Chromosome studies and genetic analyses of natural and synthetic apomictic model species

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CHAPTER 1

General introduction

One of the intriguing and enigmatic curiosities in higher plant biology is apomixis, the asexual seed production without paternal contribution. The trait was first described by Smith (1841) and has been challenging and inspiring scientists since then. Mendel too, in the latter years of his research career, was encouraged by Nägeli to study the remarkable inheritance of *Hieracium*; although he never would have been able to solve the puzzle being unaware of its apomictic trait (discussed in Henig, 2000). All apomictic systems share at least three developmental components: (i) apomeiosis, the generation of a cell capable of forming an embryo without prior (full reductional) meiosis; (ii) parthenogenesis, the spontaneous fertilization-independent development of the embryo; and (iii) the capacity to either produce endosperm autonomously or to use an endosperm derived from fertilization (Koltunow, 1993; Carman, 1997). Two basic forms of apomixis can be distinguished: **adventitious embryony** and **gametophytic apomixis**, of which the former refers to the formation of an embryo from somatic cells of the nucellus or integument, a case of sporophytic agamospermy best described for citrus. Gametophytic apomixis can be subdivided into **apospory** and **diplospory**. In apospory in which somatic cells form the embryo sac is represented by the Hieracium type and the Panicum type. In diplospory, in which the megaspore mother cell forms the embryo sac, is represented by the Taraxacum type and Antennaria type. Diplosporous apomicts display regular meiotic pairing and recombination, but meiosis undergoes an internal bypass, in the sense that the reductional part is skipped, either through first or second meiotic division restitution or through mitotic diplospory, thus giving rise to unreduced gametes. The classification given above is a very simplified view of apomixis as various cases of intermediate forms exist (reviews in Nogler, 1984a; Asker and Jerling, 1992; Savidan *et al.*, 2001). Crane (1978; 2001) classified even nine different ways for the formation of 2n embryo sacs and another five for the development of embryo and endosperm, in theory resulting in at least 45 types of gametophytic apomixis. However, only half of them were found in natural apomicts. In addition, various apomixis-like traits are known, mostly in well-studied crop plants as alfalfa, barley, lily and potato. Each of them possesses only components of the apomixis trait such as 2n gamete formation, female and male parthenogenesis, and aberrant endosperm formation. It is generally stated that apomicts are genetically identical to the mother plant whose meiosis is absent or not fully functional, but this is not entirely true for diplosporous apomicts where normal homologous recombination and chromatid exchange can lead to loss of heterozygosity in specific chromosome regions during subsequent generations (Naumova and Vielle-Calzada, 2001; Crane, 2001). The occurrence of gametophytic apomixis has been described for over 400 species in more than 40 families, but is most common in the Poaceae, Rosaceae and Asteraceae families. Apomixis is strongly correlated with polyploidy and aneuploidy such as trisomy, although a clear causality between sexuality and diploidy versus apomixis and multiple copies of genomes or chromosomes is still lacking.

Many breeding strategies used for crop improvement are based on the production of hybrid seed. Plants derived from hybrid seed have greater vigour and yield than their parents.

The production of hybrid seed depends on the generation of parental lines (true-breeding inbred lines) that are obtained only after a time consuming process of self-pollination and selection over several years. Due to the sexual nature of most cultivated crops, the agronomic vigour of hybrid seed is only maintained for one generation because the seed produced by hybrid plants is genetically variable. Thus, individual characters that were uniform in the commercial hybrid will be genetically separated in the offspring of the hybrid, causing significant losses in crop yield and value.

Apomixis, by providing a system to produce seeds genetically identical to the mother plant, will allow the fixation of hybrid vigour, maintaining indefinitely new crop varieties with valuable agronomic traits. It will also contribute to the conservation of plant genetic resources by increasing the efficiency of plant breeding, thereby allowing plant breeders to produce more diverse crop varieties using locally adapted varieties as breeding stocks. This diversity should also increase the resilience of agricultural systems in the face of new disease epidemics and changing weather patterns. These possibilities have encouraged the interest of geneticists to explore the apomixis trait in newly developed breeding systems (Spillane *et al.*, 2004). However, examples of such clonal seed reproduction still have to be claimed for a major crop. Attempts to transfer genetic loci controlling apomixis from wild relatives to cultivated crops by introgressive hybridizations have resulted in only partial fertile, agronomically unsuitable alien additions (Bicknell and Koltunow, 2004, Savidan, 2000; Spillane *et al.*, 2004).

Recent breakthroughs in understanding its genetics and molecular organization incite scientists to greater efforts for seeking novel approaches in transferring elements of apomixis from wild relatives to crops. Several of such natural apomict – crop combinations exist for a long time or are ultimate goals of long standing programmes, including *Pennisetum squamulatum* to pearl millet (Hanna *et al.*, 1998), *Tripsacum dactyloides* to maize (Sokolov *et al.*, 1998a,b), *Paspalum simplex* to rice (Pupilli *et al.*, 2001), *Poa pratensis* (and other species) to other grasses and cereals, and *Taraxacum officinale* to lettuce. In this thesis I plead for introducing a new combination of models: *Boechera holboellii* as donor of apomixis and its close relative, *Arabidopsis thaliana* as the recipient experimental "crop".

The nature and number of genes controlling apomixis has been the subject of long standing debates. Most apomicts are facultative and produce sexual and asexual seeds in the same plant. Therefore, it is tempting to believe that apomixis is a modification of the normal course of sexual reproduction in terms of temporal synchrony and local changes in gene expression profiles (Holsinger, 2000; Grimanelli *et al.,* 2001; Koltunow and Grossniklaus, 2003). Due to pluriformity and variation of the apomictic systems it is unlikely that a single ubiquitous gene for the apomixis pathway switch would exist. Moreover, it gets more obvious that apomixis is the result of a complex orchestration of genetical, epigenetical and environmental factors. Many models have been postulated accounting for the control of apomixis (reviews in Asker and Jerling, 1992; Savidan *et al.*, 2001; Bicknell and Koltunow, 2004). In the aposporous *Panicum* spp., *Ranunculus* spp. and *Hieracium* spp., the dominant

locus controlling apospory co-segregates with parthenogenesis, suggesting that a single locus, either simple or complex, controls apomixis (Bicknell *et al.,* 2000; Nogler, 1984b). According to the single locus regulation hypothesis, a master gene was assumed to trigger a cascade of various apomictic processes (Mogie, 1992). In other apomicts, the different elements of apomeiosis, parthenogenesis and endosperm formation appear to be separated from each other. Studies in *Taraxacum officinale* (van Dijk *et al.*, 1999), *Poa pratensis* (Matzk *et al.,* 2000) and *Erigeron annuus* (Noyes and Rieseberg, 2000) revealed that apomixis was controlled by separate genes for diplospory (apomeiosis), parthenogenetic development of the embryo and autonomous embryo development. In *Poa pratensis* even a five locus model was postulated (Matzk *et al.*, 2005).

It is generally assumed that apomixis is a modification of sexual reproduction rather than an entirely novel process, and hence deregulation of regulatory genes in the sexual reproductive pathway may cause apomixis (Peacock, 1992). Therefore, analysis of the sexual reproductive process may shed light on how apomictic genes function. An example is the *FIS* gene in *Arabidopsis* (Grossniklaus *et al.,* 1998). Mutation of *FIS* genes induces autonomous endosperm formation and some forms can produce embryo-like structures but do not complete to become mature seed (Chaudhury *et al.,* 1997; Chaudhury *et al.,* 1993; Grossniklaus *et al.,* 1998; Grossniklaus and Vielle-Calzada, 1998a; Ohad *et al.,* 1996). The genes encode polycomb group proteins, which function as chromatin remodelling or gene regulation factors (Grossniklaus *et al.,* 1998; Luo *et al.,* 1999; Ohad *et al.,* 1999). Several candidate genes involved in apomeiosis and parthenogenesis have been suggested (Reviews in Spillane *et al.,* 2001; Hecht *et al.,* 2001; Koltunow and Grossniklaus, 2003; Rose-Fricker *et al.,* 2002; Reik and Walter, 2001; Spillane *et al.*, 2001; 2004).

Attempts to produce apomixis in sexual species, especially in crop species, are ultimate interesting. The approaches are by introgression and genetic engineering. The introgression has been done in maize with the gene from *Tripsacum dactyloides* (Sokolov *et al.,* 1998a,b). Successful results have not been reached as only for low seed set formation (reviews in Bicknell and Koltunow, 2004). The other approach, genetic engineering of genes in sexual pathway with ectopic expression in megagametophytic tissues, could render synthetic apomicts. So far no progress of this approach has been reported.

Genetic analyses of the apomictic traits and its components require adapted approaches as normal female meiosis is lacking or incomplete. Obligate apomicts generally allow reductional meiosis and pollen formation and so the paternal parent can be used for linkage studies. In the case of facultative apomixis, the maternal parent may form three types of progeny: *i*) normal B_{II} hybrids resulting from fertilizations of reduced embryo sacs; *ii*) apomictic progeny from parthenogenetic embryogenesis of unreduced eggs; *iii*) and B_{III} progeny resulting from fertilization of unreduced eggs with reduced pollen, thus producing non-maternal types at higher ploidy levels (Savidan *et al.*, 2001). The assessment of apomixis in the parents and their offspring is tedious and /or time consuming depending on the methods employed.

Embryo sac microscopy is the first and most straightforward method to establish reduced sexual and unreduced apomictic events in differentiated megaspore mother cells on the basis of callose wall depositions, number and positions of the meiotic daughters and other derived cells in the ovule. The old-fashioned sectioning of embedded ovule tissues is being replaced by faster methods using Nomarski DIC microscopy of cleared ovules, and allows reliable spatial studies of the native pistil and ovule morphology. Genetic markers are more advantageous when screening for apomicts in larger populations. Most suitable markers are the co-dominant SSR (microsatellites), SSLPs and RFLP markers that visualize the molecular variants of both chromosomes. In Chapter 6 I will describe how such markers can be used to demonstrate heterozygosity fixation as an indication for apomictic inheritance. Such markers can also be used to establish variability in apomictic parents and their progeny, and when tightly linked to genes controlling elements of apomixis, they can be used in selection programmes and physical mapping studies (*e.g.*, Pupilli *et al.* 2001, 2004).

Cytogenetic methods are indispensable when apomicts and sexuals differ in ploidy level or chromosome number. Flow cytometry is an excellent tool to determine the approximate chromosome numbers, but becomes less robust when B chromosomes or other cases of aneuploidy are involved or when the parental genomes of alloploid apomictic hybrids have clearly different C values. A more recent method is the powerful flow cytometric single seed screen (Matzk *et al.*, 2000), which will be described in Chapter 3. Meiotic studies in diplosporous apomicts will further elucidate the role of chromosome pairing and segregation, and the nature of meiotic restitution in the production of unreduced gametes. Cytogenetic analyses are also essential in studies to confirm suppression of meiotic recombination and hemizygosity of aposporous controlling regions like the apospory-specific genomic region in *Pennisetum squamulatum* (Goel *et al.*, 2003). Establishment of chromosomes involved in the breakdown of apomixis in polyploids and their backcross derivatives (Noyes and Rieseberg, 2000; Sokolov *et al.*, 1998a,b; Nogler, 1984b) also required cytogenetic analyses. Further methods to demonstrate apomictic inheritance are controlled pollination after hand emasculation in conjunction with incompatibility and auxin tests (Asker and Jerling 1992; Savidan *et al.*, 2001).

Scope of the thesis

In this PhD thesis I present different studies on apomixis. In the first part I show the results of a chromosome study of natural apomicts of *Boechera* (*Arabis*) *holboelli*. The combination of *Boechera* and *Arabidopsis* is an obvious one: both are members of the mustard family (*Brassicaceae*) and phylogenetically very closely related. *Boechera*, although the only apomict in the family, exhibits an unprecedented variation in geographic distribution, phenotypes, ploidy level, chromosome numbers, and nuclear and chloroplast sequences. As will become clarified in this thesis it is an excellent candidate for an in-depth investigation on apomixis. However, hardly anything is known about its genomic organisation, genetics,

candidate genes and properties of the apomixis. The close relationship with *Arabidopsis* gives this natural apomict an enormous lead in developing advanced molecular and genetical tools for future research in the field. The combination of *Boechera* and *Arabidopsis* promises therefore to be an outstanding challenge for future research on the developmental biology of apomixis. In the second part I present a method of genetic screening for detecting the presence of a small number of plants that harbour a potential apomixis trait in an otherwise fully sexual *Arabidopsis thaliana* background. For this we employed *Arabidopsis* plants transformed with the AtSERK1 receptor kinase, a protein shown to be able to confer embryogenic properties to plant cells. This way we expected to induce one element of apomixis, parthenogenic embryo development, to gametophytic as well as sporophytic cells.

Chapter 2 begins with the taxonomic differences between the *Boechera* sexual species and the apomictic accessions that are used in further chapters (2-5). I show the diagnostic silique and trichome characteristics for the *B. holboellii* and *B. stricta* sexuals and their intermediate morphology in the *B. ×divaricarpa* hybrid. Chromosome analyses based on DAPI fluorescence and Fluorescence *in situ* Hybridisation (FISH) were then used to reveal the difference between the karyotypes of the diploid sexuals and the aneuploid apomictic accessions. Much attention was paid to the chromosome portraits of the aneuploid accessions with 15 chromosomes in the cell complements of which one chromosome was referred to as a B chromosome. Here I demonstrate that these aneuploid accessions actually possess two aberrant chromosome, one largely heterochromatic and one much smaller than any of the other chromosomes of the karyotype. I also show FISH experiments with *Arabidopsis* BACs in order to find support for the indications from microsatellite studies that a subset of the 15 chromosome accessions is trisomic. The striking variation in chromosome morphology and indications for structural karyotype heterozygosity inspired me to use the same plants for a study of male meiosis (**Chapter 3**). Here I show that all apomictic accessions have surprising differences in chromosome pairing, recombination and chromosome segregation. The aberrant chromosomes show clear chiasmatic bonds with other chromosomes, which shed light on their putative evolutionary origin. Some of the accessions show perfect chromosome pairing and segregation, whereas others were completely asynaptic and produced unreduced gametes. These results encouraged me to perform a series of genomic *in situ* hybridisation experiments in which I used total genomic DNA of *B. stricta* alone, or in combination with DNA of *B. holboellii* as probes. These experiments not only allowed distinction between the *stricta* and *holboellii* chromosomes in the *B. ×divaricarpa* hybrid, they also revealed that the apomictic accessions are composed of different chromosome numbers originated from the stricta and holboellii parental species. In **Chapter 5** I merge the results of the karyotype analysis, meiosis study and genome painting together and formulate some ideas for the putative origin of the apomictic accessions and in particular on the evolution of the two aberrant chromosomes. **Chapter 6** shows the results of a screening method to evaluate the potential of genes that transfer aspects of apomixis into sexual *Arabidopsis*

thaliana. I describe how a set of Single Sequence Length Polymorphism (SSLP) markers can be employed to identify individuals displaying heterozygosity fixation in segregating sexual populations as an indication of rare apomictic events. The study focuses on the *Arabidopsis thaliana AtSERK*1 gene expressed under the control of two different promoters. Finally in **Chapter 7** I will compare the results of my *Boechera* and *Arabidopsis* studies and make suggestions for future experiments.

Chapter 2

Karyotype analysis and FISH analysis of sexual diploids and apomictic accessions of *Boechera*

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Abstract

Diploid apomictic accessions of *Boechera* are characterized by strikingly variable chromosome constitutions, and most of them have an additional chromosome in their cell complements (2n=15). Using the sexual diploid species *Boechera holboellii* and *B. stricta* for comparison, we analysed DAPI stained mitotic cell complements and fluorescence *in situ* hybridisation (FISH) patterns using 45S and 5S rDNA as probes on four apomictic *B. holboellii* clones and one apomictic *Boechera* hybrid (*B. ×divaricarpa*), all of them with 15 chromosomes. Karyotype analyses of these aneuploids revealed striking differences in chromosome morphology between the apomictic accessions, which suggest that ancestral chromosomal rearrangements and substitutions have led to structural karyotype heterozygosity and to haploidization of the apomictic genomes. In the *Boechera* karyotypes we discovered two aberrant chromosomes that in previous studies were interpreted as B chromosomes. One of them measures about the same length as the shortest chromosomes of the complement, but is largely heterochromatinized (*Het*), whereas the other chromosome (*Del*) is smaller than any of the other chromosomes and is likely the result of ancestral translocation and/or deletion events. In order to test for (partial) trisomy in the 15 chromosome aneuploids we employed a bacterial artificial chromosome (BAC) painting strategy on mitotic cells, using FISH of *Arabidopsis* BACs onto *Boechera* chromosomes under adapted hybridisation and stringency conditions. We selected four *Arabidopsis* BACs, of which three contain DNA inserts corresponding to previously identified aneuploid-specific microsatellite alleles in *Boechera*, and a fourth BAC containing one of the *Arabidopsis* regions orthologous to the chromosome region in *Paspalum simplex* apomict containing putative aposporous genes. All four BACs produced bright fluorescent foci on corresponding positions of two homologous (or homoeologous) chromosomes only, and a variable number of minute foci on other chromosome sites. Hence, the FISH of these BACs did not give the expected support for putative trisomy for any of these markers, nor did it shed light on specific sequences located on the *Het* and the *Del* chromosomes.

Keywords: *Boechera*, apomixis, B chromosome, karyotypes, FISH, rDNA, BAC painting.

Introduction

Boechera, Böcher's rock cress, (formerly belonging to the genus *Arabis*) is a biennial or perennial genus of the Brassicaceae and is distributed from Alaska through a greater part of North America to Greenland. The genus is monophyletic, has a basic chromosome number of x=7 (Koch et al., 1999), and has diploid, aneuploid and polyploid forms which can reproduce through sex or apomixis. Classification of *Boechera* is mostly based on diagnostic trichome and silique morphology (Mulligan, 1995), but these characteristics are notably limited in the apomictic complex (Böcher, 1969, Dobeš et al., 2004b). The genus comprises 50-80 species (Rollins, 1993) whose remarkably high levels of polymorphism resulted from the combination of polyploidy, aneuploidy, interspecific hybridization and genome plasticity (Dobeš et al., 2005b). This abundant natural variation, along with its widespread distribution in pristine habitats, diverse modes of reproduction, hybridization, and its close relation to *Arabidopsis thaliana* makes *Boechera* one of the most promising models for research in the fields of speciation, apomixis, taxonomy, evolution and phylogeography (Koch et al., 2003).

The *Boechera holboellii* complex is composed of *B. holboellii*, *B. stricta* (syn.: *B. drummondii*) and the hybrid *B. ×divaricarpa.* The taxa are highly variable in plant morphology and occupy many different habitats from lowland to alpine zone. Morphological and cpDNA analyses (Dobeš *et al.*, 2004b) have demonstrated that *B. stricta* is monophyletic, while the more polymorphic *B. holboellii* is polyphyletic, as is evidenced by elevated chloroplast lineage diversity in the latter. Analyses of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences have demonstrated that *B. ×divaricarpa* has arisen through hybridization between sexual *B. stricta* and *B. holboellii*, or a closely related species (Dobeš *et al.*, 2004a; Koch *et al.*, 2003). Allelic variation is comparable between *B. ×divaricarpa* and *B. holboellii*, and a low number of species-specific alleles suggesting that the hybrid originated recently (Dobeš *et al.,* 2004a). ITS and microsatellite analyses have further demonstrated that *B. holboellii* and *B. stricta* are genetically differentiated, and that intraspecific hybridization, introgression, and reticulation are common in *B. holboellii* (Dobeš *et al.,* 2004a; Dobeš *et al.,* 2004b). Multiple evolutionary origins of polyploidy in *Boechera* imply that the apomictic phenotype has also been repeatedly expressed from a diploid sexual background (Sharbel and Mitchell-Olds, 2001; Sharbel *et al.,* 2005).

Geographical parthenogenesis refers to the phenomenon whereby the distributions for sexual and apomictic taxa differ, with apomicts tending to be found near the northern margins of post-glacial recolonization patterns while the ecological range of putative sexual progenitors lies north or south of the glacial rims (Savidan *et al.,* 2001). This distribution of apomictic species forms part of Carman's (1997) hybridization-derived floral asynchrony theory, which proposes that differential expression patterns of homoeologous genes in interracial or interspecific hybrids can lead to apomictic development. More specifically, Carman (1997) proposed that previously separated ancestral taxa which undergo ecological

secondary contact may be characterized by asynchrony in sexual developmental times, the result of different climatic conditions to which they are adapted. Analyses in *Boechera* using nuclear DNA have demonstrated that *B. holboellii* and *B. stricta* have recent Pleistocene origins and that *B. holboellii* has recolonized geographic areas formerly covered by the Wisconsin ice sheets, data which are consistent with part of Carman's hypothesis.

B. holboellii apomicts have variable chromosome numbers, including 2n = 14, 15, 21, 22, 28, 35 and 42. Aneuploidy is more prevalent in North American than diploidy and triploidy, while apomixis, which has been found in both diploids and triploids, is more common in the latter. Most *B. stricta* accessions are sexual diploids (2n=14), although a small number of triploid apomicts have been reported (Koch *et al.,* 2003). In an extensive microscopic study of meiosis in micro- and megaspore mother cells of diploid and triploid *B. holboellii* from Greenland and Alaska, Böcher (1954) identified apomeiosis and other meiotic variants, including abnormal pairing and skewed chromosome segregations which suggested heterozygous chromosome sets.

Another intriguing cytogenetic aspect of apomictic *Boechera* lineages is the presence of a B chromosome (Böcher, 1951; Sharbel and Mitchell-Olds, 2001; Sharbel *et al.,* 2004; 2005), which has been found in various *B. holboellii* accessions from North America and Greenland (Böcher, 1951). Flow cytometric analyses have revealed high levels of genome size variation in apomictic *B. holboellii* (Sharbel and Mitchell-Olds, 2001; Sharbel *et al.* 2005), and karyotype analyses have confirmed the presence of B chromosomes in many apomictic lineages, although it was unknown to what extent these supernumerary chromosomes have contributed to the genome size variation.

Our preliminary observations enabled us to distinguish between multiple variants of this extra chromosome, and thus we selected a number of diploid sexuals and apomictic accessions for detailed karyotype analyses using DNA specific DAPI staining in conjunction with rDNA probe hybridisation. This chapter focuses on the differences between their karyotypes, with particular attention for the morphology of the B chromosomes.

As apomixis mostly occurs in polyploid species it is generally assumed that polyploidy is prerequisite for apomixis expression. We argued that if this is the case for *Boechera* a small trisomic region controlling the apomixis trait is to be expected in the aneuploid (15 chromosome) accessions. Sharbel *et al.* (manuscript in preparation) identified three different alleles in diploid *B. holboellii* carrying an aneuploid chromosome. These possess the microsatellite loci MDC16 and AthGAPAb, which share homology with the *Arabidopsis thaliana* genome (Clauss *et al.,* 2002). Two markers, MVI11 and T1B9, adjacent to these loci, were considered associated with the presence of the B chromosome in the apomictic *Boechera* (Sharbel *et al.,* 2004). DNA sequence variation for alleles sequenced from MVI11 and T1B9 was consistent with that expected from a non-recombining chromosome, and furthermore implied that putative B chromosome alleles had originated from *B. stricta* or a closely related third species (Sharbel *et al.,* 2004). We have thus chosen the *Arabidopsis thaliana* BACs containing these loci, along with a recently described BAC corresponding to an adjacent aneu-

ploid allele region in *Boechera*, MLJ15 (Sharbel, unpublished), as probes for BAC painting onto *Boechera* chromosomes. Another *Arabidopsis* BAC (F7018) that contains sequences orthologous to putative apospory genes in apomictic *Paspalum simplex* (Pupilli, 2001; 2004; personal communication) was selected as an additional probe. Chromosome painting using pooled *Arabidopsis* BACs onto chromosome complements of unrelated species of the Brassica family has previously been shown to be successful (Lysak *et al.*, 2001; 2005), and was carried out to test whether the DNA sequences associated with aneuploid alleles (and possibly apomixis) demonstrated (partial) trisomy in *Boechera.*

Material and methods

Plant material

Most of the *Boechera spp.* seeds were provided by Prof. Thomas Mitchell-Olds (MPI for Chemical Ecology, Jena, Germany) and Dr. Tim Sharbel (Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany), and included the diploid sexual species *B. holboellii* (BH208) and *B. stricta* (BS2), the apomictic hybrid *B. ×divaricarpa* (BDi175), and four apomictic *B. holboellii* accessions (BH1, BH74, BH115, BH224). Dr. Eric Schranz (Max-Planck-Institute of Chemical Ecology, Jena, Germany) gave us *B. stricta* accession ES6, and the apomictic *B. ×divaricarpa* accession ES9. Dr. Kim Boutilier provided the apomictic accession from Greenland (GRL2). Except for the Greenland accession, which was obtained from the Botanical Garden of Copenhagen, Denmark, all species and apomictic accessions were originally collected from natural populations in North America, and

Table 2-1. Overview of all *Boechera* accessions mentioned in this thesis with their origin / place of collection.

Accession		sex/apo	Origin	chr. nr.
BH208	*B. holboellii*	Sex	Charlies Gulch, Ravalli Co, MT	14
ES6	*B. stricta*	Sex	Taylor River, Gunnison, Colorado	14
BS2 (=BS522)	*B. stricta*	Sex	Ohio City, near Gold Creek, Gunnison Co. Colorado	14
GRL2	*B. holboellii*	Apo	Greenland (Botanical Garden, Copenhagen, Denmark)	14
BH1	*B. holboellii*	Apo	Wallowa Mountains, Oregon	15
BH74	*B. holboellii*	Apo	Ranch Creek, Granit Co, MT	15
BH115	*B. holboellii*	Apo	Birch Creek, Ravalli Co, MT	15
BH224	*B. holboellii*	Apo	Bandy Ranch, Missoula Co, MT	15
BDi175	*B. ×divaricarpa*	Apo	Lower Storm Lake, Deerlodge CO, MT	15
ES9	*B. ×divaricarpa*	Apo	Vipond Park, Beaverhead, Montana	14

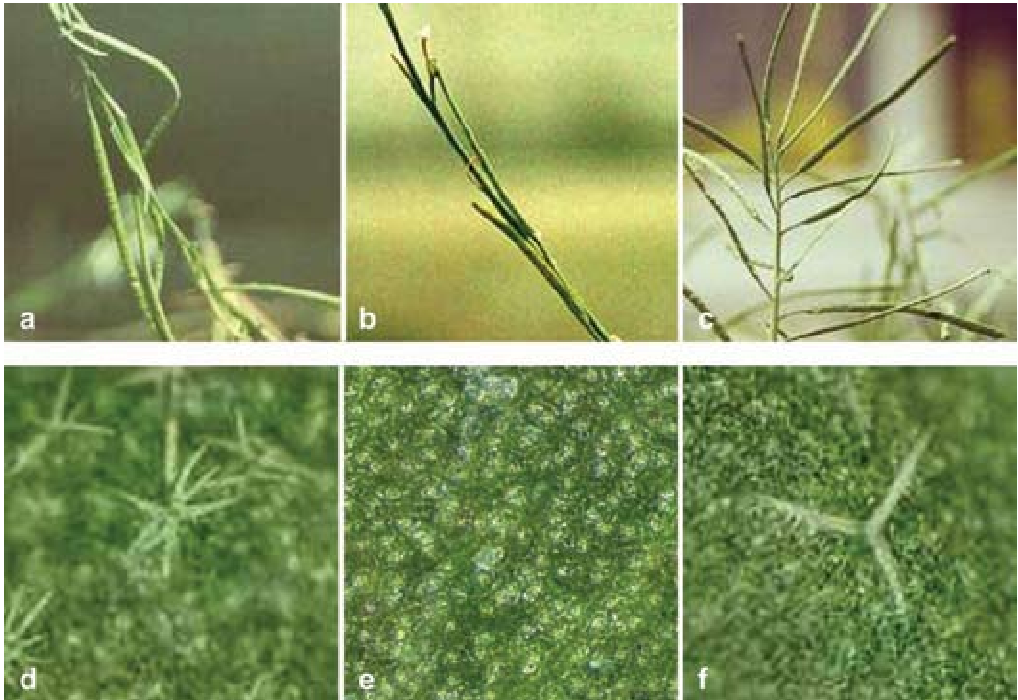


Figure 2-1. Characteristic morphological characteristics of Boechera holboellii (a,d), B. stricta (b,e) and the hybrid *B. ×divaricarpa* (c,f) based on silique position (a-c) and trichome morphology (d-f).

were propagated for several generations in the green house. Table 2-1 gives an overview of all accessions in this study with origin or place of collection.

Metaphase chromosome preparations

Seeds were cold-treated for 2 days at 4 °C, transferred to room temperature (20 °C) for 3 days to induce germination, and then the seedlings were planted into sterilized sandy soil and reared under 16/8 hour light/ dark period in the greenhouse at 25 °C. Fast growing root tips were collected between 9-10 am and were incubated in a 2 mM aqueous solution of 8-hydroxyquinoline at 15 °C for 3 hours, after which they were fixed in ethanol/acetic acid (3:1) at 4 °C for at least one day. For chromosome preparations we rinsed the root tips three times in water and one time in 10 mM citrate buffer, pH 4.5, before digesting the cell walls in a pectolytic enzyme mixture (0.3% Pectolyase, 0.3% Cellulase RS, 0.3% Cytohelicase in 10 mM citrate buffer) for 2 hrs. at 37 °C (details in Zhong *et al.*, 1996). The tips were then washed twice in water before being transferred to clean microscopic slides. Using fine needles we dissected the root tips in a small drop of 60% acetic acid on the slide while heating carefully on a hot plate at 45°C for 60-90 seconds. The material was spread by dropping about 2 mL of freshly prepared ethanol: acetic acid (3:1) onto the cells in the acetic acid solution, then

Table 2-2. Vectors containing ribosomal genes and chromosome regions of *Arabidopsis* used for preparing probes in FISH experiments on metaphase complements of *Boechera* ssp.

Plasmids / BACs	Location in *Arabidopsis*	Genes/markers	plants	references
pTA71	Chr 2 and 4	45S rDNA		Gerlach and Bedbrook, 1979
pTA794	Chr 3, 4 and 5	5S rDNA		Gerlach and Dyer, 1980
F7018	Chr 3	Aposporous marker	*Paspalum simplex*	Pupilli et al. 2001, 2004, pers. comm.
T1B9	Chr 3	AT3G07130	*Boechera* spp.	Sharbel et al. 2004; 2005
MVI11	Chr 3	AT3G19100	*Boechera* spp.	Sharbel et al. 2004; 2005
MLJ15	Chr 3		*Boechera* spp.	Sharbel, pers. comm.

dehydrated briefly in an ethanol 98% rinse and finally left to dry. The quality of the chromosome spread preparations was verified under a phase contrast microscope using a 40x objective and no cover glass. If cell spreading was insufficient or the nuclei covered with cell wall remnants we extended enzyme treatment and /or acetic acid maceration.

DAPI staining and karyotype analysis

All chromosome preparations were counterstained with 20 µL 0.2 µg/mL DAPI (4',6-diamidine-2-phenylindole) in Vectashield® Mounting Medium (Vector Laboratories). We selected well spread prometaphase and metaphase complements for measurements and karyotype analysis. Lengths of chromosome arms, heterochromatic regions and secondary constrictions (NORs) were established with the measure tool of Adobe Photoshop® and statistical analyses were performed in MS Excel. For karyotype analysis we cut out individual chromosomes in Photoshop and pasted them into Corel Draw, ordering the short arm upwards and matching chromosomes where possible on the basis of length, centromere position, heterochromatin pattern and presence of satellites / NORs.

Fluorescence *in situ* Hybridisation

We performed Fluorescence *in situ* Hybridisation (FISH) with probes obtained from 45S and 5S rDNA and five *Arabidopsis* BACs (Table 2-2). DNA was labelled either with biotin-16-dUTP or digoxiginin-11-dUTP by nick translation according to the manufacturer's protocol (Roche). FISH followed essentially the method described by Zhong *et al.* (1996). Hybridization was done overnight at 37 °C, and post-hybridization washes were done three times in 50% formamide, 2xSSC pH 7.0 at 42 °C for 5 min, and once in 2xSSC for 5 min. Background

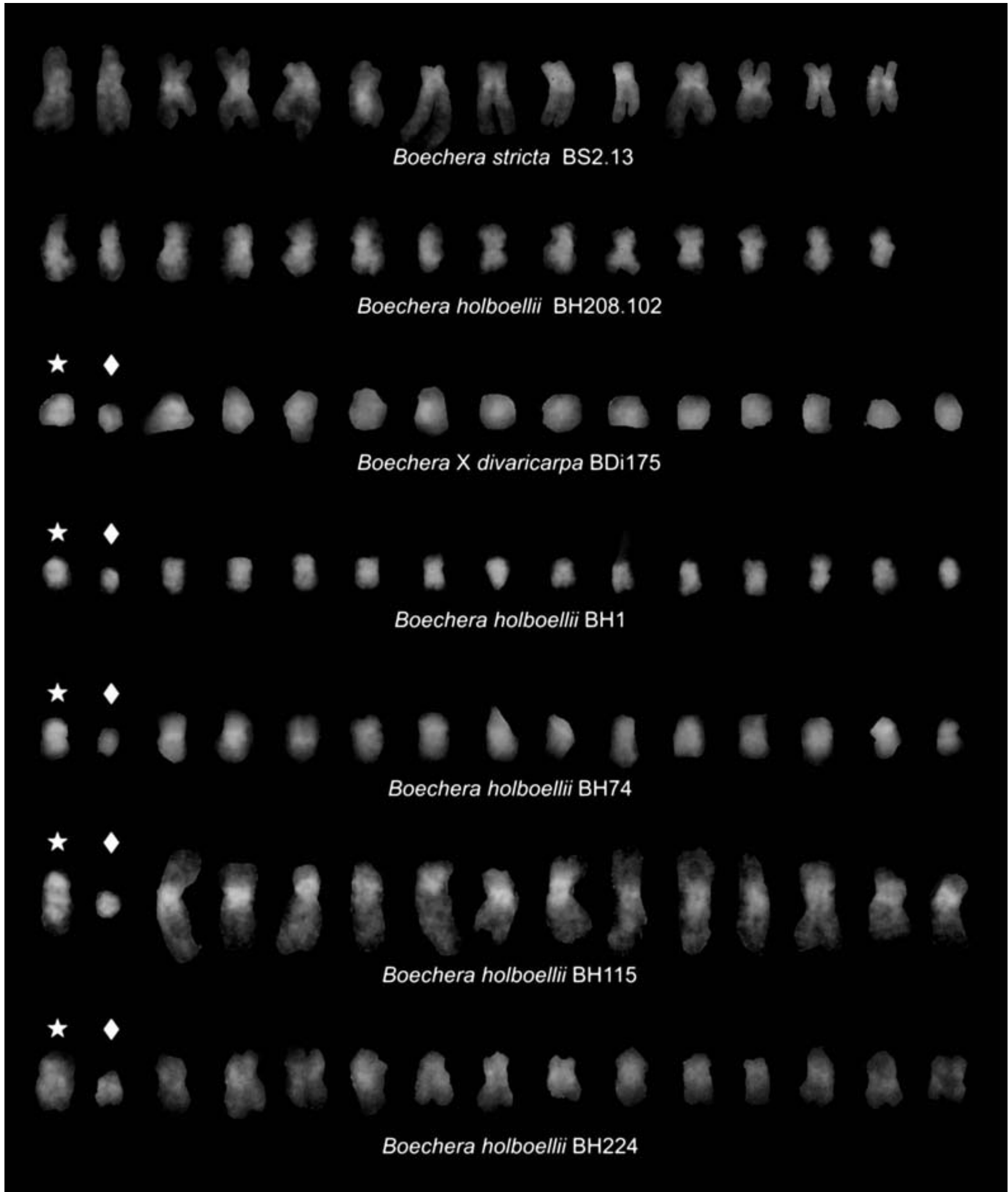


Figure 2-2. Karyotypes of sexual and apomictic accessions of the *B. holboellii* complex. Sexual diploid *B. stricta*, BS2.13, and *B. holboellii*, BH208.102 contain fourteen chromosomes (2n=2x=14). The apomictic accessions *B.×divaricarpa*, BDi175 and *B. holboellii*, BH1, BH74, BH115, BH224 have fifteen chromosomes. The asterisk and diamond indicate the heterochromatic chromosome (*Het*) and the small deletion (*Del*) chromosomes, respectively, in this and following figures. All other chromosomes were positioned in sequence of decreasing relative length (in percentage of total cell complement) and heterochromatin patterns, and were aligned with short arm upwards.

fluorescent signals was suppressed by incubating the preparation in blocking buffer (0.1M Tris-HCl, 0.15M NaCl, 1% (w/v) blocking reagent from Roche (pH 7.5) at 37 °C for 1 hour. Signal detection and amplification followed the standard protocol of the manufacturer (Roche). Digoxigenin labelled probes were detected with fluorescein-conjugated anti-digoxigenin sheep antibodies and the signals were further amplified using fluorescein-conjugated rabbit anti-sheep antibody. Biotin labelled probes were detected with Avidin-Texas Red and amplified with biotin conjugated Goat anti-Avidin and Avidin-Texas Red. Chromosomes were counterstained with DAPI in Vectashield as described above, and examined under a Zeiss Axioplan 2 Photomicroscope equipped with epifluorescence illumination and filter sets for DAPI, FITC and Texas Red fluorescence. The images were captured with a Photometrics Sensys 1,305x1,024 image array CCD camera and analysed using Genus v. 2.7 Image Analysis Workstation software (Applied Imaging Corporation). DAPI images were sharpened with a 7x7 Hi-Gauss high pass spatial filter to accentuate minor details and heterochromatin differentiation of the chromosomes. We used the levels and curves tools in Adobe Photoshop to improve DAPI heterochromatin differentiation banding and used the saturation tool too enhance colour saturation of the green and red fluorescence signals.

Results

Morphological characterization of the accessions

Accessions were classified according to the taxonomic key of Mulligan (1995) using morphology of the trichomes on the undersurfaces of the caudex leaves and silique position as most diagnostic characteristics. Species nomenclature followed Al-Shehbaz *et al.* (2003) and Dobeš *et al.* (2005). Accordingly, we identified one diploid sexual accession as *Boechera holboellii* BH208, which is characterized by downwardly positioned siliques (Figure 2-1a) and forked or arrayed trichomes (Figure 2-d). The sexual diploid *B. stricta* BS2 (Figure 2-1b and e) and ES6 have upright siliques which are generally close to the main stem. The *B. ×divaricarpa* BDi175 (Figure 2-1c and f) and ES9 hybrids exhibit intermediate morphology with spreading or ascending siliques and simple branched trichomes. On the basis of the same criteria we classified the apomictic clones BH1, BH74, BH115, BH224 and GRN2 as *B. holboellii*.

Karyotypes of the sexual *Boechera* species and apomictic accessions

Cell complements of the sexuals *B. stricta* BS2 and Es6 (not shown here), and *B. holboellii* BH208 contain fourteen chromosomes (Figure 2-2). Metaphase chromosomes of *B. stricta* measured on average 2.895 μm (1.5 – 5.8 μm) and those of *B. holboellii* on average 2.04 μm (1.2 – 3.5 μm), and could partly be arranged in putative pairs of homologues. Centromere

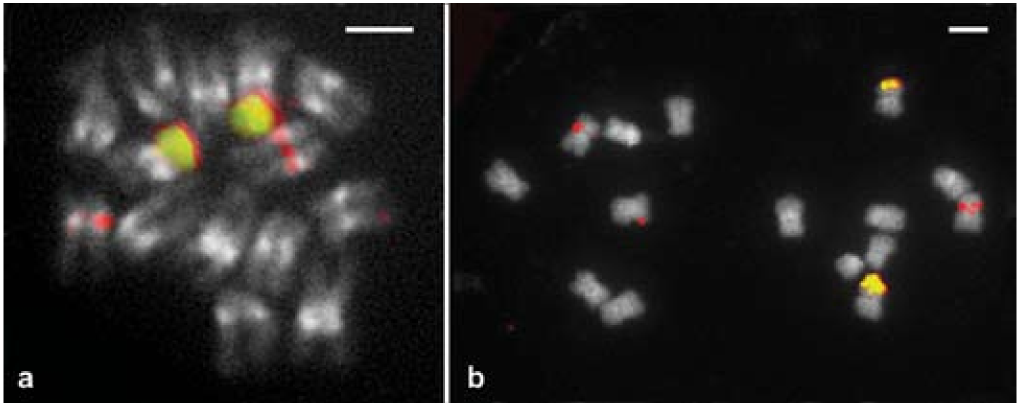


Figure 2-3. FISH with 45S (yellow) and 5S (red) rDNA. a) sexual diploid *B. stricta* (BS2) shows partly overlapping signals of the 45S and 5S rDNA repeats at the NORs of the satellite chromosomes and two signals of 5S rDNA at the pericentromeric region of a second chromosome pair. b) aneuploid apomict *B. holboellii* (BH1) demonstrates identical number and locations of 45S and 5S rDNA signals as sexual. The magnification bar equals 2 µm.

positions varied from metacentric to subtelocentric, and one of the chromosome pairs has a NOR + satellite at the short arm (Figure 2-2). The lengths of the chromosomes of the sexual *B. stricta* were significant longer than from the sexual *B. holboellii* (Anova, $P < 0.01$). We also noticed that chromosomes of *B. stricta* at late prometaphase display striking heterochromatic regions around the centromeres and nucleolar organiser regions (NORs), and that their lengths differed significantly, both within and between cells due to large contraction variation. The pericentromere heterochromatin blocks in the *B. stricta* accessions BS2 and ES6 were more pronounced and about 15% longer than those of *B. holboellii*.

The apomictic *B. holboellii* (BH1, BH74, BH115, BH224) and *B. ×divaricarpa* (BDi175) accessions were found to have 15 chromosomes (Fig 3-2). The apomictic accessions Es9 and GRN2 had 14 chromosomes, but these plants were only included in the genome painting experiments described in Chapter 5. Our analyses furthermore revealed two types of aberrant chromosomes in the apomictic accessions. The first type displayed a highly condensed structure with brightly fluorescing interstitial DAPI bands in both arms which are reflective of large heterochromatin blocks which account for 2.2 - 3.6% of the total heterochromatin content of all chromosomes. This chromosome, which is referred to as the *Het* (heterochromatic) chromosome and is indicated with an asterisk in all figures, was found in all apomictic accessions, but not in the sexual species. The heterochromatin profile of the GRN2 *Het* chromosome differed from the BH accessions and showed a larger and brighter fluorescent block in the pericentromere and flanking proximal regions (this will be further discussed in Chapter 4). The second aberrant chromosome measured on average 3.9 % of the total cell complement, and was much smaller than all other chromosomes (5.3 – 8.7%). As this chromosome most likely originated from a translocation or a large scale deletion, we refer to it as *Del* ("deletion" chromosome, indicated by a diamond in the chromosome portraits).

The pattern of brightly fluorescing heterochromatin bands differed between the apomictic accessions, making it impossible to match the chromosomes morphologically into putative homologues.

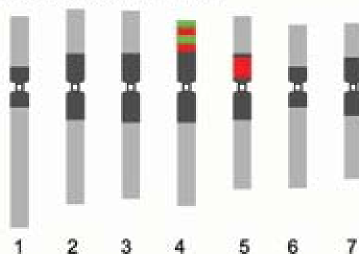
FISH analysis with the rDNA probes

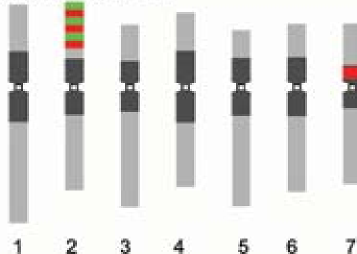
To further explore the nature of the chromosome size variation between the apomictic accessions we performed FISH experiments using the 45S and 5S rDNA probes to obtain additional chromosomal markers. In the diploid sexual *B. holboellii* and *B. stricta,* large blocks of 45S rDNA marked the short arm of one chromosome pair, including the Nucleolar Organiser Region (NOR) and the satellite (Figure 2-3). In both species these rDNA blocks partially overlap with 5S rDNA blocks. A second site of 5S rDNA was also found in the short arm heterochromatin of a second, smaller chromosome pair (Figure 2-3a: *B. holboellii* and Figure 2-3b: *B. stricta*).

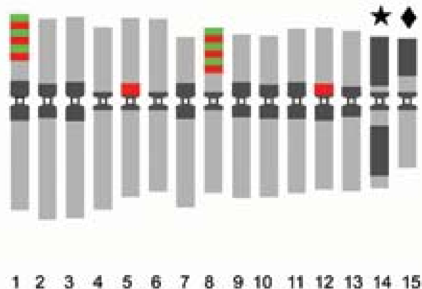
FISH with rDNA probes in the cell complements from the five apomictic accessions (BH1, BH74, BH115, BH224 and BDi175) revealed elevated variation in chromosome morphology (Figure 2-4). We arranged the chromosomes in sequence of decreasing relative length (in percentage of total cell complement) and heterochromatin patterns, with their short arm upwards. The ideograms demonstrate gradual variation in chromosome morphology with respect to the positions (i.e. relative size) of the putative homologues with the 45S and 5S signals and the size of the two aberrant chromosomes. Chromosome morphology was strikingly different between the accessions and it was not possible to match chromosomes into homologues sets.

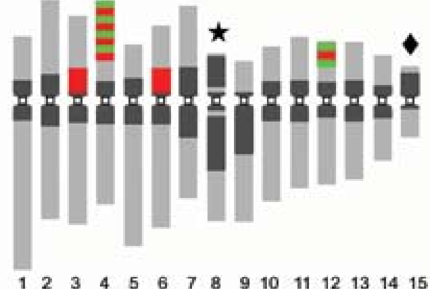
FISH detection of the *Arabidopsis* BACs

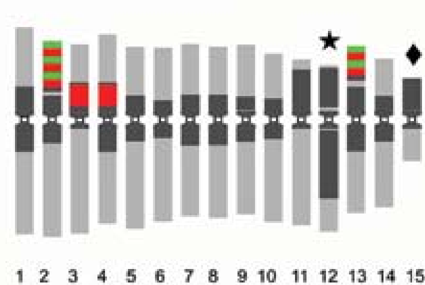
The BACs in these FISH experiments were used to screen for regions that were either potentially triplicate in the genome (on the basis of the T1B9, MVI11, and MLJ15 microsatellite markers) or containing sequences homologous to apomixis factors identified in other species (F7018). We initially attempted to identify specific clones from a *Boechera* BAC library using microsatellite loci previously shown to be diagnostic for the apomictic aneuploids (see Sharbel *et al.,* 2004; Sharbel *et al.* in prep), but the large number of positively hybridizing clones on the BAC filters suggested that common repetitive sequences in the markers made selection of these regions impossible (unpublished observations). A second series of experiments were thus developed based on FISH using *Arabidopsis* BACs containing the diagnostic microsatellite markers, *i.e.*, MVI11, T1B9 and MLJ15 (Figures 2-4a-c). A fourth marker (F7018; Figure 2-4d) containing the *Arabidopsis* region orthologous to the apospory region in *Paspalum simplex* was also employed (Dr. Fulvio Pupilli, personal communication). The post-hybridization washing step for this FISH using *Arabidopsis* probes on *Boechera* chromosomes was decreased from at the original 75% to 50% (10% formamide in 2x SSC) stringency in order to obtain sufficiently clear fluorescence signals. Most of the

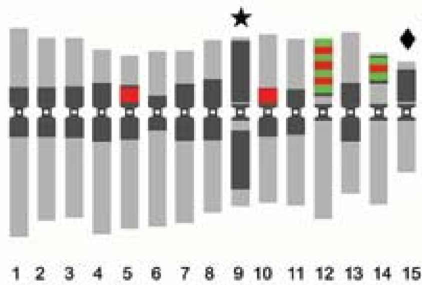

Boechera holboellii, **BH208**
1
2
3
4
5
6
7

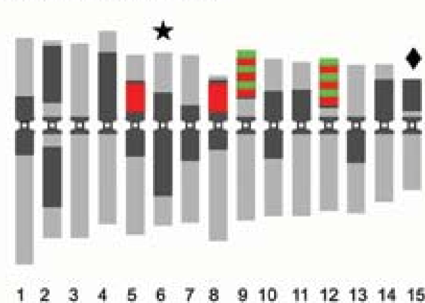

Boechera stricta, **BS2**
1
2
3
4
5
6
7

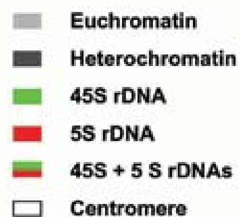

Apomictic accession **BH1**
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Apomictic accession **BH74**
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Apomictic accession **BH115**
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Apomictic accession **BDi175**
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Apomictic accession **BH224**
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Euchromatin
Heterochromatin
45S rDNA
5S rDNA
45S + 5 S rDNAs
Centromere

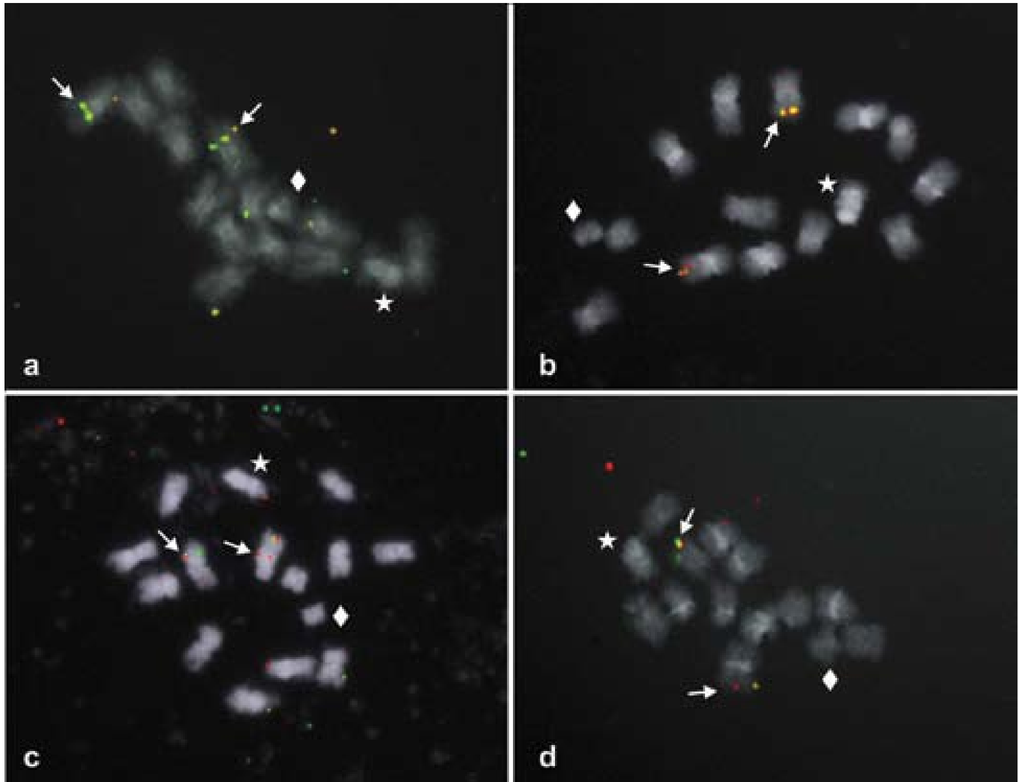


Figure 2-5. FISH painting on mitotic metaphase complements of the apomictic *B. holboellii* accession BH74 using the following *Arabidopsis* BACs: a) MVI11, b) T1B9, c) MLJ15, d) F7018.

hybridizations were carried out on mitotic metaphase spreads of the BH74 accession because of its superior chromosome morphology in which the two aberrant chromosomes were easily identifiable.

The MVI11 BAC showed major, interstitial compound signals on the short arm of two chromosomes close to the pericentromere heterochromatin, and in a few metaphase sets we observed a variable number of smaller signals on the same and other chromosomes. The minor foci occurred at identical positions on the two sister chromatids and are thus likely true signals, although they were never found in more than one metaphase set, suggesting that the hybridization sites are too small for reliable detection (Figure 2-5a). In interphase nuclei, small signals were detected which may represent repeat sequences or fragments of DNA homologous to the original *Arabidopsis* BAC. T1B9 gave one signal at the distal ends of two chromosomes (Figure 2-5b). The signals of MLJ15 were located in the middle of the

« **Figure 2-4.** Schematic representations of diploid *B. stricta* (BS2) and *B. holboellii* (BH208) haploid genomes showing nuclear organizer (NOR) and 5s rDNA regions. The ideograms of the aneuploid apomictic *B. holboellii* (BH1, BH74, BH115, BH224) and *B. ×divaricarpa* (BDi175) demonstrate heteromorphic diploid genomes containing fifteen chromosomes with two NOR and 5S rDNA signals.

short arm of the NOR chromosomes (Figure 2-5c). No hybridization signal was detected on the smallest, highly heterochromatic chromosomes (Het and Del) which are specific to the apomicts. FISH with the F7018 BAC demonstrated a single positive signal on the distal part of two chromosomes (Figure 2-5d).

Discussion

This chapter describes the first detailed morphometric chromosome analyses of aneuploid apomictic *Boechera* and demonstrates highly detailed chromosome portraits showing well differentiated heterochromatin patterns. Four results were most noticeable: *i*) *B. stricta* chromosomes have more brightly DAPI fluorescing pericentromere heterochromatin blocks than *B. holboellii*; *ii*) Chromosomes in the apomictic accessions show conspicuous variation suggesting mixtures of chromosomes from different parental species, in addition to extensive chromosomal rearrangements; *iii*) Two aberrant chromosomes were present, a brightly fluorescing (*Het*) heterochromatin chromosome and a *Del* chromosome far shorter than any other which may have possibly resulted from a deletion event and *iv*) None of the *Arabidopsis* BACs containing the putative diagnostic aneuploid-specific microsatellite locus produced triploid signals in the apomictic aneuploid *Boechera* accessions.

The chromosomes of the sexual diploid *B. holboellii* and *B. stricta* could easily be ordered based on length, centromere position and morphology, and seven pairs of homologues could be identified including one satellite chromosome pair with 45S rDNA in the short arm, and a second pair with 5S rDNA in the centromeric heterochromatin. The average chromosome length of 2.9 μm for *B. stricta* and 2.0 μm for *B. holboellii* is slightly longer than those of *Arabidopsis* (1.5 – 2.8 μm, Koornneef *et al.,* 2003), and corresponds well with the estimates 215 MB genome size for *B. stricta* (BS2) and 195 MB for *B. holboellii* (BH208), which corresponds to 1.72 x and 1.56 times the 125 MB genome size of *Arabidopsis thaliana* Columbia (R.J. Vašut, personal communication). The difference between *B. stricta* and *B. holboellii* is slightly more than the 8% in previous flow cytometry DNA measurements (Sharbel and Mitchell-Olds, 2001; Sharbel et al, 2005). The metaphase chromosome morphology of sexual *B. holboellii* and *B. stricta* are similar, but prometaphase chromosomes demonstrated more pronounced heterochromatin accumulation in *B. stricta*, which explain at least part of the genomic DNA amount difference between the two species.

In contrast to the sexual accessions, analyses of the apomictic metaphase complements did not reveal clear sets of the expected seven homologues. Both DAPI fluorescence and rDNA FISH patterns indicated elevated variation in chromosome morphology, and DAPI fluorescence banding on a number of the chromosomes strongly suggests that the genomes are composed of mixtures of chromosomes from both *B. stricta* and *B. holboellii,* or a related species. Such mixtures of assumed homoeologous or even non-homologous chromosomes demonstrate structural karyotype heterozygosity (chromosomal heteromorphy), a

phenomenon which is known from studies of ancient asexual organisms (Normark, 1999; Welch *et al.,* 2004). It explains genome-wide heterozygosity as a consequence of long evolutionary lack of meiotic recombination and accumulation of deleterious mutations. As apomixis in most of the *B. holboellii* accessions is facultative, sexual reproduction and meiotic recombination must still remain at certain levels. In the next chapter we will discuss the consequences of meiotic behaviour for these aneuploid apomicts in more detail.

An alternative explanation of karyotype heterozygosity establishment may follow the haploidization of tetraploids (de Wet, 1968; de Wet and Harlan, 1970) who presented a diploid-tetraploid-haploid cycle model to account for the dynamics of sexuality and apomixis in *Dichanthium spp.* Some autotetraploids, which behave meiotically as diploids (*e.g.,* with high levels of preferential pairing) can produce sexual haploid progeny from unfertilized egg cells. This progeny can reproduce sexually thus generating the new tetraploids that are obligate apomixis. In this case haploidization was interpreted as reintroducing sexuality back to the species, for producing new, genetically diverse clones. For *B. holboellii*, the common form is diploid and triploid, but tetraploid forms are very rare in most of its areas. Heteromorphy of individual chromosomes also results from accumulations of chromosomal mutation, which occur at all time and in both sexuals and asexuals, but the pressure of maintaining proper chromosome pairing and segregation in sexuals puts an upper limit on the amount of change which is tolerated. Asexuals do not have this limit, and thus selection does not remove the mutants from the population.

The identification of the aberrant chromosomes *Het* and *Del* in the aneuploid karyotypes has led us to reconsider our view of a single characteristic B chromosome in the apomictic populations. In our previous paper (Sharbel *et al.,* 2005) we suggested that the most aberrant chromosome in apomictic *Boechera* is a B chromosome, due to its supernumerary nature, its absence in the diploid sexuals, and differentiation at the molecular genetic level. Based upon the data presented here, we are unable to determine whether the B chromosome we previously postulated (Sharbel *et al.,* 2004; Sharbel *et al.,* 2005) is the *Het* or *Del* chromosome (or a combination of both). The *Het* and *Del* chromosomes nonetheless demonstrate some of the properties which are characteristic of the B chromosomes, including morphological variability and heterochromatin accumulation. According to the general concepts of B chromosomes (Jones and Rees, 1982; Camacho *et al.,* 2000) we thus expect the *Het* and *Del* chromosomes to be undergoing independent evolution with respect to one another and to the other autosomes, although the meiotic and transmission behaviour of these elements must be studied in order to assess if their characteristic properties meet the definition of the B chromosome. In the next chapters we will further discuss this issue.

The aneuploid apomicts in this study were collected from the ancestral areas of *B. holboellii* according to ITS sequences analysis (Dobeš *et al.,* 2004a) with a lower diversity than the southern areas, and this suggested that the population structure may indicate clonal distribution if many clones share the same characteristics. Microsatellite analysis of MVI11 and T1B9 of aneuploid in the large ancestral area showed low levels of polymorphism for

both loci. Therefore, it is more likely that *Het* chromosomes did originate from the same ancestor and spread widely in the habitat. According to wide distribution area, it suggests that the *Het* and *Del* chromosomes are evolutionary old, whereas its low level of polymorphism indicates more recent origin.

All recent studies on the extra chromosome in *Boechera* may suggest a key role of an extra chromosome(s) in the expression of apomictic elements. Much of the data collected on *Boechera* up until present demonstrate that aneuploidy is associated exclusively with apomictic individuals. The association between asexual reproduction and aneuploidy in itself is not surprising (Camacho *et al.,* 2000; Roche *et al.,* 2001), although it is intriguing that the pattern of aneuploid evolution is not random, as would be expected for a non-recombining aneuploid chromosome. For example, Sharbel *et al.* (2004) have demonstrated that genetically and geographically differentiated apomictic diploid *Boechera* are characterised by an aneuploid chromosome composed of a single genomic region. These data imply that there has been a single aneuploid chromosome origin followed by its spread throughout different apomictic lineages, or alternatively that aneuploid chromosome origin is a frequent occurrence (perhaps via non-disjunction in triploids) but that only a single aneuploid state is viable (i.e. under positive selection). Aneuploid (B) chromosomes should be lost from a population if they have neutral or deleterious effects (Camacho *et al.* 2000), and thus the apparent maintenance of a single aneuploid genomic region in different clonal lineages implies that it may be characterised by a meiotic drive mechanism, or more interestingly that it may be linked to apomictic reproduction.

In this study a first attempt was made to confirm (partial) trisomy for the cases of three microsatellite alleles on the chromosomes of the 15-chromosomes *Boechera* accessions. A direct cytogenetic assignment of such sequences would be impossible because microsatellites occupy large regions in of the chromosomal DNA. In addition, pilot experiments with BACs selected from the *Boechera* ES9 BAC library gave hundreds of positive clones strongly suggesting high amounts of repetitive sequences in these vectors (unpublished results). The tentative results with the *Arabidopsis* BACs should however, be interpreted with care: the compound foci of MVI11 on one of the chromosome pairs may indicate duplication of this region on the *Boechera* genome. In addition, the minute foci seen in corresponding positions of the sister chromatids in few of the chromosomes do demonstrate genuine targets, but are likely too small for an unequivocal interpretation or the targets may represent hybridisation with small genome duplications or uncommon repeat fraction. Two strategies are needed for a more reliable solution of this question: 1. extensive chromosome painting with pooled BACs for every chromosome region of *Arabidopsis* (cf. Lysak *et al.,* 2003, 2005) will in the long term elucidate the chromosome regions that are triplicate in the apomictic *Boechera* region. 2. More knowledge about putative genes involved in switching on apomixis in this species can be further explored in the *Arabidopsis* genome and as probes for FISH experiments show their chromosomal position. The involvement of the aberrant chromosomes will then become obvious.

Chapter 3

Microsporogenesis and flow cytometric seed screens of sexual and apomictic *Boechera* accessions

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Abstract

This study presents different characteristics of microsporogenesis and pollen of the sexual and apomictic *Boechera* species described in the previous chapter. The focus here is on chromosome pairing, chiasma formation and chromosome segregation, pollen size and viability, whereas flow cytometry of crunched seeds provided ploidy levels of embryo and endosperm nuclei for establishing the mode of reproduction. Microsporogenesis of the diploid sexual *B. holboellii* and *B. stricta* is regular as expected, with full pairing and recombination of the homologues and a balanced segregation of the four daughter cells. However, DAPI staining of pachytene complements demonstrated a 2-3 times larger amount of pericentromere heterochromatin in *B. stricta* than in *B. holboellii*, which confirms the karyotype differences as described in the previous chapter. The five apomictic accessions displayed an enormous variation in meiosis, pollen and ploidy levels of the seeds. The BH1, BH115 and BH224 apomicts have fully synapsed homologues, but only BH1 – meiotically the most regular of the studied apomicts – has a normal duration of meiotic prophase and produce functional haploid pollen. BH115 and BH224 have a strikingly longer meiotic prophase and produced unreduced male gametes. BH115 showed one quadrivalent in their metaphase I complements, which we interpreted as heterozygosity for a reciprocal translocation. Pairing is entirely abolished in the BH74 *B. holboellii* and the BDi175 *B. ×divaricarpa* apomicts, but only the former shows some anaphase I segregation and produces pollen at haploid level. The two aberrant chromosomes *Het* and *Del* could be identified in part of the pachytene and metaphase complements, by their aberrant heteropycnotic morphology and their size. In BH1, BH115, BH224 we observed heteromorphic bivalents with the *Het* chromosome and one of the normal chromosomes, whereas the *Del* chromosome formed chiasmate bonds with one other chromosome pair, thus forming a heteromorphic trivalent.

Keywords: *Boechera*, apomixis, B chromosome, meiotic aberrations, pollen viability, flow cytometry seed screen

Introduction

The *Boechera holboellii* complex is a highly polymorphic clade with several species and hybrids which differ with respect to morphology, chromosome number, ecology and reproductive mode, and is distributed from Alaska to Greenland with its main distribution in the western states of North America. Asexual *B. holboellii* are facultative pseudogamous apomicts, which on the basis of megasporogenesis analysis belongs to the *Taraxacum* type of diplospory (Böcher 1951; Naumova *et al.,* 2001; Taskin *et al.,* 2004).The meiotic and embryological mechanisms underlying its genomic variability have been extensively studied by Böcher (1951), who made detailed comparisons between the course of micro- and megasporogenesis in diploid and triploid plants with and without supernumerary chromosomes to reveal diverse meiotic aberrations which were assumed to be under genetic and environmental control. According to Böcher (1951) individual plants can produce pollen resulting from combinations of FDR and SDR. Additional evidence for this mechanism came from meiotic studies on some of the Greenland apomicts revealing combinations of FDR, SDR and post-meiotic duplication (unpublished observations). It was suggested that high levels of chromosome heterozygosity affected pairing and chiasma formation, leading to different degrees of univalent chromosome formation at metaphase I.

In spite of the plethora of meiotic and embryological disturbances in *Boechera,* little is known about the underlying mechanisms which lead to this variation. In the previous chapter of this thesis a cytogenetic study was performed on several apomictic *B. holboellii* and *B. ×divaricarpa* accessions, and two related sexual diploid *B. holboellii* and *B. stricta* accessions. Karyotype analyses of DAPI stained chromosome sets and FISH patterns with rDNA probes demonstrated high levels of chromosome variation within and between the apomictic accessions, suggesting large scale chromosome rearrangement and / or haploidization of the genomes. We also described two aberrant chromosomes, *Het* and *Del*, previously classified as B chromosomes, which were clearly distinguishable in the karyotypes of the apomicts.

In this chapter we describe a study of microsporogenesis and pollen characteristics in sexual and apomictic *Boechera* accessions. The aim was to characterise chromosome pairing and segregation, chiasma formation, and pollen size viability. In addition, we used flow cytometric seed screens (Matzk *et al.,* 2000) to assess embryo and endosperm ploidy levels in order to determine the mode of reproduction in the different accessions.

Material and methods

Plant material

We used the sexual diploid *B. holboellii* and *B. stricta*, and the apomictic accessions *B. holboellii* BH1, BH74, BH115, BH224 and *B. ×divaricarpa* BDi175. Details of their morphology

and chromosome portraits were described in Chapter 2. One year old greenhouse plants were vernalized in a cold room (+4 °C) under a 16/8 hr light/dark regime for six weeks. After being transferred to a greenhouse at 21 °C and same dark light regime, the first inflorescences appeared within a few weeks. As genotypes, season and environmental conditions strongly determined the synchronisation of pollen mother cells, we collected the very young, still unopened flower buds at different hours of the day and at different stages of development.

Chromosome preparations

Inflorescences were fixed in a freshly prepared acetic acid: ethanol (1:3) solution. For the study of meiosis we selected anthers at all stages of microsporogenesis. Details on the chromosome preparation technique, DAPI staining and image acquisition were given in Chapter 2. The only modification of the technique for this material was the extension of the enzymatic cell wall digestion to 2½ hours.

Pollen staining and measurements

Pollen preparations were made of selected anthers from the acetic acid fixed inflorescences. Pollen at the right stage were rinsed in water and then squeezed out in a drop of lacto-phenol acid fuchsin staining solution (Sass 1964) on a microscopic slide, after which a cover slip was put on the cells and a squash preparation made. Digital photographs of pollen grains from each accession were captured with a CCD camera, and morphometric measurements were performed on the imported files with the measure tool in Adobe Photoshop. Pollen size is variable within and between accessions as has been reported before (Böcher 1951) and depends to a greater extends on ploidy level. We first analysed pollen size from the sexual species with regular meiosis, and used these results as references for comparison with pollen size measurements of the apomictic accessions, considering the mode of male meiosis (in the same inflorescence) and nuclear DNA amounts by flow cytometry of the offspring seeds (see below). For the apomictic accessions we only measured viable pollen.

Seed germination test

Seeds were sterilized by incubation in 2% HCl, 4% Hypo Chlorite for 3 hours before germination on half strength MS medium under 16 / 8 hour light / dark period at 25 °C. Seedlings were counted ten days after germination.

Flow Cytometric Seed Screen

Embryo and endosperm ploidy from dry seeds were analyzed using a Partec flow cytometer PA (Partec GmbH, Münster, Germany) following the Flow Cytometric Seed Screen technique (FCSS) of Matzk *et al.* (2000). DNA content was examined in single and bulk seeds samples (15) for each accession. Briefly, seeds were crushed on dry fine grade sandpaper and

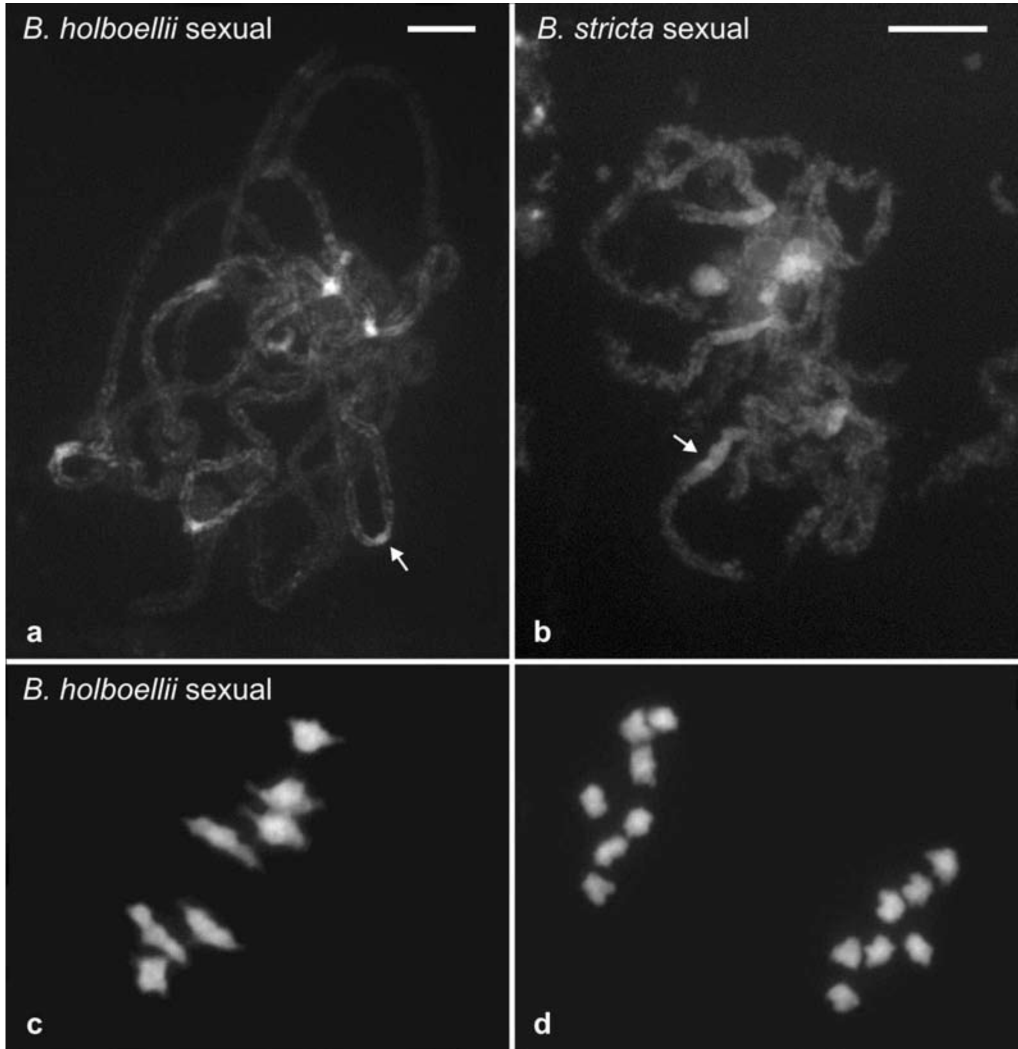

Figure 3-1. Meiosis in DAPI stained pollen mother cells of diploid sexual *Boechera holboellii* BH208 (a, c and d) and *B. stricta*, BS2 (b). (a and b) Late pachytene. Bivalents are well separated and show a characteristic pattern of brightly fluorescing heterochromatin blocks in the pericentromere regions (arrows) and weakly stained euchromatin with numerous small chromomeres. Note that the pericentromere regions of *B. stricta* are significantly larger than of *B. holboellii*. (c) Metaphase I. Seven bivalents oriented with their chiasmata in the equatorial plane. (d) Anaphase I. Equal segregation of the two sets of half bivalents to the poles. Bars equal 5 μm.

rinsed in 2 mL of the DAPI staining solution containing 5 mM $MgCl_2$, 0.1 M NaCl, 2.5 mM sodium citrate, 0.1 M Tris, 10 % Triton X-100 and 2.2 µM 4'-6-diamidino-2-phenylindole, pH 7.0. The suspensions were filtered through a 20 µm mesh nylon sieve and incubated in the dark at 4 °C for 30 min before flow cytometric analysis.

Results and discussion

Sexual diploid *B. holboellii* and *B. stricta*

Anthers collected at 1-2 pm contained pollen mother cells at all stages of microsporogenesis. In a preparation containing the pollen mother cells of a single anther we could find meiocytes ranging from the early prophase I to anaphase II stages, and thus we focused on the following three stages for our analyses: *i*) late pachytene for assessing chromosome pairing and heterochromatin morphology; *ii*) diakinesis / metaphase I to verify chiasma formation and orientation of the bivalents / univalents in the equatorial plane; *iii*) anaphase I / II for the segregation of the chromosomes to the poles. For the interpretations of meiotic stages we compared our observations with the morphology of meiotic stages of *Arabidopsis* as described by Ross *et al.* (1996) and Armstrong and Jones (2003). We furthermore analysed pollen grains to assess uniformity, size and viability of the male gametes. Representative meiotic stages of the sexual and apomictic accessions are shown in the Figures 3-1 and 3-2, and an overview of all important features is given in Table 3-1.

Pachytene chromosomes of *Boechera* resemble the typical morphology of *Arabidopsis* (Figure 3-1), showing brightly DAPI fluorescing heterochromatin blocks in the pericentromere and nucleolar organiser regions (NORs). However, unlike *Arabidopsis*, the euchromatin of *Boechera* is characterised by numerous minor heterochromatin bodies (chromomeres). At late pachytene, just before the transition to diplotene, chromosomes disjoin from their typical pericentromere clustering at zygotene and early pachytene (cf. Ross *et al.*, 1996), which makes this the optimal stage for meiotic chromosome pairing studies. The pericentromere heterochromatin regions and NORs in *B. stricta* are 2-3 times longer than in *B. holboellii* (Figure 3-1a and b), whereas euchromatin measured roughly the same in both species. Metaphase I was regular as expected, showing seven bivalents with clear, stretched centromeres facing the poles, with each bivalent containing 1-3 chiasmata. Anaphase I/II showed balanced segregation with chromosome / chromatid numbers of 7+7 and 7+7+7+7. Pollen was regularly shaped with almost 100% viability (Table 3-1, Figure 3-3). The sample of the *B. stricta* also showed some unreduced pollen.

table 3-1. Overview of meiosis, pollen and seed properties of the diploid sexual and aneuploid apomictic *Boechera*.

Accession	Sex or Apo	Meiosis			Pollen		Seed	
		Chromosome pairing	Chiasmata	Anaphase I Segregation	Size in µm (n)	Viability %, (n)	Germination %)	Flow Cytometry
B. holboellii, BH208	Sex	Pairing saturated; small pericentromere bands	1-3 chiasmata / biv.	7+7	12.4 ± 1.1 (54)	100 (40)	100 (50)	2C+3C
B. stricta, BS2	Sex	Pairing saturated; large pericentromere bands	1-3 chiasmata / biv.	7+7	12.1 ± 1.0 (79) 17.7 ± 0.6 (4)	98 (50)	100 (52)	2C+3C
B. holboellii, BH1	Apo	Pairing almost complete; Het partially synapsed	1-3 chiasmata / biv. *Het* in heteromorphic bivalent; *Del* mostly in heteromorphic trivalent	7+8	13.7 ± 1.2 (43)	100 (43)	100 (54)	2C+5C
B. holboellii, BH74	Apo	Some presynaptic alignment; no pairing (asynaptic)	*Del* recognizable 3 lagging chromosomes lying separated	Few cells with segregating chromosomes	14.1 ± 0.9 (63) 17.4 ± 1.1 (48)	98(113)	50 (60)	2C+6C
B. holboellii, BH115	Apo	Pairing almost complete apart from few, short regions	1-3 chiasmata / bivalent and 1 quadrivalent *Del* in trivalent *Het* in bivalent	Incidentally reductional; mostly FDR	21.8 ± 1.1 (52)	97(54)	100 (50)	2C+6C
B. holboellii, BH224	Apo	Pairing regular and almost complete	*Del* in triv. *Het* in biv.	Incidentally reductional; mostly FDR	15.7 ± 0.7 (4) 20.4 ± 2.1 (78)	62 (133)	100 (53)	2C+6C
B. ×divarcarpa BDi 175	Apo	Little or no pairing (asynaptic)	Very few chiasmata; 1-2 bivalents	FDR	19.8 ± 2.1 (51)	95 (51)	100 (54)	2C+6C

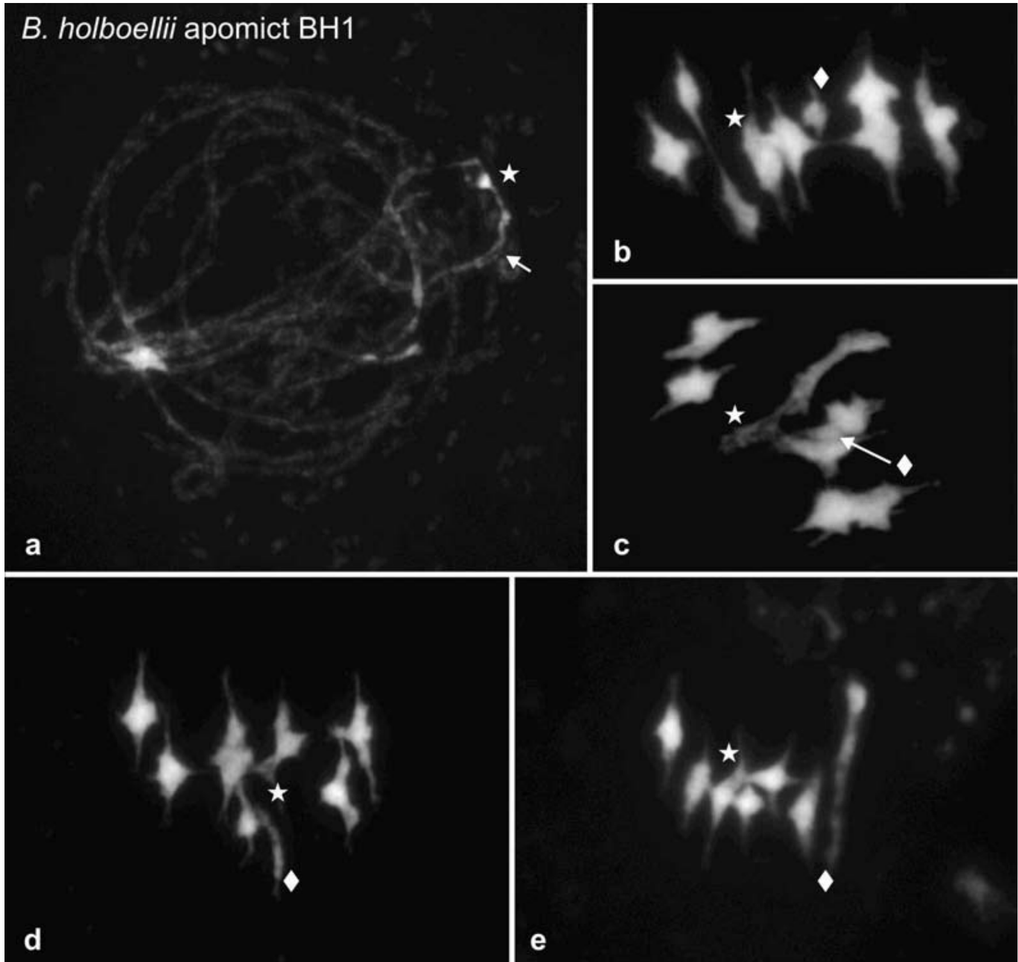

Figure 3-2. Representative meiotic abnormalities found in spreads pollen mother cell of aneuploid apomict *B. holboellii*. (a-e) BH1; (f-h) BH74; (i-l) BH115; (m-p) BH224; (q-s) BDi175. (a) Late pachytene of BH1, with some association of pericentromere regions. The asterisk points at the *Het* chromosome; the arrow shows the paired region with one of the normal chromosomes. (b-e) Examples of metaphase I complements. The *Het* chromosome (asterisk) is often clearly heteropycnotic and the *Del* chromosome (diamond) much shorter than the other chromosomes. The *Het* forms in all cases heteromorphic bivalents; the *Del* chromosome can be a univalent (see b) or attached to two other chromosomes forming a heteromorphic trivalent (see e).

Apomictic *B. holboellii* and *B.* ×*divaricarpa* accessions

B. holboellii BH1

Chromosome behaviour in the pollen mother cells of the apomictic *B. holboellii* BH1 resembled, in many respects, regular meiosis as observed by the diploid species, and showed the most normal male meiosis with respect to the other apomictic accessions (Table 3-1; Figure 3-2a-e).

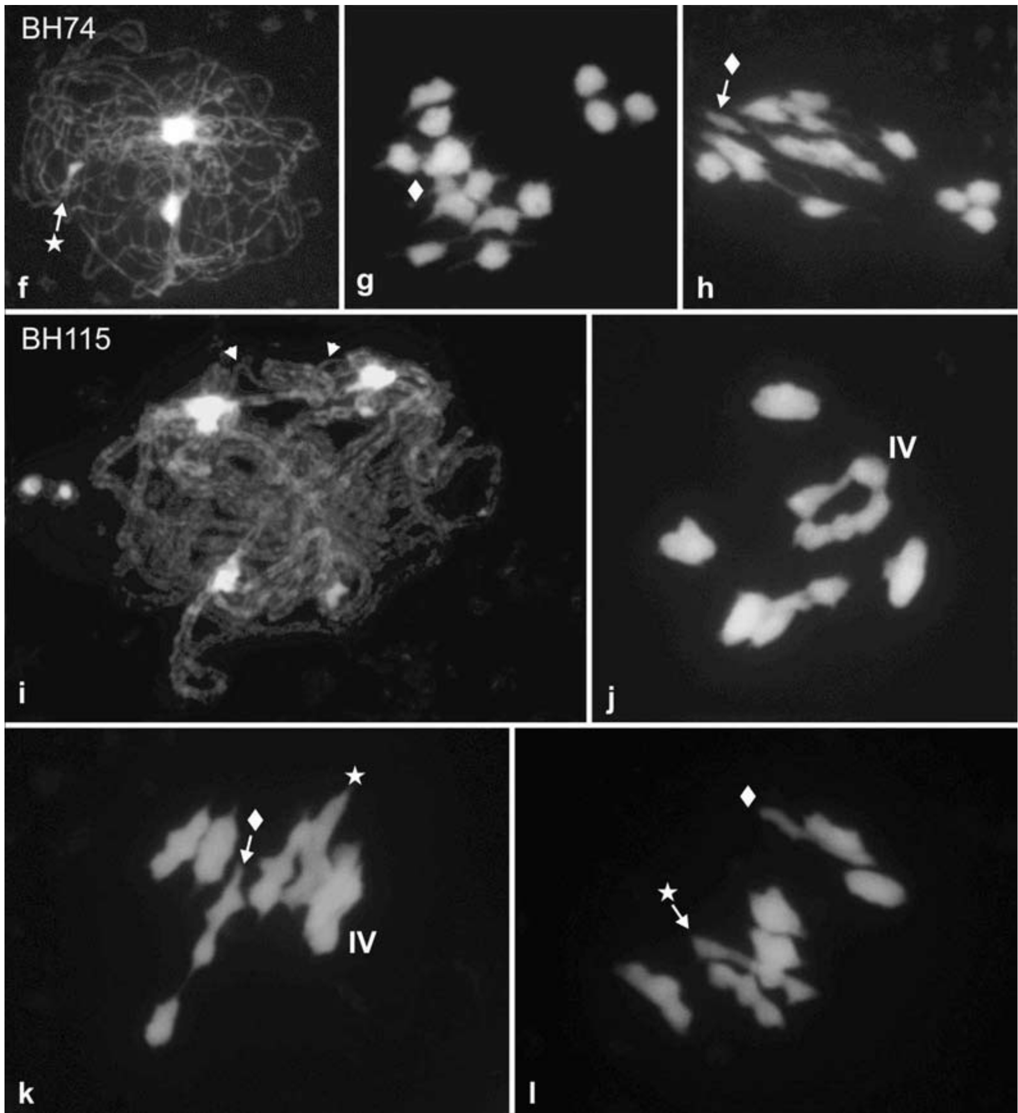

Figure 3-2, cont. (f) Asynaptic pachytene in BH74 with little or no pairing between the chromosomes. The *Het* chromosome is indicated by an asterisk. (g and h) Metaphase I. All chromosomes remain as univalents, quite often with three chromosomes apart. The *Del* chromosome is indicated. (i) Pachytene in BH115 is regular, but some short stretches of asynapsis (arrowheads) can be observed. (j) Diakinesis. This cell shows a clear adjacent quadrivalent. (k-l) Examples of Metaphase I complements showing the heteromorphic bivalent with the *Het* and heteromorphic trivalent with the *Del*.

The two aberrant chromosomes in the complement (the *Het* chromosome, which is largely heterochromatic, and the *Del* chromosome, which is shorter than any of the other chromosomes, see Chapter 3), clearly showed pairing and chiasmate bonds with other chromosomes, but to a variable extend and never with each other. At pachytene the *Het* chromo-

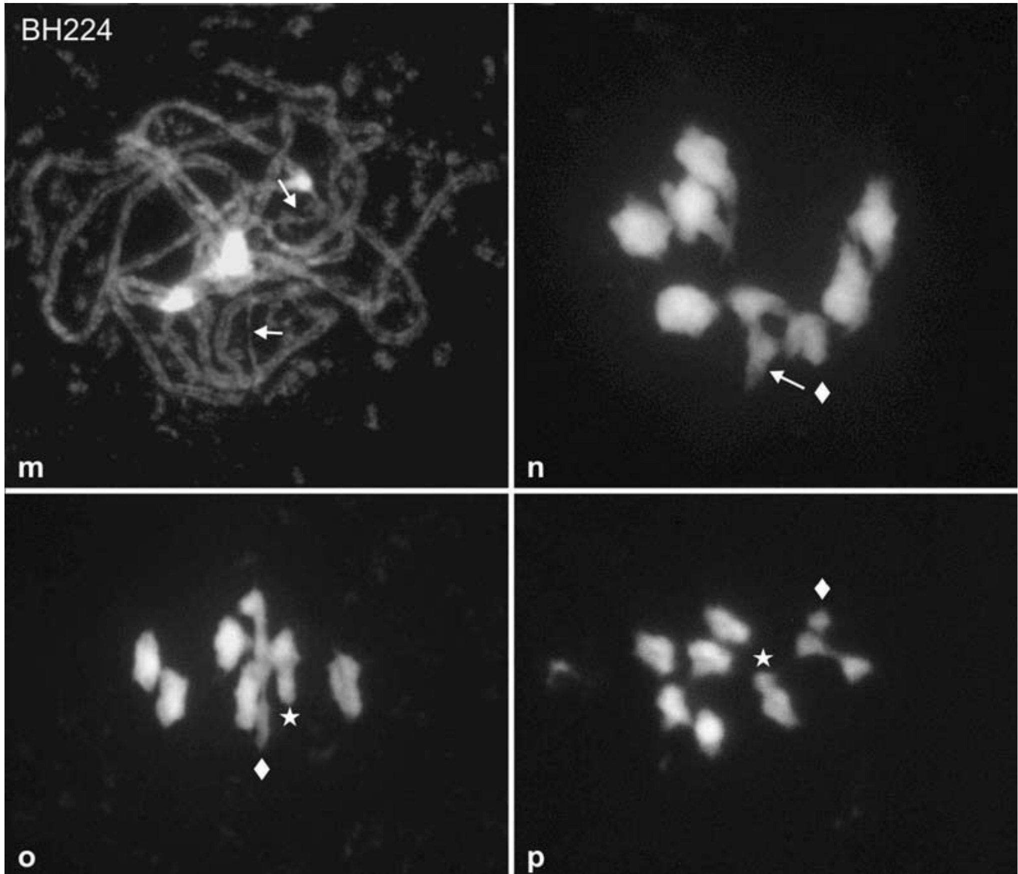

Figure 3-2, cont. (m) BH224. Late pachytene is normal with small unpaired chromosome regions (arrows). (n) Prometaphase I with clear trivalent including the *Del* chromosome. (o-p) Examples of Metaphase I complements, with the *Het* and *Del* chromosomes indicated..

some could be identified by its heterochromatin pattern and was found to pair partially with one of the other chromosomes (Figure 3-2a). This heterochromatin profile was composed of one large, brightly fluorescing and one or two adjacent smaller heterochromatin blocks. At metaphase I we distinguished both aberrant chromosomes in most cell complements (see examples in the Figures 3-2b-e). The *Het* chromosome was always found associated with one of the other chromosomes in the form of a heteromorphic bivalent. The physical association of the *Het* with its pairing partner (Figure 3-2d) suggests that pairing and recombination of this highly heterochromatic chromosome are confined to a small distal chromosome segment. This characteristic, out-of-synchrony condensation cycle of heterochromatin is called negative heteropycnotic (cf, Cattani and Papaschi, 2004) and is known among others from facultative X chromosomes in mammals and from sex and B chromosomes in insects (Brown, 1966, and references therein).

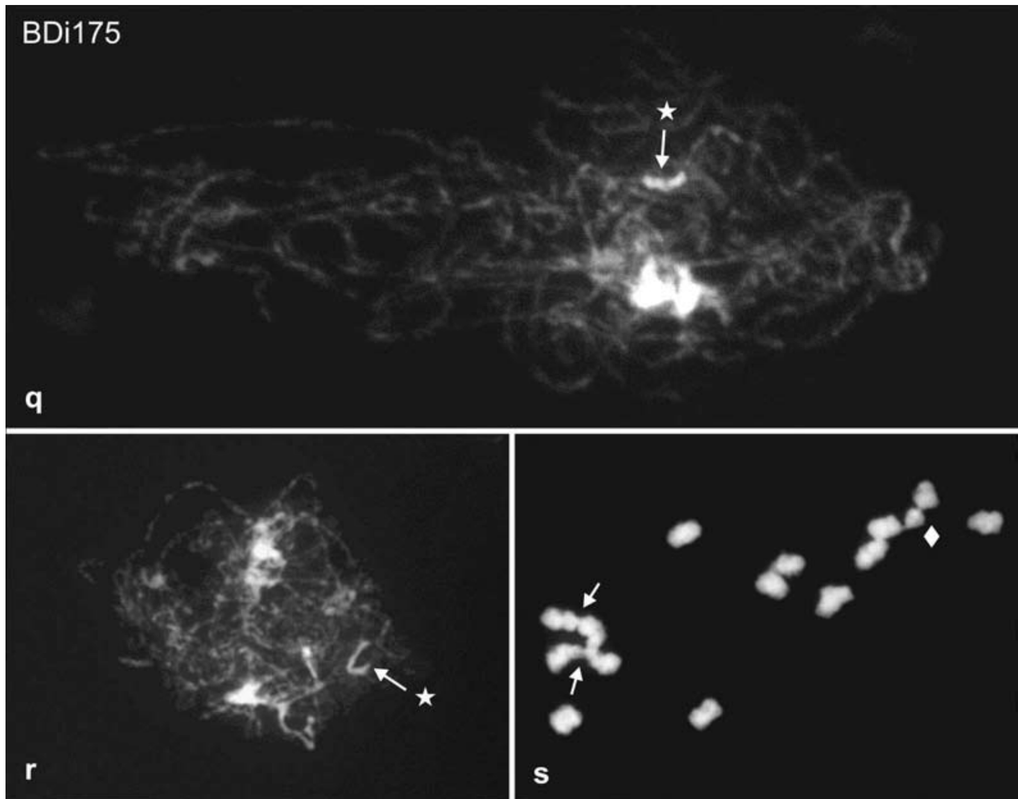

Figure 3-2, cont. (q-r) Pachytene in the hybrid *B. ×divaricarpa*, BDi175 is entirely asynaptic with only few stretches of presynaptic alignment. The arrows indicate the position of the *Het* chromosome. (s) Metaphase I. Only very few chromosomes are connected by chiasmate bonds, most others are univalents.

Although the *Del* chromosome is shorter, it resembled more closely the morphology of the other chromosomes and was found as a univalent or associated with one of the bivalents to form a heteromorphic trivalent (Figure 3-2e). Most of the trivalents showed linear orientation (with centromeres in one line perpendicular to the equatorial plane), although cases of adjacent (adjacent centromeres facing the same pole) and alternate (adjacent centromeres facing opposite poles) orientations were also recorded. The frequencies of trivalent formation differed strongly between seasons and plants, and were therefore not quantified. Only very few anaphase I cells were found and they all showed a 7 + 8 segregation. We have no quantitative data on the transmission of the *Het* and *Del* chromosomes. Pollen grains looked normal and measured roughly the same size as the diploid sexuals (Figure 3-3). Flow cytometric seed screens showed peaks for 2C (embryo) and 5C (endosperm), the latter resulting from the 4C polar nucleus and 1C male gamete, and indicates that the pollen of this accession is functional (Table 3-1). Our samples were too small to detect variation between individual plants of this accession.

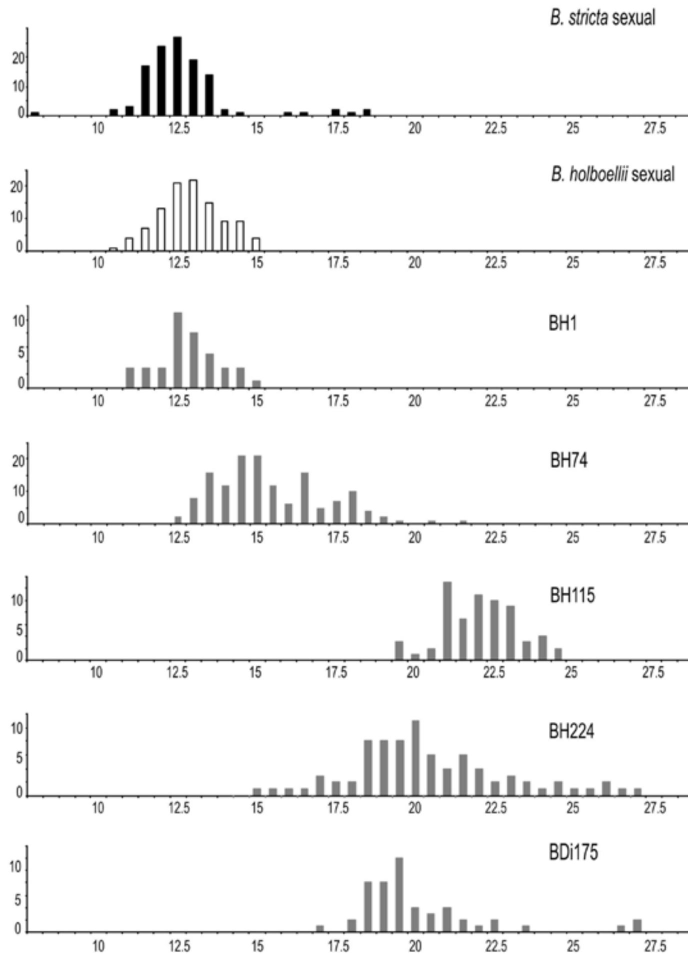

Figure 3-3. Graphical representations of pollen size distributions for the two sexual and five apomictic *Boechera* accessions..

B. holboellii BH74

Meiosis of the apomictic accession BH74 was far more aberrant. To begin with, it was very difficult to find all stages of meiosis. For example, anthers with pollen mother cells at the same stage of development which were collected over 24 hours contained only meiotic prophase I stages, suggesting that meiotic pairing and recombination take exceptionally long. Microscopic preparations of prophase I nuclei with a typical morphology and spreading of late pachytene in normal meiosis showed that chromosomes approached each other at a distance of less than 2 μm (presynaptic adjustment) but almost never come to intimate

pairing (Figure 3-2f). This behaviour, which is typical for an asynaptic mutant, was further confirmed by analyses of metaphase I cells which revealed hardly any chiasmate bonds between the chromosomes. Instead, these cells were characterised by three unassociated univalents and no stretched centromeres, suggesting that they are lagging chromosomes which are not pulled to one of the poles (Figure 4-2g and h). Only a small number of later meiotic stages, including anaphase I cells with 7+8 and anaphase II with 7+7+8+8 chromosomes, were observed. The pollen size distribution is clearly bimodal with one class of pollen with a diameter of 14.1 µm on average, slightly larger than that of the diploid sexuals (12.5 µm) and one of 17.4 µm, which we interpret as reduced (haploid) and unreduced (diploid) male gametes, respectively. Flow cytometry revealed 2C and 6C peaks in the flow histograms, indicating an apomictically produced 2C embryo and 6C endosperm fertilized by an unreduced male gamete (Table 3-1), As half of the seeds germinated we assumed that the unreduced female meiosis resulted from second division restitution of which only half of the gametes are functional or that embryos which did not receive the aneuploid chromosome aborted.

B. holboellii BH115

Meiosis in the apomict BH115 resembles that of BH1, except for the presence of a quadrivalent in metaphase I complements. With 15 chromosomes in the complement and the *Het* and *Del* chromosomes involved in the heteromorphic bivalent and trivalent, we concluded that this quadrivalent represents a set of four chromosomes forming together a translocation complex. The short single chromosome strands that we observed in the pachytene cells (Figure 3-2i) are likely the unpaired regions around the translocation breakpoint. Figure 3-2j shows an example of an adjacent configuration giving rise to unbalanced haploid complements in the case of a normal reductional meiosis. An example of alternate quadrivalent orientation as shown in Figure 3-2k will produce balanced segregation of the chromosomes in the translocation complex. Figure 3-2l shows an example of heteromorphic associations with the *Het* and *Del* chromosomes, and five more bivalents including the two translocation bivalents. The few normal cells at tetrad stage that we observed indicate that this accession can produce unreduced gametes. Also pollen and flow cytometry analyses confirmed that most male and female gametes are meiotically unreduced and most likely resulting from First Division Restitution (FDR) in which all gametes are unreduced and genetically (almost) identical (Table 3-1, Figure 3-3).

B. holboellii BH224

The apomictic accession BH224 resembles BH1 the most, and displays the characteristic heteromorphic bivalent and trivalent associations of BH1 which involve the *Het* and the *Del* chromosomes (Figures 4-2m-p). In contrast, this accession showed two partly overlapping classes of pollen size, one in the range of 14-17 µm and one class of 17-27.5 µm, showing that BH224 produces both reduced and unreduced gametes (Table 3-1, Figure 3-3). As one of the

offspring plants were found with 16 chromosomes (and only one *Het* and one *Del* chromosome) suggests that an incidental reductional meiosis can produce functional gametes.

B. ×divaricarpa BDi175
Meiosis in the apomictic *B. ×divaricarpa* hybrid BDi175 accession had a fully asynaptic pachytene stage which resembled that of BH74 the most. Metaphase I complements were as expected and showed only 0 - 2 bivalents in most cases. Pollen and flow cytometry analyses revealed that male and female gametes are unreduced (Table 3-1, Figure 3-3).

Conclusions

The comparative study of male meiosis, pollen size and viability, embryo and endosperm ploidy in sexual and apomictic *Boechera* revealed a great deal of variability. The most striking facts are:

1. **Heterochromatin differentiation of the parental species.**
   The sexual *B. holboellii* and *B. stricta* have essentially the same reductional meiosis, but the heterochromatin blocks around the centromeres in pachytene chromosomes of *B. stricta* are significantly larger than those of *B. holboellii*, and fits well with previous observations on the DAPI fluorescence banding of mitotic prometaphase chromosomes (Chapter 2). This interspecific heterochromatin differentiation implies a large scale amplification of major repeat(s) in the pericentromere and centromere areas of the *B. stricta* genome, or alternatively that *B. stricta* has acquired a novel repeat class which has a significantly higher copy number than its corresponding repeats in *B. holboellii*. This point will further be elaborated in the genome painting study of Chapter 4.
2. **Overall differences in meiotic behaviour.**
   We detected obvious differences in meiotic behaviour between the apomictic accessions. A first group includes BH1, BH115 and BH224, and is characterised by normal, (almost) saturated pairing at pachytene and regular bivalent formation at metaphase I. The duration of meiotic prophase I in BH1 is like a sexual diploid, whereas in BH115 and BH224 it takes 7-8 hours longer which is likely the result of disturbed or delayed chromosome pairing and recombination. Bivalent formation with the *Het* chromosome is normal, and the associations with the *Del* chromo some follows the behaviour of a typical translocation trisomic, which means that the extra chromosome is a translocation, or that of a telotrisomic, in which the extra arm is a telosome (Sybenga, 1992). However, a notable difference exists in later phases of meiosis, with BH1 being reductional and producing functional pollen, whereas BH115 and BH224 are apomeiotic and likely produce FDR gametes.

The second group is characterised by asynaptic meiosis, and includes BH74 and BDi175. Gamete formation nonetheless differs between the two accessions, with BH74 producing haploid pollen and BDi175 not. We have no indications though that the pollen of BH74 is functional. There are few explanations for this variation in meiotic behaviour. Firstly, accessions may differ in terms of their karyotypes which may represent chromosomal rearrangements, as was suggested in Chapter 2 of this thesis. All have the *Het* and *Del* chromosomes in common, but only BH115 is evidently heterozygous for a reciprocal translocation. Other chromosomal rearrangement may occur, but they may be too small to cause disturbed chromosome pairing and recombination. Secondly, accessions may differ in their genes expressing meiotic pairing, recombination and segregation, including apomeiosis (diplospory). Thirdly, accessions may differ in their clonal age, giving rise to diverse level of heterozygosity including global differentiation of repeats. A fourth possibility is genetic control of homoeologous chromosome pairing, as has been shown for *Ph1* in wheat (Riley and Chapman 1958; Sears and Okamoto 1958) and *PrBn* in *Brassica napus* (Jenczewski *et al.*, 2003), and thus mutation accumulation in apomictic clones could lead to pairing variation as a result. The production of functional haploid pollen in BH1 suggests that this apomictic accession has not accumulated genomic and chromosomal mutations, and hence it may be an evolutionary younger clonal lineage compared to BH115 or BDi175.

3. **Significance of functional pollen.**

   In nature, *B. holboellii* apomicts are recurrent, which indicates that pollen can introgress apomictic elements into sexual recipients. It follows that pollen should be balanced with a complete set of the haploid genome, possibly supplemented with the *Het* and *Del* chromosomes that may be essential for the apomictic trait. It follows that every newborn apomict inherits the eroded old genome from the father and highly polymorphic genome from the sexually propagating mother. In our studies we found that BH1 can produce functional haploid pollen for endosperm formation but whether the heterozygous pollen is complete enough for sexual reproduction, is still to be proven. Most *B. holboellii* in natural populations are triploid, suggesting that they arise from diploid pollen and that haploid genome is not complete enough for embryogenesis. We will further discuss these points in the general discussion (Chapter 5) of this thesis.

4. **Obligate – facultative apomixis.**

   As we discussed above, apomeiotic elements are expressed in the female line but can be transferred trough the male, although a direct genetic proof for it is lacking. In the BH74, BH115, BDi175 and BH224 accessions, we observed that functional pollen has the size corresponding to that diploid pollen, suggesting that apomeiotic

microsporogenesis is strongly expressed in these accessions. Even though, plants can produce haploid pollen, referred to pollen size, but their number is always small compared to the diploids. The different proportion of haploid and diploid pollen between BH1, BH74 and BH115, BH224 and BDi175 points at differences in genetic control of apomeiosis in the pollen mother cells. Differences may also occur between anthers or be susceptible to environmental conditions, but these effects have not been studied. From our chromosome spread preparation, we also could not identify which mechanisms produce unreduced gamete.

5. **The nature of the two aberrant chromosomes.**
Morphology and behaviour or the two aberrant *Het* and *Del* chromosomes in the aneuploid accessions were easily identifiable at meiotic prophase. The *Het* chromosome at pachytene exhibits a short stretch of few highly condensed heterochromatin blocks. At metaphase I the heterochromatin region is strikingly less condensed than the other chromosomes in the complement, whereas the euchromatin part is chiasmatically associated with one other chromosome. This asynchronous condensation behaviour of the heterochromatic part of the *Het* chromosome is known as heteropycnotic and resembles the heterochromatic Y in a dimorphic XY chromosome pair, and paired with its counterpart in a small autosomal synaptic region through a single obligate chiasma needed for proper disjunction of the *Het* and its pairing partner at metaphase I – anaphase I. As all the *Boechera* apomicts contain this *Het* chromosome it is tempting to assume that this element in some way or another represents a key role in the expression of apomixis. The *Del* chromosome is equally intriguing. As demonstrated in BH1, BH115 and BH224, it is capable of recombining with one of the chromosome pairs forming a heteromorphic trivalent. If we assume that the *Del* resulted from a deletion event, such trivalents can be interpreted as a pairing configuration resulting from an incomplete primary trisomic, closely resembling a telotrisomic. A key role in the switch to apomixis is equally attractive as it might explain local polyploidy for genes essential in the switch towards apomixis. However, a strong argument against the essential involvement of *Del* in the apomictic pathway is the fact that this aberrant chromosome was only detected in the Rocky Mountain accessions (BH1, BH74, BH115, BH224 and BDi175), and not detected in the *B. holboellii* apomicts ES9 and GNL2. This will be further discussed in Chapter 5.

Chapter 4

Genome painting of *Boechera* reveals different parental chromosomes combinations in apomictic accessions

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Abstract

In this chapter we report on a series of genome *in situ* hybridisation (GISH) experiments of apomictic *Boechera* accessions. In the first part we used fluorescence *in situ* hybridisations with total genomic DNA from the diploid sexual *B. stricta* as probe and blocked with genomic DNA from *B. holboellii.* Chromosome complements showed fluorescent signals on the pericentromere regions of only the *B. stricta* chromosomes in the hybrids. An additional two-colour genome painting was developed with the simultaneous hybridisation of both *B. stricta* and *B. holboellii* probes and blocking with total genomic DNA of *Arabidopsis thaliana* in order to further improve the discrimination of the parental species. This genome *in situ* hybridisation technique revealed that *B. holboellii* and *B. stricta* have undergone dramatic evolutionary changes in the repetitive sequences in the pericentromere regions of their chromosomes, producing species-specific bands in their chromosomes. The first group of apomictic accessions taxonomically classified as *B. ×divaricarpa* are the aneuploid BDi175 with 6 *B. stricta* and 9 *B. holboellii* chromosomes, and the euploid accessions ES9 with 7 *B. stricta* and 7 *B. holboellii* chromosomes. The second group includes the *B. holboellii* apomicts, which demonstrated different *B. stricta* chromosome numbers in the complements: varying from with five *B. stricta* and nine *B. holboellii* in GRL2, four *B. stricta* and eleven *B. holboellii* in BH1 to eleven *B. stricta* and four *B. holboellii* chromosomes in BH74. Our genome painting study therefore substantiated that apomictic accessions are all genuine hybrids but are constituted of different numbers of *B. stricta* and *B. holboellii* chromosomes that have been formed through repeated homoeologous substitutions involving single chromosomes or chromosome pairs. The aberrant chromosomes *Het*, *Het'* and *Del*, previously described in the karyotype analyses of chapter 2, all contain the *stricta* specific repeats suggesting that they originated from the ancestral sexual *B. stricta* genome.

Keywords: *Boechera*, apomixis, genome painting, GISH, chromosome substitutions.

Introduction

Various studies of the *Boechera holboellii* complex have revealed a large variation in plant morphology, chromosome counts, geographic distribution, and molecular genetic markers between apomictic lineages (Böcher, 1951; Koch *et al.*, 1999; Sharbel and Mitchell-Olds, 2001; Sharbel *et al.*, 2004, 2005; Dobeš *et al.*, 2004a, b). A detailed cytogenetic investigation of several diploid apomictic *B. holboellii* and *B. ×divaricarpa* hybrids in chapters 3 of this thesis demonstrated striking inter-individual differences in chromosome morphology, in addition to the presence of one or two aberrant chromosomes. Moreover, pollen analyses in chapter 3 demonstrated differences in chromosome pairing, chiasma potential, and unreduced gamete formation between apomictic lineages. In two of the apomictic accessions, *B. holboellii* BH74, and the hybrid *B. ×divaricarpa* BDi175, meiosis was completely asynaptic, suggesting that these apomicts were partially or entirely amphihaploid (allodiploid) with one *B. holboellii* and one *B. stricta* genome as most probable ancestral species.

One of the best tools for the elucidation of genome composition in putative interspecific hybrids is genome painting or genomic *in situ* hybridisation (GISH), originally developed by Schwarzacher *et al.* (1989). This technique is based on a fluorescence *in* situ hybridisation protocol using total genomic DNA from one of the parental species as probe, and excess amounts of unlabelled DNA from the other parental species in order to block hybridisation of common sequences. Such a cytogenetic approach not only elucidates alien chromosome transfers in introgressive hybridisation programmes between a close relative and the recipient crop, such as rye to wheat, barley to maize and tomato to potato, it is also helpful in establishing the putative parental species in natural allopolyploid hybrids (review in Raina and Rani, 2001).

The discriminative power of genome painting is correlated with the evolutionary differentiation of dispersed and tandem repetitive sequences between related species. This fraction of the genomic DNA represents 40-95% of the total nuclear DNA, and apart from the telomere and rDNA repeats, belongs to the most dynamic fraction of the genome (Bennett, 1995). Parental genomes were thus identified in diverse plant species, including those with large (*eg*, *Gibasis* hybrids; Parokonny *et al.*, 1992) and small chromosomes (e.g. *Rachypodium distachyon*; Hasterok *et al.*, 2004). Technological advances have enabled the development of multicolour GISH. For example, Krustaleva and Kik (1998) used simultaneous labelling of two parental genomes in a multicolour genome painting study of the trihybrid bridge cross *Allium cepa* X (*A. fistulosum* X *A. roylei*) to visualise the three genomes in the hybrids.

In this chapter we present the results of a genome painting study of *B. holboellii* and *B. ×divaricarpa* apomicts, using the total genomic DNA of putative parental sexual *B. holboellii* and *B. stricta* as probes in 1- and 2-colour fluorescence *in situ* hybridizations. The aim of this study was to determine if the apomictic hybrids are truly amphihaploid, as our previous studies of meiosis have suggested. We were furthermore interested in elucidating the

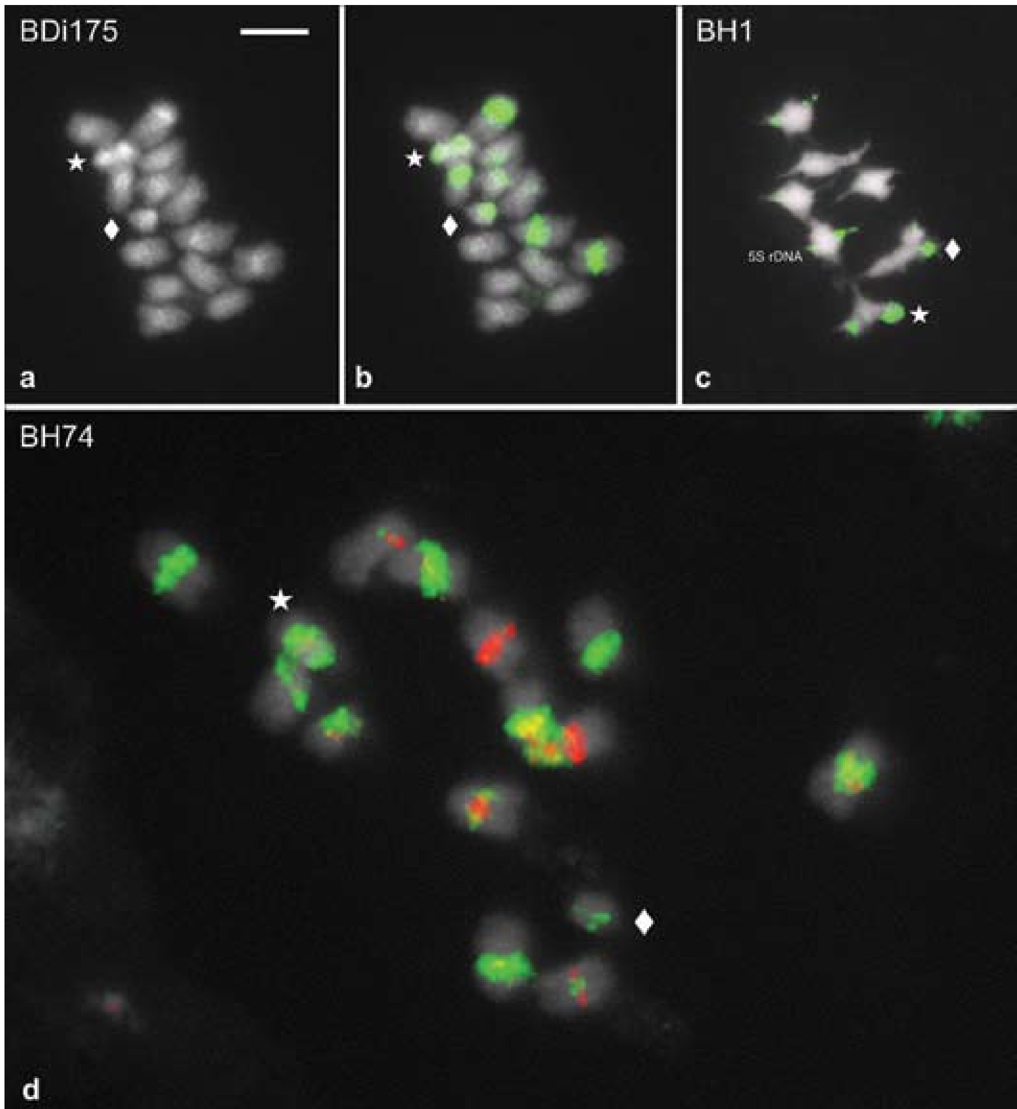

Figure 4-1. Genome painting in *Boechera* apomicts. (a) shows the DAPI fluorescence of a mitotic metaphase cell of the *B.* X *divaricarpa* hybrid BDi175 with the aberrant chromosomes *Het* and *Del* indicated. Bar equals 3 µm. (b) is the same metaphase now showing the genomic in situ hybridisation signals of the *B. stricta* probe and blocked with *B. holboellii* DNA. Six chromosomes including the two aberrant chromosomes *Het* (asterisk) and *Del* (diamond) show a clear *B. stricta* signal in the heterochromatic region. (1) shows a metaphase I complement of BH1 with the same probe and block as in Figure 1b. Here the *Het* chromosome forms a heteromorphic bivalent with one other *B. stricta* chromosome, whereas the *Del* chromosome is associated with a *B. holboellii* bivalent. (d) Two-colour genome painted mitotic metaphase complements of BH74. In this FISH we used the green fluorescing probe of *B. stricta* DNA, red fluorescing probe of *B. holboellii* DNA and blocking with *Arabidopsis* DNA.

genomic origins of the aberrant *Het* and *Del* chromosomes, as sequencing data have implied that they originated from *B. stricta* (Sharbel *et al.*, 2004).

Material and methods

Plant material

In this study we used all apomictic accessions mentioned in Chapter 2, in addition to the apomictic *B. holboellii* accessions ES6 from Colorado and GNL2 from Greenland.

Chromosome preparations

Root tips and young anthers were used for preparing chromsome preparations containing mitotic and meiotic stages, respectively. Details were described in the Chapters 2 and 3.

Genomic *in situ* Hybridisation

We used a Nucleon Phytopure extraction kit (Amersham Biosciences) to isolate total genomic DNA from the diploid sexuals *B. holboellii*, BH208, *B. stricta*, BS2, and *Arabidopsis thaliana* (Columbia accession). DNA was labelled with either digoxiginin-11-dUTP or biotin-16-dUTP using the nick translation kit of Roche (Mannheim). In the pre-hybridization step we first dried the microscopic preparations at 67 °C for 30 min before incubation with 1 µg/mL RNAse-A in 2xSSC at 37 °C for 1 hr and two wash steps in 2xSSC for 5 min at 20 °C. The preparations were rinsed in 10 mM HCl for 2 min and then treated with 100 µL pepsin (5 µg/mL) in 10 mM HCl for 5 min at 37 °C, washed three times in 2xSSC for 5 min, fixed in 10 % formaldehyde for 10 min followed by 2 washes in 2xSSC for 5 min (all steps at 20 °C). The preparations were dehydrated in ethanol series (70 %, 90% and absolute ethanol for 3 min each) and air dried. For the single colour genome painting we used a hybridization mixture of 50 % formamide, 10 % sodium dextrane sulphate, 2xSSC, 0.25 % SDS and 100 ng DNA probe (BH208 or BS2 genomic DNA) and 10 µg blocking DNA, in a total of 40 µL hybridization buffer. For the two-colour GISH, the hybridization mixtures contained both BH208 and BS2 probes and blocking DNA (1:100 total genomic DNA of *A. thaliana*). The mixture was denatured at 100 °C for 10 min and chilled immediately on ice for 10 min before use. For each preparation we added 40 µL of the hybridization mix and covered it with a 24x50 mm cover slide, followed by heating on a 80 °C hot plate for 2½ min before an overnight hybridisation at 37 °C in a humidified chamber. Post hybridization washes involved three wash steps of 50% formamide in 2x SSC (pH 7.0) at 42 °C for 5 min, and two steps in 2xSSC for 5 min at 20°C. The hybridization signals were detected with FITC-conjugated anti-dig antibodies and amplified with FITC-conjugated rabbit anti-sheep antibodies

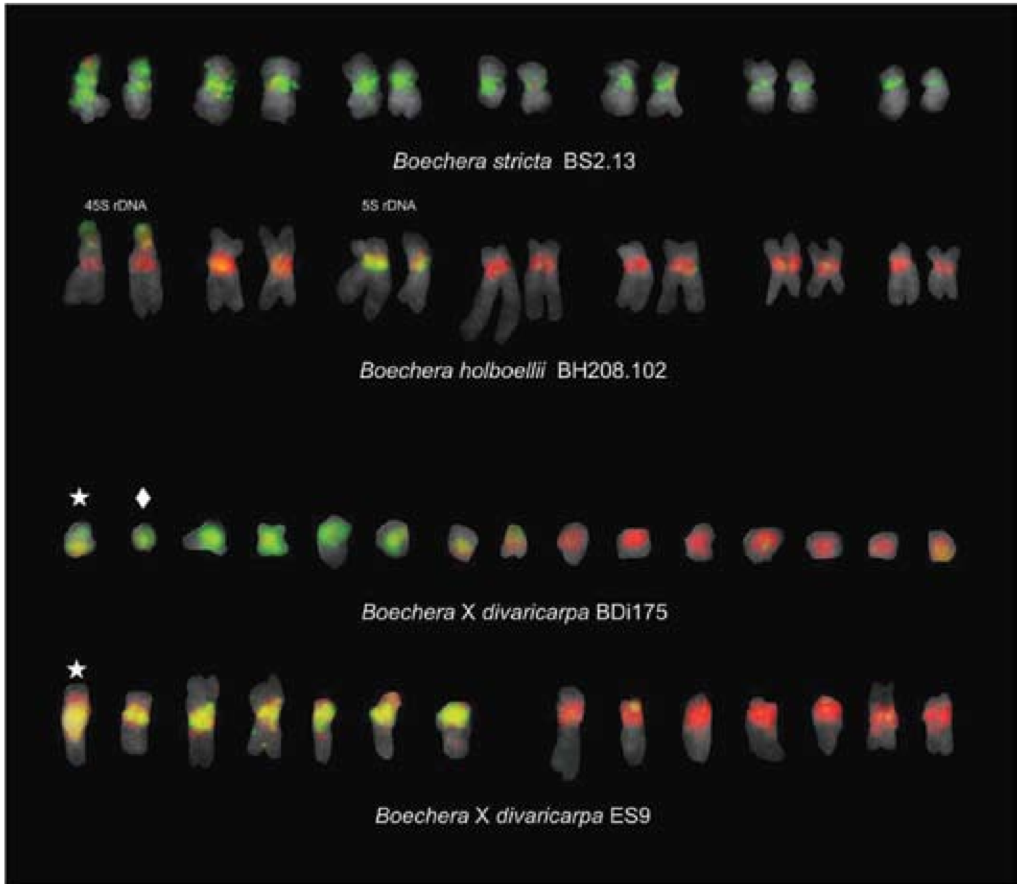

Figure 4-2a. Genome painting karyotypes of mitotic metaphase complements obtained according to the 2-colour genome in situ hybridisation scheme. (a) shows the diploid sexual species *B. stricta* and *B. holboellii* and the two *B.* ×*divaricarpa* hybrids.

for the digoxigenin-labelled probe, and with Avidin Texas-Red for the biotin labelled probe which was amplified with biotinylated anti-avidine and Avidin Texas-Red. The preparations were counterstained in 100 µL of a 2 µg/mL DAPI solution in 100 mM citrate buffer, pH 6.0 for 10 minutes in the dark and finally mounted in Vectashield (Vecta Laboratories) under a 24x50 mm cover slip.

Image acquisition and karyotype analysis

Full details were described in Chapter 2. Chromosomes were arranged according to their genome painting signals and their size.

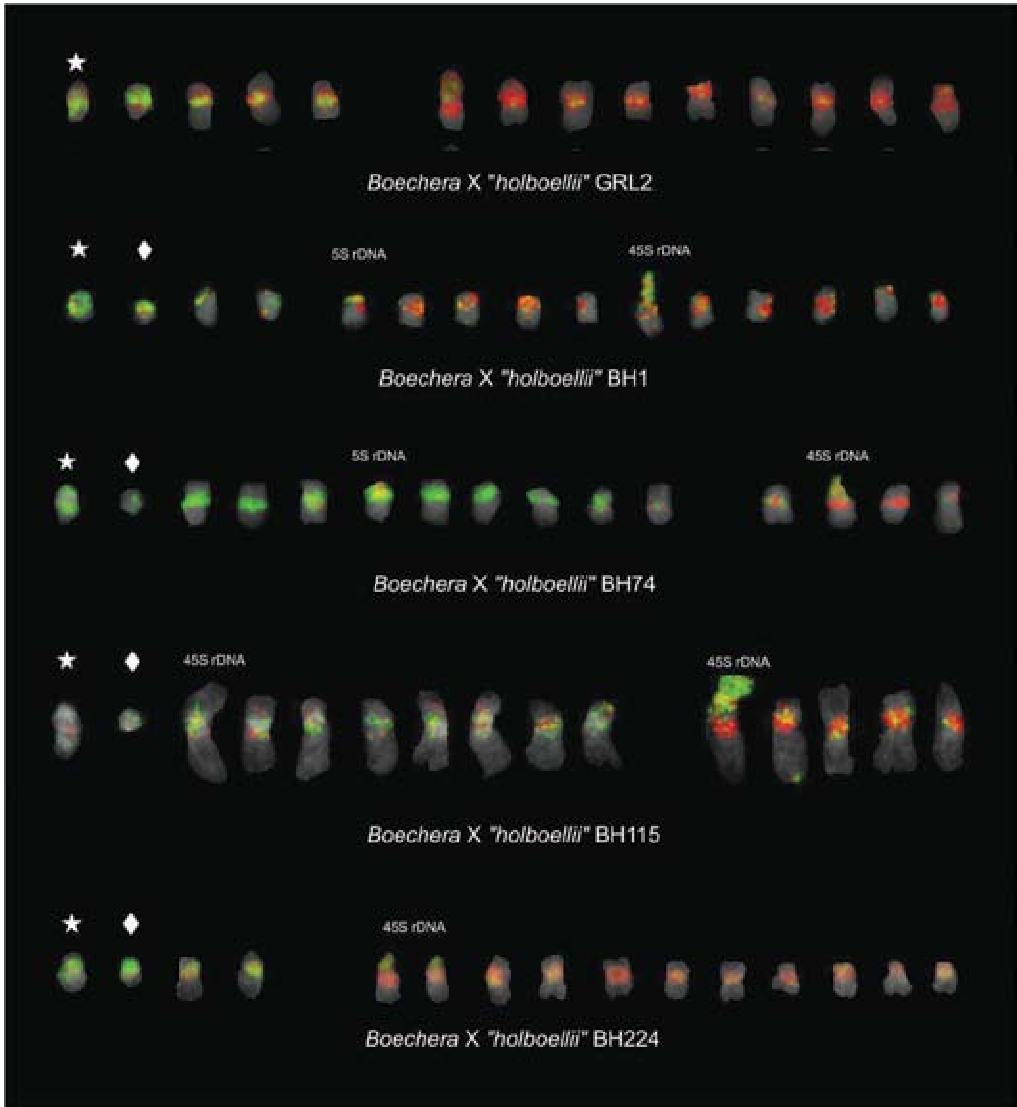

Figure 4-2b. Remaining *B. holboellii* apomicts. Chromosomes were ordered according to their *B. stricta* and *B. holboellii* signals. Some of the chromosomes could also be identified based on the basis of their 45S and 5S rDNA profiles. The *Het* and *Del* chromosomes are indicated with an asterisk and diamond symbol, respectively..

Results

The first series of experiments was based on single colour GISH using *B. stricta* total genomic DNA as probe and 100x *B. holboellii* total genomic DNA for blocking. Chromosomes were always counterstained with DAPI, but their blue fluorescence signals were displayed

Table 4-1. Overview of relative species-specific chromosome numbers, based upon distributions of *B. holboellii* (red fluorescence) and *B. stricta* (green fluorescence) probes. *Het, Het'* and *Del* are the aberrant chromosomes in the complement (see Chapter 2 for more information) and always display the green *B. stricta* hybridisation signal.

Accession	chr. nr.	Genome constitutions (chromosomes)			
		B. stricta	***B. holboellii***	**Aberrant chrs.**	**remarks**
sexuals					
BH208	14		14		
BS2	14	14			
ES6	14	14			
B. ×*divaricarpa* apomicts					
BDi175	15	6	9	*Het + Del*	
ES9	14	7	7	*Het'*	
B. holboellii apomicts					
GRL2	14	5	9	*Het'*	
BH1	15	4	11	*Het + Del*	weak overall *stricta* fluorescence
BH74	15	11	4	*Het + Del*	
BH115	15	10	5	*Het + Del*	weak overall *stricta* fluorescence
BH224	15	4	11	*Het + Del*	

in gray. Gaussian sharpening and contrast improvements using image processing software were performed as described in the Chapters 2 and 3. A representative example of a metaphase chromosome spread from the apomictic *B.* ×*divaricarpa* hybrid Bdi175 is shown in Figure 4-1a and b. DAPI fluorescence clearly shows the heterochromatin bands of the pericentromere regions and those of the *Het* and the *Del* chromosomes. The *B. stricta* probe was found on 8 of the 15 chromosomes, including the *Het* and the *Del* chromosomes, in the same positions as the brightly fluorescing heterochromatin blocks. Control experiments using the same probe and blocking DNA under identical hybridisation conditions on sexual *B. stricta* and *B. holboellii* metaphase spreads confirmed *B. stricta* specificity of the probe, with the exception of the 45S and 5S rDNA sites. More information on the chromosome specificity will be discussed below.

A pilot one-colour GISH experiment using the *B. stricta* probe on a metaphase I cell from the *B. holboellii* apomict BH1 demonstrates a heteromorphic bivalent containing the *Het* chromosome associated with its homologue, both of them displaying a clear *B. stricta* signal (Figure 4-1c). The *Del* chromosome also has a *B. stricta* signal, and is part of a heteromorphic trivalent in which the other two chromosomes lack the *B. stricta* signal. The complement also contains one or two more bivalents with a *B. stricta s*ignal in the centromere region.

To further improve the quality of genome discrimination we set up a two-colour GISH system using probe DNAs from both sexual parents and 100 times probe concentration *Arabidopsis* DNA for blocking off all common sequences. BH74 was used for the first test because of its superior chromosome morphology and well distinguishable *Het* and *Del* chromosomes (Figure 4-1d). The two-colour GISH reveals a well-differentiated pattern, with green fluorescence from the *B. stricta* probe hybridizing to the pericentromere regions of specific chromosomes, including the *Het* and the *Del*, and the red fluorescence representative of the *B. holboellii* targets. We noted that the bright DAPI fluorescing pericentromere regions always correspond with the *B. stricta* signal.

The Figures 4-2a and 2b and Table 4-1 give an overview of all two-colour genome painting experiments. We selected between five and ten well spread metaphase complements and compared the results for karyotype analysis. The diploid sexual controls demonstrate uniform staining of the pericentromere regions of all chromosomes in both species, except for green – red (yellow) overlapping signals on two chromosome pairs in *B. holboellii* which represent the 45S rDNA (first pair) and 5S rDNA sites (third pair in the karyotype). In addition, the genome painting of BH74 and BH115 made clear that one NOR chromosome originates from *B. stricta* and one from *B. holboellii*.

We also noticed that the *B. stricta* probe produced weak signals on few of the *B. holboellii* chromosomes, and vice versa. The two *B. ×divaricarpa* hybrids differ with respect to the relative number of parental chromosomes, as BDi175 has 6 *B. stricta* chromosomes, nine *B. holboellii* chromosomes, and the *Het* and *Del* elements, while ES9 has a balanced genome with seven chromosomes each of *B. stricta* and *B. holboellii*. Both DAPI fluorescence and GISH using the *B. stricta* probe demonstrated different *Het* chromosome profiles for ES9 (*Het'*) with brighter signals in the centromere region, and BDi175 whose *Het* was characterized by a more segmented pattern of two or three heterochromatin blocks. The other *Boechera* apomicts also showed different relative numbers of *B. stricta* and *B. holboellii* chromosomes, with 5 *B. stricta* and 9 *B. holboellii* chromosomes in GRL2, 4 *B. stricta* chromosomes in BH1 and BH224, 10 *B. stricta* chromosomes in BH115, and 11 in BH74. These individuals all showed green *B. stricta* fluorescence in the *Het* and the *Del* chromosomes. In addition, the GRL2 accession was characterised by a *Het* chromosome having a heterochromatin pattern similar to that of ES9. The overall green fluorescence signal of the *B. stricta* probe was clearly weaker in the apomicts BH1 and BH115 compared to the other apomicts.

Discussion

This study has shown that repetitive DNA sequences in the genomes of *B. stricta* and *B. holboellii* genomes have diverged sufficiently such that they can be distinguished in a genome painting assay of their hybrids. The two-colour GISH experiments using the simultaneous hybridisation of both genomic DNAs as probes furthermore shed light on the dynamics

of non-coding DNA in *Boechera*. For example, the fluorescence patterns clearly show that genomic differentiation is confined to the centromeres and flanking pericentromere heterochromatin. This reflects the generally known dynamic nature of (peri)centromere regions as shown for many species including Arabidopsis and related species (Kamm *et al.*, 1995; Comai 2000; Hall *et al.,* 2004). Cross hybridisation occurs mostly in the rDNA domains, as is evidenced by yellow bands in the NOR of the satellite chromosome pair, and a second, often stronger band in the pericentromere region of a second pair. The weak signals of the *B. stricta* probe on the chromosomes of the diploid *B. holboellii* and *vice versa* suggest that the differences between these repeats are quantitative rather than qualitative in nature. The co-localisation of the *B. stricta* specific repeat DNA with the dense heterochromatic pericentromere regions of *B. stricta* chromosomes suggest that the larger chromosomes and genome size (relative to *B. holboellii*; see Sharbel and Mitchell-Olds, 2001; Sharbel et al., 2005) may have resulted from the amplification of this repeat class.

The localisation of the GISH probes in the pericentromere regions is similar to the pattern of small repeat domains located around the centromeres and nucleolar organisers in *Arabidopsis* (Koornneef *et al.*, 2003) and other Brassicaceae in which the conspicuous proximal heterochromatin regions are the sites of species-specific retroelements, tandem satellites and other repeats (Comai *et al.*, 2003a; Ali *et al.*, 2004). In contrast, plant species of moderate to large genome size, like *Allium* (Khristaleva and Kik, 1998) and *Lilium* (Karlov *et al.*, 1999), have such species-specific repeats distributed throughout the genome, as is reflected by more uniform distributions of probe hybridization over their chromosomes.

The two *B. ×divaricarpa* apomicts ES9 and BDi175 were classified as hybrids, based upon silique and trichome morphology, and molecular markers (Dobeš 2004a,b; Chapter 2). Previous pachytene and metaphase I observations of the BDi175 accessions revealed an almost entire lack of chromosome pairing and recombination (Chapter 3), and we hypothesized that this reflected an amphihaploid state characterized by one complete *B. stricta* and one complete *B. holboellii* genome. However, genome painting of the fifteen chromosomes apomict BDi175 has demonstrated that the genome in this hybrid is not balanced, having nine *B. holboellii* and six *B. stricta* chromosomes, including the *Het* and the *Del* chromosome. Only the ES9 hybrid, also with fourteen chromosomes, seems balanced genomes with seven *B. stricta* chromosomes including the *Het*, and another seven chromosomes from *B. holboellii*. However no meiotic data of this hybrid is available.

Much more variation was observed in the apomicts that were classified morphologically as *B. holboellii*. The accessions BH1 and BH224 have only four *B. stricta* chromosomes, which are including the *Het* and the *Del*, although we do not yet know if the two other *B. stricta* chromosomes in these accessions are their homologues. Finally, in BH74 and BH115, the numbers of *B. holboellii* chromosomes are only four and five, respectively. The GRL hybrid with 14 chromosomes has five *B. stricta* chromosomes including the Het chromosome and 9 chromosomes with the *B. holboellii* specific repeat. In the next chapter we will discuss the possible processes that may account for these chromosome replacements.

Interestingly, we observed weaker fluorescence intensities of the *B. stricta* probe in BH1 and BH115. As the red fluorescing *B. holboellii* signals and the yellow rDNA signals were comparable to those found in the chromosomes of the other accessions, we conclude that this weaker signal is not a hybridisation artefact, but represents a true lower hybridisation signal of the *B. stricta* probe, possible pointing at different clonal age of the apomicts, or that the parental species is not *B. stricta*. Once the molecular organisation and sequences of the species specific repeats have been elucidated, a more extensive quantitative study on these species specific repeats can be carried out.

We distinguished three morphologically different aberrant chromosomes: 1) the *Het* chromosome has a bright DAPI fluorescence and consists of two or three heterochromatin blocks. 2) The *Del* chromosome is much smaller and has only one small proximal heterochromatin segment. Both *Het* and *Del* are present in the BH1, BH74, BH115, BH224 and BDi175 apomicts. 3) The heterochromatin chromosome *Het'* chromosome is also largely heterochromatic, but has one large heterochromatin block in its pericentromere and centromere region. This aberrant chromosome was only found in the fourteen chromosome ES9 and GRL2 apomicts. Genome painting revealed bright green fluorescence of the *B. stricta* probe in both *Het* and *Het'* chromosomes, and a smaller *B. stricta* signal on the *Del*, thus demonstrating that these chromosomes may have originated from *B. stricta* or a closely related species. These observations are furthermore supported by DNA sequencing of two autosomal loci, both of which are also found on at least one of the extra elements, which has shown that "extra-chromosome" specific alleles share similar polymorphism with those specific to *B. stricta* (Sharbel et al, 2004). Further support for the origin of the *Het* chromosome comes from a single genome painting experiment on meiotic prophase I cells of BH1, which shows the heteromorphic bivalent between the *Het* and another *B. stricta* chromosome (Fig. 4-1c). On the other hand, the *Del* chromosome, which has a clear *B. stricta* signal, was found associated with a bivalent which lacks a *B. stricta* signal, thus suggesting that this heteromorphic trivalent represents a translocation trisomic in which the *Del* chromosome has resulted from a translocation between *B. stricta* and *B. holboellii* chromosomes. Unfortunately no genome painting results were obtained with pachytene and metaphase I complements of the other apomictic accessions.

This study has demonstrated the enormous power of genome painting in *Boechera* apomicts. It is the first report that directly and unequivocally shows that apomictic accessions can have different numbers of their putative ancestral species, and thus proves the occurrence of widespread chromosome substitutions. It is still unclear whether such substitutions involved single homoeologous or whole chromosomes, although our meiotic and karyotype analyses of the different apomicts suggest that both processes can take place. In conclusion, this chromosome painting study has demonstrated the evolutionary origin of aberrant chromosomes from *B. stricta*. In the next chapter we will further discuss the models accounting for the chromosome substitutions and the evolutionary origin of the aberrant chromosomes in apomictic *Boechera*.

CHAPTER 5

A cytogenetic view on the origin of apomixis in the *Boechera holboellii* complex

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Abstract

The cytogenetic study as presented in the chapter 2, 3 and 4 of this PhD thesis include just two diploid sexual *Boechera* species and seven apomictic accessions, but this was sufficient to confirm the hypothesized apomictic plasticity of this close relative of *Arabidopsis*. Combining data from karyotype analyses, fluorescence *in situ* hybridisation, chromosome pairing, pollen morphology, flow cytometric seed screens and genome painting we discovered an unexpected plethora of chromosome forms, meiotic behaviour and apomixis transmission, which was unique for every accession we considered. *Boechera's* suitability to become the model species *par excellence* for apomixis research not only follows its unique position in the Brassica family, but also results from the fact that this apomict is one of the very few species that can express apomixis at the diploid level. The results of this thesis have made clear that *Boechera* apomicts are alloploid, with different balanced or unbalanced combinations of *B. holboellii* and *B. stricta* chromosomes. We hypothesized that such genomic constitutions result from recurrent diploid-polyploid and polyploid-diploid conversions, the latter with reductional meiosis generating haploid gametes with novel recombinations of homoeologous chromosomes. All *Boechera* apomicts always contain one or two aberrant chromosomes, either a single largely heterochromatic chromosome (*Het'*) as was found in the apomicts with fourteen chromosome, or two chromosomes with one heterochromatic (*Het*) but slightly different from the *Het'*, and a second, much smaller chromosome (*Del*) as was found in the apomicts with fifteen chromosomes. Both aberrant chromosomes share DNA sequences with repetitive pericentromere repeat(s) from the *B. stricta* parent suggesting that these chromosomes have originated from the sexual *B. stricta* species. Looking at only these few apomicts it is tempting to believe that the *Het* chromosome in all accessions originated from the same ancestral pro-*Het* chromosome and that this chromosome plays a key role in the apomictic trait containing genetic elements required for the apomictic pathway and that this chromosome meiotically resembles the Y-chromosome rather than the B chromosome. In this chapter we summarize all cytogenetic data from the *Boechera* sexuals and apomicts and develop models to explain the differences in chromosome constitutions of the apomicts and to shed light upon the evolutionary pathway of aberrant chromosomes and their relation to the apomictic trait.

Keywords: *Boechera*, apomixis, genome painting, GISH, aberrant chromosome, homoeologous substitution, chromosome evolution.

A. Genome plasticity in *Boechera*

Initially, the aim of this cytogenetic investigation was to shed light upon the nature of the aberrant chromosomes in the agamic *Boechera* complex. Although polyploidy is characteristic of the complex, we considered only diploids and derived aneuploids in order to simplify chromosome identification and to facilitate interpretations of the meiotic aberrations. We realized that the results would elucidate only part of the potential chromosome diversity of the *B. holboellii* complex, which encompasses a yet undetermined number of sexual and apomictic taxa of varying ploidy levels from large areas across North America and Greenland (Dobeš *et al.*, 2004a). It is nonetheless surprising that the seven apomictic accessions of this study demonstrated such a wide range of plant shape, chromosome morphology, meiotic recombination and chromosome transmission.

Karyotype variation

As discussed in Chapter 2, all apomictic accessions were characterized by karyotypes with striking differences in chromosome lengths, centromere positions, centromere bands and NOR size. Although DAPI stained cell spread preparations produce clear, well-defined chromosomes, it was impossible to arrange the chromosomes of the apomicts in putative homologous pairs. Part of this variation was further substantiated by FISH using rDNA probes, which demonstrated repeat size polymorphisms, of which the most prominent example was found in the BH74 accession. We also observed that the apomicts were characterised by different numbers of chromosomes having brightly fluorescing pericentromere bands, which in the genome painting experiments were determined to be of *B. stricta* origin. This large scale chromosome variation, referred to as structural karyotype heterozygosity, has likely resulted from multiple mechanisms of chromosomal change, including *i*) chromosome substitution between *B. stricta* and *B. holboellii* homoeologues, *ii*) quantitative and qualitative variability of pericentromere repeats, which in *B. stricta* is more than 8% larger than in *B. holboellii,* and *iii*) karyotype heterozygosity resulting from reciprocal translocation and inversions (*e.g.*, BH115, which shows translocation quadrivalents at metaphase I). Extensive painting using *Arabidopsis* BACs (Schubert *et al.*, 2001; Lysak *et al.*, 2003; Lysak *et al.*, 2005) and molecular genetic analyses (Sharbel et al., in preparation) will be required to further elucidate the various mechanisms which have led to the chromosome dynamics in the *B. holboellii* complex. Other, even smaller chromosome modifications and changes in the genetic code or epigenetic imprinting will need the help of large scale physical mapping and DNA sequencing studies.

Genomic origin of the normal and aberrant chromosomes

Variable numbers of parental chromosomes in the apomictic hybrids lead one to question whether specific combinations of *B. stricta* and *B. holboellii* chromosomes are required for viable and stable apomixis expression. The comparison of meiotic pairing and genome

Table 1. Overview of major cytogenetic and taxonomic characteristics of the *Boechera* apomicts.

accessions	Taxonomic morphology	GISH chromosome number		cpDNA	ITS
		holboellii	***stricta***		
BH208	*holboellii*	14	0	holboellii	*holboellii*
BS2	*stricta*	0	14	*stricta*	*stricta*
ES6	*stricta*	0	14	*stricta*	*stricta*
ES9	×*divaricarpa*	7	7	*holboellii*	*stricta*[1]
BH1	*holboellii*	11	4	*holboellii*	*holboellii*
BH74	*holboellii*	4	11	*holboellii*	*holboellii*
BH115	*holboellii*	5	10	*holboellii*	*holboellii*
BH224	*holboellii*	11	4	*holboellii*	*holboellii*
BDi175	×*divaricarpa*	9	6	*stricta*	*stricta*
GRL2	*holboellii*	9	5	*holboellii*	*holboellii*

1) Both NOR chromosomes come from *B. stricta*.

painting of the synaptic apomicts BH1, BH115 and BH224 (Table 3-1, Table 4-1 and Figure 3-1c) tells us that BH1 has one pair of *B. stricta* homologues (with the *Het* chromosome), one pair of *B. holboellii – B. stricta* homoeologues, one trivalent with two *B. holboellii* and the *Del* chromosome, and four pairs of *B. holboellii* bivalents. Accession BH115, with the translocation quadrivalent at meiotic prophase I, can have one, three or five *B. stricta – holboellii* homoeologous pairs and four, three and two homologues plus de *Del* from *B. stricta*. It is not known yet which chromosomes form the translocation complex, but likely only *B. holboellii* chromosomes are involved. Accession BH224 may have the same chromosome constitution as BH1 or has three homoeologues and four *holboellii* bivalents plus de *Del*. Other apomictic accessions will be needed to reveal whether particular *B. stricta* chromosomes or combinations of *B. stricta* and *B. holboellii* are involved in the expression of apomixis. We also want to learn which combinations of homologous and homoeologous chromosomes can be tolerated and what consequences it has for evolutionary aspects of the apomixis trait in the accessions. A major help in this focus is expected from two-colour genome paintings and BAC-FISH on meiotic cells, especially pachytene and metaphase I complements of accessions with synaptic meiosis.

Composition of the chromosomes in the hybrids can also be influenced by other factors like cytoplasmic factors but no direct evidence can be provided. Chloroplast analysis of our apomicts (Table 5-1) revealed that all accessions except the BDi hybrid have the *holboellii* variant, suggesting maternal origin of the apomicts (Dobeš, personal communication).

Meiotic chromosome pairing
Pairing behaviour could be influenced by structural changes or chromosomal rearrangements as mentioned before (heteromorphy) or by genes controlling homoeologous pairing like the *Ph1* locus in wheat (Riley and Chapman, 1958; Sears and Okamoto, 1958) and the *PrBn* locus in oilseed rape (Jenczewski *et al.*, 2003), but more likely is a combination of both. Meiotic chromosome pairing problems are known from various asexual taxa, both animal and plant. It is not known as to whether there is mutation accumulation in "pairing loci" which lead to variation, or if the same chromosome structural mutation influence both sexual and asexual genomes, but are only apparent in asexual genomes because sexual genomes have more stringent selection pressure? Further aspects of meiosis that deserve attention are the factors controlling chromosome pairing at pachytene and chiasma formation at diplotene – metaphase I. So far our data cannot differentiate between pairing failure resulting from specific homoeologous chromosome substitutions and the global lack of sufficient homology. Factors influencing synaptic and recombination processes may be replaced or entirely missing in the BH74 and BDi175 accessions.

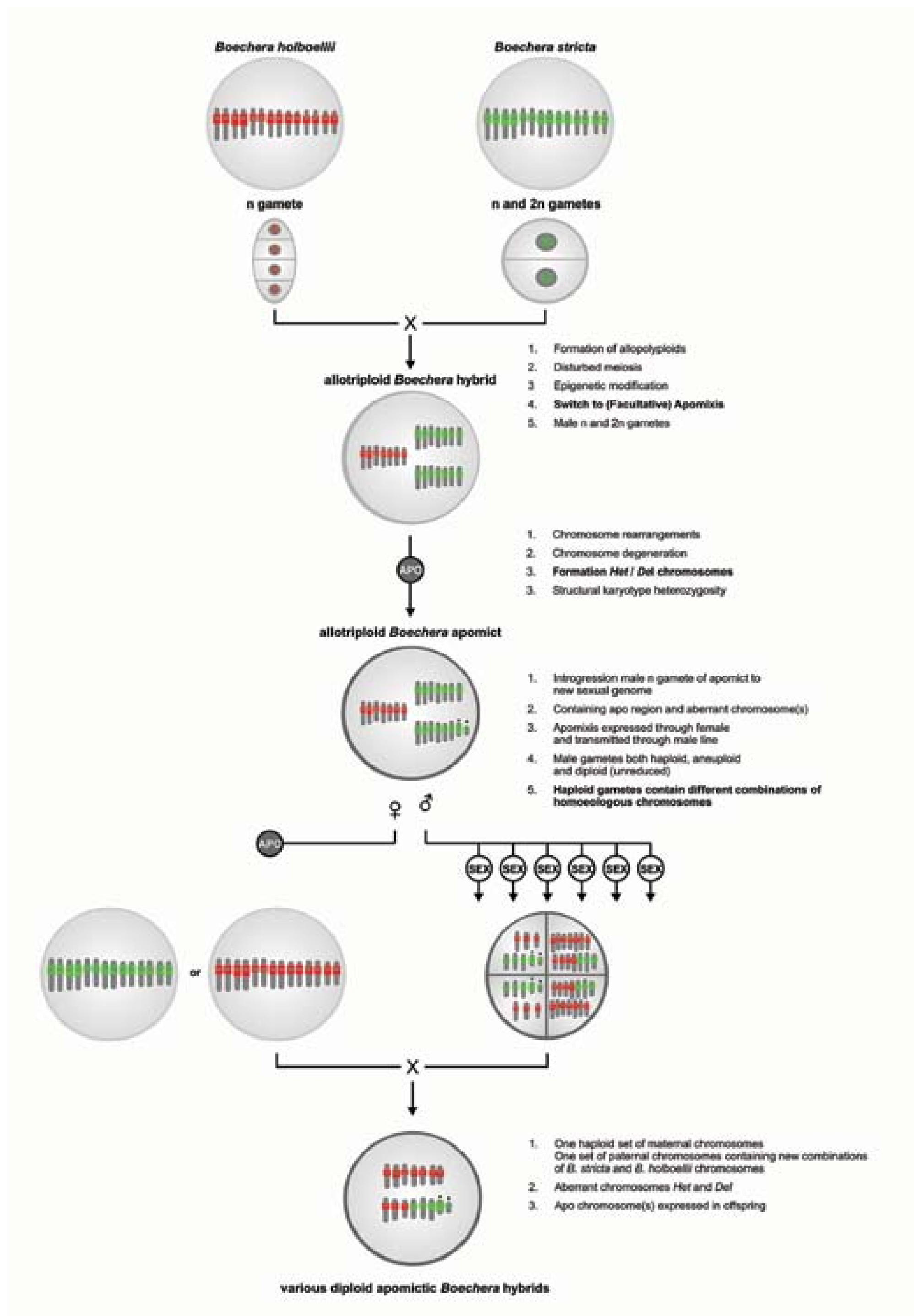
It is questionable as to whether aneuploid and polyploidy are the driving forces for unreduced meiosis (apomeiosis) in the apomicts. In BH115 and BH224, chromosome pairing was almost complete but the majority of pollen was unreduced. BDi175 showed asynapsis at metaphase I, and rarely reductional meiosis leading to *n* gametes, thus indicating that other factors are involved for producing reduced and unreduced gamete in the same accession.

Our observation of pairing and recombination between *Het* and the distal region of a *B. stricta* chromosome in BH1 clearly demonstrate that some homology exists between these chromosomes. As we will discuss later, it is conceivable that *Het* has gone through a long evolutionary process of gradual heterochromatinization and genetic degeneration for one part but retaining a small euchromatin region in the autosomal part. This characteristic resembles the evolution of sex chromosomes in several respects (Charlesworth and Charlesworth 2000; Filatov *et al.*, 2001; Vyskot and Hobza, 2004), an aspect we will clarify later.

B. Model for *Boechera* apomict evolution

Our vision of the evolutionary history of *Boechera* apomicts is based primarily on the successful distinction by GISH of *B. stricta* and *B. holboellii* specific repeat classes in the pericentromere regions of their chromosomes. The technique demonstrated that the apomicts are true hybrids, composed of a variable numbers of chromosomes from *B. stricta* and chromosomes from *B. holboellii*, or relative species. Figure 5-1 presents a schematic outline of main events and processes in the evolutionary pathway that we supposed essential for explaining the multiplicity of apomictic forms.

The first step assumes the formation of viable *B. holboellii s.l.* X *B. stricta* interspecific hybrids. These hybrids can escape from sterility by producing 2n gametes, which is supported from our observation that *B. stricta* sexuals generate male 2n gametes through FDR


Boechera holboellii
Boechera stricta
n gamete
n and 2n gametes
X
1. Formation of allopolyploids
2. Disturbed meiosis
3 Epigenetic modification
4. Switch to (Facultative) Apomixis
5. Male n and 2n gametes
allotriploid Boechera hybrid
1. Chromosome rearrangements
2. Chromosome degeneration
3. Formation Het / Del chromosomes
3. Structural karyotype heterozygosity
APO
allotriploid Boechera apomict
1. Introgression male n gamete of apomict to new sexual genome
2. Containing apo region and aberrant chromosome(s)
3. Apomixis expressed through female and transmitted through male line
4. Male gametes both haploid, aneuploid and diploid (unreduced)
5. Haploid gametes contain different combinations of homoeologous chromosomes
♀ ♂
APO
SEX
SEX
SEX
SEX
SEX
SEX
or
X
1. One haploid set of maternal chromosomes
One set of paternal chromosomes containing new combinations of B. stricta and B. holboellii chromosomes
2. Aberrant chromosomes Het and Del
3. Apo chromosome(s) expressed in offspring
various diploid apomictic Boechera hybrids

or SDR meiosis at low frequency (Chapter 3), and so can give rise to allotriploid and allotetraploid offspring. It is not known yet which combinations of *B. holboellii* and *B. stricta* are the best parentage for the interspecific hybrids and their derived apomictic accessions.

In the second step we postulate that genomic stress in interspecific hybrids induced epigenetic modification causing phenotypic instability by gene silencing by cytosine alterations and occasional gene activation as was demonstrated for *Arabidopsis* and other allopolyploids (Comai *et al.*, 2000a,b, Comai *et al.,* 2003a,b; Lee and Chen, 2001; Madlung and Comai, 2004; Madlung *et al.*, 2005; Mittelstein Scheid et al., 1996; Osborn et al., 2003; Wang *et al.*, 2004). Other mechanisms that can alter gene expression profiles in newly formed polyploids were suggested by Riddle and Birchler (2003) and Mittelstein et al. (2003), and include aberrant or novel interactions of diverged transcriptional regulators from the ancestral genomes in the polyploid. Allopolyploid formation in wheat also has lead to sequence eliminations and cytosine methylation (Feldman *et al.*, 1997; Shaked et al. 2001). If we assume that such epigenetic changes also alters the *Boechera* hybrid genome adaptive epimutations and novel phenotypes may arise (Liu and Wendel, 2003), including genes that are involved in diplosporous and parthenogenetic processes giving rise to the apomixis switch. Such process may take place stepwise and need several generations before becoming stably inherited.

The third step explains how apomicts can end up with different chromosome numbers from their putative parents. The transitions to higher ploidy levels are based on the ability of *Boechera* to produce both reduced and unreduced gametes, thus explaining the occurrence of apomicts at diploid, triploid and tetraploid level. The existence of allopolyploid apomictic clones is also confirmed by our genome painting experiments (unpublished observations) and by microsatellite marker analysis revealing multiple alleles per locus in the polyploids (Dobeš *et al.*, 2004a). As shown in our meiotic analyses (Chapter 3), such plants have the potential of producing both reduced and unreduced functional gametes with a variable mix of *B. stricta* and *B. holboellii* chromosomes. As all apomicts have at least four *B. stricta* chromosomes including the two aberrant *Het* and *Del* chromosomes, it is conceivable that a specific chromosome combination is more favourable or even essential for the maintenance of producing viable apomictic offspring.

Evolutionary, new apomictic clones are essential for refreshing the apomictic populations. The process of diploid-tetraploid-haploid cycle as purposed by de Wet (1968) indicated that occasional apomictic autopolyploid produced haploid gametes for sexual reproduction can generate new chromosome combinations in the freshly formed apomictic clones. In our model, the new clones with novel chromosome combinations also come from sexual reproduction via backcrossing between apomicts and the sexuals, which then produce prog-

« **Figure 5-1.** Model of the origin of apomixis in Boechera. All chromosomes with the red pericentromere heterochromatin bands are from *B. holboellii* and with the green bands from *B. stricta*. Cell complements with a light gray border are sexual; those with a dark gray border apomicts. All basic steps are summarized in short enumerated lists.

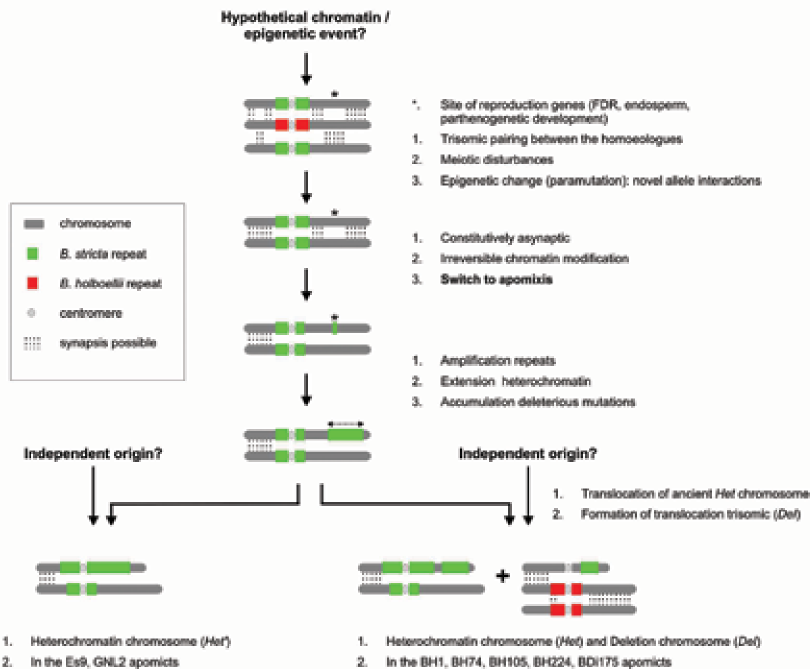


Figure 5-2. Schematic representation of the origin of aberrant chromosomes in apomictic *Boechera* accessions.

eny with diverse chromosome combinations and substitutions. In this way we explain how chromosomes can be substituted by their homoeologous giving rise to so many accessions with different numbers of *B. stricta* and *B. holboellii* chromosomes in the cell complements. It also makes clear why the *B. holboellii* apomicts BH1, BH74, BH115 and BH224 essentially are *B. ×divaricarpa* hybrids, but depending on the expression of *B. stricta* and *B. holboellii* specific alleles, may exhibit the diagnostic silique and trichome phenotype of one of their sexual parents.

C. Origin and nature of the aberrant chromosomes

The consistent presence of the aberrant chromosomes in the apomictic clones strongly suggests a key role in controlling the apomixis trait, but so far direct evidence for it is lacking. In Figure 5-2 we explain our view about the evolutionary steps of the aberrant chromosomes. We consider only the two variants of aberrant chromosomes, the *Het'* in the accessions ES9 and GRL2 and the combination of the *Het* and *Del* chromosomes in the other apomicts, but likely more exist.

Three major events are here distinguished: The first step is the pairing impairments and epigenetic settings in meiosis of a typical amphiploid hybrid (Liu and Wendel 2003), that

leads to the switch to apomixis. Further indication for an epigenetic change came from Adams *et al.* (2003) who demonstrated organ-specific gene silencing or unequal expression in one of the homoeologues in synthetic tetraploid *Gossypium* (cotton) and DNA sequence elimination in allopolyploids (reviewed in Eckhart 2001). In the second step, we hypothesize that concomitant local chromatin modifications that lead to synaptic isolation to give rise to a pro-*Het* chromosome; and in the final phase, further extension of recombination suppression (asynaptic region) is accompanied by repeat amplification, accumulation of deleterious mutations (Green 1990) and extension of the heterochromatinized chromosome region. Quite likely so, we postulate that one and the same epigenetic event initiated both the birth of the apomixis controlling region and the local pairing failure that ultimately lead to the long evolutionary process of gradual chromosome degeneration.

As explained in Figure 5-2, we assume that both *Het* and *Het'* share a common ancestral chromosome with a large *B. stricta* repeat suggesting that they have originated from one and the *B. stricta* chromosomes. As all apomicts have at least two more *B. stricta* chromosomes it cannot be excluded that (more) apomixis genes reside on these two *B. stricta* and / or other *B. holboellii* chromosomes. In the simplest explanation we begin with the idea that a single ancestral chromosome event has led to the formation of both the *Het'* in the 14 chromosome accessions and the *Het* together with the *Del* chromosome in the 15 chromosome accessions. Alternatively, and more likely, is the formation of two or more independent lines for both and other variants, involving heterochromatinization of a *B. stricta* chromosome followed by chromosomal rearrangements like translocations.

Metaphase I and genome painting analyses of the BH accessions 1, 115 and 224 in the Chapters 3 and 4 showed that the *Het* can form heteromorphic chromosome associations with a *B. stricta* chromosome, and *Del* associates through a chiasma with a *B. holboellii* bivalent, but both chromosomes have a *B. stricta* specific repeat (Chapter 3). The conspicuous *B. stricta* repeat region and heteropycnotic behaviour of this chromosome segment suggest large scale repeat amplification and heterochromatin formation, apparently accompanied with an extension of the unpaired region. The *Del* chromosome with a *B. stricta* pericentromere region and pairing capability with a *B. holboellii* pair points at a translocation chromosome resulting from a proximal *B. stricta* segment and a distal *B. holboellii* segment. No detailed meiotic data were obtained from the *Het'* chromosome in the 14 chromosome apomicts, but it is tempting to believe to genetic elements essential for the apomixis trait in the *Het'* chromosome is in some way rearranged over *Het* and *Del* in the 15 chromosome BH accessions.

A number of intriguing aspects of the aberrant chromosomes remain. The heteromorphic behaviour of the *Het* chromosome strongly suggests failure or impairment of homologous pairing of the ancestral bivalent, a widely accepted view on the evolutionary origin of heteromorphic sex chromosomes. It is imaginable that interspecific hybridization will affect species-specific chromosomes modification which has been found in uniparental gene silencing in allopolyploid *Arabidopsis* (Madlung and Comai, 2004). The modification be-

comes constitutive and inherits stably to the next generations, which causes a permanent change and gradual accumulation of heterochromatin reflective of Y chromosome evolution (Jablonka, 2004).

One of the most intriguing facts about the aberrant chromosome is its nature and function. The aberrant chromosome belongs to the group of heterochromatinized chromosomes associated with the B-chromosomes (reviews in Camacho *et al.* 2000, Jones and Rees 1982, Jones 1995), Y-chromosomes (Bachtrog and Charlesworth, 2002; Lahn *et al.* 2001) and supernumerary fragments (Roche *et al.*, 2001b). All have in common that a greater part of these chromosomes are heterochromatinized, *i.e.*, chromosome staining techniques like DAPI, Feulgen and orcein staining, and C-banding, demonstrating highly compact chromatin often with specific staining properties of Y chromosomes and supernumerary chromatin. Many, but not all studies of such chromosomes showed high proportions of repetitive DNA, methylated DNA and modified histones. It is also assumed that such chromosomes are poor in single copy sequences and that they accumulate deleterious mutations (Green, 1990). In most cases, the distinction between such cases of such (partly) degenerated chromosomes is clear: B-chromosomes are foremost parasitic, do not possess genes essential to the organism and their number may vary between individuals in the population, but they never recombine with any of the other chromosomes. Y-chromosomes are associated with the X chromosome, and are essential in the sex determination, but their number and molecular organisation differ enormously between plants and animals. Supernumerary fragments, like the apospory containing region in *Pennisetum squamulatum* (Roche et al., 2001a,b; Goel et al. 2003; Akiyama et al. 2004) are large hemizygous regions. However, a vast number of studies claimed cases of chromosomes intermediate or mixtures of properties for B-chromosomes, Y-chromosomes, or supernumerary fragment. In this respect, the aberrant chromosomes in *Boechera* share properties of the B-chromosome (extra, but not sex determining) and the Y chromosome concepts (pairing and recombination with one other chromosome). Its putative role in the expression of apomixis and the large amount of heterochromatin brings the *Het* chromosome closer to the supernumerary chromosome fragment of *Pennisetum* as two examples of apomixis controlling chromosomes in which absence of meiotic recombination have led to the previously described amplifications of repetitive sequences and heterochromatinization.

The mechanism of heterochromatic initiation sites and spreading are not randomly but under the epigenetic control of boundary proteins and small RNAs (Verdel *et al.*, 2004, Grewal and Moazed, 2003). Degeneration processes that ultimately lead to the aberrant *Het* chromosome are more likely directed by a complex and long evolutionary process of transposition insertions, accumulation of deleterious mutations and chromatin modifications, and may even involve large scale chromosomal rearrangements. We therefore do not know to what extend the molecular organization of the Het chromosomes in the different accessions have diverged.

Finally, one more fascinating controversy in the control of apomixis in *Boechera* exists. Our cytogenetic study strongly points at a diploid like organisation of the apomictic clones, apart from the *Del* chromosome which most likely represents a *B. stricta – B. holboellii* translocation, whereas the 14 chromosomes ES9 and GRL2 accessions have no cytogenetic evidence at all for (partial) trisomy. However, previous published molecular genetic data (Sharbel *et al.* 2004, 2005) and recent unpublished analyses clearly demonstrated three different alleles of specific microsatellite markers. Additional indications for (partial) polysomy in the apomicts came from *Taraxacum*, *Paspalum*, and several others. However, a first attempt to establish partial trisomy in the apomictic clones in a painting experiment with *Arabidopsis* BACs containing DNA sequences corresponding to triple microsatellites region in *Boechera* did not reveal the expected three chromosomal targets (Chapter 2). Further high-sensitivity FISH experiments will soon be carried out in search of any trisomy or duplicated segments in the chromosomes.

Chapter 6

A Single Sequence Length Polymorphism (SSLP)-based method for detection of apomictic plants in a sexual *AtSERK1* transgenic *Arabidopsis* population

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Abstract

Here we present a screening method to evaluate the potential of genes to transfer aspects of apomixis into sexual crop plants. Based on the assumption that an apomictic progeny is an exact genetic replica of the mother plant we employed a set of Single Sequence Length Polymorphism (SSLP) markers to identify individuals displaying heterozygosity fixation in segregating sexual populations as an indication of rare apomictic events. Here we present the results of such a study using the Arabidopsis thaliana SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (AtSERK1) gene expressed under the control of two different promoters: the AtLTP1 and the AtDMC1 in sexual *Arabidopsis* plants. In only one of the four tested F2 transgenic populations, i.e., expressing the AtLTP1::AtSERK1 construct we observed two plants (1.8%) with heterozygosity maintenance for the full set of SSLP markers indicating a possible clonal inheritance. However, as their offspring revealed a close to binomial segregation for number of heterozygous loci, it was concluded that these two putative apomictic plants resulted from either incidental recombination events displaying the genotype of the parent, or that they lost their clonal ability in the next generation.

Key words: Apomixis, *Arabidopsis*, SSLP, adventitious embryony, *AtSERK1*.

Introduction

Apomixis is defined as the asexual formation of a seed from the maternal tissues of the ovule avoiding the process of (reductional) meiosis and fertilization (reviewed by Bicknell and Koltunow 2004). In nature apomixis occurs in more than 400 flowering plant taxa representing 40 families (Carman 1997). Apomicts can be facultative, using a combination of sexual and asexual modes or more rarely are obligate, forming exclusively apomictic seeds (Nogler 1984). Two types of apomixis are defined: sporophytic and gametophytic apomixis. In sporophytic apomixis, or adventitious embryony, embryos arise spontaneously from ovule cells late in the ovule development. Gametophytic apomixis operates through the unreduced embryo sac (reviewed by Bicknell and Koltunow 2004). Endosperm development in both cases may be either spontaneous (autonomous) or fertilization-induced (pseudogamous) (reviewed by Koltunow 1993). Gametophytic apomixis is further subdivided into diplosporous or aposporous mode based on the cell type that gives rise to the unreduced embryo sac. In diplosporous types the megaspore mother cell (MMC) or a cell with apomictic potential is the progenitor cell of the unreduced embryo sac. That cell skips the reductional part of meiosis, typically anaphase I, and development proceeds by mitotic division to achieve embryo sac formation (meiotic diplospory). Alternatively, that cell might undergo direct mitosis to form an unreduced embryo sac (mitotic diplospory). In aposporous apomicts, one or more somatic cells of the ovule, called aposporous initials, give rise to an unreduced embryo sac. Aposporous initials can differentiate at various times during ovule development. Aposporous embryo sacs usually coexists with a reduced meiotic embryo sac which later degenerates (reviewed by Bicknell and Koltunow 2004). In apospory, mitotic diplospory and in adventitious embryony apomictic seeds are clonal progeny of maternal origin because meiosis and fertilization are absent. This however, does not hold true in apomicts with meiotic diplospory origin where recombination may occur during prophase of meiosis I (Spillane et al., 2001).

Up till now attempts to introduce apomixis into sexual species have made use of introgression of an apomictic trait from a wild apomictic relative into a recipient model or crop species (Koltunow et al. 1995). Another proposed strategy entitles cloning of the apomictic genes and their transfer into sexual plants by transformation (Koltunow et al. 1995, Vielle-Calzada et al. 1996, Grossniklaus et al. 1998a). The idea that apomixis can be successfully engineered in a sexual developmental systems is based on a number of clues. Firstly, most natural apomicts are facultative. Secondly, natural apomicts can display various ways by which sporogenesis or gametogenesis can be deregulated in a way that results into apomixis (Spillane et al. 2001). Thirdly, mutants have been described that show phenotypes corresponding to elements of apomixis, like the three *fis* (*fertilization-independent seed*) genes: *fis1/mea* (*medea*) (Grossniklaus et al. 1998b), *fis2* (Chaudhury et al. 1997) and *fis3/fie* (*fertilization-independent endosperm*) (Ohad et al. 1999), all of them with some extent of autonomous development of the central cell. A potential difficulty in engineering gametophytic or

adventitious apomixis using genes identified in sexual species is whether parthenogenesis or adventitious embryony will occur in the complete absence of functional endosperm. To avoid this, a strategy is required where the normal sexual pathway remains present without leading to parental imbalances in the endosperm caused by the fusion of an unreduced central cell with a reduced sperm (Spielman et al. 2003).

Genetic and molecular data suggest that the apomictic pathway differs from the sexual one in only a few key genes that control female gametophyte developmental (Carman 1997, Tucker et al. 2003). So far, candidate genes for apomixis have been identified mainly by comparative gene expression studies during early stages of apomictic and sexual embryo development (Chen et al. 1999, Pessino et al. 2001, Rodrigues et al. 2003, Albertini et al. 2004, 2005). Map based approaches have also identified a number of loci critical for the inheritance of the apomictic trait in different apomictic species (Pupilli et al. 2004, Vijverberg et al. 2004). Alternatively genes that are responsible for gamete reduction, parthenogenesis or autonomous endosperm formation in sexual species are regarded as more successful candidates for 'apomixis' genes. Because sexual and apomictic development are closely interrelated, deregulating such genes in the sexual model systems such as *Arabidopsis thaliana* can result in phenotypes resembling elements of an apomictic pathway. This also requires the isolation a range of cell- (*e.g.*, egg cell) and stage-specific (*e.g.*, megasporogenesis) promoters that allow missexpression of genes conferring elements of apomixis (reviewed by Grossniklaus 2001, Spillane et al. 2001).

In the context of engineering adventitious embryony several *Arabidopsis* genes promoting somatic embryogenesis such as *BABY BOOM* (*BBM*) (Boutilier et al. 2002), *WUSCHEL* (*WUS*) (Zuo et al. 2002, Gross-Hardt et al. 2002, Gallois et al. 2002), *LEAFY COTYLEDON1* and *2* (*LEC1* and *LEC2*) (Lotan et al. 1998, Stone et al. 2001) may provide a strategy to confer parthenogenesis (Spillane et al. 2001). This is further explored in the potential of another embryogenesis-related gene, the *Arabidopsis thaliana SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*AtSERK1*) to induce elements of parthenogenesis apomixis in *Arabidopsis*. Overexpression of *AtSERK1* can increase the embryogenic potential of cells in tissue culture (Hecht et al. 2001), a phenotype also observed in some mutants as *clavata* and *primordia timing* (*pt* which is allelic to *amp1* and *cop2*) (Mordhorst et al. 1998). *In planta*, expression of the *AtSERK1* gene has been detected in ovule primordia during megasporogenesis, including the functional megaspore and later in all cells of the embryo sac. After fertilization, the expression could be detected in the zygotic embryo until the heart stage (Hecht et al. 2001, Kwaaitaal et al. 2005). The C-terminal fusion of AtSERK1 with variants of the green fluorescent protein (GFP) was located in developing anthers and it confirmed the absence of *AtSERK1* expression in the integumental cell layers surrounding the embryo sac prior and around the time of fertilization (Kwaaitaal et al. 2005). Whereas in the facultative apomictic species *Hieracium*, *AtSERK1* gene was expressed in both sexual and apomictic ovules (Tucker et al. 2003), a close *SERK1* homolog was found differentially expressed in sexual and apomictic plants of *Poa pratensis* (Albertini et al. 2005). This suggested that the

signaling mediated by the AtSERK1 receptors is employed in both modes of reproduction and is an example of the molecular correspondence between both.

According to the observed ability of *AtSERK1* to increase embryogenic potential in culture we ectopically expressed the gene in *Arabidopsis* under the control of two different promoters. The *Arabidopsis thaliana* LIPID TRANSFER PROTEIN1 (*AtLTP1*) promoter (Thoma et al. 1994, Vroemen et al. 1996) was used to express the *AtSERK1* gene in epidermal layers of the integument cells. The *Arabidopsis thaliana* DISRUPTED MEIOTIC cDNA1 (*AtDMC1*) (Klimyuk and Jones 1997, Doutriaux et al. 1989) promoter was used in order to provide additional receptors in the female gametophyte.

Introducing elements of apomixis in sexual species and also understanding the different progeny types among the seedlings produced by facultative apomicts creates a strong need for a robust tool to screen for rare apomictic events. Until now, apomicts were identified through morphological scoring, chromosome counting, or progeny cross methods, which were tedious and very time consuming. In *Hieracium*, a method based on the inheritance of positive and negative selectable transgenic markers have been described (Bicknell et al. 2003). This method, based on the survival of different progeny types when placed on selective media, is however, too limited for plants that can be transformed genetically. Flow cytometric test to individual seeds has been shown to be particularly useful for the detection of apomicts from gametophytic origin (Matzk et al. 2000, 2001), but will not be useful to detect embryos that arose by adventitious embryony or parthenogenesis. The auxin test is commonly used to detect parthenogenesis of unfertilized egg cells in response to auxin in various *Poaceae* species (Matzk 1991a, 1991b).

Here we present a novel screening system for apomixis types that circumvent meiotic recombination, *e.g.*, adventitious embryony, mitotic diplospory or apospory. The method is based on heterozygosity fixation of Single Sequence Length Polymorphism (SSLP) markers. In *Arabidopsis* more that 50 SSLP markers have been described and they are routinely used as co-dominant genetic markers. SSLP detect a high level of allelic variation and they can be easily assessable by PCR (Bell and Ecker 1994, Ponce et al. 1999). The SSLP heterozygosity screen is based on the assumption that an apomictic progeny is an exact genetic replica of the mother plant. This method is essential for determining the role of any candidate apomixis gene in eventually conferring aspects of apomixis into sexual crop species.

Material and methods

Plant material

Arabidopsis accessions Wassilewskija (ws) and Landsberg *erecta* (L*er*) were used. Seeds were germinated directly in soil or were first sterilized and then germinated on one-half-strength Murashige and Skoog (½ MS) salt medium (Murashige and Skoog 1962, DUCHEFA) supple-

mented with 1% (w/v) sucrose and if desired 50mg/L kanamycin. The *Arabidopsis* seedlings were grown in soil at 22°C, with 16-h-light/ 8-h-dark periods. Rosette leaves from three-week old plants were collected for DNA extraction.

AtSERK1 ectopic expression constructs and transformation of *Arabidopsis*

The *AtLTP1* promoter region of 1.2kb and the *AtDMC1* promoter of 3.1kb were released from the Bluescript vectors pMT121 (Toonen et al. 1997) and pBSDMC1 (Klimyuk and Jones 1997) by digestion with *Hind*III/*BamH*I and *Bgl*II/*Hind*III respectively, filled in with Klenow and replaced the *Hinc*II/*Sma*I digested 35S promoter in the pRT105 vector (Hecht et al. 2001). The *AtLTP::AtSERK1::pA* and *AtDMC1::AtSERK1::pA* fusions were PCR amplified with primers RT1(TCCCCCGGGGGAAGCTTGCATGCCTG) and RT2 (TCCCCCGGGGGACTGGATTTTGGTT) designed on the pRT105 vector and containing SmaI restriction sites. The PCR fragments were then subcloned into a SmaI site of pMOG800 binary vector (Toonen et al. 1997). The constructs were verified by sequencing and used to transform *Arabidopsis* ecotype WS plants by a floral deep technique as described by Hecht et al. (2001). Nine *Arabidopsis* WS plants were used for each transformation experiments. T_1 seeds were selected on ½ MS media containing kanamycin for 10 days and the kanamycin-resistant seedlings were transferred in soil to generate the T_2 and T_3 independent lines.

Table 6-1. Sequences, fluorochrome labeling and concentrations of 14 SSLP primer sets used in multiplex PCR for heterozygous fixation analysis. Fluor. = fluorochrome; Conc. = concentration (µM).

SSLP	Fluor.	Forward primers	Reverse primers	Conc.
NGA111	TET	TGTTTTTTAGGACAAATGGCG	CTCCAGTTGGAAGCTAAAGGG	0.1
AthACS	TET	AGAAGTTTAGAAACAGGTAC	AAATGTGCAAATTGCCTTC	0.2
AthGENEA	TET	ACATAACCACAAATAGGGGTGC	ACCATGCATAGCTTAAACTTCTTG	0.1
NGA1145	TET	GCACATACCCACAACCAGAA	CCTTCACATCCAAAACCCAC	0.3
NGA1126	6FAM	GCACAGTCCAAGTCACAACC	CGCTACGCTTTTCGGTAAAG	0.1
NGA361	6FAM	ACATATCAATATATTAAAGTAGC	AAAGAGATGAGAATTTGGAC	0.3
ATHCHIB	HEX	ATGAGAAGCTATAATTTTTTCAATA	CTCATATATACAAAGAACTACTATAC	0.3
NGA1111	6FAM	AGTTCCAGATTGAGCTTTGAGC	GGGTTCGGTTACAATCGTGT	0.1
NGA1139	6FAM	TTTTTCCTTGTGTTGCATTCC	TAGCCGGATGAGTTGGTACC	0.2
NGA12	6FAM	TGATGCTCTCTGAAACAAGAGC	AATGTTGTCCTCCCCTCCTC	0.2
ATHPHYC	HEX	CTCAGAGAATTCCCAGAAAAATCT	AAACTCGAGAGTTTTGTCTAGATC	0.2
AthCTR1	HEX	CCACTTGTTTCTCTCTCTAG	TATCAACAGAAACGCACCGAG	0.1
MBK-5	HEX	GAGCATTTCACAGAGACG	ATCACTGTTGTTTACCATTA	0.1
NGA151	TET	CAGTCTAAAAGCGAGAGTATGATG	GTTTTGGGAAGTTTTGCTGG	0.2

RT-PCR analysis

Total RNA was extracted from either flowers or 7d old seedlings using the Trizol procedure (Invitrogen, CA). For every extraction we used 50 to 100 mg frozen plant material that was homogenized in 1 mL of Trizol solution for 10 sec. The mixture was then centrifuged at 1,300 rpm for 10 min at 4°C. RNA was precipitated with isopropanol after a single chloroform extraction. DNase treatment, reverse transcription, and PCR reaction were performed as described by Hecht et al. (2001). For semi-quantitative RT-PCR analysis we used serial dilutions of cDNA and collected PCR products after 28 cycles for the internal control, cyclophilin *ROC5* cDNA and after 31 cycles for the *AtSERK1* cDNA. The primers used for amplification were described in Hecht et al. (2001). The amplified PCR products were separated on 1.5% (w/v) agarose gel, transferred by alkali blotting on HybondN+ membrane and hybridized with the corresponding probes (Hecht et al. 2001). Hybridization signals were detected with a Storm 840 PhosphorImager and quantified using ImageQuant software (Molecular Dynamics). The *AtSERK1* expression levels were calculated after normalization relative to the *ROC5* expression.

Generation of the F_2 and F_3 apomictic populations

Individual T_3 plants homozygous for each *AtLTP::AtSERK1* and *AtDMC1::AtSERK1* transgene were used as a male donor in outcrosses to the L*er* ecotype to generate the respective F_1 populations. F_1 plants were selfed and flower buds at stage 11 to 12 (Smith et al. 1990), approximately 1 to 2d pre-anthesis, were dipped or sprayed in aqueous solution containing 2 μM 2,4-dichlorophenoxyacetic acid (2,4-D), supplemented with 0.04% (v/v) Triton-X100 as a surfactant as described by Vivian-Smith and Koltunow (1999). The treatment was repeated twice in two-day period and flower material was collected for RNA isolation. The 2,4-D treatment was performed to increase the chance of adventitious embryo development. The resulting F_2 populations were used for the SSLP screen. Selected individual F_2 plants were selfed and treated with 2,4-D to generate the F_3 progeny. Control experiments were carried out with F_1 plants generated by the cross between the WS and L*er Arabidopsis*. Those crosses were treated exactly as the transgenic plants and were also used for SSLP analysis.

DNA isolation, SSLP multiplex PCR, gel electrophoresis and analysis of the PCR products

Genomic DNA was isolated as described by Ponce et al. (1999). Fourteen SSLP primer pairs that are able to amplify DNA polymorphism between Ws and Ler *Arabidopsis* accessions were chosen for the SSLP analysis (Ponce *et al.* 1999). Primer sequence, labeling, and concentration used for SSLP analyses are shown in Table 6-1. PCR amplification was performed in 20 μL of reaction mixture containing 1.5 μL DNA (approximately 20 ng), 200 μM of each nucleotide, 2 μL of 10x PCR buffer, 1 mM MgCl2, 0.25 U of home made Taq enzyme and

15-45 nM SSLP primers as detailed in Table 6-1. The PCR amplification conditions were as described by Ponce et al. (1999). Electrophoresis was carried out in an ABI PRISM 377 DNA sequencer with 6% acrylamide gel. GeneScan-500 (TAMRA) was used as size standard. The samples were run in the GS36C-2400 module for 2½ hours. DNA fragment analysis was performed using GeneScan 2.1 software (Applied Biosystems). The pattern was analyzed for accession-specific polymorphic bands by using Genotyper software (Applied Biosystems).

Statistical analysis

All F2 and F3 families were tested against this theoretical distribution using the non-parametric distribution fit tool in Statistica 6.0 (StatSoft, Inc.) based on the Kolmogorov-Smirnov one-sample test.

Histological and GUS analysis

Whole-mount clearings were performed as described by Vielle-Calzada et al. 1999. After dehydration in an acetone series, specimens were embedded in Technovit^R 8100 resin (Heraeus Kulzer GmbH). 7µm thick sections were cut from the plastic blocks using a Reichert-Jung 2035 Biocut microtome. The sections were stained with toluidine blue solution (10g/L toluidine blue, 10g/L sodium tetra borate) and distained with 50 % (v/v) ethanol. The sections were observed using a Nikon Optiphot-2 microscope. Pictures were taken using a Nikon Coolpix 990 digital camera. Transgenic seeds of the Rschew ecotype, caring an Arabidopsis thaliana lipid transfer protein (AtLTP1) promoter-ß-glucuronidase (GUS) fusion were described by Vroemen et al. (1996). The histochemical localization of AtLTP1 GUS activity in unfertilized carpels and ovules was done as described by Hecht et al. (2001). The GUS stained material was fixed, embedded in Technovit® 8100 resin and proceed as descried above.

Results

Ectopic expression of *AtSERK1* using the *AtLTP1* and *AtDMC1* promoters in *Arabidopsis*

In attempt to engineer elements of the sporophytic type of apomixis in the sexual *Arabidopsis*, transgenic plants in Ws background were made harboring cDNA sequences of the *AtSERK1* gene under control of *AtLTP1* and *AtDMC1* promoters. *AtDMC1* promoter was previously found active during ovule development in *Arabidopsis* (Klimyuk and Jones 1997, Doutriaux et al. 1989). The activity of the *AtLTP1* promoter during early ovule development however was not investigated. *In situ* hybridization experiments have shown that *AtLTP1* mRNA was present in very young flower buds (stages 2-4, Smith et al. 1990) and in the

epidermal cells of fully developed ovules (Thoma et al. 1994). We further investigated the exact promoter activity during ovule development by looking at the *AtLTP1* promoter GUS expression (Figure 6-1A). As shown in Figure 6-1A I-IV the *AtLTP1* promoter was active at early stage of ovule development. In young unfertilized carpels containing ovule protrusions at stage 1-II (Figure 6-1A-I), (Schneitz et al. 1995) the *AtLTP1* promoter was first on in the epidermal layer surrounding the chalaza. Later at stages 2-II (Figure 6-1A-II), 2-IV (Figure 6-1A-III) or 3-II (Figure 6-1A-V) the *AtLTP* expression became restricted to the nucellus and the developing integuments. In mature ovules the promoter remained active in the micropylar and in the chalazal end (Figure 6-1A-IV). Histological sections did not show any GUS staining in the embryo sac (Figure 6-1A-VI). It was concluded that the *AtLTP* expression is confined to the maternal tissues of the integuments and possibly the endothelium.

Homozygous *AtLTP1::AtSERK1* or *AtDMC1::AtSERK1* T_3 progeny carrying a single insertion of each transgene was selected based on Km segregation. The presence of the constructs was additionally confirmed by PCR amplification of the Km gene (data not shown). None of the transgenic plants showed any notable phenotypic changes and they all had normal seed formation.

To select *AtLTP1::AtSERK1* transgenic lines for further SSLP analysis we first determined the expression levels of the *AtSERK1* transgene by a semi-quantitative RT-PCR. Three independent *AtLTP1::AtSERK1* transgenic lines 4.14.1, 4.17.1 and 4.18.2 were selected and the levels of *AtSERK1* expression were compared with endogenous *AtSERK1* expression in seedlings and flower buds (Figure 6-1B and 6-1C). In general, the expression of the *AtSERK1* gene in flower buds was higher than in seedlings, confirming previous observations (Hecht et al. 2001). The *AtLTP1::AtSERK1* lines 4.17.1 and 4.18.2 respectively showed a higher and an intermediate *AtSERK1* expression in flower buds and in seedling when compared to the wild type. In line 4.14.1 the *AtSERK1* mRNA level was close to that of endogenous *AtSERK1* gene. The effect of 2,4-D treatment on the mRNA levels of the *AtSERK1* gene was also determined by RT-PCR in the wild type and in the transgenic plants (Figure 6-1B and 6-1C). 2,4-D treatment did not influence the *AtSERK1* mRNA level of expression suggesting that the gene is not transcriptionally regulated by the auxin. Individual T_3 *AtLTP1::AtSERK1* lines characterized with low (no. 4.14.1), intermediate (no. 4.18.2) and high (no. 4.17.1) *AtSERK1* expression were further used in the SSLP screen for apomixis.

Design of the SSLP screen for apomixis in the *AtSERK1* expressing plants

To determine whether in the *AtSERK1* expressing plants adventitious embryony had occurred, possibly as a rare even we devised a genotypic screen based on Simple Sequence Length Polymorphism (SSLP) markers that can distinguish between plants with maternal and sexual origin. In *Arabidopsis* more that 50 SSLP markers are described and they are routinely used as co-dominant genetic markers. SSLP detect a high level of allelic variation and they can be easily assessable by PCR (Bell and Ecker 1994, Ponce et al. 1999).

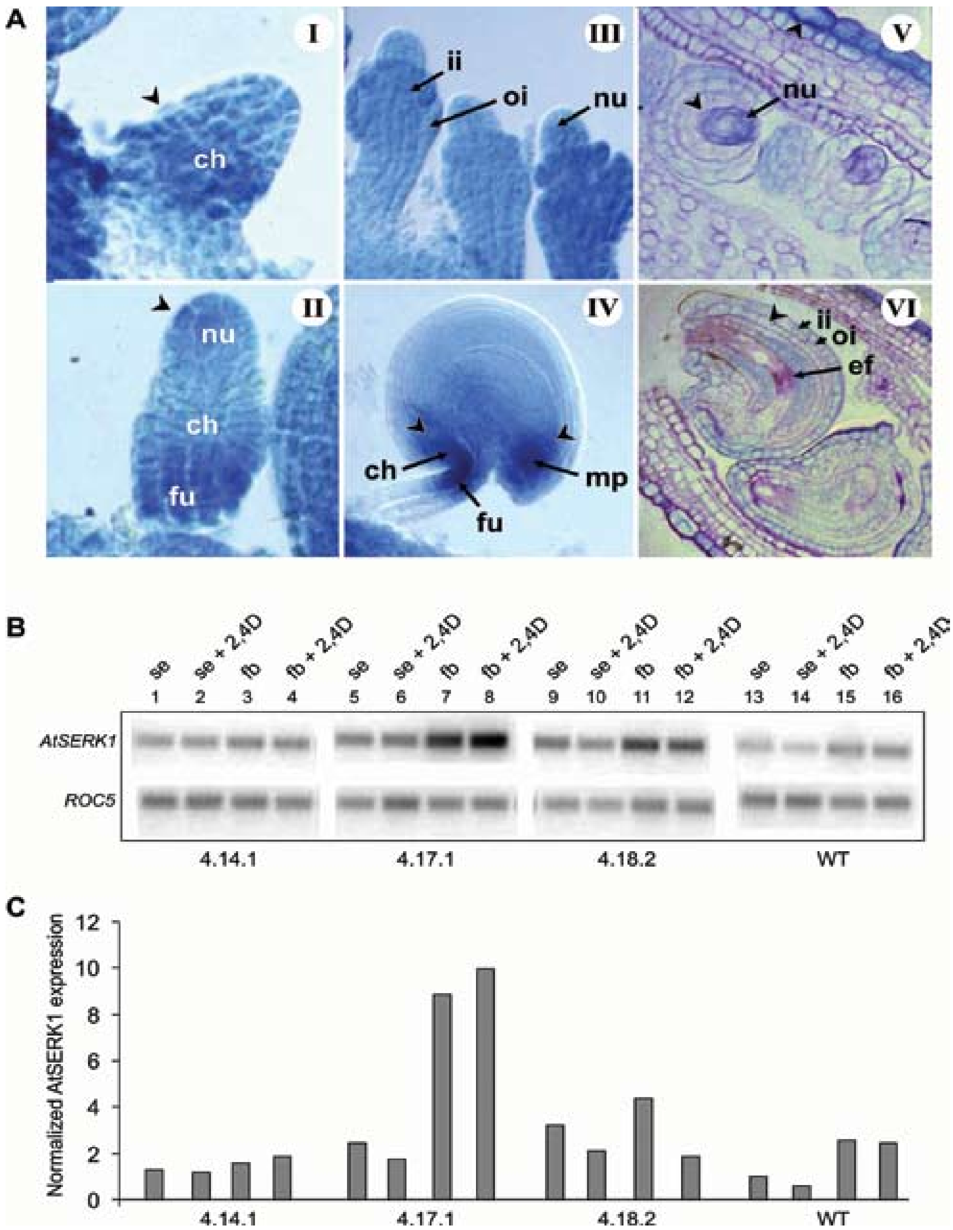

Figure 6-1. Ectopic expression of *AtSERK1* in *Arabidopsis*. A, *AtLTP::GUS* expression during ovule development in *Arabidopsis*. (A-I), ovules at stage 1-II; (A-II), ovules stage 2-II; (A-III), ovules at stage 2-IV; (A-V), ovule at stage 3-II according to Schneitz et al. 1995. (A-IV and A-VI), mature ovules. ch, chalaza; fu, funiculus; nu, nucellus; oi, outer integument; ii, inner integument; mp, micropyle; et, endothelium. (B-C) *AtSERK1* expression in seedling and flower buds of the transgenic and wild type plants with and without 2,4-D treatment by semiquantitative RT-PCR. B, *AtSERK1* expression at 30 cycles and ROC5 expression at 28 cycles. C, Relative expression of *AtSERK1*. se, seedlings; fb, flower buds.

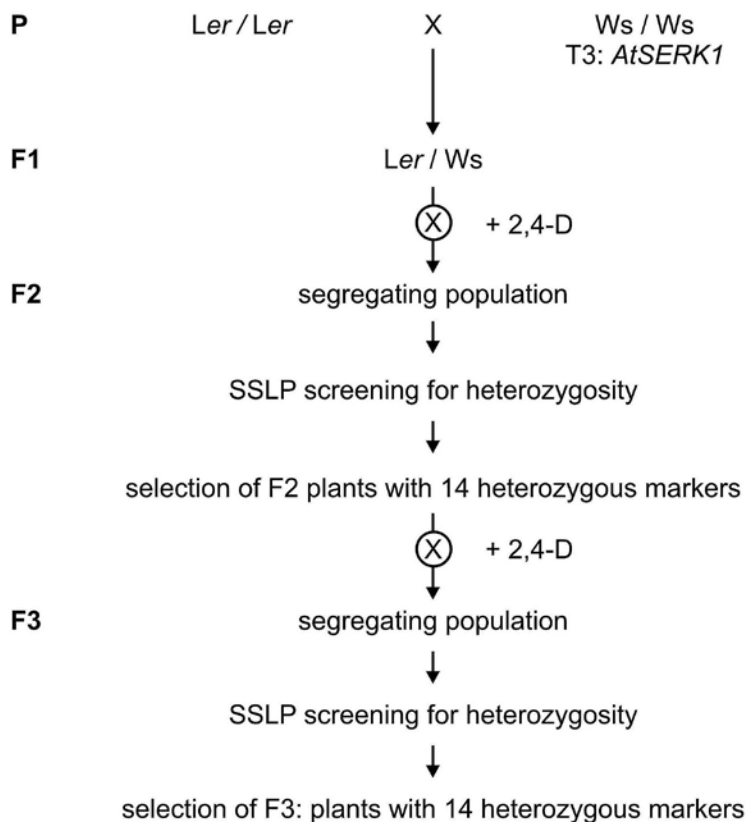

Figure 6-2. Generation of F_1, F_2 and F_3 populations containing the *AtSERK1* transgene. The *AtDMC1::AtSERK1* and *AtLTP1::AtSERK1* homozygous plants in WS ecotype were crossed with L*er* wild type to generate the heterozygous hybrid (F_1).Self-crosses of F_1 were performed to produce segregating populations for SSLP analysis detecting the hybrid with heterozygous fixation from their parents, presuming apomictic progeny. The next generations of the progeny were produced by self-crosses for investigating the apomictic inheritance.

The complete procedure for generating F_1, F_2 and F_3 transgenic generations is summarized in Figure 6-2. Individual T_3 plants homozygous for the *AtLTP1::AtSERK1* (lines no. 4.14.1; 4.18.2; and 4.17.1) and *AtDMC1::AtSERK1* (line no. 5.7.1) transgenes were used as a male donor in outcrosses to the L*er* accession. The respective F_1 populations were generated as follows: L*er* X line no. 4.14.1 gave rise to F_1-44; L*er* X line no. 4.18.2 gave rise to F_1-15, L*er* X line no. 4.17.1 gave rise to F_1-45, and L*er* X line no. 5.7.1 gave rise to F_1-33. Due to limited amount of F_1 seeds the SSLP screen was performed on the F_2 generations obtained by the selfing of the receptive F_1 plants. The F_1 plants were treated with 2,4-D after fertilization to increase the chance adventitious embryony to occur. Additional genetic crosses were performed by using wild-type ws pollen to pollinate wild-type L*er* plants that yielded the

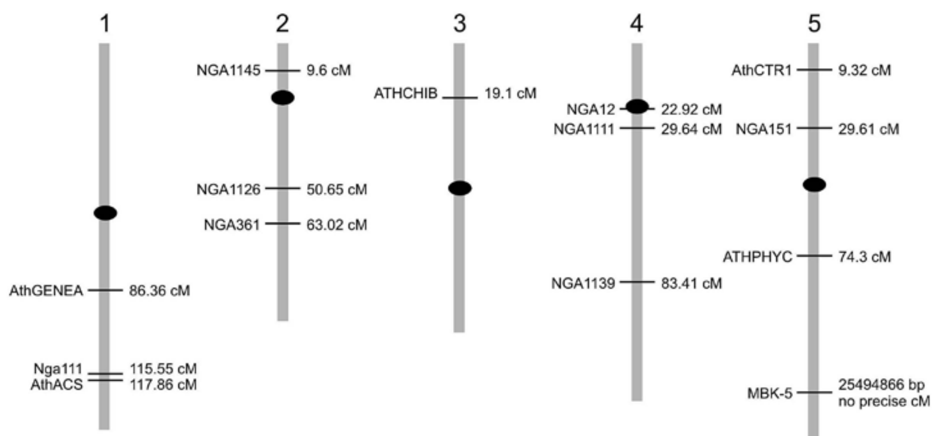

Figure 6-3. The chromosome map location of the 14 SSLP markers of *Arabidopsis thaliana* used for heterozygous fixation analysis.

control 'wild-type' F_1 plants. The control F_1 plants were selfed and two different F_2 control progenies were generated, one with and one without 2,4-D treatment. The control F_2 progenies were analyzed in identical to the transgenic plants way.

The fourteen SSLP that were polymorphic for ws and L*er*, homogeneously distributed throughout the *Arabidopsis* genome and unlinked (Figure 6-3) were used to genotype each of the F_1 plants and their F_2 progenies (Ponce et al. 1999). The SSLP profiles of all F_1 transgenic and control plants were identical, always amplifying the same 28 PCR products. They corresponded to the 14 SSLP alleles in L*er* and the 14 SSLP alleles in ws ecotypes that were present in all heterozygous F_1 plants (data not shown). F_2 plants from each transgenic experiment and from the wild-type control experiment were genotyped for all 14 SSLP markers. The SSLP profile of each F_2 plant was then compared with the SSLP profile of the corresponding F_1 mother plant. The self-cross of a sexual plant produces a segregating progeny. In the contrary, an apomict produces an offspring that is a maternal clone. Therefore, we examined the segregation pattern of each of the 14 SSLP in the F_2 transgenic populations to determine whether the plants have a maternal origin. Plants of a maternal origin will have an SSLP profile identical to the SSLP profile of the F_1 mother plant suggesting lack of recombination. The segregation of the SSLP markers in the F_2 and F_3 populations was first tested for normal disomic inheritance. We selected a set of fourteen markers that covered all five linkage groups (Figure 6-3). None of them showed tight linkage with its neighbor. On the basis of independent inheritance we assumed that the number of heterozygous loci followed a binomial distribution.

Table 6-2 The frequency of plants with 0-14 SSLP marker in control population (F2 Ler/ws) with and without 2,4-D treatment, F2 and F3 populations of AtDMC1::AtSERK1 (F2-33) and AtLTP1::AtSERK1 (F2-44, F2-45, F2-15, F3-15, no. 32 and F3-15, no. 141).

Number hetero-zygous SSLP	Populations															
	F2 Ler/ws		F2 Ler/ws + 2,4-D		F2-33 AtDMC1::At-SERK1		F2-44 AtLTP1:: AtSERK1		F2-45 AtLTP1:: AtSERK1		F2-15 AtLTP1:: AtSERK1		F3-15, no. 32 AtLTP1:: AtSERK1		F3-15, no. 141) AtLTP1:: AtSERK1	
	obs	exp	obs	exp	obs	exp	obs	exp	obs	exp	obs	exp	obs	exp	obs	exp
0	0	0.01	0	0.01	1	0.01	0	0.00	0	0.00	0	0.01	0	0.00	0	0.00
1	2	0.17	3	0.10	2	0.09	0	0.08	0	0.78	0	0.97	0	0.07	0	0.08
2	6	1.08	4	0.65	1	0.60	0	0.54	0	0.51	0	0.63	2	0.46	0	0.52
3	6	4.35	7	2.62	3	2.40	1	2.17	1	2.04	2	2.53	1	1.84	4	2.09
4	18	11.97	11	7.21	9	6.59	7	5.99	4	5.62	8	6.96	4	5.07	10	5.74
5	18	23.95	13	14.42	17	13.20	12	11.20	14	11.24	10	13.93	13	10.14	8	11.48
6	38	35.92	12	21.63	14	19.80	15	17.97	14	16.86	12	20.90	14	15.21	14	17.23
7	39	41.05	20	24.72	18	22.62	22	20.52	18	19.27	17	23.88	14	17.38	18	19.70
8	35	35.92	15	21.63	17	19.80	16	17.97	15	16.86	26	20.90	15	15.21	14	17.23
9	14	23.95	13	14.42	10	13.20	12	11.20	15	11.24	18	13.39	9	10.14	15	11.48
10	14	11.97	13	7.21	6	6.60	9	6.00	4	5.62	9	6.96	6	5.07	6	5.74
11	3	4.35	5	2.62	0	2.40	0	2.18	5	2.04	5	2.53	2	1.84	4	2.09
12	3	1.08	1	0.62	2	0.60	3	0.54	1	0.51	4	0.63	2	0.46	1	0.52
13	0	0.17	1	0.10	2	0.10	1	0.08	1	0.78	1	0.097	1	0.07	0	0.08
14	0	0.01	0	0.00	0	0.00	0	0.00	0	0.00	2	0.007	0	0.00	0	0.00

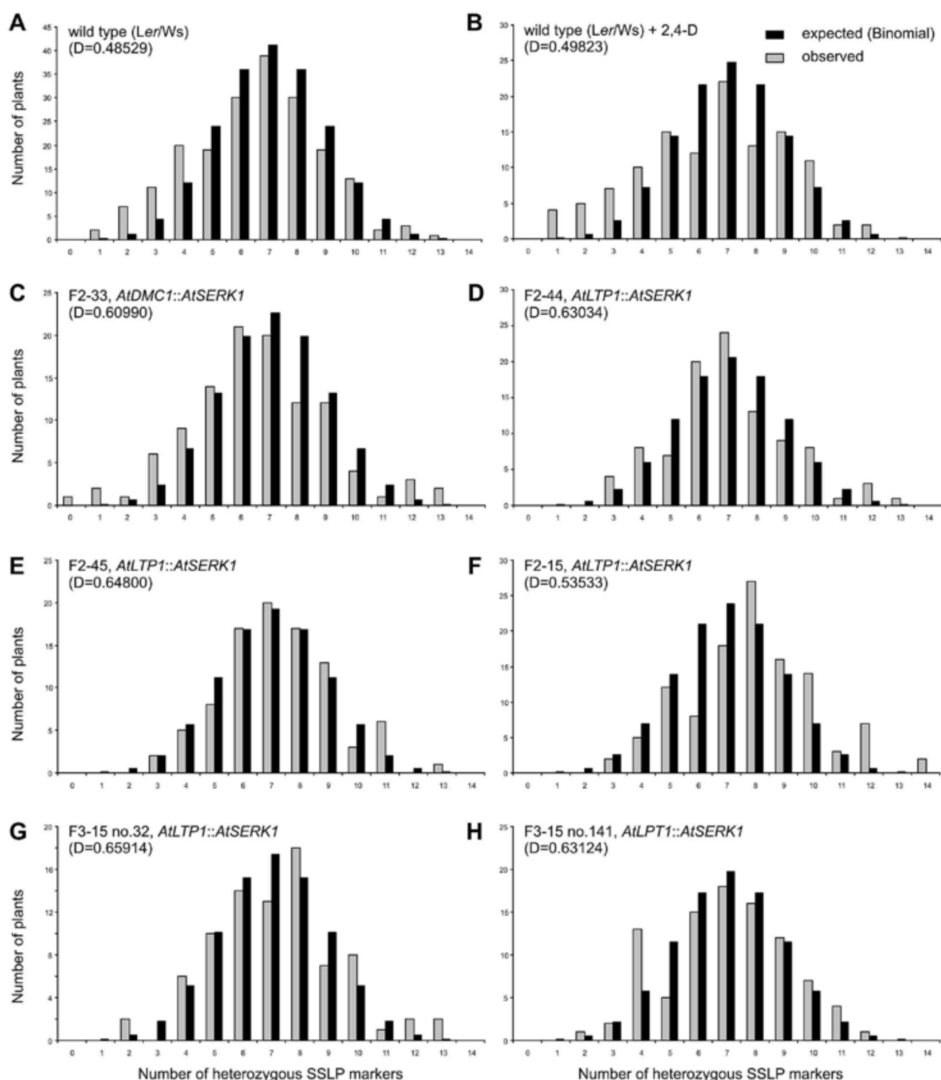

Figure 6-4. The segregation of plants with 0-14 heterozygous SSLP markers of observed (grey bars) and expected (black bars) numbers in (A-B), control populations with and without 2,4-D treatment; (C), F_2-33 *AtDMC1::AtSERK1*; (D), F_2-44 *AtLTP1::AtSERK1*; (E), F_2-45 *AtLTP1::AtSERK1*; (F), F_2-15 *AtLTP1::AtSERK1*;(G), F_3-15 no. 32 *AtLTP1::AtSERK1*and (H), F_3-15 no. 141 *AtLTP1::AtSERK1*. The values between brackets are the D statistics of the Kolmogorov-Smirnov test. All values were significant at $P<0.01$.

Segregation of the SSLP markers in the control wild-type F_2 population

In Table 6-2 and Figure 6-4A we show the distributions of heterozygous loci number of 196 F_2 plants derived from the wild-type L*er*/ws cross comprising all fourteen SSLP markers. Table 6-3 shows a notable aberration of heterozygote frequency for the markers nga111,

Table 6-3. Frequencies of heterozygosity for the individual SSLP markers. Cases of extreme values (<0.4 and > 0.6) are denoted in bold.

	F_2 L*er*/ws	F_2 L*er*/*ws* +2,4-D	F_2-33 AtDMC1:: AtSERK1	F_2-44 *AtLTP1:: AtSERK1*	F_2-45 *AtLTP1:: AtSERK1*	F_2-15 AtLTP1:: AtSERK1	F_3-15 *AtLTP1::At-SERK1* no. 32	F_3-15 *AtLTP1::At-SERK1* no. 141	Average	Variance
AthGENEA	0.536	0.466	0.509	0.582	0.576	**0.711**	**0.699**	**0.660**	0.592	0.008
nga111	**0.607**	0.551	0.564	0.520	0.576	0.473	0.470	0.447	0.526	0.003
AthACS	**0.352**	0.280	**0.278**	0.439	**0.391**	0.465	**0.205**	0.521	0.366	0.012
nga1145	0.500	0.542	0.481	0.510	0.576	0.509	0.590	0.458	0.521	0.002
nga1126	0.505	0.534	0.491	0.510	0.402	0.561	0.470	0.479	0.494	0.002
ga361	0.520	0.500	0.519	0.448	**0.359**	0.543	0.590	0.532	0.502	0.005
ATHCHIB	0.400	**0.398**	0.520	**0.602**	0.543	0.482	0.518	0.543	0.501	0.005
nga12	0.464	0.542	**0.602**	0.449	0.478	**0.623**	0.494	0.457	0.514	0.005
nga1111	0.418	0.491	0.463	0.490	0.565	**0.658**	0.494	0.553	0.517	0.005
nga1139	**0.378**	0.500	0.500	0.531	**0.641**	0.597	0.518	0.511	0.522	0.005
AthCTR1	0.510	0.483	0.417	0.469	0.491	**0.684**	0.542	0.447	0.505	0.007
nga151	0.444	0.432	0.426	0.571	0.565	0.465	**0.361**	**0.383**	0.456	0.006
AthPHYC	0.597	0.576	0.417	0.581	0.587	0.526	0.578	0.532	0.549	0.004
MBK-5	0.403	0.483	0.491	0.540	0.576	0.447	0.578	0.553	0.510	0.004
Average	0.474	0.484	0.477	0.517	0.523	0.553	0.508	0.505	0.505	0.004

AthACS, both on chromosome 1, and nga1139 on chromosome 4, with an average of 0.474 for all markers (expected is 0.5). This shift is also clearly visible in the histogram comparing observed and expected (binomial) values and was significant according to the used goodness-of-fit statistics at $P<0.01$ (Figure 6-4A). We did not find any plants heterozygous for all SSLP markers (Table 6-2). These data confirm that the wild-type *Arabidopsis* plants reproduce sexually.

We then investigated whether auxin treatment would affect the segregation of the SSLP markers in the wild- type population as stimulation of somatic embryogenesis in culture required 2,4-D application (Hecht et al. 2001). Also here we observed a comparable decrease of heterozygotes for AthACS and ATHCHIB (Table 6-3), The segregation of the SSLP markers in the F_2 population generated after 2,4-D application is shown in Table 6-3, Figure 6-4B. The results were essentially similar to the untreated control experiment, with also a slightly lower frequency of heterozygotes (0.484), and an overall significant difference for all heterozgote number classes from the expected values (Figure 6-4B). These observations suggested that a short application of auxin does not affect the segregation of the SSLP markers and by itself is not sufficient to initiate adventitious embryony in *Arabidopsis*.

Segregation of 14 SSLP markers in the *AtDMC1::AtSERK1* F_2 population

The SSLP screen of the F_2-33 progenies of *AtDMC1::AtSERK1* transgenic plants did not identify plants containing 14 heterozygous SSLP markers (Table 6-2, Figure 6-4C). However, in this population we found plants heterozygous for twelve and thirteen SSLP markers in a higher frequency then the wild-type population. In some cases the distribution of the number of heterozygotes differed significantly from the theoretical binomial distribution (Figure 6-4C). Table 6-3 also shows that extreme values for heterozygosity frequencies in this population were aberrant for the SSLP markers AthACS and nga12.

Segregation of 14 SSLP markers in the *AtLTP1::AtSERK1* F_2 and F_3 populations

F_2 populations derived from three independent transgenic lines showing low, intermediate and high *AtSERK1* expression levels (no. 4.14.1 (F_2-44), no. 4.18.2 (F_2-15) and no. 4.17.1 (F_2-45) respectively, were analyzed. The observed segregation of the 1 to 14 SSLP markers in the three transgenic populations (Figure 6-4D-F) was significantly different ($P<0.01$) from the expected binomial distribution. In the F_2 progenies of the lines with low and intermediate *AtSERK1* expression levels, 4.14.1 and 4.18.2 respectively, we observed two plants heterozygous for all 14 SSLP markers. The probability for finding two such plants on the basis of random Mendelian segregation would be 0.007 % (Table 6-2, Figure 6-4D and 6-4E). In order to see if the same phenomenon will be transmitted to the next progeny we examined the SSLP segregation in the two F_3 populations derived from these two plants (F_3-15 no. 32

and F_3-15 no. 141). The SSLP analysis of the F_3 populations showed results similar to the wild-type (Table 6-2, Figure 6-4G and 6-4H). This suggested that the phenomenom of heterozygousity fixation was not transmittable to the next generation.

Discussion

Ectopic expression of genes that influence the potential of plant cells to undergo embryogenesis in somatic cells might induce a particular apomictic component such as parthenogenesis in sexual species. In this context an apomictic embryo induction in *Arabidopsis* was employed through ectopic expression of *AtSERK1* gene under the control of female gametophyte promoters. Overexpression of *AtSERK1* using the *35S* constitutive promoter increased the embryogenic potential of cells in culture. *In planta AtSERK1* was expressed in regions of the developing ovules, including the egg cell, where it might lead to initiation of embryogenesis by the appropriate signals (Hecht et al. 2001). Expressing *AtSERK1* under the meiosis-specific, *AtDMC1* (Klimyuk and Jones 1997) and the protoderm-specific, *AtLTP1* (Thoma et al. 1994) promoters aimed to initiate adventitious embryony from the maternal tissues in ovules representing either the nucellus or the integuments. Endosperm development is required for embryo survival and thus the *AtSERK1* gene was introduced in sexual *Arabidopsis* plants. Initiation of adventitious embryony in a sexual background could be a rare even depending on successful fertilization of the adjacent reduced embryo sac and on the ability of the adventitious embryo to grow sufficiently to gain access to nutrient endosperm. This requested a reliable screening system that can differentiate between apomictic and the sexual mode of reproduction in the progeny of the transgenic plants. So far, diverse methods for apomictic screening have been reported including flow cytometry analysis (Matzk et al. 2001), transgenic approach (Bicknell et al. 2003), auxin induction (Matzk 1991a, 1991b) and morphological analysis (Koltunow et al. 1993). Those methods however are either time consuming or they are not suitable to detect the sporophytic mode of apomixis. In this study we developed and applied an SSLP screen to genetically identify individuals displaying a heterozygosity fixation as an indication for apomixis. The screen is based on assumption that seedlings with an apomictic origin will retain the genotype of the maternal parent. *AtSERK1* transgenic WS ecotype *Arabidopsis* plants were crossed with L*er* ecotype to produce heterozygous F_1 plants. Auxin application on flower buds of the F_1 plants was necessary in order to initiate embryo development as in culture. The SSLP based genotypic screen was performed on both wild type and segregating F_2 progenies deriving from *AtDMC1::AtSERK1* and *AtLTP::AtSERK1* transgenic lines. As such the distribution pattern of plants heterozygous for 0 to 14 unlinked SSLP markers was determined. In general the observed distributions of the heterozygous markers in the transgenic populations followed the wild type distribution. However in different F_2 populations some heterozygous distributions significantly differ from the expected theoretical binomial distribution with

different deviant peaks up and down (Figure 6-4) resulting in a shift in the heterozygous loci distribution. A possible explanation could be the occurrence of "an outbreeding depression". That will suggests that alleles or the chromosome segments that mark their positions, originated from the same accession may collaborate better than when obtained from different ecotypes (coadaptation).

The heterozygosity frequency on an individual SSLP marker showed extreme values in all populations to different extent, but only AthACS was found consistently aberrant in most of the populations. Plants heterozygous for 14 SSLP markers were found in the population of the *AtLTP::AtSERK1* transgene. The theoretical probability that such plants will occur by chance in a sexual population is negligible. Although it is unlikely that those plants resulted from a random meiotic recombination and segregation events we cannot exclude the possibility that a meiocyte can form chiasmata distal of each SSLP marker. To account for those recombination events increasing the number of SSLP markers is required.

The transgene effect was examined in terms of its expression level by RT-PCR. The *AtSERK1* expression was increased in the line where plants heterozygous for 14 SSLP markers were identified. However the highest overexpression line, no. 4.17.1 did not produce putative apomictic progeny. Thus in this case the potential of *AtSERK1* to induce parthenogenesis did not correlate with high expression levels. Both putative apomictic plants were explored for whether they will retain complete heterozygosity in the next generation. However none of the F_3 populations analyzed show plants with 14 heterozygous markers. The distribution of heterozygote frequency in these populations was not different from the respective F_2 population indicating that the influence of the transgene declined by generation. Although plants that retained heterozygosity were identified through the SSLP screen it is still not clear what their origin was, and whether they were produced through apomictic pathway or other means such as suppression of recombination and non-random anaphase I segregation of the half bivalents.

Chapter 7

General conclusions

This PhD thesis has pointed at several fascinating aspects of apomixis. Understanding of this complex trait requires a multidisciplinary study and must include morphological, developmental, genetical, chromosomal, and epigenetical approaches. In my thesis I have presented the outcome of the first molecular cytogenetic analysis of *Boechera holboellii* and a genetic analysis of *Arabidopsis thaliana* transformants, expressing *AtSERK1* in transgenic populations, the latter representing a first attempt towards producing elements of apomixis in a sexual background.

As to *Boechera*, the model is attractive for its impressive genomic plasticity, huge geographical distribution and an endless source of different accessions. Interestingly, both diploid and polyploid accessions can display apomixis. Apomicts possess aberrant chromosomes in their cells, which makes it interesting for segregation studies to see if this chromosome carries genes controlling apomixis. The low basic chromosome number, relatively small genome and its close relation to *Arabidopsis thaliana* make the species most useful for molecular cytogenetics and physical mapping studies. However, its basis for experimental research is still poor: there is no genetical map and the only BAC library (of the ES9 accession) yet available has still to be explored for genomics studies. In contrast, *A. thaliana* with its unprecedented leadership in experimental biology is unsurpassed in almost everything; however it is lacking natural sources of apomixis. In the following paragraphs I will focus on possible genetic experiments in the future bridging the power of both species.

Suggestions for future experiments

Nature of the aberrant chromosomes: are they the key to the apomixis region?

The presence of the aberrant chromosome(s) seems strongly correlated with the apomixis trait. However, direct evidence for apomixis genes or factors on these chromosomes still has not been presented. In Chapter 3 I have shown that meiosis in the apomictic *Boechera* accession BH1 resembles very much that of a diploid, with normal pairing, recombination and segregation, and production of unreduced gametes. Further meiotic studies of more accessions should reveal a larger number of similar genotypes with similar cytogenetic characteristics. Genome painting experiments together with chromosome-specific markers can then reveal which chromosomes are involved and how chromosomes segregate to the offspring. Such accessions are therefore potentially interesting pollinators in crossings with a sexual diploid to establish the segregation of the aberrant chromosomes to the offspring. In a later phase also the fate of the aberrant chromosomes in other meiotic types (as in the accessions GRL2, BH74, BH115, BH224, BDi175 and Es9) can be included. In a more comprehensive study, cytogenetic and genetic markers and genome painting for each chromosome can further demonstrate which parts of the genome from *Boechera holboellii* and *B. stricta* are required for the expression of the apomixis trait. A set of BACs from *Arabidopsis* chromosomes will not only reveal the genomic relationship between the two species, but can also be used as

chromosome markers for painting specific regions in *Boechera.* The development of a full genetic map will soon be started and will complement the planned linkage studies. This part will be most useful to determine the number of loci in the *Boechera* apomicts.

The birth of novel apomicts: Can apomicts be generated from new interspecific hybrids?
Hybridizations between *B. holboellii* and *B. stricta* are important, but the production of viable interspecific alloploid hybrids will be time consuming and difficult as most attempts so far produced mostly sterile progeny. A selection of 2n producing sexuals (*B. stricta* BS2 was found to produce 2n pollen at low frequency) can be useful for the ability of allotriploid hybrids. Such hybrids can be analyzed with the cytogenetic tools as described in this thesis, in order to establish genomic constitution, gamete production and the development of apomictic elements. As was discussed in chapter 5, synthetic alloploid hybrids are also more and more important to test the hypothesis that specific genes change their expression profiles under the influence of genomic stress. Such studies carried out at new interspecific hybrids and their consecutive offspring generations may possibly the birth of a new facultative apomict, and the formation of a new pro-*Het* chromosome.

Seeking for candidate genes in this research is not entirely from scratch and may be derived from different sources. Firstly, genetic analysis and DNA sequencing of apomictic regions of several apomictic models have already shown a number of putative genes involved in meiotic non-reduction (Consiglio et al., 2004) and apomixis (Spillane et al., 2001; 2004), and likely more genes will follow. Secondly, microarray studies, which have been carried out in the research group of Dr. Boutilier (Wageningen University and Research Centre) comparing apomictic and sexual ovule development have produced interesting clones which can be studied in further detail on spatial expression pattern and function.

Further exploration of candidate apomixis genes in Arabidopsis
My study on genotypic screening in the *AtSERK1* transformant offspring has not resulted in potential apomicts. However, after further refinement and automation, this system is capable of identifying a small number of plants exhibiting heterozygosity fixation in an otherwise fully sexual population. Ultimately, if genes that show the potential to confer apomixis need to be introduced in crop species one will need such screening systems operative in the normal sexual background of the crop. Future experiments of *AtSERK1* may also focus on target genes under control of AtSERK1 –mediated signalling or in combination with other genes involved in somatic embryogenesis.

Chapter 8

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Summary

Some plants have gained the ability to produce seed without fertilisation. This alternative to sexual reproduction, known as apomixis occurs most frequently in species of the families of the grasses, roses and composites, and mostly in polyploids and is considered one of the ways to escape from hybrid sterility. An impressive number of apomictic mechanisms have so far been described; most of them with different developmental modes of embryo and endosperm. The trait is potential very promising for producing uniform seeds at high efficiency and low cost, and belongs therefore since long as one to the most wanted grails of the plant breeding community. This PhD thesis points at several fascinating genetical and chromosomal aspects of apomixis using the model species *Arabidopsis thaliana* and the apomicts of the closely related *Boechera holboellii* complex. Focus is on two different studies in apomixis research: 1) unravelling chromosome organisation and genomic composition of some of the natural diploid apomicts of Boechera and 2) genetic analysis of *AtSERK*1 transformed *Arabidopsis* for engineering apomictic elements in this sexually propagating diploid.

Boechera's unsurpassed suitability for research of natural apomicts is its impressive genomic plasticity, large geographical distribution and an endless source of different accessions. Quite exceptional is that apomixis also can occur at the diploid level. The chromosome study involving the two sexual diploid species, *B. holboellii* and *B. stricta* (2n=14), and six apomictic accessions of *B. holboellii* and *B.* X *divaricarpa* (2n=14 and 15) demonstrated obvious differences between the sexual and apomictic taxa. DAPI fluorescence and FISH with rDNA probes on mitotic cell spreads clearly uncovered two different aberrant chromosome, including a largely heterochromatic chromosome (*Het*) and one much smaller chromosome (*Del*). Moreover, striking variation between the assumed homologues and great differences between the chromosome portraits of the accessions was observed, suggesting structural karyotype heterozygosity in this material. Additional studies on male meiosis of the same sexual diploids and apomicts demonstrated a plethora of meiotic variants, pollen size, seed germination and nuclear contents of the seeds. Some of the apomictic accessions displayed full chromosome pairing and recombination, and reductional meiosis, whereas others demonstrated pairing failure, and skewed chromosome segregations. The course of meiosis and pollen size indicated that both n and 2n gametes were formed. In three accessions we observed chiasmate bonds between the *Het* chromosome and one of the autosomes; whereas the *Del* chromosome was associated with two other chromosomes.

Genome *in situ* hybridization was carried out to establish the assumed hybrid composition of the apomictic *B. holboellii* and *B.* X *divaricarpa* accessions. In the first part we performed hybridisations with total genomic DNA of the diploid sexual *B. stricta* as probe and blocked with genomic DNA from *B. holboellii*. Chromosome complements showed fluorescent signals on the pericentromere regions of only the *B. stricta* chromosomes so that the parental chromosomes could be distinguished in the hybrid. An additional two-colour ge-

nome painting was developed with the simultaneous hybridisation with both *B. stricta* and *B. holboellii* probes and blocking with total genomic DNA of *Arabidopsis thaliana* in order to further improve the discrimination of the parental chromosomes. This genome painting study confirmed that chromosomes have evolved repeat differentiation at the pericentromere regions enabling a clear distinction of the *B. holboellii* and *B. stricta* chromosomes in the hybrids. This result has made clear that Boechera apomicts are alloploid, with different balanced or unbalanced combinations of *B. holboellii* and *B. stricta* chromosomes. Most likely such differences in genomic constitutions result from recurrent diploid-polyploid and polyploid-diploid conversions, the latter with reductional meiosis generating haploid gametes with novel combinations of homoeologous chromosomes.

This genome in situ hybridisation technique revealed that *B. holboellii* and *B. stricta* have undergone dramatic evolutionary changes in the repetitive sequences in the pericentromere regions of their chromosomes, producing species-specific FISH banding. The genome painting also revealed that the *Het* chromosome has *B. stricta* repeats suggesting that this highly heterochromatic chromosome originated from a *B. stricta* autosome, which shows that *Het* resembles more a Y chromosome than a B chromosome. The *Del* chromosome demonstrated only a small *stricta* segment, which at metaphase I was found associated with two *B. holboellii* chromosomes, thus suggesting that this chromosome is likely a *holboellii* / *stricta* translocation or recombinant chromosome. The outcome of this molecular cytogenetic study has enabled us to formulate a new model on the origin or apomictic accessions and evolutionary processes of the aberrant chromosomes. Essential in the hypothesis is the assumption of an epigenetic modification in the newborn (allo)polyploid hybrid. This modification is accompanied or resulted from chromosome pairing impairments at meiotic prophase, and in the longer term led to isolation from crossover recombination, accumulation of repetitive sequences and heterochromatinization of a part of that chromosome involved. Looking at only these few apomicts it is tempting to believe that the *Het* chromosome in all accessions originated from the same ancestral pro-*Het* chromosome and that this chromosome plays a key role in the genetic elements required for the apomictic pathway.

In the second part of the thesis a screening method is presented to evaluate the potential of genes to transfer aspects of apomixis into sexual crop plants. Based on the assumption that an apomictic progeny is an exact genetic replica of the mother plant we employed a set of Single Sequence Length Polymorphism (SSLP) markers to identify individuals displaying heterozygosity fixation in segregating sexual populations as an indication of rare apomictic events. Here we present the results of such a study using the *Arabidopsis thaliana* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (*AtSERK1*) gene expressed under the control of two different promoters: the AtLTP1 and the AtDMC1 in sexual *Arabidopsis* plants. In only one of the four tested F2 transgenic populations, *i.e.*, expressing the AtLTP1::AtSERK1 construct we observed two plants (1.8%) with heterozygosity maintenance for the full set of SSLP markers indicating a possible clonal inheritance. However, as their offspring revealed a close to binomial segregation for number of heterozygous loci, it was concluded that these

two putative apomictic plants resulted from either incidental recombination events displaying the genotype of the parent, or that they lost their clonal ability in the next generation. Although this genotype screening in the *AtSERK*1 transformant offspring not yet resulted in potential apomicts, further refinement and automation are expected to produce an engineered apomictic system that enables identification of a small number of plants exhibiting heterozygosity fixation in an otherwise fully sexual population. Ultimately, if genes that show the potential to confer apomixis need to be introduced in crop species one will need such screening systems operative in the normal sexual background of the crop. Future experiments of *AtSERK*1 may also focus on target genes under control of *AtSERK*1 –mediated signalling or in combination with other genes involved in somatic embryogenesis.

Samenvatting

Sommige planten hebben het vermogen ontwikkeld om zaad te produceren zonder bevruchting. Dit alternatief voor seksuele reproductie staat bekend als apomixie en komt vooral voor bij polyploïde grassen, rozen en composieten, en wordt beschouwd als een manier om te ontsnappen aan hybride-steriliteit. Een indrukwekkend aantal apomictische mechanismen is tot nu toe beschreven; en de meeste daarvan hebben verschillende manieren van ontwikkeling van het embryo en het endosperm. Apomixie is in potentie veelbelovend voor het efficiënt en goedkoop produceren van uniform zaad, en behoort daarom sinds lang als een van de grootste toekomstdromen van de plantenveredelaars. Dit proefschrift gaat over een aantal fascinerende genetische en chromosomale aspecten van apomixie, waarbij gebruik wordt gemaakt van het modelsoort *Arabidopsis thaliana* (zandraket) en de apomicten van het nauw verwante *Boechera holboellii* complex. Nadruk ligt op twee verschillende studies in het apomixie-onderzoek: 1) de ontrafeling van de chromosoomorganisatie en genoomsamenstelling van enkele natuurlijke diploïde apomicten van *Boechera* en 2) de genetische analyse van met *AtSERK* getransformeerde *Arabidopsis* voor het bewerkstellingen van apomictische elementen in deze seksueel voortplantende diploïd.

De onovertroffen geschiktheid van Boechera voor onderzoek aan natuurlijke apomicten komt vooral door zijn ondrukwekkende genoomplasticiteit, grote geografische verspreiding en schier eindeloze bron aan verschillende accessies. Nogal uitzonderlijk is dat apomictie bij deze soort ook op diploid niveau voorkomt. Het chromosoomonderzoek uitgevoerd aan de twee seksuele diploïde soorten *B. holboellii* en *B. stricta* (2n=14), en zes apomictische accessies van *B. holboellii* en *B. ×divaricarpa* (2n=14) toonde overduidelijke verschillen aan tussen de seksuele en apomictische taxa. DAPI fluorescentie en FISH met rDNA probes op mitotische celspreidpreparaten brachten twee verschillende afwijkende chromosomen aan het licht, een daarvan een groot heterochromatisch chromosoom (*Het*) en een veel kleiner chromosoom (*Del*). Verder werd opvallende variatie tussen de chromosoomportretten van de accessies aangetroffen, wat wijst op structurele karyotype heterozygotie in dit materiaal. Een aanvullende studie aan de mannelijke meiose van dezelfde seksuele en apomictische planten toonde een overvloed aan meiotische varianten, pollendiameter, zaadkieming en kerngroottes in de zaden. Sommige apomictische accessies lieten volledige chromosoomparing en recombinatie zien, terwijl andere accessies juist afwezigheid van paring en scheve chromosoomsegregaties vertoonden. Het verloop van de meiose en de grootte van de pollenkorrels toonden aan dat zowel n- als 2n-geslachtscellen werden gevormd. In drie accessies troffen we chiasmatische binden aan tussen het *Het* chromosoom en een van de autosomen, terwijl het *Del* chromosoom steeds verbonden was aan twee andere chromosomen.

Genomische *in situ* hybridisatie werd uitgevoerd om de vermeende hybride samenstelling van de apomictische *B. holboellii* en *B. ×divaricarpa* vast te stellen. In het eerste deel voerden we hybridisaties uit met totaal genomisch DNA van de seksuele *B. stricta* as probe, en geblokt met genomische DNA van *B. holboellii*. De chromosoomsets lieten zien dat fluo-

rescente signalen alleen voorkwamen in de pericentromeergebieden van de *B. stricta* chromosomen, waarmee *stricta* en *holboellii* chromosomen in de hybriden in de hybride konden worden onderscheiden. Een aanvullende twee-kleuren genoomschildering werd ontwikkeld om gelijktijdig te kunnen hybridiseren met de probes van *B. stricta* en *B. holboellii* samen met een overmaat aan *Arabidopsis* DNA, dit om het onderscheid tussen de ouderchromosomen nog verder te verbeteren. Deze genoomschildering bevestigde dat de ouderchromosomen differentiatie ondergingen van hun repetitieve sequenties in de pericentromeergebieden. Door dit verschil werd een duidelijk onderscheid mogelijk tussen de *B. holboellii* en de *B. stricta* chromosomen in de hybriden. Dit resultaat heeft duidelijk gemaakt dat *Boechera* apomicten alloploïd zijn, met verschillende gebalanceerde of ongebalanceerde combinaties van *B. holboellii* en *B. stricta* chromosomen. Waarschijnlijk ontstaan zulke verschillen in genoomconstituties uit opeenvolgende diploïde-polyploïd en polyploïd-diploïd conversies, waarbij de laatste door reductionele meiose leidt tot haploïde gameten met nieuwe combinaties van homoeologe chromosomen.

Deze genomische *in situ* hybridisatie technieken hebben aan het licht gebracht dat *B. holboellii* en *B. stricta* enorme evolutionaire veranderingen van de repetitieve sequenties in de pericentromeergebieden van hun chromosomen hebben ondergaan, wat leidde tot soortspecifieke FISH bandering. De genoomschildering toonde ook aan dat het *Het* chromosoom *B. stricta* repeats heeft wat er op wijst dat dit chromosoom uit een *B. stricta* autosoom is ontstaan. Bovendien lijkt het *Het* chromosoom meer op een Y chromosoom dan op een B chromosoom. Het *Del* chromosoom laat een klein *stricta* signaal zien, en is tijdens het metafase I stadium verbonden met twee *B. holboellii* chromosomen. Deze eigenschappen suggereren dat dit chromosoom vermoedelijk een *hoelboellii* / *stricta* stranslocatie- of recombinantchromosoom is. Het resultaat van deze moleculair cytogenetische studie heeft ons in staat gesteld een nieuw model te formuleren betreffende de oorsprong van apomictische accessies en de evolutionaire processes van de afwijkende chromosomen. Essentieel in the hypothese is de aanname van een epigenetische modificatie in nieuw gevormde (allo)polyploïde hybride. Deze modificatie gaat vergezeld van of resulteert in chromosoomparingsproblemen tijdens de meiotische profase, die op termijn leidt tot isolatie van crossover recombinatie, opstapeling van repetitieve sequenties en heterochromatinisering van het betreffende chromosoom. Het geringe aantal apomicten in dit onderzoek lijkt nu al voldoende om te stellen dat het *Het* chromosoom in alle accessies afkomstig zijn van eenzelfde oervorm van een pro-*Het* chromosoom, en dat dit chromosoom een sleutelrol speelt in de genetische elementen die voor de apomictische weg vereist zijn.

In het tweede deel van het proefschrift wordt een methode gepresenteerd voor het beoordelen van de mogelijkheid om genen die nodig zijn voor de overdracht van apomixie naar seksueel vermeerderende gewassen. Uitgaande van de aanname dat het nakomelingschap van een apomict een identieke genetische replica van de moeder is bracht ons op het idee om een set SSLP (Single Sequence Length Polymorphics) markers te gebruiken voor het identificeren van planten die in segregerende sexuele populaties heterozygotiefixatie

vertonen als aanwijziging voor zeldzame apomictische gebeurtenissen. Hier tonen we de resultaten van zo'n studie, gebruikmakend van het getransformeerde *Arabidopsis thaliana* AtSERK1 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1) gen, waarvan de expressie onder invloed staat van the AtLTP1 en de AtDMC1 promotoren in seksuele *Arabidopsis* planten. In een van de vier geteste transgene F2 populaties (met het AtLTP1::AtSERK1 construct) vonden we slechts twee planten (1.8%) met behoud van heterozygotie voor alle SSLP markers. Deze twee planten werden beschouwd als een indicatie voor mogelijke klonale overerving. Het nakomelingschap van deze planten vertoonden een bijna binomiale verdeling voor het aantal heterozygote loci, wat ons bacht tot de conclusie dat deze twee vermeende apomictische planten ontstonden uit incidentele recombinatiegebeurtenissen die het genotype van de ouder lieten zien, of dat ze het vermogen tot klonale vermeerdering in de volgende generatie hadden verloren. Hoewel deze genotype-screening in het nakomelingschap van *AtSERK1*-transformanten nog niet tot potentiële apomicten heeft geleid verwachten we dat verdere verfijning en automatisering van de gebruikte technologieën leidt tot een productie van een synthetisch apomixiesysteem. Zo'n systeem zou dan kleine aantallen planten kunnen identificeren die heterozygotiefixatievertonen in een verder volledig seksuele populatie. Uiteindelijk zal een dergelijk systeem nodig zijn om te testen hoe potentiële apomixiegenen die in een gewas geïntroduceerd worden te kunnen testen in de verder normaal seksuele achtergrond van dat gewas. Toekomstexperimenten van *AtSERK1* kunnen zich ook richten op doelgenen die onder controle staan van *AtSERK1* geleide signalering al dan niet in combinatie met andere genen die in somatische embryogenese betrokken zijn.

สรุป

พืชบางชนิดสามารถสร้างเมล็ดได้โดยไม่ผ่านการปฏิสนธิซึ่งเรียกกระบวนการนี้ว่า แอโพมิกซิส แอโพมิกซิสเป็นการสืบพันธุ์แบบหนึ่งนอกเหนือจากการสืบพันธุ์แบบอาศัยเพศ พบมากในพืชที่เป็นโพลิพลอยด์ซึ่งคาดว่าเป็นกระบวนการที่หลีกเลี่ยงการเป็นหมันของลูกผสม กลไกของแอโพมิกซิสที่ค้นพบมีอยู่หลายชนิดเกือบทั้งหมดมีข้อแตกต่างกันที่กระ - บวนการพัฒนาของคัพภะและเอนโดสเปิร์ม ด้วยลักษณะพิเศษของการผลิตเมล็ดที่เหมือนกันกับต้นแม่นี้ทำให้แอโพมิกซิสสามารถผลิตเมล็ดได้ปริมาณมากในราคาที่ถูกจึงเป็นที่สนใจอย่างมากแก่กลุ่มผู้ปรับปรุงพันธุ์มาเป็นเวลานาน ในวิทยานิพนธ์นี้ได้แสดงลักษณะพิเศษหลายๆอย่างของโครโมโซมและพันธุกรรมของพืชแอโพมิกซิสโดยศึกษาใน *Arabidopsis thaliana* พืชที่เป็นแบบจำลองทางพันธุกรรมและพืชสายพันธุ์ใกล้ชิดที่เป็นแอโพมิกซิส คือ *Boechera holboellii* การวิจัยนี้ได้ศึกษาใน ๒ หัวข้อ คือ

๑. การศึกษาการจัดตั้งรูปแบบของโครโมโซมและส่วนประกอบของจีโนมของ *Boechera* ที่เป็นดิพลอยด์และสืบพันธุ์แบบแอโพมิกซิส

๒. การวิเคราะห์พืชแปลงพันธุ์ *Arabidopsis* ด้วยยีนส์ *AtSERK1* เพื่อถ่ายตัวควบคุมลักษณะแอโพมิกซิสไปในพืชที่เป็นดิพลอยด์และสืบพันธุ์แบบอาศัยเพศ

Boechera เป็นพืชที่มีลักษณะสำคัญเหมาะแก่การศึกษาแอโพมิกซิส คือ จีโนมมีความยืดหยุ่นทางพันธุกรรมสูง มีการกระจายตัวทางภูมิศาสตร์ในระดับทวีป มีความหลากหลายทางพันธุกรรมสูง และนอกเหนือจากลักษณะข้างต้นแล้ว *Boechera* เป็นพืชที่เป็นข้อยกเว้นของแอโพมิกซิสคือเป็นพืชดิพลอยด์ การศึกษาโครโมโซมใน ๒ สายพันธุ์ของ *B. holboellii*, *B. stricta* ที่เป็นดิพลอยด์ (2n = 14) และสืบพันธุ์แบบอาศัยเพศ และ ๖ สายพันธุ์ที่เป็นแอโพมิกซิสของ *B. holboellii*, *B. X divaricarpa* (2n = 14, 15) แสดงให้เห็นความแตกต่างอย่างเด่นชัดของโครโมโซมจากต้นที่มีการสืบพันธุ์แบบอาศัยเพศและต้นที่เป็นแอโพมิกซิส ผลจากการย้อมด้วย DAPI ฟลูออเรสเซนส์ และ FISH ด้วย ไรโบโซมอลดีเอ็นเอโพรบส์ในเมแทเฟสโครโมโซมพบโครโมโซมที่มีลักษณะผิดแผก ๒ โครโมโซมในชุดของดิพลอยด์ คือ โครโมโซมที่มีเฮตเทอโรโครมาตินสูง (*Het*) และ โครโมโซมที่สั้นมาก (*Del*) นอกจากนี้ยังพบความแตกต่างระหว่างโครโมโซมที่คาดว่าเป็นคู่เหมือนและความต่างกันอย่างมากของโครโมโซมทั้งจีโนมในแต่ละสายพันธุ์ ซึ่งบ่งให้เห็นลักษณะเฮตเทอโรไซกัสทางโครงสร้างแคริโอไทป์ของสายพันธุ์ที่เป็นแอโพมิกซิส จากการศึกษาไมโอซิสในต้นที่สืบพันธุ์แบบอาศัยเพศและแอโพมิกซิส พบความแตกต่างอย่างมากในขั้นตอนไมโอซิส ขนาดของเรณู การงอกของเมล็ดและขนาดของจีโนมภายในเมล็ด แอโพมิซ์ทบางสายพันธุ์มีการเข้าคู่ของโครโมโซม รีคอมบิเนชันของดีเอ็นเอ ลดจำนวนโครโมโซม แต่บางสายพันธุ์ไม่พบการเข้าคู่ของโครโมโซมและความผิดปกติในการแยกตัวของโครโมโซม ผลของไมโอซิสและขนาดของเรณูได้ชี้ว่าการสร้างเซลล์สืบพันธุ์สามารถเป็นได้ทั้งแบบลดและไม่ลดจำนวนโครโมโซม ใน ๓ สายพันธุ์ที่ศึกษาได้พบไคแอสมาตาระหว่างโครโมโซม *Het* กับออโตโซม ในขณะที่โครโมโซม *Del* แสดงการทาบเกี่ยวกับ ๒ ออโตโซม

การเป็นลูกผสมของแอโพมิซ์ท *B. holboellii* และ *B. divaricarpa* ได้ถูกพิสูจน์โดยวิธีจีโนมอินซิตูไฮบริไดเซชัน โดยเริ่มต้นจากการไฮบริไดซ์ที่ใช้ดีเอ็นเอทั้งจีโนมของ *B. stricta* ที่เป็นดิพลอยด์และสืบพันธุ์แบบอาศัยเพศเป็นโพรบส์และ

ใช้ดีเอ็นเอทั้งจีโนมของ *B. holboellii* เป็นตัวปิดกั้นการไฮบริไดซ์ พบสัญญาณฟลูออเรสเซนส์ได้เพียงบริเวณรอบข้างของเซนโทรเมียร์ของโครโมโซมของ *B. stricta* ในลูกผสม จากนั้นได้ใช้การย้อมสองสีในจีโนมเพื่อให้ได้สัญญาณสีของทั้งสองจีโนมในเวลาเดียวกัน โดยใช้ดีเอ็นเอทั้งจีโนมของ *B. stricta* และ *B. holboellii* เป็นโพรบส์ และ ดีเอ็นเอของ *Arabidopsis thaliana* เป็นตัวปิดกั้นการไฮบริไดซ์ เพื่อให้สามารถแบ่งแยกโครโมโซมของทั้งสองจีโนมออกจากกันได้อย่างชัดเจน จากการย้อมสีบนจีโนมนี้แสดงให้เห็นการวิวัฒน์ที่ต่างกันของดีเอ็นเอรีเพททีฟที่บริเวณรอบเซนโทรเมียร์ของทั้งสองจีโนม ซึ่งสามารถใช้ลักษณะนี้เป็นตัวบ่งชี้ชนิดของจีโนมทั้งสองได้ในลูกผสม การพิสูจน์ชนิดของจีโนมในแอโพมิซ์ทได้ชี้ว่า *Boechera* มีจีโนมแบบออลโลพลอยด์ ที่ประกอบด้วยโครโมโซมของ *B. stricta* และ *B. holboellii* ที่ไม่เท่ากัน ซึ่งคาดว่ามาจากการเกิดซ้ำวงจรของดิพลอยด์-โพลิพลอยด์ และ โพลิพลอยด์-ดิพลอยด์ ซึ่งการเกิดชนิดหลังนี้เป็นไมโอซิสชนิดลดจำนวนโครโมโซมที่มีรีคอมบิเนชันของดีเอ็นเอของคู่โฮมิโอลอยส์โครโมโซม

ผลจากจีโนมอินซิตูไฮบริไดเซชั่นแสดงการวิวัฒน์ออกจากกันอย่างมหาศาลของดีเอ็นเอรีเพททีฟจนทำให้เกิดแถบจำเพาะต่อสปีชีส์บนโครโมโซม การย้อมสีบนจีโนมชี้ว่าโครโมโซม *Het* มีดีเอ็นเอรีเพททีฟของ *B. stricta* ซึ่งคาดว่าโครโมโซมนี้มีต้นกำเนิดมาจากออโตโซมของ *B. stricta* และมีคุณลักษณะที่คล้ายโครโมโซมวายมากกว่าโครโมโซมบี โครโมโซม *Del* มีส่วนของ *B. stricta* อยู่เล็กน้อย และเกิดการทาบเกี่ยวกับ ๒ ออโตโซมของ *B. holboellii* ในระยะเมแทเฟสหนึ่ง ซึ่งอาจเกิดจากทรานส์โลเคชันระหว่าง *B. stricta* และ *B. holboellii* หรือ รีคอมบิเนชันของดีเอ็นเอระหว่างสปีชีส์ ผลการศึกษาเซลล์พันธุศาสตร์ระดับโมเลกุลนี้ทำให้สามารถสร้างแบบจำลองต้นกำเนิดของสายพันธุ์แอโพมิกซิสและลำดับขั้นในการวิวัฒน์ของโครโมโซมที่ผิดแผก สมมุติฐานที่สำคัญอย่างหนึ่งคือการเกิดเอพิเจเนติซในลูกผสมที่ปืนอัลโลโพลีพลอยด์ที่เกิดใหม่ การเกิดนี้อาจเป็นการเกิดร่วมหรือเป็นผลจากการไม่เข้าคู่ของโครโมโซมในระยะไมโอซิสโพรเฟส ซึ่งในเวลานานเข้าจะไม่เกิดรีคอมบิเนชันในส่วนนี้ ทำให้มีการสะสมดีเอ็นเอรีเพททีฟและเกิดเฮตเทอโรโครมาตินในส่วนของโครโมโซมที่เกี่ยวข้อง จากการพิจารณาแอโพมิซ์ทเพียงบางสายพันธุ์พบว่าโครโมโซม *Het* ของทุกสายพันธุ์น่าจะเกิดมาจากโครโมโซมตั้งต้นเดียวกันและโครโมโซมนี้มีบทบาทและมีตัวควบคุมการเกิดแอโพมิกซิส

ในส่วนที่สองของวิทยานิพนธ์นี้เป็นวิธีการคัดเลือกเพื่อประเมินค่าความสามารถของยีนส์ที่นำลักษณะแอโพมิกซิสให้แก่พืชที่สืบพันธุ์แบบอาศัยเพศ จากสมมุติฐานที่ว่าลูกที่เกิดจากแอโพมิกซิสจะมีพันธุกรรมเหมือนต้นแม่ ทำให้เราเลือกใช้เครื่องหมายแบบ Single Sequence Length Polymorphism(SSLP) มาใช้บ่งชี้ลูกที่มีลักษณะคงที่ของเฮตเทอโรไซโกซิตีในประชากรที่มีการกระจายตัวทางพันธุกรรมอันเกิดจากการสืบพันธุ์แบบอาศัยเพศ เพื่อบ่งชี้อุบัติการแอโพมิกซิสที่เกิดในความถี่ต่ำ ยีนที่ศึกษาคือ *Arabidopsis thaliana* Somatic Embryogenesis Receptor like Kinase1(AtSERK1) ภายใต้โพรมอเตอร์ AtLTP1 และ AtDMC1 ในพืช *Arabidopsis* พบว่าหนึ่งในสี่ประชากรที่ทดสอบมี ต้นลูก ๒ ต้นที่แสดงความคงที่ของเฮตเทอโรไซโกซิตีเหมือนต้นแม่ ซึ่งอยู่ในประชากร AtLTP1::AtSERK1 ซึ่งแสดงถึงความเป็นไปได้ว่าพืชทั้งสองเกิดจากการโคลน จากการติดตามในประชากรรุ่นต่อไปของพืชทั้งสองนี้พบว่ามีการกระจายตัวทางพันธุกรรมใกล้เคียงกับแบบทวินาม ทำให้สรุปว่าพืชที่คาดว่าเป็นแอโพมิกซิสทั้งสองนั้นอาจเกิดจากความบังเอิญทางรีคอมบิเนชันที่ทำให้เกิดจีโนไทป์คล้ายต้นแม่ หรือ พืชทั้งสองสูญเสียความสามารถทางโคลนนิ่งในรุ่นถัดมา แม้ว่าการคัดเลือกแอ -โพมิกซิสไม่แสดงศักยภาพในพืชแปลงพันธุ์ด้วย AtSERK1 แต่คาดว่าการปรับปรุงและสร้างความสามารถควบคุมตนเองในกระบวนการสร้างแอโพมิกซิสที่เหมาะสมในอนาคตจะทำให้วิธีการบ่งชี้ความคงที่ของเฮตเทอโรไซโกซิตีในประชา-

กรที่มาจากการสืบพันธุ์แบบอาศัยเพศแสดงศักยภาพได้มากขึ้น หากยีนส์ที่นำลักษณะแอโพมิกซิสถูกถ่ายลงในพืชที่มีการสืบพันธุ์แบบอาศัยเพศการคัดเลือกแอโพมิกซิสในประชากรนี้จะมีความจำเป็นเป็นอย่างสูง การศึกษาต่อไปของ AtSERK1 อาจจะเน้นในการทำงานร่วมกันกับยีนส์อื่นที่ร่วมในกระบวนการส่งสัญญาณ หรือกลุ่มยีนส์ที่ร่วมในการเกิดคัพภะที่เกิดจากเซลล์ร่างกาย

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d.	**MSc courses**		
	Basic Molecular Biology techniques I (A450-220)	June, 2001	
	Basic Molecular Biology techniques II (A450-221)	June, 2001	
e.	**Laboratory use of isotopes**		
		subtotal start-up phase	8.0
2) Scientific Exposure			
a.	**EPS PhD student days**		
	PhD students day 2002, Wageningen	January 24, 2002	
	PhD students day 2003, Utrecht	March 27, 2003	
	PhD students day 2004, Amsterdam	June 3, 2004	
b.	**EPS theme symposia**		
	Theme symposium IV, 2002, Wageningen	December 20, 2002	
	Theme symposium IV, 2003, Nijmegen	December 10, 2003	
	Theme symposium IV, 2004, Wageningen	December 9, 2004	
c.	**Research discussion**		
	Weekly meetings at WU-Biochemistry and WU-Genetics	2001-2005	
d.	**National meetings**		
	ALW meeting Lunteren 2002, Plant Sciences	April 8-9, 2002	
	ALW meeting Lunteren 2003, Plant Sciences	April 7-8, 2003	
	ALW meeting Lunteren 2004, Plant Sciences	April 5-6, 2004	
	ALW meeting Lunteren 2005, Plant Sciences	April 4-5, 2005	
e.	**Seminars (series), workshops and symposia**		
	Ecological & evolutionary genomics	April 29, 2005	
	Genetics of natural variation	November 26, 2004	
f.	**International symposia and conferences**		
	2nd International Apomixis Conference, Como, Italy	April 24-28, 2001	
	B Chromosome Conference, Granada, Spain	June 26-30, 2004	
g.	**Excursion**		
		subtotal Scientific Exposure	9.2

3) In-Depth Studies			
a.	**EPS courses or other PhD course**		
	Springschool "Bioinformatics"	March 31-April 2, 2004	
b.	**Journal club**	-	
c.	**Individual research training**	-	
		Subtotal In-Depth Studies	0.6
4) Personal development			
	Skill training courses		
a.	Writing English	September, 2003	
	English presentation	December, 2004	
b.	**Organisation of PhD students day, course or conference**	-	
c.	**Membership of Board, committee or PhD council**	-	
		Subtotal Personal Development	2.0
		Total number of Credit Points	**19.8**

* A credit point represents a normative study load of 40 hours of study

Publications and manuscripts in preparation

1. Sharbel TF, Voigt M-L, Mitchell-Olds T, **Kantama L**, de Jong H (2004) Is the aneuploid chromosome in an apomict Boechera holboellii a genuine B chromosome? *Cytogenet Genome Res* 106: 173-183.

2. Sharbel TF, Mitchell-Olds T, Dobeš C, **Kantama L**, de Jong H (2005) Biogeographic distribution of polyploidy and B chromosomes in the apomictic Boechera holboellii complex. *Cytogenet Genome Res* 109: 283-292.

3. **Kantama L**, Lambert Y, Hu H, de Jong H, de Vries SC, Russinova E. (2006) A Single Sequence Length Polymorphism (SSLP)-based method for detection of rare apomictic events in a sexual AtSERK1 transgenic *Arabidopsis* population. Submitted to *Sex. Plant Reprod*.

4. Schranz ME, **Kantama L**, Pfuetzenreuter J, de Jong H, Mitchell-Olds T. (2006) Generation and analysis of triploid, aneuploid and tetraploid progeny from a cross between a sexual diploid *Boechera stricta* and an apomictic diploid *B. ×divaricarpa.* To be submitted to *New Phytologist*, Evolution section

5. **Kantama L**, Sharbel T, Schranz ME, Dobeš C, Mitchell-Olds T, de Vries SC, de Jong H. (2006). Chromosome organisation and evolution in apomicts of the *Boechera holboellii* complex. To be submitted to *Proc Nat Acad Sciences USA*.

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