Apomixis in *Taraxacum*

an embryological and genetic study

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Apomixis in *Taraxacum*

an embryological and genetic study

Apomixie in *Taraxacum*

een embryologische en genetische studie

Proefschrift

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STELLINGEN

1. - Het verstoren van de paring van homologe chromosomen tijdens de eerste meiotische profase, parthenogenetische eicel ontwikkeling en autonome endosperm ontwikkeling in apomictische paardebloemen is te verklaren door aan te nemen dat bepaalde chromosoom-specifieke eiwitten verschillen van hun "sexuele" analogen.
dit proefschrift

2. - Het grote evolutionaire succes van paardebloemen kan verklaard worden door hun vermenging van de voordelen van sexuele en asexuele reproductie.
dit proefschrift

3. - De hypothese van Vinkenoog en collega's: autonome endosperm ontwikkeling in autonome, polyploïde apomicten is mogelijk bewerkstelligd via hypomethylatie van het genoom, is zeer lastig te testen daar genoominteracties, imprinting en aanwezigheid van "modifiers", alle drie karakteristiek voor apomicten, gepaard kunnen gaan met methylatie en ook endosperm ontwikkeling kunnen beïnvloeden.
Vinkenoog et al., 2000. Hypomethylation promotes autonomous endosperm development and rescues postfertilization lethality in *fie* mutants. Plant Cell 12, 2271-2282.

4. - De verregaande specialisatie en complexiteit van de stand van het huidige onderzoek maakt het wenselijk dat wetenschappers zelf het initiatief nemen hun bevindingen en inzichten te communiceren naar het algemene publiek, bijvoorbeeld via Internet en columns in tijdschriften en kranten.

5. - Wageningen Universiteit zou zich tot doel moeten stellen met haar voorlichting en image niet louter middelbare scholieren aan te spreken teneinde de instroom van propaedeuse studenten te vergroten, maar om een duidelijk gezicht te hebben naar de gehele samenleving.

6. - De invloed van TV en overige visuele media waaronder het Internet kunnen ertoe leiden dat jongere wetenschappers minder gebruik maken van de oudere, voornamelijk tekst-georiënteerde vakliteratuur.

7. - De mate van inbedding van evolutionair-biologische theorieën in vakgebieden als genoomonderzoek en celbiologie is een maat voor de evolutie van het respectievelijke vakgebied.

8. - Wetenschap kan, net zoals de muziek, beschouwd worden als een uitdrukkingsvorm waarbij het weglaten van fragmenten gebruikt wordt om andere fragmenten meer nadruk te geven.

- *Stellingen behorende bij het proefschrift "Apomixis in* Taraxacum *– an embryological and genetic study" van Peter van Baarlen. - 11 september 2001, Wageningen*

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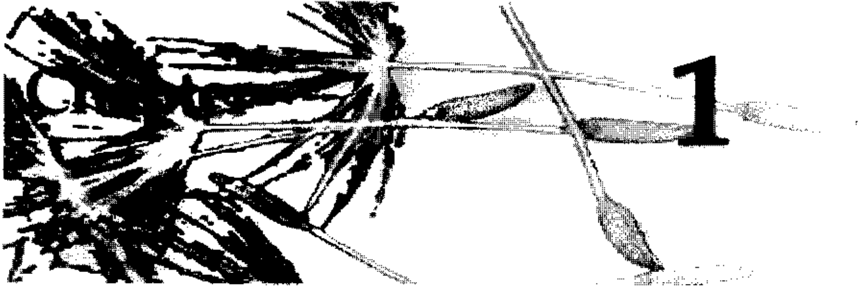


General Introduction

Peter van Baarlen

Summary
In this chapter I will present an overview of the main differences between sexual and asexual seed production in the genus *Taraxacum* (dandelions). Several cytogenetic and plant developmental biology terms and concepts that will be used throughout this thesis will be introduced. The *Taraxacum* type of apomixis will be described and I will highlight its main deviations from sexual reproduction in this species. A synopsis will be given of earlier investigations that have partially led to the research questions that form the basis of this thesis.

Sexual plant reproduction involves double fertilisation

The life cycle of diploid sexually reproducing plants alternates between a diploid vegetative or sporophytic phase and a haploid generative or gametophytic phase. Within the female reproductive centre, the megagametophyte, the nuclei from two differentiated cells, the haploid egg cell and the diploid central cell, need to be fertilised by haploid pollen sperm nuclei before reproduction can initiate. Thus, sexual plants can only reproduce after fertilisation of two distinct cell types of different ploidy level. The female gametes (egg cells) and male gametes (sperm cells) become haploid after two meiotic divisions, one reductional division, halving the chromosome number in the daughter cells and one equational division halving the chromosomes into two chromatids. Fertilisation takes place in a specialised 7-cell structure of maternal origin, the embryo sac. Egg and central cell are both located in one single embryo sac (fig. 1.1). Immediately after embryo sac formation, two haploid nuclei appear in the central cell. Prior to fertilisation, these two nuclei fuse into a diploid nucleus in several angiosperm genera (Johri, 1963; Willemse & van Went, 1984). The fertilised egg cell starts dividing mitotically to produce a diploid embryo. In the fertilised central cell, the nucleus starts dividing to give triploid endosperm nuclei. Cellularisation takes place later, resulting into the endosperm, a specialised tissue that nourishes the embryo during seed formation. Sexual plants depend on fertilisation of egg and central cell nuclei in order to set seed since fertilisation restores the somatic diploid ploidy level and triggers endosperm and seed development.

Embryo sac formation and fertilisation occur within the ovules. Megagametophyte and gamete formation starts with specialised cells within the ovules, the archespores (fig. 1.1b). Archespores transform into megaspore mother cells, the meiocytes that produce four megaspores after a standard type of female meiosis or megasporogenesis (recently reviewed in Porceddu et al., 1999; Schwarzacher, 1999). Megaspores first undergo three successive mitotic divisions without wall formation, a stage termed megagametogenesis. After this initial 8-nucleate (coenocytic) stage, cellularisation takes place and the 8-nucleate megaspores transform into embryo sacs. The type of embryo sac that applies to all plants in this study is the common Polygonum type of embryo sac (described in Johri, 1963; Willemse & van Went, 1984; fig. 1.1a & 1.2).

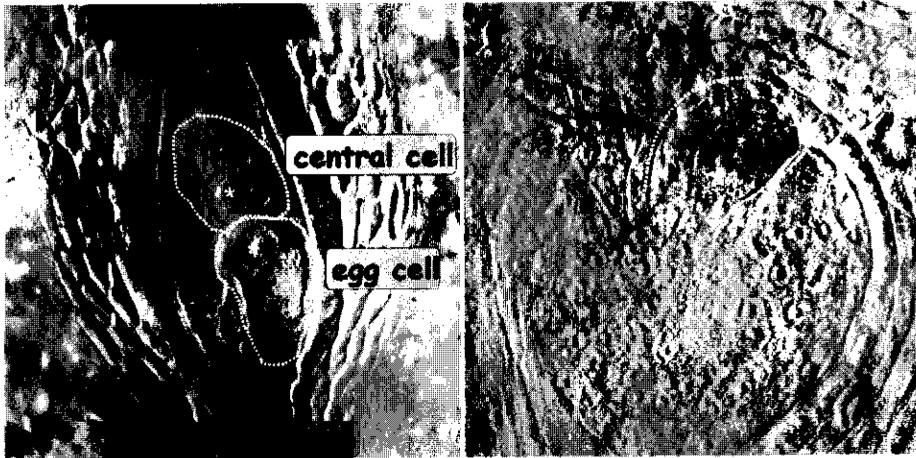


Fig 1.1a. the *Taraxacum* embryo sac. The upper chalazal and bottom micropylar region are indicated, together with the contours of the central and egg cell. To the left of the egg cell, one of the two synergids is visible (not traced).
Fig. 1.1b. the archespore (traced in white) that transforms into the megaspore mother cell. Asterisks * indicates the position of nuclei, with prominent nucleoli.

Male gamete formation takes place in the anthers. Microspore mother cells, the male meiocytes, produce four microspores after male meiosis or microsporogenesis. Microspores eventually develop into pollen grains which are surrounded by characteristic thick cell walls. Within each pollen grain, mitotic divisions give rise to one vegetative cell and two generative sperm cells, each containing one vegetative nucleus or one generative sperm nucleus. One sperm nucleus will fertilise the egg cell nucleus, the other one the central cell nucleus. This scheme of gametophyte formation followed by fertilisation of reduced haploid egg and diploid central cell nuclei by haploid pollen nuclei does not only apply to sexual diploid *Taraxacum* but to most angiosperms.

Apomixis is asexual reproduction through seed

Although most angiosperm plants form seeds exclusively sexually, approx. 0.1% of all angiosperms produce seeds without prior fertilisation of the egg cell and sometimes without fertilisation of the central cell (Mogie, 1992). This type of asexual plant reproduction through seed is termed gametophytic apomixis (Nogler, 1984) to distinguish it from vegetative asexual reproduction via production of shoots or runners. In this thesis, the conventional term apomixis will be used instead of gametophytic apomixis. Some important definitions that apply to different types of apomicts and that will be used throughout this thesis

can be found in table 1 on page 5. Although there are several types of apomictic reproduction, each with its own variations (Gustafsson, 1935b; Battaglia, 1963; Rutishauser, 1967), a shared feature of nearly all apomictic species is that they are polyploid, at least triploid (in the case of most *Taraxacum* apomicts) but usually with higher ploidy levels.

Apomixis can be subdivided in three major types: (i) adventitionous embryony, (ii) apospory and (iii), diplospory. In adventitionous embryony, embryos develop from single or multiple somatic cells within the ovary, usually near the embryo sac. One well-known example of adventitious embryony occurs in *Citrus* (Koltunow, 1993). This type of apomixis will not be discussed in this thesis.

In aposporous apomicts, unreduced embryo sacs are directly formed from somatic ovary cells, often nucellar cells. Usually, both reduced megaspores and so-called aposporous initials are present within a single ovary. Unreduced aposporous embryo sacs already start developing from the aposporous initials when the reduced megaspores in the same ovary undergo gametogenesis. Eventually, reduced embryo sacs - if formed - are displaced by already completely developed, unreduced aposporous embryo sacs. Presence of both sexual and aposporous processes within single ovaries is an important feature of aposporous apomicts (Koltunow et al., 1998). This feature does not occur in diplosporous apomicts, although incidental apospory-like phenomena have been observed in fully diplosporous *Taraxacum* apomicts (Malecka, 1973 and chapter 6). Notwithstanding these observations, there are no reports in the literature or experimental data demonstrating that aposporous-like cells (or callus-like cell conglomerates; chapter 6) can form viable embryos.

Table 1. Definitions used throughout this thesis

Apogamety : embryo development initiated by a somatic cell from within the embryo sac, often a synergid

Apomeiosis : embryo sac formation without reduction of the somatic chromosome number

Apomixis : seed production in absence of fertilisation via an asexual mechanism

Apospory : Development of an unreduced embryo sac from a somatic cell of the nucellus, chalaza or maternal somatic tissue of the ovary

Autonomous apomicts : plants that produce seeds in absence of fertilisation of egg cell and central cell nuclei (in contrast to pseudogamous apomicts, see below)

Autonomous endosperm : endosperm that is formed with no prior fertilisation of the central cell nucleus

Diplospory : Development of an unreduced embryo sac from an archespore or megaspore mother cell

Hybrids (usually as "apomictic hybrids") : plants that are the progeny of fertilisation sexual "mothers" (pistillate parents) by apomictic "fathers" or pollen donors (staminate parents)

Megasporogenesis : the female meiosis during which the diploid megaspore mother cell (the meiocyte) undergoes a reductional and equational division resulting in four haploid megaspores

Megagametogenesis : the process whereby the megaspore undergoes three successive mitotic divisions and transforms into the embryo sac

Microsporogenesis : as megasporogenesis but for the male meiosis

Parthenogenesis : embryo development initiated by an unfertilised egg cell

Pistillate parent: the parent in a cross that delivers the egg cell (the "mother")

Pseudogamy : parthenogenetic egg cell development after fertilisation of the embryo sac; fertilisation of central cell nuclei is necessary for endosperm and seed formation

Sexual - apomict crossings : crossings using a diploid sexual "mother" and a triploid apomictic pollen donor

Staminate parent : the parent in a cross that delivers the pollen grains (the "father")

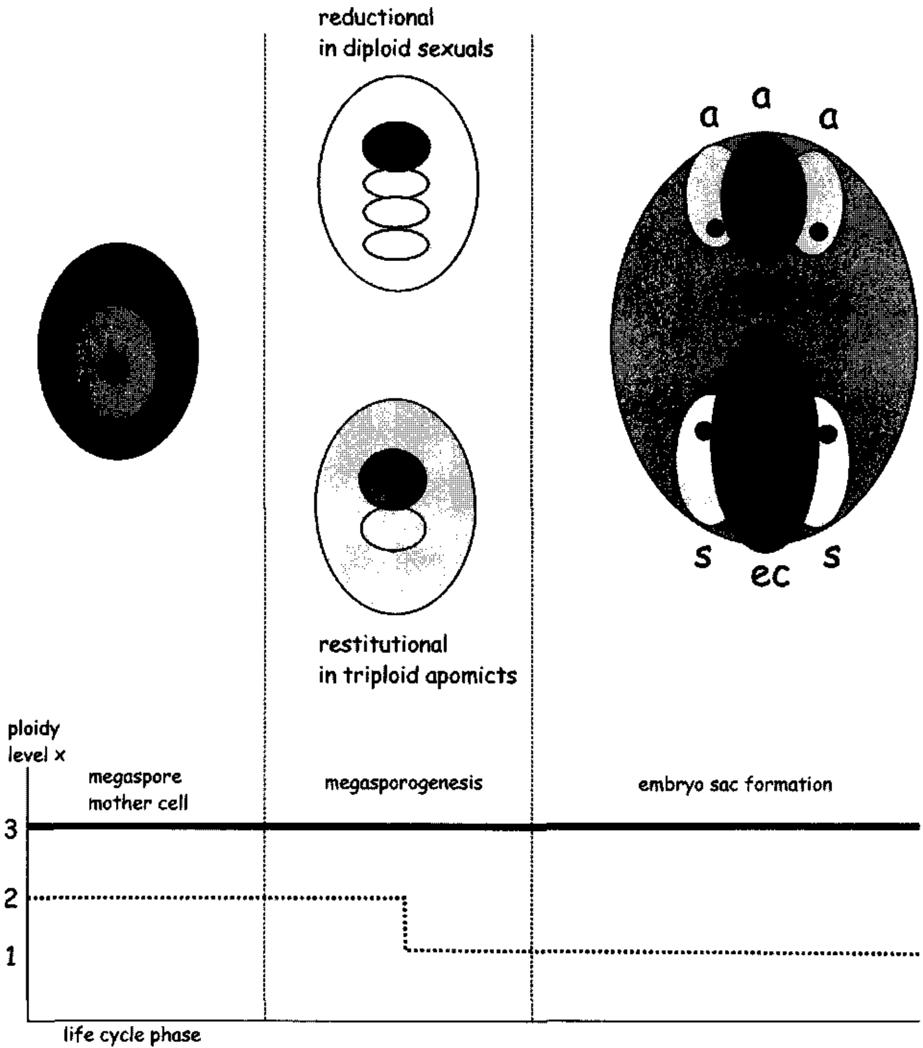


Fig. 1.2. Embryo sac formation in sexual and apomictic dandelions. During megasporogenesis, meiocytes are reduced in diploid sexuals but not in polyploid apomicts. After megasporogenesis, reduced as well as unreduced megaspores transform into similar embryo sacs but with different ploidy levels of the antipodal cells (a), central cell (cc), synergids (s) and egg cell (ec). The diagram at the bottom indicates the ploidy level of the respective nuclei. The solid bar applies to triploid apomicts, the interrupted bar to the diploid sexuals. Note that the central cell contains double the amount of nuclei as the egg cell does.

In diplosporous apomicts, unreduced embryo sacs are formed from unreduced megaspores. Nonreduction of megaspores can be achieved by two distinct pathways. During mitotic diplospory, megaspore mother cells or possibly archespores do not initiate meiosis but directly undergo the three mitotic divisions of megagametogenesis and transform into unreduced embryo sacs. During meiotic diplospory, megaspore mother cells do initiate meiosis but do not complete the first reductional meiotic division. Instead, a restitution mechanism maintains the somatic chromosome number. After completion of the second meiotic chromatid division, one of the two formed unreduced megaspores degenerates, the other one (located on the chalazal side of the ovary) undergoes the three mitotic divisions of megagametogenesis and transforms into an unreduced embryo sac. The latter pathway applies to dandelions and is termed the Taraxacum type of diplospory (*fig.* 1.2, *preceding page*).

In the Taraxacum type of diplospory, meiotic recombination can take place, and when recombined chromatids segregate, recombined allele combinations may be present in the surviving megaspore and later in the egg cell. The potential relevance of this feature for *Taraxacum* population biology is discussed in the chapters 3 and 7. A comparable type of meiosis is found in two aphid genera (see discussion in Suomalainen et al., 1976, p. 238) where mutation rates in esterase loci of 10^{-3} have been found. No such data are available for *Taraxacum*.

Restitution, the most important characteristic of megasporogenesis in all *Taraxacum* apomicts, is sex-specific: the male meiosis or microsporogenesis is reductional within the same *Taraxacum* inflorescence. In contrast, transmission is not sex-specific since reduced pollen grains from *Taraxacum* apomicts can transmit diplospory to sexual diploids (chapters 4 and 5). Another important characteristic of meiotic diplospory is that chromosome pairing in the triploid and tetraploid apomicts is disturbed (Gustafsson, 1935a; Battaglia, 1948; Malecka, 1970; van Baarlen et al., 2000; chapter 3) so that most microspores produced by *Taraxacum* apomicts are aneuploid and unfertile. Some pollen grains however are euploid, and such pollen grains can fertilise reduced embryo sacs of sexual dandelions or the infrequently formed reduced embryo sacs of apomicts (chapter 3).

Sexual and apomictic reproduction in *Taraxacum* occur within one species

The European species *Taraxacum officinale* L., the common dandelion, comprises diploid sexual and triploid or tetraploid apomictic plants. Although the apomicts mainly occur in the northern regions and the sexuals in the more southern regions, they overlap in certain regions of Europe, including some regions of Germany and the Netherlands (den Nijs et al, 1980; Jenniskens et al., 1984). Sexuals can be pollinated by apomicts; at low (up to 2%) frequencies, this gives rise to hybrid offspring (Tas and van Dijk, 1999). Apomixis was transmitted as a dominant trait in such crosses. Hybrids with a (near-) triploid or higher ploidy level displayed the capacity for apomictic reproduction in part of the flowers more often than hybrids with a diploid to hypo-triploid ploidy level (Tas and van Dijk, 1999; van Dijk et al., 1999). These crosses showed, among others, that nontransmission of the apomictic trait from the apomictic pollen donor into the sexual pistillate plant most likely results from segregational loss of apomixis factors after microsporogenesis in the apomictic parent.

The number of genes regulating apomixis has so far been a matter of controversy (van Dijk, van Baarlen and van Tienderen, in prep.; chapters 5 and 7), as is the level of complexity of apomictic reproduction (see discussion in Savidan, 2000). Apomixis in *Taraxacum* can be thought of as if it were a developmental trait with a multi-steps genetic regulation, comparable to gene cascades that occur in metabolic pathways. Mutations in each step of the pathway may not give the end result, apomictic reproduction, but lead to sterility instead. If distinct genes regulate the apomictic components diplospory, parthenogenesis and endosperm autonomy, recombination during microsporogenesis may lead to segregation of those genes so that the respective components become uncoupled. It is therefore important to analyse first-generation sexual-apomictic hybrid offspring all through the early stages of megasporogenesis up to embryo and endosperm formation in order to assess presence of components of apomictic or sexual reproduction. Detailed microscopic and genetic analysis of a few specific hybrids suggested that distinct parts of the apomictic trait: diplospory, parthenogenesis or endosperm autonomy, could be transmitted via reduced pollen (van Dijk et al., 1999). This was seen as evidence for breaking up of linkage between genes controlling diplospory, parthenogenesis and endosperm autonomy via recombination, thereby uncoupling the three apomictic elements. This finding suggests that apomixis as a trait is regulated by different genes, each one encoding one of the three components.

Can ploidy levels and sexual-apomictic genome balances influence establishment of apomixis?

Both triploid and tetraploid ploidy levels occur in *Taraxacum*. The triploid ploidy level is absent in most plant families where apomixis occurs, apart from in the Asteraceae (Nogler, 1984). This is also the family where most meiotic diplosporous apomicts are found. Meiotic diplospory seems not restricted to plants: in certain parthenogenetic insects a very similar process, ploidy stasis, which is characterised by presence of only the equational division and dependence on meiotic prophase I for egg formation (Lamb and Willey, 1987) is found.

Is it possible that there exists a link between ploidy level and apomixis? In insects (Astaurov, 1969; Suomalainen et al., 1976) and *Taraxacum* (van Baarlen et al., 2000) odd (aniso-) vs. even (iso-)ploidy levels have an influence on meiotic chromosome pairing since odd chromosome numbers interfere with pairing and recombination. Moreover, Astaurov (1969, and references in Suomalainen et al., 1976) found that heat treatments of silkworm moths (*Bombyx mori*) induced a change in the mode of meiosis from normal two-steps to one-steps equational and concomitant parthenogenetic development. Recombination in isopolyploids can give rise to multivalents that often enforce unequal chromosome segregation, aneuploidy and sterility (see for example Astaurov, 1969; chapter 6 of this thesis). In organisms where no meiotic reduction takes place, it is advantageous to develop suppression of recombination if multivalent formation leads to aneuploidy. Recombination can be suppressed by genes (Koduru and Rao, 1981) and extensive genetic divergence of parental species (Comai, 2000; Soltis and Soltis, 2000).

The natural tetraploid apomictic *Taraxacum naevosa* has a decreased frequency of meiotic recombination compared to certain tetraploid sexual - apomictic hybrids, but a higher proportion of *T. naevosa* florets reproduces via apomixis (own observations). An important part of the lower capacity for apomictic reproduction in the sexual - apomictic hybrids can be explained by sterility as a result from developmental deviations, a feature that was not observed in the natural apomicts. The possible link between intergenome balance, developmental deviations and apomixis is further discussed in chapter 6.

Autonomous endosperm mutants can be generated in sexual plants
Arabidopsis fis/medea and *fie* mutants initiate division of central cell nuclei in absence of fertilisation (Chaudhury et al., 1997; Grossniklaus et al., 1998; Luo et al, 1999). For completion of endosperm development (cellularisation), hypomethylation of the genome is required (Vinkenoog et al., 2000). The proteins encoded by wild-type *FIS* and *FIE* loci are homologues of Polycomb proteins that maintain repression states of developmental genes via heritable epigenetic "imprinting" mechanisms (Pirrotta, 1998; Preuss, 1999). It is conceivable that autonomous endosperm in *Taraxacum* apomicts is formed via pathways similar to those in the *Arabidopsis* autonomous-endosperm mutants. Parthenogenesis may also be mediated via a Polycomb-dependent mechanism. A thus far hypothetical influence of epigenetic regulation on apomixis in *Taraxacum* is explored in the chapters 5 and 7.

The evolution of the *Taraxacum* type of apomixis
Since no apomixis genes have been cloned to this date, it is not possible to look for presence of homologues in sexual plants and to investigate how apomicts have been generated from their sexual relatives. The possibility that *Taraxacum* apomixis genes have no homologues in their sexual relatives is less likely considering the results from several sexual-apomict crossings.

In *Taraxacum*, inter-species (Tas and van Dijk, 1999) and intra-species (Malecka, 1971, 1973) sexual - apomict crosses have been performed. Such crosses give hybrid offspring at variable frequencies. Marker studies employing co-dominant molecular microsatellite markers suggest that *T. officinale* apomicts are at least segmental autopolyploids with trisomic (or tetrasomic) inheritance (van Dijk et al., in prep). A comparative microscopic analysis of meiosis, embryo and endosperm development in sexual and apomictic *T. officinale* shows that sexual and apomictic reproduction differ mainly in the absence of genome reduction in the latter (Fig. 1.2; chapters 3-6). Considering the available information, it is conceivable that polyploid *Taraxacum* apomicts originate from sexual diploid siblings and that apomixis is controlled by genes that are mutated versions of genes that were present in sexual diploid progenitor dandelions.

Does apomictic reproduction in *Taraxacum* lead to decreasing fitness?

Evolutionary theory predicts that long-term asexual reproduction leads to the accumulation of mutations, most of which are supposedly deleterious (Muller, 1964; Kondrashov, 1982; Gabriel et al., 1993; Charlesworth, 1999). If *Taraxacum* apomictic lineages would be reproducing strictly asexually, they would be expected to accumulate deleterious mutations ultimately leading to extinction of the apomictic lineages as predicted by a theoretical and mathematical model known as Muller's Ratchet (Felsenstein, 1974).

How likely is extinction of apomictic lineages through the action of Muller's Ratchet? The Ratchet may lead to elimination of an asexual lineage when the following criteria are met:

- small effective population size
- no meiotic recombination
- no segregation of potentially deleterious mutations ("purging" or removing of mutation from the genome)
- a small selective disadvantage per mutation (increases the effectiveness of the Ratchet)

Asexual reproduction is expected to lead to accumulation of chromosomal rearrangements like inversions or translocations, and hence to give rise to an increasing genome instability. In addition, fitness-lowering or lethal mutations are buffered in polyploid organisms where the presence of multiple copies of genes increases the likelihood of presence of at least one functional copy of the respective genes (Vizir and Mulligan, 1999; Soltis and Soltis, 2000). As soon as genome reduction occurs, for instance during microsporogenesis, mutations are subjected to selection. Indeed, both gametophytic (Nogler, 1984; van Dijk et al., in prep.) selection against haploid transmission of apomixis genes and zygotic selection operating at the diploid ploidy level (Bicknell et al., 2000) against transmission of apomixis genes are a feature of several apomictic genera (van Baarlen et al., 1999; van Dijk, van Baarlen and van Tienderen, in prep.). But it is far from certain that mutations really do accumulate in *Taraxacum* apomicts. The Taraxacum type of diplosporous meiosis that enables recombination and segregation, in combination with the effect of mitotic recombination on DNA sequence variation (chapter 2), the generally reported high heterozygosity (Rutishauser, 1967) and large population sizes make it unlikely that Muller's Ratchet will lead to extinction of *Taraxacum* apomicts.

Organisation of this thesis

In this introduction, I have described in what way apomixis in *Taraxacum* differs from sexual reproduction in *Taraxacum*. The available knowledge of apomixis in *Taraxacum* has led to the following research questions.

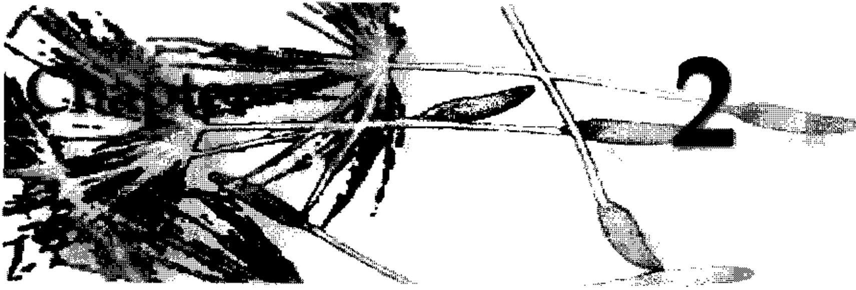
1. Is there recombination in the genome of a *Taraxacum* apomict where meiotic recombination is presumed to be absent? In chapter 2, evidence is provided for complex rearrangements in the rDNA locus where extensive sequence variation had been reported previously (van Dijk, 1997).

2. Meiotic diplospory has two major characteristics: unsaturated chromosome pairing and restitution nucleus formation. Can failure of chromosome pairing be uncoupled from restitution? In chapter 3, it is demonstrated that triploidy in itself interferes with chromosome pairing and bivalent formation but that absence of pairing is not followed by restitution nucleus formation as in apomictic triploids. The diplosporous phenotype is compared to certain meiotic mutants in plants and yeast in chapter 7.

3. What are the main differences in the cyto-embryology of sexual diploids and triploid apomicts together with their triploid and tetraploid hybrid progeny? A comparative analysis of gametophyte and embryo development of sexual diploids, apomictic triploids and their apomictic triploid hybrid progeny is presented in chapter 4. In chapter 5, a similar analysis is presented for two types of apomixis-recombinants that combine elements of sexual and apomictic reproduction. In chapter 6, the same analysis is performed using a natural tetraploid apomict and sexual - apomictic tetraploid hybrids with different genomic constitution.

4. What is the genetic basis for apomixis in *Taraxacum*? In the chapters 5 and 7, a multi-gene model for its genetic regulation is presented and the possibility of an epigenetic regulation of the genes in question is discussed.

5. What is the reason for the evolutionary success of *Taraxacum* apomicts? In chapter 7, several factors that potentially explain the evolutionary success of the genus *Taraxacum* are discussed.



Ribosomal DNA spacer variation in apomictic *Taraxacum officinale*

Peter van Baarlen, Peter J. van Dijk, Rolf F. Hoekstra, and J. Hans de Jong

Summary

Previous studies have demonstrated length variation of the 45S ribosomal DNA unit between different lineages of apomictic dandelions (*Taraxacum officinale*), sometimes even within individual plants. In this paper, we report on length variation in an IGS (intergenic spacer) subregion of individual dandelions that had been demonstrated earlier in a Southern screen of rDNA variation in field populations of apomictic dandelions. To further characterise the observed rDNA variation, we performed Fluorescence *in situ* Hybridisation (FISH) to metaphase complements and extended DNA fibres, and found striking variation in the molecular organisation of the number of IGS subregions in the nucleolar organiser regions (NORs) of the satellite chromosomes. Flow cytometry revealed that apomictic plants with such tandemly duplicated IGS repeats had a 10% higher total genomic DNA content than related dandelions without those duplications. The results show that, in line with earlier reports, the organisation of rDNA subrepeats is potentially hypervariable in apomictic lineages. As homologous NORs in the triploid and diploid dandelions do not synapse at meiotic prophase I, it is likely that non-meiotic recombination events have led to the observed rDNA variation.

INTRODUCTION

Nuclear ribosomal DNAs (rDNAs) are organised in long tandem arrays of gene units. In higher plants, the major component, the 45S rDNA, which contains the 18S, 5.8S and 25S rDNAs, is located in the nucleolar organiser regions (NORs) of one or more satellite chromosomes. Satellite chromosomes can be identified at mitotic metaphase by the presence of a thin chromatin thread, the secondary constriction, which separates the distally located satellite from the chromosome arm. At interphase and prophase the secondary constriction and (part of) the satellite are associated with the nucleolus. The 45S rDNA units in the tandem array are separated by intergenic spacers (IGS), and the internal transcribed spacers 1 and 2 (ITS1 and ITS2) separate the 18S and 5.8S, and 5.8S and 25S rDNA components, respectively (figure 2.1). In addition, plant rDNA intergenic spacers consist of several subrepeat types and intervening sequences (Hemleben and Zentgraf, 1994).

Sequence data of ribosomal genes and spacers are widely used for biodiversity estimates in population biology and taxonomic studies. Most studies have shown that there is very little rDNA variation within a species in sexual diploid organisms, especially at the level of the individual. This is unexpected since duplicated sequences should easily accumulate different mutations and diverge over evolutionary time. It is thought that the repeated rDNA genes and spacers are homogenised by unequal crossovers and biased gene conversion (Elder and Turner, 1995; Gangloff et al, 1996; Hillis and Dixon, 1991; Hillis et al., 1991). The homogenisation of tandemly repeated DNA sequences confers a greater sequence similarity of repeated units within than between species, a phenomenon known as concerted evolution (Arnheim, 1983). Concerted evolution may be interfered with when gene conversion and unequal crossing-over are not able to sufficiently homogenise a given sequence, leading to variation for that sequence. Indeed, several cases of rDNA variation within species are known. Typically, length variants of spacer regions account for the observed heterogeneity within species (e.g. Buckler et al., 1997) or between individuals (e.g. Paskewitz et al., 1993). Length variation of spacers is often based on length variants of subrepeats or the numbers of duplicated subrepeats within the spacers. Such length variation can usually conveniently be visualised by Southern hybridisation of restriction digested genomic DNA using a probe spanning the spacer regions.

In earlier studies that aimed at screening for ribosomal DNA variation in *T. officinale* populations (King and Schaal, 1990; van Dijk, 1997) variation was found within and between apomictic lineages and sometimes within individual plants. Van Dijk (1997) found exceptional *Hinf*I restriction fragment ladders on Southern blots in a few apomicts, all from the same location, when probed with the complete *T. officinale* IGS sequence. The *T.*

officinale IGS sequence (van Dijk, King et al., ms. in prep.) contains several subrepeats flanked by *HinfI* restriction sites, hereafter referred to as the HinfI region. It has been argued earlier that the molecular organisation of that specific region contributes to rDNA repeat length variation in *Taraxacum* (King and Schaal, 1990).

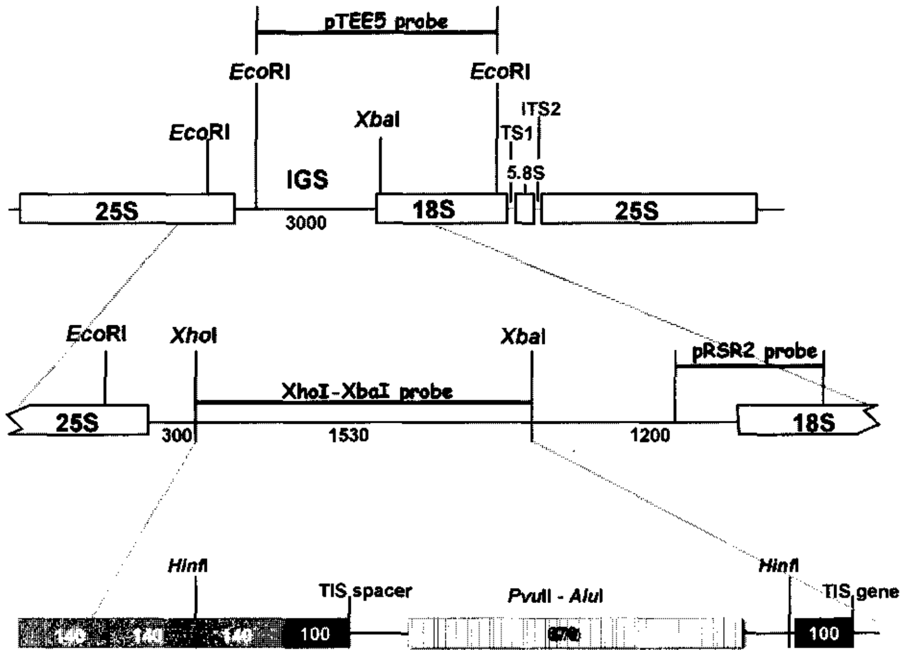


Fig. 2.1. Schematic representation of the *Taraxacum* 45S rDNA unit with detailed overview of the IGS subrepeats. Restriction sites are indicated with the abbreviation of their respective restriction enzymes. The probes used in this study are indicated as solid bars and are described in the Materials and Methods section. TIS: Transcription Initiation Site, IGS: InterGenic Spacer.

In order to study the chromosomal and molecular organisation of this region and to test the former argument, this DNA fragment was used as a probe in a Fluorescence *in situ* Hybridisation (FISH) assay to mitotic chromosome spread preparations and extended DNA fibres. A cytogenetic analysis of NOR transcriptional activity (*viz.* rDNA transcription) based on $AgNO_3$ deposition on transcriptionally active NORs (AgNOR staining) was additionally performed to extend the comparison of the plants with and without the HinfI ladder. We also isolated short DNA stretches of repetitive IGS sequences via the construction of a genomic DNA library using phage lambda and screened

this library using the HinfI probe. DNA from positive phage clones was *Hinf*I digested and Southern blotted in order to search for different *Hinf*I restriction patterns. To see if presence of a HinfI ladder was associated with a significant increase in genomic DNA, nuclear genome sizes of cells from plants with and without the HinfI ladder were compared by use of flow cytometry.

RESULTS

Molecular organisation of the *Taraxacum* 45S rDNA unit

To see whether the HinfI region, conceivably harbouring the *Hinf*I restriction fragment ladder, was also present outside the rDNA locus of the satellite chromosomes, two-colour Fluorescence *in situ* Hybridisation (FISH) was performed on mitotic chromosome spread preparations. Mitotic chromosomes were simultaneously probed with the biotinylated 18S gene probe and with the subcloned DIG-labelled *Xho*I-*Xba*I IGS fragment containing the HinfI region (fig. 2.1). A single 18S gene signal appeared as a red fluorescent dot, a single IGS fragment as a green dot (see materials and methods). Yellow fluorescent signals result from overlapping red 18S and green IGS probes. Mitotic chromosome spreads from sexual diploids, probed with the 18S and IGS fragments, showed two yellow signals (through overlap of the red and green signals) on the NORs of the satellite chromosomes. Both signals appeared as strikingly extended regions (fig. 2.2a) on prophase chromosomes and as dots (fig. 2.2b) on metaphase chromosomes due to a higher contraction of the latter chromosomes.

In the FISH preparations of mitotic chromosomes from triploid "Keyenberg" apomicts (collected from Keyenberg, Renkum, the Netherlands), three yellow signals were visible; two yellow signals traced two very long NORs of the satellite chromosomes, one compact yellow signal indicated the third much more contracted secondary constriction (fig. 2.2c) of the third satellite chromosome. "Wolf" apomicts (collected from Wolfswaard, Wageningen, the Netherlands) also displayed two long secondary constrictions at prometaphase. All green signals colocalised with the 18S probe signals and no green IGS signals were found outside the rDNA locus.

In order to study the molecular organisation of the IGS and 18S sequences in their tandemly repeated context, we probed them simultaneously on extended DNA fibres of both diploid sexuals and triploid apomicts from the Keyenberg or Wolf populations. By comparing the occurrence of the red 18S and green IGS probe signals between the sexuals and the two apomict classes

from distinct locations, differences in rDNA composition between the three groups could be visualised.

The combined red and green probe signals on the extended fibres appeared as linear tracks of yellow fluorescent dots in both sexuals and apomicts. To convert the microscopic fluorescent tracts into DNA sizes (in bp), we used the 3.27 kbp/µm conversion factor previously established by Fransz et al. (1996). The mean length of an rDNA unit converted as above was 5.9 ± 1.1 Kb for the diploid, 6.1 Kb ± 1.6 for the Wolf triploid, and 6.3 Kb ± 1.9 for the Keyenberg triploid dandelions.

We assessed rDNA heterogeneity within and between individual plants by measuring and comparing the distances between adjacent signals. The different plant samples could then be compared with respect to rDNA variation. No significant differences were found in non-fluorescent spacer lengths (measured between 200 yellow dots/plant) between the sexual (n=2) and apomictic plants from Keyenberg (n=2) and Wolfswaard (n=2) (Kruskal-Wallis ANOVA, p=0.08). However, the distribution of 18S and IGS signals in the Keyenberg apomicts differed qualitatively from the sexuals and the other apomicts. In the Keyenberg plants, the contiguous yellow signals were locally interspersed with green IGS signals (black circles in fig. 2.3b). These solitary IGS signals were found at irregular positions between colocalising probes and occurred either one or multiple consecutive times in the array. Solitary 18S signals were also incidentally observed in the Keyenberg plants.

Silver staining of mitotic chromosomes and somatic cells

In interphase nuclei from sexuals and apomicts, all NORs could be stained (fig. 2.4a and 2.4d); this was observed in 36% of the nuclei in the diploid sexuals (n=60 nuclei) and 17% of the nuclei in the triploid apomicts (n=60 nuclei). In other nuclei, rDNA activity was hardly differentially stained when compared to the background (not shown). In the mitotic chromosome preparations from diploid dandelions, two dark brown extended regions of silver deposition localised the two extended NORs (fig. 2.4b-c; n=60 cells). In apomicts, two NORs were stained and extended whereas 1 NOR appeared as a compact dot and did not stain more pronounced than the extended satellites (fig. 2.4 e-f; n=60 cells).

Flow cytometry

A flow cytometric analysis of the DNA contents relative to diploid sexual plants revealed that triploid plants from the "Keyenberg" population contained 1.6 times more DNA than the diploids (not shown). Triploid plants that were collected elsewhere had a DNA content of 1.5 relative to the diploid dandelions. Apomicts from the "Keyenberg" contain ca. 6% more DNA than the apomicts from other locations.

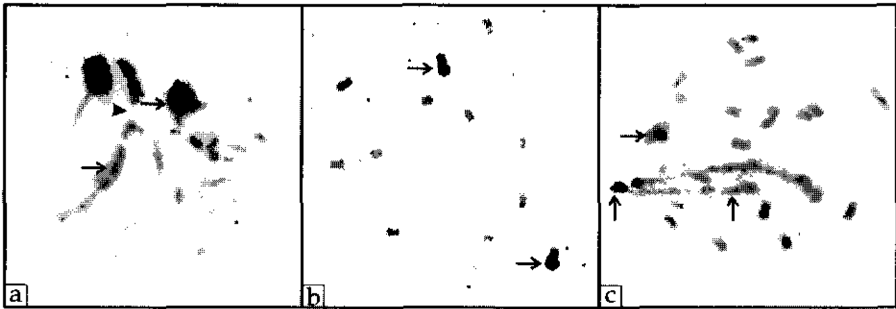


Fig. 2.2a-c. Yellow fluorescence (here in black) on mitotic chromosome spreads of sexual (2a-b) and apomictic (2c) dandelions. 2a. FISH on mitotic prometaphase chromosomes traces the elongated NOR region (the secondary constriction) and the prominent satellite. The left larger arrow points at one of the two secondary constrictions, the small arrow points at a gap in the signal tract, the right larger arrow points at one of the satellites. 2b. FISH on mitotic metaphase chromosomes. The arrows point at the two contracted NOR regions. 2c. FISH on mitotic prometaphase chromosomes. The upper horizontal arrow points at the single contracted NOR, the two vertical arrows point at the contracted satellite (left) and elongated secondary constriction (right) of the lower satellite chromosome. All images are inverted black & white images processed from the original scanned colour slides using computer software.

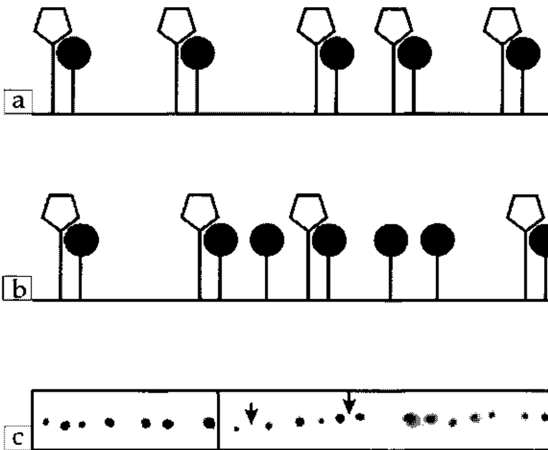


Fig. 2.3. a-b. Schematic overview of FISH 18S (polygons) and IGS (black circles) signals on linear DNA fibres in [a] Lexkesveer sexuals and apomicts and [b] Keyenberg apomicts. Whereas the 18S and IGS probe signals always colocalised on fibres from Lexkesveer dandelions, solitary IGS signals were present between the signals of colocalising 18S and IGS probe pairs on fibres from Keyenberg apomicts. c. black & white converted extended fibre images of Wolf apomicts (left panel) and of Keyenberg apomicts (right panel). Arrows point at solitary IGS probe signals.

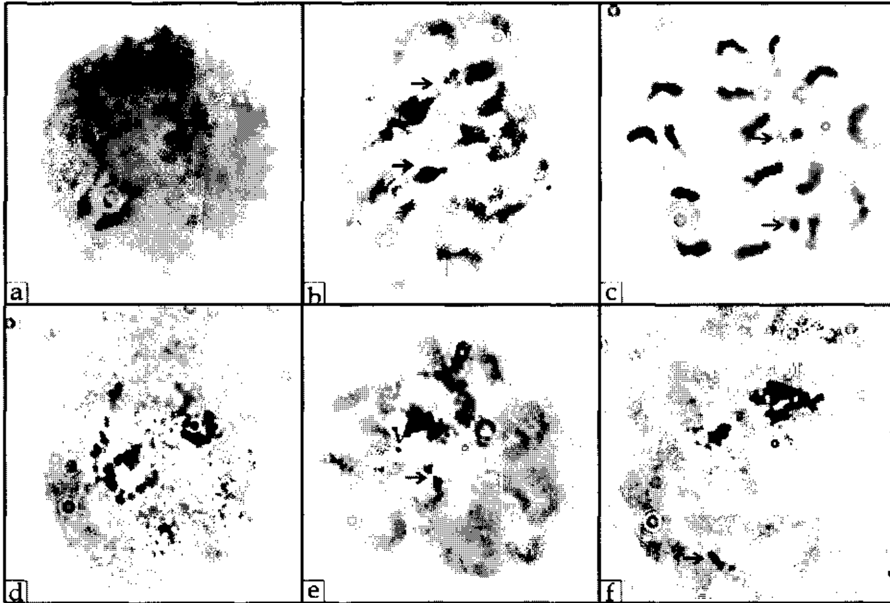


Fig. 2.4. AgNOR staining of active rDNA regions (in black) on mitotic chromosome spreads from diploid sexual (a-c) and triploid apomictic (d-f) dandelions. a. Two active rDNA regions in vegetative root tip cell. b. Mitotic cell at pro(meta)phase. Two extended NORs and two satellites are visible; the upper arrow points at one extended NOR the lower arrow points at one of the two satellites. c. Mitotic cell at a later stage than in b., before metaphase. Chromosomes are more contracted and stain more intensely; the NORs are now less stained. The arrows indicate as in b. d. Three active rDNA regions in vegetative root tip cell. e. Mitotic cell at pro/metaphase. Two NOR regions are extended (upper part) and one is contracted (arrow). f. Mitotic cell at an approx. similar stage as in e. but with more contracted NORs; the arrow points at the most contracted NOR. All images are inverted black & white images processed from the original scanned colour slides using computer software.

Construction of the Keyenberg genomic DNA library in phage lambda

A first screen of the genomic DNA library using a ^{32}P- labeled IGS fragment identified six positive clones out of > 1000 plaques. These six clones were rescreened two times. All six phages contained an insertion homologous to the XhoI-XbaI region probe. The digested DNA fragments were isolated from gel and partially digested using 0.1 units of *Hinf*I restriction enzyme during 10 min. Digested fragments were run on a 0.8% agarose gel, Southern blotted and hybridised with the ^{32}P labelled XhoI-XbaI probe. Some fragment length variation was present between the six clones after *Hinf*I digestion (fig. 2.5).

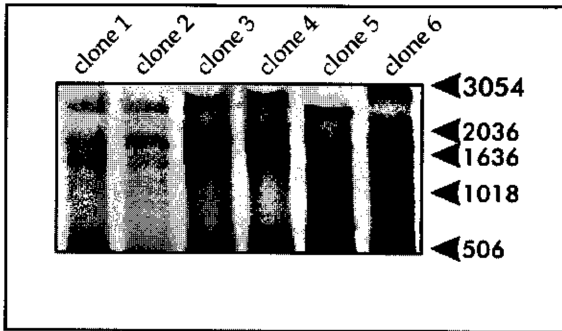


Figure 2.5. Southern blot of lambda phage clones probed with the XhoI-XbaI IGS fragment probe containing the HinfI region; the relevant region showing variation in *HinfI* fragments is shown. Fragment sizes are indicated to the right.

DISCUSSION

This microscopic and molecular study has demonstrated differences in IGS spacer organisation between apomictic and sexual dandelions. Our analysis also revealed variation in IGS spacer organisation within individual dandelions. Variation between apomictic, clonally reproducing dandelions can be explained by assuming that the lineages descended from genetically diverged parents. The observed variation in the Keyenberg apomicts is much more difficult to explain and may reflect the dynamic nature of repetitive sequences, possibly associated with transposable element activity and mitotic recombination (see below).

IGS - FISH signals are only present in the rDNA locus

The FISH images with the 18S and IGS probes to mitotic chromosomes from diploid and triploid dandelions showed that both parts of the 45S rDNA repeats were confined to the secondary constriction of the satellite chromosomes. In complements of the diploid dandelions, we observed two extended fluorescent tracts whereas we observed in complements of the triploid apomicts two extended fluorescent tracts and one compact dot. The FISH images showed an intenser fluorescence signal in the dot compared to the signals in the longer tracts suggesting that the dot contains a high concentration of fluorescent probes. One explanation for the difference in length of the secondary constriction between the satellite chromosomes in the

triploids is that there exists a different number of rDNA units in the respective satellite chromosomes (Lim et al., 2000). It is also possible that the different lengths of the secondary constriction reflect rDNA transcription and nucleolar activity (Sardana et al., 1993; Calderini et al., 1996; Lim et al., 2000). To test if nucleolar activity could be associated with a decondensed state of the NORs, silver staining of root tip cells was performed since silver deposition on nucleolar organiser regions is a measure for transcriptional activity in the nucleolus (e.g. Fernandez-Gomez et al., 1983). The morphology of silver stained mitotic chromosomes corresponded to the images of FISH on mitotic chromosomes; it is possible therefore, that the long vs. short tracts of probe signal in FISH reflect nucleolar activity and are not only caused by different lengths of the NORs. Our observations on the long and short organiser regions are comparable to those of the FISH analysis of rDNA in *Nicotiana tabacum* (Lim et al., 2000). These authors argue that long FISH tracts in the secondary constriction indicate local decondensation of rDNA chromatin which is actively transcribed.

Variable IGS subrepeat organisation in the triploid dandelions

In order to get a better insight into the molecular organisation of the HinfI region contained within the *Xho*I-*Xba*I fragment, we probed the XhoI-XbaI-IGS probe on extended DNA fibres. Tracts of numerous yellow spots were observed in diploid and triploid dandelions demonstrating colocalisation of probe signals on extended DNA fibres. The sequence of the IGS region indicates that the XhoI-XbaI-IGS and 18S probe are separated by ca. 800 bp (Van Dijk et al., in prep.) and colocalisation of probe signals that are less than 1000 bp apart has been found previously in tomato and *Arabidopsis* (Zhong, 1998). Considerable variation in the distance between the adjacent IGS and 18S targets was found in triploid apomictic dandelions and in diploid sexual dandelions. The latter finding was unexpected since Southern blots from genomic DNA probed with a complete IGS and partial 18S sequence did not show that much variation within individual diploid plants (van Dijk 1997 and unpublished data). It may be that the variation in the distances found using the fibre-FISH technique is not random but occurs at regular intervals in the locus and is not visible as such on Southern blots.

On extended fibre preparations from Keyenberg plants, single green IGS signals were observed between the yellow IGS-18S signals. The occurrence of IGS sequences within the stretches of colocalising probe signals most likely indicates duplication of (parts of) the corresponding IGS region. Duplication of IGS subrepeats is corroborated by the Southern blots of Keyenberg plants following *Hinf*I digestion (van Dijk et al.,. unpublished). The hybridisation signals from the *Hinf*I fragments on Southern blots from Keyenberg plants were far stronger compared to the non-Keyenberg plants which suggests that

HinfI fragments were present at much lower quantities in the non-Keyenberg dandelions (van Dijk et al., in prep.). The single 18S signals that were incidentally observed in the Keyenberg plants may indicate an expansion of sequences between the XhoI-XbaI-IGS and 18S regions, a duplication of the 18S gene and surrounding spacer sequences, or a deletion of the HinfI region containing the promoter sequences and transcription initiation site for the 18S gene (van Dijk et al., in prep.; a similar case has been reported in *Drosophila* by Linares et al., 1994).

We found that *HinfI* digestion of the six lambda clones containing IGS sequences gave different restriction fragments, visible after Southern blotting and hybridisation with the XhoI-XbaI IGS probe. Future restriction analysis and sequencing will reveal the subrepeat organisation of the putative IGS sequences. So far, we found only six positive clones. Agarwal and colleagues (1992) found a low number of rDNA-positive clones (less than 1%) in a similar experimental set-up using different phage vectors and speculated (i) that methylation may have hindered *Sau*3A digestion and (ii) that incorporation of functional rDNA units homologous to host rDNA rendered the host bacteria unstable.

Fast sequence divergence of the IGS in apomictic *Taraxacum*

Variation in the numbers and location of IGS subrepeats between species or populations of a species has been recognised as a general phenomenon in all plant families investigated so far (Gründler et al., 1991; Hemleben and Zentgraf, 1994) including *Taraxacum*. Sequence analysis of the *Taraxacum* IGS has shown that five major subrepeats can be distinguished (fig. 2.1; van Dijk et al., in prep.); three subrepeats are within the IGS probe that we used. Two *HinfI* restriction sites are present within the probe region (fig. 2.1), so that any variation in size and numbers of repeat blocks becomes visible via *HinfI* restriction analysis. Variation in numbers of any subrepeat may become apparent in variable lengths of the non-fluorescent regions between the probe signals. Our data on the presence of single *XhoI-XbaI* fragments within the rDNA locus of Keyenberg plants combined with the fact that apomictic dandelions from the Keyenberg had a 7% larger genome size than apomicts from other locations support an increase in copy number of one or multiple subrepeats. Theoretically, occurrence of single green IGS probe signals could have been effected by deletion of the 18S gene but this is less plausible; loss of rRNA genes has not been reported in the literature as far as we know.

It is not known what has brought about all the variation in the rDNA clusters of dandelions. Several mechanisms, some of which require ectopic meiotic recombination, have been implicated in creating rDNA variation. One conceivable explanation is activity of transposable elements (Fedoroff, 1979; Richards, 1989; Da Rocha and Bertrand, 1995). When Ty3/*gypsy* and *copia*

retrotransposon sequences from sunflower (*Helianthus*, Compositae) were aligned with the *Taraxacum* IGS sequence, four regions with 40-45% sequence similarity to the retrotransposon sequences were found (not shown). A second mechanism generating repeat expansion is mitotic recombination (King and Schaal, 1990). Intra-strand annealing of inverted repeats, which has the potential of inducing formation of larger fold-back palindromic regions, may induce recombination (Qin and Cohen, 2000). Both short inverted repeats and large fold-back hairpins are present in the *Taraxacum* IGS (van Dijk et al., in prep.). It is attractive to assume that non-meiotic events like transposition and intra-strand annealing did contribute to the IGS variation in the asexually reproducing dandelions.

Intrachromosomal processes may modulate rDNA variation

It is difficult to envision how meiotic recombination could drive concerted evolution in plant rDNA because little if any chromosome pairing occurs in the rDNA locus during the first meiotic prophase in plants (Albini, 1994; Sherman & Stack, 1995) including dandelion (own unpublished results). Absence of chromosome pairing makes homogenisation by meiotic recombination an unlikely event. Rather than meiotic recombination, mitotic gene conversion and unequal crossing-over may be responsible for IGS homogenisation. Evidence for mitotic unequal crossing-over generating IGS subrepeat variability has been found in an asexual endophyte (Ganley and Scott, 1998).

Both meiotic and mitotic recombination can disrupt rDNA units by ectopic pairing of subrepeats within the IGS (*e.g.*, the 100 bp subrepeat in fig. 2.1). Mitotic gene conversion at predefined positions (hotspots) would be a very effective way to drive concerted evolution of rDNA, with less risk for disruption of the tandemly repeated units. Such hotspots seem to exist; the mitotic recombination hotspot HOT-1 in yeast IGS is located at the 3' side of yeast IGS and promotes mitotic gene conversion of very long (more than 75 kbp) rDNA tracts (Voelkel-Meiman and Roeder, 1990). A DNA sequence with a similarity of ca. 40% to the HOT-1 sequence is present in a homologous region of the *Taraxacum* IGS (not shown). It is tempting to speculate that this *Taraxacum* IGS region might have an analogous function.

Several authors have postulated that rearrangements within individual chromosomes homogenise rDNA sequences. In *Arabidopsis*, rRNA gene variants occur clustered at specific locations in the rDNA cluster (Copenhaver and Pikaard, 1996). These variants presumably spread in the rDNA arrays by a mechanism involving slipped-mispairing of the tandemly arranged rDNA units followed by crossing over. Slipped-mispairing results in the spread of new variants near the site where they came into existence. Such a mechanism has been postulated in bacteria (Lan and Reeves, 1998) and in yeast and

Arabidopsis (discussed in Copenhaver and Pikaard, 1996). *In Drosophila,* interbreeding populations are homogeneous for different rDNA variants (Schlötterer and Tautz, 1994). This indicates that novel variants are homogenised within chromosomes by intrachromosomal recombination or gene conversion and that recombination between homologues is very rare. These mechanisms may also take place in *Taraxacum* (King and Schaal, 1990). The fact that rDNA variation in apomicts is at least as large as in sexual dandelions (van Dijk, 1997) suggests that similar mechanisms mediate and spread variation in both sexual and apomictic dandelions.

ACKNOWLEDGEMENTS

We are grateful to Professor Sacco de Vries for providing the facilities to construct and screen the lambda genomic DNA library in his laboratory and to Marijke Hartog for technical assistance. We want to express our gratitude to Dr. Paul Fransz for his expertise during the optimisation of the preparation method for extended DNA fibres and valuable discussions on the experimental data.

MATERIALS AND METHODS

Plant materials

The sexual diploid plants (2n=2x=16) Wolf 2 and Wolf 8 and natural triploid apomicts Wolf 3 and Wolf 11 were sampled from a mixed population in natural meadows on alluvial clay soils alongside the river Rhine near Wageningen, the Netherlands. We also used triploid apomicts (Kb7-2-1 and Kb7-2-2), which were sampled from a sandy soil near the "Keyenberg" estate in Renkum, the Netherlands (van Dijk, 1997).

To induce flowering, plants were vernalised in a cold room for at least three months at 4 °C and 70-80% relative humidity, and then reared in the greenhouse at a day/night regime of 16 hr 18 °C and 8 hr 16 °C. Flowers were used as a source for high-grade genomic DNA.

DNA isolation, restriction enzymes, Southern blotting

Nuclear DNA was isolated from yellow petals just prior to anthesis following the protocol of Rogstad (1992). Protocols for the rDNA-RFLP analyses were described in Van Dijk, 1997. The rDNA fragment that was used as probe was originally termed pTEE5 (King, 1993).

Construction of the genomic DNA library

Genomic DNA from a plant containing the HinfI ladder (progeny from the apomict Kb7-2) was partially digested with *Sau*3A and packaged in the Lambda FIX II vector (Stratagene) according to the manufacturer's instructions. Inserts were cut with *Hinf*I, fragments separated on 0.8% agarose gels and transferred to a Hybond-N nylon membranes (Amersham Pharmacia, Uppsala, Sweden) by vacuum blotting following the procedures recommended by the manufacturer (Pharmacia LKB-VacuGene XL, Amersham Pharmacia, Uppsala, Sweden). The XhoI-XbaI probe was labelled using the Random Primers Labeling system (Life Technologies Inc., Rockville, USA) including 20 µCi α^{32}P-ATP (Amersham, Buckinghamshire, England). Southern hybridisation was performed essentially according to Sambrook et al. (1989); posthybridisation washes were performed at 65 °C in 0.2xSSC, 0.1%SDS two times for half an hour each.

Chromosome preparations

Mitotic chromosome spread preparations from root tip cells were prepared using the protocol from Pijnacker and Ferwerda (1984). For FISH experiments, cytoplasmic protein residues were removed by incubation of the slides in 200 µl 60 % acetic acid at 46 °C for 3 min, followed by rinsing the slides in a freshly prepared ice-cold mixture of ethanol : acetic acid 3 : 1.

Nuclei isolation

Five grams of young leaves were pre-chilled for at least 3 h at 0 °C. Nuclei were isolated in 100 ml of buffer containing 50 mM Tris pH 8, 5mM EDTA pH8, 0.35 M sorbitol, 0.1% Bovine Serum Albumin (BSA), 15 mM β-mercapto-ethanol, 1 mM spermidine and 1 mM spermine by blending 2 x 5 sec in a Waring blender set at highest speed (Kemble, 1986). The homogenate was filtrated by sequential passage through 200, 120 en 50 micron filter on 100 ml beakers, chilled on ice. The filtrate was spinned down at 1200 rpm for 10 min at 4 °C and the pellet was resuspended in 300 µl nuclei isolation buffer, together with a similar volume of sterilised glycerol. Nuclei could be stored at -20 °C for 3-4 months.

Preparation of extended DNA fibres

The method for preparing extended DNA fibres is described in detail in Fransz et al., 1996. In short, the technique involves attachment of isolated nuclei (see above) to glass slides, lysis of the nuclei in a buffer, and stretching of the nuclear DNA, achieved by tilting the slides at a slight angle. For lysis of nuclei and stretching of DNA fibres, 30 instead of 10 µl of nuclei lysis buffer STE2 was used.

Probe labelling

The following probes were used (fig 2.1): *Taraxacum* probe pTEE5, spanning the complete IGS and 5' part of the 18S gene (King, 1993); probe pRSR2, a 1 kbp EcoRI fragment of the 5' side of the 18S ribosomal gene of soybean (Eckenrode et al, 1985), and the 1530 bp *XhoI-XbaI* fragment (containing the HinfI region) subcloned from probe pTEE5 into the pBluescript vector (Boehringer). For Southern blotting and screening of the genomic library, probe pTEE5 and the XhoI-XbaI fragment were labelled with ^{32}P in a standard random priming reaction. The FISH DNA probes were labelled with biotin-16-dUTP or digoxigenin (DIG)-11-dUTP in a standard nick translation assay (Boehringer) or random priming method (High Prime, Boehringer) according to the manufacturer's instructions.

Fluorescence *in situ* Hybridisation (FISH)

FISH was essentially performed as described by Fransz et al. (1996) with some modifications. The DNA probes (1.0 ng/µl for metaphase chromosomes, 0.1 ng/µl for extended DNA fibres) were boiled for 3 min prior to hybridisation. For hybridisation to extended DNA fibres, salmon sperm DNA was not needed. The DNA fibres on the slides were denatured at 82 °C for 2 min.

AgNO3 staining of active rDNA sites

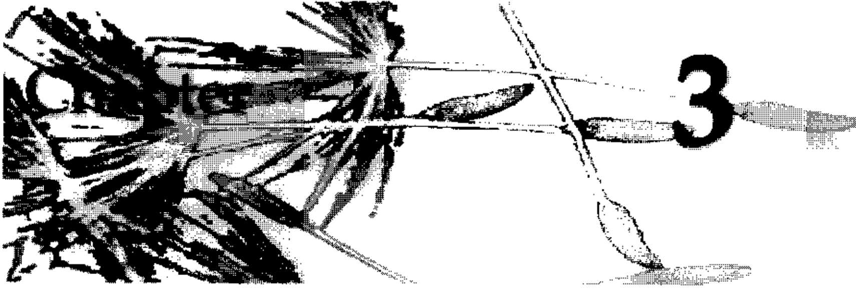
In order to assess transcription of ribosomal genes in the nucleolus, chromosome spread preparations from root tip cells (see above) were incubated in a 50% solution of $AgNO_3$ at 60 °C for 55 min in a Petri dish on a moistened filter paper, 0.2 ml $AgNO_3$ was added to each slide and a piece of nylon mesh (size 300 micron) was placed on the slide to achieve an even silver staining across the chromosome spread preparation (modified after Stack et al., 1991). After incubation, slides were washed 4 times in distilled water, air-dried and mounted in Entellan (Merck).

Flow cytometry

Protocols for measurement of DNA content using flow cytometry and the calibration of the measured DNA contents using chromosome counts are described in Tas and van Dijk, 1999.

Image visualisation

FISH signals were observed under a Zeiss Axioplan microscope equipped with epifluorescence illumination and Plan-Neofluar optics. Selected images were photographed on 400 ISO colour negative films using filters for DAPI, FITC and Texas Red fluorescence. Negatives were scanned at 1000 dpi and the digital images were optimised for contrast and brightness using Adobe Photoshop ® (Adobe, CA). $AgNO_3$ stained preparations were studied using a Nikon Optiphot microscope with Fluor optics. Images were photographed on Ektar 25 colour negative films, and selected images were digitised as described above.



Meiotic recombination in sexual diploid and apomictic triploid dandelions (*Taraxacum officinale* L.)

Peter van Baarlen, Peter J. van Dijk, Rolf F. Hoekstra, and J. Hans de Jong

Summary

Taraxacum officinale L. (dandelion) is a vigorous weed in Europe with diploid sexual populations in the southern regions and partially overlapping populations of diploid sexuals and triploid or tetraploid apomicts in the central and northern regions. Previous studies have demonstrated unexpectedly high levels of genetic variation in the apomictic populations, suggesting the occurrence of genetic segregation in the apomicts and/or hybridisation between sexual and apomictic individuals. In this study we analysed meiosis in both sexual diploid and apomictic triploid plants to find mechanisms that could account for the high levels of genetic variation in the apomicts. Microscopic study of microsporocytes in the triploid apomicts revealed that the levels of chromosome pairing and chiasma formation at meiotic prophase I were lower than in that of the sexual diploids, but still sufficient to assume recombination between the homologues. Nomarski DIC microscopy of optically cleared megasporocytes in the apomicts demonstrated incidental formation of tetrads, which suggests that hybridisation can occur in triploid apomicts.

Genome 43, 1-9 (2000)

INTRODUCTION

The species complex *Taraxacum officinale* L., the common dandelion, can be divided into the group of self-incompatible diploids (2n=2x=16) and of polyploid apomicts. The diploids have sexual reproduction and predominate in the central and southern parts of Europe, whereas the polyploids, mainly triploids, reproduce apomictically and are not only distributed in the southern and central parts of Europe where they overlap with sexual populations but also in the northern areas (Menken et al, 1995; Den Nijs and Sterk, 1980).

Meiosis in the sexually reproducing diploids is regular. At prophase I, chromosome pairing is saturated resulting in the formation of 8 bivalents at metaphase I followed by normal segregation of half-bivalents and chromatids at anaphase I and II, respectively. The four gametes are balanced with 8 chromosomes each. In the triploid *Taraxacum* apomicts, however, strongly reduced chromosome pairing lead to mainly univalents at metaphase I (Gustafsson, 1932, 1935a,b; Battaglia, 1948) and hence, to unequal chromosome segregation and gametes with variable chromosome numbers. An efficient way to avoid formation of unbalanced gametes and sterility is first division restitution (cf. Rosenberg, 1927), described for *Taraxacum* by Gustafsson (1932, 1935b). First division restitution, which involves circumvention of the first meiotic (reductional) division, results in the formation of dyads with unreduced chromosome numbers (24 in the triploids) and hence, effectively avoids sterility. In *Taraxacum*, this meiotic diplospory is common in the megasporocytes, resulting in unreduced embryo sacs with triploid egg and polar nuclei, but is rare in the microsporocytes (Gustafsson, 1932; Battaglia, 1948). Meiotic recombination is supposedly negligible and its absence explains the uniformity in the offspring of individual meiotic diplosporic apomicts.

Dandelions can be considered as obligate apomicts which means that both a viable embryo as well as a functional endosperm are formed with no prior fertilisation (Gustafsson, 1932; Malecka, 1971; Battaglia, 1963; Nogler, 1984), although occasionally facultative apomixis has been reported (discussed in Jenniskens et al., 1984). As apomicts are presumed to produce genetically identical offspring, low levels of genetic variation are expected. Paradoxically, morphological variation and aneuploidy (Sørensen and Gudjónsson, 1976), and differences between plants in pollen grain size and morphology (Chiguryaeva, 1946) are indicative for genetic variability. More recent studies with isozyme or allozyme markers (Menken et al, 1995) and DNA markers (King, 1990; King and Schaal, 1993; Van Dijk, 1997; Van der Hulst et al., 2000) revealed large genetic variation within populations of apomictic dandelions. There are several possible explanations for such high clonal diversities. When diploid sexuals and polyploid apomicts are sympatric, haploid sexual egg cells of diploids may be fertilised by diploid pollen from triploid or tetraploid apomicts, thus producing novel triploid apomictic lineages by hybridisation.

This has been demonstrated experimentally (Malecka, 1971; Tas and Van Dijk, 1999) and the sharing of rare allozymes in mixed sexual-apomictic populations (Menken et al., 1995) strongly suggests that this process also occurs in nature.

High clonal diversity in regions where sexuals are absent (King 1993; van der Hulst et al. 2000) is more difficult to explain. King (1993) suggested that new apomictic lineages originated by hybridisation between sexual and apomictic populations in the sympatric regions and migrated into the purely apomictic regions. However, genetic variation could also arise *de novo* in the strictly apomictic regions in absence of sexual diploids by autosegregation (Gustafsson 1932, 1935b). Autosegregation includes two processes (see Rutishauser, 1967; Nogler, 1984) that have the potential of generating genetic variation in asexual organisms: chromosome gain or loss and subsexual reproduction. Subsexual reproduction (Darlington, 1937) involves crossing-over between a heterozygous locus and the centromere without reduction, leading to homozygosity of genes distal of the crossovers. Sørensen and Gudjonsson (1946) found in extensive cultivation experiments of apomictic lineages occasionally morphologically deviant offspring. Part of their aberrants were due to loss of chromosomes. Other variants had the normal triploid complement of 24 chromosomes. Darlington and Mather (1952, pp. 265-268) suggested that this type of variation was caused by subsexual reproduction. In the Taraxacum type of apomixis where the first meiotic prophase is initiated so that recombination can occur (though the first reductional division does not take place) subsexual reproduction is a potential source of variation. In contrast, in other types of apomixis such as mitotic diplospory or apospory (see Nogler, 1984) the first meiotic prophase is absent so that subsexual reproduction can not occur.

Although several reports appeared on the course of meiosis in apomicts (reviewed in Rutishauser, 1967; Nogler, 1984), detailed numerical information on pairing and recombination are scarce (see Malecka, 1971 for *T. officinale*). These processes were quantified in the triploid apomicts to see if first division restitution leading to autosegregation (more specific, subsexual reproduction) could contribute to genetic diversity in the triploid apomictic populations. Ideally, meiotic processes including pairing and recombination would be quantified in the megasporocytes since only female meiocytes are relevant in obligate apomicts. However, analysing large numbers of female gametes is extremely laborious and time-consuming. Consequently, we did most of the microscopic study on pairing and recombination in spread preparations of microsporocytes. Meiotic processes are essentially the same in male and female meiosis although megasporogenesis is generally characterised by slightly higher crossover values and/or differentially located chiasmata (Callan and Perry, 1977; De Vicente and Tanksley, 1991; Koduru and Rao, 1980; Van Ooijen et al., 1994). We quantified the extent of chromosome pairing in

nuclei at pachytene, counted chiasmata and chromosome associations, and analysed chromosome segregation at later stages. We decided to estimate pairing on chromosome spread preparations rather than using electron microscopic observations of synaptonemal complexes in order to be able to distinguish between heterochromatic and euchromatic areas. Chromosome spread preparations allowed us to establish if there was a preference for pairing in euchromatic vs. heterochromatic regions. To assess the effect of triploidy on the course of meiosis, we analysed meiosis in synthetic non-apomictic triploids generated from backcrossing colchicine-induced sexual tetraploids and diploid sexuals. Moreover, meiosis was analysed in triploid hybrid offspring of sexual diploids X apomictic triploids in order to see if meiotic restitution can be transmitted to a sexual background. We applied Nomarski Differential Interference Contrast microscopy of cleared female gametophytes to see if meiotic recombination does occur during megasporogenesis and to see if reduced embryo sacs are formed in natural apomicts.

RESULTS

Chromosome numbers

All dandelions used in this study had the previously published chromosome numbers (e.g. Gustafsson, 1935b) of 2n=2x=16 for the diploid sexuals and 2n=3x=24 for the apomictic and synthetic triploids.

Microsporogenesis in the sexual diploids

Microscopic studies of microspore mother cells in the diploid plants confirmed the previously reported normal meiosis. Chromosome pairing at pachytene of the eight sets of homologues was complete, except for the NOR and adjacent satellite regions of satellite chromosomes. The distal chromosome regions were largely euchromatic except for the satellite region (the darkly stained curved region in the upper part of the configuration; Fig. 3.1a). At diplotene ring and rod bivalents appeared (Fig. 3.1g) showing 12-14 chiasmata (12.7 ± 1.0) per complement. The eight bivalents congressed and disjoined at metaphase I – anaphase I, giving rise to equal chromosome numbers of 8+8 chromosomes (figure 3.1h,k). At anaphase II equal segregation of the chromatids (figure 3.1k) produced tetrads with four regularly shaped microspores.

Microsporogenesis in the triploid apomicts

Chromosome pairing at pachytene was strongly reduced in the triploid apomicts (23% on average, see fig. 3.1b, table 3.1) and mainly confined to the distal euchromatic regions. In meiocytes at diakinesis chiasma formation was significantly lower compared to the diploids, with 3.4 ± 1.5 bivalents with 3.8 ± 1.7 chiasmata and 0.2 ± 0.4 trivalents with 0.4 ± 0.9 chiasmata per triploid complement (fig. 3.1e, table 3.1). At metaphase I univalents, bi- and trivalents were scattered over the spindle in the cytoplasm (fig. 3.1j). At anaphase II chromatid division progressed regularly. The random distribution of chromosomes during the first meiotic division and the equal distribution during the second meiotic division resulted in tetrads with four meiotic cells of different chromosome content and size. Pollen grains also differed in size (Fig. 3.2e). A proper quantification of the distribution of chromosome numbers per pollen grain was not possible since we found only eight telophase II images where we could count individual chromosomes. First division restitution was observed in 0.1-1% of the 300-500 microspore mother cells per slide. These meiotic cells are supposed to produce unreduced triploid microspores. Nomarski microscopy showed that 60-70% of the microspores (1000-2000 visually inspected per flower) have no cell content shortly after the tetrad stage. This corresponds reasonably well with the analysis of stained pollen which suggested that 1-10% of the microspores per flower (ca. 200 grains counted per flower) were fertile. Dyads, which were rarely observed in the Nomarski preparations of the natural apomicts (usually under 1% dyads observed in anthers of Lexkesveer and Keyenberg apomicts but up to 10% in intact anthers of one Keyenberg apomict; fig. 3.2 c-d), showed no signs of cell degeneration and were considered able to germinate upon pollination. Unreduced pollen grains could not reliably be identified in the standard spread preparations, so their fertility could not be assessed by pollen fertility staining.

Microsporogenesis in synthetic triploids

In the non-apomictic triploids obtained from crosses of diploid dandelions with colchicine-induced tetraploids, we observed that chromosome pairing at meiotic prophase was in the range of 20-47% (33.0 ± 13.7, see table 3.1 and Fig. 3.1c), significantly higher than in the natural apomicts. Pairing occurred mainly in distal euchromatic chromosome regions as in the triploid apomicts but was also found extending into the intermediary, more heterochromatic regions. Metaphase I complements showed 4.8 ±1.5 bivalents with 5.2 ± 1.8 chiasmata, also significantly higher than in the natural apomicts, and 0.4 ± 0.7 trivalents with 0.8 ± 1.3 chiasmata per meiotic cell. In over 90% of the microspore mother cells, chromosomes at metaphase I failed to orient in the equatorial plane and random distribution comparable to that observed in the natural apomicts was apparent. In less than 10% of microspore mother cells, a meta-

phase plane orientation was apparent (fig. 3.1i). Accurate chromosome counts in cells at anaphase I or telophase I were only possible in 20 nuclei. Two showed a 16:8 distribution, the rest were all unbalanced.

Microsporogenesis in the hybrid dandelions

Microscopic analysis of the microsporocytes of the triploid progeny of sexual x apomict crosses (described in Tas and van Dijk, 1999 and Van Dijk et al., 1999) showed that microsporogenesis is predominantly reductional. The most conspicuous difference in meiosis between the natural dandelions and the hybrid triploids was that pairing in the hybrids was significantly less saturated (32 ± 27%) than in natural sexual diploids, and ca. 10% higher (not significant) than in the natural triploid apomicts. Figure 3.1d shows a well-paired pachytene configuration in one hybrid triploid genotype; note the lightly staining euchromatic distal regions (except for the satellite arm in the upper part of the configuration) and the similarity in distribution of heterochromatic knots alongside the paired chromosome arms. In other meiocytes with less saturated pairing, pairing was again mainly observed in the distal chromosomal regions. Although a high capacity for pairing and bivalent formation were occasionally observed (fig. 3.1d,f), microspore mother cells showed random distribution of chromosomes at anaphase I and regular segregation of chromatids at the second meiotic division (fig. 1l). At metaphase we observed 4.3 ± 2.5 bivalents with 4.5 ± 2.9 chiasmata and 0.075 ± 0.3 trivalents with 0.2 ± 0.5 chiasma per meiotic cell (note the low numbers of meiocytes analysed; table 3.1). Restitution nuclei were observed with a frequency of under 0.5% per microsporogenesis preparation in all examined hybrids.

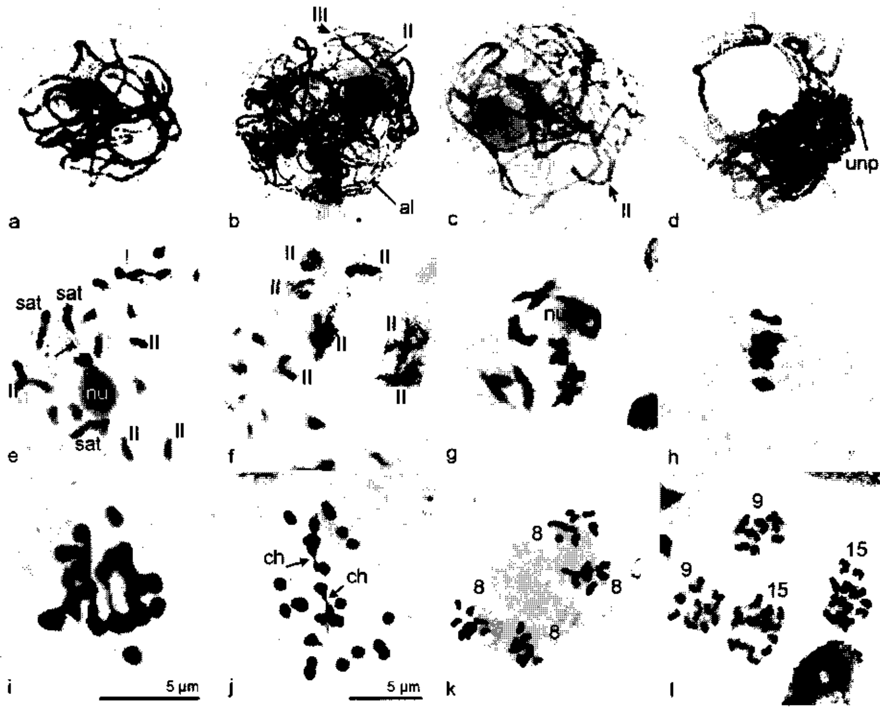


Fig. 3.1. Chromosome morphology, pairing, bivalent formation and segregation in microsporocytes of *Taraxacum officinale* L. a. Full pairing of all bivalents in a sexual diploid plant. b. Incomplete chromosome pairing in an apomictic triploid plant. c. Incomplete pairing in a synthetic triploid (colchicine tetraploid X diploid). Note that most parts of the chromosome arms remain unpaired. d. Pachytene complement of a triploid hybrid obtained from the cross diploid sexual X triploid apomict. Most parts of the chromosome arms are paired. e. Diakinesis in natural triploid. f. Diplotene (diffuse) in triploid hybrid. g. Diplotene in sexual diploid. h. First metaphase in sexual diploid. i. First metaphase in synthetic triploid. j. Anaphase in natural apomict. k. Balanced equational division in sexual diploid. l. Unbalanced equational division in hybrid triploid.
In figs. b-c, II indicates pairing and bivalents in e, f; III in b indicates triple pairing. In fig. d, unp indicates the unpaired regions. In fig. e and g, nu indicates the nucleolus and Sat indicates the satellite (NOR) chromosomes. The stopped arrow in e indicates the extended NOR region. In fig. j, ch indicates segregating bivalents. The numbers in the figs. k and l indicate the chromosome numbers.

Figures a-c and e, g-k are images of Giemsa stained chromosome spread preparations; d, f and l are obtained from DAPI stained spread preparations. The fluorescent images have been digitally inverted. Bars represent 5 µm.

The analysis of megasporogenesis

Nomarski DIC microscopy of the methyl salicylate-cleared megaspore mother cells of the sexual diploids showed that meiosis was regular (not shown) with balanced chromosome numbers at anaphase I and II. We could not quantify bivalent formation and chiasma numbers in these preparations.

In the cleared flowers of the natural apomicts, we could analyse female gametophytes from diplotene up to the end of megasporogenesis. Bivalents in the cells at diplotene and metaphase I displayed comparable low chiasma numbers as in the microsporocytes (fig. 3.2a,b). Trivalents were not observed. Chromosomes segregate randomly but meiotic restitution avoided the formation of unbalanced gametes. By the end of megasporogenesis, more than 99% (372 out of 375) of the megaspores were unreduced.

DISCUSSION

The comparisons of meiosis in the sexual, apomictic, hybrid and synthetic triploid dandelions have shown that triploidy is likely the primary determinant of pairing disturbances in triploid *Taraxacum*. The strongly reduced pairing explains the strong decrease of chiasmata in the triploids and the high numbers of univalents. We also observed a conspicuous difference between male and female sporogenesis: male meiosis is predominantly reductional leading to over 90% of unfertile gametes, whereas female meiosis effectively avoids sterility by first division restitution leading to high fertility of apomictic dandelions.

Meiosis in the diploid and triploid dandelions

Meiosis in the diploid sexual dandelions essentially follows the regular course that is described for higher plants. Meiocytes and prophase I feature full pairing and normal chiasma formation with 8 bivalents and 12.7 chiasmata at diakinesis – metaphase I, followed by the reductional division of the homologous chromosomes and the segregation of chromatids yielding 4 microspores with 8 chromosomes. Megasporogenesis was reductional as well and yielded only tetrads of megaspores (Nomarski images not shown).

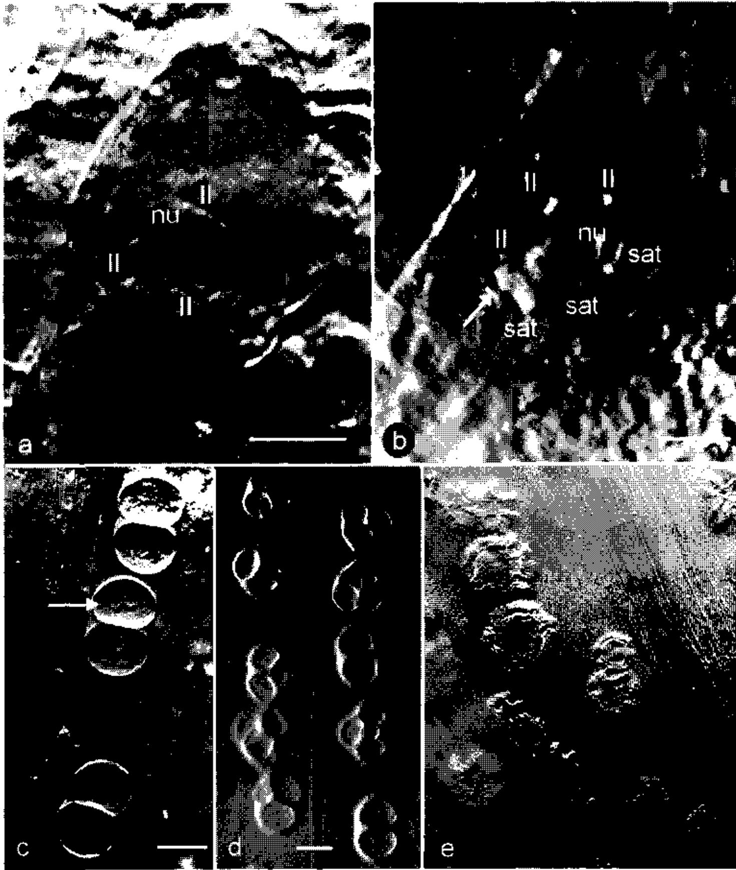


Fig. 3.2. Bivalent formation in megasporogenesis and dyad, tetrad and pollen grain morphology in microsporogenesis in triploid apomicts. a. Early diakinesis in natural apomict. b. Late diakinesis in natural apomict. c-d. Dyads and tetrads at the end of natural triploid microsporogenesis; arrow in c points at unreduced microspore with mitotic nucleus. e. Pollen size variation in natural apomicts. In figs. 2a and 2b, II indicate bivalents, nu indicates the nucleolus and Sat indicates the satellite (NOR) chromosomes. The stopped arrow in 2b indicates the extended NOR region. Figures 2a-e are Nomarski images. Not all chromosomes are in microscope plane. Note similarity in chromosome configuration and morphology in Fig. 1e and 2b. Bars represent 10 μm.

In the triploid apomicts, the strong reduction of chiasmata and bivalent formation compared to the diploids resulted in the random distribution of chromosomes during the first meiotic division and unbalanced microspores.

Comparable low figures were shown for the synthetic and hybrid triploids but it is of interest that the synthetic triploids showed significantly higher pairing percentages, bivalent and chiasma formation than the natural triploids. Few microspore mother cells in the hybrid and synthetic triploids had near-regular metaphases with relatively few univalents (fig. 3.1f,i) indicating differences in the regulation of meiotic pairing and recombination between the natural apomictic triploids and the synthetic and hybrid triploids. The fact that pairing percentages, bivalent and chiasma formation did not significantly differ between natural apomicts and the triploid hybrids also suggests genetic differences between natural apomicts and hybrids on one hand and synthetic triploids on the other hand (table 3.1). Pairing in all triploids was mainly confined to the distal chromosome regions that are largely euchromatic although differences in the organisation of the (darker staining) heterochromatic regions are visible between homologue sets (fig. 3.1a,c,d). At the moment, there is no clear visible evidence suggesting that large-scale chromosomal rearrangements are responsible for the low pairing capacity in triploid apomicts. Although genetic background may influence the regulation of meiotic processes it is tempting to argue that triploidy is the main determinant of reduced chiasmata and bivalent formation in the triploids. In this context, our unpublished observations on male meiosis in tetraploid hybrids are of special interest. These tetraploids result from fertilisation of a sexual egg with an unreduced pollen grain from a natural triploid apomict (Tas and van Dijk, 1999). Analysis of chromosome spread preparations revealed that ca. 90% of the microspore mother cells displayed 16 bivalents. Nomarski analysis of the anthers showed that the pollen grains were of similar size. The majority of the pollen grains from these tetraploid hybrids were regularly shaped and fertile when used in crossings. Ploidy analysis of the progeny from such crosses demonstrated that the pollen from these tetraploid hybrids were reduced and diploid. This relationship of ploidy levels and production of balanced gametes will be subject of further studies using more tetraploid hybrid materials.

An alternative explanation for the diminished chiasma formation in the apomicts and synthetic and hybrid triploids is that regulation of meiosis-specific or cell-cycle genes is disturbed in a triploid background or that the amount of gene product is not in balance with the chromosome number. In the tetraploid background, either gene expression, the balance between gene product/chromosome number or a combination would then be restored to the diploid level allowing proper chromosome synapsis and bivalent formation.

The use of methyl salicylate clearing with Nomarski microscopy enabled us to reliably establish stages and chromosome morphology in cells at diplotene and later stages of megasporogenic development. Eight megaspore mother cells near diplotene were studied and revealed that bivalents occurred to a comparable extent as in microsporogenesis (fig 3.1m,n). As outlined in the in-

troduction, male and female meiosis are supposed not to differ fundamentally and only slightly in their numbers and location of chiasmata. The most significant difference between male and female meiosis in *Taraxacum* is that the male meiosis is usually reductional, whereas nearly all megaspore mother cells undergo first division restitution. Only three out of the 375 megaspore mother cells in the natural apomicts formed tetrads, and so were considered to undergo reductional meiosis. The occurrence of meiotic restitution was not analysed in the synthetic triploids but there is indirect evidence that meiosis is reductional rather than restitutional in both megasporogenesis. This evidence comes from the finding that when the synthetic triploids were pollinated with haploid pollen from sexual diploids, the resulting progeny were very low in numbers, vitality and fertility (P. van Dijk, unpublished data). This indicates that most egg cells of synthetic triploids were unbalanced and sterile. In the hybrids, restitution was more common but we will present a detailed cyto-embryological analysis of hybrid dandelions in a coming paper.

Evolutionary consequences of apomixis in *Taraxacum*

Hybridisation in completely apomictic populations requires that fertile pollen and reduced egg cells are produced by natural apomicts. Fertility assays, based on a vital pollen staining and studies of cleared whole-mount sporogenic tissue indicated that up to 10% of the pollen grains was fertile. Functional evidence came from crosses of diploid sexual mothers with apomictic pollen donors that resulted in hybrid progeny with diploid and higher ploidy levels (Tas and van Dijk, 1999). The second requirement for hybridisation, the formation of reduced egg cells, was also met. Nomarski microscopy revealed low frequencies of reductional meiosis in apomicts. The reduced egg cells that need fertilisation for embryo development (Malecka, 1973) can be fertilised by pollen grains from apomicts and so, produce novel apomictic lineages, without involvement of sexual diploids. As male meiosis in the triploid apomicts generally results in unbalanced gametes, fertility of reduced egg cells is expected to be very low. Nevertheless, at evolutionary time scales a considerable clonal diversity could be generated. These observations support the likeliness of genetic exchange to occur between sexual and apomictic dandelions and, to a lesser extent, among apomicts in natural populations.

Another potential source for genetic variation within apomictic *Taraxacum* lineages is subsexual reproduction as described by Darlington (1937; see also Rutishauser, 1967). The fact that homologous recombination occurs during female meiotic prophase I and is followed by restitution and equational segregation of chromatids suggests that subsexual reproduction can occur. The significance of subsexual reproduction for generating genetic variation is at this moment uncertain. It is noteworthy that the distal euchromatic regions contain most of the chiasmata (e.g. Fig. 3.1a,d) indicating that reciprocal re-

combination followed by chromatid segregation can generate and transmit new allele combinations of functional genes. However, the resulting crossing-overs in unreduced gametes lead to homozygosity at all loci distal of the cross-over. It is generally assumed that one of the major advantages of apomixis over sexual reproduction is the fixation of heterozygosity and heterosis (Rutishauser, 1967; Asker and Jerling, 1992). Therefore, the homozygosity that is the result of subsexual reproduction may have a negative effect on fitness. Our data demonstrate that in the triploids, chromosome recombination and thus subsexual reproduction is considerably lower than in the diploids and tetraploids. Interestingly, triploidy is common in apomicts with the Taraxacum or Ixeris type of meiotic diplospory that have the capacity to undergo meiotic recombination (e.g. *Taraxacum, Chondrilla, Arabis*), whereas tetraploidy (or higher) is common in apomicts with the mitotic or Antennaria type of diplospory (e.g. *Antennaria, Tripsacum*) and in aposporous apomicts (e.g. *Hieracium, Ranunculus, Pennisetum*) (Nogler, 1984; Asker and Jerling, 1992) where meiotic recombination is completely absent. The fact that triploidy is mainly found in the meiotic diplosporous genera suggests that apomicts with the capacity for subsexual reproduction are selected for avoidance of meiotic recombination and therefore are selected for a triploid ploidy level.

But subsexual reproduction in apomictic lineages may be advantageous in the sense that it limits accumulation of deleterious mutations. As only one of the two megaspores survives, there is a probability of 1/2 that deleterious alleles end up in the degenerating megaspore. In other words, segregation of chromatids where one set is discarded in the degenerating megaspore and one set is transmitted to future offspring through the surviving megaspore allows purging of deleterious alleles. Even segregation of heterozygous non-recombinant chromatids has the potential of generating genetic variation in populations (Antezana and Hudson, 1997a,b). Altogether, subsexual reproduction may have a substantial impact on genetic variation in apomictic populations and could increase the evolutionary life-span of apomictic *Taraxacum* lineages but its potential effect must be modelled first using the available *Taraxacum* data. We will exploit these aspects in a subsequent study.

This study has shown that studying apomixis in natural plants can shed light on the complex way apomixis functions. Crossing sexuals with apomicts has demonstrated that genes mediating a restitutional meiosis can be transmitted to the progeny (Van Dijk et al., 1999). The fact that these genes from apomicts function in a sexual background allows us to characterise them in further experimental work. Study of transmission of apomixis in sexual x apomict crosses is not only of fundamental interest but also yields information that is useful to the seed industry in their attempts to introduce apomixis in sexual crops, since the Taraxacum type of apomixis has been considered closest to reproduction in sexual species (Khokhlov, 1976, p. 14).

ACKNOWLEDGEMENTS

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Table 3.1. Pachytene pairing, bivalent, trivalent and chiasmata formation compared between different plant materials

plant type	number of pro-phase nuclei	pairing (%)	number of diakinesis-metaphase nuclei	univalents per cell	bivalents per cell	Trivalents per cell	number of chiasmata[5] in bivalents	number of chiasmata[5] in trivalents
diploids[1]	56	98.4 ± 3.5[a]	120	0	8 ± 0[a]	0	12.7 ± 1.0[a]	0
apomictic triploids[2]	32	23.1 ± 17.1[b]	120	16.6 ± 3.4	3.4 ± 1.5[b]	0.2 ± 0.4	3.8 ± 1.7[b]	0.4 ± 0.9
colchicine triploids[3]	20	33.0 ± 13.7[c]	90	13.2 ± 3.2	4.8 ± 1.5[c]	0.4 ± 0.7	5.2 ± 1.8[c]	0.8 ± 1.3
hybrid triploids[4]	26	32.3 ± 27.0[bc]	40	15.3 ± 4.9	4.3 ± 2.5[bc]	0.08 ± 0.3	4.5 ± 2.9[bd]	0.2 ± 0.5

[1] natural sexual diploids from Wolfswaard population;

[2] natural triploid apomicts from Wolfswaard and Keyenberg populations;

[3] synthetic triploid progeny from a natural sexual diploid x a colchicine-induced tetraploid cross;

[4] hybrid triploid progeny from a natural sexual x natural apomict cross;

[5] following cytogenetic interpretation in Havekes et al., 1994.

The ± sign denotes SD values. Chiasma counts and measurements were made per individual meiotic cell. Different letters in % pairing, numbers of bivalents and numbers of chiasmata in bivalents denote statistical significant differences (Kruskal-Wallis ANOVA by Ranks, $p=0.05$ level)

Materials and Methods

Plant materials

The six sexual diploid plants (2n=2x=16) and 24 natural triploid apomicts came from a mixed population in natural meadows on alluvial clay soils alongside the river Rhine near Wageningen, the Netherlands. Three more triploid apomicts were sampled from a sandy soil near the "Keyenberg" estate in Renkum, on a 5 km distance from Wageningen. Four synthetic triploid non-apomictic plant lineages were obtained by bidirectional backcrossings of colchicine-induced autotetraploids with diploid "mother" plants. The synthetic triploid non-apomicts were produced as follows. One or a few drops of colchicine (0.25% in a 2% dimethylsulfoxide solution) were applied between the cotyledons of one-week old diploid sexual seedlings on two successive days. The seeds originated from the Wageningen population. This resulted in plants with tetraploid and octaploid shoots. The colchicine tetraploids did not produce seeds after style removal nor after isolation of the inflorescences with paper bags so these colchicine-induced tetraploids were considered to be sexual. Octaploids were pollen and seed sterile. Tetraploids produced triploid offspring in bidirectional crosses with diploids. These "synthetic" triploids did not show any sign of apomixis, irrespective of the cross direction, so synthetic triploids were considered sexual. Synthetic triploids produced low number of seeds in crosses with diploid pollen donors and had irregular microsporogenesis (see Results). Hybrid plants obtained from sexual X apomict crosses are described in Tas and van Dijk (1999). Additional information about the hybrids were described in Van Dijk et al. (1999). The used hybrids produced ca. 70% apomictic seeds.

To induce flowering, plants were vernalised in a cold room for at least three months at 4 °C and at a relative humidity (RH) of 70-80%. We then transferred the plants to fresh pot soil and removed most of the leaves to stimulate new growth and reared them in the greenhouse at a day/night regime of 16 hr 18 °C and 8 hr 16 °C and a RH of 80%.

Chromosome preparations and photomicroscopy

Chromosome spread preparations were prepared according to Pijnacker and Ferwerda (1984). Root tips were first pre-treated in a 1% aqueous solution of the spindle inhibitor 8-hydroxyquinoline at 6-8 °C for six hours before they were fixed in freshly prepared (3:1:4) chloroform, acetic acid and ethanol at 20°C. Flower buds containing microsporocytes at meiosis were directly fixed in a 1:3 mixture of acetic acid and ethanol (Carnoy). Cell walls of root tip and flower buds were enzymatically digested in 0.1% cellulase RS, 0.1% pectolyase Y23 and 0.1% cytohelicase in a 10mM sodium citrate buffer (pH 4.5) for 2-

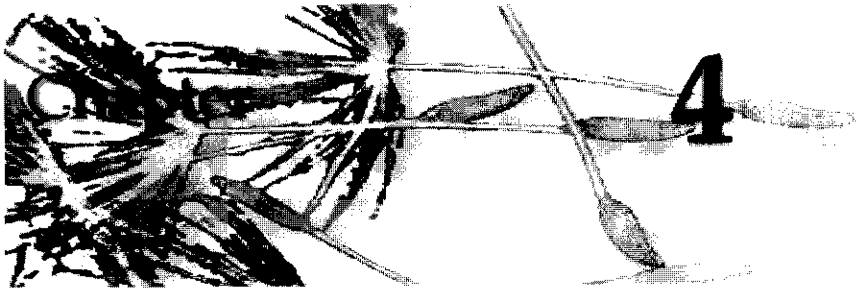
3 hours at 37 °C. Cells were dissected with fine needles in a drop of 60% acetic acid on a grease-free microscopic slide. After heating the preparation to 42 °C for 1 minute, cells were spread on a glass object slide with a drop of ice-cold Carnoy and then air-dried. Chromosome preparations were incubated in 2x SSC (pH 7.4) at 60 °C for 10 minutes, rinsed in tap water and then stained in a 4% solution of Giemsa stain (Merck) in Sörensen buffer (10 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 6.8) for 30 minutes. A few preparations were stained and mounted in 5 µg DAPI (4',6 diamidino-propyl-indole) in Vectashield (Vector Laboratories) and were studied under the fluorescence microscope. Chromosome spread preparations of single flower buds, which contained up to a few hundred microsporocytes, were used for quantitative analyses. For measuring chromosome pairing, scanned images (see below) were printed onto A4-size paper and the paired and unpaired chromosome regions were traced onto transparencies. The pachytene configurations were enlarged until they were approximately similar-sized. The lengths of the respective traced regions were obtained by retracing the chromosome arms using strings of appropriate diameter, marking the paired regions and measuring the respective regions from the strings. The measurement error (3 times repeated) was between 1 and 2%. Estimates of paired regions in the same image were repeated at least three times throughout the experimental period to see whether interpretation of the respective regions differed and were found to differ less than 4%. Later on, percentages of paired and unpaired regions were directly obtained from scanned and enlarged images instead of from transparencies. Estimations of chiasma numbers were based on the interpretation of bivalents in diakinesis and metaphase I cells according to Havekes et al. (1994). These authors describe chiasma formation in terms of rod or ring bivalents and the morphology of separating bivalents.

Qualitative analyses of micro- and megasporogenesis were performed at fixed intact flowers. The material was dehydrated through two absolute ethanol steps for at least 10 minutes each before it was optically cleared in methyl salicylate (Merck) for at least 30 min at 20 °C or one day in the dark at 4 °C. The cleared tissues were then transferred to a drop of methyl salicylate on a microscope slide, covered with a cover slip and studied under a microscope equipped with Nomarski's differential interference contrast (DIC) optics. A preparation of a cleared flower bud allowed analysis of up to a thousand microsporocytes or a single megasporocyte.

We used Kodak Imagelink® negative film rated at 25 ISO for bright field and Nomarski photomicroscopy. Films were developed in 10% Neutol paper developer (Agfa) for 7 min. at 20 °C. DAPI fluorescence images were photographed on 400 ISO colour negative films. Selected negatives were scanned with a film scanner at 1000 dpi and the resulting digital images were optimised with Adobe Photoshop® (Adobe, CA).

Assessment of pollen fertility

Pollen fertility was assessed using either Giemsa stained preparations of spread tetrads or cleared whole-mount anthers. To quantify male fertility, we also used a solution of lactophenol - acid fuchsin to stain mature pollen grains according to Sass (1964).



Comparative cyto-embryological investigations of sexual and apomictic dandelions (*Taraxacum*) and their apomictic hybrids

Peter van Baarlen, J. Hans de Jong, and Peter J. van Dijk

Summary

In autonomous apomictic *Taraxacum officinale*, common dandelions, parthenogenetic egg cells develop into embryos and central cells into endosperm without prior fertilisation. Unreduced (2n) megaspores are formed via meiotic diplospory, a nonreductional type of meiosis. In this paper, we describe the normal developmental pathways of sexual and apomictic reproduction and compare these with the development observed in the apomictic hybrids. In sexual diploids, a standard type of megasporogenesis and embryo sac development proceeds synchronised between florets in individual capitula. In contrast, we observed that megasporogenesis and gametogenesis proceeded asynchronous between florets within a single capitulum of natural triploid apomicts. In addition, autonomous endosperm and embryo development initiated independently from each other within individual florets. Parthenogenetic initiation of embryo development in outdoor apomicts was found to be temperature-dependent. Egg cells produced in natural apomicts were not fertilised after pollination with haploid pollen grains although pollen tubes were observed to grow into their embryo sacs. Both reductional and diplosporous megasporogenesis were observed in individual inflorescences of triploid apomictic hybrids. Initiation of embryo and endosperm development initiated independently from each other in natural and hybrid apomicts.

submitted to Sexual Plant Reproduction

INTRODUCTION

Gametophytic apomixis in plants, which is asexual reproduction by seeds, can be accomplished by a variety of ways (Nogler, 1984; Asker and Jerling, 1992). In autonomous apomictic polyploid *Taraxacum officinale* (common dandelions), parthenogenetic egg cells develop into embryos and central cells into endosperm without fertilisation. In *Taraxacum* apomicts, meiotic diplospory is the process whereby embryo sacs are produced from megaspores that are the result of a nonreductional form of meiosis. In contrast, diploid *T. officinale* have a regular meiosis and reproduce only after double fertilisation of the egg and central cell.

Sexual and apomictic development in *Taraxacum* has been the subject of several studies that collectively list the major differences between sexual and apomictic *Taraxacum* accessions. Juel (1906), Poddubnaja-Arnoldi and Dianova (1934) and Gustafsson (1935a,b) described the main differences in meiosis and subsequent seed development in sexual and apomictic dandelions, along with some developmental deviations (e.g. fertilisation of embryo sacs already containing an embryo) in the sexual *T. kok-saghyz*. Fagerlind (1947) studied megasporogenesis in several apomictic *Taraxacum* accessions and showed that the female restitutional meiosis in apomicts takes place asynchronously between the florets of a single capitulum, in contrast to the synchronised reductional meiosis in sexual dandelions. Likewise, Cooper and Brink (1949) showed that initiation of embryo and endosperm development occurs independent from each other within single florets of apomicts and asynchronously between florets, in contrast to the synchronous initiation in sexual dandelions.

In *Taraxacum*, Richards (1973) distinguished obligate apomixis (meaning that all seeds are formed via apomixis) and partial apomixis: some embryo sacs appeared to be reduced and depended on fertilisation for seed formation as in sexual dandelions. Similar findings were reported by Malecka (1973), who occasionally found reduced egg cells that needed fertilisation for viable seed set in apomictic triploid *T. palustre*. Moreover, it was found that unreduced egg cells may persist after anthesis; such egg cells could be fertilised by sperm from haploid pollen grains (Malecka, 1973). This event gave rise to so-called BIII-hybrids (cf. Rutishauser in Nogler, 1984) which combine the genomes of an unreduced egg cell and a haploid sperm nucleus ($2n+n$, according to the nomenclature of Harlan and de Wet, 1975) .

The latter finding is relevant for testing the hypothesis that states that fertilisation of parthenogenetic egg cells is avoided by precocious development (pre-anthesis) of these egg cells into embryos (Richards, 1973; Mogie, 1988, 1992). The occurrence of BIII hybrids (Malecka, 1973) and the fact that outgrowth of pollen tubes through the micropyle of embryo sacs has been observed in natural (Poddubnaja-Arnoldi and Dianowa, 1934;

Malecka, 1973) and hybrid (Malecka, 1971) *Taraxacum* apomicts raises the question whether precocity of embryo development is sufficient to avoid fertilisation of egg cells in apomicts.

In this paper we describe a clearing method that we used to study megagametophyte development in fully sexual and apomictic dandelions by differential interference contrast (DIC) microscopy. We also describe apomictic development in partially apomictic hybrids that result from crossing closely related sexuals and apomicts. Progeny tests had revealed that these partial apomicts were semi-sterile rather than partially sexual; pollinating the inflorescences of such hybrids (a procedure that would lead to fertilisation of sexual embryo sacs and increased seed set) did not result in a higher seed-set (van Dijk et al., 1999). To test the precocity-hypothesis, we pollinated inflorescences of outdoor apomicts displaying delayed egg cell parthenogenesis and, in addition, performed artificial bud pollination, a procedure that had yielded zygotic embryos in apomictic *Ranunculus* (Nogler, 1995) and *Paspalum* (Martinez et al., 1994).

RESULTS

Cyto-embryology of diploid sexual dandelions

An overview of developmental stages related to floral stem length in the examined sexual and apomictic plants is given in table 4.1. Analysis of megasporogenesis and megagametogenesis in the diploid sexuals revealed a high degree of synchronisation of developmental stages between individual flowers within a capitulum. Within individual florets, we found that megasporocytes were at diakinesis - metaphase I when microsporocytes were at the tetrad stage, and that tetrad wall formation had been completed in ovules when pollen walls were clearly apparent in the anthers. In inflorescences with floral stem lengths between 0-0.5 cm, megasporogenesis had progressed up to the tetrad stage. All tetrads were linear (fig. 4.1A displays a tetrad). Variation in the degree of wall formation within the tetrad, possibly associated with the amount of callose deposition, was visible (not shown). The three consecutive mitotic divisions of megagametogenesis were identified after counting the numbers of nuclei in the surviving megaspore (2, 4 and 8 respectively; figs. 4.1C1-3). Some florets at stem length 0-0.5 cm displayed cellularising embryo sacs (fig. 4.1D). At a floral stem length of 1.5 cm, cellularisation of embryo sacs had been completed. Initi-

ally, the unfused polar nuclei were close together near the embryo sac wall. At higher floral stem lengths, crystal-like inclusions (e.g. fig. 4.1H) were visible in the integument cells. In the anthers, uniformly sized pollen grains with developing characteristic thick walls were visible.

At a floral stem length of 5 cm, crystal-like inclusions were now present as a dense cloud in all cells surrounding the embryo sac. Egg cells and fused polar nuclei with a large nucleolus were visible at this stage; fused polar nuclei near the egg cell were observed in about half of the cases (8 out of 19); in the other cases, fused polar nuclei were found near the embryo sac wall. Synergids were well-developed, whereas two of the antipodals frequently appeared to be degenerating. The third antipodal cell was often visible and sometimes appeared vital judged from the presence of cell contents (figs. 4.1F). Pollen grains had already matured at this stage.

At a floral stem length stage of about 9 cm, embryo sacs had retained the same morphology as at the 5 cm stage. All central cell nuclei were near the chalazal side of the egg cell. The egg cell became ca. 1.5 times larger (fig. 4.1E). The synergids were often hard to distinguish depending on the orientation of the gametophyte on the object slide. A single antipodal cell was visible in 6 out of 10 embryosacs. All embryo sacs appeared completely viable and no degeneration was observed.

At anthesis, floral stem length 12-14 cm, the embryo sac retained the same appearance as at the 9 cm stage. No degeneration of synergids was evident at this stage and embryo and endosperm development was not observed prior to anthesis and after anthesis only after fertilisation. Pollinating flowers with haploid outcross pollen resulted in fertilisation of embryo sacs within four hours (fig. 4.1F).

Cyto-embryology of natural triploid apomictic dandelions

Megasporogenesis of apomictic dandelions, which results in dyad formation via restitution nucleus formation (Gustafsson, 1935; van Baarlen et al., 2000 and chapter 3), took place in florets with 0 to 0.5 cm floral stem length. Megaspore mother cells in apomicts did not develop synchronously as observed in diploid sexuals. In the apomicts, some megaspore mother cells had not yet completed meiosis II, whereas in other gametophytes within the same bud, the first mitotic division of megagametogenesis was visible. In the flower material from 9 apomicts, 376 dyads (fig. 4.1A) and 3 linear tetrads (0.8%) (fig. 4.1B) were found. The morphology of these tetrads was not different from those observed in the sexual diploid dandelions. After megasporogenesis, the micropylar megaspore degenerated and the chalazal megaspore underwent megagametogenesis.

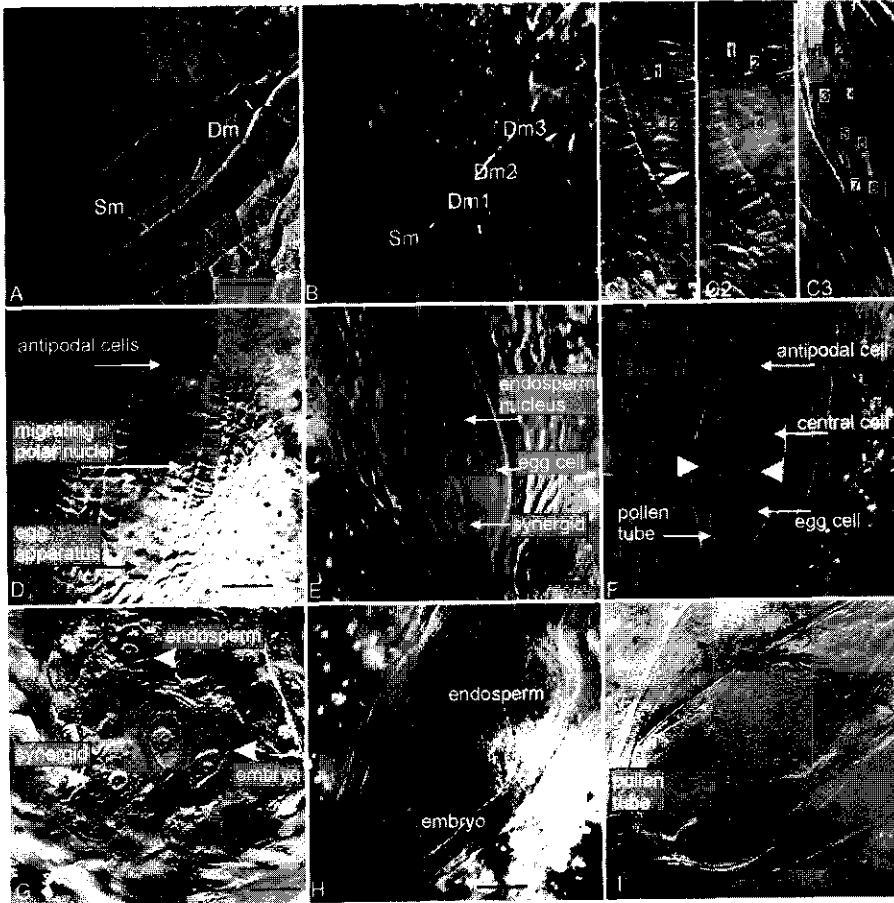


Fig. 4.1. Megasporogenesis, -gametogenesis and embryology of natural sexual diploid and apomictic triploid dandelions. A. Dyad with degenerating (Dm) and surviving (Sm) megaspore in natural apomict. B. Tetrad in natural apomict. C1. First mitotic division of megagametogenesis showing the two nuclei. Note the degenerated megaspores in the micropylar region. C2. Second mitotic division showing four nuclei. C3. Third mitotic division showing eight nuclei (not all in focal plane). D. Maturation of embryo sac; pre-cellular stage showing the formation of antipodal cells (chalazal region), the egg apparatus (micropylar region) and the two unfused polar nuclei (centre region). E. Mature embryo sac showing the egg cell, secondary endosperm nucleus and one synergid. The three antipodal cells and second synergid are not in focal plane. F. Fertilised embryo sac in sexual dandelion showing the central cell and the egg cell, the pollen tube and two sperm cells (indicated by triangles). G. Parthenogenesis of the egg cell and autonomy of endosperm in the triploid hybrid 14-4-30, two days prior to anthesis. One synergid is clearly visible on the left side, the synergid on the right is not in the same focal plane. H. Embryo and endosperm in unpollinated natural apomict. I. Embryo and pollen tube in pollinated natural apomict.All images were obtained from unstained, cleared flower material using Nomarski optics. Size bars denote 10 µm in A-C, 20 µm in 2D, 30 µm in 2E, 25 µm in 2G, 160 µm in H, and 140 µm in 2I.

At floral stem length of 0.5-1 cm, in apomictic dandelions Kb7-2, grown in the greenhouse, we observed the 2, 4 or 8-nucleate stage of gametogenesis and the later stages of embryo sac formation (cellularisation). Megagametogenesis in apomicts did not appear different from megagametogenesis in sexual dandelions. The mitotic divisions resulted in an eight-nucleate embryo sac that had the same polarity and morphology as observed in sexual dandelions. In addition to the easily identifyable synergids, antipodals, polar nuclei and the egg cell, we also regularly observed a single large central cell nucleus, after fusion of the polar nuclei.

Just before and at anthesis, no differences were observed in embryo sac morphology of outdoor growing apomicts or apomicts grown in the greenhouse. But in contrast to the sexual dandelions, embryo and endosperm formation started before anthesis in the florets of apomicts. In greenhouse-grown apomicts, nuclear endosperm development was apparent in 8 out of 48 florets (16%) one day before anthesis, but no embryo development was observed. At anthesis, embryo and endosperm development had always initiated (> 200 florets from six capitula inspected) in apomicts grown in the greenhouse.

Autonomous endosperm development was also observed in the outdoor flowering apomicts. At anthesis, endosperm nuclei near the embryo sac walls were present in 13% of the florets (n = 179). In 8% of the embryo sacs (n = 179) in florets of outdoor-flowering apomicts, embryo development at anthesis was observed. The other egg cells were delayed. One day after anthesis, globular embryos were present in all florets (fig. 4.1H).

Egg cells in natural apomicts were neither fertilised after pollination at anthesis nor after early artificial bud pollination

To test whether the egg cells of outdoor apomicts with delayed parthenogenesis could be fertilised, inflorescences of six apomicts were pollinated and mature seeds were collected from these plants. The seeds were germinated and the ploidy levels were established by flow cytometry. All plants reared from seeds were triploid ($2n$+0). In the florets sampled 30 min and 2 hrs after pollination, pollen tube outgrowth was visible after anilin-blue staining of the pollen tube callose wall and visualisation of the stained walls using fluorescence microscopy. When the pollinated flowers were examined using Nomarski microscopy, pollen tube outgrowth through the micropyle could be observed.

When inflorescences from greenhouse-grown apomicts were pollinated with haploid outcross pollen and sampled after six hours, microscopic analysis revealed that pollen tubes had penetrated the embryo sacs although these already contained embryos (fig. 4.1I). All the progeny of these greenhouse-reared apomicts had the expected triploid ploidy level ($2n$+0).

Early pollination of closed flower buds after removal of part of the stigmas did only very infrequently give rise to BIII ($2n+n$) hybrids. At the youngest stage of 4-5 cm floral stem length, no BIII hybrids were found among 45 offspring. At the medium stage, 7-9 floral stem length, no BIII hybrids were found among 125 offspring, but two of these were found to have a doubled genome (6x). Only one BIII hybrid was found among 52 offspring at the latest stage, 1 day before anthesis. These data show that egg cells produced by apomicts are only very incidentally fertilised by outcross pollen grains although pollen tubes can reach and penetrate embryo sacs.

Cyto-embryology of triploid hybrid apomictic dandelions

The triploid hybrid #14-7-25 produces viable seeds in ca. 70% of the florets. Fertility differences between inflorescences were found in this hybrid since some flower buds contained only empty and degenerating embryo sacs whereas other buds on the same plant contained normal embryo sacs. In one very young flower bud (floral stem length 0-0.5 cm) 22% of the gametophytes formed tetrads and 78% formed dyads (n = 27) formation. Megagametogenesis was seriously impaired in the same bud: no stages after the second mitotic division (19 found; 40 florets examined) were found but we found seven megaspores with 5-6 nuclei together with putative degenerated nuclei. In an older bud, floral stem length 3.5 cm, the last stages of gametogenesis and embryo sacs with a regular appearance were observed. We studied 47 florets and found that 16 of those (34%) contained megaspores that had completed the third mitotic division of megagametogenesis. The remaining gametophytes displayed apparently vital embryo sacs; there were no signs of autonomous endosperm or parthenogenesis at this stage. In a bud with a floral stem length of 8 cm, we observed several developmental deviations together with apparently viable embryo sacs. Sixteen percent of the embryo sacs (n = 53) that were analysed had a collapsed and twisted shape. In 8% of the gametophytes, endothelium cells were very thin and no egg cells, synergids, polar nuclei, antipodals or uncellularised nuclei were observed. At anthesis, 95% of the embryo sacs of a flower bud contained autonomous endosperm of which 89% contained small globular embryos (n = 60).

Similar observations were made in two other triploid hybrids. One of the hybrids, #14-4-30, produces viable seeds via apomixis in 34-56% of the florets. Unfortunately, no megasporogenesis stages were found in the available material. One young flower bud (floral stem length 1.5 cm) contained only nonviable embryo sacs (40 gametophytes inspected); most lacked embryo sac contents, other sacs apparently lacked the egg apparatus. In a different flower bud at the 2 cm floral stem length stage, we observed embryo sacs with the egg and antipodal apparatus. The polar nuclei

were fused in nearly 60% of the cases (n=22). One flower bud, collected at floral stem length 7 cm, contained apparently healthy embryo sacs but showed no signs of apomixis (35 studied). One flower bud collected ca. 1 day prior to anthesis (floral stem length 12 cm) contained regular embryo sacs. We analysed 60 of these and found that 13 embryo sacs contained autonomous endosperm nuclei (22%); 4 of these 13 embryo sacs contained globular embryos (fig. 4.1G). Hybrid #14-4-39 showed restitutional megasporogenesis stages in ovules at 0-0.5 cm floral stem length (n=42). In six ovules, ectopic location of megaspores undergoing gametogenesis at the periphery of the ovules was observed. No other stages were available from this hybrid.

DISCUSSION

Cyto-embryology of natural diploid sexuals and triploid apomicts

Megagametophyte development of diploid dandelions is of a standard type and its pathway comparable to that reported for the diploid *A. thaliana* (Schneitz et al., 1995). No restitution, autonomous embryo or endosperm formation was evident in whole-mount analysis of ovula in closed flower buds nor in unpollinated inflorescences two days after anthesis.

In natural apomicts, we found three megaspore tetrads (0.8% of 397 gametophytes) demonstrating that the female first meiotic division can be reductional in apomicts (see also Richards, 1970; Malecka, 1971, 1973). Reductional meiosis in *Taraxacum* is sex-specific, in that the female meiosis is by default restitutional, whereas the male meiosis is reductional (Gustafsson, 1935; van Baarlen et al., 2000 and chapter 3). Reductional meiosis in apomicts may lead to segregational loss of apomictic components; this will be discussed later on. Megasporogenesis in the natural triploid apomicts differs from the closely related sexuals in two aspects: the meiotic stages are not synchronised within a flower bud and the first meiotic division is restitutional instead of reductional, resulting in unreduced triploid megaspores. Lack of synchronisation of meiotic stages in apomictic *Taraxacum* had previously been found and quantified by Fagerlind (1947). Between-floret asynchronous megasporogenesis is so far typical for *Taraxacum* apomicts.

The developmental stages upon megasporogenesis in the apomicts were not different from those found in the sexuals, apart from asynchronous megasporogenesis and subsequent developmental stages between florets. Endosperm and embryo development could initiate independently

from each other in individual florets in the apomicts, although endosperm development usually initiated before embryo development. Similar observations were made by Cooper and Brink (1949) who conducted that endosperm was not necessary for the early stages of embryo development in sexual and apomictic dandelions; they argued that nutrition for the developing embryo comes from the surrounding protein-rich integument tissue since in 20% (n=1118) of *T. officinale* gametophytes, no endosperm cells were present although embryo development had clearly started.

Egg cells of apomicts are refractory to fertilisation

The egg cells of natural apomicts did not show parthenogenesis in 83% of the embryo sacs (n = 179) prior to anthesis at temperatures well under 10 °C. Egg cells at anthesis have also been found in *Taraxacum* apomicts by Cooper and Brink (1949) and Malecka (1973) and have been found in apomictic *Eupatorium* (Bertasso-Borges and Coleman, 1998). Malecka (1973) suggested that resting egg cells might have resulted from reduced megaspores that had lost the capacity for parthogenesis after segregation. However, this explanation is not plausible here because pollination of the apomictic inflorescences containing egg cells did not give tetraploid offspring, notwithstanding the observed outgrowth of pollen tubes and penetration of embryo sacs. It is possible that low temperatures and limited light had delayed egg cell parthenogenesis and autonomy of endosperm in the majority of the flowers (Malecka, 1973). The combined reports on occurrence of resting egg cells in apomicts at anthesis contradict the statement that embryo development in *Taraxacum* apomicts always initiates 48-24 hours before anthesis (Richards, 1970).

We also tested if egg cells in apomicts could be fertilised under artificial conditions, by pollinating developing flower buds after removal of the top part of the bud. The artificial bud pollination data demonstrate that egg cells in the apomicts are only very rarely fertilised by sperm nuclei (1 fertilisation event out of 122 offspring analysed).

Our observation that delayed egg cells from apomicts are refractory to fertilisation does not corroborate the hypothesis that only precocious embryo development avoids fertilisation in apomicts (Richards, 1970; Mogie, 1992). We propose that in addition to precocity, other physiological factors can inhibit fertilisation of egg cells in apomicts, for instance presence of wall structures around egg cells (see LeBlanc and Savidan, 1994). Refractoriness of egg cells to fertilisation may be a fourth element of the Taraxacum type of apomixis, next to diplospory, egg cell parthenogenesis and endosperm autonomy.

Cyto-embryology of hybrid dandelions

The single-locus model (Mogie 1988, 1992) of Taraxacum-apomixis is not corroborated by recent experimental studies performed to analyse the inheritance and segregation of apomixis in crosses between sexual and apomictic dandelions. These studies (Tas and van Dijk, 1999; van Dijk et al., 1999) have shown that apomixis can be genetically dissected into separate factors controlling diplospory, parthenogenesis and autonomous endosperm. Our microscopic data show independent initiation of embryo and endosperm development. Moreover, apart from semi-sterility, the Nomarski microscopy studies showed that reproductive development in the hybrid apomicts was not different from their apomictic staminate parents. Probable causes for the observed semi-sterility in the apomictic hybrids were the occurrence of reductional divisions which may lead to lethal aneuploidy of megaspores and segregational loss of those chromosomes that contain apomixis loci, and a defective megagametogenesis. The latter explanation is based on our observations in megagametophytes of the triploid hybrids #14-7-25 and #14-4-30 and #14-4-39 in which part of the inflorescences showed defective megagametogenesis and formation of nonviable embryo sacs in the first two hybrids.

In all viable embryo sacs, endosperm development had initiated independent from egg cell parthenogenesis. Pollinating such flowers did not lead to an increased seed set (Tas and van Dijk, 1999). It is therefore likely that the lowered fertility that is characteristic for these hybrids is accounted for by production of nonviable embryo sacs and not because of lowered capacity for egg cell parthenogenesis or autonomy of endosperm formation. It is not known what causes the improper gametogenesis and embryo sac formation in part of the florets of the triploid hybrid apomicts. Modifyers of apomixis genes (see also Koltunow et al., 2000) may be present on chromosomes from the apomictic staminate parent that have not been introgressed. Semi-sterility may also be a result of the occurrence of reductional divisions (van Dijk et al., 1999). It is very likely that reductional megasporogenesis in triploid *Taraxacum* leads to aneuploid megaspores (Malecka, 1971, 1973; van Baarlen et al., 2000 and chapter 3) and subsequent nonviable embryo sacs and egg cells.

ACKNOWLEDGEMENTS

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Table 4. 1. Overview of developmental stages of diploid sexuals and natural and hybrid triploid apomicts

floral stem length in cm	days before anthesis	diploid sexuals (2n=2x)	triploid natural apomicts (2n=3x)		apomictic hybrids (2n=3x; Tas and van Dijk, 1999)
0-0.2	7-5	tetrads	dyads, tetrads		early megasporogenesis, tetrads, dyads
0.5	7-5	mitotic divisions megagametogenesis, cellularisation	mitotic divisions megagametogenesis		mitotic divisions megagametogenesis
1.5	5-4	cellularised embryo sacs, unfused polar nuclei	mature embryo sacs, nuclear endosperm		mature embryo sacs, unfused polar nuclei
5	3	fused polar nuclei, start degeneration of 2 from 3 antipodal cells	*field*	*greenhouse*	
9	2	fused polar nuclei, egg cells, synergids, 1 antipodal cell	egg cells, nuclear endo-sperm	n.d.	
12-14	1	egg cells, synergids	egg cells, nuclear endo-sperm	n.d.	embryos, nuclear endosperm
anthesis	0	egg cells, synergids	egg cells, embryos	embryos	embryos, endosperm
6 hrs[1] after pollination - 1 day[2] no poll.	-1	embryos[1]	embryos[2]	n.d.	embryos

[1] in case of sexual dandelions
[2] in case of apomicts
n.d. = no data

Plant materials

Seeds from sexual diploid and apomictic triploid dandelions were collected from several locations near Wageningen, the Netherlands, germinated and reared in the greenhouse at 21 °C, 16 h light; 15 °C, 8h dark. In addition, hybrids between diploid sexual and triploid apomictic plants were generated in controlled greenhouse crosses and by transplanting sexual plants in completely apomictic populations (Tas and van Dijk, 1999; van Dijk et al., 1999; see fig. 4.2 and table 4.2).

Fertilisation experiments with apomicts

Two experiments were conducted to test if it was possible to fertilise egg cells of apomicts with fertile non-self pollen. A first test involved pollination of inflorescences of outdoor and greenhouse-reared apomicts at anthesis. Microscopic investigations had shown that florets of triploid apomicts growing outdoor in May 1998 still contained egg cells at anthesis. To see if these delayed egg cells would develop via parthenogenesis or could be fertilised, we pollinated six apomicts with haploid pollen at the receptive stage during the end of the morning. Four hours later, three inflorescences were sampled per plant; the remaining inflorescences were allowed to set seed. Florets were studied using Nomarski and fluorescence microscopy (see below) to investigate pollen tube formation and embryo sac fertilisation.

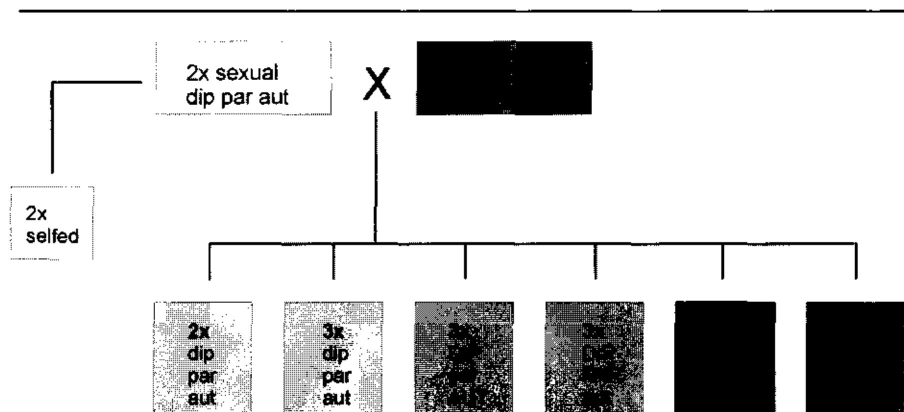


Fig. 4.2. Crossing scheme of diploid sexuals X triploid apomicts and their progeny. The classes analysed in this study are the parents and their apomictic 3x progeny (dark background, boxed).

Table 4.2. Plant materials

Plant index	no.[1]	ploidy level	reproduction	origin
Wolf 1	1	diploid	sexual	natural
Wolf 15	1	diploid	sexual	natural
S1-148	2	diploid	sexual	natural
B8-5	2	diploid	sexual	natural
Kb7-2	4	triploid	apomictic	natural
Wolf 8	1	triploid	apomictic	natural
Erf 1-5[2]	1	triploid	apomictic	natural
H2	1	triploid	apomictic	hybrid[3]
14-7-22	1	triploid	apomictic	hybrid[3]
14-7-25	1	triploid	apomictic	hybrid[3]
14-4-30	1	triploid	apomictic	hybrid[3]
14-4-39	1	triploid	apomictic	hybrid[3]

[1] no. of individual plants
[2] one series of 5 individual plants
[3] made by van Dijk and colleagues

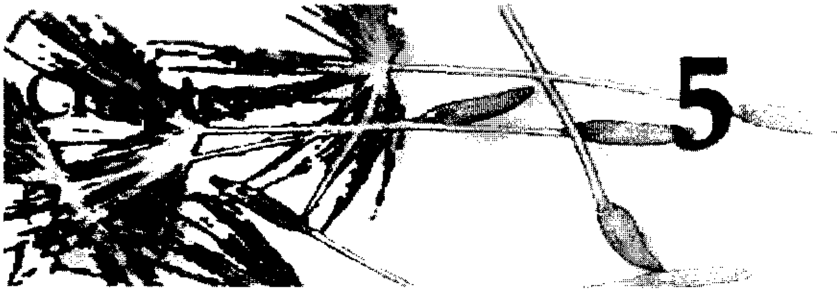
The collected seeds were germinated and the ploidy levels of the seedlings were determined via flow cytometry (PARTEC Ploidy Analyser; Tas and van Dijk, 1999). If the seeds had been produced via apomixis they would have a triploid ploidy level like the apomictic mother plants ($2n+0$); if the seeds were the result of fertilisation they would have a tetraploid ploidy level ($2n+n$).

In a second experiment, we pollinated flowers from triploid apomicts at the bud stage (Martinez et al., 1994) to see if early pollination resulted in fertilisation. To expose the stigmas of immature bud florets, the top of the bud was sliced off with a sharp razor blade under a slight angle, creating florets with styles ranging from uncut to slightly cut off. Immediately after cutting, the buds were rubbed onto the inflorescence of a sexual diploid pollen-releasing dandelion. Successful fertilisation would result in tetraploid ($2n+n$) hybrid offspring. This treatment was done at several developmental stages classified by the floral stem length of 4-5, 7-9 and 12-16 cm which corresponds to 3, 2 and 1 day before anthesis, respectively. The ploidy level of the progeny was determined via flow cytometry as above.

Microscopy procedures
For preparing floral tissues for whole-mount DIC microscopy, we used floral stem length - which was found to correspond with days before anthesis - as major characteristic for standardisation of developmental stages.

The inflorescence of *Taraxacum* is composed of 100-200 individual small flowers or florets. Each floret contains one megaspore mother cell that will give rise to one embryo sac in a single ovule, and which will be transformed into a single seed. In the bud stage, the florets are covered with bracts. These bracts and the floral stem were removed, the bud was cut in 2-4 parts and fixed in freshly prepared Carnoy (1 part acetic acid and 3 parts 100% ethanol) for at least one day at 4 °C. The flower material was dehydrated by two steps of 10 min incubation in 100% ethanol. The flowers were then incubated in methylsalicylate (Merck) for at least 4 hours. When floral stems were longer than 1.5 cm, the gametophytes were chopped lengthwise using a razor blade. Cleared ovaries were mounted in methyl salicylate on glass slides and studied under a Nikon Optiphot microscope with Nomarski differential interference contrast (DIC) optics and a 20x Nikon Fluor objective. Per inflorescence, at least 40 florets or seeds were studied using DIC microscopy but only the unambiguously interpretable preparations are used in the resulting analysis (table 4.2).

To see if pollen tubes germinated and could reach the embryo sacs of apomicts, flowers from triploids that had been pollinated with haploid pollen from diploid donors were sampled at intervals of 4, 24 and 48 hrs after anthesis and fixed in Carnoy. Outgrowth of pollen tubes was visualised in intact flowers using anilin-blue staining of callose. Intact flowers were mounted in a 2% anilin-blue, 20% K_3PO_4 solution (A. van Lammeren, pers. comm.; modified after Martin, 1959) and directly studied using a microscope with epifluorescence illumination and a DAPI (4', 6-diamidino-2-phenyl indole) filter. Pollen tube outgrowth was also studied using Nomarski microscopy after clearing the tissue in methyl salicylate.



The occurrence of reciprocal apomixis-recombinants indicates that autonomous apomixis in the common dandelion (*Taraxacum officinale*) is regulated by multiple dominant genes

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* both authors contributed equally to this paper

Summary

While sexual diploid dandelions (*Taraxacum officinale*) produce seeds after double fertilisation of the reduced egg and central cell, apomictic triploid dandelions do so without meiotic reduction and fertilisation. In apomictic *Taraxacum* meiosis is restitutional, embryo development is parthenogenetic and endosperm development is autonomous. Here we show that some hybrids obtained from diploid sexual x triploid apomict crosses combine elements of the apomictic developmental pathway with elements of the sexual pathway. These hybrids could be subdivided into two complementary groups based on the presence of parthenogenesis or autonomous endosperm formation. Recovery of reciprocal recombinants strongly suggests that elements of apomixis are regulated by different genes. Our study also indicates (i) that parthenogenetic embryo development is conditional on meiotic restitution and (ii) that early embryo development is not dependent on endosperm formation. The phenotype of one of the recombinants resembles that of *fis* autonomous endosperm mutants of *Arabidopsis thaliana*. We discuss the similarities between autonomous endosperm development in *Arabidopsis* mutants and autonomous apomixis in *Taraxacum* to develop a model on the putative action of apomixis genes.

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INTRODUCTION

Sexual seed development in the angiosperms involves the unique process of double fertilisation (Nawaschin, 1898; Willemse and Van Went, 1984). In this process the first generative pollen sperm nucleus (*n*) fertilizes the egg cell (*n*), while the second generative pollen sperm nucleus (n) fertilizes the central cell (*2n*). The zygote (*2n*) forms the embryo and the fertilized central cell and gives rise to the endosperm (*3n*), a tissue which nourishes the developing embryo. A group of angiosperms, the gametophytic apomicts, (Stebbins, 1950; Nogler, 1984; Koltunow, 1993) have lost the need of fertilisation for further embryo development. Instead the egg cell develops parthenogenetically into an embryo. Apomicts produce clonal seeds that are genetically identical to the mother plant. Most gametophytic apomicts are pseudogamous, which means that they still need fertilisation of the central cell for endosperm development. Autonomous apomicts however, have abandoned fertilisation completely and here also the endosperm develops without prior fertilisation. Autonomous apomixis is not uncommon in the Asteraceae, but rare in other plant families.

The question how autonomous apomicts have by-passed double fertilisation is not only important for the understanding of apomixis, but also for the understanding of the process of sexual reproduction in angiosperms in general. To answer this question knowledge of the genetic basis of apomixis is indispensable. Here we report on hybrids obtained in crosses between sexual diploid and autonomous apomictic triploid dandelions, *Taraxacum*, which were found to combine parts of the sexual and the apomictic reproductive pathway.

In order to retain the same ploidy level in the absence of fertilisation, gametophytic apomicts have to avoid meiotic reduction (apomeiosis). Two types of apomeiosis can be distinguished: apospory and diplospory. In aposporous species, meiotically reduced megaspores are formed but these are replaced by somatic (often nucellar) cells which form an unreduced embryo sac or megagametophyte. In diplosporous species a reductional meiosis is replaced by mitosis (mitotic diplospory) or by restitutional meiosis (meiotic diplospory).

Thus autonomous apomixis involves at least three elements: (i) apomeiosis, (ii) parthenogenetic embryo development and (iii) autonomous endosperm development. In *Hieracium piloselloides* apomixis 'as a whole' (apomeiosis, parthenogenesis and endosperm autonomy) was assumed to inherit as a single dominant trait (Bicknell et al., 2000). This suggests that the complete apomictic pathway is controlled by a single gene or by a complex of tightly linked genes. In contrast, another composite species, *Erigeron annuus*, at least two unlinked genes mediate

apomixis, one controls diplospory and another one controls parthenogenesis (Noyes, 2000; Noyes and Rieseberg, 2000).

In *Taraxacum* the genetic basis of autonomous apomixis is controversial. Richards (1970, 1973) suggested that apomixis in *Taraxacum* was controlled by two dominant genes, located on different chromosomes. This model was mainly based on the reports on the loss of apomixis in rare disomics (2n =3x-1) by Sørensen and Gudjonsson (1946) and Sørensen (1958). These authors claimed that the loss of a specific chromosome in an apomictic clone resulted in the loss of diplospory. Loss of another, non-homologous chromosome resulted in the fertilisation of unreduced egg cells. The dominant two-locus model has been challenged by Mogie (1988, 1992), mainly because of the small sample sizes analysed by Sørensen and Gudjonsson and the - according to Mogie - unreliable chromosome identification. Instead Mogie (1988, 1992) proposed a recessive single locus model for apomixis. In Mogie's model it is assumed that a single recessive gene is responsible for diplospory and that this gene has pleiotropic effects on egg cell parthenogenesis. This model has received considerable attention in the apomixis literature (e.g. in reviews by Asker and Jerling 1992; Mogie 1992; Koltunow, 1993). Experimental crossing data, supporting one or the other model, have been lacking so far.

In this paper, we describe and characterise two types of triploid hybrid dandelions which we isolated from crosses between sexual diploids and apomictic triploids. Based on progeny analysis in back-crosses with diploid sexual pollen donors, we supposed that these hybrids combined elements of sexual and apomictic reproduction (Van Dijk et al., 1999). The occurrence of apomixis-recombinants would favor a multi-gene model for control of apomixis. We show by Nomarski DIC-microscopy, flow cytometry and genetic analysis with microsatellite markers that the so-called type B hybrids have lost the capacity of autonomous endosperm development, but still can produce embryos via parthenogenesis. These hybrids are functionally pseudogamous. We also show that the recombinant type C forms autonomous endosperm, but does not form embryos in absence of fertilisation. We conclude that type B and C hybrids are reciprocal apomixis-recombinants.

The phenotype of type C hybrids resembles the autonomous endosperm mutants in sexual *Arabidopsis* in some but not all aspects. In *A. thaliana,* three genes have been characterised that control endosperm development: *MEA (MEDEA) / FIS1, FIS2;* and *FIE / FIS3* (reviewed in Preuss, 1999). In loss-of-function mutants the central cell divides without prior fertilisation. The literature reports on the action of FIS/FIE proteins give important clues for the genetic regulation of autonomous endosperm and apomixis in general. We discuss the reciprocal apomixis-

recombinants in *Taraxacum* and present a model based on the interpretation of FIS/FIE protein function.

RESULTS

Cyto-embryology of non-pollinated type B hybrids

The results of the Nomarski-DIC microscopic observations on seed development in non-pollinated type B hybrids are summarized in Fig. 5.1. At one day before anthesis half of the ovules displayed degenerated embryo sacs. Some embryo sacs lacked polar nuclei, central cells and egg cells (Fig. 5.3A and B). Others had collapsed completely, with only two endothelia layers visible (Fig. 5.3C). These non-viable embryo sacs corroborate with the low seed sets after crossing with diploid pollen donors (see below) and must have degenerated after gametogenesis, because at stem length < 0.5 cm development still appeared normal (40 analysed).

Among the viable embryo sacs at one day before anthesis, besides embryo sacs with a resting egg cell and undivided central cell nucleus (41%), also few embryo sacs (9%) with parthenogenetic embryos were observed. In this latter class the central cell nuclei had not divided and no endosperm had formed, in contrast to the pre-anthesis autonomous endosperm development in natural apomicts grown under the same conditions (Van Baarlen et al., submitted; chapter 4).

At anthesis the fraction of non-viable embryo sacs had not changed significantly (42%) whereas the number of embryo sacs with parthenogenetic embryos had increased and equalled the number with resting egg cells (both 29%). There was no endosperm development in the embryo sacs with parthenogenetic embryos. At one and three days after anthesis, the fraction of degenerating embryo sacs had increased further to 65 and 72%, respectively. At these stages over 95% of the viable embryo sacs showed no traces of autonomous endosperm development. A few embryo sacs had undergone division of the central cell resulting in 2-8 endosperm nuclei but cellularised endosperm was not observed in such embryo sacs. About two-thirds (66 %) of the viable embryo sacs now contained parthenogenetic embryos. Figs. 5.3D and E show a globular embryo with a still undivided central cell nucleus. Most embryos (>90%) just about reached the heart stage. No parthenogenetic embryos developed beyond the heart stage. The embryo sac did not expand

properly and remained globular, whereas seeds with mature endosperm are elongated at this stage (compare fig. 5.3D and E with fig. 5.3F).

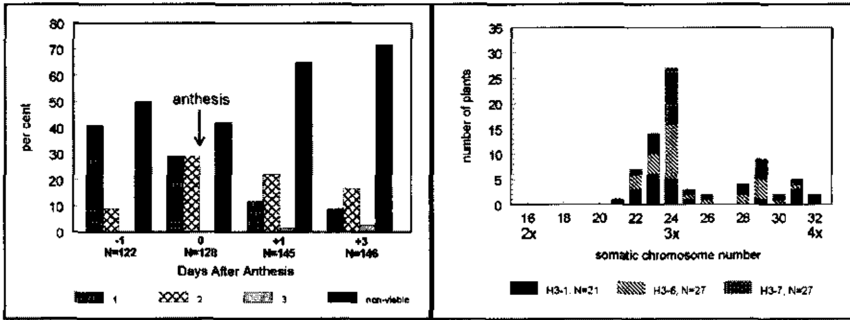

Fig. 5.1 (above, left panel). The distribution of embryo sac types observed by Nomarski-DIC microscopy in non-pollinated florets ranging from one day before anthesis (DAA-1) to three days after anthesis (DAA+3). The data of H3-1 and H3-7 are pooled. Non-viable embryo sacs were degenerating or empty. Embryo sac types: 1. resting egg cell and undivided central cell; 2. parthenogenetic embryo and undivided central cell; 3. parthenogenetic embryo and 2-8-nucleate, non-cellularised autonomous endosperm. N is the number of embryo sacs analysed.

Fig. 5.2 (above, right panel). The distribution of progeny chromosome numbers of three type B hybrids after pollination with diploid plants. Chromosome numbers were calculated from the relative DNA amounts, determined by flow cytometry.

Fig. 5.3 : see next page

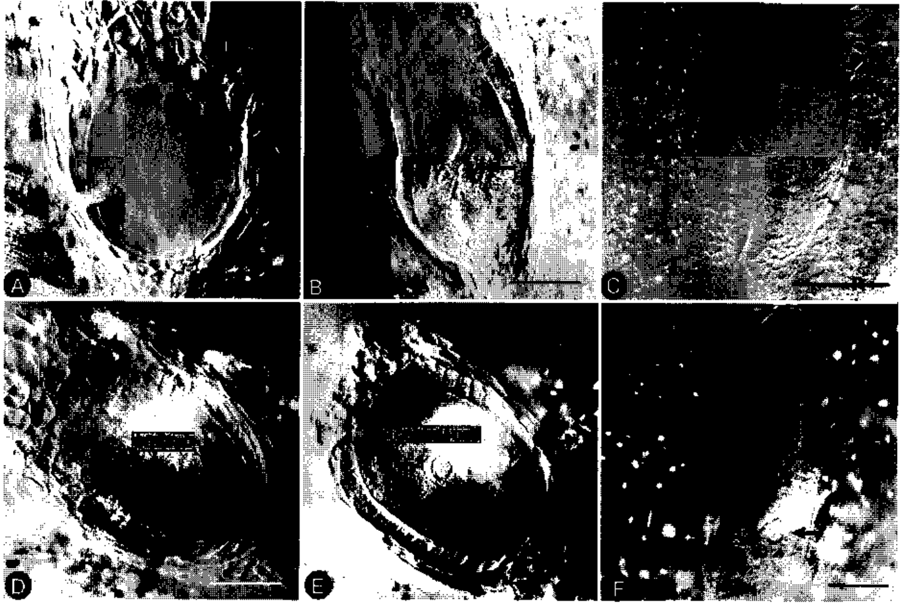

Fig. 5.3. Nomarski-DIC images of embryo sacs of non-pollinated florets one or two days after anthesis. A: Empty embryo sac with faint traces of endosperm formation in H3-1. B: Deviant type of embryo sac with large, unidentified cells in the chalazal region (small arrow) and endosperm-like cells in the central region. Note absence of the egg apparatus in the micropylar region. C: Collapsed embryo sac in H3-7; the arrow points at the "cavity". D and E: Type B, the same embryo sac at different focal planes. D: globular parthenogenetic embryo, E: non-divided central cell. F: Type C. Unreduced egg cell embedded in multi-cellular endosperm. Note that the embryo sac in F has expanded and elongated due to the endosperm growth, whereas the embryo sac in D and E is not expanded but spherical due to the absence of endosperm. Size bars in fig. A-C denote 50 micrometer, in D-E 100 micrometer and in F 500 µm.

Cyto-embryology of non-pollinated type C hybrids

Nomarski-DIC microscopy of non-pollinated flower heads revealed that the endosperm of type C plants developed autonomously. Two days before anthesis more than half of the embryo sacs (59%, N=22) already contained 2- or 4-nucleate endosperm, whereas the rest still had an undivided central cell. None of the egg cells had divided. One day before anthesis, resting egg cells were surrounded by endosperm in all 22 examined ovules. Apart from the resting egg cell, morphology of seed development in the type C hybrids was similar to that in the apomictic parent. Between one and three days after anthesis the endosperm

proliferated and became cellularized. The somatic maternal tissues had also expanded, but the egg cell remained in a resting stage (fig. 5.3E).

The origin of the progenies from pollinated type B hybrids

To determine the sexual or parthenogenetic origin of progenies after pollination, the triploid type B hybrids were crossed with diploid sexuals and the ploidy levels of the offspring were determined. In addition to the 38 already analysed progeny plants in Van Dijk et al. (1999), the ploidy levels of 33 more plants were established. Fig. 5.2 shows the distribution of the chromosome numbers of the progeny plants. All three type B hybrids produced (hypo)-triploid and hyper-triploid offspring.

Microsatellite genotypes were determined for a subset of 24 of the progeny plants with known chromosome number. Fig. 5.4 shows a typical example. All offspring plants have the maternal genotype whereas a few also show a paternal band. Table 5.1 (p. 76) gives an overview of the multi-locus genotypes, together with their inferred chromosome numbers. Most loci were tri-allelic. In the case of bi-allelic genotype, double dosages could not be distinguished from single dosages.

The results for the three type B hybrids were very similar. In total 13 (near) triploid progeny plants of these mothers were analysed for 5 microsatellite loci. In all cases the progeny genotype was identical to the mother genotype. Absence of segregation of a five locus heterozygous genotype strongly indicates an unreduced gametic origin of these triploid progenies. Two plants from H3-6 lacked a maternal allele at locus Msta61. The relative DNA contents indicated that these plants were hypotriploid, lacking at least one chromosome. None of the 13 (near) triploid offspring plants showed an additional non-maternal band. This indicates that the triploid offspring were derived parthenogenetically and were $2n + 0$ plants (classification according to Harlan and Dewet, 1975). In contrast, all 11 (near) tetraploids showed at least one additional non-maternal band, strongly suggesting that they were the result of the fertilisation on an unreduced egg cell ($2n + n$ hybrids).

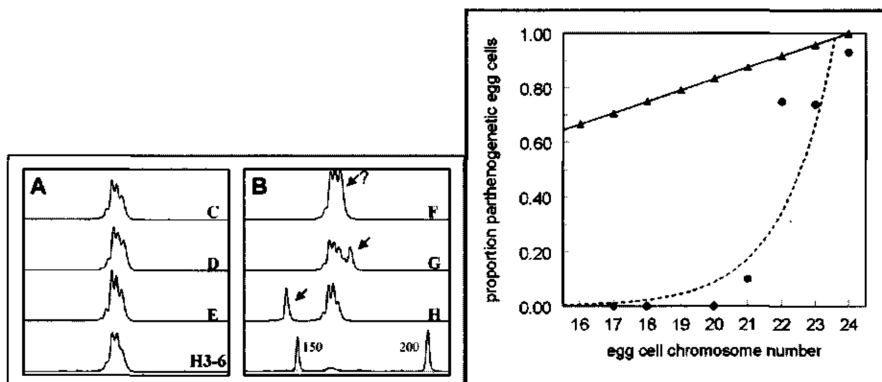

Fig. 5.4 (above, left panel). The detection of non-maternal alleles for Msta78 in the progeny of H3-6 after pollination with a diploid pollen donor. Panel A shows three (near)-triploid progeny plans and the mother. Panel B shows three (near)-tetraploid progeny plants and two molecular weight markers (150 and 200 bp, respectively). The arrows indicate additional, non-maternal alleles. The question mark for plant F indicates a possible double dose of the 166 bp allele. Plant codes correspond to table 1 (p. 76). Curve view mode of the ALFwin™ Sequence Analyser 2.00 .

Fig. 5.5 (above, right panel). The relationship between egg cell chromosome number and the probability of fertilisation in type B hybrids. A: the distribution of the egg cell chromosome numbers after pollination in parthenogenetic offspring (gray bars) and in zygotic offspring (black bars). Data for all three type B plants are pooled. B: The probability of parthenogenetic egg cell development in relation to the inferred egg cell chromosome number. The proportions are calculated from fig. 5A. The solid line with triangles indicates the expected probabilities under a model of a simplex dominant parthenogenesis genotype, Ppp, with random chromosome loss (see text). The dashed line is an exponential non-linear regression curve fitted to the observed values.

Based on ploidy levels, the fractions of triploid ($2n$+0) offspring in H3-1, H3-6 and H3-7 were 0.67, 0.67, and 0.74, respectively. These fractions are almost identical to the fractions of parthenogenetic embryos observed in the cyto-embryological investigations on non-pollinated flowers (on average 0.66 for the H3-1 and H3-7, see above). As the sexual ($2n$+ n) offspring was derived from egg cells fertilized by haploid pollen grains from diploid plants (2n=16), the egg cell chromosome number is simply the chromosome number in the progeny plants minus 8. Fig. 5.5A shows that most of the egg cells with 20 and 21 chromosomes became fertilized, whereas most of egg cells with 22-24 chromosomes developed parthenogenetically. It can be seen in Fig. 5.5B that the probability of

parthenogenetic development increased exponentially with the chromosome number of the egg cell.

Were parthenogenesis controlled by a single dominant gene (*Ppp*), then loss of the chromosome carrying its dominant allele (*P*) would provide a likely explanation why hypotriploid egg cells had lost their ability to develop parthenogenetically. However, under the assumption of random chromosome loss, a linear relationship between chromosome number and parthenogenetic development would be expected (fig. 5.5B). As the observed numbers of parthenogenetically developing egg cells for the two classes $2n < 21$ and $2n = 21$ is significantly lower than the expected numbers ($\chi^2 = 6.90$ and 6.91 respectively, df=1; $P < 0.01$ for both) this model has to be rejected.

DISCUSSION

Type B and C hybrids are reciprocal apomixis-recombinants

Our combined microscopic and genetic analyses provided a clear characterisation of the B and C type hybrids obtained in sexual x apomictic crosses. The genetic progeny analysis proved that type B hybrids produced unreduced egg cells that possessed the capacity of developing parthenogenetically into embryos. The cyto-embryological investigations confirmed the formation of parthenogenetic embryos but also revealed that type B hybrids lacked the capacity for autonomous endosperm formation. The production of viable apomictic seeds after pollination, as indicated by the microsatellite analysis, can be explained by endosperm development upon fertilisation of the central cell. Parthenogenetic embryos are rescued by sexual endosperm. Type B plants are thus pseudogamous offspring, derived from an autonomous apomictic father SE3x-6 (see materials and methods). Pseudogamy has not been reported before in *Taraxacum*.

The investigations of type B hybrids also shed light on the relationship between embryo and endosperm development. In the absence of endosperm, parthenogenetic embryos in type B hybrids developed up to the early heart stage, which suggests that endosperm is non-essential for early embryo growth. This corroborates with the study of Cooper and Brink (1949) who found that early growth of the embryo and endosperm were not correlated in apomictic *Taraxacum*. Our results indicate however, that endosperm development is essential for further embryo development

and growth as parthenogenetic embryos did not develop beyond the early heart stage.

About one-third of the unreduced viable egg cells produced by type B hybrids did not initiate parthenogenetic embryo development, but retained a resting phase. These egg cells were often aneutriploid and were fertilized after pollination. Hypo-triploidy is due to incomplete nuclear restitution (Rosenberg, 1927; Bergman, 1950) and was significantly more common in type B hybrids than in type C hybrids or the apomictic father SE3x-6 (Van Dijk et al. 1999). The relationship between aneutriploidy and loss of parthenogenesis will be discussed below.

Type C hybrids produce empty seeds in absence of pollination and can thus be considered as parthenocarpic (Van Dijk et al. 1999). As some parthenocarpic *A. thaliana* mutants can form autonomous endosperm (Ohad et al., 1996; Grossniklaus et al., 1998; Chaudhury et al., 1997), we hypothesized that type C hybrids possessed autonomous endosperm development but lacked egg cell parthenogenesis. Our microscopic observations confirm this hypothesis. Ploidy and microsatellite analysis of the progenies resulting from pollinated type C hybrids had previously established that these plants produced unreduced egg cells (Van Dijk et al., 1999). Therefore type C hybrids indeed can be interpreted as diplosporous with autonomous endosperm, but lacking egg cell parthenogenesis.

Three dominant genes may regulate apomixis in *Taraxacum*

This study allows several inferences to be made about the genetic regulation of apomixis in *Taraxacum*. Genetically and cyto-embryologically we can dissect apomixis into three main components: diplospory, egg cell parthenogenesis and autonomous endosperm development. Phenotypically sexuals are for these respective elements -/-/-, whereas apomicts are +/+/+. Type B hybrids possess diplospory and parthenogenesis, but lack endosperm autonomy: +/+/-. Type C hybrids possess diplospory and endosperm autonomy, but lack parthenogenesis: +/-/+. Hence, type B and C hybrids are phenotypically reciprocal apomixis-recombinants.

By studying the segregation of diplospory in tetraploid offspring derived from type C hybrid H6-3 we could show that diplospory is encoded by a single dominant gene, *DIPLOSPOROUS* (*DIP*), located on one of the satellite chromosomes (van Dijk et al., submitted). Based on closely linked microsatellite markers the genotype of the triploid apomictic father SE3x-6 was determined as *Ddd*. During reductional pollen meiosis both *Dd* and *dd* diploid pollen grains are produced (as well as haploid *D* and *d* pollen). This segregation explains why part of the

triploid offspring of SE3x-6 in the cross with the diploid sexuals had lost diplospory.

We presume that, analogous to diplospory, segregation also caused the loss of autonomous endosperm development in type B hybrids and the loss of parthenogenesis in type C hybrids. Recently, Noyes and Rieseberg (2000) identified a diplospory gene and an unlinked parthenogenesis gene in triploid apomictic *Erigeron annuus*, both present as simplex dominant genotypes (*Aaa*). Segregation studies have indicated that at least three unlinked dominant genes are necessary to explain the inheritance of apomixis 'as a whole' in *Taraxacum* (Van Dijk et al. in prep). We postulate, in addition to *DIP*, a gene *AUTONOMOUS ENDOSPERM DEVELOPMENT* (*AUT*) and a gene *PARTHENOGENETIC EGG CELL DEVELOPMENT* (*PAR*). Accordingly the genotype of the triploid apomictic parent SE3x-6 is *Ddd / Ppp / Aaa* and that of the diploid sexual parent is *dd / pp / aa*.

Our model of three dominant genes controlling apomixis in *Taraxacum* contradicts Mogie's single recessive gene model. Mogie's model is not supported by empirical data. In fact, the studies on the mode of reproduction of rare disomics by Sørensen and Gudjonsson (1946) and Sørensen (1958), as well as the cyto-embryological studies of partial apomicts by Malecka (1973) both suggest that apomixis in *Taraxacum* consists of several independent elements that can recombine.

Possible action of apomixis genes

As mentioned before, *Arabidopsis* mutants with autonomous endosperm development phenotypically resemble the type C hybrids (except that type C hybrids are also diplosporous). Three genes have been identified in which loss-of-function mutations cause autonomous proliferation of endosperm nuclei, growth of somatic tissues and the seed coat, i.e. *FIS* (*FERTILISATION-INDEPENDENT SEED DEVELOPMENT*)*1/MEDEA*, *FIS2*; and *FIS3/FIE* (*FERTILISATION-INDEPENDENT ENDOSPERM*) (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998). As different research groups using different mutation screens isolated the same genes, the screens seem saturated and the corresponding genes are likely to be the major genes controlling endosperm development. *MEA* and *FIE* encode proteins that are functional homologues of the *Drosophila* Polycomb group proteins (Preuss, 1999), proteins that maintain the repressed state of developmental genes (Pirrotta, 1997b). Probably the FIS proteins function in a complex that represses the transcription of seed development genes (Luo et al. 1999; Preuss, 1999). Conceivably, in *mea* and *fie* loss-of-function mutants, central cells start to divide without fertilisation because pre-fertilisation transcription blocks are incomplete.

Absence of functional transcription blocks may also be the cause of autonomous endosperm development in apomictic dandelions.

We conclude from our crosses that the *A* allele of *AUT* in *Taraxacum* is dominant, whereas *Arabidopsis mea* and *fie* loss-of-function mutations are recessive (Grossniklaus et al., 1998; Kiyosue et al., 1999; Ohad et al., 1999). Moreover, the finding that pollinating type C hybrids gives numerous viable offspring is also different from what is observed in *FIS* mutants where pollination leads to arrested seed development (Luo et al., 2000). We therefore consider it unlikely that *AUT* is an ortholog of one of the *FIS* genes. Given dominant action of the A allele, *AUT* could act upstream from the *FIS* genes and that the A allele product in fact represses *FIS* gene products or interferes with the repression block that they mediate. It has been demonstrated that paternal forms of *FIS* genes are inactivated during early seed development (Vielle-Calzada et al., 1999; Luo et al., 2000). The dominant *A* allele could thus silence one or more of the *FIS* genes. As opposed to an upstream function, it is also possible that *AUT* acts downstream of the *FIS* genes as an endosperm development gene. In sexual plants, transcription of the maternal *a* allele may be repressed by the *FIS* gene products. A non-repressed paternal allele may then be necessary to initiate endosperm development after fertilisation. A maternal *A* allele however, could be insensitive to the repression block, for example if the repression block DNA binding site were modified. Escape from repression could explain the dominant nature of the *A* allele.

In our type C hybrids, autonomous endosperm did cellularize as in wild-type. In *fie* mutants, cellularization is dependent on hypomethylation (Vinkenoog et al., 2000) showing an association between autonomous endosperm formation and the methylation status of plants. Vinkenoog et al. (2000) suggest that natural apomicts may have partially overcome the requirement for a paternal contribution to the endosperm by having a lower methylation status.

Is nuclear restitution conditional for egg cell parthenogenesis?

The probability of parthenogenetic egg cell development in type B plants increased strongly with the perfection of nuclear restitution. This cannot simply be explained by random loss of the chromosome carrying the dominant *P* allele of *PAR*. Both in *Taraxacum* (Van Dijk et al., 1999) and in *Erigeron annuus* (Noyes and Rieseberg, 2000) parthenogenesis and autonomous endosperm development are observed in diplosporous but not in meiotic plants. These observations suggest that diplospory is indispensable, but not sufficient, for the expression of parthenogenesis and endosperm autonomy. Restitution-dependent parthenogenesis may explain why some researchers (e.g. Mogie 1992) have thought that

parthenogenesis in *Taraxacum* was a direct consequence of non-reduction of egg cells. At the moment, one can only speculate why diplospory were conditional for the expression of parthenogenesis. Parthenogenesis may, similar to autonomous endosperm development, be blocked by Polycomb gene products in sexual egg cells. In *Drosophila,* chromosome pairing and the action of polycomb proteins are linked, since longer pairing interactions enforce Polycomb-mediated gene silencing (Pirrotta, 1998). Absence of chromosome pairing at meiotic prophase I, one key feature of meiotic diplospory in *Taraxacum* (Gustafsson, 1935a; Malecka, 1970; Van Baarlen et al., 2000 and chapter 3), may hinder the establishment of polycomb silencing complexes and may therefore indirectly interfere with repression of genes that should only be active after fertilisation. Alternatively, the DIP protein may be a chromosomal protein that has the potential of interfering with meiotic recombination, the first meiotic division and the overall imprinting status of chromosomes. Expression of parthenogenesis may be dependent upon this modified chromosomal imprinting. This hypothesis can be tested as soon as the *DIP* gene and its product have been identified. This work is now in progress.

ACKNOWLEDGEMENTS

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Table 5.1. Multi-locus microsatellite genotypes of type B hybrids and their progeny derived after crossing with sexual diploid pollen donors. Non-maternal alleles are shown in bold, * indicates an incomplete maternal genotype. Double or triple dosages could not be distinguished from single dosages. The somatic chromosome numbers are estimated from the relative DNA amounts as determined by flow cytometry. Allele sizes: Msta 60: a=270, b=286, c=302, d=312, e=318 bp; Msta 61: a=124, b=129, c=131, d=135, e=137, f=141 bp, g=145, h=147 bp; Msta 64: a=176, b=210, c=212 bp; Msta 72: a=172, b=176, c=182, d=186, e=190, f=194 bp; Msta 78: a=146, b=154, c=162, d=164, e=166, f=170, g=174 bp. The hybrid classifications follow Harlan and DeWet (1975).

Plant	$2n$	*Msta* 60	*Msta* 61	*Msta* 64	*Msta* 72	*Msta* 78	Hybrid type
H3-1	24	*bde*	*bg*	*ab*	*acd*	*Ceg*	
A	24	*bde*	*bg*	*ab*	*acd*	*Ceg*	$2n + 0$
B	24	*bde*	*bg*	*ab*	*acd*	*Ceg*	$2n + 0$
C	24	*bde*	*bg*	*ab*	*acd*	*Ceg*	$2n + 0$
D	24	*bde*	*bg*	*ab*	*acd*	*Ceg*	$2n + 0$
E	31	*abde*	*bcg*	*ab*	*abcd*	*Cdeg*	$2n + n$
F	31	*bde*	*bcg*	*ab*	*acd*	*Cdeg*	$2n + n$
H	31	*bde*	*bcg*	*ab*	*abcd*	*Cdeg*	$2n + n$
H3-6	24	*bde*	*bdg*	*ab*	*abd*	*Cde*	
A	24	*bde*	*bdg*	*ab*	*abd*	*Cde*	$2n + 0$
B	23	*bde*	*dg**	*ab*	*abd*	*Cde*	$2n + 0$
C	24	*bde*	*bdg*	*ab*	*abd*	*Cde*	$2n + 0$
D	24	*bde*	*bdg*	*ab*	*abd*	*Cde*	$2n + 0$
E	23	*bde*	*dg**	*ab*	*abd*	*Cde*	$2n + 0$
F	31	*abde*	*bdg*	*ab*	*abd*	*Cde*	$2n + n$
G	31	*abde*	*bdg*	*ab*	*abd*	*Cdef*	$2n + n$
H	30	*bde*	*bdg*	*ab*	*abde*	*Acde*	$2n + n$
H3-7	24	*bde*	*dg*	*abc*	*acd*	*Cdg*	
A	24	*bde*	*dg*	*abc*	*acd*	*Cdg*	$2n + 0$
B	24	*bde*	*dg*	*abc*	*acd*	*Cdg*	$2n + 0$
C	24	*bde*	*dg*	*abc*	*acd*	*Cdg*	$2n + 0$
D	24	*bde*	*dg*	*abc*	*acd*	*Cdg*	$2n + 0$
E	32	*abde*	*dfg*	*abc*	*acd*	*Cdeg*	$2n + n$
F	31	*a bde*	*dfg*	*abc*	*acd*	*Cdeg*	$2n + n$
G	32	*abde*	*dgh*	*abc*	*acd*	*Cdeg*	$2n + n$
H	31	*abde*	*adg*	*abc*	*acde*	*Acdg*	$2n + n$
I	28	*bde*	*dg*	*abc*	*acdf*	*Acdg*	$2n + n$

METHODS

Plant material

Taraxacum officinale plants were grown in the greenhouse at (21°C, 16 h light/ 15°C, 8 h dark). The origin of the plant material is described in detail in Tas and Van Dijk (1999) and Van Dijk et al. (1999). To induce flowering, the plants were vernalized for a period of two month at 4°C. Briefly, after crossing two diploid sexuals (L2x-6-1, cross 3 and L2x17-18, cross 6) with triploid apomict SE3x-6, two types of presumed triploid apomixis-recombinants were obtained: type B: four plants (H3-1, H3-6, H3-7 and H6-4) and type C: two plants (H3-9 and H6-3). Type A plants were triploids with a reductional meiosis. To investigate their mode of reproduction, type B and type C plants were pollinated with haploid pollen from diploid sexuals (Van Dijk et al., 1999).

Ploidy analysis

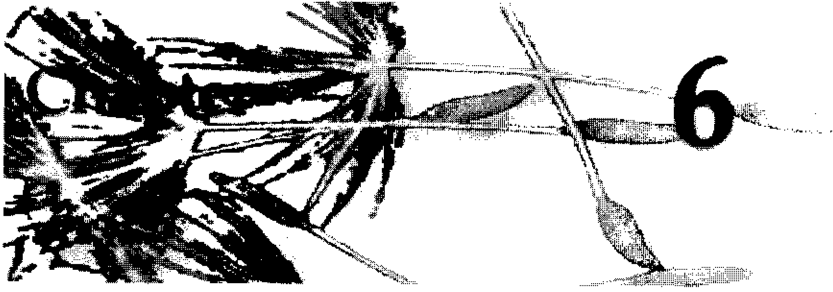
Ploidy levels of the type B X diploid offspring were determined by flow cytometry (Tas and van Dijk 1999). In a test series the relationship between somatic chromosome number and relative DNA content (to an internal diploid standard) was established as $2n = 15.38 * DNA_{2n} / DNA_{2x} + 0.53$ ($r = 0.99$; $N=42$). This formula was used for estimating chromosome numbers from cytometric data.

Microsatellite analysis

To investigate segregation of the maternal genotype and the presence of additional, non-maternal alleles the three type B mother-plants and in total 24 of their progeny plants were analysed for five microsatellite loci (msta 60, 61, 64, 72 and 78, Falque et al. 1998). The original diploid pollen donors were no longer available. Msta 64 and 72, 64 and 78 and 72 and 78 are unlinked (Falque et al. 1998), but the other linkage relationships are not known. All three B-mothers carried the msta78-a allele, which is tightly linked to the DIP-gene in SE3x-6 (Van Dijk et al., in prep.). DNA was extracted from young leaves according to the protocol of Rogstad (1992). PCR conditions were identical to these described in Falque et al. (1998), with the exception that one of the primers was labeled with fluorochrome Cy5. The PCR-products were first checked on a 2% agarose gel and then run on an ALF express II automatic sequencer (Amersham Pharmacia biotech), using a 50 bp size external molecular weight standards (Pharmacia). Microsatellite genotypes were scored in the Curve view mode of the ALFwin™ Sequence Analyser 2.00.

Cyto-embryological investigations

Seed development was studied in non-pollinated capitula. To prevent cross-pollination buds were covered with small paper bags. Flower buds from the type B (H3-1 and H3-7) and C (H6-3) hybrids in various stages of development were collected and fixed at days -1, 0, +1 and +3 days after anthesis. In this time frame parthenogenetic embryo development and autonomous endosperm formation is established in the triploid apomictic pollen donor, SE3x-6. From H3-1 one very young flower bud (floral stem length 0-0.5 cm) was sampled as well. Fixation, gametophyte clearing and Nomarski Differential Interference Contrast (DIC) microscopy techniques are described in Van Baarlen et al. (2000). One inflorescence was studied per hybrid/sample date combination; 50-80 florets were studied per inflorescence.



The influence of sexual-apomictic genome interactions on apomictic gametophyte development

Peter van Baarlen and Peter van Dijk

Summary

Previous work had demonstrated that, compared to triploid hybrids, some tetraploid hybrids displayed higher capacity for apomictic reproduction whereas others experienced relatively high levels of sterility. In order to find the cause for the observed sterility, female gametophyte development was analysed in an unpollinated tetraploid natural apomict and in hybrids with different ratios of genomes of sexual or apomictic origin. All developmental stages in the natural tetraploid apomictic *Taraxacum richardsianum* sect. *naevosa* were similar to the stages in triploid apomictic *T. officinale*. In apomictic hybrids with 1sex - 3apo genomes ($4x^{saaa}$) and in non-apomictic hybrids with 2sex - 2apo ($4x^{ssaa}$) genomes, loss of cell identity, stalled megagametogenesis and improper embryo sac formation were found to be causes of sterility. Moreover, several developmental deviations, not found in the parents, were observed in the unpollinated $4x^{ssaa}$ hybrids that were obtained after pollinating triploid type C hybrids (see chapter 5) with haploid pollen. These data suggest that there exists a critical interaction between the sexual and apomictic genomes that influences gametophyte development and thus, establishment of apomixis.

Introduction

Most European *Taraxacum* apomicts are triploid (comprising 90% of the European *Taraxacum* apomicts, according to Richards, 1973). Like the triploid apomicts, tetraploid apomicts also occur throughout Europe, the greater majority in the northern regions (den Nijs *et al.*, 1980). Triploid and tetraploid *Taraxacum* apomicts occurring in mixed populations can display all sorts of variation in reproductive system, as the fraction of restitutional sporogenesis, the regularity of pollen grain sizes (Malecka 1965, 1973), and the timing of expression of parthenogenesis and endosperm autonomy (Malecka, 1973).

If sexual diploid dandelions grow in the vicinity of triploid apomictic dandelions, tetraploids may arise as a result of hybridisation between the former two types (Malecka, 1973; Tas and van Dijk, 1998). In the Netherlands, tetraploids are seldom found in natural mixed diploid-triploid populations, suggestive of a lower fitness at the higher ploidy level. Apomictic fertility seems to be affected by the ploidy level in *Taraxacum*. Malecka (1973) observed a lower capacity for apomictic seed set in a pentaploid dandelion, compared to tri- and tetraploid dandelions growing in the pentaploid's neighbourhood (Malecka, 1973). Tetraploid hybrid apomicts obtained in sexual diploid x apomictic triploid crosses were more variable in degree of apomixis than triploid hybrid apomicts (P.J. Van Dijk, pers. comm.). During introgression studies in *Tripsacum* (Grimanelli *et al.*, 1998b) and in crossings in *Ranunculus* (Nogler, 1984b, 1994, 1995) and in *Hieracium* (Koltunow et al., 2000) similar phenomena have been found, suggesting that the capacity for apomictic reproduction in general is affected by the ploidy level.

In *Hieracium*, decrease of the capacity for apomictic reproduction at higher ploidy levels is possibly a consequence of modulation of apomictic development by modifiers from the sexual and apomictic parents, modifiers that need to be present at certain ratios in the progeny for proper apomictic gametophyte development and reproduction (Bicknell et al., 2000; Koltunow et al., 2000). Aberrant gametophyte development is likely to occur during megagametogenesis, which encompasses the three mitotic divisions of the surviving reduced or unreduced megaspore and subsequent embryo sac formation. Several gametophytic mutations lead to absence of (or improper) gametophyte or embryo sac formation and sterility in sexual species (reviewed in Drews et al., 1998; Grossniklaus and Schneitz, 1998; Yang and Sundaresan, 2000). According to Malecka (1973), the decreased female fertility in the pentaploid *Taraxacum* apomict, mentioned above, was presumably a consequence of disrupted gametophyte development.

In the previous two chapters, we described how the observed capacity for viable apomictic seed-set was lower in triploid hybrids (compared to their sexual and apomictic parents) because of an increased female sterility, rather than residual sexuality. Microscopic investigations (chapter 4) showed that sterility in triploid hybrids was due to disrupted gametophyte development. In this chapter, we describe not only comparable disruptions at the tetraploid hybrid level but also two new types of aberrations that seem to involve loss or misspecification of cell identity, and abnormal outgrowth of the embryo sac wall or endothelium together with an interrupted development of the integuments.

The tetraploid hybrids used in this study were generated via either one of two crossing schemes (scheme 6.1., next page):

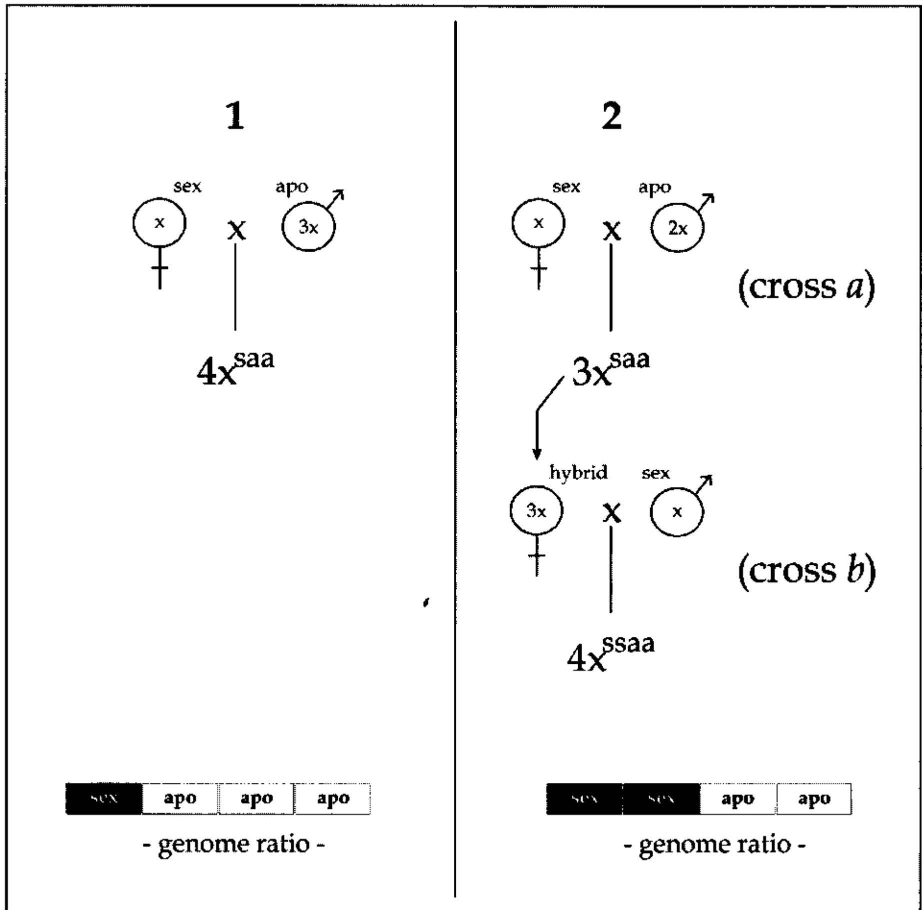
1. via fertilisation of haploid egg cells in sexuals by triploid sperm nuclei from unreduced pollen grains from the triploid apomictic father

2. via pollination of triploid non-apomictic hybrids (haploid egg cell from sexual X diploid pollen grain from apomict; cross *a* in scheme on next page) that lack capacity for egg cell parthenogenesis (hybrid type C in chapter 5) with haploid pollen grains from diploid sexual (cross *b* in scheme on next page). This category is different from the first one in that the gene mediating parthenogenesis is absent in these tetraploid hybrids (under the expectation that the gene is absent in the sexual staminate parent). One second difference is that plants from this category result from two crossing events.

If an increase of the ploidy level has no influence on gametophyte development, the DIC microscopic analysis of tetraploid hybrids is expected to show the same results as reported in the previous chapter. However, it can be expected that ploidy level influences developmental processes via influencing expression of developmental genes (Comai, 2000; Soltis and Soltis, 2000). Detailed expression studies in yeast (Galitski et al., 1999) and silkworms (Suzuki et al., 1999) have shown that changing a given ploidy level changes expression of genes from diverse functional groups, including genes involved in development (see also Hieter and Griffiths, 1999).

We observed several anomalies during gametophyte development in sexual-apomict tetraploid hybrids that were not observed in their diploid or triploid parents. Considering the literature information on gene expression during development and especially in the progeny of comparable sexual-apomict crossings (see above), we hypothesize that ectopic expression of developmental genes from the sexual and apomictic parents causes the observed developmental anomalies in the studied *Taraxacum* hybrids. Modification of expression (*viz.* after an increase in ploidy level) causes severe developmental deviations leading to sterility. We propose

that natural apomicts with high apomictic fertility have been selected for proper timing of expression of genes controlling apomixis.



Scheme 6.1. Schematic overview of the crosses generating the tetraploid hybrids from this study; the triploid hybrids ($3x^{saa}$) in crossing scheme 2 (right panel) are the type C hybrids from chapter 5. s=sexual, a=apomictic

Embryo sac formation and embryo and endosperm development in the natural tetraploid apomict

All stages from megasporogenesis and gametogenesis up to embryo and endosperm formation we observed in unpollinated florets of *T. richardsianum* (not shown) were largely similar to the stages in the triploid apomictic *T. officinale* (see Chapter 4), the main difference being a high degree of synchronisation of megasporogenesis stages (not shown), as in the sexual diploid dandelions. Female fertility was near 100%. In a few florets, parthenogenetic embryo development had not initiated and the respective embryo sacs showed signs of degeneration.

Embryo sac formation and embryo and endosperm development in 1sex : 3 apo hybrids

In most florets of the unpollinated $4x^{saaa}$ hybrids (scheme 6.1), gametophyte and embryo and endosperm development proceeded regularly as described in chapter 5, giving normal embryos and endosperm (fig. 6.2j).

In up to 40 per cent of the florets, megagametophyte development was abnormal, leading to aborted seeds. The microscopically observed percentages of aborted seeds were in agreement with the fractions of non-germinating seeds in germination tests (e.g. for H5-2, 70% and 76% respectively and for #13-9-3, 66% and 77% respectively). In these seeds, embryo development was occasionally stopped in the absence of central cell division (fig. 6.2k) or because of lack of egg cell division (fig. 6.2l). In #14-4-24, some clearly aborted and undeveloped seeds lacked nearly all embryo sac contents and displayed failure of egg cell division (fig. 6.2e). Other embryo sacs showed no traces of the egg apparatus although endosperm formation was clearly present (fig. 6.2h, i). In six embryo sacs (out of 92), the embryo sac cavity was occupied by nondescript cells of unknown identity, with large nucleoli (fig. 6.2f, g).

In florets one day after after anthesis, parthenogenetic embryo were often absent, whereas autonomously developed endosperm was nearly always present. Thus the penetrance of autonomous endosperm development in tetraploid hybrids is higher than the penetrance of parthenogenetic embryo development.

Embryo sac formation, gametophyte, embryo and endosperm development in 2sex : 2apo hybrids

Most unpollinated ovules of the $4x^{ssaa}$ hybrids developed a normal embryo sac with autonomously developing endosperm (see chapters 4-5). The egg cell remained undivided and the seed degenerated in the absence of fertilization. However, 30-40 % of the ovules showed disrupted preanthesis development. It is not clear which developmental stages these types represent since the corresponding microscopic images were very different from those corresponding to the stages described in the chapters 4 and 5.

In about 5% of the embryo sacs (n=400, pooled) the central cavity was still visible but its contents were nearly or completely degenerated (fig. 6.1j, k); in other embryo sacs (out of the 5%) the egg apparatus had degenerated although autonomous endosperm formation was visible (fig. 6.1.l). In 10-20% of the florets, structures resembling stretched embryos that we called "pseudo-embryos" were observed after dehydrating florets in ethanol prior to clearing (results not shown). DIC microscopy after clearing of such florets showed that these pseudo-embryos actually originated from outgrowth of presumptive endothelial cells, after collapse of the empty embryo sac (fig. 6.1g). Dark-field microscopy of the florets containing such pseudo-embryos lacked stretching of the integuments but not of the surrounding maternal tissue (among others the future seed coat; fig. 6.1i) in contrast to normally developing gametophytes (fig. 6.1h).

In 15% of the florets, embryo sacs had a normal appearance but displayed several anomalies in the morphology of antipodal, central, egg or synergid cells. In 12% of the florets, the central cell seemed to divide into daughter nuclei with a different localisation and morphology than normal endosperm nuclei (figs. 6.1a, e and f). In some of these embryo sacs (5%) additional unidentified cells were present in the embryo sac (6.1b-d, 6.2b).

In 3% of the embryo sacs, the egg and/or one of the synergid cells was disproportionally vacuolated (fig. 6.2c-e). Endosperm formation was not always observed in such embryo sacs. Importantly, these developmental deviations were not observed in the diploid sexual staminate parent nor in the triploid diplosporous pistillate parent (chapter 5).

Figure 6.1 (next page). Developmental anomalies in embryo sacs of 4x^ssaa^ hybrids H3-9S13 (a-j) and PA14 (k-l). Note absence of normal central cells or endosperm in all embryo sacs but 6.1.l. a. Atypical large coenocyte with prominent nucleoli in the approximate location of the central cell indicated by upper arrow; the lower arrow points at the egg cell. b. Cell conglomerate of different size compared to cells in a. (upper larger arrow). The lower larger arrow indicates the egg cell. The smaller arrow to the right points at a large uni-nucleate cell with prominent nucleolus that lies close to the endothelium (embryo sac wall). c. As in b. but different focal plane; the large uninucleate cell on the right (small arrow) in focal plane. Note difference in cell size compared to cell conglomerate. d. Different embryo sac with comparable contents as in b. and c. The egg cell (lower larger arrow) is not in focal plane. e. Coenocyte with large, prominent nucleoli (upper arrow) and egg cell (lower arrow). f. As in e. but shifted up. Note the central location of the coenocyte. g. "Pseudo-embryo"-like outgrowth of embryo sac/endothelial tissue within the integuments. The arrow points at the approximate location where some cells of the chalazal upper part of the embryo sac start proliferating (upper clump of cells). h. Dark-field microscopy image of ethanol-cleared healthy fruit. Note the outgrowth of the integuments (the darker, speckled part) within the (translucent) seed coat. i. similar image as in h. but with abortive seed formation. Note the lack of outgrowth of the integuments within the seed coat and the plumper form of the fruit. j. Sterile embryo sac lacking egg apparatus (degenerated parts indicated by lower arrow). The upper arrow points at a presumptive surviving antipodal cell. k. Sterile embryo sac devoid of contents. The arrow points at degenerated contents. l. Embryo sac with no egg cell or synergids but filling with endosperm. The upper arrow points at endosperm nucleus, the lower arrow at the location where the egg cell would be. Size bars denote 30 µm in a., 20 µm in b. and e., 25 µm in c., d. and f., 40 µm in g., 800 µm in h., 700 µm in i., 35 µm in j. and k., and 15 µm in l.

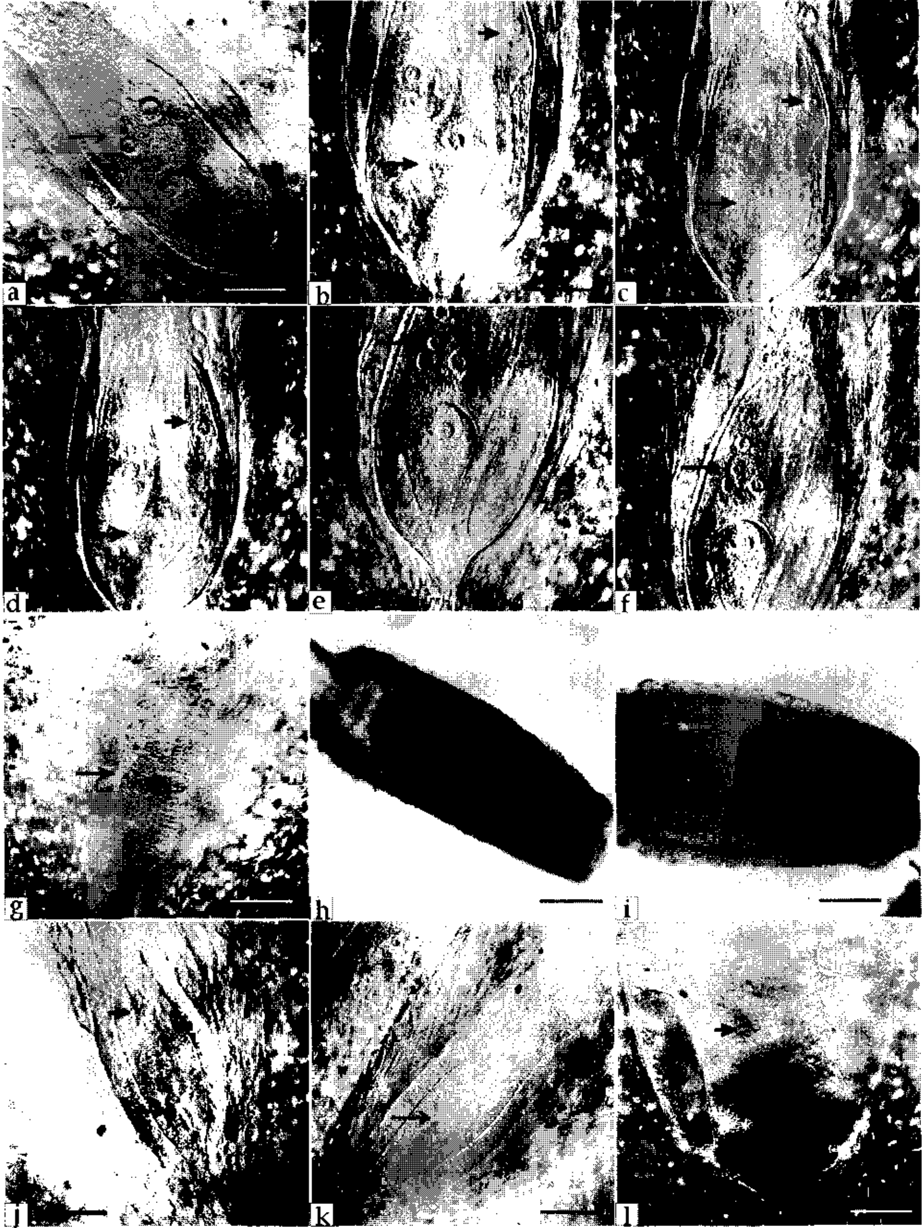

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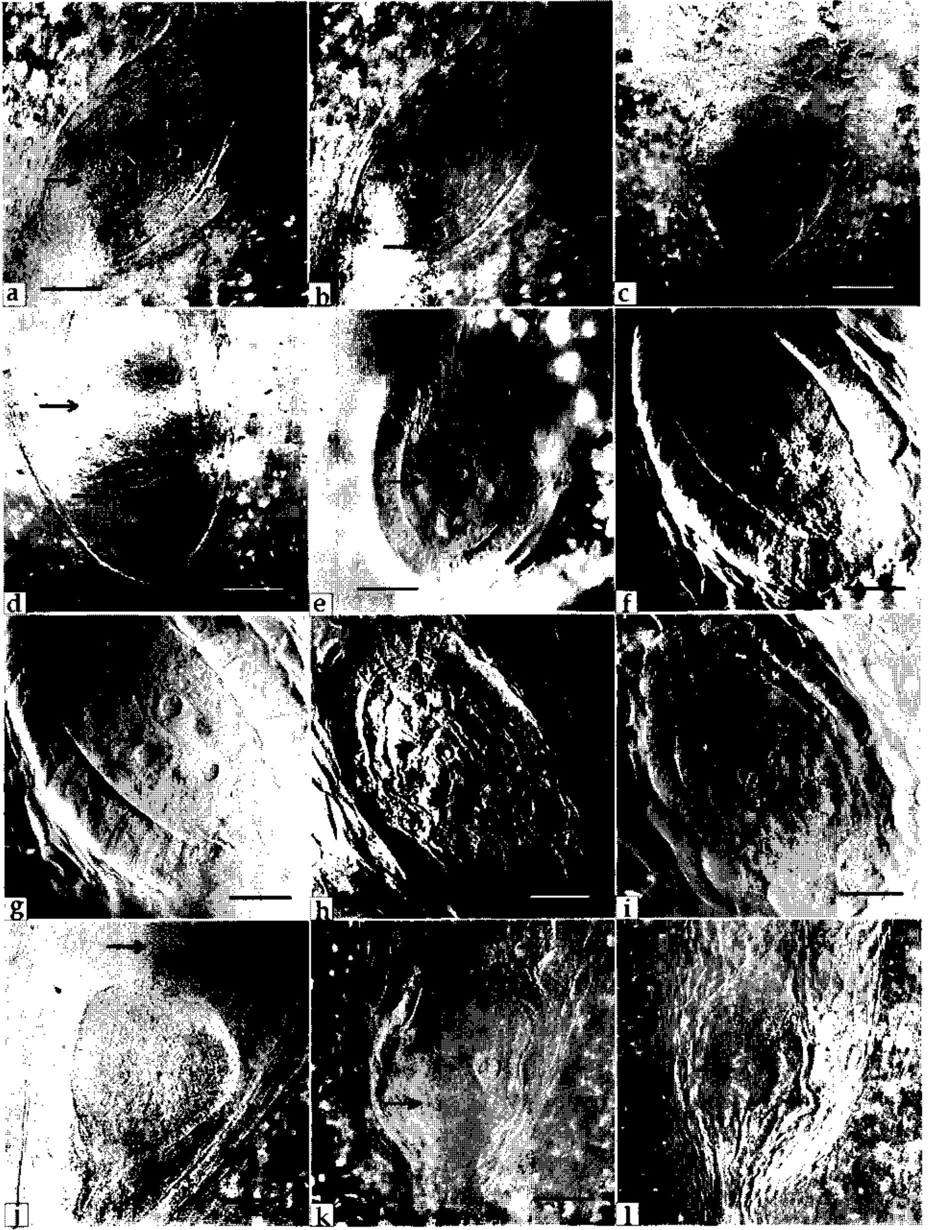
Fig. 6.2 (next page). Developmental anomalies in embryo sacs of tetraploid back-cross hybrid PA14 (a-d) and in tetraploid sexual-apomict hybrid #14-4-24 (e-i), embryo formation (j) and embryo sac degeneration (k-l) in tetraploid hybrids. a. Atypical coenocyte (upper arrow) in the location of the central cell. Lower arrow points at the egg cell. b. Same embryo sac as in a. in different focal plane. Two other single cells are indicated with the upper arrow and the lower arrow on the right. Note correspondence in appearance of the lower-right solitary peripheral cell with the solitary cell in the upper part of the embryo sac in fig. 6.1.c. and 6.1.d. c. Misshapen embryo sac with large-vacuolate egg cell (left arrow) and, to the right, large-vacuolate synergid (lower arrow). The upper arrow points at the undivided central cell (positioned upon the egg cell). d. Embryo sac with endosperm (upper arrow), large-vacuolated egg cell (middle arrow) and, to its left, large-vacuolated synergid (lower arrow). e. Aborted fruit containing egg cell (left arrow) and large synergid (right arrow). The granular contents of the embryo sac might be cell debris or endosperm remnants. f. Sterile embryo sac with unspecified cells (lower arrow), some with large vacuoles (upper arrow). g. Same embryo sac as in f. in different focal plane. The arrow points at one of the unspecified cells. h. Sterile embryo sac with endosperm but lacking the egg cell or embryo. The upper arrow points at well-developed endosperm (alveolar walls - see below -clearly visible). The lower arrow points at where the egg apparatus (or embryo) would be. i. Embryo sac comparable to the one in h. The arrow points at a developing alveolar wall. Alveolar walls are cell walls that are formed at a later stage in a coenocyte, following initial nuclear division and proliferation without cell wall formation). j. Large globular-near heart stage embryo in TM37-19. The arrow indicates endosperm wall. k. Degenerating embryo sac in TM37-19; the large arrow points at a small globular embryo, the small arrow at the undivided central cell nucleus. l. Degenerating embryo sac in TM37-23; the large arrow points at undivided egg cell, the small arrow at remnant of pollen tube.
Size bars indicate 30 µm in a-c., 20 µm in d., 50 µm in e, h. and i., 40 µm in f. and g, 150 µm in j, 80 µm in k, and 50 µm in l.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Megagametogenesis is the most critical phase for successful apomictic reproduction and can be influenced by genome interactions

Our microscopic data have shown that gametogenesis is the most critical developmental stage. Developmental anomalies were most often observed during gametogenesis and are the major cause of female sterility.

Abnormalities were observed in both types of 4x hybrids, but more severe in hybrids derived from two successive crosses with sexual diploids. In the two $4x^{ssaa}$ hybrids, several gametophyte developmental anomalies were observed that were never observed in their sexual, apomictic or triploid hybrid parents nor in the nine $4x^{saaa}$ hybrids. These observations suggest that genome interactions in 4x hybrid dandelions are able to influence progression of megagametogenesis. In line with earlier findings in *Hieracium* (see above), we consider it plausible that modifiers from both the sexual and apomictic parents influence gametophyte development in the hybrids and that the interaction between such modifiers and developmental gene products (sexual and/or apomictic) may render developing gametophytes sterile.

The developmental deviations in the backcross hybrids may be caused by genome interactions or be a result of gamete imprinting

The 4x hybrids PA14 and H3-9S13 differ from the 3x hybrids H3-6 and H3-9 (chapter 5) by one euhaploid "sexual" genome. The tetraploid hybrids are characterised by a diplosporous meiosis and produce dyads at the end of megasporogenesis (Nomarski data not shown). Some of the developmental deviations observed in these tetraploid hybrids were not observed in the apomictic and sexual parents, nor in the $4x^{saaa}$ tetraploid hybrids. It is possible that interactions of the two sexual and two apomictic genomes bring about the observed deviations, and that introgression of sexual genomes in sexual-apomict hybrids leads to an increasing developmental instability. Alternatively, the developmental aberrations may be an indirect result of the two fertilisation events combining respectively the $1sex^{maternal}$ - $2apo^{paternal}$ - $1sex^{paternal}$ genomes (see scheme 6.1). Differential germline imprinting, the phenomenom whereby male and female gametes display a different (timing of) gene expression due to parent-specific DNA modification (Vielle-Calzada et al., 2000; Grossniklaus et al., 2001), in the triploid hybrid and diploid sexual parents may have resulted in aberrant expression of developmental genes in the developing tetraploid hybrid embryos. Both explanations are not mutually exclusive.

The identity of the coenocytes and other cells present in the deviant embryo sacs (figs. 6.1a-f and 6.2a-b) is unknown but may reflect atypical division and proliferation of the central cell (nucleus). The aberrant cell types observed in the embryo sacs of the hybrid #14-4-24 (figs. 6.2f and 6.2g) may be the daughter nuclei of a functional megaspore that fail to transform into egg and central cells, synergids and antipodals because of lack of cell specification or loss of cell identity. The "pseudo-embryo"-like structures we observed in 10-20% of the embryo sacs of PA14 and H3-9S13 are remarkably similar to the "aposporic embryo sac-like formations" observed by Malecka (1973) in a pentaploid *Taraxacum* accession that probably originated in a population of apomicts as a result of fertilisation of an egg cell with delayed parthenogenesis. Interestingly, several other developmental deviations that we observed in the tetraploids PA14 and H3-9S13 were characteristic for this pentaploid accession as well (Malecka, 1973).

It is becoming more evident that gene expression is partially influenced by the ploidy level and that intergenome balances may interfere with expression of genes that are involved in development and reproduction (reviewed in Comai, 2000; Soltis and Soltis, 2000). It is reasonable to assume an influence of ploidy level and intergenome balance on apomixis. One example of the former is the expression of apomixis in autotetraploids induced by colchicine treatment of sexually reproducing diploid (or possibly dihaploid) *Paspalum* (Quarin et al., 2001).

In *Taraxacum,* several researchers have unsuccessfully tried to induce apomixis in sexual diploids via colchicine treatment (Kostoff and Tiber, 1939; Warmke, 1945; Stebbins, 1950, Richards, 1970, and Van Dijk, unpublished). However, according to Stebbins (1950), Warmke (1945) obtained low numbers of parthenogenetically developed embryos with reduced chromosome number (n+0 progeny) in autotetraploids derived from purely sexual plants. Stebbins (1950) concluded that this demonstrates that polyploidy may promote parthenogenetic embryo development. Unfortunately, Warmke (1945) does not report on this. Although Richards (1970) did not succeed in inducing apomixis by cholchicine treatment in *Taraxacum,* he observed reciprocal differences in crosses between diploid sexuals and colchicine tetraploids. When the sexual diploids were the seed parent, fertile triploids were produced, whereas the reciprocal cross yielded only sterile triploids. The difference may be explained by differential parental imprinting of the male and female gametes.

Sexual - apomictic genome interactions may influence hybrid fertility in nature

Our observations indicate that upon a rise of ploidy level after crossing sexual and apomictic dandelions, interactions between sexual and apomictic genomes may influence hybrid fertility. Such putative interactions are important for the establishment of new hybrid apomictic lineages in nature. An increased developmental instability upon introgression of sexual genomes into an apomictic background could explain why tetraploid apomicts are hardly found in mixed diploid-triploid populations: as a result of adverse genome interactions, disruption of developmental processes leads to a decreased female fertility thus lowering competitive fitness compared to the already established diploids and triploids. Megasporo- and gametogenesis in the well-established tetraploid apomict *T. richardsianum* were similar to development in triploid *T. officinale* and had a female fertility of (near-) 100%. No developmental deviations were observed in this apomict. We propose that only those newly formed hybrid apomicts that do not experience adverse effects of genome interference (*viz.* developmental aberrations) will be successful competitors under natural conditions since their female fertility will approach 100%. In other words, hybrid apomicts will be selected for absence of genome interference.

Our development data from hybrid apomicts indicate that apomictic development is under complex genetic control. Apomixis in *Taraxacum* may be regulated by major genes whose expression and function is modified by expression of minor genes or modifiers. Existence of modifiers in addition to major apomixis genes had previously been proposed by Bicknell et al. (2000) and Koltunow et al. (2000) in the aposporous composite *Hieracium*. The Taraxacum type of apomixis as a developmental trait might be shown to inherit as a quantitative trait locus (QTL) in future molecular marker studies.

ACKNOWLEDGEMENTS

We are grateful to Ron van der Hulst for providing the natural tetraploid *T. richardsianum* and tetraploid sexual-apomictic hybrids together with unpublished data.

MATERIALS AND METHODS

Plant materials

The tetraploid dandelion types studied in this study are in table 1 on page 93. The natural tetraploid apomict was collected in Wales, UK by den Nijs and van der Hulst. The hybrid TL326-16 is part of the progeny of a cross involving the male-sterile pistillate parent TJX3-20 from Beaujeu, France and the triploid apomictic staminate parent TJX4-79 from Langres, France, collected by den Nijs. The hybrids TM37-16, TM37-19 and TM37-23 are part of the progeny of crossing TJX3-20 x TLX11-38; the latter is sampled near Brehmen, Germany. The hybrid H5-3 is described in Tas and van Dijk., 1999. The other tetraploid hybrids result from uncontrolled pollinations of closely related sexual mothers transplanted into a completely apomictic population of dandelions (van Dijk et al.., unpublished) . The hybrids PA14 and H3-9_S13 are progeny of crossing diplosporous non-parthenogenetic "type C" hybrids H6-3 and H3-9, respectively (van Dijk et al., 1999; chapter 5) with sexual diploid fathers. See scheme 6.1 for the backcross scheme.

Microscopy procedures

The methods for preparing meiotic chromosome spreads from microspore mother cells are described in chapter 3. The procedures for assessing developmental stages, fixation of inflorescences, clearing of material and DIC microscopy are described in the chapters 4 and 5. For dark-field microscopy, the Nikon Optiphot microscope was equipped with Nikon phase-contrast and dark-field optics.

As in chapters 4 and 5, only those preparations that could be interpreted beyond doubt were included in the quantifications.

Table 6.1. Occurrence of diplosporous restitution* and apomixis in the tetraploid hybrids used in this study

hybrid	restitution (in %)	apomixis (in %)[4]
TK45[*]	100	98%
TM37-16[1]	100	82-100
TM37-19[1]	100	64-100
TM37-23[1]	100	82-100
TL326-16[1]	n.d.	90-100
H5-2[2]	100	76
13-9-3[2]	100	66
14-1-23[2]	70	48
14-4-24[2]	62	19
14-4-31[2]	100	41
PA14[3]	100	0
H3-9_S13[3]	100	0

* data based on Nomarski microscopy observations during megasporogenesis

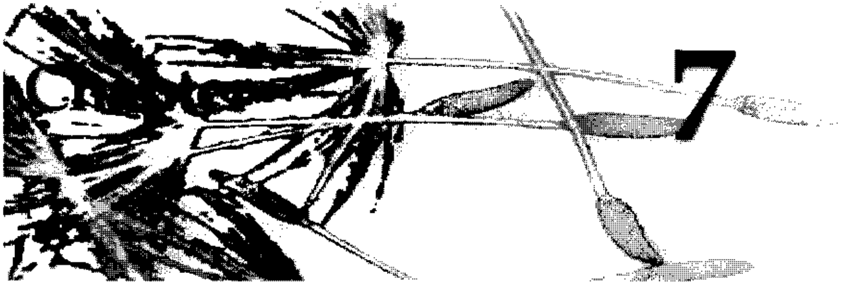
[*] natural apomict *T. richardsianum* sect. *naevosa* (UK)

[1] *T. officinale* hybrids resulting from fertilisation of reduced haploid egg cell by triploid unreduced pollen grain nuclei from triploid apomict - crosses by R. van der Hulst

[2] *T. officinale* hybrids resulting from fertilisation event as above - crosses by P. van Dijk

[3] *T. officinale* hybrids resulting from fertilisation of triploid type B hybrids (non-parthenogenetic egg cells, autonomous endosperm) by haploid reduced pollen grain nuclei from diploid sexual

[4] data on [1] based on seed formation (van der Hulst, unnpublished) and on [2-3] on seed germination (van Dijk et al., 1999 and unpublished)



General Discussion

Peter van Baarlen

In the preceding chapters, the developmental pathway of apomixis in *Taraxacum* has been described and apomictic reproduction compared with sexual reproduction. From the analysis of partially and completely apomictic hybrids, a model has been constructed that proposes multigene control of the expression of apomixis as a whole. A comparison with the available information on autonomous endosperm mutants in sexual *Arabidopsis* has given insight in the genetic regulation of endosperm formation and has provided important clues on the possible genetics of autonomous endosperm. Furthermore, several possible ways for creating genetic variation have been described. Recombination during meiotic prophase preceding restitution followed by segregation provides a means to generate genetic variation. Formation of reduced pollen grains and occasionally reduced egg cells provides an opportunity (however infrequent) for sexual reproduction.

In this chapter, the relevance of several characteristics of apomictic reproduction for the evolution of apomixis will be discussed. In addition, the phenotype of meiotic diplospory, parthenogenesis and autonomous endosperm formation will be compared with plant and yeast mutants.

The cyto-embryology of sexual and apomictic reproduction in *Taraxacum* is very similar

A comparative analysis of meiosis and embryology in sexual diploid and apomictic triploid dandelions made it possible to describe the following aspects:

- main differences in the microscopic phenotypes of meiosis and embryology between sexuals and apomicts
- the expression of diplospory, parthenogenesis and autonomous endosperm in the progeny of sexual X apomict crossings
- characteristics of the reproductive system that may contribute to the assessed variation in natural populations of *Taraxacum* apomicts

The main conclusions of this thesis are:

1. Megasporogenesis in *Taraxacum* apomicts differs from megasporogenesis in *Taraxacum* sexuals through the modification of standard meiosis to a type where chromosome pairing is interfered with and where no reductional division takes place but restitution nucleus formation instead (chapter 3)
2. Embryo and endosperm development appear similar in sexuals and apomicts although apomicts do not depend on fertilisation for the initiation of development (chapter 4)
3. The microscopic phenotypes of hybrids without the capacity for egg cell parthenogenesis resemble those of *Arabidopsis* autonomous endosperm mutants (chapter 5)
4. Successful expression of apomixis possibly depends upon genome interactions; deviations result in female sterility due to developmental deviations, especially during megagametogenesis (chapter 6)

The genetic data obtained by van Dijk and colleagues (1999, in prep.) showing that hybrid tetraploid progeny dandelions behave as autotetraploids (van Dijk et al., in prep), and the segregation data of apomixis in crossing sexuals x apomicts strongly suggest that apomixis differs from sexual reproduction by a few major genes. These genes can be uncoupled by meiotic recombination - which does occur especially during pollen meiosis and probably far less frequently during the restitutional egg cell meiosis - and "mixed" with elements of sexual reproduction (chapter 5).

Diplospory is a modification of standard meiosis

The main characteristics of meiotic diplospory are:

- sex-specific expression : by default during megasporogenesis, not during microsporogenesis
- interference with chromosome pairing at meiotic prophase
- circular orientation of chromosomes at diakinesis (e.g. fig. 3.2b).
- restitution nucleus formation, resembling an early telophase nucleus with a nuclear membrane surrounding the unreduced chromosome complement

At meiotic prophase of megasporogenesis in apomicts, recombination is observed at low frequencies (Richards, 1970; Malecka, 1971; chapter 3) notwithstanding the expression of the *DIP* gene. The meiotic pathway is nearly regular during microsporogenesis (where the *DIP* gene is by default not expressed) apart from the near-absence of homologue pairing, strongly suggesting that the meiotic machinery is intact in diplosporous apomicts but that specific parts of it are modified. Several meiotic mutants in plants and yeasts have phenotypes similar to certain features of diplospory. I will discuss a few of these mutants in greater detail.

Diplospory genes might be homologues of cohesin-complex genes

Two specific *Arabidopsis* mutants show disturbed chromosome segregation and fragmentation. The *dif1* (Bhatt et al., 1999) and *syn1* (Bai et al., 1999) mutants are presumably mutants of the same gene (P. Fransz, pers.comm.). The *syn1* mutant has several meiotic defects, from improper chromosome condensation and impaired pairing at early prophase I to chromosome fragmentation at anaphase I. Cytogenetic analysis showed that both the absence of pairing and fragmentation were associated with disturbed chromosome condensation at early prophase I (Bai et al., 1999). The DIF1 and SYN1 proteins are functional homologues of yeast mitotic Rad21 and meiotic Rec8 proteins that function as cohesins: proteins that hold sister chromatids together. The Rec8 functions explain the meiotic anomalies in the *syn1* and *dif1* mutants.

Schizosaccharomyces pombe Rec8 protein is incorporated into the centromeres and pericentromeric chromosome regions during premeiotic S-phase, is loaded onto chromosomes by the end of pachytene, plays a role during assembly of the synaptonemal complex onto chromosomes and is necessary to hold sister chromatids together at the centromeres during the first meiotic division (Watanabe and Nurse, 1999; Klein et al., 1999). After meiosis I, Rec8 protein is only present near the centromeres. Rec8 protein is present on unpaired (univalent) chromosomes in

asynaptic mutants and in the ribosomal DNA gene locus that remains unpaired during yeast meiosis (Klein et al., 1999). In *Rec8* deletion mutants, sister chromatids separate at the first meiotic division and may be distributed randomly during the second division. The viability of the resulting dyad spores is near-wildtype but viability of tetrad spores is less than 20%, very likely because of aneuploidy (Watanabe and Nurse, 1999). Other *rec8* mutation phenotypes are decreased meiotic recombination, accumulation of hyperresected chromosome fragments (as in the *syn1* or *dif1* mutants) and disruption of linear elements (Stoop-Meyer and Amon, 1999; Watanabe and Nurse, 1999). Recombination in the *rec8* deletion mutant is decreased in the centromeric regions but is much higher in the telomeric regions where Rad21 protein (the mitotic Rec8 variant) is localised (Watanabe and Nurse, 1999). Presence of Rec8 at the centromeres after anaphase I is dependent on presence of the Spo13 protein which delays the removal of the Rec8 protein from the centromeres until the second meiotic division (Klein et al., 1999). *Spo13* mutants have several characteristics reminiscent of diplospory that I will discuss in greater detail.

The *spo13* mutants of *Saccharomyces cerevisiae* produce dyads during sporulation (Klapholz and Esposito, 1980a). Although recombination in these mutants is near-wildtype (Klapholz and Esposito, 1980b), in 9 out of 10 meioses only the equational division takes place. In certain *spo13* strains, mixed segregation has been observed with some chromosomes segregating reductionally whereas other chromosomes in the same meiocyte divide equationally (Hugerat and Simchen, 1993). The *spo13-1* mutation yields fertile unreduced spores in absence of recombination (Wagstaff et al., 1982). Considering these characteristics, it is attractive to assume that functional homologues of the *SPO13* gene are involved in meiotic diplospory. Both in *Chondrilla* and *Taraxacum*, no or very little recombination does occur during meiotic prophase I and a single equational division takes place yielding dyads containing fertile megaspores. Moreover, division of chromosomes as well as chromatids in one single division has been observed in *C. juncea* (Bergman, 1950) and tetraploid *Taraxacum* apomicts (own unpublished observations). To date, no *SPO13* homologue has been found in plants. Plant Rec8-homologous SYN1 (DIF1) proteins can potentially be used in two-hybrid assays under the expectation that plant Spo13 proteins bind SYN1.

Diplospory may be a result of improper specification of mitosis and meiosis

The finding that certain plant mutants exhibit the tendency to perform mitosis instead of meiosis (e.g. the *am1* mutant; Golubovskaya et al., 1993, 1997) together with the presence of genes that have meiosis- and mitosis-specific variants suggests that specific regulators are needed in order to initiate meiosis and repress mitosis. Commitment of cells to develop into archesporial cells and megaspore mother cells requires a "programming" or balance between genes regulating meiosis and mitosis (e.g. Kleckner, 1996) that can be variable between gametophytes. Mitotic diplosporous plants often produce diplosporous and meiotic megaspore mother cells within a single inflorescence (Rutishauser, 1967), suggestive of a differential commitment of cells to the mitotic and meiotic pathways. Commitment to meiosis is possibly determined during S-phase, by incorporation of meiosis-specific proteins in chromosomes. It is attractive to assume that after incorporation of meiosis-specific proteins (e.g. Rec8 proteins) in chromosomes during S-phase, archesporial cells will be committed to meiosis and form megaspore mother cells with the capacity to undergo meiosis. Likewise, incorporation of mitotic variants into chromosomes during S-phase might "preprogram" megaspore mother cells to undergo mitosis; this hypothetical scenario might apply to mitotic diplospory.

Meiotic diplospory may depend on incorporation of variant meiosis-specific proteins in chromosomes during premeiotic S-phase that confer megaspore mother cells the capacity to initiate meiotic prophase but not efficient recombination. An hypothetical scenario is that chromosomes with variant meiosis-specific proteins do not undergo the first meiotic division but only the second mitotic-like division (since proper mitotic variants can still be present). Such a deviant meiotic pathway is presumably regulated by several gene products since meiosis-specific proteins often occur in complexes together with other proteins. In polyploids, proteins may be encoded by diverged homologues. If proteins within a complex are not capable of interacting as in either parent, the result may be a complex that performs a different function in the polyploid than in either parent at the comparable stage. This idea is intruiging since natural apomicts are nearly always polyploid.

Type C hybrids resemble autonomous endosperm mutants in *Arabidopsis*

One of the triploid hybrid types obtained by van Dijk et al. (1999), type C, seemed to combine elements of sexual and apomictic reproduction. Application of molecular markers, flow cytometry and Nomarski microscopy demonstrated that this type of hybrid had a nonreductional meiosis, produced autonomous endosperm but lacked the capacity for egg cell parthenogenesis (chapter 5; van Dijk et al., in prep.). This phenotype resembled that of the *Arabidopsis fis1/mea* or *fie* autonomous endosperm mutants (chapter 5) with the important difference that the endosperm in these mutants does not cellularise but remains mainly nuclear. Maturation of endosperm depends on the methylation status of the plant since double mutants with abolished *FIE* and *MET1* function do display cellularising endosperm (Vinkenoog et al., 2000). It had previously been shown that methylation status is important for expression of *FIS1* / *MEA* (Vielle-Calzada et al., 1999; Grossniklaus et al., 2001) and for genes that function together with *MEA* during endosperm and embryo development (Luo et al., 2000). It is clear that polycomb-dependent imprinting and methylation play essential roles during endosperm and seed formation (Sørensen et al., 2001).

In apomicts, imprinting of the maternal genome may be different from imprinting in sexual plants since in apomicts, no male genome is introduced. The methylation status in apomicts might be lower than in sexual plants considering that mature endosperm is produced in hypomethylated but not normally methylated, unpollinated *fie* mutants (Vinkenoog et al., 2000). A lower methylation status implies at first sight less repression of overall gene transcription in apomicts. However, many genes from the male genome are activated later during embryo and endosperm development in sexual diploid *Arabidopsis* (Vielle-Calzada et al., 2000). To "mimic" this condition, apomicts would have to repress transcription of large parts of their genomes at the initiation of autonomous embryo and endosperm development. This seems contradictory to the proposition by Vinkenoog and colleagues (2000). Perhaps repression of transcription is not primarily achieved via methylation but instead via chromatin modification, for instance via polycomb proteins, and methylation may serve to maintain chromatin-established imprints. One factor complicating a detailed analysis of gene expression in apomicts is their polyploid status since polyploidy has been shown to influence gene expression in yeast (Galitski et al., 1999) and plants (e.g. Mittelsten-Scheid et al., 1996; Comai, 2000; Soltis and Soltis, 2000).

Expression of parthenogenesis seems conditional on preceding expression of diplospory

All hybrids with the capacity for parthenogenetic embryo formation were diplosporous (chapter 4 and 5; van Dijk et al., 1999, submitted). A similar connection between diplospory and parthenogenesis has been found in diplosporous *Erigeron* (Noyes, 2000; Noyes and Rieseberg, 2000). Moreover, a linear decrease of chromosome numbers led to exponential decrease of the capacity for parthenogenesis in triploid apomictic *Taraxacum* hybrids (van Dijk et al., submitted). Chromosome loss in triploid diplosporous hybrids is an indication for inefficient restitution nucleus formation mediated by the diplospory gene. The finding that diplospory is linked to expression of parthenogenesis is strongly suggesting that timely expression of the *DIP* gene and concomitant timely restitution nucleus formation, incorporating all chromosomes, is necessary for parthenogenesis to become expressed. How can this dependence be explained? considering that restitution nucleus formation and parthenogenetic initiation of embryo development are not only separated by the second meiotic division but, more importantly, by the three mitotic divisions of megagametogenesis where each division is preceded by a DNA synthesis phase.

I speculated above that the DIP protein is a chromosomal protein, and now further speculate that DIP proteins are involved in chromatin remodelling and imprinting. Following this line of thinking, improper restitution indicates that the imprinting status of the surviving megaspore is different from those produced in natural apomicts, in such a way that it does not enable parthenogenetic initiation of embryo development.

Alternatively, the DIP protein may function as a cyclin or together with a cyclin, regulating some aspect of the transition from early prophase to telophase. Expression of DIP mediates or superposes a precocious telophase stage instead of the first meiotic anaphase. The two alternative hypotheses are not mutually exclusive.

A last possibility is that the *DIP* mRNA itself functions to shift the first meiotic division into restitution, analogous to the meiRNA that mediates the switch from mitosis to meiosis and is necessary for performing the first meiotic division in the yeast *S. pombe* (Watanabe and Yamamoto, 1994). Identification and characterisation of the diplospory gene and its regulation will ultimately show how meiotic diplospory is achieved in *Taraxacum*. At this moment, is is at least conceivable to envision that imprinting influences the capacity for parthenogenetic initiation of embryo development, as polycomb-dependent imprinting influences autonomous endosperm development.

Origin of apomictic reproduction in *Taraxacum*

It was argued above that the apomictic mode of reproduction in *Taraxacum* presumably diverged from the sexual mode. If apomixis in *Taraxacum* is a modification of sexual reproduction, then apomixis genes are most likely variant forms of genes present in their sexual conspecifics. The finding of *fis / mea* and *fie* autonomous endosperm mutants in the sexual plant *Arabidopsis* corroborates this hypothesis. The corresponding *Taraxacum FIS / MEA* and *FIE* homologues will be searched for in sexual and apomictic dandelions (work in progress). But the *Taraxacum* gene(s) mediating autonomous endosperm development are most likely not the *FIS / MEA* or *FIE* Polycomb homologues themselves since the autonomous endosperm trait is dominant in *Taraxacum*, also when crossed into sexual diploids, but recessive in the *Arabidopsis* mutants. The *Taraxacum AUT* gene (chapter 5) may function either upstream or downstream the *FIS / MEA* and *FIE* Polycomb genes, upstream as a life-cycle dependent negative regulator of the Polycomb genes and downstream as a gene or locus that somehow "escapes" the polycomb-imposed repression-of-expression imprint. This "escape" can be through a mutation in a polycomb-response element (the recognition site where a polycomb complex will be formed onto the DNA) so that no functional repression block will be formed, or brought about by a translocation event during which the specific polycomb-regulated locus/loci are moved to a chromosomal region that is not regulated by polycomb proteins. In theory, it is also possible that factors (DIP proteins?) are present in *Taraxacum* apomicts that interfere with polycomb proteins or repressive complex formation, by mutation of a gene from which the encoded protein should be present only after, not before, fertilisation.

Evolutionary stability of genetic variation in *Taraxacum*

Taraxacum officinale, the common dandelion, is a particularly interesting species for evolutionary studies because sexual and apomictic reproduction occur in one single species. Dandelions are highly heterozygous as has been known for most apomictic genera (Rutishauser, 1967) and genetic variation is large in sexual, apomictic and mixed dandelion populations (Menken et al., 1995; van der Hulst et al., 2000). In mixed populations, both shared and unique molecular markers have been found, suggesting gene flow between apomicts and sexuals (Menken et al., 1995). Sexual and apomictic dandelions can be successfully crossed giving a certain proportion of hybrid apomictic progeny plants (Tas and van Dijk, 1999). It is likely that novel apomictic lineages are continuously formed in nature in those areas where sexual and apomictic dandelions are flowering together. The controlled crosses also show that the genes

determining apomixis function in a sexual background, are dominant and highly penetrant.

Between sexual and apomictic dandelions, only the progression of meiosis and the requirement for fertilisation of the embryo sac is different. The diplosporous type of meiosis resembles a mutated form of the type that is found in sexual dandelions. Therefore, it is very likely that apomictic reproduction is derived from sexual reproduction in *Taraxacum*. The modification of meiosis is the most apparent change in the transition from sexual to apomictic reproduction. The variation found in apomictic populations (van der Hulst et al., 2000) can be the result of two features of the type of meiosis found in *Taraxacum* apomicts.

The first feature is a consequence of the fact that recombination does take place in male and female meiosis in apomicts and that recombined chromatids segregate during the second meiotic division (chapter 3). The second feature is that female meiosis is reductional at low frequencies (van Baarlen et al., 2000 and chapter 3). Reductional female meiosis will often lead to aneuploid gametes, as in male meiosis. Fertile (euploid) reduced egg cells have presumably lost the potential for parthenogenesis (Malecka, 1973) and can be fertilised by pollen grains. When the parental plants had diverged genomes, fusion of their gametes will give rise to apomictic lineages with novel recombinant genomes. This event is, although it takes place in purely apomictic populations, not different from regular sexual reproduction and can be seen as a form of covert sex (Hurst et al., 1992).

These features offer two relevant explanations why apomicts still produce pollen grains. First, apomixis can be transferred via reduced pollen grains from apomicts to sexuals, thereby creating novel hybrid apomictic lineages. Reduced pollen grains may have borne a lower mutational load and the hybrid apomictic lineage may have a higher fitness than the parental apomictic pollen donor. Second, pollen grains may create novel apomictic lineages in purely apomictic populations by fertilising reduced egg cells.

But why is it that male meiosis is reductional but female meiosis not? Genome reduction may provide most of the selective advantages that are always associated with meiosis (e.g. Felsenstein, 1974; Kondrashov, 1984; Bernstein et al., 1988; Otto and Barton, 1997). One selective advantage that is specific for *Taraxacum* has become clear in controlled crosses; corroborating facts can be found in natural apomictic populations. It is difficult to obtain and maintain dandelions with a higher than tetraploid ploidy level (Malecka, 1973; Tas and van Dijk, 1999; van Dijk, unpublished data). This suggests that higher ploidy levels are lowering fitness. Likewise, most apomicts found in nature are triploid or (in the northern regions) tetraploid (den Nijs and Sterk, 1980; Menken et al.,

1995). This provides a good explanation why it is advantageous to produce reduced pollen grains: it avoids the continuous increase of the ploidy level in the offspring.

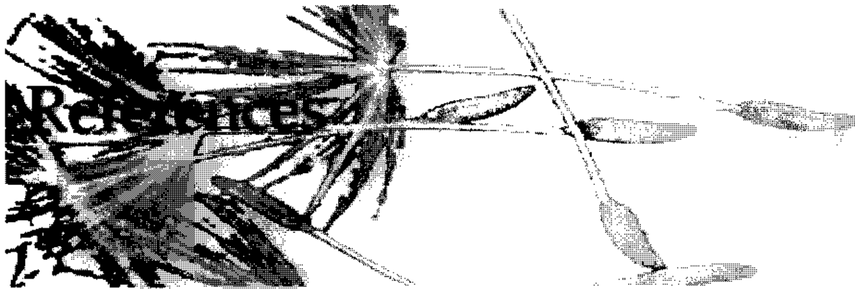
The egg cells, on the other hand, are unreduced and only the second meiotic division takes place. The evolutionary advantage of this nonreduction is that it preserves the maternal genome. Nonreduction of egg cells is a perfect way to ensure their euploidy and contain heterozygosity.

Recombination and restitution create and maintain variation in apomictic dandelions

Several researchers (Richards, 1989; King and Schaal, 1990; van Dijk, 1997 and unpublished data; chapter 2) have found unexpectedly high levels of rDNA variation in the apomictic dandelions, usually higher than in sexual dandelions. In the rDNA locus, mitotic recombination presumably creates variation, for instance via the reorganisation and duplication of repetitive sequences (see discussion in chapter 2). Absence of meiotic recombination through lack of pairing of homologous satellite chromosomes followed by restitution tends to maintain this variation once arisen. Also outside the rDNA locus, heterozygosity is maintained during meiotic diplospory because of absence of recombination (the latter would lead to homozygosity for a given sequence; see chapter 3). Avoidance of meiotic recombination has a second advantage: recombination during megasporogenesis can increase multivalent formation and concomitant problems with balanced segregation and female sterility (chapter 1). Avoidance of meiotic recombination may be achieved via two ways in *Taraxacum*.

Recombination in *Taraxacum* is significantly lower at the triploid ploidy level compared to the diploid and tetraploid ploidy level (van Baarlen et al., 2000; chapter 3). This was not only found in natural and hybrid apomicts but also in synthetic triploids that were the results of crosses between sexual diploids and tetraploids obtained by colchicin treatment of sexual diploids (chapter 3). It is very attractive to assume that there is positive selection for the triploid ploidy level in meiotic diplosporous apomicts to avoid loss of heterozygosity. This hypothesis is supported by the observation that many meiotic diplosporous apomicts are triploid, but triploidy is in itself an uncommon ploidy level in apomictic plants (Nogler, 1984). Most apomicts have tetraploid or higher, usually "even" (iso-)ploidy levels.

Not only odd ploidy levels but also specific mutations in meiotic genes that mediate recombination may abolish meiotic recombination. There is some evidence for such mutations in *Taraxacum* since chromosome pairing and bivalent formation is significantly lower in natural apomictic triploids and in sexual-apomictic hybrid triploids than in synthetic triploids (van Baarlen et al., 2000; chapter 3). Selection pressures that tend to increase or maintain variation is a trend that is expected under Weismann's hypothesis (recently discussed by Burt, 2000). Meiotic and mitotic recombination, formation of reduced gametes and delayed parthenogenesis open up several possibilities to create and maintain genetic variation, even in purely apomictic populations. These features of the Taraxacum type of apomixis make it unlikely that long-term fitness of apomictic *Taraxacum* lineages is seriously lowered by accumulation of mutations (Muller's Ratchet; see chapter 1). The fact that apomictic dandelions can be found throughout the world, that high heterozygosity is found in apomictic populations (even in regions where their sexual counterparts are not found), and that apomictic lineages can continously be formed (especially in mixed sexual-apomictic populations are all demonstrating that a combination of asexual reproduction and occasional sex can be very successful.



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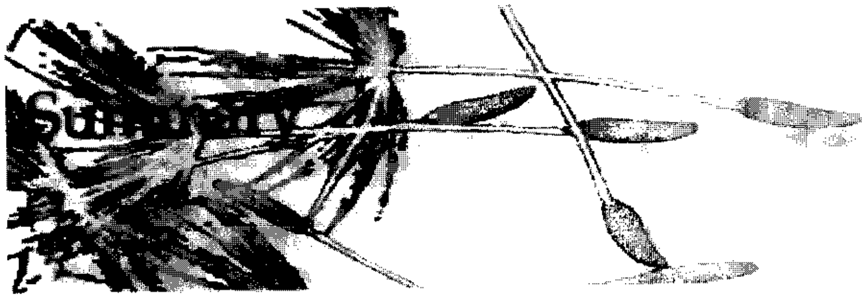


Nearly all flowering plants reproduce sexually, which means that reproduction is preceded by double fertilisation of haploid egg cells and binucleate or diploid central cells. A small fraction (approximately 0.1%) of the angiosperms can produce seeds without prior double fertilisation. This type of asexual reproduction is via a process called apomixis. Apomictic plants form the female reproductive centre or the megagametophyte, comtaining the egg and central cell, by several means. All forms of apomixis are characterised by the somatic chromosome number in the nuclei of the egg and central cell. One form of apomixis is meiotic diplospory, where megaspore mother cells (the female meiocytes) initiate meiosis but do not perform the first, reductional division. After the second meiotic (chromatid) division, the meiocytes transform into dyads with two (instead of four as in standard meiosis) megaspores, each with the somatic chromosome number. The megaspores undergo three mitotic divisions (megagametogenesis) and transform into eight-nucleate, seven-celled embryo sacs that contain (among others) the egg and binucleate central cell. The Taraxacum (dandelion) type of apomixis is one form of meiotic diplospory, and resembles sexual reproduction the most of all forms of apomixis.

The genus *Taraxacum* comprises diploid sexual as well as tri- or tetraploid apomictic dandelions. Sexual dandelions follow the standard type of angiosperm sexual reproduction and depend on double fertilisation in order to set seed. Apomicts differ in their nonreductional meiosis (diplospory) and their independence on double fertilisation for seed production. In triploid apomictic dandelions, the egg cell develops into a triploid embryo via parthenogenesis. The hexaploid central cell (which is the result of fusion of the two triploid nuclei) develops autonomously into hexaploid endosperm the tissue that provides the nutrition for the growing embryo. The genetic basis for the Taraxacum type of apomixis is unknown, as well as to what extent the genetics of Taraxacum-apomixis differs from sexual reproduction.

In the research that is now presented, the central issue was the microscopic comparison of standard (reductional) and diplosporous

meiosis, together with the male and female gametophyte and gamete development, using natural sexual and apomictic dandelions and their hybrids that were obtained after crossing natural sexual mothers and apomictic fathers. Furthermore, it was demonstrated using the FISH technique (Fluorescence *in situ* Hybridisation) that variation in ribosomal DNA within individual apomicts on the chromosomal level resulted from variable organisation of repetitive DNA fragments (chapter 2). The obtained results were also seen from an evolutionary viewpoint and gave insight into possible reasons for the successful way of life of apomictic dandelions (chapter 7).

One important result from the comparative microscopical analysis was that sexual and apomictic reproduction in dandelion are essentially similar, apart from the three elements mentioned before. In a detailed analysis of reductional and diplosporous meiosis, it could be demonstrated that recombination does occur during both forms of meiosis, and that recombined chromatids can segregate in the apomicts (chapter 3). The large genetic variation that has been found in populations of apomictic dandelions may partly be the result of recombination and possibly contributes to the evolutionary success of dandelions (also chapter 7).

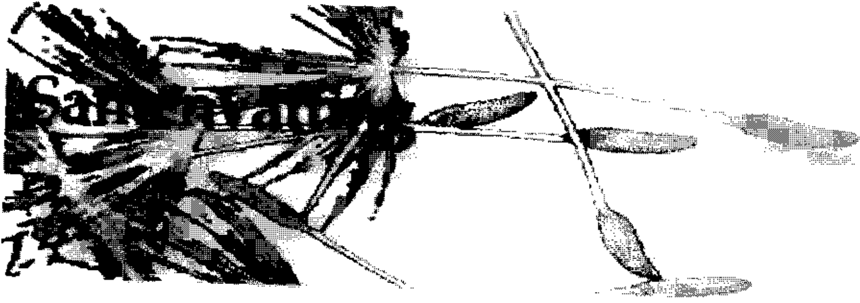
The comparison of megagametophyte formation and embryology of diploid sexual and triploid apomictic dandelions and their triploid-apomictic hybrids demonstrated that the morphology of gametophytes, did not differ, nor did the the developing embryo or endosperm. One of the conclusions was that the genetic basis for apomixis especially bears upon the initialisation of embryo and endosperm development. There may exist genes in the apomicts that do not suppress embryo and endosperm development in absence of fertilisation, while homologous genes do so in the sexual dandelions. The microscopical data, supplemented with genetic data and ploidy analyses and the interpretation of these different types of datasets are presented in chapter 4.

In addition to the completely apomictic triploid, sexual-apomictic hybrids, other hybrids were found that were shown to be dependent upon pollination for viable seed-set, even though apparently healthy seeds were produced in absence of pollination. An analysis of gametophyte-, embryo and endosperm development in these triploid hybrids showed that although gametophyte development was again as in the earlier investigated dandelions, embryo or endosperm development were or were not dependent on pollination. The interpretation of microscopical, genetic and flow cytometry data led to the conclusion that these hybrids combined elements of sexual and apomictic reproduction. The analysis of this special type of hybrids, the so-called "apomixis-

recombinants", is described in chapter 5. From this analysis, a multi-gene model for the genetic regulation of the Taraxacum type of apomixis has been composed that is being discussed in the chapters 5 and 7.

One last class of hybrids was formed by the (non-)apomictic tetraploids (4x). The 4x-hybrids that were the result of fertilisation of a haploid egg cell in a sexual mother by a triploid (unreduced) pollen grain from an apomictic father were all apomictic. The 4x-hybrids that were the result of fertilisation of an egg cell in the recombinant semi-apomictic triploid hybrid "type C" by a haploid pollen grain from a sexual dandelion were near-sterile and dependent on fertilisation for seed set. The developmental anomalies that were observed in the latter type of 4x-hybrids gave insight into the complex regulation of apomixis and suggested that interactions between genomes in hybrids can bring about sterility and are therefore important for the establishment for novel hybrid lineages in natural populations.

The multi-gene model (chapter 5) is, together with a comparison of plant and yeast mutant displaying phenotypes resembling certain characteristics of apomixis, being further described in chapter 7. In addition, the evolutionary consequences of recombination during diplosporous meiosis and the possibility of incidental fertilisation events (sexual reproduction) in populations of apomicts are being described here. The discussed characteristics of Taraxacum apomixis, dominant apomixis genes and the possibility for intercrossing apomicts and sexuals in mixed populations resulting in the formation of novel apomictic lineages collectively can explain the evolutionary success of apomixis in *Taraxacum*.



Vrijwel alle zaadplanten ondergaan sexuele reproductie, wat inhoudt dat voortplanting voorafgegaan wordt door bevruchting van een haploïde eicel en haploïd-tweekernige of diploïde centrale cel. Een klein deel (ongeveer 0.1%) van de angiospermen of bedektzadigen is in staat zaad te zetten zonder voorafgaande dubbele bevruchting. Deze wijze van asexuele voortplanting verloopt via een prodes dat apomixie wordt genoemd. Er zijn verschillende manieren waarop apomictische planten het vrouwelijk voortplantingscentrum, oftewel de megagametofyt welke de eicel en centrale cel bevat, formeren. Kenmerkend voor alle vormen van apomixie is dat de kernen van de eicel en centrale cel hetzelfde chromosoomaantal hebben als kernen in somatische cellen. Eén van de vormen van apomixie is meiotische diplosporie, waarbij megaspore moeder cellen (de vrouwelijke meiocyt) de meiose ingaan maar de eerste reductiedeling niet uitvoeren. Na de tweede meiotische (chromatide) deling transformeren de meiocyten in dyades die twee (in plaats van vier als in standaard meiose) megasporen bevatten, elk met het somatisch chromosoomaantal. De megasporen ondergaan drie mitotische delingen (megagametogenese) en transformeren in achtkernige, zevencellige embryozakken die (onder andere) de eicel en tweekernige centrale cel bevatten. Het Taraxacum (paardebloem) type apomixie is een vorm van meiotische diplosporie en gelijkt van alle vormen van apomixie het meest op sexuele reproductie.

Het geslacht *Taraxacum* omvat zowel diploïde sexuele en tri- of tetraploïde apomictische paardebloemen. De sexuele paardebloemen volgen het standaard type sexuele voortplanting van de angiospermen en zijn voor zaadzetting afhankelijk van dubbele bevruchting. De apomicten wijken daarin af in hun nonreductionele meiose (diplosporie) en hun onafhankelijkheid van dubbele bevruchting voor zaadzetting. Bij triploïde apomictische paardebloemen ontwikkelt de eicel zich via parthenogenese tot triploïd embryo. De hexaploïde centrale cel (welke ontstaan is na versmelting van de twee triploïde celkernen) ontwikkelt zich autonoom tot hexaploïd endosperm, het weefsel dat het groeiende embryo van voedingsstoffen voorziet. Het is onbekend wat de genetische basis is voor

het Taraxacum type van apomixie en in hoeverre Taraxacum-apomixie hierin afwijkt van sexuele reproductie.

Binnen het hier gepresenteerde onderzoek stond de microscopische vergelijking van standaard (reductionele) en diplospore meiose alsmede de mannelijke en vrouwelijke gametofyt- en gameetontwikkeling centraal, gebruikmakend van in de vrije natuur verzamelde sexuele en apomictische paardebloemen en hun hybrides, verkregen na kruising van de natuurlijke sexuele moeders en apomictische vaders. Verder werd aangetoond, gebruikmakend van de techniek FISH (Fluorescence *in situ* Hybridisation), dat variatie in ribosomaal DNA binnen individuele apomicten op chromosoom niveau het resultaat was van variabele organisatie van repetitieve stukjes DNA (hoofdstuk 2). De verkregen resultaten werden mede bezien vanuit een evolutionair perspectief en gaven inzicht in mogelijke redenen voor de succesvolle levenswijze van apomictische paardebloemen (hoofdstuk 7).

Een belangrijk resultaat van de vergelijkende microscopische analyse was dat sexuele en apomictische reproductie in de paardebloem in essentie gelijk zijn, op de drie eerder genoemde elementen na. In een uitvoerige analyse van reductionele en diplospore meiose kon worden aangetoond dat recombinatie in beide vormen van meiose optreedt en dat gerecombineerde chromatiden kunnen segregeren in de apomicten (hoofdstuk 3). De grote genetische variatie die gevonden is in populaties van apomictische paardebloemen kan voor een deel het gevolg zijn van recombinatie en draagt mogelijk bij tot het evolutionaire succes van paardebloemen (zie ook hoofdstuk 7).

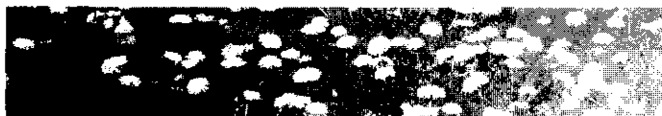
De vergelijking van megagametofytvorming en embryologie van diploïd sexuele en triploïd apomictische paardebloemen en hun triploïd-apomictische hybriden toonde aan dat er geen verschillen waren in de morfologie van de gametofyten, noch in het ontwikkelende embryo of endosperm. Eén van de conclusies die hieruit getrokken werden was dat de genetische basis voor apomixie vooral betrekking heeft op de initialisatie van embryo- en endosperm ontwikkeling. Er zijn wellicht genen aanwezig in de apomicten die embryo- en endosperm ontwikkeling niet afremmen bij ontbreken van bevruchting, terwijl homologe genen dat wel doen in de sexuele paardebloemen. De microscopische data, aangevuld met genetische data en ploïdie-analyses en de interpretatie van deze verschillende datatypes zijn gepresenteerd in hoofdstuk 4.

Naast de compleet apomictische, triploïde sexueel-apomictische hybriden zijn ook hybriden gevonden die afhankelijk van bestuiving bleken om kiemkrachtig zaad te zetten, ook al werden ogenschijnlijk gezonde zaden geproduceerd wanneer geen bestuiving werd uitgevoerd. Een analyse van de gametofyt-, embryo- en endosperm ontwikkeling in deze triploïde hybriden toonde aan dat alhoewel gametofytontwikkeling wederom

hetzelfde schema volgde als in de voorheen onderzochte paardebloemen, embryo- of endosperm ontwikkeling al dan niet afhankelijk van bestuiving waren. De interpretatie van microscopische, genetische en flow cytometrische data leidden tot de conclusie dat de bewuste hybriden elementen van sexuele en apomictische reproductie combineren. De analyse van dit speciale type hybriden, de zogenaamde "apomixie-recombinanten" , staat beschreven in hoofdstuk 5. Aan de hand van deze analyse is een multi-gen model voor de genetische regulatie van het Taraxacum type apomixie opgesteld dat in de hoofdstukken 5 en 7 wordt bediscussieerd.

Een laatste klasse van hybriden vormden de al dan niet apomictische tetraploïden (4x). De 4x-hybriden welke ontstonden na bevruchting van een haploïde eicel in een sexuele moeder door een triploïde (ongereduceerde) pollenkorrel van een apomictische vader waren alle apomictisch. De 4x-hybriden welke ontstonden na bevruchting van een eicel in de recombinant semi-apomictische triploïde hybride "type B" (zie hoofdstuk 5) door een haploïde pollenkorrel van een diploïde sexuele paardebloem waren nagenoeg steriel en afhankelijk van bevruchting voor zaadzetting. De ontwikkelingsstoornissen welke in het laatste type 4x-hybride werden waargenomen gaf inzicht in de complexe regulatie van apomixie en suggereerde dat interacties tussen genomen in hybriden steriliteit teweeg kunnen brengen en dus van belang zijn voor vestiging van nieuwe hybride lijnen in natuurlijke populaties.

Het multi-gen model uit hoofdstuk 5 wordt, vergezeld van een vergelijking van plante- en gistmutanten met fenotypes die gelijken op bepaalde karakteristieken van apomixie, verder bediscussieerd in hoofdstuk 7. Tevens worden hier de evolutionaire consequenties besproken van recombinatie tijdens diplospore meiose en de mogelijkheid van het incidentele optreden van bevruchting (sexuele reproductie) in populaties van apomicten. De besproken karakteristieken van Taraxacum-apomixie, dominante apomixie genen en de mogelijkheid tot kruising van apomicten en sexuelen in gemengde populaties met als gevolg: vorming van nieuwe apomictische lijnen kunnen gezamenlijk het evolutionaire succes van apomixie in *Taraxacum* verklaren.



Nawoord

Nu het wetenschappelijk deel achter de rug is kan het voor mij belangrijkste onderdeel gepresenteerd worden: het persoonlijke deel. De afgelopen jaren zijn een aantal mensen extra belangrijk geweest tijdens mijn onderzoeksperiode die ik nu hiervoor kan bedanken.

Hans, jou wil ik als eerste bedanken voor je inzet en continue betrokkenheid bij zowel mijn persoonlijke ontwikkeling als het onderzoek dat ik verrichtte. Zelfs mijn wildste hypothesen vonden altijd een oor bij je, en mijn vele zijpaadjes en overige bezigheden konden ook altijd op je warme belangstelling rekenen. Dit proefschrift had zonder jou nooit deze gedaante kunnen aannemen, niet qua inhoud noch qua lay-out.

Peter, ik ben blij dat ik dit onderzoek grotendeels samen met jou heb kunnen uitvoeren. Je nooit aflatende enthousiasme om in het sexleven van paardebloemen te duiken was een grote stimulans voor mij. De embryologische studies waren slechts mogelijk dankzij jouw continue ondersteuning met inzichten en plantenmaterialen en ik ben blij dat het onderzoeken van die kleine sexorgaantjes zoveel heeft opgeleverd.

Rolf, ik wil jou bedanken voor het vertrouwen dat je als promotor in mij, Hans en Peter toonde door de daadwerkelijke onderzoekslijnen goed te keuren die toch erg buiten het oorspronkelijke project zijn getreden. Verder hebben de discussies over allerlei uiteenlopende onderwerpen die ik zowel met jou als met Fons gevoerd heb mijn promotieperiode van allerlei interessante overpeinzingen voorzien, waar ik jullie beiden erg dankbaar voor ben.

Boudewijn van Veen, jij mag zeker niet in dit rijtje ontbreken. De mogelijkheden van uiteenlopende grafisch software en kleurgebruik zijn mij vooral door jou goed bijgebracht, en je stond ondanks je drukke werkzaamheden altijd klaar om me niet alleen te helpen maar ook van alles te vertellen over de achtergronden van grafisch werk.

Jannie, Henny en Paul, jullie stonden vanaf het begin af klaar om mij waar mogelijk te ondersteunen in het lab; niet alleen door technieken bij te brengen en uitleg te geven over de achtergronden daarvan maar ook door middel van het praktiseren van leuke lab-babbels die het voor mij zo saaie lableven kleur gaven. Paul, ik hoop dat ik ooit nog eens met jouw verve leer fluiten!

Mede in dit verband wil ik ook graag Sandor, Wim en Lucy bedanken; regelmatig hebben jullie mijn klaagzangen over labwerk aangehoord en geholpen waar mogelijk. Verder waren jullie ontzettend leuke mede-aio's waar ik veel mee afgeouwehoerd heb in het lab, en ik vind dan ook dat jullie alle drie te vroeg Erfelijkheidsleer verlaten hebben. Schande!

Een lab bestaat uiteraard uit een hele lading studenten, aio's, overige medewerkers en assistenten waarvan sommigen buiten Erfelijkheidsleer. Ik wil met name in Erfelijkheidsleer Jaap, Henk, Ronnie, Judith, Siemen, Olga, Klaas, Duur, Anne, Marijn, Arjan, Menno, Marlinde (die gelukkig mijn paranimf wilde worden!), Pieter, Edu, Marijke, Salah, Emile en Leonie en daarbuiten Tijs, Jim, Valerie, Jenny, Paul, Marijke en er zijn nog heel veel mensen die ik hier onmogelijk allemaal kan noemen.......bedanken voor de leuke momenten waar, hoe en wanneer dan ook (maar dan vooral in de kroeg). Ik denk dat vooral de CuliCult avondjes (onze versie van de Bonte Avond) hebben laten zien hoe belangrijk het is om ook naast het werk met elkaar creatief bezig te zijn.

De Paardebloemen-werkgroep bestond naast de mensen uit Erfelijkheidsleer uit aio's en hun begeleiders vanuit Amsterdam, Utrecht en Heteren; Ron, Carolien, Hans, Gerdien, Peter en Jos, bedankt! voor jullie bijdragen, inzichten en visies en Ron: het plantenmateriaal, dit alles heeft hoe dan ook bijgedragen tot het hier achter liggende geschrevene.

Margot, Wilbert (die uiteraard mijn paranimf wilde worden) en Saskia, jullie waren vanaf het begin van dit onderzoek nauw betrokken bij mij en mijn onderzoek; ik wil jullie dan ook graag apart bedanken voor alle steun en getoonde warme belangstelling. Zowel ikzelf als dit onderzoek had nooit hetzelfde kunnen zijn zonder jullie. Wilbert, je bent de beste alter-ego gebleken die een aio zich maar kan wensen! wat zou het leven saai zijn wanneer ik de enige Ideale Schoonzoon op deesch aardkloot zou zijn.

Ik vind het leuk dat ik nu ook in de gelegenheid ben de mensen met wie ik sinds enkele jaren met veel plezier in de band 't Hardt (thardt.musiceert.nl) muziek maak op officiële wijze te bedanken: Gern, Marjolein, Margareth, Roger, Ernst, Wiebe en Paul, bedankt! voor de leuke tijd die ik met jullie heb. Moge ik nog vaak het *a capella* verzoek krijgen: "Kan die basversterker niet wat zachter!!?"

Aan allemaal: bedankt!

Peter van Baarlen
Wageningen, 16 juni 2001

Curriculum vitae

Peter van Baarlen werd op 16 juni 1969 te Rotterdam geboren. Na het behalen van het VWO diploma aan het City College Emmaus-Franciscus begon hij in 1987 de studie Plantenziektenkunde aan de Landbouwuniversiteit te Wageningen (LUW). De doctoraalfase omvatte drie vijfmaands afstudeervakken met als onderwerpen tritrofische interacties, genetische karakterisering en identificatie van paddestoelen, en populatiegenetica van plantepathogenen. Tijdens de stageperiode, uitgevoerd bij het Horticulture Research International te Littlehampton, Engeland verkreeg hij ervaring met veldwerk op het gebied van het zoekgedrag van sluipwespen. In 1993 studeerde hij af en werkte hij achtereenvolgens bij het laboratorium voor Entomologie van de LUW en bij het Instituut voor Plantenziektenkundig Onderzoek (IPO-DLO) te Wageningen. In 1996 begon hij aan de vakgroep Erfelijkheidsleer van de LUW een promotie-onderzoek met als onderwerp: de evolutie van asexuele reproductie in paardebloemen (gefinancierd door de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO), onderdeel Aard- en Levenswetenschappen (ALW)). De resultaten van dit onderzoek zijn in dit proefschrift beschreven.

Ondertussen werkt hij tijdelijk als onderzoeker aan de populatiebiologie van plantepathogenen bij het Hilbrands Laboratorium te Wijster.