

Creating Tetraploid Parents

It is best if both parents are at the same ploidy level. The tetraploid level seems to be the natural milieu for expression of apomixis, whereas apomixis is rarely confirmed in diploids. If fully sexual tetraploids are not available, they may be produced by various strategies. Colchicine treatment of sexual diploids has created sexual tetraploids used in hybridizations (Burton and Forbes 1960; Richards 1970; Savidan 1981; Miles and Valle 1991; Valle et al. 1991). Leblanc et al. (1995a) treated embryogenic calli of sexual diploid *Tripsacum* with colchicine to induce chromosome doubling; the regenerated tetraploid plants reproduced sexually. However, when a sexual line of *Paspalum hexastachium* was doubled, the tetraploid was facultatively aposporous (Quarin and Hanna 1980). Asker (1967) started with an apomictic diploid (possibly a dihaploid?) biotype of *Potentilla argentea* and obtained a partially sexual tetraploid. Thus colchicine doubling may reveal latent capacities for apomixis or sexuality.

Tetraploids can be created by hybridization. Harlan et al. (1964) selected a completely sexual tetraploid of *Bothriochloa grahamii* from a cross between two facultatively apomictic tetraploids. Savidan's (1981) crosses of facultative tetraploids of genotype *Aaaa* yielded sexual:aposporous (S:A) progeny in the ratio of 1:3, indicating that the sexual progeny were genotype *aaaa*. The genetic analyses of Harlan et al. (1964) also used a sexual tetraploid *Dichanthium annulatum* accession that originated from a B_{III} hybridization of an emasculated diploid sexual plant with a tetraploid male. Burton and Hanna (1986) grew diploid sexual Pensacola bahiagrass (*Paspalum notatum*) in isolation with an apomictic tetraploid to produce a triploid B_{III} hybrid. Open pollination of the triploid with Pensacola bahiagrass yielded B_{III} hybrids at the tetraploid level. These facultatively apomictic lines could be used as maternal parents in crosses and selfs to create sexual tetraploids. Quarin (1992) successfully used a similar scheme.

Identification of Genomes and Chromosomes with Apomixis Genes

Interspecific or intergeneric crosses have provided evidence for apomixis genes in a genome or chromosome unique to one of the parents. Sexual tetraploid vaseygrass (*Paspalum urvillei*) with the genomic formula IIJJ was hybridized with hexaploid *P. dilatatum* biotypes with genomic formulas of IIJJXX and $IIJJXX_2$ (Burson 1992). The pentaploid hybrids were facultative apomicts, indicating a gene(s) controlling apomixis was located in the X genome.

Examinations of aneuploids indicate that genetic control for apomixis may reside in one chromosome (Sorenson 1958; Nogler 1984b; Mogie 1988). This also appeared to explain introgressive transfer of apospory into a sexual *Pennisetum glaucum* background (Dujardin and Hanna 1989). It appeared that the apomictic 29 chromosome BC_3 plant received 28 chromosomes from pearl millet and one, bearing an apomixis gene, from *Pennisetum squamulatum;* however, molecular data of Ozias-Akins et al. (1993) did not support this assumption.

RFLP markers were used successfully to locate a portion of a sexual maize chromosome homologous to the *Tripsacum* chromosome bearing the diplospory gene (Leblanc et al. 1995b; Grimanelli et al., Chap.6). Three RFLP probes cosegregated with diplospory in a maize-*Tripsacum* F_1 population that was segregating for mode of reproduction. The markers also were associated with the long arm of maize chromosome 6.

Testing Inheritance

Starting Point

Genetic relatedness, chromosomal balance, and sexuality of the maternal parent are the primary considerations in selecting parents (Savidan 1990a). Generally speaking, the best situation is to use intraspecific crosses; interspecific crosses are a second choice, and intergeneric crosses are a poor third alternative. Interspecific and intergeneric crosses of distant parents usually create abnormalities that interfere with reproduction. Selection of genetically close parents, however, must be tempered with consideration of natural crossability rather than arbitrary taxonomic distinctions. Interspecific crossing has been relatively successful among some species of the *Dichanthium / Bothriochloa / Capillipedium* complex (de Wet and Harlan 1970) and within *Brachiaria* (Valle and Miles, Chap. 10). On the other hand, a composite species such as *Potentilla argentea* may constitute a complex aggregation of sexual and apomictic biotypes that do not readily interbreed (Asker 1970a, b).

Crosses between plants at the same ploidy level are preferred in order to reduce meiotic disturbances and sterility. Hybridization across ploidy levels leads to intermediate, and often unstable, chromosome numbers. Triploid hybrids resulting from crossing diploid and tetraploid parents can express apomixis, but they will not be desirable for further crossing or selfing except for developing aneuploids.

Sexual parents should be devoid of apomixis alleles. Savidan (1990a) warns of hidden apomixis within naturally occurring tetraploids that appear to be sexual. He suggests that sexuality in natural tetraploids could be facultative, even if it looks obligate in a progeny test, because of modifying factors that influence expression of the apomixis gene. Savidan goes on to recommend creation of totally sexual tetraploids by colchicine doubling of sexual diploids.

Crossing Schemes

All apomixis inheritance studies published to date have included crosses between sexual maternal parents and apomictic pollen parents followed by characterization of the F_1. Usually, several crosses were made, and first selfed

generation S_1 plants of sexual parents were analyzed. Information from F_1s and S_1s has only limited power for testing alternative genetic models. Interpretations can be strengthened using advanced generations, as in studies by Burton and Forbes (1960); Nogler (1984b); Savidan (1981); and Valle and Miles (Chap. 10).

Classification and Grouping

How should facultative progeny displaying various degrees of apomixis be grouped when testing segregation ratios? Current practice places all plants showing any apomixis into one group (deemed apomictic) and all plants devoid of apomixis into a second group (deemed sexual) (Savidan 1981; Voigt and Burson 1983; Miles and Valle 1991; Sherwood et al. 1994). Most facultative plants produce far more apomictic sacs and progeny than sexual sacs or nonmaternal progeny, and classification is relatively straightforward. With only two groups being recognized, it is inevitable that the genetic models are for simple gene action with Mendelian interpretations. Unimodal distributions of reproductive types indicative of quantitative or polygenic inheritance have not been tested.

Testing Genetic Models

All reasonable genetic models and permutations of genotypes should be tested. Predicted ratios should consider the effects of number of loci, dominance, epistasis, and lethality. If the degree of alloploidy versus autoploidy of tetraploids is unknown, both disomic and tetrasomic inheritance and both partial and complete random assortment of chromosomes or chromatids should be considered (Sherwood et al. 1994).

Inheritance of Apomixis

Literature on inheritance of apomixis has been reviewed by Stebbins (1941), Gustafsson (1946–1947), Asker (1980), Nogler (1984a), Bashaw and Hanna (1990), and Asker and Jerling (1992). Reports issued since 1950 are summarized here, with a reinterpretation of some of the results. Symbol "*A*" is used to denote the dominant allele of the putative apomixis gene regardless of the symbols used in the original reports.

Monopolar Apospory (Gramineae–Panicoideae)

***Paspalum notatum* (bahiagrass).** Burton and Forbes (1960) crossed colchicine-induced sexual autotetraploid lines of Pensacola bahiagrass with an apomictic tetraploid line. Progeny were classified only by whole plant progeny testing. The crosses produced sexual:apomictic (S:A) segregation ratios near 3:1. Selfing the sexual progeny gave an F_2 with a ratio near 35:1. Selfing the apomictic progeny gave only apomictic F_2 progeny. Burton (1992) postulated an *A* gene dominant for apomixis and an independent *S* gene dominant for sexuality. He assigned genotype *Aaaassss* to MH, and genotype *aaaaSSSS* to the sexual parents. Some progeny from selfing sexuals appeared apomictic based on their uniformity but actually may have been sexual; this could give rise to the 35:1 ratios. In the crosses giving 3:1 ratios, some facultatively apomictic hybrid F_1 progeny may have been inadvertently classified sexual, skewing the ratios in favor of high numbers of sexual progeny. More recent data support the view that apomixis is coded for by dominant genes (Burton and Hanna 1992). The bahiagrass system deserves reexamination using modern classification methods.

***Dichanthium-Bothriochloa* (bluestem).** Harlan et al. (1964) crossed sexual tetraploids of *D. annulatum* and *D. grahamii* with apomictic tetraploid pollen parents. Crosses of sexual x sexual provided only sexual F_1s. Crosses of sexuals x apomicts gave a S:A of 1:4.1. They postulated random assortment of two disomic genes and assigned genotype $A_1a_1A_2a_2$ to tetraploid apomicts, and $a_1a_1a_2a_2$ to sexuals. If

we postulate that the tetraploids were tetrasomic with genotype *AAaa*, the observed ratios fit ratios expected for the assumption of random chromatid assortment (1:3.67) or the assumption of a recessive lethal effect of the *A* allele (1:4) (Sherwood et al. 1994).

***Pennisetum ciliare* (buffelgrass).** A naturally occurring tetraploid sexual plant, designated B-1S, was selfed and crossed with two aposporous biotypes by Taliaferro and Bashaw (1966). The S:A ratios (near 13:3 for S_1s and 5:3 for F_1s) suggest two disomic independent genes with the dominant allele of gene *A* being required for apospory, and the dominant allele of gene *B* being epistatic to *A* and conferring sexuality. Sexual parent B-1S was assigned genotype *AaBa*. Further evaluation of F_1s, F_2s, and a BC_1 from B-1S, identified true breeding sexual progeny of apparent genotype *aabb* and apomictic plants of putative genotypes *Aabb* and *AAbb* (Read and Bashaw 1969; Bashaw et al. 1970).

Crane (1992) proposed a tetraploid single gene model to explain the Taliaferro and Bashaw segregations. Three alleles were postulated: *a* (wild type sexual), *A* (aposporous), and *A+* (super sexual). Only genotypes *AAAA*, *AAAa*, and *AAaa* would be apomictic. Chromosomal segregation patterns were postulated to be preferential and to differ during microsporogenesis and megasporogenesis.

Sherwood et al. (1994) studied inheritance of embryo sac type of sexual tetraploid plant B-2S. From open pollinated B-2S, five sibling sexual lines and five sibling aposporous lines were selected as parents. Segregations were determined for crosses of sexual x aposporous lines and sexual x sexual lines, and selfs of sexual lines. Selfs and crosses of sexual plants gave only sexual progeny. F_1s from sexual x aposporous combinations segregated for S:A at ratios near 15:13 for four aposporous lines and 1:2.8 for the other line. Segregations did not fit any one- or two-disomic gene models, nor any recessive gene models. Data were compatible with a one-tetrasomic-gene model with apospory regulated by dominant allele *A*, under either of two assumptions:(*i*) random assortment of chromatids, or (*ii*) *A* acting as a recessive lethal in gametes. Sexual plants were assigned genotype *aaaa*; apomicts were *Aaaa* and *AAaa*. Data on linkage of apospory in *Pennisetum* with molecular markers (Gustine et al. 1997; see Grimanelli et al., Chap. 6) provides additional evidence that a single major locus regulates apospory in *Pennisetum*.

***Panicum maximum* (guineagrass).** Hanna et al. (1973) reported that four naturally sexual tetraploid accessions produced S_1 progenies segregating in a combined ratio of 116S:54A. Crosses of sexuals x apomicts gave 21S:28A. They proposed a digenic, disomic additive model using the assignment of *AaBb* for the sexual plants and *Aabb*, *aaBb*, or *aabb* for apomicts (two dominant doses required for sexuality).

Savidan (1981) crossed a colchicine-induced autotetraploid sexual plant and a natural apomictic tetraploid. Ten kinds of crosses were tested (Table 5.1). All the data fit perfectly with

Table 5.1 Segregations for mode of reproduction in 10 crosses of *Panicum maximum* (Savidan 1981; Savidan et al. 1989)

sexual x apomictic crosses	sum	apo	sex
F1 hybrids: S1 x A1	133	71	62
3-way hybrids: (S1 x A1)sex x A2	279	135	144
Backcross: (S1 x A1)sex x A1	26	14	12
Backcross: sexual 3-way hybrid x A2	170	73	97
sexual 3-way x apomictic 3-way	60	26	34
Backcross: S1 x (S1 x A1)apo	23	13	10
total sex x apo crosses	691	332	359
sexual x sexual crosses (or selfed)			
sexual F1 hybrids selfed	126	0	126
sexual 3-way hybrids selfed	57	0	57
sexual 3-way x sexual 3-way	82	0	82
total sex x sex crosses	265	0	265
apomictic x apomictic crosses			
apomictic 3-way x apomictic 3-way*	71	53	18

** analysis made of off-types (maternal types not counted)

the hypothesis of one tetraploid gene dominant for apomixis, with all sexual parents assigned genotype *aaaa* and all apomicts assigned genotype *Aaaa*.

***Brachiaria* (Gramineae).** Valle and coworkers (1991, 1992, 1993, and Chap. 10) conducted extensive studies of *Brachiaria* along the lines of Savidan's (1981) guineagrass program. The results pointed to a single dominant gene determining apospory with genotypes of *aaaa* for the colchicine-induced tetraploid sexual parents and *Aaaa* for the aposporous tetraploid parents.

Bipolar Apospory

***Ranunculus* (buttercup, Ranunculaceae).** Nogler (1984b) crossed diploid sexual *R. cassubicifolius* with tetraploid apomictic *R. megacarpus* (Figure 5.1). Four fertile triploid facultatively aposporous progeny were obtained and used to initiate three generations of reciprocal backcrossing to the sexual diploid. Nogler deduced that apospory is caused by a dominant factor *A* (designated A^- in Nogler 1984b), the wild allele of which (*a*) (designated A^+ in Nogler 1984b) does not enhance apospory. Dominance of *A* is incomplete and additive. Furthermore, *A*, when homozygous

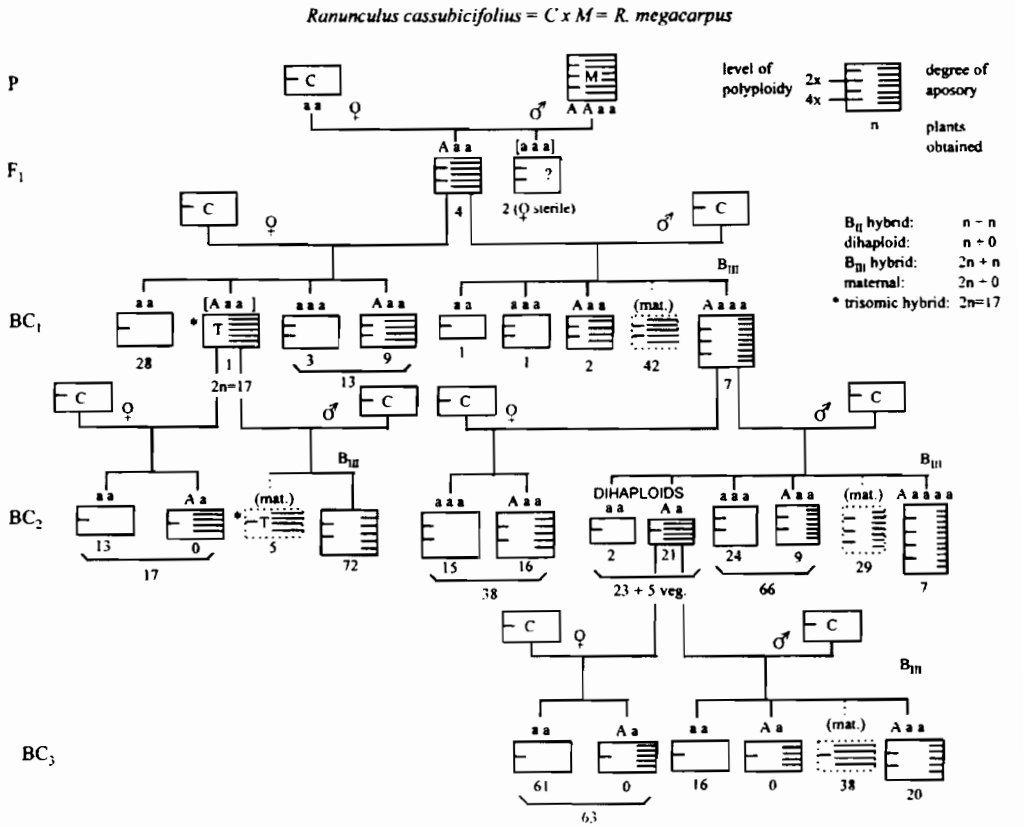

Figure 5.1 Genealogical tree of the cross *Ranunculus cassubicifoliu s* = C, 2*x* = 16, meiotic (sexual) x *R. megacarpus* = M, 4*x* = 32, partially aposporous ("totally" apomictic) and the different backcrosses with the sexual parent C. The number of plants obtained, the level of polyploidy, the approximate degree of apospory, and the genotype are indicated for each offspring.

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in the gametophyte, is lethal to the gametophyte; there are no functional *A*, *AA*, or *AAA* gametes because of recessive lethality. Gametes must carry the wild type *a* allele to be viable. Several lines of evidence point to lethality, including failure to find aposporous diploid hybrid progeny. A highly aposporous $2n + 1$ aneuploid, line T, was believed to have originated from an *a* egg and an *Aa* sperm and have genotype *Aaa*. Line T transmitted *a* and *Aa* gametes, but not *A* gametes. The data, for the most part, did not permit testing of segregation ratios, but cross *aa* x *Aaaa* and its reciprocal did yield 1:1 ratios as expected. In trisomic apomictic lines assigned genotype *Aaa*, there was declining strength of apomixis with advance from BC_1 to BC_3. Nogler believed this might be due to modifying genes for sexuality that increased with each generation of backcrossing. Within generations there was a dosage effect; for example genotype *Aaa* showed greater apospory than *Aaaa*. Some plants were identified as dihaploids with genotype *Aa* and were highly aposporous. Dihaploids are formed by parthenogenetic development of reduced *Aa* eggs of facultatively apomictic *AAaa* or *Aaaa* tetraploids; in other species, dihaploids usually are sexual (Nogler 1984a).

Line T expressed low parthenogenicity. Nogler (1984b) postulated that parthenogenicity was coded by a separate gene closely linked to *A*. Later tests revealed that apomictic line T transmitted genes for parthenogenicity. Therefore, it was not necessary to insist that apomixis and parthenogenesis were coded by separate genes (Nogler 1989, 1995). Nogler's (1984b) study encountered all the problems anticipated from interspecific crossing at different ploidy levels—poor fertility, poor seed set, facultative expression, aneuploidy, and ambiguous segregation ratios—but succeeded because of exceptional effort and insight.

***Poa pratensis* (Kentucky bluegrass, Poaceae).** There are several inconclusive and contradictory reports on inheritance of apomixis in Kentucky bluegrass. Bluegrass populations have complex arrays of polyploidy and aneuploidy, with chromosome numbers ranging continuously from 24 to 124. In addition, somatic cells within plants may vary by as many as 30 chromosomes (Huff 1992). Plants vary widely in the degree of apomixis. Almgard (1966) concluded that the presence of aposporous embryo sacs showed dominant inheritance, but retention of the maternal phenotype (functional apomixis) was recessive.

Matzk (1991b) explored regulation of the parthenogenetic capacity of *P. pratensis* using the auxin test. He concluded that the apomictic parents were heterozygous for one or more dominant alleles for parthenogenesis. The results were consistent with expectations for a single major gene.

Study of the genetic regulation of facultative expression (Nogler's modifying genes for sexuality) may be feasible using molecular and biological probes developed for *P. pratensis* (Huff and Bara 1993; Naumova et al. 1993; Mazzucato et al. 1995; Mazzucato et al. 1996). There is also an interesting report of Hieracium-type embryo-sac formation in diploid specimens of three *Tribolium* species (Poaceae) (Visser and Spies 1994).

***Hieracium* (hawkweed, Asteraceae).** From a cross of diploid sexual *H. auricula* x tetraploid apomictic *H. aurantiacum*, Christoff (1942) found 32 sexual and 27 apomictic progeny, a ratio that fits the expected 1:1 if the male has a single dominant allele for apomixis. Gadella (1987) believed results from a cross of tetraploid sexual *H. pilosella* with a pentaploid apomict could be explained by monogenic dominant inheritance with the pentaploid

having genotype *Aaaaa*. However, the observed ratio 36:*aaaa*:8*Aaaa*:5*Aaaaa*:2*aaaaa* did not fit the expected ratio 3:2:3:2.

Bicknell and Borst (1996) observed segregation for sexuality and apomixis among tetraploid regenerants of *H. pilosella* derived from reduced calli of an apomictic biotype. They considered this evidence for dominant inheritance of apomixis. Bicknell (1994, and Chap. 8); Koltunow et al. (1995) believe *Hieracium* can be a model system for studying molecular genetics of apomixis.

Mitotic Diplospory

Most reports on diplospory, summarized below, were handicapped by lack of suitable sexual parents and by unavailability of convenient classification techniques.

***Eragrostis curvula* (weeping lovegrass, Poaceae).** Crosses of naturally occurring tetraploid sexual plants with tetraploid and hexaploid apomicts gave F_1 progeny test segregations indicating that apomixis is monogenic and dominant (Voigt and Burson 1983). Results of a cross with an aneuploid indicated possible dosage effects, but may have been confounded by B_{III} hybridization or chromosome elimination.

***Tripsacum dactyloides* (Eastern gamagrass, Poaceae).** Sherman et al. (1991) crossed a sexual diploid female parent with a triploid apomict. Forty-six hyperdiploid progeny were identified as hybrids. All but two of these showed cytological indications of apomixis; the degree of diplospory ranged from predominantly sexual to highly apomictic. The authors believed this indicated that apomixis is incompletely dominant, or that minor additive genes on various chromosomes affect penetrance of apomixis. Recent production of sexual tetraploids by colchicine doubling should facilitate future study of inheritance. The tetraploid *T. dactyloides* parent genotype is simplex for the diplospory allele. Maize-*Tripsacum* F_1s segregated 1:1 for mode of reproduction (Leblanc et al. 1995b).

***Parthenium argentatum* (guayule, Asteracae).** Gerstel and Mishanec (1950) reciprocally crossed a sexual diploid ($2n = 36$) with a facultatively diplosporous hyperdiploid ($2n = 37$). With the sexual plant as the maternal parent, all F_1 diploid hybrid progeny (21 plants) were sexual. In the reciprocal, most progeny, as expected, were maternal or polyploid apomicts, but four diploid sexual F_1s were formed (Gerstel et al. 1953). They concluded that apomixis genes acted recessively but additively and postulated that polyploids with two apomixis genomes and one sexual genome were apomictic.

There is an alternate interpretation. The starting materials and results with *Parthenium* resemble those of Nogler (1984b) for aneuploid plant T of *Ranunculus* (Figure 5.1). The *Parthenium* results may indicate the same control as in *Ranunculus*, i.e., a single factor dominant for apomixis, that acts as a recessive lethal, with the polyhaploid parent being genotype *Aaa* and the diploids being *aa*.

Restitutional Diplospory

***Taraxacum* (dandelion, Asteracae).** Eutriploids ($2n = 24$) and many hypotriploids ($2n = 23$) have facultative diplospory. Certain $2n = 23$ aberrants are primarily sexual (Sorensen 1958). Mogie (1988) offered the following interpretation of earlier studies. Expression of apomictic phenotype in *Taraxacum* depends on one or more genes located on one chromosome and on dosage. At least two copies of the mutant apomixis allele are required to obtain apomixis; the allele prevents meiosis in diplosporous apomicts. The dominance relationship between the wild type and mutant allele is determined by balance and environment.

Perhaps the most significant aspect of the hypothesis is that Mogie deduced that the wild type (*a*) allele of the apomixis locus has an essential function in the plant. He suggests that it codes for meiotic reduction and that it is also involved in the control of mitosis, which would be disrupted by the expression of the mutant allele in somatic cells.

Multicellular Archesporia

***Beta* (beet, Chenopodiaceae).** At least two species in the section *Corollinae* form multicellular archesporia that show both diplosporous and aposporous development. Jassem (1990) conducted extensive analyses of crosses involving sexual and apomictic species, across ploidy levels, including F_2 and BC_1 generations. B_{III} hybridization and aneuploidy complicated the results. Although unable to draw unequivocal conclusions, she believed that apomixis genes were partly dominant and acted in a complementary fashion.

***Sorbus* (mountain ash, Rosaceae).** Liljefors (1955) used leaf morphology and chromosome pairing to deduce genomic formulas of aposporous polyploids in the agamic complex *Sorbus*. He assigned the genomic formula *BB* to totally sexual *S. aucuparea*. Species assigned genomic formulae *AAAA*, *AAAB*, and *AAB* were fully aposporous, and *AABB* was facultative. He postulated that a gene or genes for apomixis were associated with genome *A*, and that expression was dosage/balance related. However, species with genomic formulae *ABBB* and *ABB* were also highly apomictic, and he had to postulate exchange of the gene into the *B* genome. Liljefors' observations may be equally well explained using a one major-gene model with facultative expression, and postulating that genotypes *AAaa*, *Aaaa*, and *Aaa* are aposporous.

Towards a Comprehensive Model of Inheritance

Inheritance of apomixis has been explored in relatively few species, yet several genetic models have been put forward that differ widely in postulated number of loci and nature of gene action (Bashaw and Hanna 1990; Asker and Jerling 1992; den Nijs and van Dijk 1993). Is the seemingly capricious occurrence and regulation of apomixis in different taxa to be attributed to independent, random mutations at various reproductive loci leading to similar phenotypic consequences? Is there a single apomixis locus or linkage group shared by all apomicts? The reality lies somewhere between these extremes.

Regulation of Monopolar Apospory

Monopolar (Panicum-type) apospory occurs commonly throughout Panicoideae and Arundinaceae and is found nowhere else. This indicates a common genetic basis. Brown and Emery (1958) postulated that coding for the monopolar pattern arose early in the evolution of the group. Inheritance data for all the Panicoid species studied have been interpreted as indicating that expression of apomixis requires a major locus, with apomixis behaving as a dominant trait. Common gene action and phenotype in related species indicate the same linkage group may be involved. The molecular evidence for one linkage group in *Pennisetum* is impressive. However, C. F. Crane (personal comm.) cautions that this does not necessarily imply that the monopolar type evolved only once and spread laterally among related genera that are well populated with sexual species. He considers it more likely that the ancestor of the *A-a* locus has become widespread in the panicoids and chloridoids, and that apospory has emerged repeatedly by mutation of the wild type locus.

Savidan (1991a, 1992), Peacock (1993), and others suggest that a single master gene is responsible for induction of embryo-sac formation. They view induction as triggering a cascade of events that requires direction by many genes with a potential for modifying the end result. Savidan (1989) suggests that several genes controlling apomixis may be linked on one small chromosome segment or linkat. Jefferson (1993) suggests that apomixis involves phenotypic mutations at several loci acting together as a non-recombining unit and having the appearance of a single gene. Accordingly, classical genetic observations of segregations might be less informative than expected (Grimanelli et al. 1995, and Chap. 6).

Some early studies on Panicoideae interpreted the data as indicating not only a locus for apospory, but also a second, independent locus that affected sexuality (Burton and Forbes 1960, reinterpreted by Burton 1992; Taliaferro and Bashaw 1966). In the Taliaferro and Bashaw (1966) report, the gene was postulated to be epistatic to the apomixis allele. Hanna et al. (1973) proposed two loci with genes acting additively to confer sexuality. These interpretations followed observations that selfing of apparently sexual parents gave progeny segregating for mode of reproduction. The data sets from these three studies could not be fitted to the single tetraploid apomixis gene model or to any other cited model (Sherwood et al. 1994). Savidan (1982b) noted some rare facultative genotypes of *Panicum maximum* with nearly 90% sexuality. Later, Savidan (1991a, b) proposed a technical explanation for the observation of apomictic progeny when naturally occurring tetraploid sexual parents were selfed in the earlier studies. He believes the tetraploid parents were facultative apomicts of genotype *Aaaa* with a high frequency of sexual reproduction and that the parents were mistakenly classified sexual because of the limitations of the sectioning and progeny testing techniques used at the time. However, selfing or crossing *Aaaa* parents should give S_1 progeny of 1:3, S:A (Figure 5.1), not the reported ratios of 2.5:1 or 13:3, so the matter is not yet resolved.

Alternatively, the data of Hanna et al., Taliaferro and Bashaw, and those of all other studies on Panicoideae can be accommodated in one genetic model that postulates two tetrasomically inherited loci—the *A* locus with a gene dominant for apomixis (and recessively lethal), and a *B* locus with the dominant allele epistatic to *A* (Sherwood et al. 1994).

Regulation of Diplospory

Recent studies indicate that segregation for diplospory in maize-*Tripsacum* progeny involves a single Mendelian factor, with perhaps some modifying factors (Leblanc et al. 1995b; Grimanelli et al. 1995; Savidan et al. 1995). The factor appears to be an apomixis linkat. Savidan et al. (1995) stated they "may now have a series of concrete reasons to believe that apomixis is indeed controlled by something more complex than this dominant gene, including at least one recessive factor which prevents apomixis expression in diploids." See also Grimanelli et al. (Chap. 6).

Regulation of Facultative Expression

All apomictic species for which inheritance data are available show facultative expression. Degree of apomixis could be due to dosage or penetrance effects of a major gene and/or to modifying genes. Data for *Ranunculus* (Nogler 1984b) and *Paspalum* (Quarin 1986, 1992) indicate that penetrance of the *A* allele is incomplete; degree of apomixis may increase with increased number of *A* alleles. Quarin and Hanna (1980) suggested that a certain genetic threshold must be reached for apomixis to be expressed in some *Paspalum*. A single dose of the *A* allele is sufficient to support a high level of apomixis in *Pennisetum* (Sherwood et al. 1994) and *Panicum* (Savidan 1981).

Environment plays a role in expression (Nogler 1984a; den Nijs and van Dijk 1993). A short photoperiod increases the frequency of aposporous vs. sexual embryo sacs in *Dichanthium aristatum* (Knox 1967) and *Paspalum chromyorrhizon* (Quarin 1986). Salt stress affects facultative expression in *P. ciliare* (Gounaris et al. 1991).

Harlan et al. (1964) accurately assessed the relation between sexual and aposporous reproduction. "Apomixis (read apospory) and sexual reproduction are not alternative modes of reproduction, either genetically or operationally, but are simultaneous and independent phenomena. The genes controlling normal sexual reproduction are not allelic to those controlling apomixis in the conventional sense." This accounts for facultative expression of apospory. In aposporous lines, meiotic reduction of the archesporial nucleus and apomeiotic induction of apospory in nucellar cells go forward at about the same time. Aposporous initials and unreduced embryo sacs normally crowd out the reduced megaspores and sacs.

Facultatively displosporous plants also possess all of the genetic information required for completion of both meiotic and apomeiotic embryo sacs. However, in contrast to facultative apospory, the two events cannot proceed simultaneously in a facultatively diplosporous ovule, for they compete for the same site in the ovule. Events beginning in the megaspore mother cell can proceed only towards normal meiosis or apomeiosis, but not both. Variability within facultative diplosporous or aposporous types indicates that the entire apomictic developmental process cannot be explained on the basis of a single gene (Grimanelli et al. 1995; Savidan et al. 1995).

The Lethal Gene as the Basis for Heterozygosity

Nogler (1984b) concluded that functional gametes of *Ranunculus* contain a copy of the wild type *a* allele. Noirot (1993) reviewed evidence that the *A* allele may act adversely in *Panicum maximum*; his report focused on male and female sterility. Male and female sterility is encountered in spontaneous dihaploids and trihaploids of *Panicum* of putative genotypes *Aa* and *Aaa*, respectively (Combes 1975). Mogie (1988) proposed that the wild type *a* allele has a function that is essential to normal plant processes. If gametophytes and gametes bearing dominant allele *A* must also bear the wild type *a* allele to remain functional, this would account for the observation (Harlan et al. 1964) that aposporous apomicts invariably are heterozygous at the apomixis locus. No apomict has ever transmitted an exclusive capacity for apomixis to the offspring; a capacity for sexual reproduction is always also transmitted, although it may not surface until later generations. Heterozygous *Aa* gametes can be formed by dihaploids, triploids, or tetraploids. In the case of the dihaploid, the *Aa* gamete is from an unreduced (apomictic) sac, and the progeny are either maternal (no fertilization) or B_{III} hybrids (fertilization), as shown for the dihaploids of *Ranunculus* (Figure 5.1). In the case of triploids and tetraploids, the *Aa* gamete occurs in sexually reduced (meiotic) sacs, and the egg is usually fertilized (B_{II} hybridization), but occasionally may parthenogenetically form an *Aa* dihaploid as part of a diploid-tetraploid-dihaploid cycle (de Wet and Harlan 1970; Savidan and Pernes 1982). Insofar as sexual transmission of *A* is concerned, the parent must be polyploid and heterozygous; for asexual transmission, the parent must be heterozygous.

The results of the maize-*Tripsacum* backcrossing effort conform with the idea of a recessive lethal factor linked to a dominant diplospory gene (Savidan et al. 1995). No apomictic representatives were found in BC_2 progeny with $2n = 38 = 20M+18T$ chromosomes produced from F_1 of $2n = 56 = 20M+36T$ chromosomes. See also Grimanelli et al. (Chap. 6) for discussion.

Richards (1996) postulates that lethal recessive mutants might occur at loci linked with the dominant apomixis locus and accumulate in heterozygotic apomictic lines without being expressed. These harmful recessive genes may be expressed in haploid gametophytes (i.e., pollen and embryo sacs) resulting in their abortion. The implied corollary is that the apomixis allele is not, of itself, the source of recessive lethality. The evidence from studies by Nogler (1984b), Mogie (1988), Sherwood et al. (1994), and others still points to the apomixis gene as being recessively lethal. Segregation ratios from the facultative populations cited above show an absolute lack of recombination of recessive lethality vis a vis the apomixis linkat on the male side, but not on the female side.

Summary

The results of all published studies on inheritance of apospory are compatible with the hypothesis that expression of apospory requires the dominant allele of a major gene or linkat. There is no species for which the hypothesis of a major dominant apospory factor can be ruled out. Limited data available for diplosporous species also suggest a single master gene or linkat (Savidan 1989); its possible correspondence to the apospory linkat is entirely unknown. Some reports indicate that degree of expression is regulated by modifying genes that promote sexuality; the apomixis gene(s) may show dosage effects, and environment may influence expression. Several lines of evidence indicate that the wild type allele of the apomixis gene plays an essential role in cell function and that its presence is required for survival of the gametophyte. There is no clear evidence for a major gene for parthenogenesis independent of the major gene(s) for apospory or diplospory (Nogler 1984a, 1995; Leblanc et al. 1995b; Mazzucato 1996). Recombination within the linkat is rare or nonexistent. The linkat encodes sufficient information to direct the complex sequence of developmental events in embryo sac initiation, differentiation, maturation, and function.

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Chapter 6

Applications of Molecular Genetics in Apomixis Research

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Introduction

Apomixis in higher plants refers to a wide range of mechanisms of asexual reproduction through seeds (Nogler 1984a). It is found in at least 400 wild species belonging to 35 higher plant families (Richards 1986; Asker and Jerling 1992; Carman 1997). The modalities of apomictic development in the wild are nearly as diverse as the number of species studied, but in most cases, apomictic processes completely bypass meiosis and egg cell fertilization, and produce offspring that are exact genetic replicas of the mother plant.

Two major types of gametophytic apomixis have been described, namely diplosporous apomixis and aposporous apomixis, based on the origin of the megagametophytes. In aposporous apomicts, one or more unreduced female gametophytes form mitotically from somatic nucellar cells while the legitimate sexual line generally aborts. Diplospory results from meiotic failure in megasporocytes that directly develop into mature unreduced female gametophytes through three or more mitoses. Typically, apomixis is a facultative phenomenon, and an apomictic plant usually produces both asexually (apomeiotic) and sexually derived embryos.

Apomixis, for the most part, is found in wild species. In contrast, major crop plants are sexual, with only rare exceptions such as some prominent tropical forages. This could be conceived partly as a consequence of crop domestication because the process necessarily implies that early farmers had access to variability and segregation among the wild types. In *modern* agriculture, however, the ability to fix superior genotypes through generations would offer numerous advantages. Recognition of these advantages has led to a growing interest in apomixis research, and indeed, many scientists have extolled the tremendous potential that apomixis holds for plant improvement (this volume; Jefferson and Bicknell 1996; Grossniklauss et al. 1999; Savidan 2000).

Various strategies are being considered by a growing number of research groups around the world to introduce apomixis into major food crops. The oldest efforts were directed toward the introgression of the genes for apomixis from wild species into cultivated relatives (see review by Savidan 2000). As an alternative approach, the *de novo* synthesis of apomixis in sexual plants through genetic engineering is now underway through a number of intiatives (Jefferson and Bicknell 1996; Grossniklaus et al. 1999; Luo et al. 2000).

Despite this growing interest, surprisingly little is known about the biology of apomictic plants. This is certainly the primary reason why attempts to manipulate apomixis have failed to yield useful products to date, and it is clear that harnessing the potential of apomixis will strongly depend on our ability to develop a reliable understanding of the basic features of the biological processes of apomixis and its genetic control. The emergence of powerful

tools in molecular genetics now offers new approaches to gain much needed knowledge about the regulation of apomixis. In this chapter, we discuss, in detail, potential applications of molecular genetics to apomixis research. First, we define the biological aspects of the genetics of apomicts that lend themselves to analyses using molecular genetics. A discussion about different strategies for tagging or manipulating the corresponding genes then follows.

Some Biological Aspects of Apomixis Worth Studying using Molecular Genetics

Nonreduction followed by Parthenogenesis

Decades of cytoembryological observations have yielded precise descriptions of the apomictic processes (reviewed by Crane, Chap. 3). These observations have revealed both the complexity of the developmental process of apomictic reproduction and the remarkable diversity of mechanisms leading to the generation of unreduced gametes in apomictic plants. Nevertheless, to date, we do not have a clear understanding of the genetic bases of this developmental trait. According to recent work reviewed by Sherwood (Chap. 5), apospory is probably simply inherited. Much less is known about diplospory, but the literature suggests a similar working hypothesis (Leblanc et al. 1995; Noyes and Rieseberg 2000, Bicknell et al. 2000), and works on *Taraxacum* reviewed by Mogie 1988). However, those results, both on diplospory and apospory, fail to provide information about the fine genetic control of nonreduction followed by the failure of fertilization and the induction of embryogenesis. In brief, it remains unclear whether all three events rely on distinct, but linked, genetic factors, or on a single gene controlling their successive induction as a pleiotropic effect. Both hypotheses have been defended logically, but whatever the number of genes specifically transmitted to an apomict, they should either behave genetically as a single locus, or manifest as a monomorphic trait in both sexual and apomictic ecotypes.If the genes were independent, upon segregation such mutations would rapidly be eliminated because of their low individual viability. Note that exceptions to this principle have been reported (Nogler 1984b; Asker and Jerling 1992; Kojima et al. 1994; Noyes and Riesberg 2000).

In work on *Ranunculus* species, for example, Nogler (1984b) first reported a trisomic hybrid lacking the ability for parthenogenesis, despite being highly aposporous. A similar case was described by Kojima et al. (1994) for *Allium* species, and by Noyes and Rieseberg (2000) for *Erigeron annuus*. In addition, apomictic plants usually produce "off-type" progenies, in which one of the two steps is skipped. This results in dihaploids, in which there is reduction but also parthenogenesis, or in 2n + n off-types, in which there is nonreduction but fertilization. Such cases do not necessarily require different genes, but they least entail the independent expression of the putative developmental components. Finally, it should be noted that in the case of grasses, it may not be neccesary to transfer specific genes for parthenogenesis, since it is apparently a latent ability in most of them.

Whether one or several genes are involved in apomixis, many questions remain about their mode of expression and regulation. Early results from *Panicum* (Savidan 1982) and *Ranunculus* ssp. (Nogler 1984b) indicated that although the induction of apospory is under simple genetic control, the overall apomictic behavior of these aposporous species is more quantitative as evidenced by the relative proportion of ameiotic and meiotic embryo sacs, some environmental effects, etc. Modifier effects that need to be identified include the

number of genes, their relative importance, their dominance relationships, epistatic effects, pleiotropic effects, possible allelic diversity, chromosomal localization, maternal and/or paternal effects, and environmental regulation.

Expression of Apomixis and Ploidy Levels

A remarkable aspect of apomixis is its relationship to polyploidy. Except in rare cases, apomicts are polyploids while sexuality in the same species, if known, is usually found at lower ploidy levels. It is widely accepted that some type of mechanism protects diploid sexual populations from being "invaded" by apomixis.

Three broad types of hypotheses have been proposed concerning that mechanism. One school of thought assumes that the alleles controlling apomixis could eventually be transmitted to diploid plants, but that the expression of the trait is restricted to polyploids; the penetrance of the character depending on dosage effects between the various alleles at the locus or loci controlling diplospory (Mogie 1992; Noirot 1993). Noirot, assuming a single allele *A* controlling apomeiosis in a dominant manner, proposed that not more than one copy of the *A* allele would be found among every four alleles (a ratio between *A* and *a* not to exceed 0.25). The hypothesis contradicts reported cases of apomictic triploids, trisomics, and dihaploids (a ratio of up to 0.5) (Leblanc et al. 1996; Nogler 1982). Mogie (1992) proposed a different though related dosage model for the regulation of diplospory in *Taraxacum*, in which the dominance relationship between the wild type (*a*) and mutant (*A*) alleles is determined by their relative copy numbers: avoidance of meiotic reduction occurs when the mutant allele is present in more copies than the wild type *a* allele. Mogie also assumes that the a locus plays an important role in mitosis and meiosis, thus explaining why *A* is not expressed or eliminated when transmitted in the haploid or homozygous states. Mogie's data for *Taraxacum* have been challenged recently by van Dijk et al. (2000), who proposes a more complex model for the inheritance of diplospory in this genera.

The second hypothesis (Nogler 1982) is based on the assumption that apomixis is usually not transmitted to diploids. In Nogler's work with *Ranunculus* hybrids, the *A* factor was not transmitted through haploid gametes, presumably because of a lethal effect of the allele when present under haploid conditions. Noyes and Riesberg (2000), working with *Erigeron*, proposed a more complex but related explanation, in which the absence of diplospory in diploids is best explained by both the combined effect of a recessive lethal gametophytic selection against a unique parthenogenetic-controlling locus, and univalent inheritance of the region responsible for diplospory. Related data was also obtained in *Tripsacum* (Grimanelli et al. 1998a), showing that apomeiosis was generally not transmitted via haploid gametes. In *Hieracium*, however, Bicknell et al. (2000) suggest that diplosporous apomixis can be transmitted both by diploid and haploid gametes, and that the absence of diploid apomictic progenies is caused by selection against the survival of diploid zygotes, rather than against the elimination of haploid gametes. Once more, as with the control of apomeiosis, it is hard to define a model that fits all apomicts. Whether those differences are due to experimental bias or simply to more fundamental differences in the nature of the various forms of apomixis has not yet been determined.

Carman (1997) puts forward a third general hypothesis that cites differences in rates of reproductive development between different ecotypes as being responsible for multiple reproductive anomalies, among which is

apomixis. In his hypothesis, Carman assumes that polyploidy may result in asynchronous expression among the genomes contributed to the polyploid of different genes regulating megasporogenesis and megagametogenesis, and that this asynchrony might be responsible for the apomictic phenotype. According to Carman, polyploidy, or at least the existence of multiple copies of asynchronously expressed genes, is a causal factor for the expression of apomixis.

Endosperm Development

Dosage studies, mainly in maize (reviewed by Birchler 1993), have shown that a major factor influencing endosperm development is the dosage effect between the relative contributions of the male and the female genomes in the endosperm. In maize, a genomic ratio of 2 maternal doses to 1 paternal (2m:1p) is required for normal development, and even limited perturbations around that ratio can have strong deleterious effects on endosperm development and thus on the viability of the embryo. By contrast, apomictic plants seem to develop normal embryos with a great variety of maternal and paternal contributions that can strongly differ from the 2m:1p ratio needed in many sexually reproducing plants. In *Tripsacum*, for example, the endosperm seems to develop normally, even though the ratio of genomic contributions deviates from the 2m:1p ratio (Grimanelli et al. 1997). Indeed, ratios of 2:1, 4:1, 4:2, 8:1, and 8:2 can be observed, depending on both the ploidy level of the parents and mode of reproduction. Autonomous apomicts, in which the endosperm develops without fertilization of the central cell, also provide striking evidence that some adaptation to dosage response exists in apomicts.

Surprisingly little has been published about this specific aspect of apomictic reproduction. It is clear, however, that understanding the basis of endosperm development in apomicts is a critical step toward the utilization of apomixis in food crop production. Recent publications concerning the induction of seed and endosperm development in *Arabidopsis thaliana* (Grossniklaus et al. 1998; Vielle Calzada et al. 1999; Luo et al. 2000) and the specific role of Polycomb group-like proteins in the process have intensified interest in this issue. The application of these data to the production of apomictic plants, especially monocots, remains uncertain.

The Single-Gene Model Revisited

Most likely, many genes act to insure the viable development of an apomictic embryo. Most, if not all, of those genes also play a role in the development of sexual embryos, and so should be common to both apomictic and sexual development. But one or several alleles of some of these genes, or alternatively their regulation, must be specific to the apomictic plants. The challenge here is less that of understanding the fine genetic control of apomixis (the genes acting during the apomictic process), rather than identifying the specific alleles that must be transmitted to, or altered in, sexual plants in order to induce an apomictic mode of reproduction. The identification of these alleles is important for understanding the process of apomixis and crucial for the ultimate introduction of apomixis into crops.

Despite the complexity of the developmental process of apomictic reproduction, most genetic analyses of apomixis conclude that a simple mode of inheritance is involved. Studies on *Panicum, Ranunculus Hieracium, Tripsacum, Erigeron and Brachiaria* (see earlier references) show that apomixis segregates as a single, or eventually a few dominant loci. Such conclusions, however, should be taken with caution. The cited genetic analyses have been conducted mainly by crossing apomictic and sexual genotypes within species or genera,

and they are not necessarily informative when it comes to manipulating apomixis genes beyond their respective species. Indeed, it could well be that a single mutation in those species gave rise to an apomictic genotype. This does not rule out the possibility that several other genetic factors may be required to ensure the expression of apomixis. Such factors would not necessarily be detected through classical genetic analysis, simply because of a lack of polymorphism for those characteristics, but the factors would be revealed by manipulating apomixis beyond the limits of specific species or genera. Those characteristics, necessary but not sufficient, probably would have accumulated during the evolution of those species prior to their switch from sexual to apomictic modes of reproduction.

Several observations support this hypothesis. During various attempts to transfer apomixis from wild relatives to cultivated crops, the observed transmission of apomixis through generations of backcrossing did not conform to a simple genetic model. In the case of the maize-*Tripsacum* system, genetic data show that the expression of functional apomictic reproduction depends on a complex mode of inheritance (Leblanc, personal comm.; Savidan, Chap. 11). The conditions of endosperm development in pseudogamous apomictic grasses is another strong illustration of this hypothesis. Angiosperm apomicts evolved from sexual ancestors that may have been subject to dosage effects in the endosperm, as apparently many angiosperms have to a variable degree (see Birchler 1993). This suggests that some adjustments in the mechanisms governing endosperm development might have accompanied the evolution of apomixis; because the switch from sexual to apomictic reproduction simultaneously changes the genomic ratio of the endosperm, dosage requirements would have acted as a barrier against the emergence of apomixis by preventing endosperm formation. Hence, only families in which the regulation of endosperm development had somehow been modified would have been prone to the emergence of apomixis.

By the same token, it is conceivable that different families would have been inclined to different types of apomixis. Strong supporting evidence that different species are compatible with different forms of apomixis can be found in the phylogenetic pattern of distribution of the various forms of apomixis (Richards 1986; Asker and Jerling 1992; Mogie 1992; Carman 1997). Most apomictic taxa (75%) belong to only three families: Asteraceae, Poaceae, and Rosaceae, which together comprise no more than 10% of angiosperm species. Diplospory is common among the Asteraceae, but less so among the Rosaceae and the Poaceae, while apospory is common among the Rosaceae and the Poaceae, but less so among the Asteraceae. Autonomous apomixis appears to be restricted mostly to the Asteraceae and is found only infrequently in the Poaceae and Rosaceae. Clearly, the occurrence of apomixis and the distribution of its various forms are not random (Carman 1997). This might reflect (*i*) that not all taxa are compatible with the emergence of apomixis, and (*ii*) that different taxa are not compatible with the same types of apomixis.

Hence, introducing apomixis into otherwise sexually reproducing crops may depend on more than the few genes responsible for polymorphism in modes of reproduction within agamospecies. Other factors may need to be considered, such as the endosperm, that represent necessary conditions for the successful expression of the apomictic genes per se.

Applications of Molecular Genetics to Apomixis Research

What Material?

It could be speculated that the diffusion of apomixis in crops could be achieved through the isolation and manipulation of genes from a well-chosen model system. It is worth considering, then, whether this model could be defined for apomixis research. But is there solely one "universal apomixis," despite the amazing diversity of apomictic processes? In other words, should we consider the different types that have been described in the literature as different expressions of the same genetic components, or should different sources of apomixis be studied as distinct and unrelated processes? According to Sherwood (Chap. 5), a single gene might be responsible for the induction of both diplospory and apospory. Still, apomixis has occurred in a seemingly independent fashion in various taxa during their evolution through different processes, which might also be viewed as evolutionary convergence. Although answering these questions is undoubtedly an important long-term goal, given our current knowledge, the choice of a model system for apomixis research is more a matter of technical considerations. Some of those considerations are proposed by Bicknell (Chap. 8) in this volume, and include the ability for both in vivo and in vitro culture, a short generation time, easy hybridization, the availability of related sexual and apomictic biotypes, good characterization at the genome level, and ability for transgeny. Another important consideration is that using diploid apomicts greatly simplifies genetic analyses. Furthermore, access to efficient mutagenesis procedures, including transposon mutagenesis, would provide attractive tools for functional analyses.

While no known taxon fulfills all of the above criteria, researchers working on the genetics and molecular biology of apomixis have considered two alternatives. The first alternative is to use existing apomictic species that fulfill, as much as possible, the criteria described earlier. Bicknell proposed *Hieracium* as a model system and has been developing a transposon tagging approach for aposporous apomixis in that species (Chap. 8). On the other hand, the wealth of genetic information available in the grass families, including the remarkable level of genomic synteny found in the Poaceae (Bennetzen and Freeling 1993; Ahn and Tanksley 1993; Moore et al. 1995), make apomictic grasses an attractive model because the various forms of apomixis can be compared.

The second alternative relies on generating or transferring the components of apomixis into a well-characterized, easily handled organism, such as *Arabidopsis* or maize.Three approaches for this alternative have been proposed: (*i*) the transfer of apomixis from a wild species to a related and genetically well-studied crop through sexual hybridizations, (*ii*) the *de novo* generation of apomixis in normally sexual organisms by mutagenesis and manipulation of gene expression, and (*iii*) the *de novo* generation of apomixis through wide hybridization after selection of the appropriate parental reproductive phenotypes (based on Carman's hypothesis [Carman 1997, Chap. 7]). A review and discussion of the first two approaches follow later in this chapter. A description of the third approach may be found in Chapter 7.

Most current work in apomixis research essentially focuses on the very first event in the apomixis mechanism, i.e., the failure or absence of meiosis. This is partly a consequence of the prevailing hypothesis that apomixis processes in their entirety, or at the very least, apomeiosis, might depend on a single-gene regulation. As opinions evolve regarding this regulation, more effort will be

directed toward identifying the components required for the expression of functional apomixis and dissecting their genetic basis. Most of the works presented herein deal with apomeiosis. The strategies described, however, apply to most aspects of apomictic development.

Molecular Mapping of Apomixis

The first molecular work on apomixis essentially focused on the development of molecular maps and the localization of the DNA regions that control apomixis in various organisms. Part of the interest in developing genetic maps lays in the nature of molecular markers; their Mendelian inheritance is independent of either environmental conditions, or our ability to actually observe a given phenotype. Therefore, by studying their cosegregation with any trait of interest, one can identify and characterize chromosomal regions that play a role in the expression of that trait. Once mapped, any trait can theoretically be studied or followed—regardless of its expression and with a known confidence level—by detecting and analyzing the segregation of linked molecular markers.

Chapter 10 is devoted to the genetic improvement of apomictic cultivars. Most applications of molecular maps in plant improvement are also relevant to apomicts, and comprehensive reviews on such applications are readily available. Note, however, that molecular markers are particularly valuable for studying characters that are expressed late in plant development, such as apomixis or other reproductive traits. By using DNA markers, reproductive behavior can be rapidly predicted at the seedling stage, with confidence levels that depend mainly on the linkage between the marker and the mapped gene(s). Moreover, as opposed to cytoembryological tests, molecular marker analysis is not destructive.

DNA markers linked with both apospory and diplospory have been reported for apomictic *Pennisetum, Brachiaria, Taraxacum, Tripsacum, and Erigeron* species, among others (Ozias-Akins et al. 1998; Pessino 1997; Leblanc et al. 1995; Grimanelli et al. 1998b; Noyes and Rieseberg 2000). Interestingly, these diverse reports reach common conclusions about several aspects of apomixis. Taken together, they demonstrate that apomeiosis is likely controlled by one or several genes located on a single chromosome segment. Furthermore, reports on *Tripsacum* (Grimanelli et al. 1998a) *Pennisetum* (Ozias Akins et al. 1998) and possibly *Erigeron* (Noyes and Riesberg 2000) indicate that this segment might be characterized by a very strong restriction to recombination. In *Tripsacum*, where the mapping data could be compared between apomictic and sexual accessions, this restriction to recombination appears to be apomict-specific; while in the sexual forms the mapped alleles underwent a significant rate of recombination, complete linkage was observed in the apomict for the alleles detected by the same probes. Clearly, recombination is restricted at the tetraploid (apomictic) level as opposed to the diploid (sexual) level in both *Tripsacum* and maize, as seen in their RFLP maps. In *Pennisetum*, the segment itself seems to be apomixis specific, as revealed by Southern analysis.

Because the specific chromosome segment shows a restricted level of recombination, the classical model of monogenic inheritance for apomixis probably warrants a careful review, because regardless of the number of genes involved, they behave as a single locus in segregating populations. This number of genes might be particularly important within the framework of a gene isolation program.

Cloning the Apomixis Gene(s) Using Molecular Genetics Tools

A major difficulty encountered by those interested in cloning "apomixis genes" is simply defining what they are. Introducing apomixis into crops implies that specific genes are transferred or altered and expressed in the target crops. Most likely, not all of the genes involved in the apomictic process should be targeted: most, if not all, of them should already be present and playing a role in sexually reproducing plants. The issue then is which alleles of pertinent genes must be transmitted or manipulated for the induction and successful development of apomictic embryos and seeds. To date, all efforts to tag apomixis genes, including those presented in this paper, have focused on the mechanism of nonreduction, mainly because it is an excellent indicator of apomictic development and it is probably the easiest one to score. Nevertheless, it should be remembered that apomixis is probably more complex than the simple process of nonreduction. The importance of this constraint will likely emerge when attempts are made to synthesize *de novo* apomicts in sexual organisms

"Map-based" cloning in apomictic species. Once a gene has been located on a genetic map, subsequent efforts to specify its position can ultimately lead to its isolation (for the first successful efforts in plants, see Giraudat et al. 1992; Martin et al. 1994). The recent development of powerful new approaches for physically mapping chromosome segments combined with the ability to clone large DNA fragments (Burke et al. 1987; Shizuya et al. 1992), and progress in genome sequencing techniques have created new and higher standards for positional cloning in plants. It is still a laborious and risky task outside of a few well-characterized model genomes, but the number of genes cloned in this manner are rapidly increasing. However, positional cloning for apomixis is not very promising because most, if not all, of the candidate species for a map-based cloning project are highly heterozygous tetraploids, for which little genomic characterization exists.

Furthermore, when attempting positional cloning, the first step is to identify a chromosomal region, defined by two or more molecular markers, that flanks the gene under study. The precision of the estimated position of the gene is therefore limited by the smallest measurable recombination unit, meaning one recombinant in a given mapping population. Hence, the recombination level around the apomixis gene(s) presents another significant challenge: positional cloning will prove efficient only insofar as recombination can be observed near the locus of interest. As mentioned earlier, recombination near the apomictic alleles is very likely restricted, at least in *Pennisetum* and *Tripsacum*. Consequently, the smallest recombination unit defined by two markers that encompasses the apomixis locus might well be a relatively large amount of DNA.

Transposon tagging of apomixis genes. Some model plants, such as maize, rice, tomato, *Arabidopsis*, and *Petunia* have undergone extensive genome characterization. Specific approaches are available for gene tagging these plants that might be considered for tagging apomixis gene(s), provided that components of apomixis occur in one of these organisms.

A very promising approach is that of transposon tagging. Transposable elements are short DNA sequences that have the property to transpose to more or less random locations in the genome (see Walbot 1992, for a review). They were discovered in maize, but have since been identified or introduced in very diverse organisms. They have been used in a wide range of genetic studies, and have been found to be highly effective for gene tagging and cloning.

Transposon tagging in apomicts presents some constraints, including access to transposable elements and the genetic control of the trait. To the best of our knowledge, transposon activity has not been demonstrated in apomictic species. This might be overcome by introducing functional transposable elements into apomicts, either through transformation (as in *Hieracium*, Bicknell, Chap. 8) or through hybridization with a close relative (as with maize and *Tripsacum*, Grimanelli 1997). In both cases, maize transposable elements were successfully introduced into an apomictic background, and transposable activity was demonstrated.

In our view, the main issue concerning transposon tagging of apomixis is genetic control of the trait. While this approach is efficient for phenotypes controlled by single genes, it might yield no, or disappointing, results if apomixis is genetically more complex. But taken further, it would at least provide an elegant method to determine whether apomixis is controlled by one or several genes: if a single allele controls the trait, then a single mutation should allow complete reversion to sexuality; if a more complex system is involved, then individual mutations should lead to abnormal or only partial expression of the trait.

Candidate gene approaches. Although apomixis is unknown in major crop plants or other genetically well-characterized organisms, useful information can be derived from detailed analyses of the reproduction processes of select sexual organisms. For example, genes involved in the control of ovule development, the initiation of meiosis, embryogenesis, and endosperm development have been described in various organisms, and a close look at these genes might provide useful information about the regulation of apomixis. Such genes, but not necessarily their respective alleles, might represent prospective "candidates" for the apomixis gene(s), i.e., the gene(s) that would code for identical functions as their apomicitic counterparts. The best, though not the only, candidates are the yeast genes responsible for the induction of meiosis and the meiotic mutants identified in higher plants.

Major biochemical pathways involved in the regulation of the cell cycle and meiosis appear to be relatively well conserved between distant organisms such as yeast and higher plants, and the advance of whole-genome sequencing puts provides complete catalogs of putative candidate genes. This progress offers great promise, but it is tempered by the fact that it is usually difficult to verify whether a yeast gene of known function plays a similar role in plants. One powerful way to corroborate such gene functions is the so called "reverse genetics" strategy, using either insertional mutagenesis or homologous recombination. When based on transposon or T-DNA insertions, reverse genetics (or site-specific transposon mutagenesis) implies that transposon tagging is performed to identify individuals carrying a transposon insertion in a gene of known sequence. The expected function of that given gene can then be corroborated by confirming that its disruption leads to the loss or alteration of the expected function. Powerful reverse-genetic systems are available in various plant species, including maize, *Arabidopsis*, and tomato.

A specific candidate gene strategy based on comparative mapping can also be undertaken within the grass family. The identification of orthologous genes between species (i.e., genes that diverged from a common gene at the time that the species harboring them diverged) could be used to understand the relationships between the genes responsible for various components of apomixis in apomictic plants,

and meiotic or developmental mutants that are well characterized in sexual plants. Numerous mutants are known in grasses, especially in maize (Neuffer et al. 1997), for various aspects of sexual reproduction. Furthermore, large numbers of such mutants can be generated through classical (e.g., chemical) or transposon mutagenesis. Recent results of comparative mapping among grasses (Bennetzen and Freeling 1993; Ahn and Tanksley 1993; Moore et al. 1995) demonstrate that most grasses probably share the same basic set of genes, and that the obvious differences separating the species are based on allelic variations and not on their relative gene combinations.. Therefore, we suggest that the genes whose actions produce an apomictic phenotype in some grasses almost certainly can be found in sexual species. In this instance, comparative mapping could be used to identify genes in maize or some other sexual grass that are orthologous to the apomixis genes, and then use them to isolate their counterparts in the apomictic species.

The process of identifying maize orthologs of genes responsible for apomixis involves three successive steps: (*i*) candidate genes are identified through phenotypic characterization and genetic mapping; (*ii*) promising candidates are then isolated in maize; once cloned, the isolated genes are sequenced, and the sequence information is used to clone orthologous genes in the apomicts; (*iii*) the relationship between the alleles isolated in the previous steps and the expression of apomixis is confirmed using a reverse genetic strategy in apomictic plants. For step *iii*, the construction of transposon tagging populations in apomicts are of great interest to R. Bicknell and the CIMMYT apomixis team.

Three criteria can be employed to select candidate genes: (*i*) because apomixis often affects only the female function, we propose that the gene(s) responsible for the failure of meiosis have a megasporogenesis-specific phenotype, meaning that mutants of interest should affect only the female function; (*ii*) as in diplosporous plants, the candidates should affect early stages of meiosis, ideally, the induction of meiosis; meiotic mutations acting at later stages in meiosis are probably not directly related to apomixis; and (*iii*) interesting candidates should be able to produce unreduced gametes, (thus, as in apomictic plants, the completion of unreduced gamete formation implies that the checkpoints (Hartwell and Weinert 1989), which usually act during the meiotic cell-cycle to ensure the production of normally reduced haploid gametes, failed to override abnormal behavior. With aposporous-like mutants, obvious phenotypes relate to the induction of megagametogenesis in somatic cell. Sheridan et al. (1996) describe a remarkable example of this type of mutant.

Manipulation of gene expression in model species: To date, this is probably the most widely used approach for developing apomictic cultivars, (details are discussed elsewhere in this volume). Current work centers on large-scale mutant screening in *Arabidopsis* and *Petunia* (Jefferson and Bicknell 1996; Ohad et al. 1996; Chaudurhy et al. 1997; Grossniklaus et al 1999; Luo et al. 2000). The best prospect from these approaches would be the engineering of a mode of apomixis that better meets the requirements of agricultural production than the apomixis mechanisms found in the wild (see Jefferson and Bicknell 1996, and Chap. 8). The remarkable results obtained recently with a set of mutations in Polycomb-related genes in *Arabidopsis* (Grossniklaus et al. 1998; Luo et al. 2000) are very encouraging. They demonstrate that phenotypes related to apomixis may eventually be obtained by manipulating the expression of genes involved in sexual reproduction, without reference to apomixis as seen in the wild.

Conclusions

Our understanding of the genetics of apomixis is changing rapidly, from the idea that a simple genetic system might control the whole developmental process, to a more integrated conception and sophisticated models. Part of that evolution stems from the application of molecular genetic technologies to the study of apomixis. Still, many important questions and problems remain unresolved; there is no shortage of challenges in the field of apomixis research. Many serious research efforts may only serve as preliminary and somewhat academic steps toward the long-term goal of introducing apomixis into farmers' fields. To reach the distant goal of deployment to farmers, future research should include an assessment of the social and economic impact of apomixis, and a definition of adequate deployment strategies. These critical elements will strongly influence the biological aspects of apomixis research and what "kind" of apomixis should be targeted for development and deployment.

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Chapter 7

The Gene Effect: Genome Collisions and Apomixis

JOHN G. CARMAN

Introduction

In the vast majority of angiosperms, female meiosis results in the formation of a tetrad of mononucleate megaspores, of which three degenerate and one forms the genetically-reduced 8-nucleate female gametophyte (Polygonum-type embryo sac). Consistently-expressed cytological deviations from this norm occur in certain species in 506 of the 13,479 genera of angiosperms recognized by the Kew Botanical Gardens (Carman 1997). However, most species in most of these 506 genera reproduce normally. Thus, the percentage of species consistently expressing reproductive anomalies (probably < 0.3 %) is far less than the percentage of genera (3.8 %) currently known to contain anomalous species. Reproductively-anomalous species occur in at least 184 families, which is 53 % of those in which some embryological analyses have been reported, and are much more abundant in some families than in others (Carman 1997).

The reproductive anomalies considered in this chapter generally belong to three categories: gametophytic apomixis, polyspory, and polyembryony. Gametophytic apomicts produce unreduced embryo sacs that contain parthenogenetic eggs, are generally polyploid, and occur in at least 33 of 460 families of angiosperms. Diplospory and apospory are two major subdivisions of gametophytic apomixis (referred to as apomixis hereafter) and occur when unreduced embryo sacs form precociously from ameiotic megaspore mother cells (MMCs) or nearby somatic cells, respectively (Asker and Jerling 1992; Carman 1997; Peel et al. 1997a, b; Crane, Chap. 3). Polysporic species (bisporic or tetrasporic) are sexual and occur in at least 88 families. As in diplospory, embryo sacs of polysporic species form precociously from MMCs, but only portions of meiosis not critical to genetic reduction are affected (Battaglia 1989; Johri et al. 1992; Carman 1997). Polyembryony involves the formation of embryos from cells of other embryos, synergids, antipodals, nucelli, integuments, and even leaves (Tisserat et al. 1979; Johri et al. 1992). Like parthenogenesis in apomicts, polyembryony often begins before pollination (Naumova 1993). This chapter summarizes these anomalies in terms of developmental similarities, phylogenetic associations, and gene effect hypotheses, and it discusses implications of the gene effect hypotheses for future research and plant improvement. It concludes that a major purging of some widely accepted dogma concerning the evolution and genetic regulation of apomixis will probably occur as the mechanisms involved give way to more accurate evolutionary, developmental, and molecular characterizations.

Developmental Biology and Phylogeny of Reproductively-Anomalous Species

At the developmental level, some apomictic mechanisms resemble sexual polysporic mechanisms more than other apomictic

mechanisms (Figure 7.1). For example, Antennaria-type diplospory is identical to tetraspory (sexual), except that the nuclear divisions leading to a tetranucleate embryo sac are meiotic in tetraspory but mitotic in Antennaria-type diplospory. Both Antennaria-type diplospory and tetraspory occur in *Antennaria*, *Erigeron*, *Limonium*, and *Rudbeckia* (Carman 1997). In both anomalies, MMC lack callose (Peel et al. 1997a). Ixeris-type diplospory is even more similar to tetraspory because, as in tetraspory, a meiotic prophase occurs. However, in the diplosporous mechanism, a first division restitution ensues. Ixeris-type diplospory is identical to bispory (sexual) except that meiosis I fails in the former (Figure 7.1). Both Ixeris-type diplospory and bispory (and apospory and tetraspory) occur in *Erigeron*. Allium odorum-type diplospory and bispory differ only in that a chromosomal endoreduplication occurs in the former. Both are found in *Allium*. The recognition of these developmental similarities and phylogenetic associations led Carman (1997) to analyze taxonomic data for all known species that express these anomalies. It was found that apomictic, polysporic, and polyembryonic species are polyphyletic and tend to be phylogenetically related. Many highly significant associations were discovered.

The variation represented in Figure 7.1 is somewhat continuous. For example, Antennaria-type diplospory is similar to both tetraspory and apospory. In some families, both species and genera span this continuum. For example, apomixis and polyspory occur together in 13 of 127 apomixis-containing genera (*Allium*, *Antennaria*, *Burmannia*, *Cordia*, *Cynoglossum*, *Erigeron*, *Eurybiopsis*, *Leontodon*, *Limonium*, *Rubus*, *Rudbeckia*, *Sambucus*, and *Tridax*) and 18 of 33 apomixis-containing

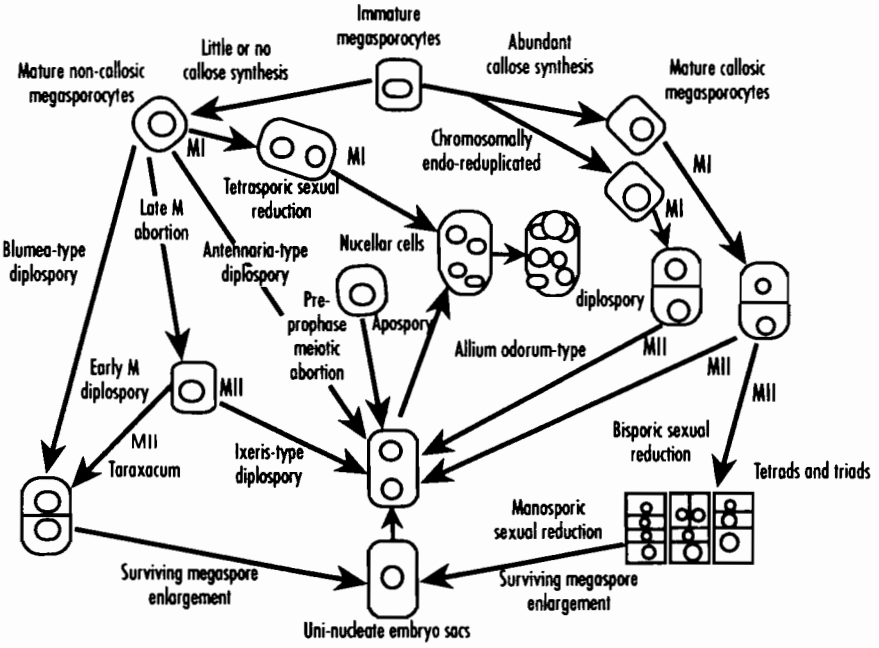

Figure 7.1 Developmental stages during megasporogenesis and embryo-sac development in selected sexual (monosporic, bisporic, and tetrasporic) and apomictic (Allium odorum-type diplospory, Antennaria-type diplospory, Taraxacum-type diplospory, Ixeris-type diplospory, Blumea-type diplospory, and apospory) angiosperms.

families. Differences between observed and expected values for these associations, assuming independent distribution, were highly significant (Carman 1997). Such developmental similarities and phylogenetic associations suggest that the gene effect mechanisms responsible for apomixis are in some way mechanistically and evolutionarily related to those responsible for polyspory and polyembryony. Hence, gene effect hypotheses that explain apomixis should also explain the related anomalies.

Two other female anomalies appear mechanistically and phylogenetically related to apomixis and polyspory. Both repeat a segment of female development in an asynchronous manner. The first, preleptotene chromosome condensation, inserts an additional mitotic prophase between premeiotic S and leptonema. The chromosomes condense to a mitotic metaphase state, decondense, and resume meiotic prophase (Bennett and Stern 1975). This phenomenon is similar to Allium odorum-type diplospory (see Crane, Chap. 3) in that a segment of the cell cycle (prophase, not S) is duplicated before meiosis. Preleptotene chromosome condensations occur in certain species of *Lilium*, *Tradescantia*, *Trillium*, *Fritillaria*, *Nicotiana*, *Vicia*, and *Psilotum*, with the first five also containing polysporic species.

In the second anomaly, observed in Rosaceae, an apparently normal MMC forms, degenerates, and is subsequently replaced by one or more secondary and fully functional MMCs. This anomaly occurs in *Alchemilla*, *Cotoneaster*, *Sorbus*, *Rubus*, *Aphanes* (Davis 1966) and *Waldsteinia* (Czapik 1985), all of which also contain apomictic or polysporic species (Czapik 1985; Carman 1997). The fact that preleptotene chromosomal condensations and repetitive MMC formations occur in genera that in almost all cases contain species expressing apomixis or polyspory is strong evidence that certain genera are particularly vulnerable to evolutionary processes that *asynchronize* ovule development. Several possible reasons for this vulnerability have surfaced from our examinations of the genomic makeup of reproductively-anomalous species.

Genomes of Reproductively-Anomalous Species

High chromosome base numbers ($x \geq 10$) suggest paleopolyploidy, which means polyploidy followed by diploidization with or without ascending or descending aneuploidy. Multiple base numbers per genus reflect diploidization following ascending or descending aneuploidy and is further evidence of paleopolyploidy (Goldblatt 1980; Lewis 1980). Carman (1997) found chromosome base numbers for 80% of all genera identified as containing apomictic, polysporic, or polyembryonic species. Statistical analyses indicated that polysporic and polyembryonic species are generally paleopolyploid (x = 15.7 and 13.2, respectively), while many apomicts, which are generally polyploid (Asker and Jerling 1992), contain primary genomes (x = 9.6). Furthermore, genera with polysporic but not apomictic species have more x values per genus (2.7 ± 0.4 SE) than genera with apomictic but not polysporic species (1.7 ± 0.1). This means apomicts tend to have balanced sets of duplicate genes (primary genomes) and polysporic and polyembryonic species tend to have imbalanced sets of genes (paleopolyploid genomes, i.e., partially duplicated or triplicated due to aneuploid series formation followed by diploidization). Thus, a distinct divergence in genome composition occurs between apomixis on the one hand and tetraspory and polyembryony on the other, even though these anomalies are phylogenetically and mechanistically related. Hence, gene effect hypotheses attempting to

explain the existence of apomixis must also address these highly significant peculiarities in genome composition, phylogenetic relatedness, and developmental (or mechanistic) affinities.

Other genome-related factors may abnormally affect female development in polyploids or paleopolyploids. For example, meiotic rates are linearly correlated with DNA content, but the regression line is much steeper in polyploids. And, meiosis in tetraploids usually requires the same period of time as in related diploids containing half the DNA. This occurs because genes for meiosis in polyploids are duplicated (Bennett 1977). That the meiotic rate to DNA content regression slopes in paleopolyploids reflect either a diploid or a polyploid condition may be critical to the evolution of apomixis and related anomalies, Examples include *Scilla nonscriptus*, $2n = 2x = 16$, which belongs to an aneuploid series with $x = 6$ to 9, 15, and 17 as stabilized base numbers, and *Convallaria majalis*, $2n = 2x = 38$, with a sole base number of $x = 19$. Both are paleopolyploid diploids with large quantities of DNA, but their meioses occur in only 50% of the time predicted for non-paleopolyploid diploids with similar amounts of DNA, i.e., they behave as polyploids. In contrast, *Ornithogalum virens* ($2n = 6$), which is at the bottom of a descending aneuploid series in which $x = 3$ to 5 and 7, *Allium cepa* ($2n = 16$), which is at the middle of an aneuploid series with $x = 7$ to 9, and *Fritillaria meleagris* ($2n = 24$), which is at the top of an ascending aneuploid series with $x = 7$, 9, and 12, are paleopolyploid diploids with slow meioses that is indicative of diploids with considerable DNA. Duplicate genes for meiosis in these species have either been lost through aneuploidy or genetically silenced. Hexaploid nulli 5B tetra 5D wheat is another example. Meiotic rates in this line reflect a tetraploid, not a hexaploid, probably because of imbalanced sets of meiotic genes (Bennett 1977).

Several questions relevant to the evolution of female developmental anomalies can be formulated from this information. For example, how is the synchrony of female development affected when some of the duplicated genes responsible for megasporogenesis, embryo-sac development, and embryony from one of two genomes are silenced or lost during diploidization (paleopolyploid formation)? What happens when there are duplicate doses of genes for certain stages of meiosis or embryo sac development and not other stages, as is anticipated in highly aneuploid paleopolyploid polysporic species? Could such imbalances cause some of the anomalies of embryo sac development observed in polysporic species, such as a precocious gametophytization of the MMC or the formation of 4 to 32 nucleate embryo sacs?

Total quantities of DNA also influence the types of life cycles angiosperms assume. For example, species of *Fritillaria* (many of which are polysporic paleopolyploids) have large amounts of nuclear DNA and their meioses may require 3–4 weeks to complete. In contrast, annuals have little DNA and very short meioses (Bennett 1977), and apomixis and related anomalies are rare among them (Asker and Jerling 1992). Hence, a minimum threshold in duration of meiosis may be a prerequisite for the evolution of certain reproductive anomalies.

Reproductive anomalies in angiosperms might also be influenced by differences in meiotic durations between genders. In cereals, female and male meioses are generally synchronous and similar in duration. However, in species in which female meiosis occurs later than male meiosis, differences in duration may be as great as 50 % (Bennett 1977). Such differences might encourage anomalous development in one gender but not the other.

Finally, ecotype divergences resulting from divergent selection pressures associated with high versus low latitudes (flowering responses to specific photoperiods), high versus low elevations, highly shaded versus full sun conditions, or moist versus dry conditions may contribute to the evolution of apomixis and related anomalies. Many plants have been categorized according to their response to photoperiod. For example, long-day plants are adapted to higher latitudes and flower in the spring and early summer when days are long. Short-day plants are often found in lower latitudes (tropics) and often flower during the tropical "winter" when days are short. Dual-day-length plants require either short or long days to induce reproductive bud formation, followed by long or short days, respectively, to cause the formed buds to mature into flowers. Intermediate-day plants will not flower if days are too long or too short. Day-neutral plants show little adaptation to day length and flower induction occurs under a broad range of day lengths. In addition, several other specialized categories exist.

Salisbury and Ross (1992) selected 78 species from among approximately 300 species of plants studied for flowering responses to different photoperiods. These 78 species were chosen independently of any reproductive anomalies that might be expressed either within themselves or within other species in the genera they represent. In contrast, they were chosen to distinctly represent specific photoperiod response categories. Sixty-seven different genera are represented by these 78 species. It is interesting to note that 33% of these genera (22 of 67) contain species with female reproductive anomalies (gametophytic apomixis, polyspory, or polyembryony; compare Salisbury and Ross 1992, Table 23-1, with the appendix in Carman 1997). This is a ninefold increase in the number of genera expected if reproductive anomalies occur independently of adaptations to distinct photoperiod responses, i.e., only 3.8% of genera are known to express reproductive anomalies. Thus, if reproductive anomalies occur independently of distinct adaptations to photoperiod, then only 3.8% (not 33%) of the genera identified as containing species with distinct photoperiod adaptations should have also expressed reproductive anomalies. This 33% breaks down as follows: 12% contain gametophytic apomicts (compared with 1% of all angiospermous genera, i.e., a 12-fold increase); 13% contain polysporic species (compared with 1.6% of all angiospermous genera, i.e., an eightfold increase); and 7.5 % contain polyembryonic species (compared with 1.7% of all angiospermous genera, i.e., a 4.4-fold increase). These associations between genera containing species that express reproductive anomalies and genera containing species that express distinct adaptations to photoperiod (different latitudes, etc.) should be studied in more detail, as they suggest that photoperiod responses either contribute to the evolution of reproductive anomalies or are at least correlated with unknown causal factor(s) (see Carman 2000).

These data indicate that apomictic polyploids could contain interracial or interspecific genomes polygenically "coadapted" (Wallace 1991) to divergent environmental conditions. If true, the sexual progenitors of apomicts could have expressed cytologically-detectable temporal divergences in gross ovule development relative to the time at which female meiosis occurs. Such differences have now been observed among the putative sexual diploid progenitors of apomictic *Antennaria rosea* and *Tripsacum dactyloides* (Carman and Kowallis, unpublished), and these divergences probably represent coadaptions to different photoperiods (latitudes or elevation), shading regimes (full sun or highly shaded environments), elevations (duration of growing season), or other environmental

parameters affecting whether meiosis and embryo-sac formation occur early or late during floral bud development. Hybridization among ecotypes expressing such divergent floral development schedules may cause apomixis in the absence of mutation (Carman 1997, 2000).

The Gene Effect Hypotheses

Insights concerning the molecular control of female development can be gleaned by studying the many developmental, phylogenetic, and genomic peculiarities that apomicts share with polysporic and polyembryonic species (Table 7.1). The gene effects that ultimately explain apomixis will probably also largely explain these peculiarities. Several gene effect hypotheses are presented below and are judged by how well they explain the data of Table 7.1.

The Callose Hypothesis

In 1984, Charles Crane, working in the author's lab, noted that MMC walls of diplosporous lines of *Elymus rectisetus* were abnormally thin. This was the first indication that diplospory might be similar to tetraspory (Rodkievitz 1970) with regard to an absence of MMC callose. The molecular sieve properties of callose (Heslop-Harrison and Mackenzie 1967) led Crane and Carman to hypothesize that the absence of callose may allow regulatory factors necessary for meiosis to diffuse away from the MMC and/or allow regulatory factors responsible for mitosis to diffuse into the MMC from the nucellus or integuments. This callose hypothesis was formally discussed in 1986 (Carman 1986; Carman and Crane 1986). Subsequent reports (Crane and Carman 1987; Carman et al. 1991) suggested that the deficiency in callose deposition around MMCs of apomictic *E. rectisetus* may only be coincidental to a more fundamental genetic lesion that causes apomixis. The latter report (Carman et al. 1991) documented that sexual MMCs from diplosporous lines of *E. rectisetus*, which usually constitute less than 5% of all MMCs in this facultative apomict, are richly enveloped by callose, as in normal sexual species.

Table 7.1 Phylogenetic, genomic, and developmental peculiarities that hypotheses for the genetic regulation of apomixis and related reproductive anomalies must explain

Phylogenetic
Apomixis, polyspory, and polyembryony are rare yet polyphyletic
Apomixis, polyspory, and polyembryony tend to occur in the same species/genera/families
MMC degeneration/replacement occurs in apomictic or polysporic genera
Preleptotene chromosomal condensations tend to occur in polysporic genera The majority of apomicts evolved during the past three million years, i.e., during the last 2% of the evolutionary existence of angiosperms (see Carman, 2000).
Genomic/developmental
Chromosome base numbers are low in apomicts and high in polysporic/polyembryonic species
Apomicts tend to be genome-balanced, polysporic/polyembryonic species tend not to be
Apomixis, polyspory, and polyembryony are generally absent in annuals (low amounts of DNA/rapid meioses)
Paleopolyploids may behave as diploids or polyploids with respect to meiotic duration
Polygonum-type reproduction is facultative in apomictic, polysporic, and polyembryonic species
Tendencies to apomixis are observed in some wide hybrids including *Raphanobrassica* (consistent aposporous embryo sac formation)
Male and female meioses in some species start at different times and have different durations
Apomixis, polyspory, and polyembrony tend to occur in genera capable of strong adaptations to photoperiod
Apomixis, polyspory, and polyembryony are characterized by asynchronously-expressed substitutions, replacements, or duplications of discrete reproductive phases Most apomicts display relaxed endosperm balance number requirements (see Grimanelli et al., Chap 6; Grossniklaus, Chap 12)

Since these first reports, the absence of callose around diplosporous MMCs has been documented in *Poa nemoralis* (Naumova et al. 1993), various *Tripsacum* species (Leblanc et al. 1995; Peel et al. 1997a), *Eragrostis curvula* (Peel 1997a), and *Antennaria rosea* (Carman, unpublished). Although usually present, callose envelopment of MMCs is often abnormal in aposporous apomicts (Naumova et al. 1993; Naumova and Willemse 1995; Peel 1997a), and these abnormalities may be related to the time at which aposporous embryo sacs are initiated (Peel et al 1997a).

The callose hypothesis states that a genetic lesion(s) frequently prevents or reduces callose deposition in the walls of MMCs. This causes apomixis by allowing developmental signals to move in a less restricted manner through the ovule, thus confusing development. If this underlying concept is correct, i.e., if a role of callose is to contain developmental signals, we would expect to see this mechanism associated with other reproductive anomalies. However, MMC callose is generally not lacking in apospory or bispory, and no apparent connection exists between absence of MMC callose and parthenogenesis or the proliferation of embryo-sac nuclei in various forms of tetraspory (Johri et al. 1992). The callose hypothesis also fails to address (*i*) genome differences between apomicts and polysporic or polyembryonic species, and (*ii*) the occurrence of "tendencies for apomixis" that occur in many wide hybrids (Asker and Jerling 1992; Carman 1997).

The Precocious Induction Hypothesis

That apospory is caused by the precocious and ectopic expression of a normal master gene for embryo sac formation (embryo sac induction gene, *Esi*) was proposed by Peacock (1993), who suggested that the precocious expression of *Esi* may be caused by a single mutation. If this is correct, inducing mutations in maize, rice, or *Arabidopsis* could produce apomixis. The precocious induction hypothesis is attractive because it explains precocious initiation of embryo-sac formation in apospory, diplospory, tetraspory, and bispory. However, the single-mutation-based induction of *Esi* fails to explain other features of female reproductive anomalies not temporally or spatially associated with embryo-sac induction (Koltunow 1993).

In many reproductive anomalies, two or more developmental processes occur simultaneously and asynchronously (Figure 7.1). During apomixis, meiosis and embryo-sac formation often occur simultaneously, and parthenogenesis is often initiated before fertilization of the central cell. Preleptotene chromosomal replications and condensations, MMC abortions and subsequent replacements, and elimination or duplication of nuclear divisions resulting in from 4 to 32-nucleate tetrasporic embryo sacs would require additional mutations. The mutation-based nature of this hypothesis also fails to explain why reproductively-anomalous taxa are phylogenetically related and why apomicts are generally polyploid with balanced genomes and low chromosome base numbers, while polysporic and polyembryonic species are typically paleopolyploid with unbalanced genomes, high chromosome base numbers, and multiple base numbers per genus (see Table 7.1 for other phylogenetic, genomic, and developmental correlations and phenomena not readily explained by simple mutation). Thus, while the developmental displacement component of this hypothesis is intriguing, its mutation-based explanation is questionable.

The Hybridization-Derived Floral Asynchrony Theory

The hybridization-derived floral asynchrony (HFA) theory expands on the developmental displacement component of the precocious induction hypothesis but suggests a non-mutation origin. The foundation of this theory

was developed in 1994 by the author while attempting to reconcile simple inheritance for apomixis (the prevailing opinion at the time; Nogler 1984; Asker and Jerling 1992; Mogie 1992) with (*i*) the many apparent asynchronous replacements, competitions, and duplications of discrete developmental segments in reproductively-anomalous species (Figure 7.1; many phenomena in addition to embryo-sac induction), and (*ii*) the fact that nearly all apomicts are polyploid. The author concluded that such a reconciliation is unreasonable. According to HFA theory, apomixis occurs when hybrids are produced from ecotypes that are distinctly divergent with respect to their start times and rates of MMC formation, meiosis, embryo sac formation, and embryogenesis relative to gross ovule development. Such "genome collisions" (terminology suggested by Sven Asker, personal comm., 1997) explain the abundant duplicity and asynchrony of development depicted in Figure 7.1.

In 1994, the author conducted a preliminary search of the literature to determine if polysporic and polyembryonic species contain multiple genomes, i.e., whether they are polyploid. A negative result was soon obtained, which seemed to deal a fatal blow to this fledgling multi-genome "asynchrony hypothesis." However, in studying the genome composition of the polysporic and polyembryonic diploids, it was found that these "diploids" generally have high chromosome base numbers indicative of paleopolyploidy. The author concluded that if the HFA theory is correct, the base number trends observed in the preliminary 1994 study should hold in a large-scale study of all known apomictic, polysporic, and polyembryonic species. The theory survived the large-scale examination, was refined, and additional hypotheses concerning the origins of apomixis and its role in the evolution of some reproductively-novel polysporic and polyembryonic species and genera were developed (Carman 1997; Peel et al. 1997a, b).

The HFA theory states (*i*) duplicate sets of genes encoding female developmental pathways exist in interracial or interspecific hybrids, polyploids, mesopolyploids, and paleopolyploids; (*ii*) polygenic "heterozygosity" for photoperiodic floral induction and start times and durations of MMC formation, megasporogenesis, embryo-sac formation, endosperm formation, and embryony, is the primary cause of apomixis, polyspory, polyembryony, and related anomalies; (*iii*) allopolyploidy or segmental allopolyploidy is often required for apomixis because it prevents or greatly reduces the incidence of genetic recombination between genomically-isolated sets of parental genes, which otherwise would lead to recombination among the many genes required for apomixis, resulting in reversion to sexuality (Carman, in preparation); (*iv*) polyploidy also influences apomixis by influencing the timing and duration of meiosis (Bennett 1977) and because divergent genomes are probably more prone to be physically partitioned in the nucleus (Leitch et al. 1990) when present as homologous pairs and thus more functionally independent (Carman 1997); and (*v*) mutations are of secondary importance and may improve reproductive fitness through null-allele formation in one or both genomes. This theory is consistent with current models of developmental gene expression, including (*i*) the ABC model, in which floral genes from a B cassette are expressed only when genes from an A cassette are expressed (the expression of C genes requires expression of B genes, etc.) (Theissen et al. 2000), and (*ii*) checkpoint models, in which precocious expression of checkpoint genes causes developmental phases to be skipped, e.g., fusing G_1-phase yeast cells with M-phase cells causes G_1 nuclei of

heterokaryons to skip S and G2 and proceed precociously to mitosis (Lewin 1994).

Many examples of checkpoint phenomena can be hypothesized from an examination of Figure 7.1. For example, if embryo-sac development signals from one genome are superimposed on megasporogenesis signals from another genome, meiosis may be skipped (diplospory), similar to the heterokaryon examples described above, or embryo-sac development may be ectopic (apospory). Accordingly, apomictic-like tendencies would occur in polyploids only if major differences in timing of megasporogenesis and embryo-sac development (relative to other ovule and ovary tissues) exist among the ancestral ecotypes or species (Figure 7.2). Such natural variation may be infrequently found in highly cosmopolitan genera, i.e., genera with broad latitudinal and ecological distributions, but possibly absent in less cosmopolitan genera.

The HFA theory was refined using criteria tabulated in Table 7.1. Hence, it explains these criteria as well as many inconsistencies in the apomixis literature. For example, apomixis, polyspory, and polyembryony are rare but tend to occur together in cosmopolitan families, such as Poaceae, Asteraceae, and Rosaceae, because sufficient ecotypic variation in reproductive start-times, etc., is rare in most families but high in these cosmopolitan families. Sexual reproduction of the monosporic Polygonum-type occurs facultatively in apomictic and polysporic species because, barring deletions or mutations, each parental genome contains genes required for normal reproduction, and growing conditions may occasionally favor the expression of one genome over the other, causing sexual development to occur. Facultativeness may be influenced by (*i*) differential silencing of genomes (epigenetic

Figure 7.2 Model of how asynchronously-expressed duplicate genes cause diplospory and apospory in polyploids containing two genomes divergent in the temporal expression of female developmental schedules (floral induction, megaspore formation, gametophyte development, and embryony). Bolded developmental phases are skipped as described below.

	Developmentally-critical stages*			
Genome	**1**	**2**	**3**	**4**
Genome I (unmodified)	Archespore	**Meiosis**	**Embryo sac**	**Double fertilization/ early embryony**
Genome I (modified)		Embryo sac	**Double fertilization/** early embryony	Fertilization of central cell only
Genome II	**Meiosis**	Embryo sac	**Double fertilization/** early embryony	Fertilization of central cell only

* Ovule development is initiated by developmental signals from genome I, which polygenically encode adoptations for late meiosis relative to gross ovule development, and genome II, which polygenically encode adaptations for early meiosis relative to gross ovule development

I. 1. At the beginning of stage 1, genome II produces signals for meiosis, which fail because the MMC is not ready for meiosis, i.e., it develops at an intermediate rate dictated by the intermediate phenotype.

II. 2. At the beginning of stage 2, embryo sac development signals from genome II may synchronize genome I with genome II in a manner similar to that observed in asynchronous yeast heterokaryons (reviewed herein). If meiosis is successfully preempted, one of several forms of diplospory (Fig 1) occurs, i.e., an embryo sac forms precociously from the megasporocyte (*Antennaria*-type diplospory) or young female meiocyte (*Taraxacum* or *Ixeris*-types of diplospory). If meiosis is unsuccessfully preempted, either facultative sexuality or apospory (Fig 1) occurs. In the latter case, one or more embryo sacs form from adjacent nucellar cells. This occurs primarily in species containing multiple or ill-defined archegonial cells. In both apospory and diplospory, a genetically unreduced embryo sac develops. Development of the nongametophytic tissues of the ovule and ovary continues to occur at a normal rate. In contrast, embryo sac development continues to occur precociously because of the precociously-expressed embryo sac development genes of genomes I and II.

III. 3. Precocious signals from the two synchronized genomes induce egg formation and parthenogenesis, both of which occur precociously relative to the development of nongametophytic ovule and ovary tissues.

4. Pollination occurs according to the intermediate phenotype schedule, but the egg is no longer receptive and in many cases has already divided. The central cell, if not autonomous, is fertilized, and the endosperm and parthenogenetic embryo develop.

effects), which could be caused by differences in genetic background, or (*ii*) environmental factors that reduce the degree of asynchrony by accelerating or decelerating gene expression from one genome relative to that of another (photoperiod or temperature responses, e.g., as occurs in *Dicanthium, Themeda*), thus allowing sexual development to occur facultatively (Carman 2000).

According to the HFA theory, polyspory and polyembryony result from the competitive expression of grossly imbalanced genomes (incompletely duplicated sets of reproductive genes) in which some checkpoint systems are missing. In contrast, competitive expression among genomes is terminated by checkpoint genes in apomicts, which generally contain balanced sets of reproductive genes (Carman 1997), thus allowing a somewhat smooth transition to apomixis (Figure 7.2).

Apomixis is much more prevalent among outcrossing species than inbreeding species (Asker and Jerling 1992). This is consistent with the HFA theory because outcrossing species are much more prone to form interecotypic or interspecific polyploids when secondary contacts occur, e.g., during the numerous major climatic shifts associated with the Pleistocene glaciations (Frakes et al. 1992; Carman 2000). Likewise, more apomicts are allopolyploid than autopolyploid because polyploidization by B_{III} hybrid formation is expected to occur more frequently in nature in interspecific hybrids than interracial hybrids. Similarly, the chances of B_{III} hybrid formation occurring in interecotypic or interspecific F_1 hybrids that are sterile and annual are low compared to their formation in sterile perennials, which may flower annually for many years. This factor limits the chances of annuals becoming apomictic and explains their low frequency in nature.

The HFA theory also predicts ambiguous outcomes regarding the sexuality of progeny when an apomict is crossed with a sexual or with another apomict, regardless of the closeness or wideness of the cross. The mode of reproduction expressed in the progeny will depend on how the added or removed genome(s) affect asynchrony, and this cannot be predicted without some a priori knowledge of the female developmental schedules encoded by the involved genomes (Carman 1997). That these many inconsistencies in the apomixis literature are explained by the HFA theory is strong evidence for its validity.

Testing the Gene Effect Hypotheses

If apomixis is the result of one or a few mutations, similar artificially induced mutations might produce apomicts from sexual species. Research programs currently exploring this possibility are reviewed by Bicknell (Chap. 8), Grossniklaus (Chap. 12), and Praekelt and Scott (Chap. 13). Likewise, simple inheritance should permit transfer of apomixis gene(s) to sexual species. To date, introgression projects have failed to confer apomixis upon sexual species by adding anything less than at least one complete alien chromosome (Savidan, Chap 11). Kindiger et al. (1996) reported a condition that might lead to an exception. They isolated, from their maize-*Tripsacum* backcross program, a line (30 Mz + 9 Tr chromosomes) that appears to contain a maize *Tripsacum* translocation possessing gene(s) for apomixis. However, Blakey et al. (1997, reviewed below) determined that the genes required for apomixis occur in five distinct *Tripsacum* linkage groups that are syntenic to regions on three maize chromosomes. These data cast doubt on Kindiger's simple inheritance model.

If apomixis is caused by hybridization, the parental phenotypes (divergent floral induction stimuli and meiotic start times in ovules, etc.) may be identified by a combination of phenological and cytological studies. Because many developmental pathways occur asynchronously in apomicts (Figure 7.1), it may be possible to use molecular probes to determine if the asynchronous signals originate from the same genomes or different genomes. For example, do both genomes in a tetraploid apomict produce both meiotic and embryo-sac development signals at the same time, or does one genome produce meiotic signals (and not embryo-sac development signals) at the same time the other genome is producing embryo-sac development signals (see Figure 7.2)?

Many criteria should be considered in testing for such asynchrony. First, it would be desirable for the apomict to (*i*) be allotetraploid with known sexual diploid progenitors, (*ii*) contain well-mapped genomes, (*iii*) be amenable to transposon tagging, (*iv*) be easily grown with a short vegetative phase, and (*v*) have ovules readily accessible to cytological analyses. At present, no apomict meets all of these criteria. Second, molecular probes that recognize mRNAs specific to different developmental stages would need to be produced, and the genes from which they are developed would need to contain adequate intergenomic sequence divergence such that probes unique to each genome could be produced. Such probes may currently be under development.

Portions of meiotic prophase and early embryo-sac development occur simultaneously in most aposporous apomicts and all Taraxacum- and Ixeris-type diplosporous apomicts (Carman 1997; Peel et al. 1997a). The HFA theory would be confirmed if the following two conditions are observed: (*i*) a probe unique to meiotic prophase of genome A plus a probe unique to early embryo-sac development from genome B detect their respective genome-specific mRNAs in ovules fixed and sectioned during meiotic prophase, and (*ii*) the probe for meiotic prophase from genome B does not detect its respective genome-specific mRNA product (or vice versa). This would confirm that one genome codes for meiosis (but not embryo-sac development) at the same time the other genome is coding for embryo-sac development. This test would not be valid if the mRNA synthesis identified by the respective probes is produced in response to *trans*acting regulatory genes.

Implications of the HFA Theory

If the HFA theory is correct, much of the apomixis literature will need to be reinterpreted. This includes interpretations of how apomixis evolved, the role of apomixis in evolution, the genetic control of apomixis, and how apomixis might be transferred to (or induced to occur in) sexual species.

Evolution of Apomixis and Related Anomalies

According to HFA theory, many secondary contacts must have occurred between ecotypes (or closely related species) that had been isolated from each other for many years along latitudinal or other ecological gradients. Major climatic shifts could account for such secondary contacts (Carman 2000). The distribution patterns of most apomicts indicate a Pleistocene origin (Stebbins 1971; Asker and Jerling 1992), i.e., the geographic distributions and centers of diversity of many apomicts are centered near the margins of the Pleistocene glaciations, but their ranges often encompass the ecological ranges of the putative sexual progenitors, which lie north and south of the glacial margins. Eight major glaciations, which covered as much as 20% of the earth's surface, occurred during the Pleistocene. These were separated by warm interglacial periods lasting

for several thousand to a hundred thousand years each. Likewise, most of the major glacial events consisted of glacial advances interrupted by minor interglacial periods that lasted for a few thousand years (Frakes et al. 1992). Hence, during the Pleistocene, the high latitudes of both hemispheres were repeatedly deglaciated and revegetated by cosmopolitan taxa capable of adapting to cool climates, long days, and short growing seasons.

A long-day flowering response and a precocious meiosis and embryo-sac development in young ovules of sexual *Antennaria*, and probably many other taxa, are adaptations to short summers in high latitudes or altitudes (Carman, unpublished). Glacial advances, which followed the numerous interglacial periods, cooled the midlatitudes, permitting higher latitude flora to invade midlatitude flora. This provided opportunities for ecotypes with a putatively-precocious female development (from higher latitudes or elevations, i.e., temperate to arctic climates) to form polyploids with ecotypes (or related species) with a putatively-delayed female development (from lower latitudes, i.e,. tropic to full-sun temperate climates). Such polyploids may have given rise to modern apomicts (Carman 1997, 2000).

This interpretation is consistent with the observed effects of climatic factors on facultativeness in certain apomicts. For example, exposing *Dicanthium annulatum*, *D. intermedium*, and *Themeda triandra* to short days and low temperatures increases the frequency of apospory. The opposite conditions increase the frequency of sexuality in a partially-sexual *Dicanthium* hybrid (reviewed by Asker and Jerling 1992; Carman 2000). Such shifts in facultativeness are expected if signal transduction pathways vary among genomes in sensitivity to morphogens (hormones, etc.) that accumulate in response to changing seasons and photoperiods.

Once asynchrony is initiated, further shifts in facultativeness might occur in response to growing conditions. G. Ledyard Stebbins (personal communication, 1997) suggests that conditions favoring rapid growth (high temperatures, moisture, and light) might enforce competition between asynchronous genomes causing an increased frequency of apomixis. This prediction was observed in clones of F_1 hybrids obtained between wheat and diplosporous *Elymus rectisetus*. Clones grown under favorable conditions (high temperatures and light intensities) produced more apomeiotic-like MMCs (Peel et al. 1997b). Additional research should be conducted to elucidate such effects on facultativeness (Asker and Jerling 1992). Such research could provide clues concerning the nature of the divergent parental phenotypes that may have produced apomicts upon hybridization and polyploidization during the Pleistocene.

The HFA theory also suggests that the role of apomixis in evolution is more prominent than previously supposed. What happens if some of the many checkpoint genes permitting a reasonably smooth replacement of developmental segments (resulting in apomixis) are mutated or lost during diploidization? Phylogenetic and genomic evidence suggests that such confusions of development may manifest themselves as polyspory or polyembryony. Thus, apomixis, instead of being an evolutionary dead end, may occasionally serve as an evolutionary springboard in the evolution of normal or developmentally-novel paleopolyploid sexual species and genera (Carman 1997).

Mendelian Analyses of Apomixis

Essentially all Mendelian analyses of apomixis face reinterpretation if the HFA theory is correct. Data from a variety of apomicts have on one or more occasions (or when grown in certain environments) tended to fit a tetrasomic inheritance model with apomixis

conferred by a single dominant gene "*A*" (or linkat). The putative linkat encodes sufficient information to ... t embryo-sac formation and function (Sherwood, Chap. 5). However, such "segregation ratios" are in some cases influenced by the environment in which the putative segregants are grown, much like the *Dicanthium* hybrid (reviewed above), which is sexual when grown in one environment and facultatively apomictic when grown in another.

Dujardin and Hanna (1983) concluded that *Pennisetum squamulatum* is heterozygous for apomixis because some F_1s in a cross between aposporous *P. squamulatum* and sexual tetraploid inbred pearl millet (*Pennisetum americanum*) were mostly sexual. This conclusion may not be warranted. Six of 20 F_1s obtained in the study were highly apomictic, but 13 of the 14 remaining F_1s were facultative apomicts with, on average, 3.2% of nonaborted ovules containing aposporous embryo sacs. No aposporous embryo sacs were observed in one of the 20 F_1s, but only 64 nonabortive ovules from this F_1 were studied. It is possible, given the small sample size and the possible influence of environment on facultativeness, that this F_1 also was facultative.

Many published studies may prove useful in interpreting how genetic background and environment shift degrees of facultativeness. According to HFA theory, if genetic background favors the expression of one genome over another, sexual development will be favored. A genetic background favoring both genomes would favor apomixis, and a genetic background that completely silences reproductive signals from one genome would enforce sexuality.

In the Dujardin and Hanna (1983) study, a third category consisting of about five of the 20 F_1s was observed. This category occurred primarily among weakly apomictic F_1s and was defined by a very high level of ovule abortion (48–68%). The apomictic *P. squamulatum* parent is an autoallohexaploid and contains four copies of the S genome and two copies of an S′ genome (Patil et al. 1961). The sexual inbred autotetraploid pearl millet parent contained four copies of the A genome. From this information, several reasonable scenarios involving "intergenomic heterozygosity" could be proposed for the expression of apomixis in the F_1s.

One scenario is that the A and S genomes are similar with regard to reproductive timing, but both differ from the S′ genome. Thus, the F_1 genome combination AASSS′ would be apomictic. However, because the S genomes in *P. squamulatum* are heterozygous, shifts in facultativeness are expected because of recombination among S genome chromosomes prior to fertilization. This may explain the three reasonably distinct reproductive phenotypes observed: strongly apomictic (6 of 20 F_1s, both genomes strongly expressed); weakly apomictic/highly abortive (5 F_1s, one genome expressed more strongly than the other); and weakly apomictic/weakly abortive (9 F_1s, one genome expressed much more strongly than the other). In this regard, it is interesting that most of the apomictic progeny are late-maturing relative to pearl millet (Hanna et al. 1992), which suggests they are expressing intermediate phenotypes with regard to reproductive development.

Replicated Mendelian analyses with consistent segregation ratios have been conducted in *Panicum* (Savidan 1982), *Tripsacum* (Leblanc et al. 1995b), and *Brachiaria* (Valle and Miles 2000, Chap 10), and each study suggests apomeiosis (detected cytologically) is controlled by a single dominant allele. However, other recent studies challenge this conclusion, e.g., the apomeiosis "allele" in the *Tripsacum* accession studied by Leblanc et al. (1995b) is part of a

large linkage group in which recombination is suppressed (Grimanelli et al. 1998), and a similar linkage group appears to exist in apomictic *Pennisetum* (Grimanelli et al. 2000, Chap 6), *Cenchrus* (Roche et al. 1999), and *Brachiaria* (Pessino et al. 1999). Such linkage groups may contain multiple genes required for apomeiosis (Grimanelli et al. 1998, 2000).

Two groups are introgressing apomixis into maize from *Tripsacum*, and neither has reported its expression in addition lines with less than nine *Tripsacum* chromosomes. In one group, two apomictic maize triploids containing nine *Tripsacum* chromosomes ($3x + 9$) were produced. Cytogenetic and molecular studies strongly suggested that the nine *Tripsacum* chromosomes in each line were the same (Kindiger et al. 1996). A third triploid addition line, again with nine *Tripsacum* chromosomes ($3x + 9$), was produced by the same group. However, many of the nine chromosomes in this line differed from the nine chromosomes of the two former lines. The maize chromosomes were the same for all three lines. The latter $3x + 9$ plant was also apomictic, but the frequency of apomixis was only 10–15%, compared to 95–100% for the two former lines (Victor Sokolov, personal comm., 1997). These data and unpublished findings from the other group attempting to transfer apomixis to maize (Grimanelli et al. 2000, Chap. 6) suggest a much more complex mode of inheritance for apomixis.

In another study, sexual *T. dactyloides* diploids were crossed with highly apomictic *T. dactyloides* triploids (%) to produce aneuploids. All but three of 46 F_1s showed tendencies for apomeiosis (determined cytologically). However, the highly apomeiotic F_1s contained seven or more chromosomes above the diploid level, and all F_1s with chromosome numbers near the diploid level were sexual (Sherman et al. 1991), which also suggests complex inheritance. Finally, genes essential to the expression of apomixis in artificially produced *Tripsacum* triploids cosegregated with five *Tripsacum* linkage groups that are syntenic with regions from three maize chromosomes (Blakey et al. 1997).

These latter studies infer (*i*) the interaction of multiple genes from multiple chromosomes (at least in *Tripsacum*) is required for the expression of apomixis; (*ii*) many genes affect facultativeness and behave additively; (*iii*) some *Tripsacum* chromosomes affect facultativeness much more than others; and (*iv*) alleles from at least three maize chromosomes fail to substitute for their homeologous (syntenic) counterparts from *Tripsacum* in conferring apomixis. These inferences evoke the following questions: How many genes are required to efficiently express apomixis in an alien genetic background? What selective pressures cause the accumulation of appropriate combinations of alleles that confer apomixis and regulate facultativeness? Can such selective pressures predispose sexual plants to apomixis upon inter-ecotypic hybridization? What role does polyploidy play? Does mutation play any role at all? The HFA theory answers these questions.

That Mendelian analyses of some apomicts consistently result in expected ratios does not prove apomixis genes exist. Apomixis in such species may be largely controlled by one recombinationally-isolated linkage group with many minor unlinked genes affecting facultativeness (Carman 2000). According to HFA theory, such linkage groups do not contain apomixis genes per se and will not confer apomixis when syntenic regions of a sexual species are thereby replaced. Such linkage groups mimic heterozygous dominant gene action because of genomic configurations that isolate them from recombination. This isolation maintains the intergenomic (or intersegmental) polygenic heterozygosity required for apomixis and is critical to the

stabilization of apomixis, i.e., the ability of apomicts to facultatively produce progeny sexually without such progeny being sexual revertants (Carman, in preparation). The mapping of genes or linkage groups with strong effects on apomixis penetrance is important, and, as in many other breeding studies, segregation ratios and levels of facultativeness should be verified by testing putative sexual segregants for apomixis (cytology of 100–200 ovules and/or progeny tests involving molecular markers) over multiple years and multiple locations. Putative segregants should be considered unreliable for mapping studies until such tests are performed. Finally, because levels of apomixis expression (facultativeness) often are highly variable in Mendelian analyses (reviewed by Carman 2000), QTL analyses should be conducted to estimate the number of genes involved.

Making Crops Apomictic

According to HFA theory, "apomixis genes" that confer apomixis when transferred to sexual species do not exist. In contrast, heterozygosity at many loci is required; this heterozygosity involves traits such as floral induction stimuli and stages of ovule development in which meiosis, embryo-sac development, and embryony occur. Transferring such linkage groups to sexual species will not confer apomixis unless the timing of female reproductive development encoded by the recipient plant is appropriately asynchronous with that encoded by the alien segment. Hence, HFA theory predicts that programs designed to introgress apomixis into crops will experience difficulties producing apomictic lines that possess anything less than one to a few alien chromosomes.

It should be possible to produce apomicts in at least some crops by (*i*) selection for appropriate parental phenotypes (divergence in female developmental schedules), (*ii*) crossing the appropriately-divergent phenotypes, and (*iii*) stabilizing apomixis through cytogenetic modifications that isolate the responsible heterozygous loci from recombination (polyploidy or inversion or translocation heterozygosity) or through transgenic modifications that obligately abort female meiosis. The success of this approach will depend on the existence in primary gene pools of sufficient genetic variability for female developmental schedules and on correctly identifying the appropriate cytological parental phenotypes. It may be possible to enhance insufficient variability in some crops by outcrossing to the secondary gene pool. According to HFA theory, the required genes are not apomixis genes per se, but consist of normal genes with multiple ecotype-specific alleles, which when found in specific combinations confer temporally-divergent schedules of sexual female development to natural ecotypes.

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Chapter 8

Model Systems to Study the Genetics and Developmental Biology of Apomixis

Ross A. Bicknell

Introduction

Apomixis is a diverse topic of study, encompassing the cellular and sub-cellular events associated with the trait, its inheritance across taxa, the taxonomic, ecological, biogeographical and evolutionary consequences of its expression, and the potential economic impacts of its incorporation into crop species. For each intended purpose, different characteristics are required in the study material, so the choice of an experimental system will vary between research groups. Historical reasons, access to material and expertise, and funding opportunities are clearly also critical factors in such decisions. For this brief chapter, I intentionally restricted my focus to the choice of model systems for studying the developmental biology of apomixis and the genetics underlying its expression and inheritance. Furthermore, characteristics of experimental merit have been ranked above those with more immediate commercial value. It is appreciated that this introduces a bias that is not appropriate for all researchers, however, it is hoped that the considerations discussed remain relevant across different program aims.

Why Use a Model System for Apomixis?

Until very recently, most published studies regarding apomixis focused on either describing the cellular mechanisms underlying asexual seed formation or on the ecological implications of the trait for many different species. Much of the available data on apomixis are therefore quite diverse. This has been valuable for identifying elements common to different types of apomixis, however, it has also led to difficulties when comparing results from widely disparate systems. Consequently, it now seems appropriate to concentrate research effort on a smaller number of systems that may serve as models for the trait. The benefits of using model systems are clearly recognized in most fields of biology. Concentrating on a single system makes it possible to establish research networks; accumulate information on the experimental manipulation of the organism; develop genetic maps; maintain repositories for associated materials (such as genetically characterized plants, DNA libraries and probes); and to devote limited resources to developing a comprehensive understanding of a representative system(s).

As other chapters in this book make clear, many critical questions in apomixis research remain unanswered, such as the number and nature of the major genes involved and the role of epistasis, with regard to modifiers and the interaction with polyploidy. Temporal and spatial gene regulation clearly occur within the ovule to determine tissue fate, and timing appears to be critical in determining the relative importance of the meiotic and apomeiotic pathways of seed formation in facultative systems (Savidan 1989; Koltunow

1993). Before the trait can be successfully commercialized, more information is required about the impact of environmental variables on the expression of apomixis and their interaction with sexuality, particularly with regard to fertility and resource allocation. Ultrastructural studies are also required to more clearly elucidate the cytological and histological events involved in apomixis, such as the role of differential callose deposition (Carman et al. 1991; Leblanc et al. 1995a; Peel et al. 1997).

Attributes of A Model System

The innate advantages and limitations of any system will dictate the available research opportunities and impact on the rate of possible progress. Therefore, before discussing candidates, it is helpful to consider the features that would facilitate their use, specifically in a study of the developmental biology and molecular genetics of apomixis.

Biological Attributes

To facilitate experimental progress, there are a number of cultural characteristics to consider in choosing a model system. For simplicity, the plant should be easy to cultivate, both in vivo and in vitro. Ideally it should also be a perennial that can be easily propagated vegetatively to permit the maintenance of sterile or self-incompatible sexual biotypes. A small, compact plant stature, which does not require training, will facilitate the manipulation of large populations, such as those used during mutant screens and inheritance studies. For the rapid turnover of experimental populations it is convenient to use a species with a short generation time, abundant seed set, and adequate seed fertility. This is particularly important for studying apomixis because plants are assessed at the time of flowering or seed formation. For the evaluation of apomixis, it is advantageous to use a species in which apomixis can be easily assessed, preferably in a format that can be quantified, to facilitate the study of allelic differences and epistasis.

The inheritance of apomixis is typically assessed by crossing sexual and apomictic biotypes, often using the sexual plant as the maternal parent. Although it is usually also possible, and sometimes necessary, to perform the reciprocal cross, it requires the separation of recombinant (B_{II} and B_{III} hybrids) from non-recombinant (maternal and polyhaploid) progeny. Inheritance studies, therefore, require that cross-compatible sexual and apomictic biotypes are available, and if a sexual recipient is used, that microsporogenesis and microgametogenesis are functional in the apomictic biotypes used. Sexual biotypes are also useful in molecular studies for assessing the developmental importance of reintroduced, putative control sequences.

Types of Apomixis

Two principal mechanisms of apomixis have been reported: (*i*) adventitious embryony, in which the maternal embryo arises directly from a somatic cell, and (*ii*) gametophytic apomixis, in which the maternal embryo is derived from an egg cell within an unreduced embryo sac. Gametophytic apomixis is further divided into diplosporous and aposporous mechanisms, depending on whether the unreduced embryo sac arises from a modification of the meiotic apparatus or from a separate cell, respectively. Intermediates between the two forms of gametophytic apomixis have been reported (Gustafsson 1946), indicating that they possibly represent modifications of a single developmental mechanism. For more detailed descriptions of mechanisms, see Nogler (1984a) and Koltunow (1993). Most current research efforts on native apomixis focus on gametophytic mechanisms, including studies of both aposporous and diplosporous species.

For an experimental system using a native apomict, it is convenient to use a facultative species in which both maternal and nonmaternal ("aberrant") progeny can be harvested from the same plant. Rutishauser (1948) identified three aberrant types among the progeny of these plants: B_{II} hybrids, derived from the fusion of a reduced egg cell and sperm nucleus; B_{III} hybrids, derived from the fusion of an unreduced egg cell and sperm nucleus; and polyhaploids, which arise through parthenogenesis from a reduced egg cell. Aberrant progeny are often very useful for studying the genetics of apomixis. Hybrid progeny permit the evaluation of cytoplasmic-inheritance and the hybridization of pollen-sterile biotypes such as interspecific hybrids (Savidan et al. 1994). Similarly polyhaploids have been used to study the expression of apomixis in diploids (Nogler 1982; Bicknell 1997).

Competence to form both meiotic and apomeiotic seed is also invaluable for mutation screening. Dominant inheritance (see below) suggests that the selective inactivation of either developmental pathway by a mutation will lead to the exclusive expression of the other. This dual competency provides a useful internal control. The continued formation of at least one class of seed indicates that the mutation(s) is specific to an event(s) associated with apomixis and that related requirements for floral development, megagametogenesis, and embryogenesis remain intact. Similarly, as facultative mechanisms incorporate a balance between the utilization of parallel developmental pathways, they can be used to study factors that influence that balance, such as the impact of physiological stress and of interactions between genetic modifiers.

Finally, as one clear goal of this research is to incorporate apomixis into crop populations, a facultative mechanism is likely to provide the maximum flexibility for farmers and plant breeders alike. Fortunately, most gametophytic apomicts of all types appear to be facultative, although they differ in the relative importance of the meiotic and apomeiotic pathways of seed formation.

The formation of the endosperm may also be an important consideration in the choice of an experimental system. The endosperm may either form spontaneously, as in "autonomous" apomicts, or require the fertilization of the polar nuclei by a sperm nucleus (pseudogamy). As pseudogamy only requires spontaneous embryogenesis and not spontaneous endosperm formation, pseudogamous species may be considered potentially simpler models to study. This possible advantage, however, is offset by experimental constraints associated with pseudogamy. In these plants, pollination is required for seed formation, so it becomes necessary to demonstrate that the applied pollen led only to the fertilization of the polar nuclei and not of the egg. This difficulty has been largely overcome, however, in species that can be assessed for apomixis using a correlated cytological character, such as callose deposition in diplosporous species of *Tripsacum* (Leblanc et al. 1995a) and four-celled embryo-sac formation in aposporous species of *Panicum* (Savidan 1980). Finally, there may be end uses for which autonomous apomixis is more suitable, such as the production of male sterile or self-incompatible lines, the avoidance of pollinators, or for crops in which the endosperm is commercially important, such as grains.

Genetic Attributes

Apomixis appears to be relatively simply inherited either as a one- or two-gene trait, at least in the small number of cases studied. Examples include apospory in *Ranunculus auricomus* (Nogler 1984b); *Panicum maximum* (Savidan 1980, 1982); *Pennisetum squamulatum* (Dujardin and Hanna 1985); *Cenchrus ciliaris*

(Sherwood et al. 1994); *Brachiaria* sp. (Valle and Savidan 1996); *Hieracium* (Gadella 1991; Bicknell et al. 2000); and diplospory in *Tripsacum dactyloides* (Leblanc et al. 1995b; Grimanelli et al. 1998); *Taraxacum* (van Dijk et al. 1999); and *Erigeron* (Noyes and Rieseberg 2000). Earlier work by Döpp (1939) indicates that it may even be true for the fern *Dryopteris*. Simple inheritance would be particularly valuable for analyzing the molecular biology of the trait.

For the advancement of a molecular research program, it is particularly advantageous if a model system can be genetically transformed. This permits the introduction of marker genes to follow segregation and recombination and mutagenic sequences such as T-DNA tags and transposons to assist in the cloning of sequences associated with the expression of apomixis. Furthermore, transformation permits expression studies about putative regulatory elements by their fusion to marker sequences and the functional testing of putative control genes by their introduction into sexual or mutant genotypes.

It would also be preferable to use a species with a small genome size, such as *Arabidopsis*, to facilitate the identification of critical loci. Similarly, an ideal system would already be characterized with respect to genomic sequence and mapped morphological and molecular markers (deletions, translocations, RFLPs, RAPDs, transposons, etc.) for the localization of loci. Finally, it would be advantageous, although not essential, to use a crop species to assist in the transfer of research findings into practical outcomes.

Experimental Methods

The success of any program on apomixis will depend on both the natural attributes of the species used and on the power of the techniques available to manipulate it experimentally. For some species, techniques have already been documented, while for others they would need to be established. Tissue culture and transformation techniques are particularly valuable. They permit the rapid micropropagation of selected types, the retention of somatic genotypes through in vitro shoot regeneration, and the maintenance of valuable genotypes through long-term cold storage. It is also possible to recover interspecific hybrids using embryo rescue, and to manipulate ploidy in culture through the use of anther or microspore culture to isolate reduced genotypes and by colchicine application for chromosome doubling.

As the initiation of the embryo sac is the first decisive event of the female reproductive phase, a thorough embryological analysis is an indispensable base for an investigation of apomixis, while embryology continues to be necessary at all stages of the research. Reliable cytological techniques are therefore essential for this work. Fortunately, this is one area of apomixis research that is well documented, particularly with respect to the use of versatile, routine clearing techniques (Leblanc et al. 1995a) that enable the use of thick sections to visualize the complex internal structure of the ovule.

Quantifying Apomixis

Most studies of apomixis require a method for determining the presence of apomixis, and when possible, for quantifying its extent, either with respect to prevalence in a population or level of expression in a single genotype. Measuring apomixis, however, is a difficult task. When considered as a genetic event, apomixis is reproduction through seed without either allelic segregation or recombination. Assessment, therefore, should be strictly based on whether allelic rearrangement occurs. This is seldom practical experimentally and most systems for assessing apomixis use a correlated character. The reliability of these correlations is clearly

an important concern in interpreting data collected from any system. For example, in most apomicts demonstrating apospory of the Panicum-type, maternal embryos typically arise from four-celled, unreduced embryo sacs, while reduced embryo sacs are usually eight-celled. Apomixis can therefore be readily scored in these plants by determining the relative abundance of the different structures. The correlation between the form of the embryo sac and nuclear state appears to be very good, but the technique is also based on the assumption that the number of unreduced embryo sacs is a direct reflection of the number of maternal embryos that will reach maturity. As the assessment is conducted before the requisite events involved in apomixis have occurred, an overestimate of intact apomixis is possible. In contrast, asexual seed forms without pollination in autonomous apomicts, therefore, apomixis can be quantified in these species from the seed that sets without pollination. This is attractive experimentally, but it presents a conservative bias because individuals with only partial apomixis are easily scored as either sexual or sterile. It is clear in every case that results should be verified by either using more than one method for quantifying apomixis or by establishing the credibility of the results with either an embryological or a genetic study of the system.

Candidate Systems

Two approaches have been taken in developing model systems for studying apomixis: (*i*) the modification of an established model plant species, and (*ii*) the experimental manipulation of a known apomict.

The first approach has the advantage of immediate access to extensive records on the genetics, biochemistry, and developmental biology of the system. Experimental methods have been devised, genotype collections are available, DNA libraries, probes, map markers, and sequenced genomic fragments are accessible, and existing laboratory collaborations are present for cross-referencing findings. Conversely, existing model plant systems have been chosen for reasons other than apomixis. This approach, therefore, dictates targeting the mechanism of apomixis, depending on the nature of the available mutations. It is also based on the assumption that apomixis is a derivative of sexuality and/or that it can be derived artificially from sexuality. While this currently appears intuitive, it should be noted that this assumption remains unproven.

In contrast, the use of a naturally apomictic species enables the selection of both the apomictic mechanism for its applicability and the plant type for its amenability. The model plant systems currently used to study developmental biology, however, are obligate sexuals. Consequently, this approach requires the establishment of experimental methods for the selected species, the collection and characterization of critical genotypes and other experimental tools, the maintenance of those collections, and the establishment of new collaborative networks.

Modification of an Existing System

Several genera have been used as models to study plant reproductive biology including *Antirrhinum*, *Arabidopsis*, *Capsella*, *Daucus*, *Hordeum*, *Lemna*, *Lolium*, *Lycopersicum*, *Nicotiana*, *Perilla*, *Petunia*, *Pharbitis*, *Sinapis*, *Xanthium*, and *Zea*. Recently, with an increased emphasis on molecular genetics, the most important model system has become the cruciferous species *Arabidopsis thaliana*, with less emphasis on the solanaceous genera *Lycopersicum*, *Nicotiana*, and *Petunia*, and the monocotyledonous genus *Zea*. These plants reproduce through seed by obligate sexuality. Two approaches have been taken to use obligate sexual species as models for apomixis research. Some scientists have attempted to "synthesize" the trait directly, either through

induced mutation or by the accumulation of mutant alleles. A second approach has been to attempt the transfer of the trait by introgression from an apomictic relative.

Two important advantages of mutagenesis are that it can be conducted on a species without any known close apomictic relative and it has the potential to rapidly provide the experimental material required in a characterized genetic background. The principal difficulty with this approach is that methods must be developed for screening mutant populations for apomixis without any foreknowledge of the developmental mechanism(s) that will arise. In particular, it is not known whether a single mutagenic event can cause an unambiguous phenotypic change that can be identified as either an intact apomixis or a recognizable component of the trait. The screening technique must therefore be based both on the appearance of developmental anomalies that are characteristic of apomixis and, ultimately, on the retention of the maternal genotype. One good example of this approach is the mutant screening of *Arabidopsis thaliana* for mutations leading to the formation of seed without fertilization (*fie* and *fis* mutations) (Ohad et al. 1996; Chaudhury et al. 1997). The screens were based on the identification of plants that developed elongated siliques without prior fertilization. The advantages of *Arabidopsis* for this approach are highlighted by the authors' use of male sterile mutants (*pop*1 and *pistillata*) to avoid self-fertilization, and the use of *GUS* as a paternal marker in test crosses with the mutants. The description of these mutants is a particularly exciting outcome, providing evidence for the involvement of chromatin remodeling factors in the control of cell division at the time of fertilization (Grossniklaus et al. 1998; Kiyosue et al. 1999). Furthermore, recently reported data indicates that methylation is critical to the regulation of these genes, specifically with their expression during gametogenesis and early embryogenesis and that this may be associated with mechanisms of maternal inheritance (Vielle-Calzada et al. 1999).

When mutations that alter megasporogenesis, megagametogenesis, and embryogenesis are already known, it may be possible to create an apomictic mechanism by the accumulation of appropriate alleles within a single genotype. In potato, the homozygous representation of the *ds*-1 allele significantly reduces chiasmata frequencies on all chromosomes during both megasporogenesis and microsporogenesis, leading to high levels of $2n$ gametes through first division restitution (FDR) (Jongedijk et al. 1991). If this could be combined with parthenogenesis, true potato seed could be generated through a synthetic diplosporic mechanism (Hermsen et al. 1985).

When an apomictic relative is available, it may be possible to transfer the trait into a characterized system by introgression. This typically involves a backcrossing program using the sexual species as the recurrent parent. Introgression has been used to attempt the introduction of apomixis into several crop species, normally as part of a crop improvement program (Asker and Jerling 1992). Examples of this approach include attempted transfers from *Pennisetum squamulatum* to cultivated pearl millet (Dujardin and Hanna 1985), *Elymus rectisetus* to *Triticum* (wheat) (Torabinejad and Mueller 1993), and *Tripsacum dactyloides* to *Zea* (maize) (Leblanc et al. 1995b). Similarly, it may be possible to transfer the trait from apomictic crucifers, such as *Arabis holboellii* (Roy 1995) or *Draba oligosperma* (Mulligan and Findlay 1969), to *Arabidopsis thaliana* to take advantage of the versatility of this model system. Unlike synthesis, transfer has the advantage that the program is based on a known, functional apomictic mechanism. Furthermore, the

inheritance of that trait can be monitored through each generation, providing information on its genetic basis in the system under study. Finally, as indicated above, when the recipient species is a crop, the product may be of immediate commercial value. The principal disadvantage of transfer is that the availability of apomictic relatives typically dictates the mechanism used. It has also proven difficult to incorporate the trait into obligate sexual crops with a significant level of expression. The reason for this difficulty is unclear. It may be associated with the inheritance of modifiers or an important association with polyploidy, which is lost during the backcrossing program. Alternatively, the difficulties encountered may be more a problem of experimental scale. Most crop species do not have a close apomictic relative so introgression requires wide crossing. This often results in poor fertility among the progeny and little, if any, crossing over during meiosis. Large populations need to be formed and assessed for ploidy and apomixis, but traditional methods of chromosome counting and thin sectioning are too labor-intensive to be practical for most research teams. Recent advances in DNA quantification through flow cytometry and analytical cytology using clearing techniques are overcoming these difficulties. Over the past two years, researchers in the maize/*Tripsacum* program have screened more than 200,000 plants for chromosome number using flow cytometry (Savidan, personal comm.) and the experimental populations have been advanced to the BC_5 generation.

Development of a Model System from an Existing Apomict

Apomixis occurs throughout the plant kingdom. Species utilizing various forms of asexual reproduction that involve gametophytic structures have been recorded among the algae, pteridophtyes, and in more than 400 species of flowering plants from more than 35 families (Asker and Jerling 1992; Carman 1997).

Which is the best to study? Different species clearly have different advantages. The unique biology of ferns, for example, presents some unusual opportunities to study apomixis (Sheffield and Bell 1981). The events of megasporogenesis and megagametogenesis are physically separated in these plants, permitting the study of the individual component processes of apomixis in isolation. Unlike the angiosperm embryo sac, the free living fern gametophyte is isolated from parental influence, and, in some systems, parthenogenetic development of the sporophyte can be induced in vitro (Sheffield and Bell 1987). Furthermore, the sporogenic tissue is relatively exposed in ferns, simplifying the study of events associated with the avoidance of meiosis. It is interesting to note that Manton (1950) reported that unreduced spores of *Crytomium* arose from tissue immediately adjacent to meiotic tissue in the sporangium, a situation closely analogous to the development of aposporous initials in the angiosperm ovule. Finally, as the fern sporophyte develops without the need for an endosperm, difficulties associated with the development of that tissue do not arise. There are, of course, several limitations in using ferns as model systems, particularly for a molecular study of development. They are often large slow-growing plants that can be difficult to cultivate, and they present some real challenges when conducting controlled fertilizations with motile sperm cells. Finally, very little is known of the molecular biology of this group, which would impede progress in any molecular study of apomixis.

Apomixis occurs throughout the angiosperms including representatives of both monocotyledonous and dicotyledonous genera. Many of the most comprehensive

studies of apomixis used monocotyledonous species, principally relatives of the cereals, such as *Elymus* (Torabinejad and Mueller 1993), *Tripsacum* (Leblanc et al. 1995a), *Panicum* (Savidan 1982), and important forage grasses, such as *Pennisetum* (Dujardin and Hanna 1985; Ozias-Akins et al. 1998)), *Brachiaria* (Valle et al. 1994), and *Paspalum* (Bonilla and Quarin 1997).

The inheritance of apomixis has been particularly well characterized for *Panicum maximum*. Savidan (1990, 2000) partially ascribed his success with this system to the use of apomictic and sexual forms from within the same species and at the same ploidy level. This important advantage is shared with only a very small number of studied apomictic taxa. In contrast, most known apomictic species appear to have either evolved away from their immediate sexual progenitor(s) or their progenitor(s) no longer exists. As a result, apomicts have often been crossed with a related but distinctly different sexual species. In such studies, the subsequent analysis of the progeny must consider the inheritance of the breeding system while also allowing for unrelated effects resulting from interspecific hybridisation. Despite this caveat, however, mapping strategies have led to the isolation of molecular markers linked to apospory in the grass genus *Pennisetum* (Ozais-Akins et al. 1993, 1998). In a similar approach, colinearity between grass genomes has also been used to propose a linkage to apomeiosis in *Tripsacum* (Leblanc et al. 1995b) and *Brachiaria* (Pessino et al. 1997; see Grimanelli et al., Chap. 6).

Some dicotyledonous species have been used previously as model systems, most notably *Potentilla* (Rutishauser 1948; Asker 1970, 1971); *Taraxacum* (Richards 1973; van Dijk et al. 1999); *Hypericum* (Noack 1939); *Ranunculus* (Nogler 1984a); and *Hieracium* (Bicknell et al. 2000). The inheritance of apospory in *Ranunculus* has been particularly well studied (Nogler 1984b) through four generations of crosses and backcrosses. The results indicate that apospory is inherited as a simple dominant Mendelian trait in this system, however, the allele conferring apomixis could only be transferred in a diploid or polyploid gamete. Nogler noted that this mechanism could explain the very close association observed between polyploidy and apomixis in native systems. In contrast, recent results with the diplosporous genus *Taraxacum* (van Dijk et al. 1999) indicate that a two loci model better fits the data obtained from controlled crosses within this taxon. *Taraxacum* is an attractive experimental system because it is a well studied ecological model (Richards 1986) and it has also been successfully transformed (Song and Chua 1991).

One dicotyledonous taxon that appears to be well suited for use as a model system is *Hieracium* subgenus *Pilosella* (Koltunow et al. 1995). The plants are small herbaceous perennial daisies that are easily propagated and maintained in the greenhouse. *Hieracium* is a long-day plant, flowering in response to extended photoperiods (Yeung 1989). Photoperiodicity is a very useful experimental tool as it enables the programmed production of flowers at any time during the year using day-length extension lighting. Both *H. pilosella* and *H. aurantiacum* set seed within 3–4 months of germination, allowing 3–4 generations per annum (Bicknell 1994a).

Apomictic biotypes of *Hieracium* subgenus *Pilosella* develop seed by facultative apospory coupled to autonomous endosperm development. Pollination is therefore not required for the formation of maternal seed, and apomixis can be scored by quantifying the seeds that set after the exclusion of pollen. As with *Taraxacum*, the simplest method of excluding pollen is to decapitate the immature bud, removing both the anthers and stigmas (Ostenfeld 1906; Richards 1986).

The embryology of *Hieracium* is now well documented (Koltunow et al. 1998) and a range of tissue culture techniques have been described, including methods for shoot regeneration from leaf tissue (Bicknell 1994b), the recovery of reduced genotypes from anther culture (Bicknell and Borst 1994), and an efficient genetic transformation system (Bicknell and Borst 1996).

Summary

The choice of experimental system is a fundamental decision in any research program. The relative strengths and weaknesses of the chosen system determine the opportunities available to the researcher, and often dictate end uses for the information gathered. Although different research objectives often lead to the selection of different systems, there is a need to develop either one, or a small number of, model system(s) for studying the developmental biology and molecular genetics of apomixis. Several systems have been proposed for this purpose, including some modified sexual systems such as *Arabidopsis*, and others that are naturally-occurring apomicts. Irrespective of the plants used, it is important that collaborative links are established and maintained between teams working on apomixis research in order to expedite the advance of our knowledge in this fascinating field.

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Chapter 9

Screening Procedures to Identify and Quantify Apomixis

Olivier Leblanc and Andrea Mazzucato

Introduction

Mendel was no doubt puzzled when he studied the progenies from crosses in the *Hieracium* species to confirm his *Pisum* experiments: F_1 families were highly variable and some F_1 hybrids produced homogeneous progenies (Mendel 1870). So what about the world famous laws of inheritance worked out on *Pisum*? In fact, the laws still held. What the great geneticist did not know was that he had made the first progeny tests with apomictic species.

The term "apomixis" initially covered all of the mechanisms of asexual reproduction (Winkler 1908), but today it is applied strictly to asexual reproduction through seeds (Nogler 1984). There are two main types of apomixis, based on the origin of the embryo: adventitious embryony, in which the embryo forms directly from the sporophyte (the gametophyte phase is bypassed), and gametophytic apomixis, in which the embryo develops parthenogenetically from an unreduced female gametophyte (Gustafsson 1947; Stebbins 1950). Apomictic developments bypass meiosis and fertilization, the bases of sexual reproduction and genetic recombination, and therefore, offspring are genetically identical to the mother plant.

Apomixis has been widely identified in the plant kingdom (Asker and Jerling 1992; Carman 1997), and occurs in families of economic importance (Rutaceae, Poaceae, Rosaceae). Moreover, it appears to be a very common mode of reproduction in the Panicoideae subfamily of Poaceae (Brown and Emery 1958), which includes several major grain crops. Apomictic processes are still poorly understood, but the potential impact of apomixis on agriculture appears great, provided that it is proven to be ecologically safe (Vielle-Calzada et al. 1996; van Dijk and van Damme 1999; Toenniessen, Chap. 1). Identifying sources of apomixis, understanding its inheritance, and breeding and manipulating apomictic species will require reliable and efficient procedures to screen for mode of reproduction.

This chapter concentrates on identifying and quantifying gametophytic apomixis, but for the most part, the procedures are the same for adventitious embryony. After presenting basic features of apomixis, screening procedures for the reproductive mode are described and the various challenges encountered by scientists working with apomixis are discussed.

Apomictic Mechanisms as Potential Screening Indicators

Seed production through gametophytic apomixis requires production of embryo sacs with unreduced nuclei unreduced female gamete (no reduction of chromosome number or apomeiosis), followed by embryogenesis without fusion of nuclei of the male and female gametes (parthenogenesis). The regulatory and quantitative aspects of parthenogenesis in unreduced egg cells have been poorly

documented (Asker 1980; Nogler 1984; Mogie 1988), but fertilization-independant mutants for both seed and endosperm development, recently described in *Arabidopsis thaliana* (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998), might provide new insights into embryogenesis in apomicts. Pseudogamy is the most common path of seed development, but autonomous apomixis occurs in some cases (Nogler 1984). By contrast, apomeiosis is well documented and may follow different pathways. Several reviews (Nogler 1984; Asker and Jerling 1992; Koltunow 1993; Crane, Chap. 3) provide detailed descriptions of most types of apomixis that occur in the wild.

The two types of apomeiosis—aposporY and diplospory—and their characteristics are briefly described in this chapter in order to highlight differences with sexual reproduction that are pertinent for the development of screening tools. In adventitious embryony, both megasporogenesis and megagametogenesis are bypassed; this type of apomixis has been extensively reviewed by Naumova (1992) and Koltunow et al. (1995a).

Types of Meiotic and Apomeiotic Embryo Sac Formation

Sexual reproduction starts with the differentiation of one hypodermal archesporial nucellar cell into a megaspore mother cell (MMC). See Figure 9.1. This MMC enlarges and produces a dyad of megaspores through the first meiotic division and a tetrad of megaspores through the second division. At least two biochemical pathways have been reported as critical to or associated with meiosis: callose synthesis throughout megasporogenesis in angiosperms that produce mono- and bisporic ESs (Rodkiewicz 1970), and altered expression of plasma membrane arabinogalactan protein (Pennell and Roberts 1990).

Mature meiotic ES structure varies among taxa, but it generally displays antipodal cells, two polar nuclei within the central cell,

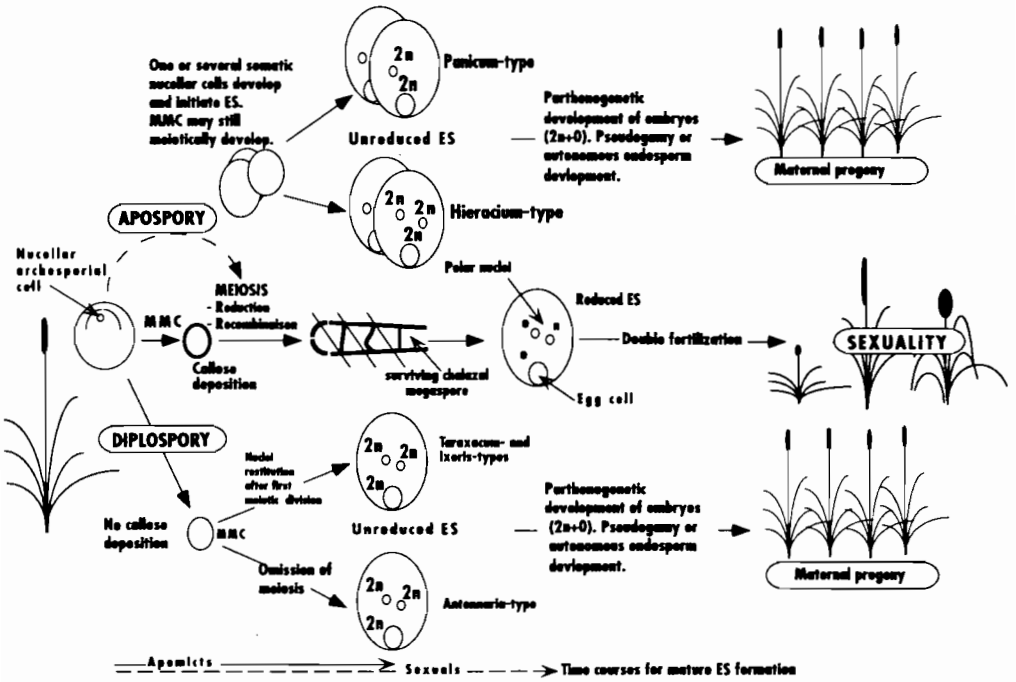

Figure 9.1 Mechanisms of pseudogamous gametophytic apomixis: consequences and comparison with sexual reproduction.

synergid(s) and one egg cell (Polygonum-type development). All nuclei from the meiotic ES are reduced (n chromosomes). Double fertilization is required for embryo and endosperm development to begin. Each of the sperm cells is involved in different fusion events: fusion with the egg restores the sporophytic chromosome number, and fusion with the central cell produces a nutritive tissue, the endosperm.

In diplospory, meiosis is totally omitted (e.g., Antennaria-type) or perturbed (e.g., Taraxacum-, Ixeris- or Allium-types). In both cases, ESs form through three or more mitoses: (*i*) from MMCs behaving as unreduced megaspores (Antennaria-type); (*ii*) from unreduced megaspores after restitution nucleus (Taraxacum- and Ixeris-types); or (*iii*) from 2n megaspores after premeiotic chromosome doubling (Allium-type). The characteristic meiotic sequence (MMC, dyad, tetrad) is absent and callose deposition does not occur (Naumova et al. 1993; Leblanc et al. 1995a) or is strongly disturbed in diplosporous pathways (Carman et al. 1991; Peel et al. 1997). Lack of callose deposition also has been reported in meiotic mutants of *Medicago sativa* that produce unreduced embryo sacs (Barcaccia et al. 1996) and in apomictic *Antennaria* hybrids obtained from parents displaying floral asynchrony (Carman 2000). In apospory, several ESs generally differentiate from nucellar (somatic) cells. In contrast to diplospory, which seems to result from genetic lesions directly affecting meiosis, some authors have stated that meiotic and apomeiotic developments are independent in apospory (Harlan et al. 1964; Nogler 1984). Both developments can theoretically occur at the same time within the same ovule, but usually the legitimate sexual line is eliminated in subsequent developmental stages. Abnormal patterns of callose deposition have been observed in various aposporous species (Naumova et al. 1993; Peel 1993; Peel et al. 1997), but little light was shed on their reproductive behavior. (Peel 1993; Peel et al. 1997). However, recent studies on callose deposition patterns and the dynamics of beta-1,3-glucanase (HpGluc) expression in aposporous *Hieracium* provide new insights into the role of callose (Tucker et al. 2000): both altered patterns and persistence of callose during megasporogenesis occur in apomictic plants when compared to sexual ones. In addition, the HpGluc enzyme might also play a role in promoting the aposporous pathway over megasporogenic callose dissolution.

Megagametogenesis in apomicts and in their sexual counterparts is usually similar. One exception is the aposporous ES structure in the Panicoideae and Andropogoneae subfamilies (Poaceae), which is a 4-nucleate ES (Panicum-type, after Warmke 1954), the sexual counterparts producing 8-nucleate ESs (Polygonum-type).

Embryo and Seed Formation

The formation of viable seeds usually requires endosperm differentiation. This is achieved in apomicts through (*i*) pseudogamy (single fertilization of polar nuclei) or (*ii*) autonomous endosperm development (central cell develops autonomously). Most apomicts are pseudogamous.

Megagametogenesis in both reproductive modes appears to take the same amount of time, but time periods for megasporogenesis differ. Such differences have been documented for several aposporous species (*Ranunculus auricomus*, Nogler 1984; *Panicum maximum*, Savidan 1982a; *Paspalum notatum*, Martínez et al. 1994) and two diplosporous species (*Tripsacum zopilotense* and *T. dactyloides*, Leblanc and Savidan 1994). The complete maturation of the apomeiotic ES before the meiotic ES may contribute to the failure of unreduced egg cell fertilization: by the time the pollen tube reaches

the ovule, unreduced egg cells may not be receptive. This loss of receptivity is not yet well understood, but several hypotheses have been proposed, including chemical or mechanical barriers (e.g., a complete cell wall around the egg) and a temporal window of receptivity, among others.

Consequences of Apomictic Seed Formation

In sexual reproduction, the two gametes that fuse are produced through meiosis. Sexual development allows genetic recombination and segregation, thereby enhancing genetic diversity. Aside from strict autogamy and from the very specific case of permanent translocation heterozygosity (Ellstrand and Levin 1982), offspring from sexual plants are new genotypes. Apomictic pathways are characterized by unreduced egg cell parthenogenesis, resulting in offspring that are exact genotypic replicas of the mother plant. However, genetic recombination may occur during apomictic reproduction in plants that show partially synaptic and restitutional meiosis or somatic DNA rearrangements (Richards 1997).

Complete (100%) maternal progenies are recovered when the mother plant reproduces through obligate apomixis. But generally, apomixis is facultative and progenies comprise various types, each resulting from a different combination of failure or success in meiosis and fertilization (Table 9.1). A fairly strict genetic control for both the formation of unreduced ES (reviewed by Sherwood, Chap. 5) and the degree of apomixis (Asker 1980) has been reported in most taxa studied.

Table 9.1 The four theoretical offspring classes in progenies from facultative pseudogamous apomicts. Note that apomeiotic mechanisms can induce chromosome losses and result in unbalanced unreduced female gametes.

	Egg cell development after	
Egg cell origin	Fusion with a sperm cell (+n)	Parthenogenesis (+0)
Reduced megaspore after meiosis : n gamete	Sexuality n+n offspring New genotypes	(Poly)haploid production n+0 offspring New genotypes
Apomeiosis (apospory or diplospory): 2n gamete	"Genomic accumulation" 2n+n offspring New genotypes	***Apomixis*** 2n+0 offspring Maternal genotypes

Levels of Screening and Related Tools

There are several indicators of apomixis, including high frequency of multiple seedlings, high seed fertility in plants expected to be sterile (e.g., wide hybrids, triploids, autopolyploids, and aneuploids), homogeneous progenies, etc. (Bashaw 1980; Hanna and Bashaw 1987; den Nijs and van Dijk 1993). They are sometimes difficult to use in the case of wild materials and, in all cases, further investigation is required to assess apomixis type and level of expression. The relative advantages or disadvantages of the screening procedures presented here are discussed further in "Choosing Suitable Procedures."

Analyses at the Plant Level

1. Molecular markers cosegregating with apomixis. To date, the identification of isozymic or molecular markers strongly linked with apomixis is the only procedure for detecting apomixis prior to flowering.

Molecular marker-based analyses in apomicts were conducted either to investigate the molecular basis of apomixis, to assist in transferring apomixis into crops, or to ultimately isolate the gene(s) responsible for its regulation. Segregating progenies or bulk segregant analyses were used after determining the reproductive behavior on the basis of cytoembryological observations or progeny testing. Because of conflicting results, debate continues over whether apomixis is controlled by a single locus or by multiple loci.

However, molecular markers (RFLP, SSR, and AFLP) for apomixis, apomeiosis, and parthenogenesis have been reported for several aposporous genera (*Pennisetum*: Ozias-Akins et al. 1993, 1998; *Cenchrus*: Gustine et al. 1997; Roche et al. 1999; *Brachiaria*: Miles et al. 1994; Pessino et al. 1997, 1998; *Poa*: Barcaccia et al. 1998; *Erygon*: Noyes and Rieseberg 2000), and in diplosporous *Tripsacum dactyloides* (Leblanc et al. 1995b; Kindiger et al. 1996; Blakey et al. 1997; Grimanelli et al. 1997a).

2. Cytoembryology. Cytoembryological differences between sexual and apomictic developments appear at different times. Observations to determine the origin of ESs are therefore based on differences in megasporogenesis (i.e., MMC behavior, occurrence of meiosis products, see Table 9.2) in the case of diplospory, on nucellar cell initiation in the case of apospory, and on differences in mature ESs for apospory of the Panicum-type. To standardize time of sampling, pistils can be classified according to pollen developmental stage if the flowers are hermaphrodite, or by size if they are monoecious. In pseudogamous species showing early embryo divisions before anthesis and endosperm formation (precocious embryony), cytoembryological observations within ESs can also help identify and quantify parthenogenesis (Kojima and Nagato 1992a; Naumova et al. 1993).

Table 9.2 Main characteristics of megasporogenesis and megagametogenesis during both sexual reproduction and gametophytic apomixis

	Meiosis	Megasporogenesis Callose	Cytoembryology	Megagametogenesis	References
SEXUALITY	Completed. The chalazal megaspore of the tetrad develops into an ES. Altered expression of arabinogalactan protein was shown to be associated with sexual development in *Pisum*. [1].	Callose deposition in Angiosperms producing mono- and bisporic embryo sacs [2].	Meiotic sequence (MMC, dyad, tetrad).	Mature 8-nucleate ES forms from chalazal reduced megaspore through 3 (Polygonum-type) or more mitoses. Mature ES are produced latter than in apomeiotic (meiosis delays megagametogenesis)	[1] Pennel and Roberts, 1990. [2] Rodkiewicz, 1970. -Herr, 1971. -Russel, 1978. -Dumas and Mogensen, 1993.
APOMIXIS					-Nogler, 1984. -Crane, chap. 3.
Apospory	Meiosis is initiated but generally fails soon or latter.	Yes (meiotic products). D Disturbed callose patterns may indicate apospory.	Concomitant development of the reproductive cell through meiosis (sexuality) and somatic cell(s) through mitoses after enlargement.	ES forms from somatic cells through mitoses. Polyembryony: Several somatic cells may develop. Reduced ES can be formed. Panicum-type: 4-nucleate ES. Hieracium-type: ES are similar to sexuals.	-Müntzig, 1940. -Warmke, 1954. -Burson and Bennett, 1970. -Young et al., 1979. -Savidan, 1982b. -Naumova et al., 1993. -Tucker et al., 2000
Diplospory	Antennaria-type: meiosis is totally over-passed. Taraxacum- and Ixeris-types: meiosis fails early producing a restitution nucleus. Allium-type: endomitosis	No callose deposition in megasporocyte cell walls.	MMC enlarges (*Tripsacum* spp., *Eragrostis curvula*) or elongates (*Elymus rectisetus*). Relation between enlargement/ Antennaria type and elongation/Taraxacum type?	ES forms from the reproductive cell. No polyembryony. Generally similar to sexuality. Binucleate ES shape can be characteristic (*Tripsacum*) Four nucleate ES described in *Eragrostis curvula*	-Voigt and Bashaw, 1972. -Crane and Carman, 1987. -Carman et al., 1991. -Kojima and Nagato, 1992b. -Peel et al., 1997

Paraffin sectioning methods (Figure 9.2) combined with staining (e.g., safranin-fast green stain, Johansen 1940; or aniline blue, Russel 1978) have been used over the last century for cytoembryological studies of reproductive development and in apomixis research (e.g., Snyder 1957; Voigt and Bashaw 1972; Burson et al. 1990). However, preparing paraffin sections is arduous and time consuming, and interpretations may be difficult. Clearing procedures (Figure 9.3) were described more than 90 years ago (Strasburger and Hillhouse 1900), but have been recently rediscovered and greatly improved (see Crane, appendix of Chap.3). They do not require sectioning or squashing and thus allow ovules to be observed in situ in three dimensions, making interpretations easier than from a series of sections. Squashing techniques, generally combined with staining, were developed for studies of megagametogenesis or megasporogenesis in various species (Hillary 1940; Bradley 1948; Saran and de Wet 1966; Darlington and La Cour 1966), but have proven only moderately successful. Nevertheless, improved squashing techniques combined with clearing procedures provide good results when analyzing female meiosis (Jongedijk 1987; Kojima et al. 1991a; Kojima and Nagato 1992b).

Clearing techniques using non-aqueous fluids (Herr 1971; Young et al.1979; Crane and Carman 1987) now represent the best tool for observing ovule details during both megasporogenesis and megagametogenesis in aposporous and diplosporous materials. Procedures combining Mayer's hemalum staining with methyl-salicilate clearing have been successfully used for observations within whole ovules of *Solanum* (Stelly et al. 1984) and *Medicago* (Tavoletti et al. 1991). These techniques are of great interest for embryological analyses in apomicts because they do not require the use of special optics. Clearing procedures combining aqueous solution (sucrose, KI) and aniline blue have recently been developed for observation of callose deposition during megasporogenesis (Carman et al. 1991; Leblanc et al. 1995b; Peel et al. 1997).

3. Egg cell parthenogenetic capacity. Egg cells produced through apospory or diplospory should be better able to differentiate parthenogenetically than those produced through sexual development, because of the apparent linkage between the two steps of apomictic development. Matzk (1991) recently proposed a new procedure to identify and quantify parthenogenesis for a wide range of cool season grasses. The technique, known as the auxin test, involves applying a synthetic auxin compound a few days before anthesis to induce parthenocarpic development in unpollinated ovaries. Auxin induced grains will lack endosperms, because the fusion of the sperm and polar nuclei is no longer possible, but egg cells with parthenogenetic capacities will develop into embryos. Studies in *Poa pratensis* using the auxin test to estimate the degree of parthenogenesis in various genotypes showed good reliability and low variation across years and environments (Mazzucato et al. 1996).

Progeny analysis

In classical progeny testing, one compares the mother plant with its offspring and/or evaluates heterogeneity within progeny. Offspring from apomictic plants are expected to be genetically identical to the mother plant; therefore phenotypic identity with the maternal type suggests apomictic reproduction, whereas variations indicate sexuality, recombination, and/or fertilization. Traditionally, progeny tests based on gross morphology have been used in apomixis research because they are easy to perform (e.g., Duich and Musser 1959; Burton et al. 1973; Gadella 1983), but many other descriptors may

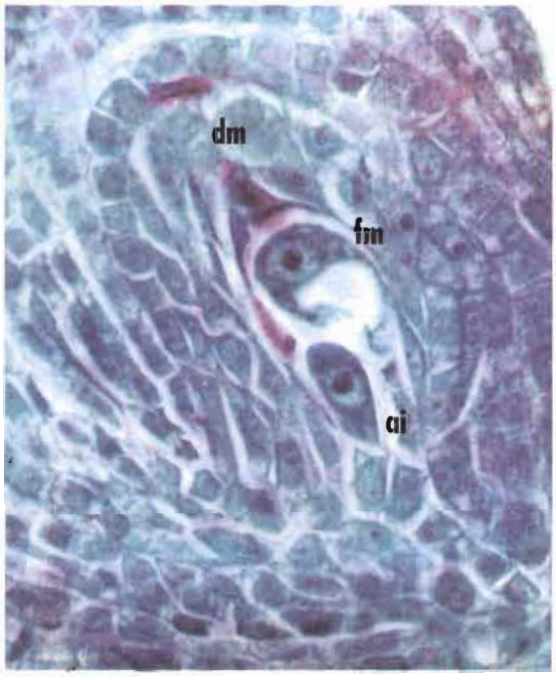

Figure 9.2 Aposporous development of Hieracium-type in *Poa pratensis* (sectioning and staining procedure). Macrospore degeneration on the micropylar side (dm), enlarged functional megaspore (fm), and aposporous initial in lateral-chalazal position (ai) (800x). (Mazzucato et al. 1995).

Figure 9.3 Clearing techniques in *Tripsacum* spp. a. Diplosporous enlarged megaspocyte observed under interference-phase contrast after a methyl benzoate-dibutyl phthalate clearing procedure (600x). (Leblanc et al. 1995a). b. Callose deposition during megasporogenesis in a sexual line after a sucrose-aniline blue clearing procedure (250x).

be useful for progeny tests and should therefore be considered. Progeny tests are usually performed on seedlings or fully-grown plants, but other tissues from earlier developmental stages, such as ovaries, endosperms or seeds, can also be used.

1. Analysis of pollinated ovaries or seeds. Determining ploidy levels in pollinated ovaries or seeds (albuminated) provides information on both reduction (meiosis) and fertilization events. Ratios between endosperm and embryos and between female and male contributions to the endosperm in apomicts often differs from those in sexual plants except for the Panicum-type aposporous development (Figure. 9.1). For many other apomictic pathways, these ratios differ. For example, endosperms found in tetraploid diplosporous apomicts are higher than in their sexual tetraploid counterparts for identical pollen donors (i.e., 10x versus 6x if the pollen is 2x); endosperm/embryo ratio for autonomous apomixis is 2:1 and 5:2 [(4x + 4x) + 2x / 4x + 0] for tetraploid pseudogamous apomicts (tetraploid pollen donor). Fertilization by unreduced pollen (Chao 1980; Huff and Bara 1993) and endopolyploidization, which sometimes occurs during endosperm development, is also possible and may further complicate analyses. However, endosperm ploidy level(s) may suggest apomictic reproduction or allow the quantification of facultative apomixis. Nevertheless, it cannot reveal the precise nature of the apomictic mechanisms involved.

Ploidy level in fertilized ovaries or immature seeds cannot easily be determined using classical chromosome counting methods, but flow cytometry now permits rapid measurement of DNA content in a variety of plant tissues, including single embryos, young endosperms, or seeds (Galbraith et al. 1983; Kowles et al. 1990; Hignight et al. 1991). Analyses in numerous apomictic species have proven flow cytometry to be a rapid and reliable procedure for determining the mode of reproduction (Mazzucato et al. 1994; Brautigam and Brautigam 1996; Grimanelli et al. 1997b; Gupta et al. 1998; Naumova et al. 1999; Matzk et al. 2000). Another option for DNA content estimation of the endosperm nuclei is to combine staining with 4'-6-diamidino-2-phenylindole (DAPI), fluoresence microscopy, and image analysis (Naumova et al. 1993; Sherwood 1995; Caceres et al. 1999).

2. Ovule regenerated plants. In tetraploid accessions of *Allium tuberosum*, Kojima and Kawaguchi (1989) reported a high frequency of tetraploid regenerated plants from unpollinated cultured ovules, suggesting apomixis expression. This indicator could be applied in screening because, in similar culture media, sexual plants would generate few (poly)haploids, whereas apomeiotic ovules would grow mostly into plantlets with the same number of chromosomes as the mother plant.

3. Analysis of progeny plants. Progeny tests must clearly identify either hybrid offspring (n + 0 types are generally poorly represented) or seed production in absence of pollination when pseudogamous apomixis or autonomous apomixis, respectively, are suspected. Hybrids can be identified using (*i*) morphological descriptors, (*ii*) cytological data, and/or (*iii*) marker analyses, if the origin of the progeny is appropriate.

Remarks on progeny size. The use of progenies from controlled crosses is recommended. Male parents bearing discriminating traits (dominant traits, different chromosome numbers, etc.) should be chosen when available, limiting possible confusions between selfed and hybrid progenies. However, open pollinated progenies can be used when mother plants are sufficiently heterozygous to detect

segregation after selfing and when there is significant diversity in the surrounding field collection, as is the case for most apomictic species, which are generally polyploid, polymorphic, and highly heterozygous.

Identifying or quantifying apomixis does not require the same number of progeny. To detect apomixis, a relatively small number of progeny (15–25) can be analyzed. Aberrant rates typically are a:n ratios with 'n' the progeny size and 'a' the number of aberrants observed in the progeny. Statistically, such samplings are binomial; 'p' (aberrant rate) is the ratio to be estimated for a given value of n (progeny size) on the basis of an observed value for a (number of aberrants detected within the progeny). Confidence limits for p in a binomial sampling are given in Figure 9.4 for various values of n (a = 0.025). Note that for n>30, confidence limits can be estimated using formulas for the normal distribution. Curves shown in Figure 9.4 clearly indicate that, up to n = 100, the information obtained is poorly significant regardless of the value of a. Finally, to obtain good estimations of aberrant rates (i.e., less than 10% confidence limits), it appears that a high number of individuals is required.

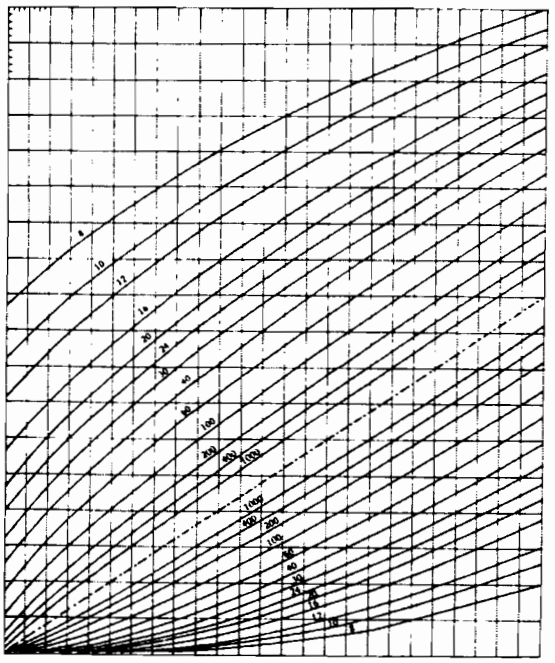

Figure 9.4 Confidence limits (α=0.025) for p in binomial sampling, given a sample fraction a/n. The numbers printed along the curves indicate the sample size, n. For a given value of a/n (abscissa), limits for p (p_A and p_B) are the ordinates read from the appropriate lower and upper curves ($Pr\{p_A \leq p \leq p_B\} \leq 1\text{-}2\alpha$).

Chromosome number determination within progenies. The sexual or asexual origin of offspring is not detectable from crosses made at the same level of ploidy, but 2n + n and n + 0 off-types are easily detected even at the seedling stage. Interploidy-level crosses could be used to detect all classes of offspring, but information can be biased by disturbances caused by unstable ploidy levels or ploidy barriers. Chromosome counts can be made from root tips, microspores, or any somatic tissue using flow cytometry (Hignight et al. 1991).

Detection of seed production in absence of pollination. Species carrying non-hermaphrodite flowers obviously represent the easiest situation, the only precaution required being to avoid pollen contamination. Contrarily, emasculation will be required unless an appropriate genetic system that ensures male sterility can be developed. Such systems require thorough knowledge of the genetics and genetic stocks of the material under study, making their application very limited in natural populations. They have been exclusively developed in experimental mutagenic populations of sexual model species (*Aradidopsis thaliana*, *Petunia hybrida*), with the aim of identifying mutants that reproduce through autonomous apomixis (Koltunow et al. 1995b; Chaudhury et al. 1997; Ramulu et al. 1997).

Markers for hybrid detection. Traits under simple genetic control are ideal for progeny testing by crossing recessive maternal genotypes with homozygous dominant testers (Hanna et al. 1970; Bashaw and Hanna 1990). Models for estimating levels of apomixis by following marker segregation have been developed (Marshall and Brown 1974), however, recombination can occur without fertilization, and the presence of dominant traits in progeny tells nothing about the origin of the off-types (n + n or 2n + n) in the absence of cytological data. Moreover, identification of such "ideal" markers in apomictic species or agamic complexes is not necessarily easy, because traits in polyploid apomicts are difficult to analyze genetically.

Morphological descriptors are the easiest means for conducting progeny tests. If the tester (pollen donor) differs significantly from the progeny-tested plant, hybrids will vary sufficiently from the maternal type to allow detection. In the case of selfing, because apomicts are generally highly heterozygous, offspring arising through sexuality will vary sufficiently from the mother plant to be scored as off-types. In most species, (poly)haploids are easily detected because of their particular phenotypes and the low vigor they exhibit (Asker and Jerling 1992). However, when using morphological descriptors, it is often not possible to distinguish between sexuality (n+n) and genomic accumulation (2n + n). But when morphological and cytological (chromosome number) data are combined, the identification of all classes is theoretically possible. Analysis of seedlings has the major advantages of timeliness and saving space, but the most informative descriptors for screening purposes are usually expressed at maturity. There are few reports of successful progeny testing for morphology on seedlings after interspecific crosses (Williamson 1981).

Isozymes or molecular markers can be used to assess variation in progenies (fingerprinting analyses; Nybom 1996). Finding good polymorphic isozyme systems, RFLP probes, or primers for PCR as candidates for fingerprint experiments is not a major obstacle. Although genetic analysis is still hindered by polyploidy, any variation in isozyme or DNA patterns indicates off-type production, provided that somatic recombination does not occur frequently in the material under study. Esterase and peroxydase were the first systems used to isolate sexual plants from *Panicum maximum* (Smith 1972). Apomixis expression was also confirmed or quantified using isozymes in *Taraxacum* (Ford and Richard 1985), *Arabis holboellii* (Roy and Rieseberg 1989), *Allium tuberosum* (Kojima et al. 1991b), *Poa pratensis* (Wu et al. 1984; Barcaccia et al. 1994), *Tripsacum* spp. (Leblanc 1995), and *Malus* sp. (Ur-Rahman et al. 1997).

Mazzucato et al. (1995) showed a slightly higher capacity of RAPD markers in discriminating off-types in progenies from the same species, when compared with three polymorphic isozyme systems or with analysis of traditional morphological traits. Although still seldom used, molecular markers have been successfully used for progeny fingerprinting (e.g., *Poa pratensis*: Huff and Bara 1993; Barcaccia et al. 1997; *Paspalum notatum*: Ortiz et al. 1997).

Choosing Suitable Procedures

Analyses at the Plant Level versus Progeny Tests

1. Nature of the information obtained. Apomixis results from apomeiotic processes (apospory or diplospory) that produce unreduced ESs, and parthenogenetic embryo development from unreduced eggs. Although nonreduction and parthenogenesis are thought to be closely linked in apomicts, observations and/or analyses of the plant itself

obviously provides insights only about apomeiotic or meiotic events, not about the complete process of apomixis. Data on the next generation (progeny test) must be collected to study fertilization and parthenogenesis events as well as the degree of apomixis. The choice of the level of analysis (apomeiosis / parthenogenesis / apomixis) depends on the objectives of the research, i.e., whether one wishes to determine only cytological processes, study parthenogenesis, or investigate apomixis in its entirety.

2. Comparing results. Limited information is available on diplosporous development, but cytological analyses of parent plants compared with progeny tests generally show good agreement between apomeiosis and apomixis screenings in *Eragrostis curvula* (Voigt and Burson 1981), *Allium tuberosum* (Kojima and Nagato 1992b), and *Tripsacum* spp. (Leblanc 1995). By contrast, the situation in aposporous species appears more complex: cytoembryological analyses generally revealed higher sexual potential than did morphology-based progeny tests in *Panicum maximum* (Savidan 1982b), *Poa pratensis* (Nygren 1951), and *Bothriochloa-Dichanthium* (Harlan et al. 1964). The same tendency was also observed by Mazzucato et al. (1996) in *Poa pratensis*, when auxin tests and field data were compared. However, using progeny tests on more than 100 *Brachiaria* F_1s, Miles and do Valle (1991) classified ten plants that were highly facultative apomicts as sexual, according to cytoembryological tests. Sexual potential in aposporous tropical grasses has generally been scored according to the formation of 8-nucleate ESs that may develop concomitantly with several apomeiotic (4-nucleate) ESs. The competition among ESs—more favorable to apomeiotics (Savidan 1982a)—and the possible weakness of certain hybrids that are eliminated early, may explain the overestimation of sexuality in facultative apospore as measured using cytoembryology (Clausen et al. 1947; Kojima and Nagato 1992b). This was confirmed by Savidan (1982a) in one *Panicum maximum* accession: sexual potential was estimated using a clearing procedure at 22.5%, but only 3% of the open pollinated adult progeny, were off-types. Elimination of hybrid offspring occurred at germination (-7%) or after transferring plants to the field (-12.5%), because of their inbred nature (resulting from selfing or hydridization with genetically close genotypes in the collection). On the other hand, after self- or sib-pollination, the lack of heterozygous loci in segregation may cause an overestimation of apomixis, with progeny tests showing the presence of "apparent apomixis" (Bayer et al. 1990).

Screening Procedures: Advantages and Constraints

Until recently, screening tools for mode of reproduction were limited to easy-but-late morphological progeny tests or skill-demanding and time-consuming cytological sectioning methods (see Table 9.3). During the past 15 years, new tools in molecular and cell biology have made screening for mode of reproduction more efficient, rapid, and reliable. These techniques include ovary progeny testing, flow cytometry for determining ploidy level, auxin test, and molecular markers that cosegregate with reproductive mode. The major disadvantage of the new methods is their expense. In addition, though the methods seem to agree with cytological and/or field observations, additional data are needed to confirm their reliability.

1. Apomixis identification and characterization. As mentioned, apomixis may be detected in various ways, but cytoembryological observations are ultimately needed to confirm the origin of the ES and to determine the type of apomixis. Clearing techniques are now quick and easy but require the use of phase-contrast or differential

interference contrast optics, both entailing considerable expense. Stain clearing techniques that allow observation of ovule details under traditional optics are less expensive. Molecular markers that cosegregate with apomixis, which enable analysis at earlier growth stages than cytoembryology, require the development of special plant materials and protocols, and the cost of associated supplies is often beyond the means of many research groups. Moreover, they may not be used with materials that differ in origin from the materials used to identify the markers, especially in the case of the highly cross-specific RAPDs (Williams et al. 1993). Morphological progeny tests are time- and space-consuming because good descriptors are usually expressed in adult plants and a minimum of 15 to 25 offspring are needed. However, these tests do not require much equipment or technical skill, and can thus be managed everywhere. Their main drawback is that they produce frequent errors because facultative apomixis occurs more often than previously thought. Moreover, progeny with sexual origin may resemble the mother plant in morphology, leading to misclassification and to an overestimation of the degree of apomixis. The existence of this gray area in progeny plant classification was reported by Williamson (1976), after extensive progeny testing in *Poa* sp. This makes morphological progeny tests unreliable when apomixis is highly facultative, but more efficient as apomixis expression increases. Early progeny tests using isozymic or molecular markers can be conducted for apomixis detection on 15–25 offspring. Only a few isozyme systems are

Table 9.3 Advantages and disadvantages of important procedures for the investigation of modes of reproduction at the plant and progeny levels. * See Ragot and Hoisington (1993) for RFLP and RAPD costs.

	Plant level analyses			Progeny tests			
Procedures	Cytoembryology (clearing procedures)	Molecular markers co-segregating with apomixis*	Auxin tests	Chr. counting in ovaries or seeds	Adult Plants Chr. counting*	Morphology	Fingerprinting
Information expected	Apomixis type determination and sexual potential estimation.	Depends on the nature of the marker(s) identified (to date linkage with apomeiosis).	Indication of apomixis expression; estimation of the degree of parthenogenesis	Indication of apomixis expression; estimation of the degree of apomixis.	Off-types of 2n+n and n+0 nature detection.	Apomixis identification and quantification; off-types nature if combined with chromosome counting.	
Plant materials required	15 to 100 flowers, depending on the objectives.	Already determined materials in segregation for marker identification.	100 flowers.	50 to 100 ovaries/seeds.	Apomixis identification: 15 to 25 offspring. Apomixis quantification: at least 100 offspring.		
Advantages	Easy and quick to perform after flowering.	Analyses can be performed early.	Easy and quick to perform after flowering	Easy and quick to perform after pollination.	Easy if flow cytometry (embryo, endosperm, plantlets).	Easy	Analyses on young offsprings possible.
Constraints	Expensive equipment for microscopy.	Preliminary work to determine materials. Use of the markers across accessions of different origins? Expensive.	The auxin test has been mainly used to date in cool-season grasses.	Expensive equipment for flow cytometry.	Time consuming (classical methods) or expensive if flow cytometry is used.	Time and space consumming. Morphological tests: unreliable if apomixis is highly facultative.	

required to indicate apomixis and determine the nature of the hybrids detected. RFLPs and RAPDs can also be used in the same way, but at greater expense.

2. Degree of apomixis expression. Many offspring are needed to obtain a good estimate of the degree of apomixis. Both auxin tests and flow cytometric analyses of pollinated ovaries or seeds provide good estimates of sexual potential, though distinguishing 2n + 0 from n + n offspring might be difficult in certain cases. In contrast, systematic chromosome counting within progenies is useful for detecting 2n + n and n + 0 off-types, but it does not separate 2n + 0 from n + n offspring, and without flow cytometry it becomes tremendously time consuming. Progeny tests combining cytology and marker analyses represent the best option for identifying the different classes of offspring within apomictic progenies. To limit cytology work (when flow cytometry is not available), markers can be applied first to separate maternal offspring from (poly)haploids or hybrids. The origin of the latter may be determined according to the patterns they produce (i.e., 2n + n off-types must carry all bands from the mother plant, plus extra bands from the pollen), and then cytologically confirmed.

Choosing a Procedure

There are four main areas of apomixis research, each with distinct constraints and objectives: (*i*) the search for apomixis or elements of apomixis in new taxa, coupled with genetic studies in wild populations, (*ii*) germplasm characterization of apomictic species, (*iii*) genetic and biological studies for further manipulation of apomixis, and (*iv*) breeding of apomicts and introduction of apomixis into sexual crops.

Since gametophytic apomixis is formidably limited to perennial, polyploid, and outcrossing species, the search for apomixis in additional species should begin with taxa presenting these traits. The very first screening can be based on the expression of the already mentioned "indicators of apomixis," while more discriminative procedures may be applied to promising specimens. For germplasm evaluation, a representative sample of the collection must be chosen on the basis of morphological and cytological data, and traits of agronomic value such as disease resistance. Chromosome number, repro-ductive development, and degree of apomixis are the primary factors for which basic data must be collected to develop strategies for further research. Genetic studies also may be attempted to genetically dissect apomictic mechanisms (number of genes involved and their effects). Following this preliminary work, appropriate tools for larger-scale screening should be developed or chosen according to the apomixis characteristics of the collection (e.g., callose patterns for diplospory, ES clearing for apospory of the Panicum-type, etc.).

Sexual parents involved in crosses for apomixis inheritance studies must be carefully chosen using cytoembryology. Highly facultative apomicts are easily misclassified as sexuals using progeny tests. This causes distortions of segregation ratios for mode of reproduction among progeny. In the same way, looking for differences between sexual and apomictic development at the molecular level requires the analysis of genotypes that are well characterized for mode of reproduction. This may allow the development of near isogenic lines, an important step in identifying the gene(s) controlling apomixis.

Before apomixis can be transferred into crops or used in breeding programs, researchers need procedures to identify apomictic genotypes (see do Valle and Miles, Chap. 10; Savidan, Chap. 11) and to quantify apomixis in genotypes selected for varietal release. Progeny

testing in such programs may help identify apomixis, because offspring are necessarily produced as part of breeding schemes, but an entire plant cycle must pass before data are obtained (a serious drawback in the case of annual plants). Notwithstanding, in some cases—especially when low female fertility is affecting the plants (e.g., interspecific or intergeneric hybrids)—this may be the best way to test for mode of reproduction. Because in most species apomixis and sexuality do not express at the same ploidy level, estimating chromosome number within progenies using flow cytometry allows easy identification of apomictic genotypes in early backcross generations, but becomes less effective when chromosome numbers close to that of the recurrent parent are recovered. Appropriate cytological procedures or marker-assisted selection may also be used to identify apomixis.

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Chapter 10

Breeding of Apomictic Species

CACILDA BORGES DO VALLE AND JOHN W. MILES

Introduction

From a plant breeding perspective, apomixis may restrict genetic recombination, but it also provides a unique mechanism for developing superior cultivars and preserving these genotypes indefinitely. Apomictic plants, like sexual plants, develop seed in the ovule of the flower, but egg and sperm nuclei do not fuse to form an embryo. Therefore, the embryo of an apomictic plant receives all of its chromosomes from the mother plant. Unlike most asexually propagated plants—such as banana, potato, or horticultural crops that are propagated from vegetative parts of the mother plant—an apomictic plant is propagated through the very convenient vehicle of seed.

Early investigators (e.g., Darlington 1939) were led to believe that apomixis was an evolutionary "blind alley" due to the apparent lack of variation in natural apomictic populations. Indeed, obligate apomixis poses a formidable barrier to plant breeding: without the new gene combinations that result from sexual cross breeding, genetic improvement cannot occur, except by rare, random, and generally deleterious mutations. In truth, sexual or partially sexual plants have been found in native populations of most apomictic species, generating sufficient genetic variation to maintain the species under changing environments and providing germplasm for plant improvement.

Aside from citrus fruits (which exhibit apomictic reproduction through seed, but are generally propagated vegetatively), only a few forage and turf grasses have active apomictic breeding programs; these include species of *Eragrostis, Paspalum, Poa, Panicum, Pennisetum, Cenchrus,* and *Brachiaria.* These species (or agamic complexes) have many common attributes, which will be addressed in a general manner later in this chapter. Bashaw and Funk (1987) reviewed many aspects of breeding apomictic forage grasses, and recent papers have specifically considered plant breeding in the genus *Paspalum* (Savidan 1987; Burton 1992); in *Cenchrus ciliaris* (Bashaw and Funk 1987); in *Panicum maximum* (Savidan et al. 1989); in *Pennisetum* (Hanna et al. 1992); and most recently in *Brachiaria* (Miles and Valle 1996). In this chapter we focus on the *Brachiaria* breeding programs in Brazil and Colombia to illustrate pertinent aspects of apomixis vis a vis breeding programs.

Prerequisites for an Effective Forage Breeding Program

Beef production, especially in the tropics, largely depends on pastures, either native or planted to superior introduced species. Scientific research on forages is relatively recent compared to field crops. Experience with tropical forages is even more limited; the few commercially available cultivars are little more than "side of the road" collections, which were often accidentally introduced, mostly from Africa, evaluated in small plots, and then multiplied for release.

Cameron (1983) posed a Shakespearean question: "To breed or not to breed," in reference to Australian investment in breeding tropical forage plants utilizing limited genetic resources. To quote Harlan (1983): "It is fruitless to engage in plant-breeding programs with inadequate germplasm collections. . . ." Representative collections for most of the tropical apomictic grasses are limited, therefore, a key prerequisite for effective tropical forage breeding projects is to acquire diverse germplasm from the centers of origin of the target genus/species.

Panicum maximum was extensively collected by French and Japanese geneticists (Combes and Pernes 1970; Nakajima et al. 1978); the resulting germplasm collections are representative of the natural variation (Savidan et al. 1989). An extensive collection of *Brachiaria* was undertaken by the Centro Internacional de Agricultura Tropical (CIAT) in 1984–85 (CIAT 1986). Other important apomictic tropical forage genera (*Hyparrhenia*, *Melinis*, *Urochloa*, *Cenchrus*, and *Pennisetum*) still need to be collected to broaden variability and to identify sexual accessions to facilitate breeding.

Surveys of closely related species are relevant when sexual plants are not available in the apomictic species of interest or when other desirable traits cannot be found in the primary gene pool. In sexual crops, the search for apomixis may involve other species or genera in order to find cross-compatible wild relatives, as seen in the *Zea* x *Trypsacum* transfer program (Savidan, Chap. 11). To accomplish hybridization, research is needed to establish phylogenetic relationships and to overcome differences in ploidy level, genome relationships, and gene pools (Hanna and Bashaw 1987). If the species relationship is sufficiently close, and given that apomixis tends to restore fertility, one should be able to produce useful obligate apomictic, interspecific hybrids with good seed set.

An extensive species relationship survey was carried out on *Paspalum*, a large grass genus with tropical and subtropical adaptations (Burson 1983; Quarin and Norrmann 1987; Burson 1989; Quarin 1992). Two species are particularly important as forage grasses, *P. notatum* and *P. dilatatum*, and several others have shown promising results in agronomic and grazing trials (Grof et al. 1989b; Fernandes et al. 1992; Pizarro and Carvalho 1992; Batista and Godoy 2000). Approximately 400 species have been described taxonomically, and about 80% of these are polyploids. The genus has sexual diploids and apomictic and sexual polyploids, which range from triploids to 16*x* (Quarin 1992). Diploid species reproduce sexually and have regular meiosis (bivalent chromosome pairing and normal distribution). Polyploidy, apomixis, and irregular meiotic chromosome associations are highly correlated (Quarin and Norrmann 1987). Valuable information has been gathered about this genus, leading to more effective interspecific hybridization that may result in superior apomictic genotypes (Quarin 1987).

A second fundamental prerequisite for breeding is adequate knowledge of biology, cytology, and reproduction of the material at hand (Asker and Jerling 1992). Breeders have long been challenged by the problems of reproductive isolation resulting from apomixis and polyploidy. Efforts directed at determining the genetic basis of apomixis in several species have generally shown it to be under simple genetic control (see Sherwood, Chap. 5), e.g., *Bothriochloa-Dichanthium* (Harlan et al. 1964), *Panicum* (Savidan 1982), *Cenchrus* (Sherwood et al. 1994), *Paspalum* (Burton and Forbes 1960), *Brachiaria* (Ndikumana 1985; Valle et al. 1993b; Valle and Savidan 1996), *Tripsacum* (Leblanc et al. 1995b), and possibly *Eragrostis* (Voigt and Burson 1992). Hence it should be possible to manipulate apomixis in a breeding program once cross-compatible sexual or highly sexual

facultative apomicts are found (Harlan et al. 1964; Voigt and Bashaw 1972; Bashaw 1980; Savidan 1983; Hanna and Bashaw 1987).

Differences in ploidy level are common among sexual and apomictic species of tropical grasses (Burton and Forbes 1960; Carnahan and Hill 1961; Dujardin and Hanna 1983; Norrmann et al. 1989). However, in groups in which apomixis is found, diploid accessions are generally obligatory sexual while polyploids display different degrees of apomixis ranging from essentially sexual to obligate apomicts (de Wet and Harlan 1970; Quarin and Norrmann 1987; Valle et al. 1989; Valle 1990; Asker and Jerling 1992). In species with higher ploidy levels (6x or 7x), such as *B. humidicola*, sexuality may be found at the tetraploid level (Valle and Glienke 1991).

Sexually reproducing genotypes in the tropical forage grasses outcross and are highly heterozygous (Bashaw and Funk 1987). Some degree of self-incompatibility or strong inbreeding depression is common (Bashaw and Funk 1987). Rates of self-fertility in sexual *B. ruziziensis* were not affected by chromosome doubling and ranged from 7.2 to 8.4%, according to Lutts et al. (1991). When hybridization with apomicts has been possible, resulting progenies are highly variable owing to segregation in the heterozygous parents.

Since hybridization and production of fertile progeny are more effective when progenitors are at the same ploidy level, basic studies leading to the determination of chromosome number should be undertaken early in the program to enhance the chances of successful recombination of attributes by conventional crossing.

A third prerequisite for efficient breeding of apomicts, as in any plant improvement program, is the establishment of clear, achievable objectives, and the identification of sources of the desired attributes in the existing germplasm. This presupposes intimate knowledge of the species of interest, in order to identify limiting factors not readily overcome by simple selection of superior genotypes from the available germplasm or amenable to improved cultural practices. Once a constraint has been identified (e.g., disease susceptibility or low forage quality), the natural germplasm needs to be screened to identify candidates for hybridization. Ideally, the desired attribute(s) can be found in apomictic or cross-compatible sexual accessions with a superior agronomic background.

General Structure of a Breeding Program

A general selection and breeding scheme for apomictic forage species is presented in Figure 10.1. Note that *Brachiaria* serves as the example for the topics under discussion.

Brachiaria is native to the tropical savannas of Africa (IBPGR 1984), encompassing about 90 species with wide morphological and phenological differences (Clayton and Renvoize 1982; Renvoize et al. 1996). Apomictic cultivars of some of these species,

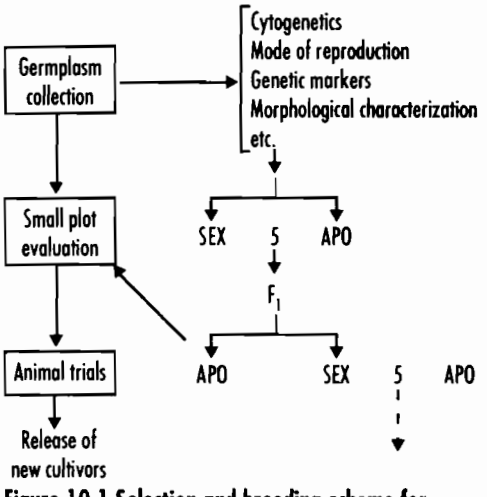

Figure 10.1 Selection and breeding scheme for apomictic forage species.

derived directly from natural germplasm, are widely sown because of their excellent adaptation to infertile acid soils, good forage value, and abundant seed production (Bogdan 1977; Keller-Grein et al. 1996). Cultivated pastures cover more than 45 million hectares of acid savannas in Brazil alone (Anuário Estatístico do Brasil 1995) and cultivars of two *Brachiaria* species (*B. decumbens* cv. Basilisk and *B. brizantha* cv. Marandu) are grown on more than 85% of that area. Genetic uniformity (associated with inability to generate new genotypes due to apomixis) resulted in a massive failure of cv. Basilisk in the Brazilian Amazon, stemming from its susceptibility to spittlebugs (Homoptera: Cercopidae), (Seiffert 1984).

Objectives

In the *B. decumbens-B. brizantha* agamic complex, we are seeking apomictic genotypes adapted to, and persistent on, low-fertility acid soils, with high levels of antibiotic resistance to a range of species and genera of spittlebug (Homoptera: Cercopidae). For continuously grazed pastures, stoloniferous growth and strong competitive ability are desired. If a new *Brachiaria* cultivar is used in a lay farming system in rotation with annual crops, genotypes with less "weedy" characteristics may be preferred (Valle, unpublished). Forage yield and quality are clearly important attributes of any forage plant cultivar, especially tropical grasses.

Achieving breeding objectives depends on the ability to reliably measure attributes of interest in large segregating populations. For example, a greenhouse screening methodology for resistance to spittlebug was developed at CIAT about a decade ago (Lapointe et al. 1989, 1992), but its utility for breeders was limited by its low capacity. More recently, an improved methodology was implemented to allow faster and more efficient screening for spittlebug resistance (CIAT 1998; Cardona et al. 1999). Another line of research now being pursued is based on identifying molecular markers that cosegregate with the resistance to spittlebugs (CIAT 1998).

Screening for nutritive value and consumption potential is also a laborious, costly, and time-consuming endeavor, many times depending on grazing trials. New procedures are being developed in an attempt to identify promising genotypes early in the breeding program. Such identification entails correlating measures of physical traits (shearing strength and grinding resistance), anatomical characteristics (patterns of lignin deposition and cuticle and epidermis width), and gas production potential with nutritive value (Hughes et al. 1998, 2000; Sabatel et al. 1999).

Germplasm Acquisition and Evaluation

Plant introduction programs and subsequent agronomic evaluation were required for *Brachiaria*, as they are for most tropical forage grasses. Prior to 1984, the small "world collection" of *Brachiaria* accessions numbered no more than 150 (Keller-Grein et al. 1996), and the desired combination of attributes had not been identified.

Extensive collection of *Brachiaria* was undertaken in 1984–85 in East Africa, the center of origin and diversity for the main species of agronomic importance. Nearly 700 new accessions in 24 species were collected in a joint CIAT/IBPGR (International Board for Plant Genetic Resources) venture, with the support of ILCA (International Livestock Center for Africa) in Ethiopia and Kenya, and national institutions in East Africa, including Zimbabwe, Grassland Research Station Marondera; Burundi, Institute des Sciences Agronomiques du Burundi (ISABU); Rwanda, Institute de Sciences Agronomiques du Rwanda (ISAR); and Tanzania, Tanzania Livestock Research Organization (TALIRO) (CIAT 1986).

As accessions were transferred to CIAT-Colombia and released from quarantine, large portions of this collection were subsequently forwarded to Brazil (approximately 400 accessions), Costa Rica (approximately 280 accessions), and Peru (approximately 260 accessions) for agronomic evaluation (Grof et al. 1989a; CIAT 1992). Because large numbers of accessions were involved, evaluation methodology needed to be simple and efficient, such as that proposed by Toledo (1982), in order to discard poorly performing materials. Since then, more detailed and intensive evaluation has been conducted at the Embrapa Beef Cattle Research Center in Campo Grande, MS, Brazil. Agronomic evaluation began with accessions planted in small plots with three replications. A periodic cutting regime was imposed for three years to estimate overall and seasonal production, regrowth vigor, seed production, and resistance to spittlebug and diseases (Valle et al. 1993a). At one harvest each year, samples were analyzed for crude protein content and in vitro digestibility. The range of variation observed within this collection is remarkable (Table 10.1). Nineteen selected accessions were then evaluated in regional trials in different ecosystems and superior genotypes were identified (Valle et al. 1997). The next evaluation step involved studying the effects of livestock on the pasture. Eight apomictic accessions were compared in paddocks to the commercial cultivar. Four of these were selected (Euclides et al. 2001) to undergo animal performance trials, the last step prior to release as a new cultivar.

Morphological characterization, applying numerical taxonomy and using 26 descriptors, was carried out for all 340 accessions in the Brazilian *Brachiaria* collection (Valle et al. 1993c). The objectives were to study the diversity of the accessions, analyze the dispersion and genetic distance between accessions and species, and organize the germplasm into groups of morphologically similar accessions, regardless of taxonomic classification. This type of study helps researchers define closely related accessions within and among groups from which individual progenitors may be selected for future crosses. This analysis revealed the continuous polymorphism that exists among three species (*B. decumbens*, *B. brizantha*, and *B. ruziziensis*) and clearly separated typical accessions of *B. humidicola*, *B. dictyoneura*, and *B. jubata* (Figure 10.2). The selection of accessions for pasture trials was based on an association of agronomic traits with morphological characteristics.

Cytology, Reproductive Mode, and Inheritance of Apomixis

Basic information about mode of reproduction and cytogenetics of sexually reproducing accessions was also ascertained from the

Table 10.1 Agronomic evaluation of *Brachiaria* accessions in Brazil

	N	LDMY (kg/ha)	%DSP	R
B. brizantha				
range	96	2040 - 9420	9 - 27	1.9 - 3.8
average collec.	96	4797	19	2.6
average select.	10	7503	18	3.1
B. decumbens				
range	35	1348 - 5543	10 - 25	1.5 - 3.2
average collec.	35	2369	16	2.0
average select.	5	4063	18	2.7
B. humidicola				
range	21	1908 - 4435	9 - 18	2.3 - 3.7
average collec.	21	3242	13	2.7
average select.	4	4358	15	2.6
B. ruziziensis				
range	20	1563 - 2685	9 - 19	1.4 - 2.7
average collec.	20	2160	13	1.1
average select.	2	3099	19	2.1
B. jubata				
range	11	1281 - 2320	7 - 22	1.5 - 3.1
average collec.	11	1327	16	2.3
average select.	4	1864	18	2.7

N. = number of accessions; LDMY = Leaf dry matter yield; %DSP = Percentage dry season production; R = regrowth during rainy season (0-6 max). Values are 3-year averages.

experimental plots. Previous reports on some species of this genus established the basic chromosome number as $n = 9$, and the most common ploidy level among commercial cultivars as $2n = 4x = 36$ (Schank and Sotomayor-Rios 1968; Ferguson and Crowder 1974; Valle 1986). *B. ruziziensis* is the only commercially cultivated species that is diploid and obligately sexual, with normal chromosome behavior at meiosis. Other species are polyploid ($4x$ or $6x$) and have irregular meiotic configurations. These polyploids are apomictic, with apospory characterized by a 4-nucleate embryo sac of the Panicum-type. One egg-cell and one (occasionally two) conspicuous polar nucleus can be observed in cleared ovaries. The two synergids are rarely seen. Meiotic embryo sacs of the Polygonum-type with an egg-cell, two large polar nuclei, and multiple antipodal cells are found in the sexual accessions and also in the apomicts, in differing proportions. *Brachiaria* is pseudogamous, therefore, pollen production results from normal meiosis and is abundant both in apomictic and sexual plants.

The diversity of the introduced collection justified a thorough search for sexuality. The mode of reproduction was determined by examination of embryo sacs for 427 accessions of 15 different species in Colombia and Brazil (Table 10.2). Flowers were fixed in FAA for 24 hours and later transferred to 70% ethyl alcohol. Ovaries were then extracted under a stereoscope and cleared using dehydration and methyl salycilate (Young et al. 1979). Structures were mounted on slides and examined with interference contrast microscopy. Results include discovery of obligate sexual accessions in species previously considered obligate apomicts, such as *B. decumbens*, *B. dictyoneura*, and *B. brizantha*, and determination of mode of reproduction for species never before studied, such as *B. serrata*, *B. platynota*, and *B. subulifolia* (Valle 1990).

Chromosome counts were taken on microsporocytes of various sexual accessions using traditional acetocarmin squashes. It was determined that the one sexual *B. brizantha* and all sexual *B. decumbens* accessions were diploids, whereas the majority of apomictic accessions of these two species were tetraploid.

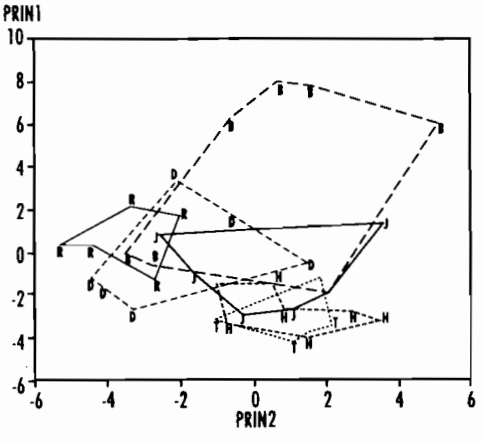

Figure 10.2 Distribution of 253 accessions of *Brachiaria* (B = *B. brizantha*; D = *B. decumbens*; R = *B. ruziziensis*; H = *B. humidicola*; J = *B. jubata*; T = *B. dictyoneura*) in two planes (PRIN1 and PRIN2) generated by Principal Component Analysis using seven morphological descriptors.

Table 10.2 Mode of reproduction of 15 species of *Brachiaria*, based on embryo-sac analysis

Species	no. accessions	Range sex	SEX	APO
B. arrecta	3	79 - 90	3	0
B. bovonei	4	7 - 27	0	4
B. brizantha	235	0 - 94	1	234
B. decumbens	54	0 - 100	22	31
B. deflexa	1	91	1	0
B. dictyoneura	6	0 - 96	1	5
B. dura	1	93	1	0
B. humidicola	52	0 - 100	2	50
B. jubata	34	0 - 94	5	29
B. miliiformis	1	6	1	0
B. nigropedata	3	5 - 20	0	3
B. platynota	3	3 - 97	2	1
B. ruziziensis	24	40 - 100	24	0
B. serrata	2	30 - 100	2	0
B. subulifolia	4	7 - 38	0	4
Total	426		65	361

The sexual *B. humidicola* accession is tetraploid, with regular bivalent pairing, whereas the few apomict *B. humidicola* examined are hexaploid (Valle et al. 1989; Valle and Glienke 1991). These materials are relevant for breeding purposes and also for studies of phylogenetics and polyploidization within the genus.

More recently, a thorough survey of ploidy levels of 435 accessions in 13 species of *Brachiaria* in Campo Grande, Brazil, was accomplished by means of flow cytometry (Penteado et al. 2000). Traditional cytology was used to verify chromosome numbers in specific situations. Despite a predominance of tetraploidy, variation in ploidy level within species was confirmed and levels never before described, such as pentaploids in *B. brizantha* (Letteriello et al. 1999) and heptaploids in *B. jubata* and *B. humidicola*, were observed. Cytometry is a valuable tool in breeding programs involving polyploid species because a large number of hybrids must be quickly and accurately screened for use in additional crosses.

Early hybridization studies indicated the need for ploidy compatibility to effectively produce hybrids in *Brachiaria*. Attempts by Ferguson and Crowder (1974) to hybridize a sexual diploid *B. ruziziensis* with an apomictic tetraploid *B. decumbens* proved unsuccessful. More recently, Hacker (1988) crossed a diploid sexual *B. decumbens* with a tetraploid apomictic cytotype and recovered a single, totally sterile triploid hybrid. In Belgium, natural diploids of *B. ruziziensis* were successfully polyploidized using colchicine (Sweene et al. 1981; Gobbe et al. 1981). The resulting induced-autotetraploids maintained the obligate sexuality of the original diploid material. Recently, three diploid accessions of *B. decumbens* were duplicated in vitro as described by Leblanc et al. (1995a), opening the possibility of intraspecific hybridizations (Pinheiro et al. 2000).

Ndikumana (1985) obtained the first interspecific *Brachiaria* hybrids by crossing the artificially-induced sexual tetraploid *B. ruziziensis* to natural tetraploid apomictic accessions of *B. decumbens* or *B. brizantha*. The 35 hybrids obtained were screened for mode of reproduction and chromosome behavior. Of 29 hybrids with *B. decumbens*, 15 were sexual and 14 apomictic. Six hybrids with *B. brizantha* broke down into four sexual and two apomictic. The ratio of sexual:apomictic obtained was close to 1:1, which pointed to a simple model of inheritance of apomixis. The relative ease of crossing after ploidy barriers were removed, together with the chromosomal configuration of hybrids, suggests a probable agamic complex involving these three species. Further support for this comes from work undertaken in Colombia and Brazil since 1988. Large numbers of crosses have been made under greenhouse and field conditions between sexual tetraploid *B. ruziziensis* (R) and different apomictic genotypes of *B. decumbens* (D) and *B. brizantha* (B) (Valle et al. 1991; CIAT 1992). The objectives of these hybridization programs were to explore the potential for manipulating apomixis in applied *Brachiaria* breeding programs, to study the inheritance of mode of reproduction, and to enlarge the tetraploid sexual pool of *Brachiaria*. To this end, the experimental scheme crossed different clones of sexual tetraploid *B. ruziziensis* (derived from the original Belgian material) to *B. decumbens* cv. Basilisk (which has excellent adaptation to acid soils and vigorous stoloniferous growth) and to *B. brizantha* cv. Marandu (which has spittlebug resistance and vigorous tufted growth) (Figure 10.3).

Greenhouse crosses were accomplished without prior emasculation of potted plants of *B. ruziziensis* or the sexually-reproducing hybrids. Inflorescences were brought from the field on the afternoon before pollination and

kept in vases of water. On the day of pollination, inflorescences were shaken over petri dishes to collect pollen, which was used on flowers from which stigmas had just extruded. The inflorescence from the sexual plant was prepared by removing unopened and old flowers. After brushing the turgid stigmata with pollen from the apomictic parent, the racemes of the sexual plant were individually bagged and labeled. Bags were collected when seed shattering started. Scarified seeds were individually germinated 4–6 months later in styrofoam trays with a sand:perlite mixture (2:1) or in petri dishes, and then transferred to plastic bags with soil, from which they were later transferred to the field (Valle et al. 1991).

Mode of reproduction was determined by embryo-sac analysis on 30–40 ovaries of 376 individual first-generation hybrids from greenhouse crosses in Brazil. No reliable genetic marker yet exists to determine hybrid nature of the progeny, therefore attempts to discriminate among individuals were made using morphological characteristics and/or electrophoresis. Whenever parental materials display wide differences in morphology or in band patterns, hybrids showing intermediate characteristics can be readily identified.

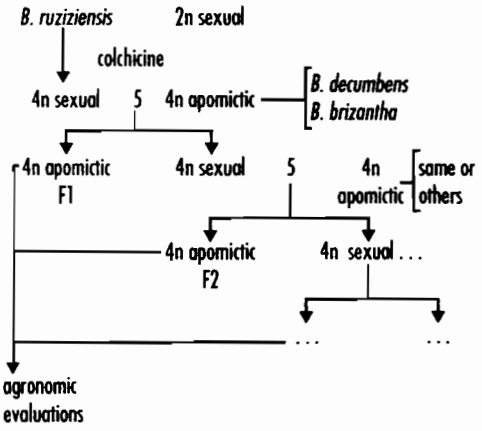

Figure 10.3 Hybridization scheme for breeding *Brachiaria* (adapted from Gobbe et al. 1983).

Studies conducted at CIAT identified an alpha-beta esterase system capable of discriminating among putative hybrids of carefully selected progenitors (Cruz et al. 1989a, 1989b; Calderón and Agudelo 1990).

Second generation crosses in Brazil included sexual x apomictic backcrosses, crosses between half sibs, full sibs, selfing of sexual F_1s, 3-way hybrids, and facultative apomictic x apomictic crosses. Results from this experiment (Table 10.3) point to a single dominant gene determining apomixis, as proposed for *Panicum maximum* (Savidan 1983), for *Brachiaria* (Ndikumana 1985), and *Cenchrus* (Sherwood et al. 1994). The excess number of sexual plants observed in some crosses may be due to crossing procedures; sexual maternal plants were not emasculated, and no special precautions were taken to avoid pollen circulation in the greenhouse, except after pollination when flowers were bagged.

Table 10.3 Segregation for mode of reproduction in *Brachiaria* hybrids

Type cross	SEX	APO	STER	Abn
F_1				
B.ruziziensis x *B.decumbens*	79	49		
B.ruziziensis x *B. brizantha*	125	123		
F_2				
B.ruziziensis x *B.decumbens*	2	0	2	0
B.ruziziensis x *B. brizantha*	7	0	0	0
BC				
B.ruziziensis x *B.decumbens*	10	9	0	0
B.ruziziensis x *B. brizantha*	9	7	0	0
3-W				
B.ruziziensis x *B.decumbens*	24	21	9	1
B.ruziziensis x *B. brizantha*	31	6	0	0
FS				
B.ruziziensis x *B.decumbens*	38	27	4	0
B.ruziziensis x *B. brizantha*	24	32	3	1
HS				
B.ruziziensis x *B.decumbens*	51	53	6	8
B.ruziziensis x *B. brizantha*	61	23	6	0

BC: backcross, 3-W: three-way hybrids, FS: full-sibs, HS: half-sibs.

The mode of reproduction of an independent set of 107 first generation *Brachiaria* hybrids was determined by progeny tests and by embryo-sac analysis in Colombia (Table 10.4). Embryo-sac analysis determined that 56 of the plants were sexual and 51 were apomicts (Miles and Valle 1991), a finding that agrees with the proposed hypothesis of simple inheritance. Although interspecific crosses may not be ideal for studying inheritance of apomixis, work on the agamic complex formed by *B. ruziziensis, B. brizantha, and B. decumbens* indicates simple genetic control for apomixis. Sexual x apomictic crosses released a large amount of phenotypic variation in the progeny (plant morphology, growth habit, and flowering time).

Progeny tests of the 107 open pollinated, first-generation interspecific hybrids were also used to assess reproductive behavior (Miles and Valle 1991). Seeds harvested from individual plants were sown in five-plant plots, in one to four replicates. The mode of reproduction of the mother plant was inferred from the relative uniformity or heterogeneity of the open pollinated progeny. The results were later compared to microscopic examination of embryo-sac structures of the hybrid mother plants. The two methods agreed closely, except for ten of the progenies, in which the degree of sexuality (determined by embryo-sac analysis) ranged between 10 and 77%. (Table 10.4). The degree of effective sexuality as detected by the progeny test was not closely associated with the proportion of sexual embryo-sacs observed microscopically. Whereas facultative apomictic hybrids with 10 or 17% sexuality produced heterogeneous progenies, other hybrids, in which sexual embryo-sacs were observed in up to 73% of the progenies, appeared to behave as obligate apomicts in the test. It is unclear what factor(s) contributes to determining effective reproductive behavior. Elucidation of the mechanism of apomixis might help explain its expression under different circumstances.

Table 10.4 Comparison between progeny test and embryo-sac analysis for determination of mode of reproduction for first-generation interspecific *Brachiaria* hybrids

	Mode of reproduction		Rate of sexuality (%)
	progeny-test	embryo-sac analysis	embryo-sac analysis
54 hybrids	sexual	sexual	
37 hybrids	apomictic	apomictic	10 - 73
4 hybrids	unclassified	apomictic	7 - 83
2 hybrids	unclassified	sexual	
Facultative apomicts first classified as sexual			
541-03	sexual	apomictic	10
544-04	sexual	apomictic	50
549-02	sexual	apomictic	17
554-02	sexual	apomictic	63
554-03	sexual	apomictic	77
683-01	sexual	apomictic	52
687-01	sexual	apomictic	70
693-02	sexual	apomictic	47
694-07	sexual	apomictic	43
702-06	sexual	apomictic	30

At CIAT and the Institute for Grassland and Environmental Research (IGER), Aberystwyth, Wales, U.K., a molecular marker for the apomixis gene(s) is being sought, which could prove potentially useful for determining reproductive mode. Pessino et al. (1997), using a bulk segregant analysis and RFLPs and RAPDs, were able to identify molecular markers cosegregating with apomixis in a small (n = 43) *Brachiaria* F_1 population. Two clones (umc147 and umc72) belong to a duplicated linkage group that maps to the distal part of maize chromosome-1 long arm and chromosome-5 short arm. Another, (OPC4), previously reported as a potential marker for apospory in *Pennisetum*, also cosegregated well in *Brachiaria*. In later work, Pessino et al. (1998), using RFLPs and AFLPs, generated a complete map for the region in maize chromosome 5, identifying at least two markers closely linked to the apospory region.

Markers PAM52-5 and PAM49-13 were located respectively at 1.2 cM and 5.7 cM, on either side of the target locus. The map shows close synteny to regions of maize chromosome 5 and rice chromosome 2. If proven that apomixis is, in fact, conditioned by a single dominant gene, and these markers prove to be tightly linked, then it should be possible to determine the reproductive mode of a hybrid plant before flowering or even before transplanting into the field. This would substantially improve the genetic efficiency of breeding schemes (see also Leblanc and Mazzucato, Chap. 9).

Brachiaria breeding involves interspecific hybridization because compatible sexual plants could not be found in the agronomically important species. Wider crosses may also be required to transfer important traits (such as complete antibiosis present in one accession of apomictic *B. jubata* [Lapointe et al. 1992]) to the commercially important apomictic cultivars, using sexual plants of different species as "bridges." *B. humidicola*, for instance, is well adapted to waterlogged soils, however, it has proven impossible to hybridize the only sexual tetraploid *B. humidicola* accession with the tetraploid apomictic varieties of *B. decumbens* and *B. brizantha*. Further phylogenetic studies need to be conducted to determine possible compatible materials with which to attempt crosses.

When conventional sexual hybridization is impossible, direct transfer of DNA between species may be considered. Protocols for callus induction and regeneration have been developed for five *Brachiaria* spp. (CIAT 1993) and a system for genetic transformation using particle bombardment has been established (Lennis 1998; Galindo 1997).

Breeding Plans

The delineation of clear breeding objectives, the identification of sources of desired attributes in apomictic *Brachiaria* accessions from the newly enhanced germplasm collection, and the creation of a cross-compatible sexual material has led to the possibility of developing large-scale, applied plant breeding projects for this important forage grass.

The fundamental objective of any plant breeding program for an apomictic species in which genetic recombination can be achieved is the identification among segregating progenies of superior, true-breeding apomictic genotypes suitable for cultivar status. Breeding plans that are being implemented for *Brachiaria* assume (*i*) simple (probably monogenic) control of apomixis and (*ii*) predominantly allogamous reproduction with high levels of self-incompatibility or strong inbreeding depression.

Information regarding inheritance of traits is limited to recent data showing a strong correlation between the reaction of spittlebugs to a series of parents and their top-cross progenies (Miles et al. 1995). Eleven apomictic accessions, chosen to represent a range of spittlebug reactions, were each crossed to the same susceptible sexual clone to generate 11 segregating, F_1 families. Spittlebug reaction was assessed on apomictic clones and on ten random sibs in each of the 11 top-cross families. The close parent-progeny correlations found for percentage of nymphal survival [$r = 0.95$ ($P<0.0001$)] or duration of nymphal stage [$r = 0.83$ ($P<0.001$)] suggest strong genetic control of spittlebug resistance. We assume that spittlebug resistance and most, if not all, additional attributes of interest are of complex quantitative inheritance. Hence, the basic approach has been the development and improvement of broad-based *Brachiaria* populations through various recurrent selection schemes.

Most of the small number of plant breeding projects conducted (or proposed) for apomictic

species rely on large-scale hybridization between sexuals (facultative apomicts) and obligate apomicts to produce large populations, from which superior apomictic hybrids are isolated (e.g., Burton and Forbes 1960; Taliaferro and Bashaw 1966; Gobbe et al. 1983; Bashaw and Funk 1987; Savidan et al. 1989). Three-way or double crosses involving more than one apomictic male may be required, depending on the distribution of desired attributes among available apomictic genotypes. These schemes will require careful selection of parents and the evaluation of large populations to find the desired combination of characteristics in a true breeding apomictic genotype. Such approaches offer the opportunity to generate novel apomictic genotypes, however, they are essentially conservative in the longer term because the opportunity for genetic gain is eventually exhausted.

To continue genetic advances, a systematic scheme for recycling selected hybrid genotypes will be required (i.e., population improvement by recurrent selection). Any *Brachiaria* breeding population must obviously include sexual genotypes to ensure genetic recombination. An important consideration in the development of populations is whether to attempt to include and maintain apomictic genotypes in the populations. Several authors have suggested improvement of a sexual population (e.g., Pernes et al. 1975; Miles and Escandón 1997). The scheme for sexual population improvement proposed by Pernes et al. involves recurrent crossing to elite apomicts. Therefore, the superior sexual hybrid genotypes in each crossing cycle need to be identified to resynthesize a fully sexual pool.

Miles and Escandon (1997) proposed recurrent intrapopulation improvement of a heterogeneous sexual population developed from sexual segregants selected from progeny of an initial series of crosses of a sexual tetraploid biotype with apomictic genotypes. In the case of *Brachiaria* (and other species with a similar genetic control of reproductive mode), the second scheme would have the important advantage of obviating the need to determine reproductive mode in each generation. As the frequency of favorable alleles is increased in the sexual population, hybridization with elite apomictic genotypes will generate an array of improved apomictic and sexual segregants while the sexual population remains fully sexual, i.e., advanced from purely sexual clones selected from within the population. Superior apomicts in the hybrid populations would be candidates for cultivar release. They could also be used in the subsequent cycle of sexual x apomictic crosses, although it would not be expected that crossing back to the parental sexual pool would lead to maximum expression of heterosis. A sexual population based on selected first cycle sexual x apomictic hybrids is being developed at CIAT. Thirty-two such hybrids, involving a total of ten apomictic paternal parents crossed to the same sexual tetraploid *B. ruziziensis*, were initially selected. The selection was subsequently reduced to 30, when it was determined that one clone was very susceptible to a virus and another exhibited a low, but consistent, percentage of apomictic embryo sacs. Each clone was vegetatively propagated ten times and genotypes were planted in random spacing in an isolated field plot. This population will be managed by standard half sib or mass selection (Figure 10.4).

A second population containing both sexual and apomictic genotypes has been formed from sexual x apomictic hybrids (Figure 10.5). In each generation, two types of progenies are planted in alternating positions in a square grid: (*i*) apomictic progenies of selected apomicts (reproductive mode determined by

embryo-sac analysis) and (*ii*) open pollinated progenies of selected sexual segregants. The open pollinated progenies of sexuals will

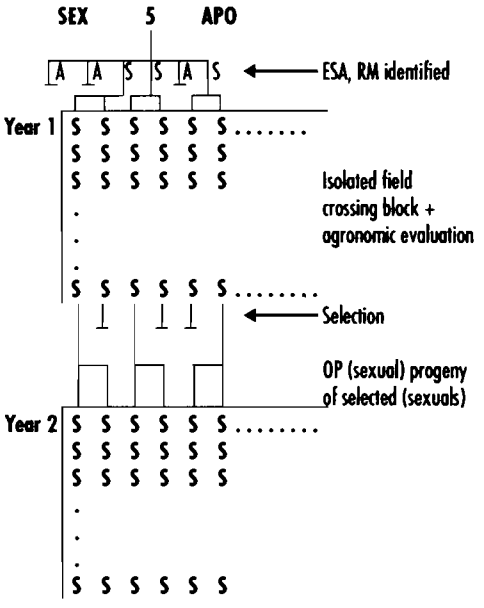

Figure 10.4 Simplified diagram of recurrent mass selection employed in SEX *Brachiaria* population.

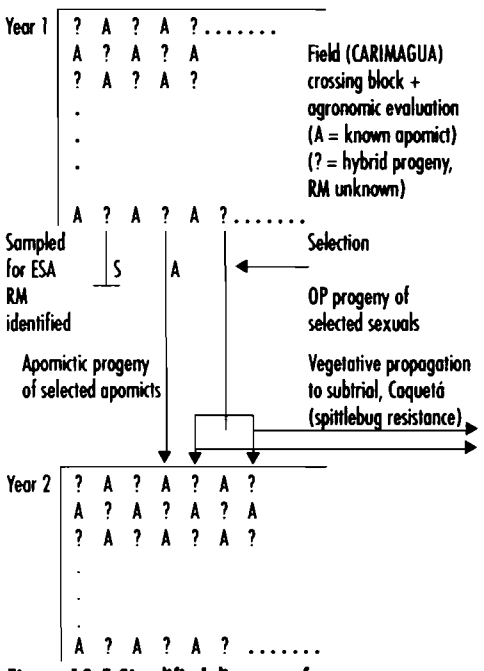

Figure 10.5 Simplified diagram of recurrent selection scheme employed in APO/SEX *Brachiaria* population at CIAT.

contain both sexual and apomictic genotypes. Seed harvested from a sexual segregant will represent a new half sib family, the result of pollination by both sexual and apomictic plants in the crossing block. Seed harvested from the apomictic segregants will produce a uniform apomictic progeny. As reproductive mode of selected individuals in the open pollinated progenies is determined, the recombinant open pollinated progenies and the apomictic progenies will be defined for the subsequent year's crossing block.

In this scheme, both sexual and apomictic genotypes are being improved simultaneously, and new apomictic recombinants—candidates for new cultivars—will be identified in each cycle. However, the expense of determining reproductive mode with every generation is substantial. A critical assessment of the relative efficiency, genetic and economic, among alternative breeding schemes is needed.

More complex schemes might be envisioned. Since the desired cultivar is essentially a hybrid, a recurrent selection scheme based on performance of hybrid genotypes, such as reciprocal recurrent selection (Hallauer and Miranda 1988) or recurrent selection on specific combining ability (Hull 1945) may be appropriate. In the latter scheme (Miles and Escandón 1997; Miles 1995, 1997), the sexual population would advance in isolation from the apomictic tester, which over cycles of selection would allow the development of heterotic interactions with one or more apomictic tester clones. With each cycle of selection, the scheme would generate a number of apomictic genotypes in the test-cross progenies for further testing and possible elevation to cultivar status.

We need to know much more about the genetics of important traits and the inheritance of apomixis in each species (or agamic

complex) of interest. The design of breeding schemes that approach "optimum" efficiency—genetically and economically—will depend, as well, upon the cost and reliability of the method(s) available to assess reproductive mode in segregating populations.

An important obstacle in the recurrent selection programs is the difficulty of achieving a full generation each year. Most attributes of interest in perennial *Brachiaria* spp. are difficult or impossible to reliably assess in a single season. Seed dormancy, which is typical in tropical wild grasses, also delays the breeding cycle and poses an obstacle that has not been overcome, even at the experimental level. A detailed understanding of the factor(s) causing physiological dormancy in *Brachiaria* seeds is essential to the rational design of dormancy-breaking treatments. In vitro techniques of embryo rescue were developed in Brazil, and Rodrigues-Otubo et al. (2000) established the age and culture medium for first generation interspecific hybrids of *Brachiaria*. Embryos that were drawn for use 9–12 days after pollination presented the highest percentage of direct regeneration, although survival rates were clearly genotype dependent. Embryo rescue resulted in significantly higher numbers of hybrid plants being recovered when compared to direct germination of hybrid seeds. This technique requires expertise and a fully operational tissue culture laboratory, and therefore is it justified only when special circumstances arise, such as the incompatibility of highly promising progenitors or when many progenies of a specific cross are needed.

Concluding Observations

Experience to date with apomictic forage grasses suggests that apomixis is simply inherited and can successfully be manipulated in an applied plant breeding program aimed at developing superior new commercial cultivars. Areas for future research and development include the design of breeding schemes that are both genetically and economically efficient and that account for the special complications—and opportunities—offered by apomixis. Methods of assessment of reproductive mode, although much improved, are still too costly and time-consuming for a large-scale breeding program and need yet more improvement. Once synthetic apomictic annual crops are developed, the substantial experience from current endeavors in the breeding of natural apomicts should be extremely useful for the design and implementation of their respective breeding programs.

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Chapter 11

Transfer of Apomixis through Wide Crosses

YVES SAVIDAN

Introduction

Interspecific hybridization has been used extensively to transfer agronomically important genes that control resistances to diseases and insect pests. Recent advances in tissue culture, especially in molecular biology, have further widened the scope of alien gene transfer—and the outlook for wide hybridization in crop improvement seems more promising than ever. But does that outlook also apply to the transfer of sequences involved in plant reproduction, especially those involved in apomixis?

The interface between conventional cytogenetical approaches and new molecular techniques makes the "conventional" wide cross approach very competitive when the trait is simply controlled and the gene(s) to be transferred is (are) available in a species that belongs to the secondary gene pool. The genetic analyses reviewed by Savidan (2000) and Sherwood (Chap. 5) suggest that apomixis a good candidate and offer support for the ongoing wide cross projects. Such projects have encountered unexpected difficulties, and several papers have questioned the ultimate likelihood of transferring apomixis to any crop. Nevertheless, knowledge gathered through the quest for wide crossing apomixis into useful crop species, which relates to the genetic control, transmission, and expression of the trait (Grimanelli et al., Chap. 6), has proven extremely valuable for those investigating other approaches. Accordingly, three paths are now being pursued in the effort to introduce apomixis into major crops: (*i*) the wide hybridization, (*ii*) the identification, isolation, and manipulation of sequences from wild apomicts, and (*iii*) the creation of an apomictic reproduction *de novo*, from individual mutations (Grossniklaus, Chap. 12; Praekelt and Scott, Chap.13). In this chapter, I review progress to date and the problems or questions that have emerged from work aimed at wide crossing of the apomictic trait.

Scientists have tried for decades to use wide crosses to transfer the apomixis trait into valuable food crops, including wheat, maize, and pearl millet. The first attempt involved maize and was initiated approximately forty years ago (Petrov et al. 1979, 1984). Crossing a tetraploid maize ($2n = 4x = 40$) with a tetraploid *Tripsacum dactyloides* ($2n = 4x = 72$), the Russian scientists successfully produced maize-*Tripsacum* F_1s and BC_1 hybrid derivatives that, according to progeny tests, reproduced apomictically. The BC_1 plants combined 20 maize chromosomes with one complete set (18) of *Tripsacum dactyloides* chromosomes. Efficient techniques for evaluating chromosome numbers, embryo-sac analysis, etc., were not available, making screening of large numbers of progenies for apomixis difficult. Consequently, little progress was made in this transfer effort. Recently, the Russian materials were transferred to the United States, and introgression efforts were reinitiated. An important piece of information generated by

these renewed efforts relates to the facultativeness of apomixis: because maize-*Tripsacum* hybrids and hybrid derivatives are completely male-sterile, progress in backcrossing is linked to the degree of facultativeness, and especially the presence of B_{II} or $n + n$ off-types as a requisite for a possible return to a normal maize chromosome number. According to Kindiger and Sokolov (1995) and Kindiger et al. (1996), the *Tripsacum dactyloides* lines they are using never produce such off-types, making the transfer impossible by conventional means only.

A program initiated in pearl millet at the end of the 1970s may still be considered the most advanced and possibly the most promising (Hanna et al. 1993). Early constraints to the program (the availability of genetic resources of the apomictic wild relatives and the limitations of the available screening tools in the 1970s) slowed progress in its early years. Recent molecular mapping activities, however, have provided intriguing new data (Ozias-Akins et al. 1993, 1998; Leblanc et al. 1995; Grimanelli et al. 1998a, and Chap. 6) and offered encouragement to scientists working in this area.

Efforts to wide cross apomixis to wheat have struggled to bypass crossing barriers and very low apomixis expression in the wheat genome background. Carman (1997, Chap. 7) now concludes that apomixis can only be expressed from interspecific hybridizations using progenitors with contrasting timings of megagametophyte development.

The maize-*Tripsacum* introgression project developed jointly by IRD (formerly ORSTOM) and CIMMYT, which serves as the focus of this chapter, has passed the BC_5 generation. Early steps and progress are reported in the following pages, as are several fundamental questions that must be addressed before apomixis can be successfully transferred to major crops.

Source of Apomixis and Choice of Parental Materials

Basic Traits to Consider

Identifying a material for molecular studies, especially for apomixis gene isolation, is discussed in other chapters (especially Bicknell, Chap. 8). Choosing a progenitor specifically as a source of apomixis for wide cross transfer, however, is a somewhat different venture. In this instance, such work should take into account the following:

1. Genetic resources available. With only a few exceptions (*Elymus rectisetus*, *Tripsacum* spp.), collection of the wild apomictic relatives of important food crops has been notably inadequate. Consequently, wide cross projects have been forced to rely on a limited number of introductions. This in turn means that the diversity present in the wild relatives may go undetected, and that scientists do not gain access to the components of this diversity that offer optimum crossability with the crop species. A preliminary effort to collect such genetic resources of interest is critically needed in most cases.

2. Chromosome number of the potential donor species. An important factor for wide crossing apomixis is whether the basic chromosome number is the same in the donor and crop species. Crosses between related species with different basic chromosome numbers are generally considered less likely to succeed. Ploidy level is another point to consider, since wild apomixis is found almost exclusively in polyploids (for exceptions, mostly in the dicots, see Asker and Jerling 1992).

3. Genome homoeology. Chromosomal exchanges are more likely to occur when the chromosomes of the two species show some degree of pairing. Molecular genetics, seldom available at the beginning of such projects, can likely provide more detailed information about chromosome homoeology than classical

cytogenetics, e.g., in *Tripsacum*, Galinat (Galinat et al. 1970; Galinat 1971) described four maize chromosomes that are capable of pairing with *Tripsacum* chromosomes. Meanwhile, mapping analyses (Grimanelli et al., Chap. 6) suggest a more widespread colinearity between the maize and *Tripsacum* genomes.

4. Pollen fertility. Except for the highly facultative apomicts, first generation hybridizations between the crop and donor species must use the latter as male. Several apomictic species have been described with greatly reduced male fertility (e.g., *Elymus rectisetus*, the apomictic wild relative of wheat); in such cases, a preliminary selection is needed.

5. Type of apomixis. Apospory has always been presented as an easier type of apomixis to work with, being associated with 4-nucleate embryo sacs in tropical and subtropical grasses and transmitted as a single dominant gene (Savidan 1982a; Nogler 1984; Asker and Jerling 1992; Savidan 2000). Recent studies on diplospory in *Tripsacum* strongly challenge this view, bolstered by flow cytometry, which can be used to analyze modes of reproduction (Grimanelli et al. 1997), and screens that use different types of molecular markers. Nevertheless, the type of apomixis must still be considered, as different types of screens may be applied to different types of apomixis. Whether one type of apomixis than another is more likely to be expressed in a particular crop background is still largely speculative.

6. Degree of apomixis (or degree of facultativeness). The degree of apomixis appears to be a major factor related to the feasibility of wide cross transfer of apomixis. An obligate apomixis cannot be used unless some degree of male fertility is recovered in the F_1s, which is seldom the case in interspecific hybrids; but to produce near obligate apomictic crops, facultativeness must be low and well controlled. This factor is addressed in more detail later in this chapter.

7. Agronomic characteristics. A species with poor agronomic traits will produce hybrids and hybrid derivatives that may conserve undesirable traits for several generations, slowing the progress of the transfer.

8. Previous knowledge. Previous knowledge concerning the interspecific or intergeneric hybridization under consideration is a definite advantage. For example, knowing the number of backcrosses needed to go from the maize-*Tripsacum* F_1s to a 20-chromosome recovered maize (Harlan and de Wet 1977) was important in developing the first work plan for the IRD-CIMMYT apomixis team and in maintaining its confidence about the feasibility of its approach.

Case History: *Pennisetum*

Pennisetum glaucum, a cultivated pearl millet, has a basic chromosome number of $x = 7$. The only known and widespread tetraploid wild species with the same basic chromosome number is *P. purpureum* ($2n = 4x = 28$). Though described as aposporic by Brown and Emery (1958), this species appears to be entirely sexual, as confirmed by a cyto-embryological survey made in morphologically uniform wild populations from West Africa (Y. Savidan, unpublished). Apomixis has been described in several other *Pennisetum* species, all of which belong to the secondary or tertiary gene pools and share a basic chromosome number of $x = 9$. Dujardin and Hanna (1989) demonstrated that three out of the seven apomictic species tested were capable of producing F_1 hybrids with pearl millet. The genus *Pennisetum*, however, is one of the most complex in the grass family. In addition, the number of species varies greatly according to the taxonomist, the most conservative estimates being approximately 100 different species (Purseglove 1972), most of which are perennial, polyploid, and likely apomictic. Because of a lack of available germplasm, no extensive

search for an opitimum apomictic donor for pearl millet has been conducted. Three species studied by Dujardin and Hanna (1989) that show crossability with the crop are *Pennisetum orientale*, a tetraploid with $2n = 36$; *P. setaceum*, a triploid with $2n = 27$; and *P. squamulatum*, a hexaploid with $2n = 54$. They all reproduce apomictically and their apomixis was described as obligate, which means that 100% of the observed progeny appeared to be maternal in field tests (Dujardin and Hanna 1984a, b).

Advantages of *P. squamulatum* as a donor species for apomixis include good pollen fertility, 4-nucleate embryo sacs, and a unique

Issue # 1. Obligate vs. Facultative Apomixis: An Artifact?

The facultativeness of apomixis has been considered to be a disadvantage (Bashaw et al. 1970; Bashaw 1975) because (*i*) it may result in uncontrolled variation in the progeny while farmers require homogeneous varieties and (*ii*) it is apparently quantitatively inherited, i.e., under a complex, yet unknown genetic control. Nevertheless, facultativeness may be needed in attempts to transfer apomixis to crops through wide hybridization. Wide crosses generally produce highly sterile hybrids that can only be backcrossed by using them as female. If these hybrids are obligate apomicts, the wide cross approach for transferring apomixis is a dead end. But is obligate apomixis ever totally obligate?

Asker (1979) assigned a question mark to obligate apomixis. The developmental process has been described at the ovule level, where meiosis succeeds or fails. At the plant level, obligate apomixis is already questionable. At the level of the population or species, obligate apomixis is likely an artifact of the screening tools (see Leblanc and Mazzucato, Chap. 9).

A large number of ovules in the case of *P. squamulatum* (Dujardin and Hanna 1984a) have been examined and 8% were classified as aborted based on the absence of a normally developed embryo sac. In *Panicum maximum*, another aposporous tropical forage grass, ovules with no sac could be either abortive, in which case they show enlarged nucellar cells with little or no cytoplasm in an overall shrivelled ovary, or in early meiotic development stages. Sexual embryo sacs (ES) were significantly late as compared to the nucellar unreduced ES (Savidan 1982a). Differences in timing of development between meiotic and apomeiotic embryo sacs should be considered in order to provide an accurate estimate of the degree of facultativeness. This difference has been found in several aposporous species aside from *Panicum*, e.g., *Ranunculus auricomus* (Nogler 1984), *Brachiaria* spp (Ndikumana 1985), *Paspalum notatum* (Martinez et al. 1994), and diplosporous *Tripsacum* species (Leblanc and Savidan 1994), among others. Dujardin (personal comm.) confirmed that the nucelli from ovules he classified as aborted were perfectly normal, hence apomixis in the *P. squamulatum* introduction was perhaps not as obligate as originally thought.

Recent data (Hanna et al. 1993) showing high degrees of facultativeness in later generation hybrid derivatives can possibly be reinterpreted in the light of this hypothesis. Modification of the genetic or epigenic background is known to affect facultative apomixis expression, with an extremely high rate of sexuality possibly being observed. This was seen in guineagrass (*Panicum maximum*), in one natural interspecific hybrid with *P. infestum* (see also Berthaud, Chap. 2).

Though apomixis is probably always facultative in the wild to some extent, the facultativeness of the donor species in a transfer attempt should be limited and/or controllable for apomixis to be properly manageable in agriculture. Therefore, a compromise must be found between the facultativeness required for male sterile hybrids to be backcrossed, and the final objective of relative homogeneity in the farmers' fields.

potential, among the few species tested, for giving some female *and male* fertility to the F_1s. Disadvantages include the requirement of a bridge species, *P. purpureum*, the different basic chromosome number ($x = 9$, as compared with $x = 7$ in pearl millet), and the hexaploid level of ploidy. Progress made on mapping apomixis in *Pennisetum* and its implications for our understanding of the genetic control are presented in Grimanelli et al. (Chap. 6).

Case History: *Tripsacum*

Numerous maize x *Tripsacum* hybrids have been produced since the pioneering research of Mangelsdorf and Reeves more than 70 years ago (Mangelsdorf and Reeves 1931). Extensive hybridization studies have been carried out by Galinat (1971), Harlan and de Wet (1977), James (1979), and Bernard and Jewell (1985), among others. The main objective of these studies was to evaluate the potential role of *Tripsacum* in maize evolution and/or the feasibility of gene transfer, though not necessarily for apomixis. Claims of introgression have been made (Simone and Hooker 1976; de Wet 1979; and Bergquist 1981), but the *Tripsacum* progenitors involved were not tested beforehand for the target traits; consequently, the same traits could presumably have been present in neighboring maize collections. However, all these studies showed that from a maize-*Tripsacum* F_1 hybrid it was possible, in a few generations, to recover a 20-chromosome maize with some morphological features that were not present in the original maize progenitor. Most of these studies were based on using a diploid sexual *Tripsacum*, and most concentrated on a single species, *T. dactyloides*. Between 1990 and 1992, maize was successfully crossed with 66 apomictic populations, representing eight different species and intermediate forms between species (Table 11.1); 895 F_1 hybrids with $2n = 46 = 10M + 36Tr$ were obtained from these crosses. Most of these (598, or 66.8%) involved *T. dactyloides* subspecies or interspecific-like accessions involving some form of *T. dactyloides*. This confirmed high crossability for *T. dactyloides*. The number of F_1 plants per number of pollinated ears, however, showed a higher crossability between maize and *T. zopilotense*, which has the smallest area of distribution in Mexico (being found only in the Cañon de Zopilote, between Mexico City and Acapulco).

Advantages of using *T. dactyloides* as the donor species include good pollen fertility and an apomixis characterized by an absence of callose around the megasporocyte and subsequent cells, which is easily detected in fluorescence microscopy (Leblanc et al. 1995b; Leblanc and Mazzucato, Chap. 9). Diplospory is further characterized by endosperms with a ploidy level different from that of sexual seeds, resulting from the fertilization of two

Table 11.1 Crossabilities between maize and wild *Tripsacum* species and presumed natural interspecific hybrids

code	nb.pop	ears	emb.	cult.	F1s	F1s/ear
ZP	2	41	860	573	118	**2.88**
DT	2	92	324	169	97	**1.05**
iMZ	2	23	1119	140	20	**0.87**
iIT	6	103	1143	427	83	**0.81**
iDH	7	132	1527	452	84	**0.64**
iPL	4	65	2169	257	33	**0.51**
DH	30	776	10816	2892	386	**0.50**
PL	1	4	10	-	1	**0.25**
IT	5	132	779	123	32	**0.24**
iDM	3	75	2513	444	14	**0.19**
DM	7	121	3655	813	17	**0.14**
LC	1	10	38	5	1	**0.10**
BV	5	96	2091	390	7	**0.07**
iBV	2	62	1847	352	2	**0.03**
PR	1	-	-	-	20	-
average		1732			895	**0.52**

nb.pop.= number of populations studied; ears= number of maize ears pollinated with the *Tripsacum* species; emb.= number of counted embryos, three weeks after pollination; cult.= number of embryos cultured; F_1s= number of F_1 hybrids grown to maturity. Species codes: ZP= T.zopilotense; DT= T.dactyloides dactyloides (US types); MZ= T.maizor; IT= T.intermedium; DH= T.dactyloides hirsutum; PL= T.pilosum; DM= T.dactyloides mexicanum; LC= T.lanceolatum; BV; T.bravum; PR= T.peruvianum; i= intermediate forms (presumes natural interspecific hybrids).

unreduced polar nuclei. This trait can also be used for screening modes of reproduction in segregating populations by means of flow cytometry (Grimanelli et al. 1997). Previous studies showing that 5–6 backcrosses are needed to produce introgressed 20-chromosome maize plants provided another advantage to using this species. Disadvantages include total male sterility, which is seemingly retained until reaching addition forms with very few *Tripsacum* chromosomes, and the difference in basic chromosome numbers (x = 18 compared to x = 10 in maize).

Production of Interspecific or Intergeneric F_1 Hybrids

Several procedures are available to produce hybrids between cultivated and distantly related wild species. Special techniques, including chromosome manipulation, bridging species, hormonal treatment, embryo rescue, ovary culture, and in vitro pollination, are available for overcoming the cross incompatibility and the sterility of the F_1s. The presence of apomixis makes the cross more difficult because it can only be performed in one direction, with the apomixis progenitor being used as pollinator. Therefore, the donor must exhibit good pollen fertility. Because most apomicts require fertilization with reduced pollen to produce endosperm, pollen quality is generally not affected by apomixis. An exception to this rule is *Elymus rectisetus*, in which male infertility is a problem with most accessions (J. G. Carman, personal comm.).

Crossing Techniques

Most of the crossing techniques are common to intra- and interspecific crosses. A prerequisite is good knowledge of the self-sterility or self-incompatibility systems existing within the crop. For most crops, however, hand emasculation is preferred.

Crossing species with different flower sizes and shapes may require special tricks, e.g., in the case of maize x *Tripsacum*, more hybrids are produced if the silks are shortened to about 2–3 cm. Most wide crosses require embryo rescue techniques, using classical media such as MS (Murashige and Skoog 1962) or N_6 (Chu et al. 1975). Small embryos from maize x *Tripsacum* F_1s grew better on 50 g/l sucrose as compared with standard embryo culture medium containing 30 g/l sucrose. Several environmental factors can further affect the production and culture of hybrid embryos. As a result, the production of hybrids may be good one year, but poor the next.

When apomixis is not found in wild relatives, transfer may be attempted from a more distant apomictic species by using protoplast fusion. Such a transfer was started for sorghum using apomixis from *Cenchrus ciliaris* (Bharathi et al. 1991). However, no reports of plant regeneration have surfaced to date, apomictic or not, from such protoplast fusions. A more recent approach, developed by Ramulu et al. (1996), explores the production of microprotoplasts containing only one or two alien chromosomes and the direct production of monosomic addition lines after fusion with protoplasts from the receptor species.

Sterility of the F_1s

Sterility in interspecific and intergeneric F_1s and subsequent backcross generations is a characteristic of wide crosses. Restoring fertility of the F_1 hybrids through chromosome doubling is the most common approach. In both pearl millet and maize transfer attempts, however, F_1s from some wild species accessions were totally sterile, while those obtained from other accessions showed some degree of fertility, making the chromosome doubling unnecessary.

The transfer programs in pearl millet and wheat have produced F_1 hybrids with some

degree of male fertility. However, as described below for *Tripsacum*, this is not an absolute requirement. Nevertheless, it obviously helps, because the F_1s generally have morphological features close to that of the wild progenitor, e.g., a limited number of fertile flowers to pollinate. In maize, the F_1s have less than 20 flowers per inflorescence, while the recurrent maize parent, if it could be used as female (i.e., if the F_1 hybrid had some male fertility), would offer hundreds.

Pennisetum setaceum ($2n = 3x = 27$) was the first apomictic species crossed with pearl millet. F_1 hybrids had $2n = 25$ chromosomes, were male sterile, but reproduced apomictically (Hanna 1979). This interspecific cross was abandoned because of male sterility. *Pennisetum orientale* ($2n = 4x = 36$) was then crossed with pearl millet. F_1 hybrids had $2n = 25 = 18$ *P. orientale* (Or) + 7 pearl millet (Pm) chromosomes (Hanna and Dujardin 1982). They were male sterile, but backcrossing was attempted using pearl millet as the pollinator.

Pennisetum squamulatum ($2n = 6x = 54$) was successfully used to pollinate tetraploid pearl millet. Crosses with diploid pearl millet failed (Dujardin and Hanna 1989). Of 20 F_1 hybrids, 15 were facultative apomicts, based on embryo-sac analyses. One F_1 was classified as an obligate apomict, although 35% of the ovules were considered aborted. This may possibly be interpreted in another way if the timing of sexual and aposporic pathways of development is different (see *Issue # 1*). Pollen fertility of this hybrid was surprisingly high (66%) and therefore it was used to pollinate tetraploid pearl millet to produce a BC_1 progeny. The BC_1 plants were totally male sterile. The breakthrough was found in making a tri-specific hybrid. The pearl millet x *P. squamulatum* male fertile F_1 (classified as an obligate apomict) was used to pollinate a pearl millet x napier (*P. purpureum*) F_1, and 1,730 hybrids were produced. A sample of 64 segregated 31 apomictic (30 classified as obligate) and 30 sexual, which suggests dominance of apomixis over sexuality.

Relative crossabilities in maize x *Tripsacum* and pearl millet x wild species of *Pennisetum* are shown in Tables 11.1 and 11.2, respectively. According to J. G. Carman (personal comm.), the crossability between wheat and apomictic *Elymus rectisetus* as measured by the same F_1s/ear ratio was less than 1%. Differences in crossability may possibly be due to relative differences in genetic distance between the crop and its wild relatives or to genetic effects.

Production of Apomictic Progenies through Backcrossing

Facultativeness becomes especially important when interspecific or intergeneric hybrids are totally male sterile. Dujardin and Hanna (1989) considered male sterility as an impediment to the transfer of apomixis because their progenitors were apparently obligate apomicts. This was certainly reasonable based on the available techniques and limited number of plants used for analysis at the beginning of their project in the early 1980s. In the progenies of the maize x *Tripsacum* BC_3

Table 11.2 Crossabilities between pearl millet and three apomictic wild *Pennisetum* species

Cross combination	ears	F1s	F1s/ear
pearl millet ($2n = 14$) x P.orientale ($2n = 36$)	88	20	**0.23**
pearl millet ($2n = 28$) x P.orientale ($2n = 36$)	70	2	**0.03**
pearl millet ($2n = 14$) x P.setaceum ($2n = 27$)	7	28	**4.00**
pearl millet ($2n = 28$) x P.squamulatum ($2n = 54$)	59	337	**5.71**
average	224	387	**1.73**

ears = number of pearl millet inflorescences pollinated with the Pennisetum wild species; F_1s= number of F_1 hybrids grown to maturity.

Issue # 2. Is facultativeness controllable?

Bashaw et al. (1970) and Bashaw (1975) presented facultative apomixis as a difficult trait to manipulate in breeding because of uncontrolled variation (off-type frequency) that may result from crossing such apomicts with sexual plants. Our experience with aposporous *Panicum maximum* suggested that facultative apomixis, when the rate of facultativeness was low (1–5%), could be maintained with the same or even lower rate of sexuality through consecutive generations of hybridization. In such cases, the F_1 and BC_1 hybrids between sexual and apomictic guineagrass accessions had the same degree of facultativeness as their apomictic progenitor (Savidan 1982b). On the other hand, crossing a highly facultative apomict with sexual guineagrass accessions produced a large variation for the rate of facultativeness among the apomictic hybrids (Savidan 1982b). Whatever the complexity of the genetic control of facultativeness, it seemed to be transmitted as a cluster along with the control of apomeiosis (Savidan, 1982). *Tripsacum* diversity was not screened for facultativeness. Whether sexual x apomictic *Tripsacum* intra- or interspecific crosses may result in a similar conservation of the degree of facultativeness is therefore unknown. Maize x *Tripsacum* hybrid derivatives could exhibit contrasting rates of facultativeness, despite having originated from the same apomictic F_1 hybrid. Given our current state of knowledge, this may be either a characteristic of *Tripsacum* apomixis or only a consequence of the intergeneric, genetic, and / or epigenetic backgrounds.

hybrid derivatives, only 0.9% of the plants apparently resulted from fertilization of a reduced egg cell, i.e., the rate of diplospory in BC_3s was 99.1%, which would probably not be detectable if only 30 or 40 plants were analyzed in a progeny test.

The obligate nature of apomixis may be overestimated because of the population size, e.g., Burton et al. (1973) classified approximately 80% of their *Panicum maximum* accessions as obligate apomicts based on 10-plant progeny tests. Savidan (1982b), however, found only 20% of such obligate apomicts using a 100-ovary embryological analysis for each accession. Therefore, the male-sterile apomictic interspecific F_1 hybrid may probably always be used as female in the backcross, provided progenies of sufficient size can be screened. One can expect that a few off-types will be produced from sexual reproduction ($n + n$ combinations) to help bypass the sterility barrier. Some may reproduce apomictically, assuming the apomixis "allele" is dominant and simplex, as observed in all sexual x apomictic hybrids produced so far in the grass family (see Nogler 1984 for review; Sherwood, Chap. 5). In *Pennisetum*, male sterile apomictic hybrids could have been a good starting point for the transfer of apomixis if flow cytometry had been available for screening of large progenies, but the technology only became available to plant scientists several years after the project began (Galbraith et al. 1983).

The BC_1 plants from pearl millet x *Pennisetum orientale* hybrids had 23, 27, or 32 chromosomes. The latter were $2n + n$ off-types with 25 + 7 Pm, as pearl millet was used as pollinator. The 23-chromosome plants were described as facultative apomicts, with a low rate (or expression) of apomixis.

From the crosses with *P. setaceum*, a $2n = 27$ BC_1 plant appeared to be totally male sterile, but could be pollinated by pearl millet or *P. setaceum*. Pollination with pearl millet produced no seed, while pollination with *P. setaceum* produced four plants, three maternal and one $2n + n$. The *P. orientale* pathway was considered unsuitable for apomixis transfer because of the low expression of apomixis or complete male sterility in the BC_1 derivatives.

Hybrids that are totally male sterile and obligately apomictic are indeed dead ends: pollinating such hybrids with the crop pollen will produce only maternal offspring, i.e., perfect copies of the sterile F_1. However, if apomixis is slightly facultative, off-types can be produced, some of which may be $n + n$ and still apomictic, representing progress toward a return to the chromosome number of the crop. The rate of facultativeness has to be low, however, if one expects the backcross procedure to eventually produce an apomictic crop germplasm with a high degree of apomixis. Analyses made on *Panicum maximum* (Savidan 1982a,b) show that the rate of facultativeness, and more precisely of $n + n$ off-types, may remain relatively conserved through generations of hybridization. It was therefore suggested that a limited range of variation could possibly allow selection back to obligate apomixis. In the intergeneric background of maize x *Tripsacum* hybrid derivatives, the variation observed (Table 11.3) appeared less stable, possibly because the apomictic *Tripsacum* progenitor was already much more facultative than the guineagrass accessions used by Savidan (1982). By selecting among *Tripsacum* accessions for their ability to produce hybrid derivatives in backcrossing F_1s with maize, the team possibly selected one of the most facultative of the apomictic tripsacums.

Table 11.4 shows the cumulative result of the analysis of approximately 6,000 progenies produced from maize x *Tripsacum* BC_1s with

Issue # 3. Can apomixis be expressed at the diploid level?

In the wild, apomixis is found only among polyploids (although a few, questionable exceptions have been cited, see Asker and Jerling 1992). Population geneticists have suggested that sexuality would be eliminated if apomixis could be expressed at the diploid level (Pernès 1972; Marshall and Brown 1981). Nogler (1984) claimed, with little evidence to support it, that apomixis is probably linked to a lethal factor expressed at the haploid (gamete) level only. After obtaining 23-chromosome pearl millet x *P. orientale* BC_1 plants, Hanna et al. (1993) stated that polyploidy is probably not needed for the expression of gene(s) controlling apomixis, because these 23-chromosome plants had only one (simplex) set of nine *P. orientale* chromosomes. The genomic structure of these plants is likely 14 Pm + 9 Or however, suggesting that the locus involved could possibly be present in triplicate. Another such case of apomictic expression in a nonpolyploid form was previously reported (Dujardin and Hanna 1986), which related to a polyhaploid plant from a pearl millet x *P. squamulatum* F_1 hybrid which had $2n = 41 =$ 14Pm + 27Sq. This haploid had $2n = 21$ chromosomes. Again, as the $2n = 21$-chromosome plant likely had seven chromosomes from pearl millet and 14 from the wild species that had a basic chromosome number of nine, the locus involved was possibly in triplicate and not in duplicate.

In the *Tripsacum* project, a few polyhaploids were obtained in the progeny of $2n = 56 =$ 20m + 36tr BC_1s (Leblanc et al. 1996). These plants have one set of maize and one set of *Tripsacum* chromosomes, as confirmed by in situ hybridization (Leblanc et al. 1996), and some of them could express apomixis. Whether they represent exceptional cases of recombination between apomixis and a lethal system linked to it is open to speculation (see Grimanelli et al. 1998b). Grimanelli et al. (1998b) suggest, however, that apomixis can be expressed even when the allele(s) involved are in a duplex situation, a position that rejects the hypotheses of dosage effect presented earlier by Mogie (1988) and Noirot (1993), and suggests that the transmission barrier, whatever its nature, may be overcome through haploidization to produce functional diploid apomicts.

$2n$ = 56 chromosomes, i.e., 20 maize + 36 *Tripsacum* chromosomes. Note that the average rate of facultativeness at that level was very close to that of the *Tripsacum* progenitor, although variation was important.

A few dihaploids have been obtained from the progeny of $2n = 56$ BC_1s, as $n + 0$ off-types (Table 4). They grew well, flowered, and produced a good seed set. Their progeny were 80% maternal and 20% $2n + n$ hybrids with $2n$ = 38 chromosomes.

The backcross series was continued in an attempt to recover apomictic maize plants with only a few *Tripsacum* chromosomes. At each generation, plants were screened for apomixis and chromosome number. Embryo-sac analyses, which have been used extensively in several genetic analyses (Sherwood, Chap. 5), cannot be applied to intergeneric hybrids or hybrid derivatives in which inflorescences are too precious to be destroyed. Modes of reproduction are therefore estimated using progeny tests, e.g., a $2n$ = 38 maize x *Tripsacum* BC hybrid that produces mostly $2n$ = 38 progenies is likely to be apomictic, while a $2n$ = 38 maize x *Tripsacum* BC hybrid that produces progeny ranging from $2n$ = 22 to $2n$ = 32 is sexual. An alternative can be offered by using markers linked with apomixis, provided that apomixis is indeed controlled by one gene or small segment of DNA, and that such markers are closely linked.

A 1:1 segregation for apomixis and sexuality was observed among maize x *Tripsacum* F_1s, as 31 hybrids were classified as apomictic and 30 as sexual, based on embryological analyses. These plants were used for a bulk segregant analysis (see Grimanelli et al., Chap. 6) aimed at identifying molecular markers that cosegregate with apomixis. Three RFLP markers were first identified as linked with apomixis; these belong to the same linkage group in maize and are located on maize chromosome-6 long arm (Leblanc et al. 1995). Other markers were subsequently added (Grimanelli et al. 1998a, and Chap. 6).

Using both flow cytometry and marker-assisted screening for apomixis, rare but useful apomictic plants can be selected among many at each generation. A source population must be grown to constantly produce new progeny until the next generation population is large enough to enable progress to be achieved in the backcross program. With a rate of only 3% useful plants, we decided to raise the BC_1 population to 3,500 plants. After about 6,000 progeny had been analyzed, we substituted this BC_1 nursery with a BC_3 nursery obtained from in vitro multiplication of the $2n$ = 38 apomictic off-types produced by the BC_2 polyhaploids ($2n$ = 28). More than 2,500 BC_3s were established in the field. The analysis of a 125,000-

Table 11.3 Facultativeness of apomixis and diplospory rate in the *Tripsacum* accession used in the backcross transfer of apomixis into maize and three BC_1 progenies, showing variation for this rate. D: diplospory rate.

	No. of progenies	2n+0 maternal	2n+n off-types	n+n off-types	others	D %
T.dactyloides						
#65-1234	98	69	26	3	0	96.9
BC_1-6-82	55	40	15	0	0	100
BC_1-6-52	98	73	22	1	2	99.0
BC_1-5-45	78	63	6	8	1	89.7

Table 11.4 Chromosome numbers of BC_1 ($2n = 56$) progenies as estimated by flow cytometry

Progenies total no.	maternal 2n+0=56	off-types 2n+n=66	off-types n+n=38	off-types n+0
6259	5006	1024	218	11
%	80.0	16.4	3.5	0.2

plant progeny is shown in Table 11.5, in which the rate of $n + n$ off-types was below 0.2%. Almost 200 hybrid derivatives have been produced and classified as BC_4, with chromosome numbers ranging from $2n = 20$ to $2n = 36$. Modes of reproduction could be being determined for some of them by RFLP markers linked with apomixis, by progeny-tests, or by ploidy of the endosperms evaluated through flow cytometry (Table 11.6). The progeny size was recently increased further.

Screening the modes of reproduction through flow cytometry is a unique opportunity offered by diplosporous species such as *Tripsacum dactyloides*. In sexual plants, triploid endosperms result from the fertilization, by a reduced pollen, of two reduced polar nuclei. Diplosporous plants form endosperm as a result of the fertilization of two unreduced polar nuclei by a reduced pollen. The difference is shown in Figure 11.1. Diploid sexual plants have triploid endosperms (peak 2 in Figure 11.1a), while tetraploid apomictics produced endosperms (peak 2 in Figure 11.1b), with a DNA content 2.5 times that of the embryos (Grimanelli et al. 1997).

Preliminary data indicated apomixis could be transmitted to the BC_4 generation, although no fertile apomictic BC_4 had been confirmed as combining 20 maize chromosomes with less than 16 *Tripsacum* chromosomes (Table 11.6). Increasing the progeny size did not change the trend, an observation suggesting that the original transfer scheme (Figure 11.2) had to be reconsidered, especially since its 38-chromosome plant step could not produce the addition lines that were expected.

Table 11.5 Maize x *Tripsacum* BC_3 progenies, in which the BC_4s are the $n + n$ category

Progenies total no.	maternal 2n+0=38	off-types 2n+n=48	off-types n+n=20-36	off-types n+0=10, 28	others*
125916	114602	10778	158	78	300
(%)	91.01	8.56	0.12	0.06	0.24
reproduction	apomictic	apomictic	segregating	sexual, apo	apomictic

* mostly 4n (restitution nuclei)

Table 11.6 Maize x *Tripsacum* BC_4 with known mode of reproduction

Plant	2n	ISH*	RFLP	Endo	PGT	plant	2n	ISH	RFLP	Endo	PGT
1496	20		Sex			1457	27	13M+14Tr	Sex		
1500	20		Sex			1476	27		Apo		
1502	20	20M	Sex			1460	28	20M+8Tr?	Sex		Sex
1503	20		Sex			1484	28	20M+8Tr?	Sex		Sex
1516	20		Sex			1348	30		Apo		
1529	20		Sex			1346	31		Apo		
1454	21		Sex		Sex	1347	31		Apo		
1482	21		Sex		Sex	1439	31				Apo
1489	21		Sex			1453	31		Apo		
1492	21		Sex		Sex	1479	31	17M+14Tr	Apo		
1535	21		Sex		Sex	1276	32		Apo		
1275	22				Sex	1339	32		Sex		
1338	22		Sex		Sex	1426	32		Apo		
1345	22		Sex			1306	33		Sex		
1422	22		Sex			1349	33	18M+15Tr	Apo	Apo	Apo
1499	22	20M+2Tr			Sex	1493	33		Apo		Apo
1534	22		Sex			1532	33		Apo		
1393	23	20M+3Tr	Sex		Sex	1313	34				Sex
1515	23		Sex			1394	34	16M+18Tr	Sex?		Apo
1229	24		Sex			1494	34	16M+18Tr	Apo	Apo	Apo
1425	24		Sex		Sex	1517	34		Apo		Apo
1481	24		Sex			1521	34		Sex		Apo
1526	24	20M+4Tr	Apo	Apo	Sex?	1522	34		Apo		Apo
1528	24	20M+4Tr	Sex		Sex	1523	34		Apo		
1471	25	20M+5Tr	Sex		Sex	1544	35				Apo
1501	25	20M+5Tr			Sex	1308	36	20M+16Tr	Apo		Apo

*ISH: in situ hybridization data; RFLP: use of markers linked to apomixis; Endo: flow cytometry analysis of the ploidy of the endosperms; PGT= progeny-test (chromosome counts).

Transfer of Gene(s) for Apomixis from an Alien Chromosome to the Crop Genome

Possibilities of recombination between maize and *Tripsacum* chromosomes are extremely limited before the BC_3 generation. As shown in the scheme presented in Figure 11.2, the only meiotic event prior to this level occurs with BC_1 plants. However, pairing is preferentially maize-maize (M-M) or *Tripsacum-Tripsacum* (Tr-Tr) (Engle et al. 1974), although trivalent and tetravalent associations have been infrequently reported (Engle et al. 1973). In the BC_3s, 20 chromosomes of maize are associated with one haploid set of *Tripsacum* chromosomes, and some M-M-Tr pairing may occur. The same may happen in later generations with less *Tripsacum* chromosomes. Associations between maize and *Tripsacum* chromosomes have been reported to increase with each BC generation (Engle et al. 1973), however, they seem to involve a limited number of maize chromosomes.

Addition lines with $2n = 21$ to 24, whenever and whatever way they are produced, are expected to show some degree of male fertility, as observed in all previous studies. Levels of fertility may vary according to the number and quality of these alien chromosomes. Most of their progeny, using them as male, will likely be $2n = 20$ because of chromosome elimination and pollen competition.

The next step in transferring apomixis to maize is still to produce fertile addition lines with one to three *Tripsacum* chromosomes. This on its own remains a large challenge, although several indirect avenues are presently under investigation. Pairing and recombination

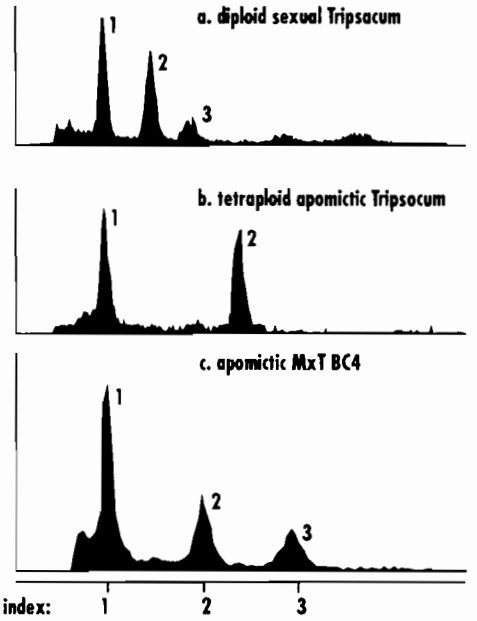

Figure 11.1 Flow-cytometric analyses on entire seeds. a. $2n = 36$ diploid sexual *Tripsacum*; peak 1: embryo ($2n = 36$), peak 2: endosperm ($2n = 54$), peak 3: duplicated cells from the embryo (G2 stage of cell cycle); b. $2n = 72$ tetraploid apomictic *Tripsacum*; peak 1: embryo ($2n = 72$), peak 2: endosperm (relative DNA content suggests $2n = 10x = 180$); c. $2n = 24$ BC4 maize-*Tripsacum* hybrid; peak 1: embryo ($2n = 24$), peak 2: duplicated cells from the embryo (G2 stage of cell cycle), peak 3: endosperm (relative DNA content suggests $2n = 2x + 2x + x + x = 68$)

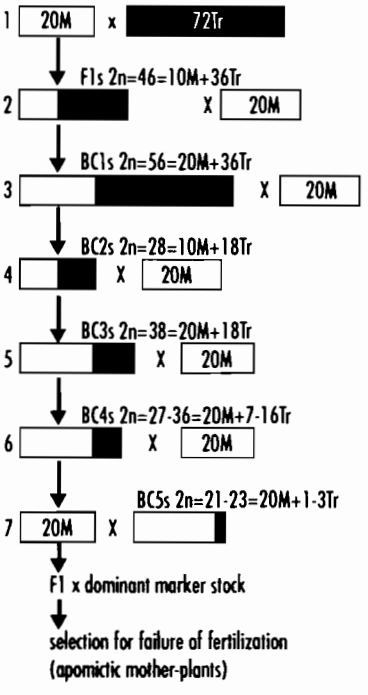

Figure 11.2 Backcross scheme for the transfer of apomixis from *Tripsacum* into maize.

between the *Tripsacum* chromosome-arm controlling apomixis and its homologous segment in maize may not occur spontaneously if recombination around apomixis is limited or impossible. Apomixis would then require the artificial induction of such a recombination. Several agents are available to induce artificial translocations. Final screening will have to be made on very large-scale progenies, and flow cytometry will be of little value because of the small differences in chromosome numbers and the small size of *Tripsacum* chromosomes. Dominant marker systems would be useful in screening between plants derived from fertilized eggs and those derived from parthenogenetic and unreduced eggs. Adequate stocks can be built up, taking advantage of available maize collections.

Is the transfer of apomixis to maize through wide hybridization feasible? Could it possibly result in a functional diploid apomictic germplasm? When? These questions remain

Issue # 4. Can a diploid apomict produce a normal seed?

Most apomicts require fertilization of the polar nuclei to produce a viable endosperm, a process known as pseudogamy. Few others, mostly Asteraceae, do produce endosperm in the absence of fertilization, a process known as autonomous apomixis. The latter, to our knowledge, is not found in the grass family, where fertilization of the endosperm is an absolute requirement related to dosage effects between alleles of maternal (m) vs. paternal (p) origin (2m:1p ratio) (for review, see Birchler 1993).

Experiments on wild apomictic grasses have revealed several types of endosperm formation. Interestingly, apomixis is especially frequent in the Panicoideae subfamily, in which embryo sacs are 4-nucleate, a single polar nucleus is fertilized, and the 2m:1p ratio is conserved. Other cases reported in the literature (Nogler 1984; Savidan 2000) in which the 2m:1p ratio is recovered, found that even in the presence of two unreduced polar nuclei (*i*) they remain unfused and are fertilized by one male nuclei each or (*ii*) the fused polar nuclei are fertilized by two male nuclei (double-fertilization). In both cases, an interesting consequence is that the most frequent off-types, the B_{III} or $2n + n$ hybrids, are eliminated from the progenies with the two male nuclei being used for endosperm formation.

Wild apomictic *Tripsacum* form 8-nucleate embryo sacs and the endosperm results from a simple (single) fertilization, making the ratio between maternal and paternal component either 8x:2x (or 4m:1p) when the tetraploid apomict is fertilized by the pollen of another tetraploid or its own pollen, or 8x:1x when the pollinator is a neighboring diploid. A complex series of this ratio can be found in a *Tripsacum* nursery where levels of ploidy from 2x to 6x are mixed together without significantly affecting the seed set and germination. Therefore, *Tripsacum* seems unaffected by an abnormal dosage effect, contrary to most other grasses (Grimanelli et al. 1997).

A few BC_4 plants with high seed set were analyzed. Preliminary data suggest that endosperms could result from a double-fertilization of the polar nuclei (Figure 1c). Part of the BC_3 seed already exhibited this endosperm structure. An attempt to correlate presence/absence of such an endosperm structure with grain gross morphology, however, proved disappointing.

If creating an autonomous apomixis *de novo* is possible, which is still in question given the apparent complexity of the control in wild apomicts (as suggested by molecular analyses) (Grimanelli et al., Chap. 6), it is also questionable whether it could be satisfactorily expressed in any grass species, especially the grain crop species, because of their imprinting requirements.

unanswered. However, more progress has been achieved toward producing an apomictic grain during the last ten years than ever before, mostly because of the development and application of new techniques. As molecular dissecting tools continue to improve, we will see great progress in our understanding of how apomixis is controlled and the isolation and manipulation of its components. Another promising avenue, approaches based on mutagenesis, is discussed in the following two chapters. These approaches will undoubtedly better our understanding of the regulation of reproduction as a whole. In the end, apomixis certainly cannot be manipulated without a thorough understanding of how it is controlled in the wild.

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Chapter 12

From Sexuality to Apomixis: Molecular and Genetic Approaches

Ueli Grossniklaus

Introduction

Sexual reproduction usually produces genetically diverse progeny, a feature that has been exploited in the selection and improvement of agricultural crops over the centuries. In contrast, apomixis results in the production of genetically uniform progeny. Apomictic embryos are derived from an unreduced cell lineage and develop independent of fertilization (Gustafsson 1947; Nogler 1984a, b; Asker and Jerling 1992; Koltunow 1993). Thus, apomictic seeds are genotypically identical to the mother plant and usually form a genetically stable clone. The clonal nature of apomictic offspring bears tremendous potential for seed production and crop improvement. It offers the possibility for the immediate fixation of any desired genotype and its indefinite clonal propagation. The transfer of apomixis to sexual crops will completely transform current breeding strategies and seed production (Hanna and Bashaw 1987; Savidan 1992; Savidan and Dujardin 1992; Dickinson 1992; Jefferson 1993; Hanna 1995; Koltunow et al. 1996; Vielle-Calzada et al. 1996; Jefferson and Bicknell 1996; Grossniklaus et al. 1998a; Savidan 2000). The resulting agricultural, commercial, and social benefits for both industrialized and developing countries would be enormous (Jefferson 1994; Koltunow et al. 1995; Grossniklaus et al. 1998c; and Toenniessen, Chap. 1).

Apomixis is an asexual form of reproduction through seeds and occurs in more than 400 species (Bashaw and Hanna 1990; Asker and Jerling 1992; Carman 1995, 1997). Having been described in plants belonging to almost 40 different families, it is thought to have evolved independently in several taxa from sexual ancestors. Apomixis can be viewed as a developmental variation of the sexual reproductive pathway in which certain steps are short-circuited (Koltunow 1993; Vielle Calzada et al. 1996; Grossniklaus et al. 1998a). Thus, apomictic and sexual reproduction are closely related to one another and share many regulatory components. It is very likely that the genes controlling apomixis also play crucial regulatory roles during sexual development. Therefore, the engineering of apomixis will require a better understanding of the genetic basis and molecular mechanisms that control sexual plant reproduction. Whereas megasporogenesis and megagametogenesis have been studied extensively at the morphological and ultrastructural levels (e.g., Cass and Jensen 1970; Willemse and van Went 1984; Russell 1985; Mogensen 1988; Huang and Russell 1992; Schneitz et al. 1995; Christensen et al. 1997), the molecular and genetic basis controlling these key steps in sexual reproduction are almost entirely unknown.

The engineering of apomixis in sexual crops will only be possible through an interdisciplinary and multifaceted approach to studying the regulation of reproduction at the genetic and molecular levels in both sexual and

apomictic species. Current research focuses on four main complementary strategies: (*i*) characterization of the genetic regulation of apomixis (reviewed in Nogler 1984a; Hanna 1995; Savidan, 2000; Sherwood, Chap.5; Grimanelli et al., Chap.6); (*ii*) development of apomictic model systems for molecular genetic studies (reviewed in Koltunow et al. 1995; Jefferson and Bicknell 1996; Bicknell, Chap. 8); (*iii*) analyses of the genetic basis and molecular mechanisms controlling megasporogenesis, megagametogenesis, and seed development in sexual species (reviewed in Drews et al. 1998; Grossniklaus and Schneitz 1998; Yang and Sundaresan 2000); and (*iv*) development of the molecular tools needed to introduce and control the expression of candidate genes (reviewed in Gatz and Lenk 1998). In addition to identifying key regulatory genes controlling the sexual pathway, the identification of mutants that display certain aspects of apomictic reproduction is one of the most promising of the approaches using sexual model systems (Chaudhury and Peacock 1993; Ohad et al. 1995; Chaudhury et al. 1997; Ramulu et al. 1997; Grossniklaus and Vielle-Calzada 1998; D. Page, R. Pruitt, S. Lolle and U. Grossniklaus, unpublished data).

Apomictic reproduction is under genetic control (Nogler 1984a; Savidan 2000; Sherwood, Chap. 5). In studies on its genetic regulation, it was found to behave as a single dominant Mendelian trait (Nogler 1973,1975, 1984b; Savidan 1980, 1982; Gadella 1987; Ozias-Akins et al. 1993, 1998; Miles et al. 1994; Sherwood et al. 1994; Leblanc et al. 1995; Kindiger et al. 1996; Pessino et al. 1997; Grimanelli et al. 1998; Barcaccia et al. 1998; van Dijk et al. 1999; Bicknell et al. 2000; Noyes and Rieseberg 2000). Ideally, the gene(s) controlling apomixis would be isolated and characterized in an apomictic species. However, molecular genetic analysis in apomicts is difficult because of their poor characterization at the molecular and genetic levels, and the obstacles posed by their clonal mode of reproduction. Genetic analysis, which relies largely on recombination, can only be studied in hybrids of sexual and apomictic genotypes between species or genera. Moreover, apomixis is tightly associated with polyploidy, thereby making genetic analysis difficult. The advent of physical mapping has had a great impact on work on apomictic species and their relatives. Recent advances in the establishment of molecular marker systems and the development of apomictic model systems are discussed elsewhere (Savidan 2000; Grimanelli et al., Chap. 6; Bicknell, Chap. 8).

In this chapter, I focus on efforts to use well-established sexual model systems to elucidate the molecular mechanisms controlling plant reproduction. A better understanding of the genes and molecules involved in the sexual pathway and the isolation of mutants relevant to apomixis will play an important role in the transfer of apomixis to sexual species. Since this chapter focuses on the use of sexual systems, the developmental events occurring during sexual reproduction and their genetic control will be reviewed in detail. I compare sexual and apomictic reproduction and discuss mutants relevant to these developmental processes. Then, some of the recent advances in the molecular and genetic characterization of sexual reproduction in *Arabidopsis thaliana* will be described. Finally, various approaches to introducing apomixis into sexual species are surveyed and the implementation technologies required to engineer apomixis in a useful manner are identified.

Developmental Aspects of Sexual and Apomictic Reproduction

The plant life cycle alternates between a diploid sporophytic and a haploid gametophytic generation, a feature with important implications for the formation of the

gametes and embryogenesis (Walbot 1996). The meiotic products of plants undergo several division cycles to form a multicellular haploid organism. The gametes differentiate later in gametophyte development. In angiosperms, double fertilization concludes the gametophytic phase and marks the beginning of the next sporophytic generation. In angiosperm apomixis, the plant life cycle is short-circuited and an unreduced cell lineage gives rise to a megagametophyte (gametophytic apomixis) or directly to an embryo (sporophytic apomixis). Because of the close developmental and evolutionary relationship between apomictic and sexual reproduction, a better understanding of the fundamental biological principles governing female gametogenesis and seed development will provide invaluable tools for the manipulation of the sexual reproductive system towards apomixis.

Sexual Model Systems

Two well-established sexual model systems, *Arabidopsis thaliana* and *Zea mays* (maize), and a rapidly emerging system, *Oryza sativa* (rice), are of particular interest for genetic and molecular investigations. All three are well characterized at the genetic level and offer a vast array of powerful genetic and molecular techniques (Freeling and Walbot 1993; Meyerowitz and Somerville 1994; Tanksley and McCouch 1997; McCouch et al. 1997). Versatile transposon systems for insertional mutagenesis and gene tagging are available and offer the opportunity for reverse genetic approaches (Walbot 1992; Dellaporta and Moreno 1994; Feldmann et al. 1994; Shimamoto et al. 1993; Shimamoto 1995; McKinney et al. 1995; Sundaresan 1996; Parinov et al. 1999; Speulman et al. 1999; Tissier et al. 1999; Meissner et al. 1999; Krysan et al. 1999).

Maize, as an agriculturally important member of the grass family (http://www.agron.missouri.edu), has some advantages for apomixis research. It can be hybridized with its apomictic relative *Tripsacum dactyloides* (e.g., Mangelsdorf and Reeves 1931; Harlan and de Wet 1977; Petrov et al. 1984; Savidan, Chap. 11), and the extensive synteny among the grasses (Bennetzen and Freeling 1993; Moore et al. 1995; Gale and Devos 1998; Keller and Feuillet 2000) allows for comparative genomic analyses between sexual and apomictic grass species. The *Mutator* transposon system offers highly efficient methods for insertional mutagenesis (Chomet 1994) and for site-specific transposon mutagenesis by reverse genetic approaches (Das and Martienssen 1995; Bensen et al. 1995; Mena et al. 1996; Rabinowtiz and Grotewold 2000), originally pioneered in the fruit fly *Drosophila melanogaster* (Ballinger and Benzer 1989).

The small plant *Arabidopsis thaliana*, a member of the Brassicaceae, has been widely adopted as a model system for the developmental biology and genetics of flowering plants (Meyerowitz 1989). The small size of the plant, its rapid life cycle, and the large number of seeds it produces make it ideal for the isolation and study of mutants that affect biochemical and developmental pathways. The small genome size (~125 Mb), high percentage of single copy DNA (Pruitt and Meyerowitz 1986), large number of molecular markers (http://www.arabidopsis.com), and the complete genome sequence (*Arabidopsis* Genome Initiative 2000) make *Arabidopsis* a powerful system for molecular studies. Highly efficient T-DNA-based transformation methods (Bechthold et al. 1993) and heterologous transposon systems for targeted gene tagging, genome wide insertional mutagenesis, and reverse genetics are available (Feldmann et al. 1994; McKinney et al. 1995; Sundaresan 1996; Parinov et al. 1999; Speulman et al. 1999; Tissier et al. 1999; Meissner et al. 1999; Krysan et al. 1999). The *Arabidopsis* genome is the first plant genome to be completely sequenced (*Arabidopsis*

Genome Initiative, 2000). In light of existing synteny between dicotyledonous and monocotyledonous taxa (Paterson et al. 1996), this genome information will also be of great importance for molecular studies in cereals. The amenability of *Arabidopsis* to genetic and molecular analysis makes it an ideal system for the identification and molecular isolation of genes controlling sexual reproduction and for the isolation of mutations that interfere with this developmental process.

That said, however, attention should be drawn to the previously mentioned efforts in rice, which have been greatly stimulated by the Rockefeller Foundation's International Program on Rice Biotechnology. Rice has many of the advantages of *Arabidopsis*, including a very small genome (~400 Mb), a true diploid genetic constitution, a rapid life cycle, and well developed genetics (http://ars-genome.cornell.edu/grasses.html). In addition, rice not only shows synteny on a broad scale, but microsynteny with all the grasses, ranging from apomictic forages (e.g., *Brachiaria*) to other important grain crops such as maize, wheat, barley, and sorghum (e.g., Kilian et al. 1995). The rapid adoption of rice as a model plant and private and public sector work on the rice genome will result in rice being the second completely sequenced plant genome. Given the importance of endosperm development and the peculiarities associated with cereal endosperm, similar developmental genetic studies on rice offer even more direct advantages for investigating the control of apomixis when the technologies are similarly mature. Existing technologies in rice are now approaching those of *Arabidopsis*, including low-copy *Agrobacterium*-mediated transformation, transposon insertional mutagenesis, and high-density maps of molecular markers required for positional cloning (e.g., Shimamoto et al. 1993; Shimamoto 1995; McCouch et al. 1997).

Sexual Reproduction

The formation of ovules, where megasporogenesis, megagametogenesis, and double fertilization occur is a key step in sexual reproduction. For more than a century, much attention has been given to the structure and development of the ovule and female gametophyte (e.g., Hofmeister 1849; Maheshwari 1950; Jensen 1965; Russell 1985; Reiser and Fischer 1993; Herr 1995; Schneitz et al. 1995; Gasser et al. 1998; Schneitz 1999). In maize, rice, and *Arabidopsis*, as in the majority of all angiosperms, monosporic development leads to the formation of an embryo sac of the Polygonum-type (Maheshwari 1950; Russell 1979; Bedinger and Russell 1994; Webb and Gunning 1990, 1991).

1. Megasporogenesis. Within the developing ovule primordium, a single hypodermal cell enlarges to form the archesporial cell. The archesporium differentiates directly into the megaspore mother cell (megasporocyte) and undergoes meiosis to produce four megaspores (Misra 1962; Davis 1966; Webb and Gunning 1990; Huang and Russell 1992; Hill and Lord 1994). Although usually only a single cell adopts a meiotic cell fate, about 5% of *Arabidopsis* ovules contain two megasporocytes, but only one appears to form a functional megaspore since twin-embryo sacs are not observed (Schneitz et al. 1995; U. Grossniklaus and J. Moore, unpublished data). Whereas the *Arabidopsis* megaspore mother cell is in direct contact with the epidermal cell layer of the nucellus (tenuinucellate), the megaspore mother cell in maize becomes more deeply embedded in the nucellar tissues (crassinucellate) as a consequence of divisions in the epidermal layer (Randolph 1936; Kiesselbach 1949; Maheshwari 1950; Misra 1962; Figure 12.1a, d). At maturity, the megasporocyte is characterized by a polar distribution of the organelles that accumulate at its chalazal pole (Russell 1979; Webb and

Gunning 1990). The megaspore mother cell is surrounded by extensive callose depositions, which isolate it from the sporophytic tissues of the ovule (Rodkiewicz 1970).

In *Arabidopsis*, the two meiotic nuclear divisions occur before cytokinesis, leading to the formation of tetrads with a multiplanar or, more rarely, a linear arrangement (Webb and Gunning 1990; Schneitz et al. 1995). In contrast, cytokinesis accompanies meiosis in maize, first producing two dyad cells and finally the four megaspores, which form a usually linear tetrad (Figure 12.1) (Weatherwax 1919; Kiesselbach 1949; Russell 1979). Little information is available on rice megasporogenesis, but its development is likely to be similar to that observed in maize. Only the chalazal-most megaspore survives (functional megaspore) whereas the other three undergo programmed cell death and degenerate. A certain variability with respect to the form of the tetrads and cytoplasm allocation to the functional megaspore has been observed (Webb and Gunning 1990; Bedinger and Russell 1994). Degenerating and surviving megaspores are initially similar at the ultrastructural level except for an enrichment of organelles in the functional megaspore. However, only the degenerating megaspores are surrounded by a callose rich cell wall, whereas the functional megaspore remains in direct contact with nucellar tissues (Rodkiewicz 1970; Webb and Gunning 1990; Russell and West 1994). It has been proposed that the pattern of callose deposition during megasporogenesis plays a crucial role for the differentiation and survival of the chalazal megaspore, which eventually forms the megagametophyte (Haig and Westoby 1986).

2. Megagametogenesis. The functional megaspore gives rise to a mature embryo sac of the Polygonum-type by three consecutive mitotic divisions that occur in a syncytium (Randolph 1936; Kiesselbach 1949; Misra 1962; Poliakova 1964; Diboll and Larson 1966; Russell 1979; Huang and Sheridan 1994; Webb and Gunning 1994; Schneitz et al. 1995; Christensen et al. 1997; Moore et al. 1997; reviewed in Grossniklaus and Schneitz 1998; Drews et al. 1998). After the first division, the nuclei migrate to opposing poles of the developing megagametophyte, and a prominent large vacuole forms in its center. A second vacuole at the chalazal pole is found in *Arabidopsis* and some genotypes of maize (Vollbrecht and Hake 1995; Christensen et al. 1997). As the embryo sac enlarges, the integuments grow to envelop the nucellus. Asymmetric growth of the integuments gives the *Arabidopsis* ovule its characteristic anatropous shape (Misra 1962). The nuclei at each pole undergo two synchronous divisions to form the 4- and 8-nucleated embryo sac. A single nucleus at the chalazal pole starts migrating toward the micropylar pole and becomes one of the two polar nuclei in the central cell. Cellularization leads to the formation of seven cells: an egg cell and two synergids at the micropylar pole, three antipodals at the chalazal pole, and a central cell harboring the two polar nuclei (Figure 12.1). In *Arabidopsis*, the nucellar tissue is absorbed as the embryo sac grows and expands. At maturity, remnants of the nucellus are only present at the chalazal pole. The endothelial tissues, which are in direct contact with the megagametophyte, are of integumental origin. In maize and rice, ovule morphogenesis is characterized by an initial intensive proliferation of the nucellar tissue, such that at maturity the embryo sac is still embedded in this tissue.

The two synergids and the egg cell are arranged in triangular configuration at the micropylar pole to form the egg apparatus (Mansfield et al. 1991; Webb and Gunning 1988; Diboll and Larson 1966; Russell 1979;

Vollbrecht and Hake 1995). The synergids are highly specialized cells thought by some to provide constraints for pollen tube attraction and the transport of the sperm cells to the egg and central cell (Huang and Russell 1992). Their cytoplasm is highly polarized with a chalazally located vacuole and, typically, a centrally located nucleus. A highly specialized cell wall, the filiform apparatus, is associated with the micropylar-most region of the synergids (Jensen 1968; Mogensen 1988; Russell 1993). One of the synergids typically degenerates prior to fertilization, but the moment for initiation of the degenerative process varies (Russell 1992; Christensen et al. 1997).

The egg cell is located at a slightly chalazal position with respect to the synergids. The distribution of the egg cytoplasm is asymmetrical with a highly vacuolated micropylar pole (or a single large vacuole in this position) and a chalazally located nucleus (Russell 1993). In rice, the nucleus is in a more central position and the egg appears less polarized than in other species (Jones and Rost 1989). Nevertheless, the micropylar-chalazal (proximo-distal) axis first eminent when ovule primordia emerge is maintained throughout megasporogenesis and megagametogenesis at the level of the megasporocyte, the embryo sac, and its constituent cells. The polarity of the egg cell may have important implications for early

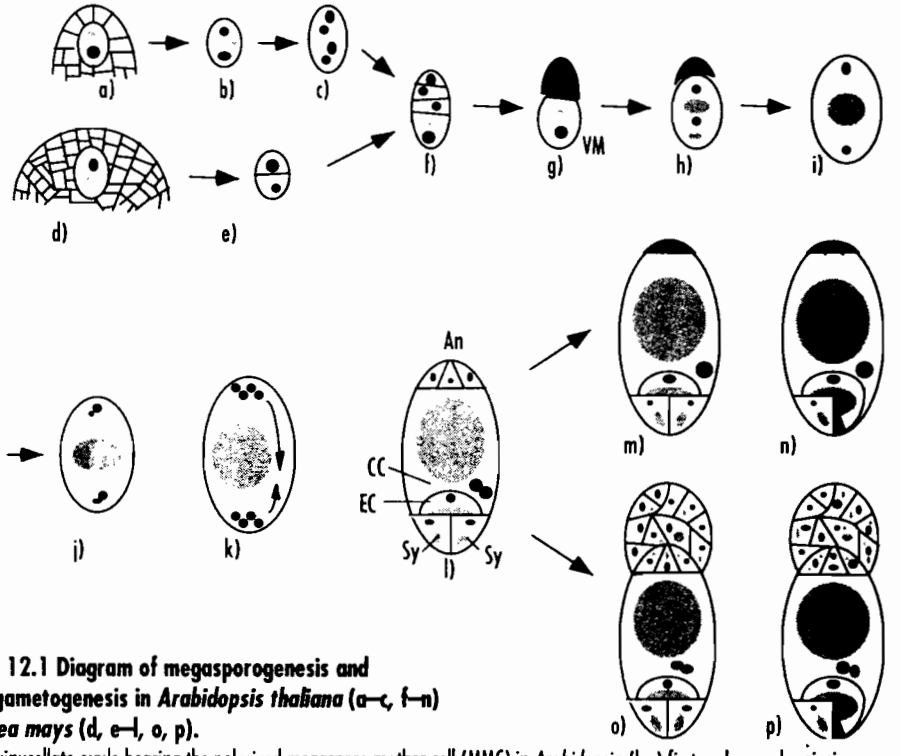

Figure 12.1 Diagram of megasporogenesis and megagametogenesis in *Arabidopsis thaliana* (a–c, f–n) and *Zea mays* (d, e–l, o, p).

(a) tenuinucellate ovule bearing the polarized megaspore mother cell (MMC) in *Arabidopsis*; (b,c) first and second meiosis occur without cytokinesis; (d) crassinucellate ovule bearing the MMC in maize; (e) dyad formation after the first meiotic division; (f) linear tetrad with the proximal-most megaspore receiving the majority of the organelles; (g) degeneration of the three distal megaspores and differentiation of the viable megaspore (VM); (h) 2-nucleate embryo sac; (i) migration of the nuclei to the poles and formation of a central vacuole; (j) 4-nucleate embryo sac; (k) 8-nucleate embryo sac, migration of the two polar nuclei; (l) cellularized 7-celled embryo sac with the four differentiated cell types: antipodals (An), central cell (CC), egg cell (EC), and synergids (Sy); (m) formation of the 4-celled embryo sac through degeneration of the antipodals, fusion of the polar nuclei prior to fertilization in *Arabidopsis*; (n) degeneration of one synergid prior to fertilization; (o) proliferation of the antipodal cells in maize; (p) degeneration of one synergid prior to fertilization, the polar nuclei fuse together with the sperm nucleus.

embryogenesis, which is initiated by a highly asymmetrical division of the zygote (Willemse 1981; Willemse and van Went 1984; West and Harada 1993).

The large central cell is highly vacuolated and contains the two polar nuclei originating from opposite poles. It shares a common cell wall with both the egg apparatus and the antipodals. The polar nuclei will fuse prior to fertilization to form the homo-diploid nucleus of the central cell; in maize and rice, nuclear fusion is partial and is not completed until the arrival of the sperm cells.

Three antipodal cells differentiate at the chalazal pole. In *Arabidopsis*, they usually degenerate after the fusion of the two polar nuclei (Webb and Gunning 1988, 1990; Murgia et al. 1993; Schneitz et al. 1995; Christensen et al. 1997). In contrast, the antipodals of the Poaceae are the only cells of the megagametophyte that proliferate after cellularization. In maize, they form a cluster of up to 40 cells that are often cytoplasmically connected (coenocytic) to some degree (Kiesselbach 1949; Diboll and Larson 1966; Vollbrecht and Hake 1995). In rice, 10 to 15 antipodals, which appear to have a highly active metabolism, are present in mature embryo sacs (Jones and Rost 1989). The ephemeral nature of the antipodals in *Arabidopsis* is intriguing and a clear function of these cells has not yet been established.

3. Double fertilization. At fertilization, the pollen tube penetrates the receptive synergid and delivers the two sperms cells (Russell 1993). They migrate to the chalazal pole of the synergid to fuse with their targets, the egg and central cell. Prominent actin coronas at the presumptive site of fusion indicate a possible involvement of actin filaments for sperm cell migration and fusion (Russell 1993). Subsequently, fertilization of both the egg and central cell gives rise to the diploid zygote and the triploid primary endosperm nucleus, respectively. The site of gametic fusion is characterized by poorly developed cell walls, such that the cell membrane of the synergid is in direct contact with the membrane of the egg and central cell. After fertilization, the cytoplasm in the zygote undergoes extensive reorganization (Jensen 1968; Olson and Cass 1981; Russell 1993). The zygote elongates but does not divide for some time. In contrast, the primary endosperm nucleus divides syncytially a few times before the first asymmetric division of the embryo occurs (Randolph 1936; Kiesselbach 1949; Jones and Rost 1989; Mansfield and Briarty 1990a; Sheridan and Clark 1994; Schneitz et al. 1995; Berger 1999). The development of the endopserm is initially syncytial. The nuclei then migrate to characteristic positions at the periphery of the embryo sac and finally cellularize in a distinct developmental pattern (McClintock 1978; Marsden and Meinke 1985; Mansfield and Briarty 1990a,b; Walbot 1994; Berger 1999; Olsen et al. 1999). Successful seed development requires the coordinate morphogenesis of embryo, endosperm, and the integumental cell layers that form the seed coat (Rutishauser 1969).

Apomixis

During apomictic reproduction, the sexual pathway described above is short-circuited. A subsequent developmental event is initiated before the previous one is completed. This developmental heterochronicity is a hallmark of apomictic reproduction and various models have been developed to account for the developmental displacement of events during megasporogenesis, megagametogenesis, and fertilization (e.g., Mogie 1992; Peacock 1993; Koltunow 1993; Carman 1997; Carman, Chap. 7). The developmental processes leading to apomictic reproduction are diverse and have been described in detail elsewhere (Nogler 1984a; Asker and Jerling 1992; Koltunow 1993;

Naumova 1993; Crane, Chap. 3). An ultrastructural characterization of apomictic development is discussed by Naumova and Vielle-Calzada (Chap. 4). Here, I will briefly describe the main developmental features of apomixis in order to facilitate a comparison with the sexual pathway.

Two fundamentally different classes of apomictic development can be distinguished (Gustafsson 1947; Nogler 1984a; Koltunow 1993) (Figure 12.2). In sporophytic apomixis, an embryo forms directly from a nucellar or integumentary cell in the ovule (adventive embryony). Although adventive embryos are not derived from gametophytic cells, their development depends on the presence of a megagametophyte, because they usually rely on sexually derived nutritive endosperm tissue. Sporophytic apomixis will not be considered further in this chapter because an engineered switch between sexuality and gameotphytic apomixis appears more attractive for breeding purposes. However, it should be kept in mind that in sporophytic apomixis only embryo initiation is affected and, thus, it may be easier to tackle sporophytic apomixis at the molecular level.

In gametophytic apomicts, the embryo results from the parthenogenetic development of an egg cell produced by an unreduced embryo sac. The unreduced gametophyte can originate either directly from nucellar cells (apospory) or from a megaspore mother cell that has undergone no or an aberrant meiosis resulting in the formation of one (mitotic diplospory) or two unreduced megaspores (meiotic diplospory). Aposporous embryo sacs form mitotically from nucellar cells that develop during or after megasporocyte differentiation and are similar in appearance to the megaspore mother cell. Often several aposporic embryo sacs are present in a single ovule in addition to the sexually derived one. In diplosporous development, a variety of cytologically distinct processes lead to a failure in meiosis; the megasporocyte switches from a meiotic to an

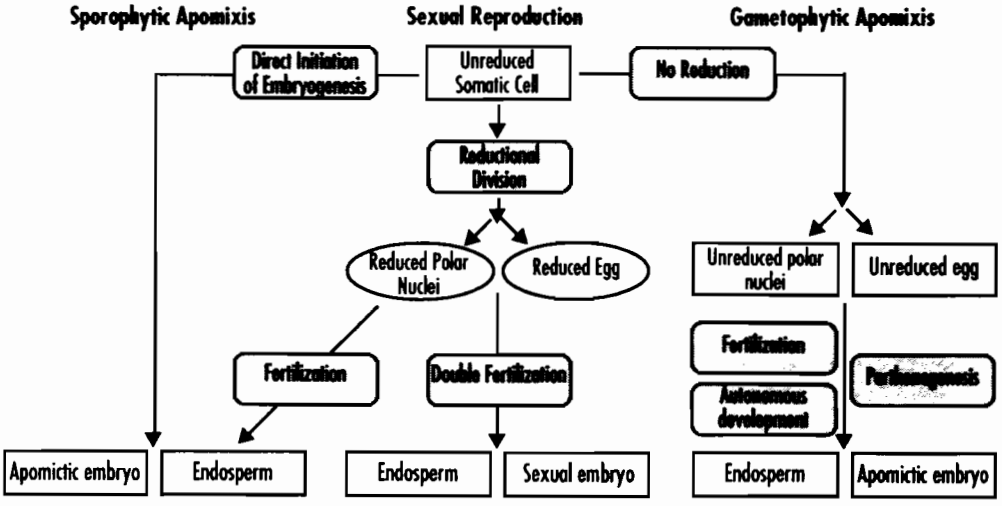

Figure 12.2 The main developmental features of apomixis in relationship to the sexual pathway.
Unreduced cells are in rectangular boxes, reduced cells are in oval boxes and key events are in darkly shaded boxes. In sexual plants, the megaspore mother cell undergoes meiosis and one of the reduced megaspores forms the embryo sac. Embryo and endosperm are formed by double fertilization. In gametophytic apomixis, reduction is avoided and embryo sac development initiated from an unreduced diplospore or an aposporic initial cell. The embryo develops parthenogenetically from the unreduced egg while the endosperm forms either autonomously or through fertilization of the central cell (pseudogamy). In sporophytic apomixis, the embryo forms directly from an unreduced nucellar initial cell. The apomictic embryo relies on sexually produced endosperm.

apomictic pathway with the net result of producing an unreduced functional megaspore (see Crane, Chap. 3).

Unlike in sexual development, the megaspore mother cell of diplosporous species is not surrounded by callose. In aposporous species, callose deposition around the sexual megasporocyte can be abnormal (Crane and Carman 1987; Carman et al. 1991; Leblanc et al. 1993; Naumova et al. 1993; Naumova and Willemse 1995; Peel et al. 1997). As is typical for the functional megaspore in sexuals, aposporic initials, which will form apomictic embryo sacs, are devoid of callose (Naumova and Willemse 1995). The marked difference of callose deposition between sexual and diplosporous species is intriguing, but most likely is not causal, instead being the consequence of a more fundamental lesion (Crane and Carman 1987; Carman et al. 1991; Carman, Chap. 7).

The unreduced megaspore of diplosporous apomicts divides mitotically to give rise to a mature embryo sac. Usually, only one megaspore of the dyad initiates embryo sac development and the other degenerates, but bisporic development, in which two unreduced nuclei are present in the same spore, also occurs (Ixeris-type). In some apomicts, the developing megagametophyte undergoes only two mitoses to form a 4-nucleated embryo sac where no antipodals form (Panicum-type) (Gustafsson 1947; Nogler 1984a, b; Crane, Chap. 3). For instance, sexual megagametophytes in *Pennisetum ciliare* are of the typical seven-celled Polygonum-type, whereas aposporic embryo sacs carry only four nuclei and typically form one egg cell, two synergids and one polar nucleus (or a variation thereof) but no antipodals (Taliaferro and Bashaw 1966; Vielle et al. 1995). The egg cell of an apomictic embryo sac initiates embryogenesis autonomously in the absence of fertilization. In *Pennisetum*, the aposporic egg cell is completely covered by a cell wall (Vielle et al. 1995) whose presence may prevent the fusion of the apomictic egg with a sperm cell (Asker and Jerling 1992; Savidan 1992). Some apomictic species are truly autonomous and do not require fertilization at all; both embryo and endosperm develop autonomously. In contrast, seed development in most apomicts depends on the fertilization of the central cell to produce the nutritive endosperm, which is required for successful seed production (pseudogamy) (Nygren 1967; Asker 1979, 1980; Nogler 1984a; Richards 1986; Bashaw and Hanna 1990; Asker and Jerling 1992). Unlike in sexual species, the apomictic egg cell often initiates embryogenesis before the first endosperm division occurs.

Interrelationship of Sexual and Apomictic Reproduction

Sexual and apomictic reproduction are developmentally and evolutionarily related. Apomixis can be viewed as a developmental variation of the sexual pathway. Apomictic and sexual modes of reproduction are not mutually exclusive. Whereas obligate apomicts produce exclusively clonal progeny, both forms of reproduction coexist in facultative apomicts (Asker 1980; Richards 1986; Bashaw and Hanna 1990). They form both reduced egg cells that are fertilized to produce genetically diverse progeny as well as apomictic embryo sacs that give rise to clonal offspring. Apomictic and sexual embryo sacs occur in the same plant or even within the same ovule (Asker 1980; Nogler 1984a; Vielle et al. 1995). Facultative apomicts, benefiting from the advantages of both modes of reproduction, may have an evolutionary advantage and are more common than obligate apomicts (Nogler 1984a; Richards 1986; Asker and Jerling 1992). The degree of sexuality versus apomixis in facultative apomicts is influenced by a variety of environmental factors, the effects of which on the reproductive system are not well

understood (e.g., Knox and Heslop-Harrison 1963; Knox 1967; Frost and Soost 1968; Cox and Ford 1987; Hussey et al. 1991).

The developmental regulation of sexual reproduction appears to be preserved during apomixis. Although an apomictic gametophyte or embryo has a distinct developmental origin, the sexual developmental program is largely conserved: megagametophyte development, embryogenesis, and the development of the endosperm and seed coat are identical in sexual and apomictic genotypes. At the level of gene expression, very few differences can be detected between obligate apomictic and sexual genotypes of *Pennisetum ciliare* (Vielle-Calzada et al. 1996). In apomixis, the sexual pathway is altered at the transitions between the two phases of the plant life cycle, meiosis and double fertilization (Figure 12.2): (*i*) meiosis is aberrant or absent leading to the production of an unreduced cell acting as the functional megaspore; (*ii*) the egg cell initiates embryogenesis parthenogenetically or embryos form directly from a sporophytic initial (adventive embryony); and (*iii*) the endosperm develops either autonomously or, in pseudogamous species, fertilization of the central cell is required for endosperm formation and successful seed development. In pseudogamous species, special adaptations may be required for successful endosperm formation. Thus, apomixis can be viewed as a short-circuited sexual pathway (Koltunow 1993; Vielle-Calzada et al. 1996; Grossniklaus et al. 1998a) in which part of the sexual developmental program is initiated at the wrong time or in the wrong cell. Thus, apomixis is characterized by a relaxation of the spatial and temporal constraints on the reproductive developmental process. It is likely that apomictic reproduction results from the heterochronic or heterotopic expression of regulatory factors that control megasporogenesis, as well as egg and central cell activation in sexual species (Mogie 1992; Peacock 1992; Koltunow 1993; Grossniklaus et al. 1998a).

Whereas nonreduction and parthenogenetic embryogenesis as two of the key components of apomixis have been discussed extensively, endosperm formation has attracted less attention. In pseudogamous apospecies, mechanisms preventing the fertilization of the egg cell but allowing fusion of sperm and central cells may rely on the formation of a complete egg cell wall prior to sperm arrival (Savidan 1992; Vielle et al. 1995). However, specific adaptations to maintain the endosperm balance number (Johnston and Hanneman 1982; Ehlenfeldt and Ortiz 1995) may be required to ensure normal seed development. In maize, endosperm formation is strictly dependent on the presence of maternal and paternal genomes in a ratio of 2m:1p, due to differential imprinting of the parental genomes (Lin 1984; Kermicle and Alleman 1990). This requirement is likely to exist in many plant species, but may be relaxed or absent in some (Haig and Westoby 1991; Messing and Grossniklaus 1999). Since apomictic species produce normal pollen, the fertilization of an unreduced central cell with a single reduced sperm cell would violate the endopserm balance number and lead to seed abortion. Endosperm formation is an important process that must be considered for the transfer of apomixis into sexual species (Grossniklaus et al. 1998a; Spillane et al. 2000; Savidan, 2000; Grossniklaus et al. 2001; Grimanelli et al., Chap. 6).

Models for Apomixis: Heterochronic Initiation of Development

A developmental analysis of apomictic events clearly indicates that several developmental processes occur simultaneously or asynchronously. Meiosis and embryo-sac formation may occur at the same time: the apomictic initial initiates embryo-sac

development without entering meiosis or after premature meiotic abortion and nuclear restitution (Crane, Chap. 3). Likewise, parthenogenetic embryogenesis is usually initiated prior to anthesis and often before fertilization of the central cell in pseudogamous species. Thus, it appears as if specific developmental events are initiated prior to completion of the previous ones. Heterochronic development is a hallmark of apomixis, with which specific developmental events are replaced asynchronously or coexist and compete with events occurring in normal sequence.

In addition to a change in the temporal order of developmental processes there is also a relaxed constraint on cell fate decisions. Whereas in sexual species a single nucellar cell is usually committed to the meiotic pathway, several nucellar cells in apomictic species have the potential to form unreduced gametophytes. The regulation of individual developmental events appears to be conserved between apomictic and sexual pathways. Therefore, it is likely that key regulatory genes playing essential roles in sexual development are misregulated in either space and/or time leading to the developmental alterations observed in apomicts. Precocious initiation of megagametogenesis and the premature activation of the egg cell could be caused by misexpressed regulatory genes that perform the same functions during sexual reproduction. Thus, the gene(s) controlling apomixis does not necessarily encode altered gene products, but rather could be under relaxed or aberrant temporal and/or spatial control.

Several models accounting for the precocious induction of developmental events and the interrelationship with the sexual pathway have been proposed (Peacock 1993; Koltunow 1993). Developmental checkpoints similar to the ones proposed to control proper progression through the cell cycle (Hartwell and Weinert 1989; Murray 1992) may ensure a strict sequential order of developmental steps during sexual reproduction. In apomicts, developmental checkpoints and feedback mechanisms may be ignored or altered, leading to the initiation of a developmental event before the completion of an earlier one (Koltunow 1993).

Alternatively, rather than misexpression of regulatory genes in the nucellus, more general changes in the cellular machinery could cause apomixis. For instance, an increase in the duration of the cell cycle may allow genes to be expressed at an earlier time in development than usual. Such a situation has been observed for genes with large introns in *Drosophila*. The genes *knirps* (*kni*) and *knirps-related* (*knrl*) encode highly similar proteins, but *knrl* contains a large 19 kb intron (Nauber et al. 1988; Oro et al. 1988; Rothe et al. 1989). Thus, *knrl* is only functional at nuclear division cycle 13 during cleavage, when the cell cycle has become long enough to allow RNA-polymerase to transcribe the entire *knrl* transcription unit before the initiation of M-phase (Rothe et al. 1992). In contrast, *kni* is expressed already at nuclear division cycle 9. An intron-less *knrl* gene can fully rescue the *kni* embryo lethal phenotype (Rothe et al. 1992). Two mutants have been isolated that allow for the functional substitution of *kni* by *knrl*. Both act by lengthening the cell cycle and, thus, allow *knrl* to be transcribed at an earlier stage of development than usual (Ruden and Jäckle 1995). A similar situation may be encountered in apomictic species, with duplicated genes being activated heterochronically. The duplicated genes may be paralogs present in the same genome or orthologs from two genomes brought together through hybridization.

The models discussed above do not take into account the tight association of apomixis with polyploidy, although they are certainly

compatible with it. Several models that consider the relationship between polyploidy and apomixis have been put forward (Nogler 1982; Mogie 1992; Noirot 1993; Carman 1997; Grimanelli et al., Chap. 6). For instance, an alteration of cell cycle length as hypothesized above may be caused by polyploidization or wide hybridization. In many species with isolates of several ploidy levels, diploids are usually sexual and polyploids apomictic (Asker and Jerling 1992; Leblanc et al. 1995). Autoploidization of sexual diploids has resulted in apomictic tetraploid plants in some species (Burton 1992). It is attractive to speculate that the cell cycle length is altered in response to changes in ploidy. However, no experimental data is available to support this hypothesis since essentially nothing is known about the regulation of the cell cycle during reproductive development in plants. The association of polyploidy with apomixis may, however, be a secondary effect caused by deleterious mutations that accumulated in the genome of apomicts. This is supported by the recent isolation of diploid apomicts in *Hieracium* and *Allium* (Bicknell 1997; Kojima and Nagato 1997). These and earlier findings suggest that polyploidy is not an absolute requirement for apomixis (Savidan 1980; Nogler 1982; Hashemi et al. 1989).

An attractive hypothesis, which takes both developmental and genomic peculiarities into account, has recently been proposed by Carman (1997; Chap. 7) based on earlier models put forward by Ernst (1918). In short, the duplicate-gene asynchrony hypothesis states that duplicate sets of genes regulating reproductive development exist in polyploids. Polyploidy originally arose through hybridization, such that the regulatory control of development originating from the two genomes may not be in synchrony. The resulting intergenomic regulatory conflict may then lead to the developmental aberrations observed in apomicts and other reproductive anomalies. An important aspect of this theory is that apomixis results from the conflicting action of genes that usually play a regulatory role during sexual development, i.e., the same wild-type genes (not mutant forms) control both sexuality and apomixis. This reinforces the need for a better understanding of the molecular and genetic basis of sexual reproduction for the engineering of apomixis in sexual crops.

Another consideration that could influence research strategies was raised by Jefferson and Bicknell (1996). At present, there is no evidence to indicate that apomixis is controlled by a *trans*-acting gene product rather than by a *cis*-acting element. One can envision an alteration of a *cis*-acting element, for instance a binding site for a *trans*-acting factor (e.g., for a transcription factor or chromatin component), with altered affinity or copy number that could cause apomixis by changing the concentration of the *trans*-acting factor in the cell. For example, the factor could be titrated out by an increase in the copy number of its binding sites, which in turn would result in precocious or inappropriate development of the embryo sac. Thus, a dominant locus could readily be explained. The recent observation that large genomic regions that are associated with the inheritance of apomixis are not present in sexual relatives (Ozias-Akins et al. 1998; Roche et al. 1999) is consistent with such a mechanism.

Genetic Control of Reproduction and Candidate Genes for the Engineering of Apomixis

To date, no fully apomictic mutants have been recovered in sexual species, however, several mutants and spontaneously occurring variations of sexual reproduction display individual components of apomixis. These include the production of unreduced spores

(Rhoades and Dempsey 1966; Franke 1975; Harlan and de Wet 1975; Jongedijk 1985; Kaul and Murthy 1985), the formation of parthenogenetic haploids (Kimber and Riley 1963; Turcotte and Feaster 1963; Sarkar and Coe 1966; Chase 1969; Hagberg and Hagberg 1980), and the autonomous activation of endosperm development (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus and Vielle-Calzada 1998; D. Page, R. Pruitt, S. Lolle and U. Grossniklaus, unpublished data). The versatility found in sexually reproducing plants suggests that sexual and apomictic modes of reproduction share many genetic regulatory components. The engineering of apomixis will require a better understanding of the regulatory control of female reproductive development in sexual plants at the molecular and genetic level. What determines the commitment of a cell to a particular developmental pathway such as meiosis or megagametophyte development? What are the events leading to egg cell activation and the initiation of embryogenesis? How are these processes connected to the control of the cell cycle? Answers to these and related questions will constitute an important step for our understanding of the reproductive system and its manipulation.

Genetic analysis of female reproduction has mainly focused on the characterization of female sterile mutants that disrupt morphogenesis of the sporophytic tissues of the ovule (Robinson-Beers et al. 1992; Lang et al. 1994; Léon-Klosterziel et al. 1994; Modrusan et al. 1994; Ray et al. 1994; Gaiser et al. 1995; Reiser et al. 1995; Klucher et al. 1996; Eliott et al. 1996; Villanueva et al. 1999; Schiefthaler et al. 1999; Yang et al. 1999). Whereas these studies have led to the formulation of genetic models for ovule development (Angenent and Colombo 1996; Schneitz et al. 1997; Baker et al. 1997; Grossniklaus and Schneitz 1998; Gasser et al. 1998; Schneitz 1999), the genetic basis and molecular mechanisms controlling megasporogenesis, megagametogenesis, and fertilization are almost completely unknown. This section reviews some of the genetic components involved in female gametogenesis, with a particular emphasis on mutants that are relevant to apomixis research.

Megasporogenesis and Nonreduction

Megasporogenesis is a complex process characterized by the determination of the megaspore mother cell, meiosis, and the selection and differentiation of the functional megaspore. Gametophytic apomixis involves the production of an unreduced gamete and its parthenogenetic development either with or without fertilization of the associated central cell to produce the endosperm. Thus, an important developmental decision is whether the megaspore mother cell or its apomictic counterpart undergoes a reductional division. To better understand the nature of the "decision" to undergo meiosis, it is helpful to consider two aspects. The first is the positional aspect of how a nucellar cell is selected to commit to the meiotic pathway. The second pertains to what steps in the meiotic pathway are essential to its irreversible commitment for further development into a functional megaspore and eventually an embryo sac. The first issue is unique to seed plants, and has little in common with organisms with dedicated germ lines, or those in unicellular models, such as yeast. The second point, which may be causally linked to the first, can be rephrased in the terminology of the cell cycle: Are there developmental checkpoints during megasporogenesis that can be simulated or bypassed to induce an unreduced cell to initiate megagametogenesis?

Insights into the early steps of megagametogenesis can be gained from an analysis of mutants affecting megaspore mother cell differentiation and meiosis. Despite the isolation of a large number of female sterile mutants in maize and

Arabidopsis, relatively little is known about the genetic control of megasporogenesis. Recently, the isolation of 270 *Arabidopsis* mutants with defective spore development (*megasporogenesis-defective*, *msd*) was reported (Schneitz et al. 1997). Mutants of the *msd* class do not produce a megagametophyte, however, sporophytic ovule development proceeds normally. The developmental defects during megasporogenesis have not been characterized in detail. All of these mutants also affect microsporogenesis and, therefore, are male and female sterile. They may affect meiosis per se rather than female specific processes.

Usually, only a single archesporial cell, and consequently a single megasporocyte, differentiates in an ovule. However, the occurrence of multiple megaspore mother cells in some species (Eames 1961; Walters 1985; Sumner and van Caseele 1998) and of two megasporocytes in about 5% of the wild type *Arabidopsis* ovules suggest that several nucellar cells have the potential to enter the meiotic pathway. Once a cell is committed, it appears to inhibit neighboring cells from doing the same (Grossniklaus and Schneitz 1998). This view is supported by a recently identified mutant in maize. Plants homozygous for *multiple archegonial cells1* (*mac1*) contain between three and 21 megasporocytes in a single ovule (Sheridan et al. 1996). Thus, *mac1* is only likely to be involved in megaspore mother cell determination. The phenotype shows certain similarities to apospory, in which multiple aposporic initials form around the sexual megaspore mother cell. However, unlike in apomicts where microsporogenesis is usually unaffected, *mac1* mutants also show abnormal male sporogenesis (Sheridan et al. 1999).

The genetic regulation of meiosis has been extensively studied in maize and the yeast *Saccharomyces cerevisiae* (Golubovskaya 1979; Golubovskaya et al. 1992; Mitchell 1994; Roeder 1995). In yeast, a large body of knowledge on the molecular mechanisms controlling meiosis has been amassed. Many yeast mutants that regulate the entry into meiosis and differentiate between meiotic and mitotic division have been isolated. These mutants share some characteristics with apospory or diplospory of the *Antennaria* type (Koltunow 1993), and their plant homologs could be instrumental in the engineering of apomixis.

Many genes that play roles in yeast meiosis have been characterized, generally by identifying mutants with specific meiotic defects and studying the level of transcripts of the corresponding genes during meiosis (Mitchell and Bowdish 1992; Mitchell 1994). These meiotic genes act at different stages of meiosis. Genes acting early in the pathway and regulating the decision between mitotic and meiotic divisions are of particular interest. Among the products of early meiotic genes, the meiotic activator *IME1* is a master control gene required for the expression of the genes acting in the early phase of meiosis (Kassir et al. 1988; Smith and Mitchell 1989; Mitchell et al. 1990; Kawaguchi et al. 1992). To be functional, *IME1* has to become phosphorylated by *RIM11* (Bowdish et al. 1994). Upon phosphorylation, the early meiotic genes are activated and a starved diploid cell undergoes meiosis to produce four haploid spores. In the fission yeast *Schizosaccharomyces pombe*, the *Mei3* gene is induced by nutrient deprivation. *Mei3* inhibits the protein kinase *Pat1*, that then triggers the entry into meiosis (reviewed by Yamamoto 1996). The *Pat1* kinase, in turn, represses the *Mei2* protein, which is an essential positive factor for entry into meiosis. Thus, regulatory networks involving phosphorylation and dephosphorylation events responding to environmental signals play a crucial role in the commitment to the meiotic pathway.

Meiosis is an almost universal feature of eukaryotic organisms, suggesting that the key regulatory events are conserved at the molecular level. A search for homologs of the meiotic genes in yeast could prove particularly useful for the engineering of apomixis. Indeed, a putative *RIM11* homolog from rice has recently been isolated by polymerase chain reaction (PCR) (Jefferson and Nugroho 1998). The deduced amino acid sequence is 68% similar and 50% identical to yeast *RIM11*, with conserved protein kinase subdomains. Experiments to investigate expression and function of *RIM11* in rice are ongoing and will reveal whether this gene plays the same regulatory function in plants and yeast (S. Nugroho and R. Jefferson, personal comm.). However, there are a very large number of kinases that are similar to RIM11 in the *Arabidopsis* genome, indicating that the identification of the ortholog will require a detailed analysis and functional tests. Similarly, several groups aimed at the isolation of *Mei2* homologs (Hirayama et al. 1997; I. Siddiqi, personal comm.; B. Tinland, personal comm.), but the presence of eight homologous genes in the *Arabidopsis* genome complicates the identification of the functional homolog. Despite the conservation of some meiotic genes between yeast and plants at the sequence level, it should be remembered that the molecular aspects of the control of meiosis are not conserved between *S. cerevisiae* and *S. pombe* and that the regulatory mechanisms in plants could be completely different.

In maize, mutants that influence the "decision" between meiosis and mitosis have also been identified, but their molecular nature is unknown. In plants homozygous for the *ameiotic 1* (*am1*) gene, meiosis does not occur and is replaced by a mitotic division (Palmer 1971). The *am1* gene appears to control the switch from the mitotic to the meiotic cell cycle and is important for the initiation of meiotic prophase I (Golubovskaya et al.1992). In plants homozygous for certain *am1* alleles, the megasporocyte does not divide at all, whereas in others meiosis is replaced by one or several mitotic division cycles (Golubovskaya et al. 1993, 1997). In *absence of first division* (*afd*) mutants, the first meiotic division is replaced by a mitosis (Golubovskaya 1979). This reversion to mitosis of a cell already committed to meiosis shows similarity to apomixis characterized by a restitution nucleus at meiosis I (Taraxacum- and Ixeris-type).

Several mutants in plants and yeast produce two unreduced spores reminiscent of diplospory. The yeast mutant *spo12* (Klapholz and Esposito 1980), the *elongate1* (*el1*) mutant of maize (Rhoades 1956; Rhoades and Dempsey 1966) and *triploid inducer* (*tri*) in barley (Ahokas 1977; Finch and Bennett 1979) affect the second meiotic division. Thus, they produce genetically diverse progeny. Whereas *el1* affects both sexes, *tri* is specific to the female and regular reduced pollen is produced leading to the formation of triploid embryos upon self-fertilization. In *Arabidopsis*, the *dyad* mutant produces a dyad of megaspores rather than a tetrad (Siddiqi et al. 2000). Based on microscopical analysis of chromosome segregation and the expression of meiosis-specific markers, the first meiotic division seems to occur normally in *dyad* mutants but then meiosis arrests (Siddiqi et al. 2000). To my knowledge, *dyad* is the first *Arabidopsis* mutant affecting sporogenesis in a sex-specific manner. Unlike in *el1* and *tri* where unreduced viable megagametophytes are produced, *dyad* mutants are fully female sterile. Although the megaspores of the dyad sometimes undergo additional divisions, no functional embryo sacs are formed (Siddiqi et al. 2000). Based on its genetic mapping position, *dyad* may be identical to the female-specific *switch1* (*swi1*) gene whose megaspore mother cell undergoes

mitotic divisions similar to the phenotype described for *am1* in maize (Motamayor et al. 2000). The difference in phenotype may be due to allelic variation as was observed for *am1*, in which different alleles have quite distinct phenotypes, or to genetic background effects.

In the yeast mutant *spo13*, meiosis I is omitted and a dyad of unreduced spores is formed by an equatorial division (Klapholz and Esposito 1980) in a process closely resembling the Taraxacum-type of diplospory (Koltunow 1993). To date, no homologs of *spo13* have been reported in other species, and we could not detect cross-hybridizing DNA in *Arabidopsis* or the closely related budding yeast *Kluveromyces lactis* by low stringent hybridization (H. Sims and U. Grossniklaus, unpublished data). Indeed, no sequence with significant homology is present in the *Arabidopsis* genome. Other approaches such a functional complementation with cDNA expression libraries (Minet et al. 1992; Hirayama et al. 1997) may be successful for isolating plant homologs of yeast meiotic mutants. It is important to stress that although meiosis in *spo13* mutants closely resembles an apomictic process, recombination still occurs. Therefore, the progeny are genetically diverse if the parental diploids show a certain degree of heterozygosity. Only a combination of *spo13* with a recombinationless mutant would produce clonal offspring. Mutants that reduce the frequency of recombination have been extensively studied in yeast (e.g., Malone et al. 1991), and homologs of *DMC1* have recently been isolated from *Lilium longiflorum* (Kobayashi et al. 1993, 1994)) and *Arabidopsis* (Sato et al. 1995; Klimyuk and Jones 1997; Doutriaux et al. 1998). Mutants in the *Arabidopsis AtDMC1* gene affect male and female meiosis, and the presence of 10 univalents in *dmc1* meiocytes suggests that *DMC1* is required for bivalent formation and/or stabilization and, thus, indirectly for recombination (Couteau et al. 1999).

Megagametogenesis

Transmission studies of chromosomal deletions (Patterson 1978; Coe et al. 1988; Buckner and Reeves 1994; Vizir et al. 1994) and deficiency analysis in maize (Vollbrecht and Hake 1995) suggest that a large number of loci essential for embryo-sac development are dispersed throughout the genome. Nevertheless, gametophytic mutants have rarely been isolated and little is known about the genetic control of morphogenesis and differentiation in the megagametophyte. Such mutants are of great relevance to apomixis research because the genes that lead to heterochronic initiation of megagametogenesis and embryogenesis in apomicts are likely to act in the megagametophyte of sexual plants.

Mutants affecting the megagametophyte are characterized by semisterility and non-Mendelian segregation, phenotypes that allow the efficient isolation of mutants affecting the gametophytic phase of the life cycle by insertional mutagenesis (Feldmann et al. 1997; Moore et al. 1997; Bonhomme et al. 1998; Christensen et al. 1998; Howden et al. 1998; Grini et al. 1999). During the last few years, we have used a transposon-based mutagenesis system (Sundaresan et al. 1995) to identify mutants that affect megagametogenesis. Nearly 60 mutants have been identified, 15 of which have been characterized at the genetic and cytological level. They affect the various developmental steps of female gametogenesis: initiation of megagametophyte development, free nuclear divisions cycles, nuclear migration and differentiation, cellularization, and double fertilization (J. Moore and U. Grossniklaus, unpublished results). A characterization of these and other mutants currently being isolated in several laboratories will shed light on the molecular mechanisms controlling the initiation of megagametogenesis, the specification of the gametophytic cell types, and their specific functions for fertilization and seed development.

To date, the phenotypes of about a dozen megagametophytic *Arabidopsis* mutants have been described in the literature (reviewed in Drews et al. 1998; Grossniklaus and Schneitz 1998; Yang and Sundaresan 2000). In *Gametophytic factor* (*Gf*) (Redei 1965; Christensen et al. 1997), *andarta* (Howden et al. 1998), *tistrya* (Howden et al. 1998), *female gametophyte2* (*fem2*) and *fem3*, *gametophyte factor4* (*gfa4*) and *gfa5* (Christensen et al. 1998), the functional megaspore does not initiate megagametogenesis. In *prolifera* (*prl*) (Springer et al. 1995), *cdc16* (Yang and Sundaresan 2000), and *hadad* (*hdd*) (Moore et al. 1997), the syncytial mitotic divisions are affected and embryo sacs show an early developmental arrest. *PRL* as a member of the MCM2-3-5 family and a putative component of DNA Replication Licensing Factor, is an essential gene required in all dividing cells (Springer et al. 1995). *CDC16* is another gene required for the normal operation of the cell cycle machinery. In *gfa2*, *gfa3*, and *gfa7* mutant embryo sacs, the two polar nuclei do not fuse, a phenotype also observed in some of our mutants (J. Moore and U. Grossniklaus, unpublished results).

In maize, megagametophytes carrying *indeterminate gametophyte* (*ig*) or the *r-X1* deficiency undergo abnormal mitotic divisions and are transmitted through the female gametophyte at a reduced frequency (Kermicle 1971; Lin 1978, 1981; Weber 1983; Huang and Sheridan 1996). We identified an *Arabidopsis* mutant, *haumea* (*hma*), sharing some of these aspects with *ig*, namely additional division cycles during megagametogenesis (J. Moore and U. Grossniklaus, unpublished data). Embryo sacs mutant for *lethal ovule* (*lo1* and *lo2*) do not produce viable seeds (Singleton and Mangelsdorf 1940; Nelson and Clary 1952). The *lo2* embryo sacs show a defect in nuclear division and migration, and arrest predominantly at the 1- and 2-nucleate stages, although some *lo2* megagametophytes undergo all three division cycles (Vollbrecht 1994; Sheridan and Huang 1997).

In *hdd* embryo sacs, nuclear divisions at the micropylar and chalazal pole are asynchronous, and some of the mutant megagametophytes cellularize prematurely. Thus, nuclear division and cellularization are regulated independently. However, these cells do not differentiate into a particular gametophytic cell type, suggesting that cell specification may depend on the correct spatial context and/or the presence of neighboring cells. Premature cellularization and asynchronous divisions at the poles have also been observed in segmental deletions in maize (Vollbrecht and Hake 1995), suggesting that several loci, including *hdd*, are involved in the spatial and temporal coordination of cellularization, nuclear division, and migration. Whereas these processes are normally tightly coordinated, they are uncoupled of each other in *hdd* mutant embryo sacs, indicating that several independent developmental programs control the different processes during embryo-sac development. They are usually highly coordinated, possibly by checkpoint mechanisms and regulatory feedback loops. Developmental aberrations may relax this coordinated control, and the various developmental programs can occur independently of their normal context.

Egg Activation and Parthenogenesis

Fertilization and egg activation have been studied extensively in animal systems at the physiological, cellular, and molecular level (reviewed in Nucitelli 1991; Whitaker and Swann 1993; Jaffe 1996). Numerous studies have shown that the adhesion of the sperm to the egg cell triggers a transient rise in free calcium ions and initiates a cascade of downstream events after fertilization (Jaffe 1991, 1996; Whitaker and Swann 1993; Homa et al. 1993). The release of calcium ions is

absolutely essential for egg activation. Case in point: the introduction of calcium into the egg is sufficient to induce parthenogenetic activation of sea urchin and mammalian eggs (Steinhardt and Eppel 1974; Uranga et al. 1996). Recently, in vitro injection experiments with mouse oocytes have shown that a truncated c-kit receptor tyrosine kinase leads to parthenogenetic egg activation that requires both calcium and phospholipase C activity (Sette et al. 1997).

In plants, our understanding of the events following fertilization is very limited. Experimentation with angiosperm gametes has been difficult because of their inaccessibility and the complex milieu within the megagametophyte where double fertilization occurs (Russell 1993; Dumas and Mogensen 1993). Specific fusion of isolated maize gametes has recently been achieved and offers great promise for the study of molecular and cellular events underlying fertilization and egg activation in vitro (Faure et al. 1994; Dumas and Faure 1995; Tirlapur et al. 1995; Kranz and Dresselhaus 1996; Rougier et al. 1996). One of the first visible changes after fertilization of an isolated maize egg cell is the formation of a cell wall (Kranz et al. 1995). In the brown algae *Fucus*, cell wall secretion depends on an increase in the cytosolic calcium concentration (Roberts et al. 1994; Roberts and Brownlee 1995; Belanger and Quatrano 2000). A transient rise of the cytosolic calcium concentration has recently been reported for the first time in angiosperms after in vitro fertilization of a maize egg cell (Digonnet et al. 1997; Antoine et al. 2000). These observations suggest that plant and animal egg activation may be similar, however, the role played by the release of calcium in the fertilized plant egg and what events it triggers are currently unknown.

Parthenogenetic egg activation is a key component of apomixis and occurs in many plant species, both spontaneously (Kimber and Riley 1963; Turcotte and Feaster 1963; Chase 1969) and after induction in isolated ovaries and ovules. Certain genetic backgrounds and mutants produce a high percentage of parthenogenetic haploids in their progeny. A better understanding of the genetic basis of parthenogenetic development will prove instrumental for the engineering of apomixis. In maize, parthenogenetic haploids are produced spontaneously at a frequency of about 0.1% (Chase 1969), and as high as 3.2% in certain genetic backgrounds (Coe 1959; Sarkar and Coe 1966). In plants homozygous for *ig*, the frequency of maternal haploids is increased fourfold to 0.6% as compared to isogenic lines homozygous for the wild type *Ig* allele (Kermicle 1969). Interestingly, *ig* also conditions the production of patroclinous (androgenetic) offspring at a frequency of more than 2% as compared to 0.001% (1/80,000) in wild type stocks (Chase 1963; Kermicle 1969, 1994). Thus, *ig* not only permits extra rounds of mitosis in the embryo sac (Lin 1978, 1981; Huang and Sheridan 1996) but it also promotes the formation of embryos in the absence of karyogamy. Extremely high frequencies of haploid production are found in barley plants homozygous for *haploid initiator* (*hap*) and in the Salmon system of wheat. The *hap* mutation conditions the formation of 30% haploid embryos when homozygous (Hagberg and Hagberg 1980; Asker et al. 1983). The sperm is prevented from fertilizing the egg by an unknown mechanism whereas the central cell gets fertilized normally to produce the nutritive endosperm (Mogensen 1988).

Salmon wheat lines can produce up to 90% parthenogenetic haploids (Matzk 1995). Both cytoplasmic and nuclear determinants are involved in parthenogenetic activation: the presence of the wheat-rye translocation chromosome 1BL-1RS in the *Aegilops* cytoplasm of *caudata* or *kotschyi* Salmon leads to haploid production, whereas either the translocation or the *Aegilops* cytoplasm alone

do not display parthenogenetic properties (Kobayashi and Tsunekami 1978; Tsunewaki and Mukai 1990; Matzk et al. 1995; Matzk 1996). The nuclear factors involved in the Salmon system have been defined genetically and shown to involve *Ptg*, a gene that induces parthenogenesis, and a *Spg*, a supressor of parthenogenesis. Successful parthenogenesis depends on the presence of the *Aegilops* cytoplasm and *Ptg*, and the absence of *Spg*. The Salmon system offers unique opportunities to study parthenogenesis at the molecular genetic level by comparing isogenic lines carrying either the *Triticum* (sexual) or *Aegilops* cytoplasms (parthenogenetic); various approaches to studying the molecular basis of parthenogenetic activation have been initiated (Matzk et al. 1995; Matzk 1996; Matzk et al. 1997).

Endosperm Development and Genomic Imprinting

Double fertilization involves two pairs of gametic cells. One sperm cell fuses with the egg to form the diploid zygote whereas the other fuses with the central cell to generate the usually triploid endosperm. The endosperm provides nutrients during seed development and synthesizes storage reserves required for post-germination development. Different modes of endosperm development have been described (Vijayraghavan and Prabhakar 1984), the most common of which is nuclear. The primary endosperm nucleus undergoes several division cycles without cytokinesis to form a large number of free nuclei, which then migrate to the periphery of the central cell and cellularize in a specific developmental pattern, usually from the periphery to the center. The nuclear type of development is typical of cereals such as maize and rice (Brink and Cooper 1947) and also for the model plant *Arabidopsis* (Mansfield and Briarty 1990a, 1990b). The interactions between embryo, endosperm, and maternal tissue are a complex and poorly understood aspect of seed development. There are many developmental patterns of endosperm development and the relative importance of the different components in the seed with regard to nutrient synthesis and acquisition varies accordingly. The origin, development, and function of the endosperm has recently been reviewed in detail (Lopes and Larkins 1993; Berger 1999; Olsen et al. 1999). In this section only aspects of endosperm development that are of direct relevance to apomixis research are discussed.

1. Interrelationship of embryo and endosperm development. In apomictic species, normal development of the endosperm is required for the formation of viable seeds. Although recent studies suggest that the morphogenesis of the embryo is largely independent of endosperm development (Sheridan et al. 1995), endosperm forms in all apomictic species studied to date. This is not only true for gametophytic but also for sporophytic apomicts, in which adventive embryos depend on a sexually produced endosperm for their pre- and/or post-germination development (Asker and Jerling 1992). Endosperm formation is achieved either by allowing fertilization of the central cell (pseudogamy) or by autonomous division of the polar nuclei to form the endosperm tissue. It is likely that the formation of a complete cell wall around the egg cell prior to anthesis prevents the egg from being fertilized (e.g., Vielle-Calzada et al. 1995), but allows the fertilization of the central cell in pseudogamous apomicts. In addition to this spatial block there may also be important temporal controls. For instance, the fertilization of the central cell occurs after the parthenogenetic activation and autonomous divisions of the egg cell in many apomicts (Asker and Jerling 1992). This is in contrast to sexual species, in which the endosperm nucleus divides several times before the first zygotic division. In autonomous apomicts, the central cell develops parthenogenetically and

the developmental program for endosperm development is activated in the absence of fertilization.

It is possible that the same genetic control is responsible for autonomous endosperm and egg activation. This would be in agreement with the hypothesis that the endosperm is evolutionary and derived from a second embryo, as first proposed by Sargant (1900). This hypothesis is supported by morphological analyses of fertilization in nonflowering seed plants of the genera *Ephedra* and *Gnetum* (Friedman 1990, 1992; Carmichael and Friedman 1995). In addition, molecular and genetic investigations have shown that there is a large overlap in gene activity between the endosperm and embryo. For instance, among the 855 characterized *defective kernel* mutants in maize (representing about 285 loci), the vast majority affect both the embryo and endosperm and very few are potentially endosperm-specific (Neuffer and Sheridan 1980). Similar results were obtained in a study of *defective kernel* mutants in barley (Bosnes et al. 1987), suggesting that a very large percentage of seed-specific genes are expressed in both embryo and endosperm, despite their different development and physiology. In contrast to this extensive overlap in gene expression between embryo and endosperm, studies on several recently isolated *Arabidopsis* mutants that allow fertilization-independent endosperm formation but not embryogenesis suggest that endosperm activation may be controlled by different developmental programs (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus and Vielle-Calzada 1998; Luo et al. 1999; Kiyosue et al. 1999; D. Page, R. Pruitt, S. Lolle, and U. Grossniklaus, unpublished data).

The importance of the endosperm for seed development varies among species depending on its developmental pattern. In some species of the Orchidaceae the endosperm undergoes only a few division cycles or does not divide at all. In many dicotyledonous species, including *Arabidopsis*, the endosperm forms but is essentially degraded by the time seed maturation is initiated. In contrast, the endosperm of cereals is persistent and of great economic value. Therefore, an engineered apomixis in grain crops will have to allow for normal development of the endosperm. In an ideal situation, endosperm formation in engineered apomictic crops could be induced autonomously. However, successful formation of endosperm in cereals depends on the specialized cytoplasm of the central cell and requires contributions from both maternal and paternal genomes. This may be because some genes are imprinted, that is their activity depends on their parental origin (Kermicle 1970; Kermicle and Alleman 1990; Messing and Grossniklaus 1999). Thus, engineered apomictic grain crops are likely to require the fertilization of the central cell. The vast majority of apomictic Gramineae are pseudogamous; possible autonomous apomixis has been observed in very few species, including *Calamagrostis*, *Poa nervosa* and *Nardus stricta* (Johri et al. 1992).

2. Genomic imprinting. Imprinting in plants is usually regarded as specific to the endosperm (Kermicle and Alleman 1990; Walbot 1996; Alleman and Doctor 2000). Formation of androgenetic and gynogenetic haploids in many species (Kimber and Riley 1963; Sarkar and Coe 1966; Kermicle 1969) and of asexually derived embryos in apomicts suggest that imprinting does not play a crucial role for embryogenesis in these species, although it may exist in others. The development of embryos from somatic tissue (Zimmerman 1993; Mordhorst et al. 1997) and through anther culture (Zaki and Dickinson 1990) is taken as further evidence that imprinting is not involved in plant embryogenesis. However, the initial

development of such embryos is distinctly different from sexually derived embryos and it is not clear whether the same developmental programs control embryogenesis in these different contexts. It is possible, for example, that imprinting requirements are suppressed under certain culture conditions. Furthermore, it is likely that the importance of imprinting for embryo and endosperm will differ among species depending on the respective roles of these tissues in the production and acquisition of nutrients (Messing and Grossniklaus 1999). For instance, the *Arabidopsis* gametophytic maternal effect mutation *medea* (*mea*) drastically affects cell proliferation in embryo and endosperm, resulting in seed abortion (Grossniklaus et al. 1998b). Genetic and expression studies suggest that *MEA* is regulated by genomic imprinting and expressed in both embryo and endosperm at early stages of seed development (Vielle-Calzada et al. 1999). At later stages, the imprint at the *mea* locus occasionally breaks down, but reports as to which tissues are affected differ (Kinoshita et al. 1999; Luo et al. 2000). That the genetic background has strong effects on the *mea* phenotype suggests that these differences may be ecotype dependent (Vielle-Calzada et al. 1999; Grossniklaus et al. 2001). Currently, however, it is not clear which fertilization product is primarily affected, but it is likely that *MEA* is required in both the embryo and endosperm. Genetic interactions of *mea* and similar mutants with mutants that affect DNA methylation and/or chromatin remodeling have been reported (Vielle-Calzada et al. 1999; Luo et al. 2000; Vinkenoog et al. 2000; Grossniklaus et al. 2001). We are currently using *mea* as a starting point to isolate additional genes involved in the genomic imprinting process through second-site modifier screens.

Genomic imprinting has not been studied in many plant species, but it has been unequivocally demonstrated in the endosperm of maize at the genomic, chromosomal, and individual gene levels (Kermicle and Alleman 1990; Messing and Grossniklaus 1999). In maize, proper development of the endosperm is strictly dependent on the presence of maternal and paternal genomes in a ratio of 2m:1p (Lin 1982, 1984; Birchler 1993). Any deviation from this ratio leads to a failure in endosperm formation and consequently to seed abortion. Interspecific and interploidy crosses suggest that this is likely to be true for other species including most agriculturally important grain crops (Nishiyama and Yabuno 1978; Johnston et al. 1980; Haig and Westoby 1991). In contrast, endosperm development in *Arabidopsis* does not require a 2m:1p ratio because interploidy crosses involving diploid and tetraploid plants produce viable seeds (Redei 1964; Grossniklaus et al. 1998b), but there are distinct parent-of-origin dependent effects on seed size in interploidy crosses (Scott et al. 1998).

3. Imprinting barriers to the introduction of apomixis into sexual species. Imprinting phenomena may be behind the high degree of sterility observed in hybrids between sexual and apomictic genera, and so, should be given consideration in efforts to introduce apomixis into sexual species. In gametophytic apomixis the female reproductive cells are unreduced whereas microsporogenesis is unaffected and the male gametophytes are reduced. Thus, fertilization of the central cell generates a ratio of 4m:1p, which is expected to result in seed abortion. However, apomictic species do not show strongly reduced fertility, suggesting (*i*) that the constraints for imprinting are relaxed in apospecies or (*ii*) that the mechanisms of fertilization have been modified. Apomicts do indeed show a relaxed requirement for imprinting, which is supported by the finding that the ploidy level of the endosperm in apomictic species can be quite variable (Johri et al. 1992). A recent study by Grimanelli et al. (1997) clearly demonstrates that endosperm

development in *Tripsacum* is normal under a wide range of ratios of maternal to paternal genomes. Similarly, apomictic *Paspalum* species are insensitive to an imbalanced genome ratio in the endosperm while the sexuals maintain this requirement (Quarin 1999).

Altered modes of fertilization that are expected to maintain the endopserm balance number have been reported in several cases. This can be achieved if either both sperm cells delivered by the pollen tube fuse with the central cell, or if only one of the two polar nuclei and a single sperm nucleus participate in karyogamy (Rutishauser 1954; Reddy and d'Cruz 1969; Nogler 1972, 1984a). Alternatively, unreduced pollen could serve as the male parent (Chao 1980). As a very successful alternative to relaxed imprinting requirements, many apomictic grasses show apospory of the Panicum-type, where 4-nucleated embryo sacs are formed, which most often contain only one polar nucleus that fuses with a single sperm nucleus (e.g., Savidan 1980). Sexual individuals of these agamic complexes usually produce 8-nucleated Polygonum-type embryo sacs with two reduced polar nuclei. Thus, fertilization of both sexually and apomictically derived central cells produces endosperms with balanced parental genomes (Reddy 1977).

Apomictic species may be evolutionarily derived from predisposed genera that had relaxed imprinting requirements or, alternatively, evolved specific adaptations of the fertilization mechanism that maintained the imprinting requirements (see also Grimanelli et al., Chap. 6). Such predispositions and adaptations are not thought to exist in most sexual species and imprinting may pose a serious problem to the introduction of apomixis into sexual crop plants (Grossniklaus et al. 1998a; Spillane et al. 2000; Savidan 2000; Grossniklaus et al. 2001). Indeed, apomictic maize-*Tripsacum* and pearl millet-*Pennisetum* hybrids show a high degree of seed abortion (Grimanelli et al. 1995; Dujardin and Hanna 1989) that is likely to result from a genomic unbalance in the endosperm (Grossniklaus et al. 1998a; Morgan et al. 1998). Crosses with pollen donors of higher ploidy that maintained the endosperm balance number could possibly restore fertility. At present, our understanding of imprinting and its importance for seed development is very limited. A sustained effort toward a better understanding of the genetic and molecular basis governing imprinting is required to overcome the potential constraints to the engineering of apomictic crops.

Genetic Screens for Mutants Displaying Apomictic Traits in Sexual Model Systems

In previous sections, I discussed a number of genes that control sexual development that could serve as powerful tools for the engineering of apomixis. As an alternative, a screen for mutants that display apomictic traits in a sexual species could directly lead to the identification of key regulatory components (Peacock 1992). This approach has been taken in several laboratories using *Arabidopsis* as a model system. Although no apomictic species have been described in this genus, the close relative *Arabis holboelli* is apomictic (Asker and Jerling 1992). While direct experimentation with *Arabis* is difficult because of its poor genetic characterization and long generation time, its close relationship to *Arabidopsis* may be useful for comparative and wide hybridization approaches.

Arabidopsis Mutants with Autonomous Seed Development

Screens for *Arabidopsis* mutants that allow seed development in the absence of fertilization have been performed in several laboratories. These screens take advantage of male sterile mutants and aim to identify second site mutations that pseudo-suppress sterility. In

Arabidopsis, unpollinated pistils do not elongate, so that pistil elongation is an easily scored phenotype correlated with seed development. Different male sterile mutants have been used, including *pistillata* (*pi*), a homeotic flower mutant that lacks stamen (Chaudhury and Peacock 1993; Koltunow et al. 1995; Chaudhury et al. 1997); the wax biosynthetic mutant *eceriferum6* (*cer6*) (Dellaert 1979; Preuss et al. 1993; Hülskamp et al. 1995), a conditional male sterile that is fertile under high humidity (Ohad et al. 1996); and the temperature sensitive mutant *TH154*, isolated in R. Pruitt's laboratory, which is male sterile at 25°C but fully fertile at 18°C (D. Page, R. Pruitt, S. Lolle, and U. Grossniklaus, unpublished results). Silique elongation under the restrictive condition indicates an asexual mode of reproduction with full or partial seed development, or the development of a fruit without concomitant seed production (parthenocarpy).

Using this type of screen with more than 15,000 M1 plants, we identified three classes of mutants (D. Page, R. Pruitt, S. Lolle, and U. Grossniklaus, unpublished results): (*i*) mutants suppressing the male sterility defect, which can easily be identified because they produce functional pollen (some of these will be true revertants of *TH154*); (*ii*) mutants displaying parthenocarpy; and (*iii*) mutants displaying apomictic traits with autonomous development of seed-like structures in the absence of pollen production. The latter are rather rare and, to date, mutants in only three loci have been reported. These have been characterized in more detail at the genetic and morphological level. In the *fertilization-independent endosperm* (*fie*), mutant endosperm develops autonomously to the free nuclear stage, the seed coat develops normally, but no embryo forms (Ohad et al. 1996). Likewise, *fertilization-independent seed* mutants (*fis1*, *fis2*, *fis3*) form autonomous endosperm, which was shown to be diploid and can progress to the cellular stage in *fis1* and *fis2*. The seed coat develops properly but no embryos form (Chaudhury et al. 1997). Structures that resemble an elongated zygote have been observed in *fis1* and *fis2* at a low frequency. In *fie*/*fis* plants, the mutant allele is either very poorly transmitted or not transmitted at all through the female gametophyte and can only be recovered through the pollen, because pollinated seeds derived from a mutant megagametophyte abort (Ohad et al. 1996; Chaudhury et al. 1997). Thus, *fie*/*fis* mutants display a gametophytic maternal effect on seed development, which is similar to the phenotype observed for *mea* that also shows fertilization-independent endosperm development (Grossniklaus et al. 1998b; Grossniklaus and Vielle-Calzada 1998). Indeed, *fis1* and two other mutants were found to be allelic to *mea* (Kiyosue et al. 1999; Luo et al. 1999), as were *fis3* and *fie* (Chaudhury et al. 1997; Ohad et al. 1999).

MEA and *FIE* encode members of the Polycomb group, proteins thought to regulate gene expression by modulating higher order chromatin structure (Grossniklaus et al. 1998b; Ohad et al. 1999). *FIS2* encodes a putative DNA binding protein with a Zn-finger domain (Luo et al. 1999). As with their animal counterparts, the MEA and FIE proteins interact directly in a protein complex (Luo et al. 2000; Spillane et al. 2000; Yadegari et al. 2000). The function and regulation of *MEA*, *FIE*, and *FIS2* have been extensively reviewed (Goodrich 1998; Ma 1999; Preuss 1999; Mora-Garcia and Goodrich 2000; Russinova and de Vries 2000; Grossniklaus et al. 2001) and will be summarized only very briefly. *MEA* was shown to be regulated by genomic imprinting, thus explaining its maternal effect on seed development (Vielle-Calzada et al. 1999; Kinoshita et al. 1999). While *FIS2* also seems to be regulated by genomic imprinting (Luo et al. 2000), *FIE* appears to be expressed biparentally later during seed development (Luo et al. 2000; Yadegari et al.

2000). At earlier stages, a FIE::GUS fusion protein was not expressed from the paternal allele, as was observed for a large number of genes expressed early during seed development (Vielle-Calzada et al. 2000), but paternal FIE::GUS activity becomes detectable later on (Luo et al. 2000), suggesting that it is regulated in a manner different from *MEA*.

Although the *mea*, *fie*, and *fis2* mutants do not produce fertile asexual seeds, they show characteristics of autonomous reproduction. It is possible that some of the yet uncharacterized mutants with fertilization-independent endosperm and maternal effect seed abortion will not only allow the autonomous endosperm formation, but also full or partial embryogenesis. Alternatively, the *mea*, *fie*, and *fis2* mutants can be used as the starting material for second site modifier screens that could eventually lead to the identification of additional mutants that allow the formation of fertile seeds.

Screen for Pseudogamous Apomixis in Cereals

Although the engineering of autonomous apomixis in which both embryo and endosperm develop without fertilization might be considered the ideal situation, it may be extremely difficult to achieve in cereals. As outlined earlier, autonomous apomixis is rare in nature and the imprinting requirements for successful endosperm development pose a serious barrier to the introduction of autonomous apomixis into sexual crops. Therefore, the engineering of apomixis in sexual species will have to target a pseudogamous mode of apomictic reproduction. A search for apomictic mutants in sexual model systems that allow fertilization of the central cell may be more appropriate than screens for fully autonomous apomicts.

Screens for *Arabidopsis* mutants displaying pseudogamous apomixis have been proposed (Chaudhury and Peacock 1993). The self-fertilizing reproductive behavior of *Arabidopsis*, however, makes such screens labor intensive because they are based on outcrossing and scoring the progeny for exclusively maternal inheritance. In maize, similar screens are greatly facilitated by the natural outcrossing mode of reproduction, the availability of embryo-specific markers that can be scored on whole kernels, and the multitude of genetic tools available to the geneticist. Over the last few years, I have developed maize stocks to perform a genetic screen aimed at isolating mutants with characteristics of pseudogamous apomictic reproduction.

The screen is based on the *Mutator* transposon system, which is used as a potent mutagen and allows for easier cloning of newly isolated mutants (Chomet 1994). The genetic screen exploits the imprinting barrier for endosperm development and an easily scored embryo and endosperm marker. Lines that are homozygous for a recessive allele of the *red color* or *r* locus and have *Mutator* activity serve as female recipients. An allele of the *r* locus, which pigments both the crown of the kernel (aleurone layer of endosperm) and the embryo (*R-nj*, Figure 12.3, p. 197), is used as a dominant paternal marker. Screens aimed independently at nonreduction and parthenogenesis have been devised. Both rely on the *R-nj* paternal marker gene in tetraploid configuration. If a mutation causes nonreduction during meiosis, the resulting central cell carries four maternal genomes, and only endosperms receiving two paternal genomes (from the tetraploid pollen donor) maintain the endopserm balance of 2m:1p (here 4m:2p) and develop normally. All seeds derived from a reduced female gametophyte will abort such that only kernels derived from a nonreduced embryo sac will develop (or a seed, where the requirement for a balanced genome ratio in the endosperm has been abolished). Thus, the imprinting requirement provides a powerful selection

system to identify mutants that lead to the formation of an embryo sac from an unreduced cell lineage. In addition, full-sized kernels can be scored for absence of the dominant paternal *R-nj* marker in the embryo, which indicates parthenogenetic development.

Because obtaining a mutation that causes both nonreduction and parthenogenesis may be extremely difficult, a second screen aimed at the isolation of parthenogenetic mutants has been developed. It makes use of the *el1* mutation, which, when homozygous, produces a large fraction of unreduced embryo sacs. In *el1* homozygotes, independent assortment during meiosis I is not affected and the resulting gametes are not genetically identical (Roades and Dempsey 1966). Nevertheless, it provides a reliable source for unreduced female gametes. Lines homozygous for *r1* and *el1* and displaying high *Mutator* activity have been constructed to serve as female recipients. Kernels derived from crosses with a tetraploid *R-nj* pollen donor can be scored for rejection of the *R-nj* marker, i.e., for the maternal phenotype, in order to identify embryos that developed without a paternal contribution. Such embryos will be diploid (through the action of *el1*), which greatly facilitates subsequent genetic characterization. These genetic screens aimed at the isolation of mutations that lead to nonreduction, parthenogenesis, or a combination of both aspects will provide useful material to further our understanding of these processes at the molecular and genetic level.

Enhancer Detection as a Powerful Tool to Study Sexual Reproduction in *Arabidopsis*

The molecular and genetic bases of megasporogenesis and megagametogenesis are poorly understood. To date, only one female-specific mutant that affects sporogenesis has been identified (Siddiqi et al. 2000; Motamayor et al. 2000) and attempts to isolate genes that regulate the developmental events initiating female gametogenesis and embryo development are just beginning. As an alternative to the isolation of mutations, we identify genes expressed specifically during megasporogenesis and megagametogenesis. The inaccessibility of the developing embryo sac and the small number of cells involved make this a difficult undertaking using conventional molecular methods such as differential screening techniques. Therefore, we use a novel technology, enhancer detection, which allows the identification of developmentally regulated genes based on their pattern of expression. Enhancer detection is one of the most powerful tools to identify tissue specifically expressed genes and their regulatory sequences. Application of this approach in angiosperms will lead to the identification of many genes that control gametogenesis and cellular differentiation in the female gametophyte. In addition, it will identify many cell type- and tissue-specific regulatory regions that will be required to express candidate genes in a precise temporal and spatial fashion.

Enhancer Detection and Gene Trap Systems

Enhancer detection was first developed in the fruit fly *Drosophila melanogaster* and relies on a mobile genetic element carrying a reporter gene under the control of a weak constitutive promoter (O'Kane and Gehring 1987). This minimal promoter is usually not active but ideally it can be activated in all tissues and at all developmental stages. If it comes under the control of genomic *cis*-regulatory elements such as enhancers, the reporter gene is expressed in a specific temporal and spatial pattern (Figure 12.4, p. 197). This pattern reflects the expression of a nearby gene controlled by the same regulatory elements and, thus, allows the identification of genes

based on their pattern of expression rather than on a mutant phenotype (Bellen et al. 1989; Bier et al. 1989; Grossniklaus et al. 1989; Wilson et al. 1989). Enhancer detector screens have been extremely successful in *Drosophila* developmental genetics and similar approaches were rapidly adapted to other model systems. Because of the large intergenomic regions in mice, gene traps were developed that depend on a modification of this approach involving the generation of transcriptional fusions to the reporter gene (Gossler et al. 1990; Skarnes 1990; Friedrich and Soriano 1991). In *Arabidopsis*, similar systems based on T-DNA insertional mutagenesis (Topping et al. 1991; Fobert et al. 1991; Kertbundit et al. 1991) or the *Ac/Ds* transposable element system from maize (Sundaresan et al. 1995; Springer et al. 1995; Smith and Fedoroff 1995) have been developed.

Enhancer detection and gene trap systems offer an added benefit: they allow the identification of genes that are not readily amenable to classical genetic analyses (Bellen et al. 1989; Bier et al. 1989; Grossniklaus et al. 1989; Wilson et al. 1989). They have been especially useful in studying developmental processes occurring late in development, i.e., after the effective lethal phase of a corresponding mutation. For example, a gene that is required for essential steps during both embryo and ovule development would be identified as an embryo lethal mutation and its function during ovule development would be masked. Enhancer detection allowed identification of the first embryo lethal genes in *Drosophila* that are also required for oogenesis or eye development (Grossniklaus et al. 1989; Mlodzik et al. 1990). The dissection of processes characterized by functional redundancy and high complexity is also greatly facilitated by enhancer detection (Wilson et al. 1990; Bellen et al. 1990).

Enhancer detection has some important advantages over classical genetic screens for the identification of genes required in the gametophytic phase of the life cycle: (*i*) many genes that encode components of the basic cellular machinery display a gametophyte lethal phenotype if disrupted. Essential genes are expected to show widespread although not necessarily ubiquitous expression, whereas expression in particular cell types of the megagametophyte suggests a function in cell specification and differentiation; (*ii*) genes required for both micro- and megagametogenesis can be isolated, because a large percentage of enhancer detector insertions do not disrupt gene function. Mutants affecting both male and female gametophytes can usually only be recovered as rare partially penetrant mutations or in genomic regions that can be covered by a duplication (Vollbrecht 1997); (*iii*) enhancer detection is the only efficient technique that allows the identification of genes expressed in very few or even single cells. By focusing on the cells and tissues where a gene is expressed, subtle phenotypes can be identified that may not easily be recognized in phenotypic screens; and (*iv*) most importantly, enhancer detector and gene trap transposons greatly facilitate the molecular cloning of genomic sequences flanking the insertion site. In addition, they allow a detailed genetic analysis of the detected gene through remobilization and the recovery of additional alleles, regional chromosomal rearrangements, and revertant sectors (e.g., Grossniklaus et al. 1992; Springer et al. 1995; Tsugeki et al. 1996; Grossniklaus et al. 1998b).

Generation of Transposants and Ongoing Screens

To identify genes involved in female gametogenesis, we have generated nearly 4,300 lines carrying randomly inserted enhancer detector or gene trap transposons (U. Grossniklaus, J. Moore, W. Gagliano, J-P. Vielle Calzada, unpublished data). We are using the

system developed by Sundaresan et al. (1995), which is based on the *Ac/Ds* transposon of maize and allows the recovery of unlinked transposition events throughout the *Arabidopsis* genome. In brief, an enhancer detector or gene trap transposon is mobilized by crossing a starter line homozygous for *Ds* to a line carrying a stable *Ac* transposase source. Self-pollination of the F_1 plants, which contain both the *Ds* starter locus and the *Ac* transposase construct, results in some F_2 progeny carrying a transposed *Ds* element (transposants). By positively selecting for the presence of *Ds* but negatively against the donor *Ds* locus and *Ac*, unlinked stable transposition events can be recovered (Sundaresan et al. 1995). Negative selection against the donor locus ensures the recovery of unlinked or loosely linked transposition events, a prerequisite for genome-wide random insertional mutagenesis. Similar strategies are currently being developed for rice (Chin et al. 1999; R. Jefferson, personal comm.).

Using six independent starter lines, we generated approximately 45,000 F_1s, of which more than 35,000 were grown to maturity to harvest their F_2 seeds. About 23,000 of the F_2 families have been put through the positive/negative selection process to recover transposants. Between 20% and 25% of the F_2 families yielded an unlinked transposition event. The transposant library of about 4,300 lines that we generated serves as the basis for four large-scale screens aimed at identifying genes involved in female reproduction.

Two of the screens we are performing are designed to identify genes expressed during ovule and megagametophyte development. The first one targets early ovule development, which encompasses the key events of megasporogenesis (Vielle-Calzada et al. 1998). More than 1,000 transposants have been screened and about 30 lines have been identified with a restricted expression pattern in young ovule primordia (J-P. Vielle Calzada and U. Grossniklaus, unpublished data). Many of the expression patterns reflect the highly polar organization of the ovule and may be involved in establishing or interpreting positional information. Other patterns are associated with the meiotic cell lineage and are of particular interest to the engineering of apomixis. The second screen is aimed at the identification of genes expressed in individual cell types of the embryo sac and concentrates on late stages of ovule ontogeny, including post-fertilization stages (R. Baskar, J. Moore, W. Gagliano, U. Grossniklaus, unpublished data). Some of these patterns are specific to the individual cell types of the embryo sac including the egg cell and will serve as important tools to direct expression of candidate genes to the megagametophyte. The results of this screen are discussed in more detail in the next section.

Independently, our transposant library is being used for classical genetic screens to isolate insertional mutants that affect fertility. The first phenotypic screen identifies mutants that disrupt the development or function of the female gametophyte. These mutants are identified in a two-step screen for reduced fertility and a non-Mendelian segregation ratio, both indicating a gametophytic defect (Moore et al. 1997; Feldmann et al. 1997; Howden et al. 1998; Christensen et al. 1998; Bonhomme et al. 1998; Grini et al. 1999). In a second screen we identify families segregating sterile plants that show a sporophytic requirement. Of 3,200 families that were screened, nearly 40 sterile mutants were identified (Vielle-Calzada et al. 1998). Reciprocal outcrosses to wild type showed that most of these mutants are male sterile or affect both sexes, but six were found to be female-specific. These recessive female sterile mutants affect ovule morphogenesis or megasporo-

genesis and are currently being analyzed in more detail at the molecular and genetic level (J-P. Vielle-Calzada and U. Grossniklaus, unpublished data).

For the rapid isolation and sequencing of genomic regions flanking the *Ds* insertion we adapted a PCR-based procedure, TAIL-PCR (Liu et al. 1995), to be used in conjunction with *Ds* elements (see also Tsugeki et al. 1996). Using a set of *Ds*-specific primers (Grossniklaus et al. 1998a) in combination with arbitrary primers, we isolated at least one flanking fragment for more than 150 lines that we identified in various screens. Using two sets of arbitrary primers, the success rate was greater than 95% (Grossniklaus et al. 1998a). We have identified insertions into a multitude of genes that encode essential proteins involved in basic metabolic and cellular processes, putative regulatory proteins, and several novel sequences of unknown function. Based on sequence information, the majority of the detected genes appear to be involved in gene regulation and signaling processes.

Identification of Developmentally Regulated Genes and Their Promoters

Very few genes expressed in the megagametophyte have been described (Nadeau et al.1996; Belostotsky and Meagher 1996), and genes expressed in individual cells of the embryo sac have not previously been identified and characterized. Recently, cDNA libraries obtained from isolated egg cell and in vitro fertilized zygotes of maize have been generated, leading to the identification of genes expressed in the embryo sac and embryo (Dresselhaus et al. 1994, 1996, 1999a,b). Although no cell type-specific genes have been isolated yet, this approach holds great promise for the identification of embryo sac-specific genes. We use enhancer detector and gene trap transposons carrying the *uidA* reporter gene encoding b-glucuronidase (GUS) (Jefferson et al. 1986; Jefferson 1987). The expression of GUS can be visualized by histochemical staining (Jefferson et al. 1987; Kavanagh et al. 1988). To identify genes expressed during ovule development and female gametogenesis, we analyzed GUS expression in maturing ovules of about 2,300 transposants (R. Baskar, J. Moore, W. Gagliano, U. Grossniklaus, unpublished data). Between 9% and 10% of the enhancer trap lines and 2% to 3% of the gene trap lines show spatially restricted GUS expression in mature ovules. Approximately half of these enhancer detector lines show expression restricted to sporophyte and gametophyte, respectively, whereas very few show regional expression in both gametophytic and sporophytic tissues.

Although many *Arabidopsis* promoters have been found to be highly compact (e.g., Dwyer et al. 1994; Thoma et al. 1994; Xia et al. 1996), enhancers that drive reporter gene expression could be at a considerable distance from the site of insertion. This makes the isolation of the detected gene and its promoter more laborious, requiring that the expression pattern of an isolated gene be confirmed. To date, we have analyzed the expression of three genes expressed in the megagametophyte; in situ hybridization indicates that they are indeed expressed as expected (Vielle-Calzada et al. 2000; R. Baskar, J-P. Vielle-Calzada and U. Grossniklaus, unpublished data). It would be preferable to identify gene trap insertions with cell type-specific expression because they have to be inserted within the transcription unit in order to function. However, because gene traps must integrate within the gene in the correct orientation (Sundaresan et al. 1995) and GUS activity is often weak, the frequency at which highly specific expression patterns are recovered is very low. The screening process for cell-type specific expression in the ovule and megagametophyte is extremely laborious, requiring preparations for high-resolution light microscopy. Therefore, we

concentrate our current screens on enhancer traps because the recovery of highly specific patterns using gene traps requires screening a many more transposants.

About half of the enhancer transposants with GUS activity in the ovule are expressed in the megagametophyte. Some are expressed in all cells of the embryo sac (Figure 12.5, p. 197), whereas in others, GUS expression is shared by only a subset of cells, for example, the three cells of the egg apparatus. Most importantly, we also identified transposants with expression in individual cells of the megagametophyte, such as the synergids, the egg cell, and the antipodals. More lines were expressed in the synergids than in any other cell type of the embryo sac. This finding is consistent with earlier reports suggesting that the synergid is, metabolically, the most active cell of the megagametophyte (Jensen 1974; Russell 1993). The synergid serves important functions in pollen tube guidance, sperm discharge and transport, and fertilization (Jensen et al. 1985; Dumas and Mogensen 1993; Russell 1993). Egg cell-specific expression and expression in the central cell are very rare.

Some of the genes expressed in individual cell types of the female gametophyte may serve important regulatory functions during sexual reproduction and could be involved in cell specification and differentiation processes. If the corresponding genes control important developmental decisions during megagametogenesis, as suggested by their expression pattern, mutant phenotype and/or sequence, they may be useful for the engineering of certain aspects of apomictic reproduction. Most importantly, the regulatory regions of these genes will prove to be invaluable tools for the misexpression of candidate genes in particular cell types. Regulatory regions that direct embryo sac-, egg apparatus-, and egg cell-specific expression have been identified (R. Baskar and U. Grossniklaus, unpublished data; W. Yang, R. Jefferson, and U. Grossniklaus, unpublished data). We intend to use these to probe the potential of the egg cell for autonomous activation through misexpression of candidate genes such as cell cycle control genes and growth regulators. Enhancer detection is the most promising technique for providing numerous temporally and spatially regulated promoters for use in modifying the reproductive system.

Introduction of Apomixis into Sexual Species

The introduction of apomixis into sexual crop plants can be achieved through two distinct routes: (*i*) the study of the genetic control of naturally occurring apomicts could provide us with the molecular tools necessary to introduce apomixis into sexual species, or (*ii*) apomixis could be engineered by synthesizing individual traits such as nonreduction, parthenogenesis, and normal endosperm development through the introduction of mutations and/or transgenes controlling these processes. The versatility of the reproductive system and the interrelationship between sexuality and apomixis suggest that this can be achieved even if naturally occurring apomixis is controlled by an entirely different and possibly complex mechanism(s). However, knowledge of the genetic and molecular basis controlling apomixis and sexuality does not directly lead to the ability to manipulate or reconstruct the trait in a sexually reproducing plant. The technological constraints to implementation are substantial and must not be underestimated in development of a suitable research strategy.

Introgression and Genetic Synthesis

The introduction of apomixis to sexual species through classical genetic means is particularly attractive because it does not require prior knowledge of the molecular nature of the gene and can be achieved even if the genomic

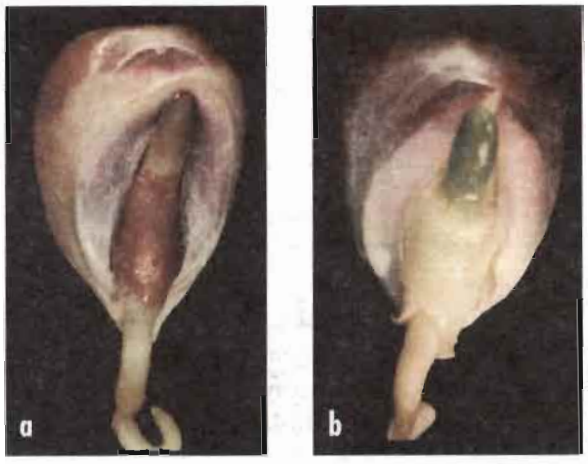

Figure 12.3 The *R-Navajo* (*R-nj*) dominant maker system for embryo and endosperm. (a) Both embryo and endosperm carry the *R-nj* marker and show anthocyanin pigmentation. (b) Only the endosperm carries the *R-nj* marker while the embryo does not, i.e., is not pigmented. This kernel was obtained from a mixed pollination through heterofertilization, but a parthenogenetically formed embryo will also lack the paternal *R-nj* marker.

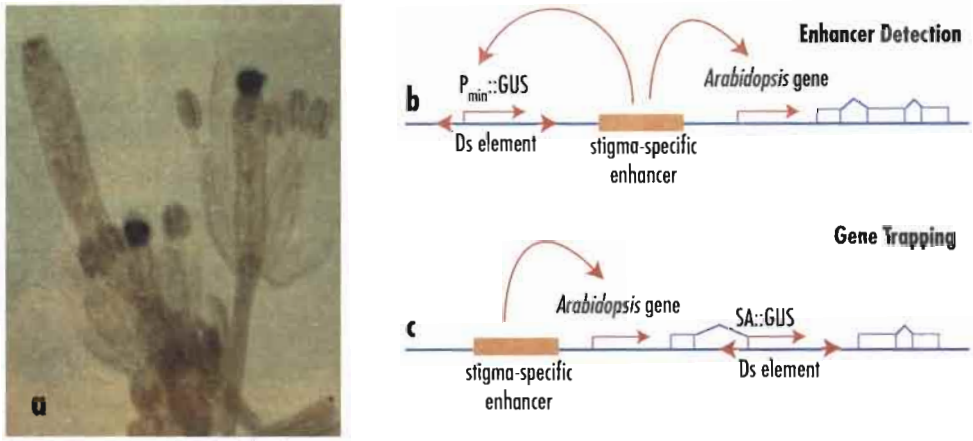

Figure 12.4 The principle of enhancer detection and gene trapping. (a) an inflorescence of an enhancer detector transposant with reporter gene activity specifically in stigmatic papillar cells of mature flowers. (b) enhancer detection relies on a *Ds* element carrying a reporter gene (*GUS*) under the control of a minimal promoter (P_{min}). If the P_{min} is influenced by genomic *cis*-regulatory sequences, e.g., a stigma-specific enhancer, the *GUS* gene is expressed in a tissue- and time-specific manner in the same way as the gene that is usually controlled by the detected enhancer. (c) gene traps are a modification of enhancer detectors relying on splice acceptor (SA) sites in all three reading frames. If the gene trap inserts into a transcriptions unit *trans*-splicing can produce a GUS fusion protein that reflects the expression of the detected gene.

Figure 12.5 Enhancer detector transposant with *GUS* expression restricted to the megagametophyte. Abbreviations: Ch, chalaza; Mi, micropyle; ES, embryo sac; OI, outer integument; II, inner integument.

regions controlling these traits are physically very large. Three different approaches that rely on classical breeding are being pursued: (*i*) introgression of apomixis from wild apomictic relatives through wide crosses (Savidan, Chap. 11); (*ii*) generation of apomicts through hybridization of sexual progenitors (Carman, Chap. 7); and (*iii*) construction of apomixis by combining reproductive mutants that display certain aspects of apomixis (Asker et al. 1982). Except for the second strategy, these approaches are only applicable if wild apomictic relatives or mutants displaying apomictic characters are available for a given crop species. Thus, a broad introduction of apomixis into sexual crops will depend on biotechnology and isolation of the genes that manipulate the reproductive system.

Although apomixis is found in many plant families, it has been described in only a small number of agriculturally important species. These include several forage grass crops (Bashaw and Hanna 1990; Savidan 2000; do Valle and Miles, Chap. 10), horticultural crops such as *Citrus*, apple, mango, and mangosteen, as well as orchids (Wakana and Uemoto 1987; Naumova 1993; Koltunow et al. 1996). Importantly, apomixis has also been described in relatives of a few important grain crops, notably maize, pearl millet, and wheat (Bashaw and Hanna 1990). Several breeding programs have focused on the introgression of apomixis from wild relatives through wide crosses (Dujardin and Hanna 1989; Petrov et al. 1994; Savidan and Berthaud 1994; Savidan 2000; Savidan, Chap 11). Although apomictic maize-*Tripsacum* and pearl millet-*Pennisetum* hybrids have been generated they are characterized by a high degree of seed abortion, which is likely caused by an endosperm defect stemming from an endosperm imbalance (GrossniKlaus et al. 1998; Morgan et al. 1998; Grimanelli et al., Chap. 6). Imprinting-related phenomena in sexual crops will pose a serious barrier to the introgression of apomixis and must be addressed at the genetic and molecular levels to make both introgression and biotechnological approaches possible.

An attractive alternative to introgression approaches is suggested by Carman, based on the genome collision theory for the evolution of apomixis. He proposes that the presence of duplicate genomes controlling the reproductive pathway in hybrids cause reproductive anomalies such as apomixis. The theory predicts that hybridization of two reproductively divergent ecotypes could be used to generate apomictic hybrids. Hybridization as the cause for apomixis was first proposed by Ernst (1918), but the mutational hypotheses for apomixis soon became more popular. The occurrence of apomictic hybrids in the offspring of certain interspecific crosses that involve only sexual parents has indeed been reported and is reviewed by Carman (Chap. 7).

The genetic synthesis of apomixis by combining reproductive mutants that display nonrecurrent forms of apomixis has been attempted in barley by Asker et al. (1982). A mutant that produces a large number of unreduced gametes, *tri*, was combined with *hap*, a mutant that gives rise to parthenogenetic haploids at a high frequency. Although the *tri* mutation is not suitable for the fixation of heterosis (because of restitution at the second meiotic division) and no apomictic double mutants have been generated, plants showing aspects of both phenotypes have been recovered. It should be noted, however, that very few reproductive mutants have been described to date and that it is essential to systematically search for additional mutants that affect megasporogenesis, parthenogenesis, and endosperm development. To my knowledge, no systematic screens directly targeted at nonreduction or parthenogenesis

have been conducted in angiosperms and the mutants described so far have been identified fortuitously. Well-defined sexual model systems are best suited for such screens. Rather than performing large-scale genetic screens for reproductive mutants in many different crop plants, it may be easier to isolate the relevant genes from *Arabidopsis* (or maize) and mimic the mutant phenotype in crops through genetic engineering.

De Novo Engineering of Apomixis through Biotechnology

As outlined above, the introduction of apomixis into a wide variety of sexual crops will be most efficiently achieved through genetic engineering. To maximize its usefulness and versatility in a bioengineered form apomixis will have to be dominant; otherwise the fixation of hybrid genotypes will be very slow. The engineering of apomixis through biotechnology will require a concerted effort in three main areas: (*i*) the identification and characterization of candidate genes that can be used to manipulate the reproductive system; (*ii*) the isolation of promoters that allow a precise control of gene expression at the spatial and temporal levels; and (*iii*) the development of efficient technologies to introduce and control transgenes and/or to perform targeted mutagenesis of endogenous genes.

The identification of regulatory genes that can be used to control apomixis is being pursued by a variety of approaches using both apomictic and sexual systems. Insertional mutagenesis in *Hieracium* and *Tripsacum* (Bicknell, Chap. 8; Grimanelli et al., Chap. 6) or positional cloning based on mapping approaches and comparative genomics will lead to the identification of the components controlling apomixis in natural apomicts. In sexual model systems, the characterization and molecular isolation of existing reproductive mutants that show individual components of apomixis is underway and will provide us with novel tools to manipulate sexuality towards apomixis. These efforts are being complemented by new screens that specifically target relevant aspects of reproduction in sexual species. Several laboratories are also trying to isolate plant homologs of yeast mutants that could play crucial roles in determining the meiotic lineage and nonreduction. It must be emphasized that our understanding of the molecular mechanisms that control plant reproduction are still extremely limited.

In addition to regulators of the sexual pathway, many other genes may be useful for the engineering of apomixis. Such genes include glucanases that degrade callose, the absence of which serves as a consistent indicator of cells initiating megagametogenesis. Genes promoting cell wall formation could also be useful to prevent fertilization of the egg cell and promote parthenogenesis. Another important class is made up of genes that control the cell cycle. Heterochronic or heterotopic expression of such regulators could potentially be used to trigger cell division and initiate developmental events such as megagametogenesis and embryogenesis. Recent studies in *Arabidopsis* have shown that misexpression of *cyclin1At* in root cells can trigger extra rounds of cell division (Doerner et al. 1996) and that *cyclinD* controls the growth rate in tobacco (Cockcroft et al. 2000). It will be interesting to see whether similar experiments can induce cell proliferation in the egg cell. Other growth regulators, such as plant hormones, have not been studied in detail during sexual reproduction, but may play crucial roles in initiating developmental programs relevant to apomixis.

Targeted misexpression of candidate genes will require well-defined regulatory sequences that can be used to drive transgene expression. To date, only a few promoters have been

described that are active in the ovule, and promoters specific to the gametophyte and its constituent cells have just been isolated (R. Baskar and U. Grossniklaus, unpublished data). Enhancer detection bears great potential for the identification of genes that play crucial roles during sexual reproduction, and also because it serves as an entry point to isolate a multitude of highly specific promoters that are restricted to specific cell types and regions of the ovule and female gametophyte.

Although the introduction of transgenes is now readily achieved in many crop species, present technology only allows for the introduction of dominant traits. Transgene activity is based either on overexpression or on homology dependent gene silencing phenomena, such as antisense suppression or sense cosuppression (e.g., Matzke and Matzke 1995; Jorgensen 1995; Jorgensen et al. 1996). By virtue of the epigenetic nature of these phenomena, it may prove difficult to efficiently and stably introduce the traits that control apomixis. Currently, it is not routinely possible to disrupt endogenous genes, and the lack of efficient homologous recombination techniques, which would allow us to mutate or otherwise modify endogenous genes, remains a serious obstacle to genetic engineering in plants. However, recent reports on successful homologous recombination in *Arabidopsis* (Miao and Lam 1995; Kempin et al. 1997) may soon lead to the development of more efficient protocols for targeted gene disruption. In *Arabidopsis* and maize, gene disruption by site-specific transposon mutagenesis has become an efficient way to inactivate specific genes. Although this approach has been adapted to rice (Izawa et al. 1997), it is unlikely that it will be implemented in a wide variety of other crop species.

Field-level Regulation of Apomictic Traits

To maximize benefits, it will be necessary to control the expression of apomixis such that a choice can be made between the sexual and apomictic modes of reproduction at any stage of a breeding program. Apomixis as a constitutive trait could potentially pose a threat to genetic diversity, which would preclude the use of apomixis for crop improvement in a versatile and creative way. For instance, apomixis would constitute the default condition, wherein application of an exogenous condition or compound would suppress apomixis to allow for sexual breeding to introgress new germplasm and create segregating populations. Alternatively, sexuality could be the default condition, addressing the concern that apomixis could pose a threat to biodiversity (van Dijk and van Damme 2000), and apomixis would only be induced at specific steps of the breeding program and for seed production.

Although several inducible systems have been described and shown to work efficiently under laboratory conditions, none of these systems would allow the induction or suppression of a trait under field conditions. These systems allow either repression or induction of a gene upon addition of a compound. All of the existing inducible (suppressible) systems, including the tetracycline repressor (Gatz et al. 1992; Weimann et al. 1994), the copper inducible system of the yeast metallothionin gene (Mett et al. 1993; Mett et al. 1996), and the glucocorticoid receptor (Schena et al. 1991; Aoyama and Chua 1997) have serious drawbacks for field use, although they are potent systems for use in the laboratory and greenhouse. These systems often have a high background of noninduced expression and use expensive and/or environmentally unacceptable inducers that often have poor mobility in the plant.

As a prerequisite for the use of apomixis in the field, optimal control systems will have to be developed that have low background activity but that can get potently induced. As outlined by Jefferson and Nugroho (1998), the inducer should be water soluble, stable, readily translocatable, and should not impair the fitness of the plant. The inducer should also be safe to use, biodegradable, and have no adverse affects on the ecosystem. Recently, two inducible systems that may be developed into agriculturally applicable systems have been described. Research at CAMBIA focuses on the development of a system that is based on the glucuronide repressor, *gusR*, which is a potent repressor in the absence of glucuronides but disengages from the DNA when a wide variety of glucuronides are added (Jefferson and Nugroho 1998). Glucuronides meet many of the criteria for ideal inducers being inexpensive, benign, stable, phloem-mobile, soluble, and not endogenous to plant cells. Another recently described system that has potential for field use is the ethanol inducible system derived from the ethanol regulon on *Aspergillus nidulans* (Caddick et al. 1998). The system is based on the *alcA* promoter and its transcriptional activator *AlcR*, which was shown to lead to about a hundredfold induction upon the addition of ethanol in transgenic plants. Whether it will be practicable to spray or water plants with ethanol in the field is not known, but the fact that *AlcR* responds to a variety of other alcohols and ketones opens many possibilities for adapting this system for field use (Gatz 1998).

Conclusions and Prospects

The introduction of apomixis into sexual crops will require a multifaceted approach that builds on the use of both sexual and apomictic systems. It is very likely that genes involved in the sexual pathway are also crucial for apomictic development. Therefore, genetic and molecular studies that concentrate on the dissection of the sexual pathway will provide invaluable tools for the engineering of apomixis through biotechnology. Several long-neglected aspects of sexual reproductive development will have to be addressed, such as the control of megasporogenesis, egg activation, and imprinting requirements for endosperm development. Studies on the control of reproductive development in apomictic species such as *Hieracium* and *Tripsacum* will have to be complemented by a detailed characterization of the developmental events during sexual reproduction and the molecular mechanisms controlling them.

Even if apomictic species have evolved complex and elusive mechanisms that control apomixis, which have no immediate molecular counterpart in sexual relatives, as suggested by recent findings in *Pennisetum* and *Cenchrus* (Ozias-Akins et al. 1998; Roche et al. 1999), apomixis could be engineered through a synthetic approach by targeting the key regulatory steps. This view is supported by the recent findings that the genetic control of apomeiosis and parthenogenesis can be separated in *Taraxacum* and *Erigeron* (van Dijk et al. 1999; Noyes and Rieseberg 2000). The engineering of individual elements of apomixis will require a sustained effort to identify more reproductive mutants that affect the relevant developmental processes in those sexual model systems that are easily amenable to molecular techniques. Classical genetic screens targeted at nonreduction, parthenogenesis, seed development (*mea*, *fie*, *fis2* mutants), and regulators of imprinting hold great promise for identifying the candidate genes required for the introduction of apomixis into a wide variety of sexual crops. Enhancer detection provides a powerful alternative to classical genetic screens and allows rapid cloning of genes involved in sexual reproduction and their promoters.

Two additional points should be stressed. First, the technologies needed to engineer and control apomixis under field conditions are not yet available and must first be developed, a point that cannot be overstated as the identification of potential regulatory genes progresses rapidly. Second, this review of plant reproduction has focused on a genetic perspective with Mendelian traits controlling sexual and apomictic development. However, gene regulation by genomic imprinting may not be the only epigenetic mechanism that should be considered. Current knowledge of the regulation of apomixis is fully compatible with an epigenetic view of this trait and we should keep our minds open to alternative explanations that are epigenetic in nature. If apomixis is, for instance, due to epigenetic interactions between genomes, the engineering of apomixis in sexual species will be much more complex.

Finally, the accessibility of apomixis technology to a broad community of plant breeders in the public and private sector worldwide must be ensured. If universal and equitable access to apomixis technology cannot be achieved through innovative patenting and licensing, then the exciting science discussed in this volume will have little positive impact (http://billie.btny.purdue.edu/apomixis/).

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Chapter 13

Induction of Apomixis in Sexual Plants by Mutagenesis

UTA PRAEKELT AND ROD SCOTT

Introduction

The ability to manipulate reproduction in crop plants from the sexual to the apomictic mode, and vice versa, is highly desirable (Hanna 1995; Jefferson and Bicknell 1996). Several chapters in this volume deal with the various approaches that have been taken toward this goal. The most promising strategy so far has been the transfer of natural apomixis genes from wild species into related sexual crop plants by introgression (Hanna et al. 1992; Leblanc et al. 1995; Savidan, Chap. 11). Unfortunately, this is likely to remain limited to those crops that have apomictic relatives, and therefore will not be widely applicable. In light of this situation, efforts are being made to identify the gene(s) that confer apomixis, both to gain a better understanding of the genetic regulation of the trait and to eventually facilitate transfer to a wider range of species by genetic engineering methods. As described by Grimanelli et al. (Chap. 6), one key step toward gene isolation is the genetic mapping of apomixis genes, and considerable progress has been made with some species (Kindiger et al. 1996). However, because of the intrinsic difficulties in mapping apomicts, and because recombination around the apomixis locus appears reduced (Grimanelli et al., Chap. 6), map-based cloning of the apomixis gene(s) is likely to proceed slowly.

Recently, an alternative approach, using mutagenesis, has been considered by several groups both for the identification of natural apomixis genes and for the *de novo* induction of apomixis in sexual plants (Koltunow et al. 1995). Mutagenesis has been widely and successfully applied to the study of many aspects of plant growth and development. Thanks to rapidly advancing methods in all areas of DNA technology, improvements in plant transformation methods, and the accumulation of mapping and sequencing data, the isolation of genes via their mutant alleles has become a feasible approach in many areas of plant research. Bicknell (Chap. 8) describes the development of *Hieracium* as a model apomict (see also Bicknell 1994a,b,c; Bicknell and Borst 1994). The aim is to induce reversion to sexual reproduction by insertional mutation of the responsible gene(s) and to isolate them via the inserted sequence.

In this chapter we describe the reciprocal approach, the mutagenesis of a model sexual plant in an attempt to induce apomixis *de novo*. Mutated alleles conferring apomixis or individual components of apomictic development can be identified with relative ease in a model plant, and the cloned genes would then be available for transfer to other species. The possibility of a mutagenic approach resulting in the isolation of the desired mutants greatly depends on the methodology employed. Therefore, in order to maximize the opportunities for the induction and detection of these mutants, several considerations must be taken into account. Details of the mechanisms of apomixis have

been described elsewhere in this volume, therefore only a brief summary is given here of the individual components of apomictic versus sexual development that could be separately affected by mutations. We summarize some of the earlier work with mutagenesis and describe some of the most interesting mutants with elements of apomixis that have been isolated in various plants. The fact that none of these potentially useful mutations has been characterized at the DNA level underlines the importance of using well-characterized model plants for this work. The feasibility of obtaining mutants of *Arabidopsis* with apomictic characteristics has been confirmed recently by the identification of several mutants with partial seed development. We describe the various approaches currently underway in several laboratories, which are aimed at the induction of apomictic characteristics in model sexual plants.

Considerations

Components of Apomixis

The two major types of gametophytic apomixis that can be distinguished, diplospory and apospory, differ from sexual development in more than one aspect (for reviews see Asker 1980; Asker and Jerling 1992; Nogler 1984; and Crane, Chap. 3). Since mutants with individual components of apomixis have been identified previously, and can be expected in future mutagenesis experiments, the main differences are summarized briefly. Also, as pointed out below, apomictic tendencies are occasionally observed in sexual plants and can be influenced by environmental stimuli.

1. Avoidance of meiosis. In diplosporous apomicts, meiosis is avoided by two principally different mechanisms: in the first, meiosis of the archesporial cell is directly replaced by a mitotic division; in the second, failure of chromosome association or synapsis is associated with nuclear restitution, and followed by equational division of the entire chromosome complement in the second meiotic division. Subsequent development leads to the formation of a functionally normal embryo sac with an unreduced egg and central cell. Sexual plants occasionally produce unreduced gametes, and through fertilization these give rise to the polyploid series that can be observed in many plant species (Asker 1980). The production of unreduced embryo sacs in some plants, including *Brassica* species, is revealed after distant or "prickle" pollination (which stimulates parthenogenesis; see below) by the appearance of matromorphous progeny at considerable frequencies (Eenink 1974a).

2. Formation of aposporous embryo sacs. In aposporous apomicts, unreduced embryo sacs arise directly by mitosis from nucellar cells, usually in addition to a sexual, reduced embryo sac with normal meiosis. In facultative apomicts, sexual and apomictic embryo sacs develop side-by-side or in separate ovules and give rise to a mixture of sexual and maternal progeny. Although female meiosis is mostly normal, in many cases the aposporous embryo sacs, which are not delayed by meiosis, develop at a faster rate than the products of meiosis, which frequently degenerate at the megaspore stage.

3. Parthenogenesis. The unreduced egg cell in both diplosporous and aposporous embryo sacs develops directly into an embryo, without fertilization by a sperm nucleus. It is not clear whether fertilization is prevented actively or as a consequence of precocious parthenogenesis. Again, parthenogenesis occasionally occurs in sexual plants, and can be induced in many plants by certain stimuli such as prickle pollination. Of particular interest to the present investigation is the frequent occurrence of matromorphy, or diploid parthenogenesis, in *Brassica oleracea*, a species that belongs to the same family as *Arabidopsis*, the Brassicaceae.

Crosses with pollen from different species result in various proportions of matromorphs arising from the parthenogenetic development of unreduced egg cells (Eenink 1974b). Thus in *Brassica*, two of the most important ingredients of apomixis are revealed by distant pollination: (*i*) the presence of unreduced embryo sacs, and (*ii*) the inherent capacity for parthenogenesis. Haploid parthenogenesis has recently been induced both in *Arabidopsis* and in *Brassica juncea* by the application of brassinolide, a steroid hormone first isolated from *Brassica* pollen (Kitani 1994).

4. Endosperm development. In autonomous apomicts, endosperm development occurs spontaneously, but in the case of pseudogamous apomicts, it depends on fertilization of the central cell nucleus by a sperm nucleus. In some cases, pollination without fertilization has been suspected of triggering endosperm development. Endosperm plays a crucial role in the formation of viable seed, and requires special consideration in the design of a mutagenesis screen. The problem of the endosperm is discussed later in more detail.

Genetic Control of Apomixis

With perhaps one exception (Carman 1997; Carman, Chap. 7), it is now generally accepted that apomixis has evolved from sexual ancestors by mutation rather than being a consequence of polyploidization and heterozygocity. This is an important assumption for mutagenic approaches to the study of apomixis. Much discussion focuses on whether apomixis is regulated by a single gene and could therefore be induced by a single mutation in a sexual plant. Given the different components of apomixis, it seems more likely that a number of mutations were needed in the evolution of a viable apomict from a sexual ancestor. The apparent single locus inheritance that has been reported in several cases could be explained by the tight linkage of several genes, a possibility that is consistent with the lack of recombination observed between molecular markers associated with apomixis (Grimanelli et al., Chap. 6). Even if a single mutation, perhaps resulting in the inhibition of meiosis, was responsible for the evolution of apomixis, it is likely to have occurred in a background that permitted its expression (Mogie 1988) and has therefore evolved only in a subsection of genera and families. For these reasons, we do not expect that a single mutation in a sexual plant could result in the production of viable seed in the absence of fertilization. However, given the variety of apomictic forms that can be distinguished, such as in diplospory and apospory, apomixis has probably arisen independently in different species and perhaps involves different genes in each case. Consequently, there may be ample opportunities for the induction of some element of apomixis in a sexual plant by mutations in a number of different genes.

An important aspect to consider is the apparent dominance, in many cases, of apomixis over sexuality (Nogler 1984; Mogie 1988; Leblanc et al. 1995). Mutations that completely abolish gene function, such as deletions, can be dominant only if expression of that gene is subject to gene dosage. One of the hypotheses that have been put forward on the control of apomixis is that the responsible gene(s) encode regulatory functions that initiate or repress certain developmental programs (Koltunow et al. 1995). If apomictic development is repressed in sexual plants by a negative regulatory protein whose concentration is crucial, then a reduction in the level of this protein could be sufficient to induce a developmental pathway that is suppressed only when two copies of the gene are present. The fact that many apomicts are facultative, and that the proportion of apomictic progeny can be influenced by environmental factors, supports the hypothesis

of a finely tuned regulatory function for apomixis genes.

An alternative route to a dominant allele is a mutation leading to a change in function of the protein, e.g., an altered specificity. Mutations of this type could be very rare indeed. However, many regulatory proteins are active only after forming a complex with other proteins, and changes in many amino acid residues, particularly those exposed on the surface, could alter their binding affinity, resulting in reduced activity of the entire complex. These possibilities have to be taken into account in the design of any mutagenesis experiments, as they may influence the choice of mutagen (see below).

How Important is Polyploidy?

The majority of apomicts are polyploid. If polyploidy is a prerequisite for apomixis, as has been suggested by some researchers, screening for apomictic mutants in a diploid species is not a promising proposition. Fortunately, there are a few examples of diploid apomicts, the most relevant to this work being *Arabis*, a genus comprising species with a range of base numbers and ploidy levels (Böcher 1951; Carman 1997). Some of the diploids are sexual, while others are pseudogamous diplosporous apomicts. The observed variation in ploidy of pollen nuclei of sexual and apomictic species, and the occasional occurrence of unreduced embryo sacs in the sexual species, form the basis for the evolution of this agamous complex, and support the view that polyploidy is a result, rather than a prerequisite, of apomixis. However, according to Carman (personal comm.), care should be exercised in assigning a particular ploidy to sexual or apomictic plants because an apparently diploid species may represent a diploidized polyploid (paleopolyploid). Notwithstanding this cautionary note, one of the most encouraging examples of apomixis at the diploid or near diploid level is the recent development of a polyhaploid hybrid between diploid (sexual) maize and tetraploid (diplosporous) *Tripsacum*, containing one complete set of chromosomes from each of the parent species. Although male-sterile, this autoallotriploid hybrid (three genomes) reproduces apomictically after pollination with maize, demonstrating that a genetic locus conferring apomixis normally in a tetraploid can also function in a diploid(-like) background (Leblanc et al. 1996).

In addition to being a consequence of apomixis, polyploidy has an important function in apomicts. As discussed by Nogler, the apospory "factor" or allele in *Ranunculus auricomus* can only be transmitted by diploid or polyploid gametes, and acts as a recessive lethal factor in haploid gametes. Apparently, the normal (sexual) allele is required for some aspect of gamete formation or function. This suggests that, while not essential in an individual plant that produces unreduced gametes heterozygous for the apomixis allele, polyploidy may be important for the maintenance and spread of apomictic populations.

The Problem of the Endosperm

There is overwhelming evidence for the importance of a balanced ratio between the maternally and paternally inherited genomes in the endosperm of many species. Endosperm is usually triploid, resulting from the fusion of two maternal polar nuclei with one pollen nucleus, producing a ratio of two maternal to one paternal genome (2m:1p). Deviation from this ratio often leads to embryo abortion or to subfertile, abnormally shaped seed. The most plausible explanation for these observations is the parental imprinting of genes, which, unlike in animals, does not affect the embryo but does affect the endosperm of the developing seed. Various strategies seem to have evolved during the evolution of apomixis to ensure normal endosperm development. In

a minority of apomicts, endosperm production is autonomous, i.e., it does not require fertilization, and here the endosperm is diploid with no paternal contribution. However, the majority of apomicts are pseudogamous and require fertilization of the central cell. Because most apomicts produce normal reduced male gametes, the expected m:p ratio in these endosperms is 4m:1p, given that the polar nuclei are diploid. This ratio, however, has not been observed in many of the examined cases, and it appears that the correct (ancestral) ratio is obtained in a variety of ways. In *Dichanthium annulatum*, the unreduced polar nuclei remain unfused at fertilization and either (*i*) a reduced sperm fuses with only one polar nucleus and the other polar nucleus degenerates, or (*ii*) each polar nucleus is fertilized with a reduced sperm (Reddy and d'Cruz 1969). Similarly, in *Ranunculus auricomus*, two fused unreduced polar nuclei are fertilized by two reduced sperm (Rutishauser 1954). A common strategy among apomictic grasses is to produce unreduced embryo sacs with four rather than eight nuclei; the single polar nucleus is fertilized by a reduced sperm (Warmke 1954).

These observations are probably the most convincing evidence that the evolution of both autonomous and pseudogamous apomicts must have occurred in a genetic environment containing either pre-adaptations or additional mutations. Autonomous apomicts may have evolved in a background where the stringent requirement for a balanced endosperm had been relaxed.

It was recently shown that in *Arabidopsis* the presence in endosperm of maternal or paternal excess, resulting from reciprocal crosses between diploid and tetraploid parents, greatly affects seed size (Scott et al. 1998). Seeds with maternal excess (4m:1p) are smaller, but seeds with paternal excess (2m:2p) are larger than normal. However, these seeds have normal viability. As indicated earlier, haploid seeds can be induced in *Arabidopsis* and *Brassica juncea* by the application of brassinolide to the stigmas of emasculated flowers. Endosperm formation in these seeds occurs in the absence of any paternal contribution, and as expected, the seeds in both cases are smaller than normal diploid seeds; however, they give rise to stable haploid plants. These are important observations with respect to the screens for apomixis in *Arabidopsis*, because they suggest that, should a mutation lead to parthenogenetic embryo development, an imbalance in the m:p ratio of the endosperm should not hinder viable seed production, provided of course that the endosperm proliferates and supports seed production. The effect on seed size by an imbalanced endosperm could, furthermore, be exploited for the screening of mutants. However, a mutation resulting in parthenogenetic embryo development does not necessarily induce endosperm proliferation. This may require an additional stimulus, such as that normally provided by pollination or a second mutation.

The problem of the endosperm is one of the most important aspects to consider in a mutagenesis program, and because of it, we do not expect to be able to induce viable forms of apomixis by a single round of mutagenesis of sexual plants. The aim of several mutagenesis and screening programs currently in progress is to facilitate the identification of mutants that have only some characteristics of apomixis, such as parthenogenesis or autonomous endosperm development, and which do not necessarily produce fertile seed in the absence of pollination.

Which Mutagen?

The choice of mutagen is an important consideration because it determines the types of mutations obtained; here we need only distinguish between the two categories of "change of function" and "loss of function"

mutations. Change of function mutations are the result of amino acid substitutions or of changes in gene expression level. Loss of function mutations abolish the gene product altogether. As discussed earlier, apomixis may be induced by a dominant allele, and this is more likely to be the result of a change of function mutation. There are numerous reports of dominant mutations resulting from single amino acid substitutions that have been obtained by EMS or by in vitro mutagenesis (e.g., Hemerly et al. 1995; Kim et al. 1996; Wilkinson et al. 1997). For this reason, it could be important to use a mutagenic agent that can induce subtle mutations. Ethylmethane sulfonate (EMS) has been shown to be the most versatile mutagen, as it causes point mutations at a high frequency, and whilst most of these should result in amino acid substitutions, they may also create stop codons, and thus lead to loss of gene function (for a summary and references, see Feldmann et al. 1994). However, as already described, some useful mutants have been obtained by radiation treatment, which frequently causes large deletions, and therefore the value of mutagens that can cause gene disruptions or deletions should not be dismissed.

A widely used method of mutagenesis is insertional inactivation, or gene tagging, with T-DNA or transposons (Feldmann 1991; Lindsey et al. 1993; Bouchez et al. 1993; Aarts et al. 1995). As with deletions, this type of mutagenesis most likely will create loss of function mutations due to insertion into the coding region of a gene, although it is conceivable that changes in gene expression levels could result from insertion into a gene promoter. However, a major advantage of gene tagging is the ease with which the mutated gene can subsequently be identified. Its application to the study of apomixis in *Hieracium* is examined by Bicknell (Chap. 8). We briefly summarize below the work by Ramulu (1997) on transposon mutagenesis in *Arabidopsis* and *Petunia*. For the purpose of inducing apomixis in *Arabidopsis*, it may be important to choose a mutagen that is capable of producing change of function mutations. *Arabidopsis* is the best-characterized plant species, and map-based cloning of nontagged mutant alleles is no longer a major obstacle.

Some Early Work with Mutants

Induction of Sexuality in Apomicts

Improving cultivars of apomictic crop species by breeding is difficult, and therefore many attempts have been made to increase the frequency of sexual reproduction in apomicts by mutagenesis (Bashaw and Hoff 1962; Hanson and Juska 1962; Gustafsson and Gadd 1965; Asker 1966). Complete reversion to sexuality was not observed in any of these studies. In the few cases where sexuality was observed, it was transient and plants reverted to apomixis in subsequent generations. A number of factors probably contributed to this lack of success. First, the induction of mutants in polyploid species is inherently difficult because mutations can be masked by additional copies of wild type genes. Second, in many experiments, the number of seeds mutagenized and screened (200 per treatment in one case) was probably inadequate for detecting potentially rare mutations. Whether the choice of mutagens, in most cases irradiation, also contributed to the lack of success depends on whether a change of function rather than a loss of function mutation would be required for a reversal to sexuality. We do not believe that the lack of success to date indicates that efforts to induce sexuality in apomictic species must necessarily fail. Researchers will need to work with a much larger number of mutants, to choose an apomict that has a low ploidy level, and perhaps to use a more subtle mutagenic treatment that induces both change and loss of function mutations.

Mutants of Sexual Plants with Apomictic Characteristics

1. Meiotic mutants. Mutants have been induced in crop plants for many years, and, more by serendipity than design, several interesting mutants with characteristics of apomictic reproduction have been discovered. Because of the numerous genes that control meiosis, mutants with defects in meiosis are frequently obtained in any screening program, where they are easily recognized as segregants in the M_2 generation, with reduced fertility or often complete sterility (Gottschalk and Klein 1976; Curtis and Doyle 1992). The largest proportion of meiotic mutants are defective in the first meiotic division, as a result of either asynapsis (the complete or partial failure of homologous chromosome pairing) or desynapsis (the failure of chiasma formation following normal pairing of chromosomes) (Baker et al. 1976). Both of these defects are normal for those diplosporous apomicts that form first division restitution nuclei, and are therefore constituents of apomixis.

Attempts have been made in several plants to utilize such mutants as the basis for the "synthesis" of apomixis, by combining them with the other required elements. For example, the desynaptic *ds-1* mutant of potato (Jongedijk and Ramanna 1988) has been combined, by conventional crosses, with factors that increase the production of unreduced megaspores (Jongedijk et al. 1991). Although pseudogamous seed development could be induced by "distant" pollination with marked *S.phureja*, the establishment of pseudogamous apomixis would require the addition of genes controlling pseudogamous seed production.

Three asynaptic mutants have been characterized from *Brassica campestris* (rapeseed) (Stringam 1970), in which restitution nuclei are formed following failure of synapsis. Unfortunately, in this case, only microspores were investigated and no information is available on megasporogenesis. If the megaspores are functional and embryo-sac formation is complete, then crossing with a tetraploid plant for the production of tetraploid progeny is a possibility.

In maize, at least 20 loci have been identified that directly affect meiosis, most of which were originally isolated by virtue of their reduced male fertility (Curtis and Doyle 1992). Of particular interest are four mutants, ameiotic-1 (*am1*), ameiotic-2 (*am2*), desynaptic (*dsy*), and plural anomalies of meiosis-1 (*pam1*), which produce unreduced eggs at high frequency. These unreduced eggs were recovered as occasional triploid progeny after crossing with diploids. Significantly, after crossing with tetraploids, their recovery in the form of tetraploid progeny was dramatically increased, presumably due to the formation of genomically balanced hexaploid endosperm.

Another maize mutant, elongate (*el*), produces unreduced eggs in various proportion with haploid eggs (Rhoades and Dempsey 1966; Nel 1974). When fertilized by haploid pollen, the unreduced egg produces a triploid embryo, and the resulting kernel is shriveled and has low viability. When fertilized by diploid pollen from a tetraploid plant, on the other hand, a plump kernel with tetraploid embryos is obtained.

The triploid (*tri*) mutant of barley is very similar to the maize *el* mutant (Ahokas 1977; Finch and Bennett 1979). Homozygous *tri* mutants produce triploid and normal diploid progeny in approximately equal proportion. The triploid seeds are easily recognized by their shrunken endosperm, which was found to be pentaploid, presumably resulting from fusion of the unreduced polar nuclei with a haploid pollen nucleus. This mutation specifically affects female gametogenesis as meiosis on the male side is normal.

The above mutants of maize and barley illustrate that mutations at a number of loci can induce one element of apomixis—the formation of unreduced gametes. Further development is dependent on fertilization, and normal endosperm development depends on a balanced maternal to paternal ratio.

The preferential detection of unreduced eggs by crosses with tetraploids is a useful reminder of the importance of choosing a suitable pollen parent for mutagenesis studies involving pollination. On one hand, mutants with unbalanced endosperm may be difficult to isolate initially because of low viability; on the other hand, the shrunken endosperm phenotype could serve as a useful criterion in the selection for meiotic mutants.

2. Parthenogenetic mutants. The EMS-induced *hap* mutant of barley was initially isolated in the form of a chlorophyll deficient mutant containing at least three linked mutations, *tig*, *let* (pollen lethality), and *hap* (Nielsen 1974). Presence of the *hap* allele results in a low frequency of haploid progeny. After separation of *hap* from the other two mutations, Hagberg and Hagberg (1980) showed that *hap* is incompletely dominant over the wild type allele: heterozygous (*hap* /+) plants produce 3–6% haploid progeny, whereas homozygous (*hap/hap*) plants produce up to 40% haploid progeny. Perhaps not surprisingly, crosses between a homozygous mutant (*hap/hap*)and wild type (+/+) plants produce different results in the F_1, depending on whether the mutant is the male or the female parent: a *hap/hap* female plant pollinated by wild type (+/+) pollen results in a high frequency of haploid F_1 progeny, whereas no haploids are produced when a wild type (+/+) female plant is crossed by a *hap/hap* male. This indicates that the *hap* locus acts only through the maternal tissue, either to prevent fertilization of the egg cell or to stimulate the egg cell nucleus to divide prematurely. In this mutant, parthenogenesis is the only element of apomixis that has been induced. The formation of a perfectly well-developed endosperm, which supports the production of a viable seed, is presumably facilitated by normal fertilization events in the central cell that result in a genomically balanced endosperm.

3. Aposporous mutants. In pearl millet, two mutants that produce aposporous embryo sacs have been reported. The first, *female sterile* (*fs*), is a recessive mutation induced by radiation treatment (Hanna and Powell 1974; Arthur et al. 1993). Homozygous mutants are female-sterile but produce normal viable pollen. Mutant ovules are small and immature compared with the wild type, and only about half of them contain embryo sacs. Of these, the majority are multiple embryo sacs that appear to be aposporous, although sexual embryo sacs are observed in some ovules. Only a very small proportion of ovaries display any endosperm or pro-embryo development, and all ovules degenerate five days after pollination. Pollen tube growth in the mutant is abnormal, and the inhibition of fertilization has been proposed to explain the absence of seed set.

The second, *stubby head*, was discovered in progeny of seed treated with both thermal neutrons and diethyl sulfate (Hanna and Powell 1973). This recessive mutation causes a pleiotropic phenotype, including twin ovules, shortened internodes, flattened stems, and a stubby inflorescence. It produces both normal sexual embryo sacs in some ovules and multiple embryo sacs, which arise from nucellar cells, in others. Test crosses confirmed that stubby head is a facultative apomict, producing maternal progeny at frequencies ranging between 23% and 77%. Mircrosporogenesis in this mutant is normal, however, seed set is low; this has been attributed partly to nonfertilization because of competition between the multiple embryo sacs.

Stubby head could indeed be a very useful mutant, as it produces viable seed by apospory. Although the nature of the mutation is not known, the mutation affects several elements of apomixis in a single step. Whilst this could be due to a single gene mutation, the pleiotropic nature of the mutant phenotype could well indicate that it is caused by a deletion encompassing a number of genes.

4. Conclusions. The above examples of mutants with apomictic characteristics illustrate a number of points relevant to mutagenic approaches in model plants.

The most important conclusion is that all the elements of apomixis can be induced by mutation in sexual plants. In one case, stubby head, several elements were induced simultaneously to produce a viable form of facultative apospory. It would be very interesting to analyze these mutants at the DNA level, but the isolation of genes from these crop species would not be a simple task. Unlike *Arabidopsis*, the cloning of genes via their mutant alleles is not routine.

None of the mutants described herein were originally isolated in screens for apomictic characteristics. The majority were isolated as reduced fertility mutants and some as pleiotropic mutants. Therefore, it seems worth considering which kinds of mutant phenotypes, other than apomixis itself, could be screened for in *Arabidopsis*. First, it might be worthwhile to screen for reduced fertility mutants, a simple screen that would involve testing for seed set in the M_2. A large number of male-sterile mutants have been isolated from *Arabidopsis*. Some are present in the collection of T-DNA tagged lines (available from the Arabidopsis Stock Centers at Nottingham, U.K. and in Ohio, U.S.A.); these could all be screened for maternal progeny after pollination with a dominantly marked paternal line. Second, the *tri* and *el* mutants both give rise to shrunken seeds, and since it has been shown that an unbalanced ratio affects seed size in *Arabidopsis* too, this phenomenon could be used as a screen for the isolation of *Arabidopsis* mutants that produce unreduced egg cells.

Current Approaches to the Isolation of Apomictic Mutants in Model Sexual Plants

The most important precondition for the large-scale screening of apomictic mutants is the availability of male sterile lines that do not set seed in the absence of pollination. *Arabidopsis* is ideally suited for such an undertaking because it offers several ways of establishing male-sterile lines. The most useful of these causes conditional male sterility, which allows the propagation of homozygous seed by selfing under permissive conditions. In addition, *Arabidopsis* provides easily scored dominant and recessive markers for subsequent screening of mutagenized progeny.

The most obvious screen for apomictic mutants of a male-sterile sexual plant is for seed set in the absence of pollination. However, there have been no reports of mutants (in any model plant) that produce viable seed in the absence of pollination. This may well be because the number of progeny screened in this way was not large enough to detect such mutants, as these could indeed be very rare. For the reasons already outlined, however, it also seems likely that more than one gene mutation is required to induce a viable form of apomixis.

Current efforts in several laboratories are directed toward the induction of partial apomictic development. Basically two main types of screens are being conducted. The first identifies mutants that show partial development of fruits in the absence of

pollination. Normally, the pistils do not elongate in unfertilized flowers of *Arabidopsis*, whereas fertilized flowers produce long fruits (siliques). In the expectation that partial seed development would lead to visible silique elongation, the screen is simply for elongated siliques on male-sterile plants under nonpermissive conditions. Whether these are due to autonomous embryo or endosperm development or both must be established by further cytological analysis of the siliques. The second screen is for mutants that produce autonomous embryos but which require fertilization of the polar nuclei for endosperm development, i.e., for pseudogamous apomicts. The screen is simply for maternal progeny among a population of male-sterile mutants that are pollinated by pollen with a dominant marker.

Screening for Elongated Siliques in the Absence of Pollination

Two laboratories have recently reported the isolation of *Arabidopsis* mutants that show partial seed development in the absence of fertilization (Ohad et al. 1996; Chaudhury et al. 1997). In one case, the parental material was a conditional male-sterile *pop1* mutant, which is fertile at high humidity but does not produce functional pollen at low humidity (Ohad et al. 1996). Homozygous *pop1* seeds were mutagenized with EMS, and 50,000 M_1 plants were screened for silique elongation under nonpermissive conditions. A total of 12 lines were isolated, each of which had partially elongated siliques appearing as sectors on the M_1 plants. The characteristics of one of these mutants, *fie* (for fertilization independent endosperm), were described in detail. Because this mutant, now called *fie3*, as well as two more *fie* mutants now appear to be allelic to the mutants subsequently obtained by Chaudhury, they will not be described separately.

Chaudhury used as the parental plant a *pistillata* (*pi*) mutant, which has no petals or stamens. Since this plant is male-sterile in all conditions, it was mutagenized in the heterozygous F_1 population after crossing to a wild type. Again, 50,000 plants, in this case the petalless portion of the M_2 generation, were screened for elongated siliques, and six mutants were obtained. Three of these (*fis1* to *fis3*—fertilization independent seed) were characterized in detail.

All three *fis* mutations are gametophytic, giving rise to seed-like structures from 50% of ovules in heterozygous *fis/FIS* plants. These mutant seeds contain diploid endosperm that develops up to the stage of cellularization and then atrophies. The majority of mutant seeds have no embryos, and only a small proportion of *fis1* and *fis2* seeds contain proembryos arrested at the globular stage. However, after pollination of mutant flowers, each of the mutant seeds contains an embryo at the more advanced torpedo stage, including *fis/fie3*, which shows no embryo development in the absence of fertilization. By testing for a dominant pollen marker (*GUS*), it could be shown that these torpedo-stage embryos consist of a mixture of zygotic and maternal embryos. Even after fertilization by a wild type sperm nucleus, the resulting embryo is aborted, suggesting that the wild type FIS/FIE allele is essential for female gametophyte development. Thus, the *fis/fie* mutations can only be transmitted via pollen, by fertilization of a wild type ovule. Ohad had previously shown that the endosperm of *fie3* too is fertilized in the presence of pollen, suggesting that the mutation does not present a block to fertilization, either of the egg or the central cell.

The primary effect of the *fis/fie* mutations is the premature proliferation of the endosperm in the absence of pollination. A secondary effect is the formation of a normal seed coat from the integuments of the ovule and the

elongation of the silique. As discussed by Ohad et al., these two processes both involve maternal developmental programs that must be induced by signals from the developing gametophyte. The discovery of embryoless seeds with normal seed coat and elongating siliques suggests that the signal originates in the endosperm rather than in the embryo.

It is not clear what the relationship is between *fis/fie* and the genes controlling apomixis. The fact that the mutations affect a late developmental stage after embryo-sac differentiation make it unlikely that they are alleles of the apomixis genes per se. They could, however, be the targets of apomictic regulatory genes. It is noteworthy that *fis/fie* are female lethal mutations, as this is consistent with the observation that apomixis appears to be controlled by a dominant gene, the recessive (sexual) allele of which may be required for some function in development. It would be interesting to test the effect of these mutations in a tetraploid background to see if diploid embryo sacs heterozygous for *fis/fie* have autonomous endosperm development and are able to transmit the mutant allele.

The three mutants described above were identified in two independent large-scale screens of M_1 and M_2 progeny, respectively; each mutant was isolated in the form of several allelic mutations, suggesting that the level of mutagenesis must have been near saturation. The fact that all mutants detected in these two screens show autonomous endosperm, and that many of the seed-like structures contained no embryos, could mean that a mutation resulting in autonomous embryo development, but one that does not activate endosperm, would not be detected in a screen for elongated siliques.

Finally, a screening program for elongated siliques in *Arabidopsis* has been described by Ramulu et al. (1997). Again, the conditional male-sterile seed parents are waxless *eceriferum* mutants, *cer1* and *cer6-2 (pop1)*, which are self-fertile at high humidity and male sterile at low humidity. M_1 plants have been screened for sectors with elongated siliques, and M_2 families derived from selfed individual M_1 plants have been screened for segregation of plants containing elongated siliques.

Screening for Dominant Mutations in the M_1 after Pollination

We are currently conducting a screen for dominant mutations that result in the production of autonomous embryos but that may require fertilization of the central nucleus for the development of endosperm. Although most natural pseudogamous apomicts have fertile pollen, induced mutations that give rise to autonomous embryos as a result of meiotic disturbances could also have defective pollen. Such mutants would not be recovered in the M_2 generation after selfing. For this reason, and to take advantage of the smaller number of plants that need to be screened to obtain a mutant, we decided to screen in the M_1.

In one version of the screen, mutants are detected in the form of maternal progeny after pollination by a pollen parent containing a dominant marker. This screen depends on the formation of fertile seed and would not detect mutant apomictic seeds that fail to germinate. Therefore, we devised a second version that allows the detection of autonomous embryos at an immature seed stage, and which can be employed should the first version fail.

The seed stock used for mutagenesis is a conditional male sterile line (DTA Q3) in the *Arabidopsis* C24 background. This line is homozygous for a transgene encoding a temperature sensitive diptheria toxin under the control of the tapetum-specific promoter A9. Growth at 18°C results in male sterility due to the absence of functional pollen. At 26°C, the plants are fertile, allowing the propagation of homozygous seed by selfing.

Seeds were mutagenized with EMS for 6 hours, washed, and dried for storage on filter paper. The seeds were sown in batches of 100–200 at regular intervals, and seedlings were grown to flowering in individual pots. After growth at 18°C for at least one week, the plants were cut back to two mature flowers on a single inflorescence. These M_1 flowers were pollinated by a transgenic line in the WS background homozygous for a Basta herbicide resistance gene. Progeny resulting from cross-fertilization are resistant to Basta, whereas maternal progeny are Basta-sensitive (Figure 13.1). However, since Basta-sensitive plants cannot be rescued, this selection presents a problem in cases in which only a small number of seeds are available. We are therefore currently using a simplified selective criterion, spoecifically, the dominant leaf morphology of the pollen parent (Figure 13.1). The WS ecotype produces a highly characteristic leaf rosette consisting of tightly-spaced round leaves with abundant trichomes that are distinguishable from the smooth elongated leaves of C24 at an early stage of growth. Heterozygous seedlings are indistinguishable from the homozygous pollen parent WS.

As the screen is conducted in the M_1, mutants that give rise to maternal progeny are most likely to be heterozygous for the mutation. Maternal progeny could potentially arise in a number of different ways: (*i*) the parthenogenetic development of a reduced egg in combination with pseudogamous endosperm resulting in a haploid seedling; (*ii*) the parthenogenetic development of an unreduced egg cell with pseudogamous endosperm and

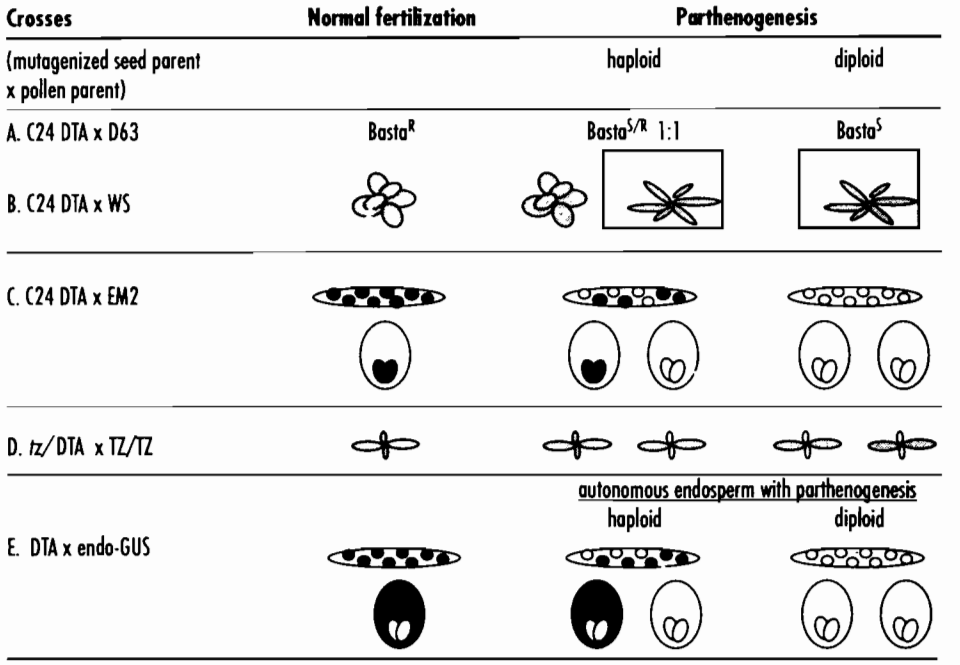

Figure 13.1 "Uncoupling" genetic screens for apomictic mutations in *Arabidopsis*.
The generation of *Arabidopsis* mutants expressing autonomous apomixis is regarded as unlikely for reasons outlined in the text. We therefore propose a number of screens that remove the requirement for mutations simultaneously conditioning both parthenogenesis and autonomous endosperm development. These "uncoupling" screens aim to identify porthenogenetic mutants in the presence of a sexual endosperm and visa versa. (Seed parents: C24 DTA = C24 ecotype with elongated leoves, male sterile at 18 x C. *tz* / DTA = thiamine auxotrophic, male sterile at 18 x C. Pollen porents: D63 = WS ecotype homozygous for Basta herbicide resistance. EM2 = promoter-trap line expressing GUS only in embryos. TZ/TZ = any wild type. endo-GUS = transgenic for GUS reporter under the control of endosperm-specific promoter. For more details and references, see text)

resulting in a diploid seedling; and (*iii*) selfing of the conditional male-sterile plant by infrequently fertile pollen. As illustrated in Figure 13.1, haploid parthenogenesis arising in the M_1 should produce equal frequencies of maternal and hybrid progeny. In the case of diploid parthenogenesis resulting from a dominant mutation, all progeny could be maternal. However, in both cases the expected frequency would be less if the mutation results in facultative parthenogenesis or if the mutation had incomplete penetrance.

Considering pseudogamous development of the endosperm, both haploid parthenogenesis and selfing would result in a balanced maternal: paternal ratio, and therefore the size and shapes of seeds are expected to be normal. However, in the case of diploid parthenogenesis, the endosperm may have either a balanced or unbalanced m:p ratio, depending on whether one or both sperm nuclei fertilize the central cell. Therefore the resulting seed could be either of normal size or smaller (maternal excess phenotype). Small seeds might be distinguishable at the time of sowing, and appropriate steps could be taken should they not readily germinate on normal soil.

The scale of any screening program involving pollination is limited by its labor intensity and by the space required for growing the progeny. For future screens, we have therefore incorporated a recessive marker into the seed parent that is detectable at a very early stage of seedling growth. We crossed the conditional male sterile line DTA Q3 with the thiamine auxotrophic *tz* mutant, and selected progeny homozygous for both conditions (DTA *tz*). After pollination with wild type pollen, heterozygous progeny are thiamine prototrophic, but maternal progeny are auxotrophic (Figure 13.1). Homozygous *tz* mutants emerge with green cotyledons, but the first true leaves are white. These seedlings can be rescued by spraying with thiamine. The advantage of this system is the early detection of maternal progeny that allows screening at a much higher density and provides results soon after sowing. If this screen for maternal progeny mutants is not successful, the second version is employed that allows the identification of autonomous embryos (Figure 13.1). For this purpose, mutants are pollinated by a pollen parent transgenic for an embryo-specific *GUS* reporter gene (Topping et al. 1994). *GUS* expression is easily detectable in the developing seeds from eight days after pollination, well in advance of seed ripening. *GUS*-negative embryos again could arise from haploid or diploid parthenogenetic egg cells or from selfing of the plant.

In principle, the viable and nonviable seed screens could be carried out simultaneously by combining the use of the thiamine-auxotrophic male-sterile seed parent with a pollen donor containing the embryo-specific *GUS* reporter. One of the siliques resulting from the cross could be stained for *GUS* activity at an immature stage and the other left on the plant until seed maturation. Putative apomictic candidates can be further tested to confirm whether endosperm development is autonomous or pseudogamous (Figure 13.1). For this purpose, a pollen donor line has been established in the ecotype WS that is transgenic for an endosperm-specific marker gene, the *GUS* gene under the control of a high molecular-weight glutenin wheat gene (Colot et al. 1987). This is expressed only in the cells of the developing endosperm. After crossing with this marker line, *GUS* expression is tested in siliques at an immature stage, before endosperm absorption. This system could also be used to screen for autonomous endosperm mutants independent of embryo formation.

If the cited screens for viable and nonviable apomictic seed prove unsuccessful, a screen involving pollination will be conducted in an

M_2. This allows the detection of recessive as well as dominant mutants. Also, it is well known that mutations occur in sectors of M_1 plants, and by screening only a single inflorescence per plant, many mutants may be lost. This loss can be avoided by screening in the M_2.

Transposon Mutagenesis for the Isolation of Apomictic Mutants of *Arabidopsis* and *Petunia*

Transposon mutagenesis, like T-DNA tagging, creates mutations by insertion of the transposon into a gene. Its advantage is that tagged genes can be isolated by using the inserted sequence as a molecular probe. Also, a large number of mutants can be produced simply by repeated selfing of the plants. This approach is currently being applied to *Arabidopsis* and *Petunia*, as described in detail by Ramulu et al. (1997). We shall briefly summarize the main points.

For *Arabidopsis*, a two-element system, derived from the maize transposable element *En- I*, was used (Aarts et al. 1995). The maize transposon has a 13 bp inverted repeat at each terminus and encodes a transposase required for transposition. The two-element system consists of a nonautonomous "wings-clipped" *En*-transposase under the control of the CaMV 35S promoter and a nonautonomous mobile I-element with flanking inverted repeats that has been inserted into a kanamycin resistance (*nptII*) gene. Both elements are contained within a T-DNA that also carries a hygromycin resistance marker for selection of transformants. Several lines that contained about 20 I-elements and the *En*-transposase were crossed with homozygous *cer1* and *cer6-2* mutants, and homozygous male sterile lines were selected from the segregating F_2 population. Propagation of these lines for several generations under permissive conditions is expected to create a large number of new mutations, which can be screened under nonpermissive conditions for apomictic mutants.

For transposon mutagenesis in *Petunia*, Ramulu et al. (1997) are using a two-element transposon system found in *Petunia*, which consists of a nonautonomous element, *dTph1*, and an autonomous element carrying the transposase, *ACT1* (Doodeman et al. 1984; Gerats et al. 1990). A line containing more than 200 copies of *dTph1*, which produces a high frequency of unstable mutations in selfed progeny, was used to establish a number of transposon genotypes, which were each crossed with a conditional male-sterile plant. Male sterility in this line results from the absence of flavonols, which is caused by a chalcone synthase antisense gene (Ylstra et al. 1994). The application of flavonols, which are required for pollen tube growth, restores fertility and allows selfing. Plants homozygous for the male sterility phenotype will be selected in F_2 populations, and screening for apomictic mutants will be conducted on a large number of F_3 and F_4 plants in the absence of flavonols.

Branching Out in the *Brassicas*

A benefit of mapping data from several important crop plants and from *Arabidopsis* has been the discovery that groups of genes within large segments of the chromosomes are arranged in the same linear order between related species regardless of differences in genome size (Flavell and Moore 1996). In many cases, molecular markers identified for one species are found to map to corresponding locations in a related species. This high level of synteny can be exploited for the isolation of genes from species with large genomes. The homologous gene can first be isolated from a related species with a small genome, such as *Arabidopsis*, where fine mapping and chromosome walking are feasible; it can then be used as a probe for direct isolation of the gene from the species of interest.

Of particular relevance to our current efforts is the observed synteny between the genomes of *Arabidopsis* and *Brassica*, a genus that contains many important crop species and, like *Arabidopsis*, belongs to the family Brassicaceae (Kowalski et al. 1994). By extension, we expect that there is synteny between *Arabidopsis* and *Arabis*, another genus of the Brassicaceae containing several apomictic species. We have initiated a molecular study of *Arabis* with a view to exploit the mapping data available from *Arabidopsis* to isolate the apomixis gene(s).

The so-called *Arabis holboellii* complex is a collection of closely related species at several base numbers and ploidy levels from diploid to triploid, tetraploid and hexaploid. Apomicts are common, particularly in the triploid species, but are also found at the diploid ($2n$ = 14) level (Böcher 1951; Roy 1995). However, as pointed out by Carman (personal comm.) this diploid species is likely to be paleopolyploid. Apomixis is of the diplosporous type, and endosperm formation is pseudogamous. It is not clear, however, whether pollination results in fertilization of the endosperm nucleus or is only required to trigger endosperm development (Roy 1995).

Pollen viability is high in apomictic *Arabis* spp., and therefore the diploid apomict would be an ideal candidate for the establishing mapping populations with the sexual species, such as *A. drummondii*. The collection of molecular markers available in *Arabidopsis* can be used to identify markers associated with apomixis in *Arabis*. Alternatively, if none of the *Arabidopsis* markers show heterozygocity between the *Arabis* species to be mapped, new markers can be identified in *Arabis* and subsequently mapped to the *Arabidopsis* genome. Much of the *Arabidopsis* genome is already available in the form of yeast or bacterial artificial chromosomes (YACs or BACs), and sequencing of the genome will be complete in a matter of a few years. Therefore, it will be possible to use the markers that cosegregate with apomixis in *Arabis* for the identification of the sexual alleles corresponding to the apomixis locus, and consequently, the apomixis genes themselves.

Conclusions and Perspectives

The various examples of mutants in non-model species and the recently discovered *Arabidopsis* "autonomous endosperm" mutants illustrate a number of points about mutagenesis for the induction of apomixis in sexual plants. First, it seems that all elements of apomixis can be induced in sexual species by mutagenesis. Which element of apomixis can be identified is clearly dependent on the kind of screen employed. Many serendipitous discoveries have been made from pleiotropic effects of the mutations, such as shriveled seed, small plant size, or male sterility, which suggests that these parameters should be included in screening programs. In *Arabidopsis*, previous efforts have relied on screens for elongated siliques and have resulted in a single category of mutants that produce autonomous endosperm. Whilst these mutants have provided important insights into the interrelationship between fruit development and endosperm proliferation, they illustrate the necessity for more sophisticated screens to isolate mutants with autonomous embryos. One of these, involving post-mutagenesis pollination, has been described in detail and is expected to identify new mutants not related to *fis/fie*.

Evidence gathered so far from mutants in the Brassicaceae suggest that (*i*) the diplosporous type of apomixis could be induced more easily in *Arabidopsis* than apospory, since diplospory is found in *Arabis* , and (*ii*) the Brassicaceae may be predisposed to the pseudogamous type of apomixis as found in *Arabis*. That *Arabidopsis* might be predisposed to

pseudogamy can be concluded from the induction of haploid parthenogenesis at high frequency by the application of brassinolide, the steroid hormone present in pollen that may be the trigger for endosperm development following normal pollination (Kitani 1994).

The fact that to date mutagenesis has not resulted in fertile maternal seed confirms the hypothesis that viable apomixis can only be obtained in species that have acquired the necessary preadaptations. A long-term consideration in our pursuit of apomictic mutants in *Arabidopsis* may well be to combine mutations obtained from different screening procedures. For example, it is possible that a mutation that produces unreduced embryo sacs, in combination with a *fis/fie* mutation (perhaps in the heterozygous state), would result in a viable form of apomixis. Although such experiments could be carried out without further knowledge of the genes involved, an important step toward a controllable system of apomixis will be the isolation and characterization of the mutations and their wild type alleles. The mutant genes could be transferred to normal *Arabidopsis* via T-DNA, to determine what effect they have in a background where the wild type allele is also present. It would be interesting to determine if the *fis/fie* mutations are transmissible by female gametes and, if dominant, give rise to seeds with autonomous endosperm and embryos.

Efforts to identify apomixis genes from *Arabis*, as well as other apomicts, should eventually come to fruition. An intriguing area of inquiry, when that comes to pass, will be the nature of the relationship between these natural apomixis loci and any mutations conferring apomictic characteristics that have been identified in the related sexual species. The combined strategies of mutagenesis in sexual plants to induce apomixis, and in apomictic plants to identify natural apomixis genes, should eventually enable us to understand the regulation of these traits and to manipulate them in the best interests of agriculture.

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Chapter 14

Genetic Engineering of Apomixis in Sexual Crops: A Critical Assessment of the Apomixis Technology

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Introduction

According to projections, world population will increase from six billion people today to eight billion in 2020, stabilizing at 9–11 billion people around the middle of the 21st century (Lutz et al. 1997; Evans 1998; Toenniessen, Chap. 1). Profuse quantities of high quality and safe food products will be required to feed this growing population. At the same time, strong pressures are at work demanding that this food be produced in an environmentally friendly manner, e.g., using less agrochemicals. In Europe, agricultural production has steadily increased while population has begun to decrease, resulting in an overproduction of food products. By contrast, the developing world will need to produce two or three times as much food as it does today (Toenniessen, Chap. 1). By 2020, cereal production, for example, will need to increase by 41%, and root and tuber production by 40% (Spillane 1999). To meet this dramatically increasing demand, new plant varieties are needed that are both higher yielding and better adapted to specific climatic conditions. Essentially, this challenge must be met without a significant expansion of agricultural area.

Although less agricultural production will be needed in the developed world, new products, so-called 'novel foods,' 'functional foods,' 'designer foods,' as well as renewable raw materials will soon gain more agricultural market share. It is expected that most of these new products will be produced through biotechnology. Therefore, it is not surprising that the global market for agricultural biotechnology products is expected to increase from US$500 million in 1996 to US$20 billion within the next 15 years (James 1997).

One biological process in particular—apomixis—could revolutionize 21st century agriculture in both developed and developing countries. The harnessing of apomixis is expected to launch a new era for plant breeding and seed production. Mastering apomixis would allow (*i*) immediate fixation of any desired genetic combination (genotypes, F_{1s} included); (*ii*) propagation of crops through seed that are currently propagated vegetatively (seed is easier to transport and to sow); (*iii*) faster and less expensive plant breeding and seed production (e.g., hybrid seeds could be easily produced); (*iv*) a larger pool of germplasm to be used to create more locally adapted varieties (once apomixis is integrated into breeding schemes); and (*v*) a carryover of beneficial phytosanitary side effects through seed propagation, because very few pathogens are transferred through seeds (Grossniklaus et al. 1998a; Bicknell and Bicknell 1999). Furthermore, exploiting apomixis would allow breeding with obligate apomictic species (e.g., *Pennisetum spec.*), where introgression of new traits is currently very limited (do Valle and Miles, Chap. 10), and the use of male sterile plants for seed production. In turn, this would prevent the migration of transgenes from crop plants to wild relatives.

All these advantages taken together undoubtedly would lead to large increases in agricultural production and prompted Vielle-Calzada et al. (1996a) to coin the term "Asexual Revolution" to describe the potential impact of the technology.

The possible economic benefits of the technology are also considerable. In rice, added productivity would total more than US$2.5 billion per year (McMeniman and Lubulwa 1997). It is projected that the heterosis effect alone would result in yield increases of more than 30% (Yuan 1993; Toennissen, Chap. 1). Of today's US$15 billion global market in commercial seed, hybrid seed accounts for 40% of sales (Rabobank 1994), a further indication of the enormous economic potential of apomixis for agricultural enterprises.

Unfortunately, scientific and economic potential shed little light on the actual intricacies of how the genes involved in apomictic reproduction work. Many have concluded that the genes that control apomixis are also crucial for sexual development, indicating that apomixis is a short-circuited sexual pathway (Koltunow et al. 1995; Grossniklaus, Chap. 12). The genetic engineering of apomixis, therefore, requires a better understanding of both apomictic and sexual pathways of reproduction.

In general, apomixis is thought to occur in polyploid species (Asker and Jerling 1992), especially in the Rosaceae, Asteraceae, and in the Poaceae (for review see Berthaud, Chap. 2). For most species in which apomixis has been described, diploids reproduce sexually, while polyploids of the same species are apomictic. Most natural apomicts reproduce through facultative apomixis (Asker and Jerling 1992; Berthaud, Chap. 2). The degree of apomictic reproduction is influenced by the genetic background, ploidy level, modifier genes, and the environment. There is also a great diversity of apomictic behavior: nine types of gametophytic apomixis have been described in addition to sporophytic apomixis (adventitious embryony) (Crane, Chap. 3).

Unfortunately, apomixis is not found in the most important cultivated crops, which could be a result of crop domestication, selection, and segregation analysis (Grossniklaus, Chap. 12). There are three main options for the engineering of apomixis into sexual crops: (*i*) transfer the trait into crops from wild, naturally apomictic relatives through numerous backcrossings, (*ii*) screen sexual crops for apomictic mutants, and (*iii*) *de novo* synthesize the apomictic trait directly into crops. These approaches will be discussed in the following pages.

Transfer of the Apomixis Trait to Sexual Crops

Breeding and Introgression from Wild Relatives

Generally, breeding apomictic species is very difficult, consequently, there have been only a few breeding programs, and these focused on a very limited number of tropical grass species. The basic structure of such breeding programs is described in this book, using *Brachiaria* as an example, an important forage grass in South America, (do Valle and Miles, Chap. 10). Obligate apomicts cannot serve as maternal plants and breeding of such species is therefore impossible. The polyploid and highly heterozygous nature of most apomictic plants further complicates genetic analysis. In addition, controlled pollination is needed to analyze reproductive behavior (methods are described by Sherwood, Chap. 5). Additional techniques are needed to monitor reproduction behavior in progeny plants of new varieties. Such techniques are described in this book by Berthaud (Chap. 2), Crane (Chap. 3), and Leblanc and Mazzucato (Chap. 9). The techniques described include chromosome counting, flow cytometry, clearing and

squashing techniques, sectioning, molecular markers, and the "auxin test." Ultrastructural studies using electron microscopy (Naumova and Vielle-Calzada, Chap. 4) reveal even more information, but are very laborious, time-consuming, and poorly suited to large-scale progeny analysis. Flow cytometry analysis of seeds is a fast and easy tool and thus probably the method of choice for first progeny testings. This is because large numbers of progeny populations have to be produced and investigated at each generation in order to analyze reproductive behavior (Matzk et al. 2000; Savidan, Chap. 11).

Several sexual crop plants are closely related to wild apomicts, and introgression of the apomixis trait through wide crosses has successfully been performed with wheat, maize, and pearl millet (reviewed by Bicknell, Chap. 8; Savidan, Chap. 11). Nevertheless, there are some limitations: total male sterility was observed frequently in F_1 hybrids of wide crosses, representing a dead end once the apomixis trait is obligate. In wide crosses between *Tripsacum* and maize, fertile apomictic BC_4 with less than 11 *Tripsacum* chromosomes could not be identified (Savidan, Chap. 11), resulting in maize lines devoid of agronomic value. Another disadvantage of this approach is that transfer of natural apomixis genes from wild species into related sexual crops by introgression is likely to remain limited to those crops that have apomictic relatives and so will not be applicable to other species.

Mutagenesis Approaches

Mutagenesis approaches have been described in great detail earlier in this book by Grossniklaus (Chap. 12) and Praekelt and Scott (Chap. 13). Therefore, we will discuss only the main conclusions here.

The basis for all mutagenesis approaches is the assumption that apomictic reproduction pathways are developmental variations of the sexual pathway, thus a short-circuited sexual pathway. Mutant screens have therefore been designed to induce sexuality in apomicts and apomictic mutants in sexual plants by the inactivation of genes. Many mutants were identified as being defective in meiosis, megasporogenesis, and gametogenesis (for review, see Yang and Sundaresan 2000; Grossniklaus, Chap. 12). Mutant analysis of megagametogenesis, for example, suggests that a large number of loci are essential for embryo-sac development. Other mutants are described as displaying autonomous embryo and/or endosperm development. The corresponding genes have been recently cloned. *Mea/fis1* (*medea/fertilization independent seed 1*) is a gametophyte maternal effect gene probably involved in regulating cell proliferation in the endosperm and also partially in the embryo (Grossniklaus et al. 1998b; Luo et al. 1999). *Fis2* shows a similar mutant phenotype and encodes a putative zinc-finger transcription factor (Luo et al. 1999). Autonomous endosperm development was observed in the *fie (fertilization independent endosperm/fis3)* mutant. *Mea/fis1* and *fie/fis3* display homology to Polycomb proteins (Grossniklaus et al. 1998b; Ohad et al. 1999), which are involved in long-term repression of homeotic genes in *Drosophila* and mammalian embryo development (Pirrotta 1998).

The most important conclusion derived from the description of these mutants is that all the elements of apomixis can indeed be induced by mutations in sexual plants. In addition, it is obvious that more than one mutation will be necessary to obtain vital apomictic seeds in sexual crops. Nevertheless, a combination of such isolated genes could be used for known gene approaches, but additional genes will be needed to obtain fully developed seeds. Until now, most mutagenesis screens have concentrated on the partial or complete inactivation of the genes that are needed for

progression or inhibition of development. Future screens will also include activation tagging in order to induce genes under a spatial, temporal, or developmental regime that differs from that in the sexual wild type plants.

Known Gene Approaches

Known genes used for genetically engineering the apomixis trait should lead to the following biological processes:

(1) avoidance and bypassing of meiosis (apomeiosis);
(2) formation, ideally, of one functional unreduced embryo sac within each ovule;
(3) autonomous development of the unreduced egg cell by parthenogenesis;
(4) development of a functional endosperm—this could be autonomous or pseudogamous after fertilization of the central cell; and
(5) an inducible/repressible system that is necessary to switch between apomictic and sexual reproduction pathways, because sexuality and recombination will be required for the introduction of new traits into crops, which will result in new and improved plant varieties.

Based on analyses of mutants in apomictic and sexual plant species, it is unlikely that the apomixis trait can be engineered using a single gene. This is supported by the fact that in most cases apomixis is facultative and that the proportion of apomictic progeny can be influenced by different factors, e.g., by environmental factors. Variability within the different apomictic reproduction pathways further indicates that asexual seed development cannot be explained on the basis of a single gene.

One possibility for engineering apomixis is based on isolating the apomixis gene(s) from natural apomicts and inserting them into sexual crops. Molecular mapping of apomixis genes and gene isolation by map-based cloning or transposon tagging (described by Grimanelli et al., Chap. 6) are performed in various laboratories, but until now no apomixis genes could be isolated and markers still lie within cM distance. One major problem with several apomicts is suppression of recombination around the apomixis loci (e.g., *Pennisetum* and *Tripsacum;* Grimanelli et al., Chap. 6). In addition, apomictic species do not belong to the classical model plant species, and therefore positional cloning is difficult because of the relatively low number of available markers, which are needed to "walk" to the apomixis gene(s). Transposon tagging is not possible for most apomicts (*Tripsacum* is an exception because it can easily be crossed with maize lines carrying active transposon elements), and for the near future, T-DNA tagging will remain restricted to dicotyledonous apomicts such as *Hieracium*, which are accessible to *Agrobacterium tumefaciens* transformation (Bicknell, Chap. 8). Moreover, it is also possible that because of the polyploid nature of natural apomicts, no such phenotype exists.

Known genes/promoters from sexual species that could be used for genetic engineering include those involved with (*i*) ovule development, (*ii*) initiation of meiosis, (*iii*) female gametophyte development, (*iv*) parthenogenesis, and thus autonomous embryo development, and (*v*) initiation of endosperm development. Grossniklaus (Chap. 12) speculates that the genes controlling apomixis are under relaxed or aberrant temporal and/or spatial control, thus developmental checkpoints and feedback mechanisms may be ignored or altered, leading to precocious development of the megaspore mother cell and/or the unreduced egg cell.

Ovule- and nucellus-specific genes/promoters are now available as tools (see Tables 14.1 and 14.2). The molecular control of meiosis is well characterized in yeast (Vershon and Pierce 2000) and some animal systems, e.g.,

Caenorhabditis elegans (Zetka and Rose 1995), and many genes have been isolated and characterized during the last few years. Much less is known about the genes involved in plant meiosis. However; the first homologs to yeast meiosis genes were recently isolated (reviewed by Grossniklaus, Chap. 12), and many meiosis mutants remain available for further characterization (e.g., in maize and *Arabidopsis*; Neuffer et al. 1997; Yang and Sundaresan 2000). Genes that are expressed during the induction of meiosis have been identified in lily (Kobayashi et al. 1994). Most work on meiosis in plants has been accomplished through investigating male meiosis, but for genetic engineering, female meiosis genes will be of particular interest. Some genes involved with female gametophyte development have been identified, of which some are specifically expressed in different cells of the female

Table 14.1 Examples of isolated genes and their promoters that might be useful as tools for *de novo* synthesis of the apomixis trait in sexual crops

Process to be manipulated *Gene* (expression/function)	(Origin)	Reference
'Apomixis genes'		
not isolated yet (?)		
Ovule and nucellus-specific target gene expression		
FBP7 promoter (ovule-specific)	(*Petunia*)	Colombo et al., 1997
DEFH9 promoter (ovule-specific)	(*Anthirrhinum*)	Rotino et al., 1997
WM403 promoter (nucellus-specific)	(water-melon)	Shen et al., unpublished
Nucellin cDNA (nucellus-specific)	(barley)	Chen and Foolad, 1997
Prevention of meiosis/apomeiosis		
diverse cDNAs (early meiosis-specific)	(lily)	Kobayashi et al., 1994
pAWJL3 cDNA (early meiosis-specific)	(wheat)	Ji and Langridge, 1994
DMC1 gene (MMC*-specific)	(*Arabidopsis*)	Klimyuk and Jones, 1997
SYN1 gene (chrom. condensation/pairing)	(*Arabidopsis*)	Bai et al., 1999
Parthenogenesis (autonomous embryo development)		
SERK gene (competence to form embryos)	(carrot, *Arabidopsis*)	Schmidt et al., 1997
LEC1 gene (competence to form embryos)	(*Arabidopsis*)	Lotan et al., 1998
BBM1 gene (competence to form embryos)	(*Brassica, Arabidopsis*)	Boutilier et al., unpublished
ZmES1-4 promoter (embryo sac-specific)	(maize)	Amien and Dresselhaus, unpublished
(Autonomous) endosperm development		
MEA/FIS1 gene (suppressor)	(*Arabidopsis*)	Grossniklaus et al., 1998b Luo et al., 1999
FIS2 gene (suppressor)	(*Arabidopsis*)	Luo et al., 1999
FIE/FIS3 gene (suppressor)	(*Arabidopsis*)	Ohad et al., 1999
ZmES1-4 promoter (embryo sac-specific)	(maize)	Amien and Dresselhaus, unpublished
Imprinting		
MET1 a/s (hypomethylation)	(*Arabidopsis*)	Adams et al., 2000 Vinkenoog et al., 2000
Inducible/repressable systems		
Steroid-inducible promoter	(mammals)	Schena et al., 1991
Copper-inducible promoter	(yeast)	Mett et al., 1993
Tetracycline-inducible/-inactivatable promoter	(bacterium)	Weinmann et al., 1994
Ethanol-inducibele promoter	(fungus)	Caddick et al., 1998

*MMC: Mega- and Microspore mother cells.

Table 14.2 Examples of patents linked with the engineering of the apomixis trait in sexual crops.
Sources: Intellectual Property Network (*http://www.delphion.com*), European Patent Office (*http://ep.dips.org/dips*), and Bicknell and Bicknell (1999).

Apomixis technology Patent number* (Publication date)	Title (and content)	Applicant(s)
Breeding strategies		
WO8900810 (Feb. 9, 1989)	Asexual induction of heritable male sterility and apomixis in plants (use of male sterility factors).	Maxell Hybrids INC.
CN1124564 (June 19, 1996)	Hybrid vigor fixing breeding process for rice apomixis (breeding and selection strategy).	Chen J.
US5710367 (Jan. 20, 1998)	Apomictic maize (introgression of apomixis from *Tripsacum* to maize).	USDA
WO9710704 (Sep. 22, 1998)	Apomixis for producing true-breeding plant progenies (introgression of apomixis from *Pennisetum squamulatum* to cultivars).	USDA
WO9833374 (Aug. 6, 1998)	Methods for producing apomicitic plants (breeding program).	University of Utah State
WO0007434 (Feb. 17, 2000)	Novel genetic material for transmission into maize (introgression of apomixis from *Tripsacum*).	Eubanks M.W.
Stimulation of apomictic reproduction		
EP0127313 (Dec. 5, 1984)	The production of haploid seed, of doubled haploids and of homozygous plant lines therefrom (causing apomixis by applying an apomixic agent).	Rohm & Haas
SU1323048 (July 15, 1987)	Stimulator of floral apomixis (no file available).	Poltav Selskokhoz IG Nikitskij
US4818693 (April 4, 1989)	Methods and materials for enhanced somatic embryo regeneration in the presence of auxin.	PGS
US5840567 (Nov. 24, 1998)	Simplified hybrid seed production by latent diploid porthenogenesis and parthenote cleavage (induced by controlled environmental conditions).	University of California
De novo synthesis of apomixis (genes and promoters)		
WO9743427 (Nov. 11, 1997)	Production of apomictic seed (using a SERK gene for embryogenic potential).	Novartis and inventors
WO9808961 (March 5, 1998)	Endosperm and nucellus specific genes, promoters and uses thereof.	Doan, D.N.P., Olsen, O.-A. and Linnestad, C.
WO9828431 (July 2, 1998)	Transcriptional regulation in plants (using a meiosis specific promoter).	John Innes Centre Innov. LTD and inventors
US5792929 (Aug. 11, 1998)	Plants with modified flowers (modifying flower cells after transformation with foreign DNA).	PGS
WO9836090 (Aug. 20, 1998)	Means for identifying nucleotide sequences involved in apomixis (isolation and modification of sexual genes for the expression of apomixis in *Gramineae*).	IRD and CIMMYT-ABC
WO9837184 (Aug. 27, 1998)	*Leafy cotyledon1* genes and their use (using embryo specific genes and their promoters).	University of California
US5907082 (May 25, 1999)	Ovule-specific gene expression (using ovule-specific genes).	University of California
WO9935258 (July 15, 1999)	Nucleic acid markers for apospory-specific genomic region (from the genus *Paspalum*).	University of Georgia Reseorch Found. INC.
WO9953083 (Oct. 21, 1999)	Seed specific polycomb group gene and methods of use for same (using repressors of embryo and endosperm development).	Cold Spring Harbor Lab.
WO0024914 (May 4, 2000)	Apomixis conferred by expression of SERK interacting proteins (see above WO9743427).	Novartis

* WO, US, EP, CN and SU refer to World patents, US-, European, Chinese and former Sowjet Union patents.

gametophyte (Grossniklaus, Chap. 12; Cordts and Dresselhaus, unpublished results). Through the use of mutant approaches (Vollbrecht and Hake 1995; Drews et al. 1998; Yang and Sundaresan 2000; Grossniklaus, Chap. 12; Praekelt and Scott, Chap. 13), we can anticipate that many more genes involved in female gametophyte development will soon be isolated. Gene trap screens such as T-DNA insertional mutagenesis, transposon mutagenesis, and enhancer detection (Grossniklaus, Chap. 12) are very powerful molecular tools for isolating the corresponding genes and/or their promoters from sexual model plants like maize and *Arabidopsis*. Further tissue/cell-specific genes and their promoters will be isolated by transcript profiling methods (e.g., Liang and Pardee 1992; Welford et al. 1998; Matsumura et al. 1999) and from tissue/cell-specific cDNA libraries (e.g., Dresselhaus et al. 1994; Diatchenko et al. 1996). Initial attempts have been made to compare gene expression profiles between sexual and apomictic lines within the same species. A few genes that are specifically expressed in the ovules of either sexual or apomictic lines were isolated (Vielle-Calzada et al. 1996b). These genes may eventually be useful tools for inducing apomictic development in sexual lines or sexual development in apomictic lines.

Parthenogenetic embryogenesis from unreduced eggs is the next required step for successfully engineering the apomixis trait. Whether this will occur spontaneously once the egg is diploid has yet to be shown. Quarin and Hanna (1980) found that doubling a sexual diploid *Paspalum* line generated a tetraploid that was facultative aposporous, thus unreduced egg cells developed parthenogenetically into embryos. Spontaneous parthenogenetic development was observed at a low frequency in maize (Chase 1969; Bantin and Dresselhaus, unpublished results). Wheat lines have been described that produced up to 90% parthenogenetic haploids (Matzk et al. 1995). Very little molecular data concerning parthenogenesis are available for higher plants. One protein (a-tubulin) was identified whose expression is associated with the initiation of parthenogenesis in wheat (Matzk et al. 1997). And auxin (2,4 D) treated sexual eggs from maize can be triggered to initiate embryo development at a low frequency (Kranz et al. 1995), however, the molecular mechanism is not understood. Three genes were used to successfully initiate the formation of embryo-like structures on vegetative tissue (*lec1*: *leafy cotyledon1*, Lotan et al. 1998; and *bbm1*: *baby boom1*, Boutilier et al., unpublished results) or to enhance the rate of somatic embryos in culture (*SERK1*: *somatic embryogenesis receptor-like kinase 1*, Hecht et al., unpublished results), respectively. It remains to be demonstrated whether these genes are also useful for inducing embryo development in reproductive cells.

Parthenogenesis may also arise as a function of timing, taking into account that parthenogenetic embryogenesis is usually initiated before anthesis. In contrast to sexual eggs, parthenogenetic eggs (e.g., *Pennisetum ciliare* and wheat) contain ample amounts of ribosomes and polysomes and a large number of cristae in mitochondria, thus suggesting a highly active metabolic status prior to pollination (Naumova and Vielle-Calzada, Chap. 4; Naumova and Matzk 1998). In contrast to sexual eggs, degeneration of synergids in aposporous *Pennisetum ciliare* female gametophyte was precocious and rapid. In addition, a complete cell wall around the eggs was already generated before the arrival of the pollen tube (Vielle et al. 1995). In maize, zygotic gene activation (ZGA), the switch from maternal to embryonic control of development, occurs soon after fertilization (Sauter et al. 1998; Dresselhaus et al. 1999; Bantin and Dresselhaus, unpublished). Precocious expression of zygotic genes before pollination/fertilization could thus eventually

be used as a tool to induce parthenogenetic development of sexual eggs, and perhaps those same genes might be useful for inducing endosperm development. Although the existence of repressor molecules that prevent unfertilized eggs from initiating embryo development has not been proven, it is reasonable to postulate their reality. Once isolated, they might be a useful tool for engineering parthenogenetic embryo development as a component of apomixis.

Induction of endosperm development will probably be the biggest obstacle to the utilizing apomixis in sexual crop species (discussed further under "Main Limitations"). Nevertheless, an in vitro system for endosperm development in maize was reported recently (Kranz et al. 1998), providing impetus to molecular investigations about gene expression and regulation during the earliest steps of endosperm development.

Transformation and Inducible Promoter Systems

Tremendous progress has been made in plant genetic engineering since the first reports of successful plant transformation appeared in the early 1980s, and many commercially relevant genes have been transferred to crop plants (Christou 1996). *Agrobacterium*-mediated transformation has been the method of choice for introducing exogenous DNA into dicotyledonous plants. *Agrobacterium* transformation has proven difficult with cereals, and consequently, alternative methods such as particle bombardment have been employed. Nevertheless, because *Agrobacterium*-mediated gene delivery offers many advantages (easy protocols, often low- or even single-copy integrations, mostly full-length integration of transgenes, short or no tissue culture period), considerable effort has been dedicated to establishing this method for cereals (Komari et al. 1998). *Agrobacterium* transformation of rice is now routine, while successful transformation of maize and wheat has also been reported (Ishida et al. 1996; Cheng et al. 1997). Even so, particle bombardment of wheat and maize immature scutellum tissue remains the most widely used method in most public laboratories. Relatively efficient transformation systems are now available for all major crops as well as some forage grasses (Spangenberg et al. 1998). Development of transformation systems for apomictic species is in progress, and transformation protocols for pearl millet will be established once interesting apomixis genes become available (P. Ozias-Akins, personal comm.). Transformation of *Brachiaria* and *Tripsacum* are foci of apomixis programs at the International Center for Tropical Agriculture (CIAT) and the International Maize and Wheat Improvement Center (CIMMYT), respectively.

A major problem related to transgene activity is the instability of expression (Jorgensen 1995; Matzke and Matzke 1995). Often inactivation of transgene expression is accompanied by an increase in DNA methylation (Meyer 1995). In addition, transgenes may be integrated in hypermethylated chromosomal regions displaying a spatial and temporal change of methylation during plant growth and development (position effect). Transgenes with homologous sequences to endogenous genes may be silenced through the cosuppression effect (Jorgensen 1995; Matzke and Matzke 1995). All the same, plants stably expressing the transgenes can be selected over generations, although this is time-consuming and expensive. Suggestions have been made as to how vectors used for genetic transformation can be optimized in order to minimize the cosuppression effect (Meyer 1995). Single-copy integration of transgenes will be enabled by the deployment of *Agrobacterium*-mediated gene delivery. This in turn will increase the rate of plants that stably express the transgenes. Gene targeting by homologous recombination, i.e., the

generation of null mutants, is probably the ideal way to stably silence genes. The deployment of this approach, however, is still relatively limited for higher plants (Puchta 1998). An alternative is homology-dependent gene silencing (HDGS; for review, see Kooter et al. 1999), especially through the use of double-stranded RNA (RNAi: RNA interference technology) as a template for gene silencing (Bass 2000). Gene silencing at rates up to 100% was reported with transgenic plants using the latter approach.

Inducible/repressible systems are necessary to engineer the apomixis trait, because genetic recombination through sexual crossing will always be required for the introduction of new traits into crops. In a panel discussion with industrial representatives during the Third European Apomixis Workshop (April 21–24, 1999, Gargnano, Italy), it became very clear that inducible systems for engineering the apomictic trait are highly desired (http://www.apomixis.de; see workshops), mainly because they serve as a natural means of protecting intellectual property rights (see "Intellectual Property Rights," this chapter). The question is whether such systems are practically possible, given the problems encountered with the application of gametocides. Various chemical inducible systems have been reported, e.g., the tetracycline inducible/inactivatable promoter system, and steroid-, copper- and ethanol inducible promoter systems (for review, see Gatz and Lenk 1998). Whether these systems are applicable and acceptable for use under field conditions is doubtful; spraying antibiotics, steroids, and heavy metals is environmentally unacceptable. Ethanol systems might offer an alternative. Most of these systems, however, are leaky and have some background activity, or they may be too sensitive. In addition, there is the question of how homogeneously the induction works in different organs, especially in embedded cells like megaspore mother cells and the cells of the embryo sac, which are the main target cells for the genetic engineering of different apomixis components. Seed producers anticipate efficiency rates as high as 99% for such systems (http://www.apomixis.de; see panel discussion during the Third European Apomixis Workshop). Existing systems, therefore, must be optimized, or preferably, new systems using natural, easily biodegradable, and harmless chemicals as inducers must be developed to satisfy seed producer demands and environmental necessities.

Main Limitations

Perhaps the biggest obstacle to genetically engineering apomictic grain crops is that fertilization of the central cell is likely to be required because of dosage effects (Birchler 1993; Savidan, Chap. 11) and because autonomous endosperm development occurs at low frequencies in cereals. A balanced maternal:paternal genome ratio (2m:1p) is an absolute requirement for endosperm development in cereals (Birchler 1993). In most cases, deviation from this ratio leads to embryo abortion or seeds with diminished fertility (Birchler 1993; Praekelt and Scott, Chap. 13). In contrast to cereals, Scott et al. (1998) have shown that in *Arabidopsis*, 2m:2p, 4m:1p and 4m:2p ratios are allowed. Also observed in most pseudogamous apomicts are ratios of 4m:1p and 4m:2p. In apomictic lines of the maize relative *Tripsacum*, Grimanelli et al. (1997) identified 2m:2p, 4m:1p, and 8m:1p ratios. Imprinting of gametic nuclei is the genetic reason behind this phenomenon: one set of alleles is silenced on the chromosomes contributed by the mother, while another set is silenced on the paternal chromosomes. Each genome thus contributes a different set of active alleles (Vinkenoog et al. 2000; Alleman and Doctor 2000). A few imprinted loci have

been investigated in plants (e.g., Kinoshita et al. 1999; Vielle-Calzada et al. 2000; Alleman and Doctor 2000; Crane, Chap. 3), but we are just beginning to understand the molecular mechanisms underlying these processes. Nevertheless, the combination of maternal hypomethylation in combination with a loss of *fie* function was recently shown to enable the formation of differentiated endosperm without fertilization in *Arabidopsis* (Vinkenoog et al. 2000). It remains to be demonstrated whether this approach is also feasible for crops, especially cereals, but it represents a promising step in assembling the many components needed to engineer apomixis into sexual crops.

Another obstacle that needs to be overcome is the relatively high number of genes/promoters that are required; in addition to inducible/repressible systems, it is likely that the precise and controlled interaction of many genes will have to be engineered. In natural apomicts, genes from different chromosomes are required for the expression of apomictic reproduction pathways. Blakey et al. (1997) have shown that in apomictic *Tripsacum*, genes required for seed set are located on at least five *Tripsacum* linkage groups, which are syntenic to four maize chromosome arms. Sherwood (Chap. 5) observes that the expression of apospory requires the dominant allele of a major gene or linkat and that the degree of apomixis may be further influenced by many other genes (e.g., modifiers). Fewer data are available for diplospory, but in this case as well, a single master gene or a number of genes that behave as a single locus may be required for the expression of apomixis. The technical difficulties of introducing multiple genes within a single transformation event were successfully resolved recently using *Agrobacterium*-transformation with rice (Ye et al. 2000). Four genes were integrated on one construct; by crossing transgenic lines carrying other transgenes, a whole biosynthetic pathway was engineered into rice endosperm (Ye et al. 2000).

To sum up, our understanding of the molecular regulation of apomictic and amphimictic reproduction pathways in crops, especially cereals, is still in its infancy, and thus, due to the complexity of these biological processes, modifying or controlling the pathways will probably not be achieved within the next five years.

Intellectual Property Rights

Intellectual property rights (IPR) are a means of promoting commercially relevant innovation and for sharing resources. The IPR owner obtains the right to use the intellectual property (IP) exclusively, license it, or not use it at all for a limited period (e.g., 20 years). In agricultural biotechnology and plant breeding, both scientific knowledge and its commercial applications are increasingly being claimed by companies, but also by public institutions such as universities and research centers (Spillane 1999). With hundreds of millions of dollars invested every year in plant biotechnology and breeding research, companies need effective IP protection to provide an incentive for making large research investments. These research results offer enormous benefits for agrochemical and seed companies, farmers, and the society as a whole. In the United States, IPR include (*i*) general utility patents, (*ii*) Plant Variety Protection (UPOV), and (*iii*) plant patents for asexually reproduced plants (Jondle 1999).

Given this context, it is not surprising that IPR for methods and genes/promoters that are useful for the genetic engineering of apomixis have been claimed (Table 14.2). Most of the patents were filed during the last five years, probably because of improvements in plant gene technology and in recognition of the enormous economic potential of utilizing

apomixis for crop improvement. These apomixis patents raised concerns about the use of apomixis technology. The Rural Advancement Foundation International (RAFI), a nongovernmental organization, recently expressed the concern that apomixis IPR could wind up in the hands of only a few dominate global agrobusiness players, and that farmers in both developed and developing countries might become totally dependent on their seed products. Other concerns are that genetic diversity could significantly decline and that developing countries will not have access to this technology because they will be unable to afford the required rights and licenses (RAFI 1998). The latter concern is shared by leading apomixis researchers and was formalized in 1998 in the Bellagio Apomixis Declaration (for full text, see http://billie.harvard.edu/apomixis). Signatories to the declaration were interested in how to develop novel approaches for generating the enabling technology, and how to patent and license it. Currently, patents related to apomixis enabling technology are dispersed among many parties (Table 14.2). Furthermore, it is expected that the number of patents will greatly swell as numerous public and private research institutions continue investigating different aspects of apomictic and amphimictic reproduction pathways using different species and approaches (see e.g., Bicknell and Bicknell 1999).

Another negative impact stemming from apomixis patents is that communication of research results to the scientific community is either delayed until patents have been filed or they are simply not communicated at all. A widespread phenomenon in today's biomedical research is that while IPR is growing rapidly, scarce resources are poorly utilized because too many patent owners are blocking one another. Paradoxically, more IPR may lead to fewer useful products for the improvement of human health (Heller and Eisenberg 1998). In regards to apomixis, it is unlikely that the situation will change in the near future because it is still possible to file very broad apomixis patents.

The question of whether farmers in developing countries will get access to disclosed apomixis technology remains unanswered. One can hope that many of the relevant patents will be secured by public organizations such as the Consultative Group on International Agricultural Research (CGIAR) and other public institutions (see Hoisington et al. 1999), thus giving interested parties in developing countries the possibility of acquiring free access to this powerful technology. Certainly, the public image of the big agrobusiness players would benefit from freely licensing the technology to CGIAR institutions or directly helping farmers in developing countries use this technology. The bulk of profits, after all, will be earned in the more developed countries. Introducing the apomixis trait into local varieties would give farmers in developing countries access to powerful and productive hybrid technology (Hoisington et al. 1999). To some extent, these farmers should have the right to save seed for subsequent replanting, thus allowing them to significantly increase their crop yield and personal income.

Risk Assessment Studies

Risk assessment research and studies relate to the use and or release of genetically modified organisms (GMOs) into the environment. Since the first release of genetically modified plants (GMPs) some twelve years ago, many short-term studies have been conducted (de Vries 1998). Short- and long-term risk assessment studies are also needed to evaluate the environmental implications of novel apomictic crops. One key issue for investigation is whether the apomixis trait can move to the landraces and wild ancestors of food crop

plants, and if so, what would be the impact. This issue is especially important in the centers of origin for the crop plants. Furthermore, the issue of how apomixis might affect genetic diversity, and whether it would increase or decrease monoculture farming needs to be explored. Based on field studies on herbicide and/or insecticide resistant plants, we can probably expect engineered apomixis genes to move through vertical gene transfer (transfer of a gene from plant to plant via sexual reproduction/pollen) (Lutman 1999). The rate of horizontal gene transfer (asexual gene flow between organisms) is relatively low and the risk negligible, however, microbiological risk assessment studies in this area could be useful (Syvanen 1994). Given our current knowledge, it appears unlikely that microorganisms could gain some advantage over wild relatives after uptake of apomixis genes.

If apomixis is controlled by multiple genes, the probability of diffusing this trait to wild relatives is extremely low. The transfer of several genes to a wild plant should lower its fitness to a level unacceptable for survival in the wild (Berthaud, Chap. 2). If apomixis is controlled by a single gene, which would result in obligate apomictic wild races, these races would lose their potential to evolve. If dominant, an apomixis gene could rapidly become fixed in an outcrossing sexual population. Therefore, in theory, apomixis transgenes could possess advantages that might result in the uncontrollable spread of the transgenes (van Dijk and van Damme 2000). Inducible apomictic systems and male sterility might circumvent these problems. Nevertheless, the described possibilities indicate that risk assessment studies and research to investigate the ecological implications of novel apomictic crops (once available) to the environment are an absolute necessity. In addition, socioeconomic studies on the positive and negative implications of this technology for breeders, seed companies, and farmers in both developing and developed countries (see also IPR) will be required, and the research results should be communicated to all potential users.

Summary

The extensive introduction of apomixis into sexual crops will undoubtedly rely on genetic engineering, as we anticipate that more candidate genes (especially regulatory genes and tissue/cell-specific promoters) and enabling techniques will be identified and developed in the near future. Transformation technology for all major crops is now available and inducible systems are currently being developed and optimized, allowing the control of transgene expression and activity even under field conditions. Adventious apomixis using already described or novel genes under the control of ovule-, nucellus- or archespore-specific promoters is probably the easiest way to engineer the apomixis trait. Plant breeders and seed producers would like to generate inducible obligate mitotic diplospory in combination with autonomous endosperm development. The latter is probably the most difficult aspect of engineering apomixis, especially for cereals such as wheat, rice, and maize, because of dosage and imprinting effects.

Although apomixis is a hot topic in plant research, our current understanding of both apomictic and amphimictic reproduction pathways in higher plants is still extremely limited. The economic potential of apomixis might provide the impetus to bring apomictic crops to the marketplace, and in the process it may well contribute significantly to our future understanding of the molecular regulation of the many different sexual and apomictic plant reproduction pathways.

International and interdisciplinary approaches and efforts are now needed to study and manipulate seed reproduction. It will be

necessary (*i*) to characterize the genetic regulation of apomixis and isolate the responsible genes, (*ii*) to analyze the genetic and molecular bases of sexual reproduction and to isolate the corresponding genes, and (*iii*) to produce the tissue/cell-specific and inducible/repressible promoters that will be needed to control the expression of the target genes. Concerted international research efforts have been made in Europe aimed at understanding apomictic and sexual reproduction pathways in order to develop tools for the manipulation of the apomictic trait (e.g., an E.U. Research Technology and Development (RTD) project entitled "The manipulation of apomixis for the improvement of tropical forages," coordinated by M. D. Hayward; a RTD project entitled "Apomixis in agriculture: a molecular approach," coordinated by M. van Lookeren Campagne; and a Concerted Action Project entitled "Introducing and controlling asexual reproduction through seeds in apomictic systems and sexual crops," coordinated by T. Dresselhaus). In 1999, a transatlantic consortium was initiated between two public institutions (CIMMYT and IRD) and three private companies (Pioneer Hi-Bred, Novartis, and Group Limagrain). This is just a beginning and more concerted projects are needed in order to reach the ambitious aim of manipulating the apomixis trait in crops.

Apomixis technology will offer many exciting opportunities for the agriculture of the 21st century, and indeed many patents already have been filed with many more yet to come. It is critically important that these patents be held and used for the good of all. Public institutions in particular must safeguard the access of developing countries to these enabling technologies. In all likelihood, constraints to the broad and generous use of apomixis technology will be political and economic rather than technical in the future.

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