Chapter 20
Apomixis in the Era of Biotechnology

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20.1 Introduction

The adaptive success of living organisms depends on the maintenance of a dynamic equilibrium between creating new genetic combinations and fixing those which are more adapted to the present environment. Sexual reproduction is universally the main route to recombine genes in the short term; on the other hand, different strategies have been adopted to fix the genetic composition of individuals demonstrating high fitness. In plants, genotypes may be “immortalized” via vegetative propagation or “photocopied” by selfing of highly homozygous individuals. A third, more technically sophisticated pathway is represented by the implementation of apomixis, where a functional sexual machine is short-circuited to asexually produce embryos with the fixed genotype of the mother plant. This developmental sophistication represents a challenging research field for the reproduction biologist and a desirable trait for the plant breeder to be used in seed production schemes of elite varieties.
20.2 General Definitions and Apomixis Mechanisms

The phenomenon of apomixis, its cyto-embryological pathways and the perspective of using apomixis as a means for cloning plants by seeds have been reviewed extensively in the last decade (Savidan 2000, Savidan et al. 2001; Spillane et al. 2001; Grimanelli et al. 2001a; Koltunow and Grossniklaus 2003; Bicknell and Koltunow 2004; Ozias-Akins 2006; Hörandl and Paun 2007). However, for many aspects, the most comprehensive dissertations of apomixis terminology, mechanisms and evolution rely on a few key reviews published some 20–25 years ago (Asker 1980; Marshall and Brown 1981; Nogler 1984a; Bashaw and Hanna 1990; Asker and Jerling 1992; Koltunow 1993).

As apomictic reproduction entails the development of an embryo from a cell with a somatic chromosome number, several ways exist to produce embryos of apomictic origin. The simplest pathway avoids the production of a gametophyte, and a maternal embryo originates from one or more somatic cells of the ovule. This process is known as adventitious embryony, and can be either nucellar or integumental, depending on the tissue from which the embryogenetic somatic cell differentiates. Adventitious embryony seems to have evolved more frequently in tropical than in temperate flora. Moreover, it is more represented in diploid species, whereas other forms of apomixis are more frequent in polyploids. Among the agriculturally important species, adventitious embryony is found in several *Citrus* species, in mango (*Mangifera indica*) and in orchids. The most comprehensive treatise on adventitious embryony was published by Naumova (1992).

When the maternal embryo originates from a diploid egg cell differentiated in an unreduced embryo sac, the apomictic pathway is referred to as gametophytic apomixis. Sexual reproduction is based on the alternation of a diploid (sporophytic) and haploid (gametophytic) generation, both of which are bounded by events entailing a shift in ploidy, namely meiosis and fertilization. In gametophytic apomixis, both edge events are short-circuited; the gametophytic generation proceeds with the maternal ploidy level (and genomic composition) and the embryo is generated without the contribution of a male gamete (Fig. 20.1a). More specifically, meiosis is altered or omitted, and 2n female gametophytes and gametes are formed (apomeiosis) which then undergo embryogenesis autonomously without fertilization by a male gamete (diploid parthenogenesis).

As this combination maintains the original ploidy and may theoretically be indefinitely reiterated, it was referred to as recurrent apomixis (Nogler 1984a). In fact, the sexual program may be short-circuited in only one of the two fundamental steps; thus, it may happen that a reduced egg cell develops in the absence of fertilization, giving rise to a (poly)haploid individual (haploid parthenogenesis). In its commonest occurrence, haploid parthenogenesis takes place from the egg cell (gynogenesis); more rarely, a haploid embryo develops autonomously from a sperm nucleus (androgenesis). Conversely, the partial short-circuiting of sexual reproduction may affect only the meiotic step. Thus, an unreduced egg cell may be fertilized by a reduced male gamete, giving rise to a 2n+n hybrid or “BIII hybrid” (Rutishauser 1948),
rather than normal n+n (B_{II}) hybrids (Fig. 20.1a). All these pathways may occur concurrently in the same taxon and even within the same plant, as in *Poa pratensis* (Grazi et al. 1961; Barcaccia et al. 1997) and *Hieracium* (Bicknell et al. 2003), amongst others (Fig. 20.1a).

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**Fig. 20.1 a** Different combinations in the occurrence of meiosis and parthenogenesis give rise to sexual and asexual pathways in plant reproduction. **b** Schematic representation of female sporogenesis and gametogenesis in sexual plants and short-circuited alternative pathways in the most common forms of apomixis
Because haploid parthenogenesis and genome accumulation, an alternative term given to the occurrence of BIII hybrids (Leblanc and Mazzucato 2001), entail shifting of the original ploidy level, they cannot reiterate themselves and, for this reason, have been referred to as “non-recurrent apomixis” (Nogler 1984a). Although not offering a stable means for genotype propagation, non-recurrent apomixis has likely been an important player in the evolution of polyploid species and is regarded as a useful tool to scale-up or -down the chromosome number in breeding programs.

### 20.3 Embryological Pathways of Gametophytic Apomixis

In gametophytic apomixis, the unreduced embryo sac may arise from a somatic nucellar cell which acquires the developmental program of a functional megaspore, a mechanism referred to as apospory. Alternatively, if the embryo sac forms from a megaspore mother cell with suppressed or modified meiosis, the pathway is called diplospory (Fig. 20.2b). These two pathways, leading to the production of 2n egg cells and broadly referred to as apomeiotic pathways, offer a variety of different developmental schemes which have been the object of several descriptions and reviews (Nogler 1984a; Asker and Jerling 1992; Crane 2001).

In apospory, when the unreduced embryo sac develops into an eight-nucleate, seven-celled gametophyte (similar to the *Polygonum*-type found in sexuals), this is referred to as *Hieracium*-type apospory (Fig. 20.1b). This scheme was first described in *Hieracium* a century ago (Rosenberg 1907) but, subsequently, it was found in other Compositae (e.g. *Crepis*) and in the Poaceae (e.g. *P. pratensis* and *Hierochloe* spp.), also in genera belonging to different families, such as *Hypericum*, *Ranunculus* and *Beta* (reviewed in Nogler 1984b). Usually one or, more rarely, a few aposporic initials differentiate from one or more nucellar cells which are in contact with the differentiated meiocyte or its derivatives, and which enlarges to form a large vacuole at the chalazal pole. This represents the best moment to recognize aposporic activity in species with *Hieracium*-type apospory (Fig. 20.2a), as mature embryo sacs of sexual or apomeiotic origin are difficult, if not impossible, to differentiate. However, a frequent occurrence inside the same ovule is the development of both the reduced and the unreduced embryo sac to produce multiple mature gametophytes and polyembryony in the seed.

In an alternative pathway, the aposporic initial cell behaves as in the former case but gametogenesis involves only two free divisions, resulting in a mature four-nucleate, four-celled embryo sac. This so-called *Panicum*-type embryo sac shows a three-celled egg apparatus, a single unreduced polar nucleus and no antipodals (Fig. 20.1b). *Panicum*-type apospory is frequent in the Paniceae (genera *Brachiaria*, *Cenchrus*, *Eriochloa*, *Panicum*, *Paspalum*, *Pennisetum* and *Urochloa*) and Andropogoneae (the *Bothriochloa-Dichanthium-Capillipedium* agamic complex and the genera *Chloris, Heteropogon, Hyparrhenia, Sorghum* and *Thedema*)
tribes of the Poaceae (Nogler 1984a; Bashaw and Hanna 1990). As in the Hieracium type, in these species the legitimate lineage may also develop alongside the aposporic initials. However, it appears that normally all sexual megaspores

Fig. 20.2  a Occurrence of an aposporic initial (arrow) in Poa pratensis. b Diplospory in the TNE Medicago falcata mutant: unreduced FDR-type monad due to omitted or modified meiosis I (left), binucleated (centre) and non-polarized ES, and polarized ES containing unreduced nuclei (right), and c embryo developed to the globular stage (arrow) before fertilization of polar nuclei in P. pratensis. Bar ¼ 10 mm

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Degenerate. In this intra-ovular competition, it seems that the timing of differentiation of aposporic initials is the crucial factor; the earlier the differentiation, the higher the competitive superiority of apospory versus sexuality. For scoring apospory, the Panicum-type pathway allows recognition of the unreduced embryo sac also at maturity. Although the model for apospory development is strictly species-specific, cases have been reported of Paspalum species in which both four- and eight-nucleate embryo sacs are found (Reusch 1961; Quarin et al. 1982).

Diplospory offers a richer repertoire of developmental pathways. When meiosis is completely bypassed, it is referred to as Antennaria-type or mitotic diplospory. Mitotic diplospory is widely distributed, e.g. in Antennaria, Eupatorium, Poa alpina, Parthenium, Eragrostis and Tripsacum (reviewed in Nogler 1984a) and, owing to the absence of meiosis, it represents a form of apomeiosis which fully guarantees the fixation of the maternal genome (Figs. 20.1b, 20.2b).

In the Taraxacum-type diplospory (aneuspory), by contrast, meiosis begins but is largely asynaptic (cf. absence of pairing between homologous chromosomes) and a restitution nucleus in the first division produces a dyad of unreduced megaspores. The chalazal megaspore generally produces the unreduced embryo sac. Whether the maternal genome is fixed or not depends on whether any bivalents have formed and crossover has taken place. In addition to Taraxacum, this type of diplospory has been found in Erigeron, Boechera (formerly Arabis), Agropyrum and some Paspalum species (reviewed in Nogler 1984a). In other diplosporic pathways (Ixeris, Datura and Allium types), the first meiotic division is carried out and the unreduced megaspores present the maternal genome with the effects of recombination. These pathways have been described in detail by Asker and Jerling (1992). In contrast to what happens in adventitious embryony and apospory, in diplospory the occurrence of more than one embryo inside the same seed (polyembryony) is, theoretically, excluded.

Irrespective of how the unreduced embryo sac has formed, the second component of (recurrent) gametophytic apomixis consists of autonomous egg cell development in the absence of fertilization (diploid parthenogenesis). In sexual species, diploid parthenogenesis occurs rarely and is called matromorphy, examples of which have been reported mainly in Brassica but also in Fragaria, Raphanobrassica and other species (reviewed in Asker and Jerling 1992). It is thought that the mechanisms controlling diploid parthenogenesis are not different from those responsible for the autonomous development of a reduced egg cell. However, compared to apomeiosis, the study of parthenogenetic mechanisms has received less attention.

The third (and last) component for the production of a functional apomictic seed is functional endosperm formation. In many apomicts, the endosperm is initiated autonomously without any contribution from male gametes. Autonomous endosperm development is widely spread in the Compositae and common among species showing diplospory and adventitious embryony. Endosperm ploidy may vary depending on whether or not the unreduced polar nuclei fuse before initiating
the endosperm divisions. In autonomous species, the synergids remain intact and the male organs are often not functional.

More frequently, endosperm development requires a pollination stimulus to occur (pseudogamy). This is found more commonly in aposporic species, including the families Rosaceae and Poaceae. It is rare in the Compositae. The pathway for endosperm formation is usually conserved at the genus level. Pseudogamy may be characterized by simple pollen tube growth in the style, cytoplasmic penetration or, most frequently, true fertilization of the polar nuclei in the embryo sac. In some pseudogamous species, parthenogenetic development precedes secondary fertilization to give rise to precocious embryony (or proembryony; Fig. 20.2c). In pseudogamous species, the endosperm ploidy level is more variable than in autonomous species; the expected level of the 4(n) maternal:1(n) paternal ratio is often altered by the variable number of polar nuclei involved, the extent of their fusion, and the number and ploidy level of the male gamete(s) which operate fertilization. Thus, in many cases the 2:1 maternal-to-paternal ratio of the endosperm is maintained, as is the case for Panicum-type apospory in which a single 2n polar nucleus is fertilized by a reduced male gamete) or, in Hieracium-type apospory, if the polar nuclei do not fuse and are each fertilized by a single sperm cell.

20.4 Genetic and Epigenetic Control of Apomixis

Several models for the genetic basis of apomixis have been proposed, including divergence in the number of genes, their function and allelic relationships, and dominance over sexuality (Asker and Jerling 1992; Koltunow et al. 1995; Carman 1997; Grimanelli et al. 2001a; Noyes 2005). Genetic analysis in several species (Table 20.1) has consistently demonstrated a simple inheritance system and a few Mendelian genes controlling the expression of apomixis or its components (Barcaccia et al. 2000; Grimanelli et al. 2001a; Bicknell and Koltunow 2004; Catanach et al. 2006; Schranz et al. 2006; Noyes et al. 2007). By contrast, molecular and cytogenetic analyses of the chromosomal region(s) carrying the determinants of apomixis in several species have unveiled attributes indicative of a complex genetic control and/or a system of polygenes, in addition to mechanisms involving lack of recombination, transacting elements for gamete elimination, supernumerary chromatin structures and DNA rearrangements (Leblanc et al. 1995a; Grimanelli et al. 1998; Roche et al. 1999; Noyes and Rieseberg 2000; Goel et al. 2003; Matzk et al. 2005; Calderini et al. 2006).

Recent data collected in species forming seeds through distinct asexual pathways, such as Hieracium spp., P. pratensis and Tripsacum dactyloides, suggest that gametophytic apomixis relies upon either spatial or temporal misexpression of genes acting during female sexual reproduction (Grimanelli et al. 2003; Tucker et al. 2003; Albertini et al. 2004). However, although genes showing differences in spatial and
Table 20.1 Genetic inheritance and molecular mapping of apomixis components (apomeiosis and parthenogenesis). Genetic models are based on the segregation analysis of progenies from crosses between sexual and apomictic genotypes and are supported by the co-segregation of tightly linked molecular markers.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of apomixis</th>
<th>Endosperm development</th>
<th>Parental ploidy and type of crosses</th>
<th>Deduced genotypes</th>
<th>Linked markers</th>
<th>Suppressed recombination</th>
<th>Main references</th>
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</thead>
<tbody>
<tr>
<td><strong>Apomeiosis</strong></td>
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<tr>
<td><em>Ranunculus auricomus</em></td>
<td>Apospory</td>
<td>Pseudogamous</td>
<td>2x–4x Intragenic</td>
<td>Aaaa</td>
<td>–</td>
<td>–</td>
<td>Nogler (1984a)</td>
</tr>
<tr>
<td><em>Panicum maximum</em></td>
<td>Apospory</td>
<td>Pseudogamous</td>
<td>4x,–4x Intraspecific</td>
<td>Aaaa</td>
<td>–</td>
<td>–</td>
<td>Savidan (1982)</td>
</tr>
<tr>
<td><em>Pennisetum squamulatum</em></td>
<td>Apospory</td>
<td>Pseudogamous</td>
<td>4x,–6x Intragenic</td>
<td>Aaaaa</td>
<td>0 cM</td>
<td>Yes</td>
<td>Ozyas-Akins et al. (1998)</td>
</tr>
<tr>
<td><em>Brachiaria brizantha</em></td>
<td>Apospory</td>
<td>Pseudogamous</td>
<td>4x,–4x Intragenic</td>
<td>Aaaa</td>
<td>1.2 cM</td>
<td>No</td>
<td>Pessino et al. (1998)</td>
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<tr>
<td><em>Paspalum simplex</em></td>
<td>Apospory</td>
<td>Pseudogamous</td>
<td>4x,–4x Intraspecific</td>
<td>Aaaa</td>
<td>0 cM</td>
<td>Yes</td>
<td>Labombarda et al. (2002)</td>
</tr>
<tr>
<td><em>Hypericum perforatum</em></td>
<td>Apospory</td>
<td>Pseudogamous</td>
<td>2x–4x Intraspecific</td>
<td>Aaaa</td>
<td>0 cM</td>
<td>–</td>
<td>Barcaccia et al. (2007)</td>
</tr>
<tr>
<td><em>Poa pratensis</em></td>
<td>Apospory</td>
<td>Pseudogamous</td>
<td>4x,–5x Intraspecific</td>
<td>Aaaaa</td>
<td>–</td>
<td>–</td>
<td>Albertini et al. (2007)</td>
</tr>
<tr>
<td><em>Tripsacum dactyloides</em></td>
<td>Diplospory</td>
<td>Pseudogamous</td>
<td>2x–4x Intragenic</td>
<td>Dddd</td>
<td>0 cM</td>
<td>Yes</td>
<td>Grimanelli et al. (1998)</td>
</tr>
<tr>
<td><em>Erigeron annuus</em></td>
<td>Diplospory</td>
<td>Autonomous</td>
<td>2x–3x Intragenic</td>
<td>Ddd</td>
<td>0 cM</td>
<td>Yes</td>
<td>Noyes and Rieseberg (2000)</td>
</tr>
<tr>
<td><em>Taraxacum officinale</em></td>
<td>Diplospory</td>
<td>Autonomous</td>
<td>2x–3x Intraspecific</td>
<td>Ddd</td>
<td>0.2 cM</td>
<td>No</td>
<td>Van Dijk and Bakx-Schotman (2004)</td>
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<tr>
<td><strong>Parthenogenesis</strong></td>
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<tr>
<td><em>Poa pratensis</em></td>
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<td>4x–6/8x Intraspecific</td>
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<td><em>Erigeron annuus</em></td>
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<td>7.3 cM</td>
<td>No</td>
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<tr>
<td><em>Taraxacum officinale</em></td>
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<td>Autonomous</td>
<td>2x–3x Intraspecific</td>
<td>Ppp</td>
<td>–</td>
<td>–</td>
<td>Van Dijk and Bakx-Schotman (2004)</td>
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*a* Parental ploidy: i, induced. -, not determined
temporal expression patterns between apomicts and their sexual counterparts have been reported (Pessino et al. 2001; Rodrigues et al. 2003; Albertini et al. 2005; Chen et al. 2005), their functions remain largely speculative. In the current view, gametophytic apomixis is thought to rely on three genetically independent Mendelian loci, each exerting control over a key developmental component, these being the formation of apomeiotic megaspores, the parthenogenetic capabilities of unreduced egg cells and modified endosperm development (Noyes and Rieseberg 2000; Albertini et al. 2001a; Grossniklaus et al. 2001a; Koltunow and Grossniklaus 2003; Bicknell and Koltunow 2004; Vijverberg and van Dijk 2007).

The importance of parent-of-origin effects and, more generally, of epigenetic factors during sexual reproduction and early seed development has emerged recently in apomicts (Guitton and Berger 2005; Köhler and Grossniklaus 2005; Autran et al. 2005; Takeda and Paszowski 2006; Xiao et al. 2006; Feil and Berger 2007; Nowack et al. 2007). Although Grossniklaus and Schneitz (1998) and Grossniklaus et al. (2001a) proposed that the regulation of apomixis might depend on heritable epialleles and that relaxation of genomic imprinting is a requirement at least for endosperm development in apomicts, the relevance of epigenetics in apomictic developmental patterns remains largely unexplored (Koltunow and Grossniklaus 2003; Ranganath 2004).

In most apomicts, apospory and diplospory have been proven to be simply inherited on the basis of the segregation of the trait in crosses between sexual seed parents and apomictic pollen parents. Since apomixis is always associated with hybridity and heterozygosity, segregation for the mode of reproduction as well as co-segregation of molecular markers have been studied in most species by adopting pseudo-testcross mapping strategies (Barcaccia et al. 1998; Ozias-Akins et al. 1998; Pessino et al. 1998; Noyes and Rieseberg 2000; Van Dijk and Bakx-Schotman 2004). In some species, both female (sexual) and male (apomict) genetic maps have been constructed (Porceddu et al. 2002; Jessup et al. 2003; Pupilli et al. 2004).

Apospory has rarely been shown to segregate from parthenogenesis, behaves as a dominant trait, and is inherited in a Mendelian fashion, although sometimes subject to segregation distortion (reviewed in Ozias-Akins 2006). This pattern of inheritance has been observed for *Pennisetum squamulatum* (Dujardin and Hanna 1983; Ozias-Akins et al. 1998), *Cenchrus ciliaris* syn. *Pennisetum ciliare* (Sherwood et al. 1994; Jessup et al. 2002), *Panicum maximum* (Savidan 2000; Ebina et al. 2005), *Brachiaria* spp. (do Valle et al. 1994; Miles and Escandon 1997), *Paspalum notatum* (Martínez et al. 2001), *Ranunculus* spp. (Nogler 1984b), *P. pratensis* (Albertini et al. 2001a) and *Hieracium* spp. (Bicknell et al. 2000). A more complex genetic model, advanced for the evolution of apomixis from sexual plants (Holsinger 2000), was recently postulated for *P. pratensis* which includes single, unlinked genes for initiation of apospory, apospory prevention, parthenogenesis initiation and parthenogenesis prevention, as well as a megaspore development gene (Matzk et al. 2005).

A single regulatory gene has been proposed as sufficient for the induction of apomixis (Peacock 1992) and, although simple genetic inheritance appears to support this hypothesis, molecular evidence suggests that more complex genetic
control of the entire apomixis process cannot be discounted. In particular, the linkage groups typically transmitted with apospory display large blocks of non-recombining molecular markers, leading to speculation that adapted gene complexes within supernumerary chromatin might be required for the function of at least certain types of apomixis (Roche et al. 2001; Ozias-Akins et al. 2003). The association of apomixis with a chromosomal region lacking genetic recombination was first described in *P. squamulatum* (Ozias-Akins et al. 1998). Extensive characterization of this chromosomal region using RFLP (restriction fragment length polymorphism) markers and FISH (fluorescence in situ hybridization) of apospory-linked clones has shown the region to be extremely large in size, heterochromatic, and highly hemizygous (Ozias-Akins et al. 1998; Roche et al. 1999; Goel et al. 2003; Akiyama et al. 2004). A heterochromatic and hemizygous region was also found in the polyploid apomicts *P. squamulatum* and *C. ciliaris*, and is indicative of heteromorphism between the homologous chromosomal pairing partners which has apparently resulted from an insertion in both species, combined with an inversion/translocation in the former (Akiyama et al. 2005). Lack of genetic recombination and hemizygosity are not confined to the genus *Pennisetum* and close relatives but have also been found in *Paspalum simplex* (Labombarda et al. 2002). The association of apospory with a heterochromatic region of the genome, rich in retrotransposons, raises the intriguing possibility that DNA structure and/or RNA interference (Lippman et al. 2004) could play a role in the control of expression of apomictic-related genes.

In *T. dactyloides* (Grimanelli et al. 2003) and *Hypericum perforatum* (Barcaccia et al. 2006), it has been shown that apomeiosis, i.e. displospory in the former and apospory in the latter, and parthenogenesis are developmentally uncoupled, supporting the hypothesis of two distinct genetic factors controlling these traits in both apomictic species. A clearly documented case of recombination between apospory and parthenogenesis is found in *P. pratensis* (Barcaccia et al. 2000; Albertini et al. 2001a, b). Independence between diplospory and parthenogenesis was also reported for *Taraxacum officinale* (Hass and van Dijk 1999; van Dijk et al. 1999; van Dijk and Bakx-Schotman 2004), where autonomous endosperm development was shown to segregate independently from diplospory and parthenogenesis (van Dijk et al. 2003; Vijverberg et al. 2004). Similarly, progeny from a cross of sexual diploid and apomictic triploid genotypes of *Erigeron annuus* showed a range of chromosome numbers which were not predictive of reproductive mode, even though a single locus model for diplospory was supported by segregation data (Noyes and Rieseberg 2000). While all parthenogenetic plants were diplosporic, several diplosporic plants were not able to form embryos, suggesting that a genetic component for parthenogenetic development of egg cells had been eliminated. Genetic mapping of the segregating population provided several AFLP (amplified fragment length polymorphism) markers linked to either diplosporic apomeiosis or parthenogenesis, providing support that the two apomixis components were segregating independently (Noyes and Rieseberg 2000).
20.5 Evolution of Apomixis and Population Genetics in Apomicts

The origin and persistence of asexual reproduction remains one of the most challenging phenomena in evolutionary biology (Bell 1982). In plants and animals, asexuality is derived from sex (amphimixis), and not only has it originated independently between different species but it has also evolved recurrently within certain species. Hence, in certain contexts, natural selection has repeatedly favoured the switch to asexual reproduction. Despite hypothesized disadvantages associated with asexual reproduction, including limited genetic diversity and mutation accumulation, asexual plants and animals are surprisingly stable from an evolutionary perspective, and thus questions regarding the origin and evolution of asexuality confront evolutionary and population biologists.

Many wild apomictic species are characterized by hybridity and polyploidy (Richards 2003) and, interestingly, these characteristics are also shared by a majority of asexual animal taxa. It is still unclear what the relative contributions of hybridization and polyploidy are to asexual lineage origin and evolution, as both phenomena can have diverse regulatory consequences (Comai et al. 2003; Osborn et al. 2003; Swanson-Wagner 2006) which could conceivably lead to coordinated deregulation of the sexual pathway in a sexual ancestor. In addition, both naturally occurring and induced mutants demonstrating the individual components separately have been identified (Curtis and Grossniklaus 2007; Ravi et al. 2008), implying that many taxa have the potential to express apomixis-like traits, in addition to supporting the hypothesis that each component is under independent regulation. The actual molecular mechanisms underlying apomixis expression are the focus of intense research described in more detail in other sections of this chapter.

Apomictic taxa are often members of “complexes” and, as the name implies, this involves species which are characterized by a complicated mixture of inter- and intraspecific genetic and phenotypic variation and gene flow. Most apomictic taxa are facultative, meaning that a single individual can produce seeds through both sexual and apomictic pathways. Furthermore, apomicts and their sexual relatives are often sympatric (but not necessarily syntopic) and morphologically difficult to differentiate. Closely related sexual and asexual taxa frequently have different, although overlapping, ranges of adaptation, referred to as “geographical parthenogenesis” (Vandel 1928). More specifically, compared to their sexual relatives, apomictic plants typically have (1) larger geographic ranges, (2) ranges which extend into more elevated latitudes and altitudes, and (3) better abilities to colonize previously glaciated regions (Bierzychudek 1987). With reference to apomictic plants, geographical parthenogenesis applies only to gametophytic apomixis and not to adventitious embryony, as the latter tends to be prevalent in tropical species and is characterized by different mechanisms and ecological constraints (Richards 1997). Apomicts differ from their sexual relatives not only in reproductive mode but, in most cases, also in ploidy (cf. apomicts are polyploid). Hence, the phenomenon of geographical parthenogenesis could equally be explained by ploidy
differences as it could by reproduction (Bierzychudek 1987). While a number of factors likely contribute to these geographic differences between amphimictic and apomictic plants, including the Pleistocene origins of apomixis in conjunction with hybridization and polyploidy, unidirectional gene flow, niche targeting by asexual clones, and limited biotic interactions in regions of glaciations, the relative influence of each is probably species-specific (Hörandl 2006).

Asexual taxa are typically thought to be better colonizers than their sexual counterparts. In animals, this has been associated with the “two-fold cost of sex”, as sexuals require two individuals to reproduce (male and female), while asexuals require only one. Hence, an asexual population has the potential to grow faster (Maynard Smith 1978). Apomicts, by nature of being hermaphroditic, do not suffer this two-fold cost and, although there appears to be decreased selection pressure on the male line (Voigt et al. 2007), some functionality must nonetheless be maintained in order to fertilize the primary endosperm nuclei, i.e. pseudogamy. Within this context, apomicts can more easily found new populations (as do selfing sexual plants), since only a single individual is required, and this is reflected by the invasiveness of some apomictic taxa, e.g. *T. officinale* (Brock et al. 2005) and *H. perforatum* (Vilà et al. 2003).

A biologist who is planning a population level study of an apomictic taxon is thus faced with two problems. One must first be able to differentiate between sexual and apomictic individuals which may or may not be morphologically distinct and share similar geographic ranges. Secondly, as wild apomicts are typically facultative, assessing variations in sexual and apomictic seed production within individuals is essential for understanding population dynamics and gene flow. Assuming a difference in ploidy between apomicts and sexuals, time-consuming karyological or cell size-based analyses can be made of each collected individual. Developments in flow cytometry have facilitated analyses of ploidy on the population level and, today, literally 1,000s of individuals can be analyzed in a few days (Sharbel and Mitchell-Olds 2001). Isolating flowers which have been emasculated can be used to identify seed formation through autonomous apomixis (Richards 1997), whereas differentiating between pseudogamous apomicts and selfing individuals in this way is more difficult in cases of self-compatibility. Apomictic reproduction should be characterized by fixed heterozygosity, while selfing, which is also reproduction in the absence of cross pollination, leads to homozygous offspring. Thus, comparison of genetic markers between parents and offspring can be used in order to identify apomictic offspring having identical fixed heterozygous genotypes. Such an approach is, of course, time-consuming and potentially problematic, if seeds cannot be collected and germinated in the glasshouse. Alternatively, if one uses a “normal” diploid sexual population and its expected population genetic parameters, e.g. linkage equilibrium, random mating or Hardy-Weinberg equilibrium as a point of reference, then deviations from expected variation can be a signal that apomixis has played a part in influencing population structure (Halkett et al. 2005). For example, the identification of fixed genotypes within a population can be used to infer apomixis, although the confidence of such analyses will be influenced by the choice of genetic marker, e.g. dominant AFLPs versus codominant microsatellites (Leblanc and Mazzucato 2001; Arnaud-Haond et al. 2007).
The problem with using indirect methods to identify apomictic individuals is that they are subject to ascertainment bias. Conclusions can be erroneously drawn as a result of the type of method used to screen for apomixis, as well as the schemes employed to choose both population and tissue samples for analysis. More recently, the “flow cytometric seed screen” (FCSS; Matzk et al. 2000) has been developed as an effective, cost-efficient and rapid way to directly measure seed formation in both sexual and apomictic plants. The FCSS method uses a flow cytometric profile of individual seeds to infer the mechanisms of seed production (Matzk et al. 2000). In a normal diploid sexual plant, a reduced (C) egg cell and a reduced central cell with two polar nuclei (C+C) are fertilized by two sperm cells to form a 2C embryo and 3C endosperm. Depending upon the taxon, apomixis is characterized by the formation of reduced and/or unreduced embryo sacs which will be fertilized by reduced or unreduced sperm cells (Matzk et al. 2000). Hence, the FCSS serves to identify deviations from the typical sexual 2:3 embryo:endosperm ratio. Using FCSS, the dynamics of seed formation in an apomictic plant can be precisely determined, and a wide range of different reproductive pathways identified (Matzk et al. 2001; Naumova et al. 2001). Importantly, analyses of large numbers of individual seeds per plant demonstrate genotype-specific quantitative variation for sexual and apomictic seed formation (O.M. Aliyu and T.F. Sharbel, unpublished data) and, furthermore, have enabled the identification of relatively rare phenomena, e.g. autonomous endosperm formation and fertilization of the apomeiotically derived egg cell (Voigt et al. 2007). Finally, FCSS analyses of seeds harvested at different stages, i.e. immature versus mature, have also shown that an apomictic plant has the potential to produce much larger levels of variation than expected if only mature dried seeds are measured (Voigt et al. 2007). Thus, it may be useful to differentiate between primary and secondary apomixis phenomena, since most of the potential variability produced by an apomict can be truncated by downstream developmental effects which are not directly associated with apomeiosis, the first and most important step (Voigt et al. 2007). This has significant implications for ongoing genomic and transcriptomic projects whenever statistical correlations with phenotypic data are made, as imprecise assessments of quantitative variation could potentially lead to spurious associations with marker data. The use of molecular markers can thus shed light upon the presence of apomixis within populations but, more importantly and in conjunction with precise methods of measuring quantitative variation in apomixis seed production (Matzk et al. 2000), inferences can be made regarding aspects of the origin and evolution of different apomictic lineages.

Sexual reproduction is hypothesized to be advantageous, since it is a mechanism through which genetic diversity can be generated and maintained at the population level. Conversely, asexually reproducing organisms are expected to be genetically homogenous and, thus, relatively static, since evolution requires genetic variation in order to proceed. Furthermore, asexual lineages are expected to accumulate deleterious mutations each generation, and are doomed to eventual extinction, i.e. Muller’s Ratchet (Muller 1964; Kondrashov 1994). A few rare cases of ancient asexuality—aexual scandals (Rice and Friberg 2007)—nonetheless exist and, while these do present challenges to accepted concepts, interesting molecular
mechanisms have been uncovered which likely counteract the effects of an absence of sex (Welch et al. 2004; Pouchkina-Stantcheva et al. 2007).

Strictly speaking, it is almost inappropriate to discuss apomixis in terms of “populations”, as no gene flow sensu stricto should occur between different apomictic individuals. In many cases, the more accurate scenario is a sexual swarm from which asexual clonal lineages arise recurrently through time. From a molecular genetic perspective, an analysis of naturally occurring apomictic complexes will uncover two kinds of genetic variability. First, consider two different apomictic lineages which arise independently at different times and/or in different geographical locations. As the lineages arise from local sexual gene pools which may also vary in space and time, they will each be established with a sampling of alleles from their respective locations (or time) and, hence, exhibit differing founding genotypes. Since their first appearance, the asexual lineages will have accumulated mutations, thereby introducing a second level of variation which differentiates them. Contrasts between these two types of variation are essential for answering questions of clonal origin and longevity, and a number of analytical approaches have been taken to classify both levels using population genetic data (Mes 1998; Meirmans and Van Tienderen 2004; Halkett et al. 2005). A further confounding effect is the instability of the asexual genome, which is no longer constrained by meiosis. Hemizygosity and chromosomal heteromorphy, both of which have been described in numerous asexual plants and animals, are examples of how homologous regions on sister chromosomes can diverge physically from one another (Birky 1996). Duplications may also accumulate in asexual genomes, leading to confusing patterns of microsatellite variation, and difficulties in assuming homology of allelic size variants (Corral et al. 2008). The degree to which all such effects influence the evolution of asexuality must be considered within the context of the taxon being studied, and Halkett et al. (2005) have attempted to outline a logical approach as to how studies of asexual systems should be conducted.

Finally, as in other fields, the application of new massively parallel sequencing, transcriptomic and proteomics technologies to wild asexual taxa will undoubtedly demonstrate that asexuality is not static on the individual and population levels. Such approaches will not only help to elucidate why asexuality has remained a successful form of reproduction but, in addition, analysis of genomes which are relatively unconstrained by the effects of meiosis will contribute to our understanding of the molecular dynamics and evolution of “normal” sexual genomes.

### 20.6 Transferring Apomixis in Crops from Wild Relatives, Molecular Mapping of Apomixis Components and Map-Based Cloning of Candidate Genes

The transfer of apomixis into crops from wild relatives has been performed mainly in pearl millet from the aposporous *P. squamulatum* and has been attempted in maize from the diplosporous *T. dactyloides*. In a research program to transfer
apomixis from \textit{P. squamulatum} to pearl millet, a polyhaploid plant (2n = 3x = 21) was discovered in the uniform open-pollinated progeny of an apomictic interspecific hybrid between pearl millet and \textit{P. squamulatum}. The polyhaploid plant was shorter, less vigorous and smaller than its maternal parent. It probably originated by parthenogenetic development of a reduced egg cell in the apomictic interspecific hybrid. The polyhaploid plant was male-sterile and partially female-fertile, having multiple aposporic embryo sacs in 95\% of the ovules. Seed set was as low as 3\% when open-pollinated and 33\% when pollinated with pearl millet, due to competition among multiple embryos developing in the same ovule. Seventeen progeny plants from seed produced under open-pollination on the polyhaploid each had 21 chromosomes and were morphologically uniform and genetically identical to the maternal parent (Dujardin and Hanna 1983, 1984).

These results demonstrated the possibility of conferring the apomictic trait to a plant which normally reproduces sexually, such as pearl millet, by introducing the desired gene(s) controlling apomixis. This information was patented as a protocol to generate an apomictic hybrid plant which produces progeny identical to itself, by transferring an apomictic mechanism from a wild species to a cultivated plant (US Patent 5811636; Ozias-Akins 1998). In particular, this procedure has been reported as exploitable for breeding apomictic pearl millet–\textit{P. squamulatum} hybrids which are more genotypically millet-like. Seeds can be multiplied by crossing an apomictic plant with a nurse cultivar as a pollen source for endosperm formation in seeds.

The genus \textit{Tripsacum} includes wild relatives of maize (\textit{Zea mays} L.) widely distributed across the American continent, and highly variable in many aspects (Randolph 1970; Berthaud et al. 1997). Efforts towards allele mining from this diverse genetic reservoir have been limited, with one notable exception concerning apomixis (reviewed in Savidan 2000). Within the tribe \textit{Maydae}, apomixis occurs only in \textit{Tripsacum} (Brown and Emery 1958), making the genus an important candidate to elaborate strategies for transfer of apomixis to maize, either through breeding or by genetic engineering. \textit{Tripsacum} species typically form an agamic complex (sensu Babcock and Stebbins 1938), whereby diploid individuals (2n = 2x = 36) are sexual and polyploid individuals (2n = 3x to 6x) reproduce apomictically. Apomixis is diplosporic of the \textit{Antenaria} type (Farquharson 1955; Leblanc et al. 1995b; Grimanelli et al. 2003). The megaspore mother cell “skips” meiosis and differentiates directly into a uninuclear embryo sac functional (Grimanelli et al. 2003). Further differentiation into embryo sacs resembles that of the \textit{Polygonum} type. Activation of unreduced egg cells through unknown developmental alterations in the embryo sacs may induce embryogenesis in the absence of fertilization (Farquharson 1955; Bantin et al. 2001). However, the developmental pattern of maternal embryos is interrupted after a few rounds of mitotic divisions, resulting in quiescent proembryos within unfertilized embryo sacs (Grimanelli et al. 2003). Pollination, followed by the delivery of two sperm cells into the mature embryo sac and fertilization of the central cell only, is required for seed development. Besides the apomictic pathway, reproductive traits which allow genetic variation have been preserved through evolution in \textit{Tripsacum}, as in many other apomicts. The most documented cases result from partial or complete restoration of
sexual programs (Asker and Jerling 1992; Bicknell and Koltunow 2004) but other mechanisms, such as incomplete nucleus restitution during meiosis abortion, mitotic and meiotic non-disjunction, somatic recombination and gene mutation, have been reported as well (Hair 1956; Richards 1996; Noyes 2005).

Diplospory was determined to be under a simple genetic control in a cross between Z. mays and T. dactyloides, and several RFLP markers, known to be positioned on the long arm of maize chromosome 6, were found to be strictly cosegregating with diplospory (Leblanc et al. 1995a; Grimanelli et al. 1998). Since the days when maize and Tripsacum were hybridized for the first time (Mangelsdorf and Reeves 1931), pathways for introgressing Tripsacum genetic material into the crop have been investigated extensively (e.g. Harlan et al. 1970; Harlan and DeWet 1977). Nevertheless, in spite of several decades of effort (Petrov et al. 1984; Leblanc 1995a, 1996; Kindiger and Sokolov 1997), maize germplasm expressing some level of apomixis has not yet been recovered. Conventional backcrossing strategies using T. dactyloides as an apomictic donor yielded facultative apomictic hybrids possessing two maize genomes and one genome from T. dactyloides, i.e. 2n=38=20+18 (Leblanc et al. 1996). The localization of apomixis to a maize–Tripsacum chromosome translocation supported the conclusion that only a single Tripsacum chromosome transmitted apomixis (Kindiger et al. 1996).

A detailed understanding of the inheritance of apomixis in model apomicts is required for the identification of candidate genes and eventual transfer of this valuable trait into species which naturally propagate sexually. Detailed genetic mapping analysis is extremely difficult, due to the association of facultative apomixis with polyploidy and variable but elevated levels of heterozygosity. Nonetheless, the chromosomal regions associated with apomixis factors have been characterized in several species, and molecular markers tightly linked to putative apomeiosis and/or parthenogenesis loci have been identified. Molecular differential screening of plants with contrasting modes of reproduction is still considered one of the most powerful tools for identifying, mapping and isolating the gene(s) underlying the expression of apomixis. Even in remarkably complex genomes like those of apomictic species, the visualization of molecular markers in combination with bulked segregant analysis (Michelmore et al. 1991) was shown to be effective for detecting gene polymorphisms and genome sequences useful for positional cloning (Barcaccia et al. 1998; Ozias-Akins et al. 1998; Pessino et al. 1998). In the case of facultative apomicts, such a method relies on pooling genomic DNA subsets from progeny plants showing extreme classes for the mode of reproduction, and then screening for molecular polymorphisms between apomictic and sexual individuals using DNA markers. This approach enables the analysis of a large number of genomic traits and increases the reliability of polymorphisms linked to apomixis and its components (Labombarda et al. 2002; Vijverberg et al. 2004).

The experimental evidence for simple inheritance of apomixis components is supported by a number of genetic mapping studies using molecular markers in both aposporic and diplosporic species. Mapping results have confirmed the simple, dominant inheritance of apomixis components, corresponding to one chromosomal region or a few chromosomal blocks (Table 20.1). With the exception of
T. officinale (van Dijk et al. 2003), strong suppression of recombination around the loci linked to apomeiosis has been found in all documented cases. In particular, the association of apospory and diplospory with chromosomal regions showing suppressed recombination has now been observed in aposporic (P. squamulatum and P. simplex) and diplosporic (E. annuus and T. dactyloides) species (reviewed in Ozias-Akins 2006; Vijverberg and van Dijk 2007). Surprisingly, the pattern of low recombination has been “broken” upon construction of a saturated molecular linkage map of the diplospory region in Taraxacum (Vijverberg et al. 2004).

A bulked segregant analysis was used to identify and map molecular markers in the region harbouring the diplospory locus and spanning a length of about 20 cM, although none was found to be strictly linked with diplospory. This species provides a unique case of genetic recombination in a chromosomal region carrying genes for apomeiotic embryo sac formation. Molecular markers linked with parthenogenesis have been identified in P. pratensis (Barcaccia et al. 1998) and E. annuus (Noyes and Rieseberg 2000), although no evidence for suppression of genetic recombination was found in the corresponding chromosomal regions (Table 20.1).

An important contribution to the mapping of the apomixis trait was given by synteny between specific chromosomal regions in apomicts and sexual crops and by the exploitation of heterologous probes. As far as apospory is concerned, genetic mapping studies were performed in Brachiaria brizantha (Pessino et al. 1997, 1998), Paspalum simplex (Pupilli et al. 2001, 2004; Labombarda et al. 2002), P. squamulatum (Ozias-Akins et al. 1998) and P. pratensis (Barcaccia et al. 1998). In B. brizantha, an intrageneric cross at the tetraploid level (2n=4x=36) was investigated mainly using RFLP markers with heterologous maize probes related to the short arm of chromosome 5. The apomixis locus was mapped in a genetic window longer than ~20 cM, with six markers all positioned at one of the flanking sides (Fig. 20.3a). Two additional AFLP markers co-segregating with apomixis were mapped at 1.2 and 5.7 cM from the apomixis locus, spanning the
trait in a total length of 6.9 cM (Pessino et al. 1998). In *P. simplex*, a progeny segregating for apomixis was obtained by backcrossing an intraspecific tetraploid hybrid (2n=4x=40). Five RFLP markers detected by using heterologous rice probes, spanning a 15-cM region in the long arm of chromosome 12, were mapped at 0 cM from the putative apomixis locus (Fig. 20.3b). Four additional AFLP markers were found tightly clustered at this locus, one of which tested to be hemizygous, being present only in the apomicts (Labombarda et al. 2002). A comparative mapping analysis revealed the apomictic chromosomal regions in *P. simplex* to be largely and partly conserved in the close relatives of *P. melacophyllum* and *P. notatum* respectively (Pupilli et al. 2004). This result suggested that a relatively small proportion of the chromosome carrying the apomixis locus is structurally and functionally conserved in *Paspalum*. In *P. squamulatum*, 12 random amplified polymorphic DNA markers were found strictly co-segregating with apospory (Ozias-Akins et al. 1998). Of the six low-copy number DNA clones derived from these markers, four appeared to be hemizygous, and all were found to be conserved in apomictic individuals of the close relative *Cenchrus ciliaris* (Roche et al. 1999). Subsequent FISH experiments with the apospory-related BACs (bacterial artificial clones) supported the conservation of the apospory-specific genomic region in *P. squamulatum* and *C. ciliaris* (Roche et al. 2002) and revealed the localization of this region on the short arm of a single chromosome (Goel et al. 2003). Sequencing work revealed a high abundance of repetitive elements from a retrotransponson family in the apospory-specific chromosomal region (Akiyama et al. 2004). Parthenogenesis has been mapped in *P. pratensis* only, with nine AFLP markers equally distributed on both sides of the putative locus, being the closest markers found at 6.6 and 8.8 cM (Barcaccia et al. 1998; Albertini et al. 2001b).

For diplospory, genetic mapping studies were performed in *T. dactyloides* (2n=4x=72) using a segregating population from an intergeneric cross with *Z. mays* (2n=2x=20; Grimanelli et al. 1998), in *E. annuus* (2n=3x=27) using a segregating population from an interspecific cross with *E. strigosus* (2n=2x=18; Noyes and Rieseberg 2000) and in *T. officinale* using a segregating population from an intraspecific cross between diploid sexual and tetraploid aposporic plants of common dandelion (van Dijk and Bakx-Schotman 2004; Vijverberg et al. 2004). In *Tripsacum*, three RFLP markers detected by means of heterologous maize probes, spanning a ~40 cM region in the long arm of chromosome 6, were found to be strictly inherited with the apomixis trait (Fig. 20.3c), although they showed recombination on the corresponding maize homologues (Grimanelli et al. 2001b). In *Erigeron*, as many as 11 AFLP markers closely co-segregated with diplospory and one additional marker was mapped 2 cM apart from the target locus, while four AFLP markers co-segregated with parthenogenesis and spanned a 20-cM distance on a different linkage group (Noyes and Rieseberg 2000). In *Taraxacum*, a linkage group showing a total length of 18.6 cM was constructed with markers found on both sides of the diplospory locus in regions 5.9 and 12.7 cM long (Vijverberg et al. 2004). No AFLP markers fully co-segregated with diplospory, and the closest AFLP markers were located at 1.4 cM on both flanking sides. Several additional AFLP markers were later mapped in the same region using a larger segregating
population. The results were consistent with the lack of suppressed recombination in the chromosomal region surrounding the diplospory locus (Vijverberg and van Dijk 2007).

Further cytogenetic characterization of apomixis chromosomal regions was carried out in a few species such as *P. squamulatum* (Ozias-Akins et al. 1998), *P. simplex* (Labombarda et al. 2002) and *T. officinale* (Vijverberg and van Dijk 2007), by performing FISH experiments using apomixis-associated BACs. With the exception of *Taraxacum*, overall results confirmed the existence of a strong suppression of genetic recombination in all species, supporting physical lengths of \( \sim 50 \) Mbp (Akiyama et al. 2004) up to \( \sim 100 \) Mbp (Calderini et al. 2006). This suppression of recombination has hindered subsequent high-resolution genetic mapping and map-based cloning strategies aimed at isolating the genes for apomixis in these species.

In conclusion, genomic loci for apomixis as a whole or apomeiosis alone are defined by large chromosomal regions in most species, suggesting the presence of several linked genes, rather than a single one. The strong suppression of genetic recombination found in most apomixis chromosomal regions mapped so far by means of molecular markers demonstrates both diversification of allele sequences at these loci, compared to homologous regions in sexual relatives, and violation of synteny between apomictically reproducing species and phylogenetically correlated sexual species, e.g. *P. squamulatum, P. simplex* and *T. dactyloides*. In fact, those species which lacked evidence for strong suppression of genetic recombination in the apomixis chromosomal regions, i.e. *T. officinale* for diplospory, *B. bryzantha* for apospory, and *E. annuus* and *P. pratensis* for parthenogenesis, were characterized by the independent inheritance of apomeiosis and parthenogenesis. This finding indicates that, in species or genera where apomicts and close sexual relatives still exist, genetic divergence between apomictic and sexual forms is limited. By implication, relationships between parental lines and types of crosses can influence the success of genetic linkage mapping studies and apomixis gene cloning strategies.

Attempts to introgress apomixis from natural apomicts into crop species have failed (Spillane et al. 2001), and efforts to identify apomixis genes in natural apomicts by map-based cloning have been hampered by the finding that apomixis is associated with large genomic regions which are repressed for recombination (Grimanelli et al. 1998; Ozias-Akins et al. 1998; Pessino et al. 1998; Pupilli et al. 2004).

**20.7 Advanced Biotechnological Approaches: Looking for Candidate Genes and Engineering Apomixis**

Although many years of descriptive studies have provided a solid documentation of the types of apomictic processes occurring in a wide variety of plant species, molecular studies aimed at understanding the basis of apomixis have shed little
information on its central mystery, partly because the majority of apomicts do not constitute agriculturally important crops and, with few exceptions (e.g. *Tripsacum* and maize), do not have agriculturally important relatives (Bicknell and Koltunow 2004; Albertini et al. 2005). Zygotic embryogenesis (sexuality) and apomeiotic parthenogenesis (apomixis) are thought to follow similar pathways during embryo and seed production. Specific genes are activated, modulated or silenced in the primary steps of plant reproduction to ensure that functioning embryo sacs develop from meiotic spores and/or apomeiotic cells. As additional genes may be specifically or differentially expressed in sexually and apomictically reproducing plants, and operate during embryo development, we would be better equipped to understand apomixis if the genes responsible for controlling the specific and differential expression in embryo sac and embryo formation were to be detected. Chaudhury and Peacock (1993) hypothesized that genes isolated in model species, such as *Arabidopsis* (*Arabidopsis thaliana*), would be important for the study of apomixis. The advantages of such a strategy reside in (1) the possibility of using, in apomictic species, molecular tools developed in Arabidopsis and other model species, as associated with the reproduction system and (2) trying to understand the function of genes putatively involved in apomixis by studying these in the model sexual species. For this reason, advanced research on apomixis is generally divided into two complementary approaches: (1) analysis of the trait in natural apomictic species and (2) functional analysis of genes involved in female sporogenesis and seed development in species which normally form seeds by sexual reproduction.

Mutagenesis approaches aimed at identifying genes deregulating steps which are fundamental in circumventing meiotic reduction (apomeiosis), in activating embryo development without fertilization (parthenogenesis), and in initiating and maintaining the formation of functional endosperm have served to isolate mutants with apomictic characteristics in *Arabidopsis* and other model species by loss-of-function mutagenesis screenings. These approaches are generally based on either allowing fertilization and then screening for purely maternal inheritance in the progeny, or preventing fertilization and isolating pseudo-suppressors which allow seed development to take place in the absence of fertilization (Curtis and Grossniklaus 2007). Of these, the screens for fertilization-independent seed development have led to the identification of mutants now known as the fertilization independent seed (*fis*) class mutants (Grossniklaus et al. 2001b), all of which are able to initiate endosperm development in the absence of fertilization. In particular, proteins codified by three of these genes, FIS1 (or MEA), FIS2 and FIS3 (or FIE), repress cell proliferation in the central cell in sexual plants in the absence of fertilization (Ohad et al. 1996; Grossniklaus and Schneitz 1998; Kiyosue et al. 1999; Luo et al. 1999; Vielle-Calzada et al. 1999; Grossniklaus et al. 2001b; Lohe and Chaudhury 2002; Hsieh et al. 2003; Guitton and Berger 2005). This suggests that a number of developmental checkpoints must be deregulated in the sexual process before viable seed is generated in the absence of fertilization (Curtis and Grossniklaus 2007).

Nowack et al. (2007) demonstrated that it is possible to obtain viable fertilized seeds with uniparental diploid endosperm of maternal origin when the maternal FIS machinery is impaired. It has also been demonstrated that loss of function of
MET1, leading to hypomethylation of the maternal gametophyte in fie1 mutants, gives rise to an endosperm formation very similar to that associated with sexual reproduction. Other proteins interacting with MEA (FIS), such as the origin recognition complex (ORC), might also play a role in the apomictic mode of reproduction. Another gene, identified with a loss-of-function approach, is Multicopy Suppressor of Ira1 (MSI1). Guittton and Berger (2005) demonstrated that msi mutants are characterized by spontaneous division of the egg cell, even though parthenogenetically derived embryos aborted early in development and did not form viable seeds.

An alternative approach is to generate synthetic apomictic traits using the gain-of-function approach, which seems very promising because the genes controlling elements of apomixis behave as dominant factors in crosses with sexual relatives (Savidan 2000; Grimanelli et al. 2001a; Grossniklaus et al. 2001a, Richards 2003; Curtis and Grossniklaus 2007). One of the simplest strategies is to place a candidate gene under the transcriptional control of a heterologous promoter (Curtis and Grossniklaus 2007), but the identification of candidate genes in sexually reproducing plants has been a difficult task. In fact, it has resulted in the isolation of only a small number of genes involved in the acquisition of embryogenic competence from somatic cells, e.g. somatic embryogenesis receptor-like kinase (SERK; Schmidt et al. 1997; Hecht et al. 2001), and spontaneous induction of embryo production when overexpressed (LEC1, LEC2) or repressed (PKL; Ogas et al. 1999). An activation tagging approach was used by Zuo et al. (2002) to identify genes of which the overexpression could induce the formation of somatic embryos in Arabidopsis tissues without the need for external hormonal treatments. This resulted in the isolation of an allele, PGA6, which was found to be identical to WUSCHEL (WUS), a homeodomain protein previously shown to be involved in specifying stem cell fate in shoot and floral meristems. WUS PGA6 presumably promotes a vegetative-to-embryogenic transition and/or maintains embryonic stem cell identity (Feher et al. 2003). Another candidate was identified by induction in microspore cultures of Brassica napus undergoing somatic embryogenesis (Boutilier et al. 2002). The gene was named babyboom (bbm) because, when overexpressed under the control of the 35S promoter, it led to the ectopic formation of embryos and cotyledons on leaves. Genes sharing similarity with BBM have been isolated in several other species but maybe the most important finding is the isolation of the ASGR-BBM in the apospory-specific genomic region (ASGR) of P. squamulatum (Conner et al. 2007). The ASGR-BBM transcript encodes a 545-amino acid protein containing two AP2 domains which are 96% similar to the AP2 regions of BnBBM. Outside of the AP2 domains, similarity of ASGR-BBM to BnBBM declines significantly (35% similarity upstream and 27% similarity downstream; Conner et al. 2007).

Studies have also been performed on other species carrying mutations resembling components of apomixis. For example, two genes classified as MOB1-like have been identified in an apomeiotic mutant of Medicago sativa (TWO-N-EGG; Citterio et al. 2005) as being involved in cell proliferation and programmed cell death within reproductive organs. It has been demonstrated that, in addition to
alfalfa, other plant genomes—e.g. the sexual diploids Arabidopsis and rice, and the apomictic polyploids *P. pratensis* and *Hypericum* spp.—contain MOB1-related genes (Barcaccia et al. 2001; Citterio et al. 2005).

Diploid cells in place of normal haploid megaspores have been observed recently in Arabidopsis, resulting from mutations of the *SWI1* (*SWITCH1/DYAD*) gene (Ravi et al. 2008). The occurrence of apomeiosis by mutation of a single gene coding for a phospholipase C which controls sister chromatid cohesion and centromere organization during sporogenesis was demonstrated. These findings represent a significant step towards the synthesis of apomixis by the manipulation of genes which function in normal sexual development.

The second main approach requires searching for candidate apomixis genes in species where the trait occurs naturally. For this reason, transcriptional profiling procedures have been proposed to compare transcripts of sexual and apomictic reproductive cell types, but these are always hampered by the low accessibility of the female gametophyte and by the high ploidy level of apomictic species. Molecular differential screening of plants with contrasting modes of reproduction is one of the most powerful tools which can be applied to identifying, mapping and isolating the gene(s) putatively involved in apomixis. Many new techniques have been designed in recent years (Green et al. 2001). All assess new genes but while some focus on obtaining expression data and high-throughput data, others aim at identifying new and rare, differentially expressed transcripts. Some require large amounts of material to be analyzed and pre-existing genomic knowledge. One of the new techniques is based on microarrays (Brown and Botstein 1999), which allows a genome-wide expression profile of thousands of genes to be performed in one experiment. Though powerful, this approach is expensive and can be readily applied only to model species for which significant genomic information is available (Baldwin et al. 1999). Unfortunately, genetic annotation in higher eukaryotes is limited to a few models and information on less well-characterized species is poor, and likely to remain so for some time. Moreover, because rarely expressed transcripts are usually missing from cDNA libraries due to overrepresentation of abundant messengers, microarrays could fail to detect genes which are rare but fundamental for traits like apomixis. Differential display (DD), PCR-derived techniques which share gel separation and visualization procedures, but differ in the methods adopted for generating amplified cDNA fragments, would be more suitable for identifying low-expressed genes (Reijans et al. 2003). mRNA fingerprinting strategies permit a large number of fragments to be analyzed, and increase the reliability of differentially expressed transcript detection which starts from very small amounts of messengers (Bachem et al. 1996). This feature is essential when DD is applied to tissues where it is hard to isolate stage-specific mRNAs, such as small florets. cDNA-AFLP (Bachem et al. 1996) has proved the most popular procedure because of its ability to detect differentially expressed genes. It has good reliability and sensitivity, and correlates well with Northern analysis (Durrant et al. 2000; Jones et al. 2000; Barcaccia et al. 2001; Donson et al. 2002; Cnudde et al. 2003). The reproducibility is very high compared to that of microarray and GeneChip technologies (Reijans et al. 2003). A possible drawback of the technique
is that more than one band is expected to be visualized for each transcript (Matz and Lukyanov 1998). However, redundancy can be very informative in cases of alternative splicing.

Comparative gene expression studies have been carried out during the early stages of apomictic and sexual embryo sac development in *Panicum maximum* (Chen et al. 1999), *Brachiaria* species (Leblanc et al. 1997; Rodrigues et al. 2003), *Pennisetum* (Vielle-Calzada et al. 1996; Jessup et al. 2003) and *Paspalum* (Pessino et al. 2001). However, most of these were based on subtractive hybridization techniques and isolated only a few genes to which, disappointingly, no clear function could be assigned. Hybridization-based studies, even if negative in context, add support to the proposal that sexual and apomictic developmental pathways differ primarily in their ability to regulate common elements (Bicknell and Koltunow 2004). In support of this hypothesis, Tucker et al. (2003) and Albertini et al. (2004) have demonstrated that the developmental program is highly conserved during zygotic embryogenesis and apomeiotic parthenogenesis and, on the basis of available results, natural apomixis does not seem to result from the failure of a single gene of the reproductive pathway, but rather from epistatic, possibly silencing action, exerted on the normal sexual reproduction pathway by a set of genes inherited as a unit and evolved in polyploid plants (Ozias-Akins et al. 1998).

Laspina et al. (2007) carried out a full-transcriptome survey in order to isolate genes differentially expressed in immature inflorescences of sexual and aposporous *P. notatum* genotypes. Differential display experiments were used to check the expression of about 10,000 transcripts, and led to the identification of 71 unigenes expressed either in the aposporous or in the sexual genotype, whereas functional annotation was achieved for 39 of them.

Perhaps the most thorough study using a transcript profiling approach comes from *P. pratensis* where cDNA-AFLP analysis resulted in the isolation of fragments which were specific to carefully staged florets of either a sexual or apomictic genotype and were not present in leaves (Albertini et al. 2004). Most of the cDNA sequences were not specifically expressed in apomictic or sexual genotypes, but rather their expression was differentially modulated or quantitatively different (Albertini et al. 2004, 2005), lending additional support to the hypothesis that apomixis results from a deregulated sexual pathway (reviewed in Ozias-Akins 2006). In particular, PpSERK and APOSTART were characterized (Albertini et al. 2005), and they seem to be involved in cell-to-cell interaction for both the signalling pathway and hormone stimulation. These authors proposed that PpSERK gene activation in nucellar cells of apomictic genotypes is the switch which channels embryo sac development and that it could redirect signalling gene products to compartments other than their typical ones. The SERK-mediated signalling pathway may interact with the auxin/hormonal pathway controlled by APOSTART. Moreover, since BLAST analysis of sequences revealed that homologies of APOSTART, PpSERK, PpMET, PpARM and other genes are tightly linked in a small chromosome region of Arabidopsis, *M. truncatula* and rice, attempts were made using the physical mapping in *P. pratensis* to determine whether the linkage was maintained. Preliminary results indicate a strong co-segregation of clones carrying
PpSERK, APOSTART and other genes such as PpMET (Albertini et al. 2007). In addition, partial/complete cDNA fragments showing homology to APOSTART have also been isolated from *P. squamulatum, C. ciliaris* and *H. perforatum* by other research groups, and spatial/temporal characterization studies are in progress. In fact, if these genes are truly involved in apomixis, then irrespective of the species under study, they should conserve their involvement in this modification of the reproductive system.

In *H. perforatum*, a transcription profile approach of sporogenesis and gametogenesis performed by mRNA profiling of unripened anthers and unpollinated pistils led to the isolation of several transcripts specifically expressed in pistils of the highly apomictic ecotype, including an EST showing similarity to a gene coding for an ATPase RNA helicase responsible for an embryo defective phenotype in *Arabidopsis* (MEE29, i.e. maternal effect embryo). This gene, termed *HpMEE29-like*, was found to be differentially expressed between aposporic and meiotic plants of *H. perforatum* (Galla and Barcaccia, unpublished data). Moreover, a RING-finger gene (i.e. *HpARIADNE*), the DNA markers of which were found to be in strong linkage disequilibrium with the apomixis trait, is also under study in *H. perforatum* (Barcaccia et al. 2007).

More recently, the apomixis research group of IPK has applied a high-throughput differential display approach (SuperSAGE) to study naturally occurring quantitative variations in gene expression between ovules of apomictic and sexual *Boechera holboellii* genotypes (Sharbel et al. 2009). Using SuperSAGE, they have identified over 6,000 differentially expressed mRNA tags in ten microdissected ovules from two sexual and two diploid apomictic accessions. The genes to which the mRNA tags belong were determined by homology searches to sexual and apomictic flower-specific transcriptome libraries which were sequenced using 454 technology. Comparisons between the sexual and apomictic ovules show that many of the differentially expressed mRNAs are of low copy number. Nevertheless, both allele-specific expression and microduplication can explain the observed variation between reproductive modes. The use of deep transcriptomic analyses of living microdissected tissue, in conjunction with massively parallel transcriptome sequencing, has thus enabled the identification of a large set of candidate alleles which will be the subject of subsequent analyses of expression profiles at different developmental stages and in different genetic backgrounds.

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