

# Apomixis: An enigma with potential applications

V. Bhat<sup>1,\*</sup>, K. K. Dwivedi<sup>2</sup>, J. P. Khurana<sup>3</sup> and S. K. Sopory<sup>4</sup>

<sup>1</sup>Department of Botany, University of Delhi, Delhi 110 007, India

<sup>2</sup>National Research Centre for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India

<sup>3</sup>Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

<sup>4</sup>Plant Molecular Biology, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India

**Apomixis has been the focus of research in plant sciences in recent years with lot of scope for crop improvement. It results in clonal progeny without fertilization, having maternal genetic constitution. The impact of introducing apomixis in crop plants could be significant mainly for its use in fixation of hybrid vigour. Because of epigenetic barriers, introgression of apomixis from a close relative to a sexual crop plant by conventional plant breeding methods could not generate expected results. Recent developments in plant molecular biology and biotechnology can help in developing potential strategies. This article summarizes various aspects of apomixis research that are being followed in India and abroad.**

**Keywords:** Agamospermy, apospory, diplospory, parthenogenesis, epigenetics.

APOMIXIS is an asexual mode of reproduction in which an ovule develops into a seed without involving meiosis and fertilization. It is now used synonymously with 'agamospermy'<sup>1</sup> meaning asexual reproduction by seeds or seed apomixis. The prospect of introducing apomixis into sexual crops could be so revolutionary that it justifies a sustained international scientific effort. If apomixis could be generated with a sufficiently high degree of flexibility, its impact on agriculture could be profound and its scope extremely broad to include: (i) immediate genetic fixation of any desired plant; (ii) revolutionize breeding procedures, by helping to move from family-based strategies to individual plant-based strategies; (iii) preparation of number of hybrid cultivars from every crop species, facilitating the development, mass production, and maintenance of elite parental lines and their derived hybrids; (iv) replace the need for cuttings, artificial seeds or other vegetative propagules; (v) environmentally and ecologically sound protection from horizontal transfer of transgenic characters into neighbouring populations, by introducing autonomous apomixis into male-sterile varieties (<http://billie.bny.purdue.edu/apomixis/apotech.html>). The most comprehensive account of apomixis was given by P. Maheshwari in his classical book on embryology<sup>2</sup>. In a recent review, Maheshwari *et al.*<sup>3</sup> have elaborated various options for plant molecular biologists to engineer apomixis in the crop plants.

## Types of apomixis

A defined sequence of events must be completed to result in the generation of a fertile and genetically unique seed that is the end product of sexual reproduction in angiosperms. This sequence comprises the following events: megaspore mother cell differentiation from the nucellus, megaspore production by meiosis (megasporogenesis), megaspore selection, embryo sac development by mitotic processes (megagametogenesis), embryo sac maturation, double fertilization, endosperm development and embryo formation. In contrast to sexual reproduction, apomictic processes can completely omit some of the events in this sequence and still produce a fully formed, viable embryo within the confines of the ovule. Cytological studies have shown that apomictic processes always deviate from sexual reproduction in more than one respect<sup>4-7</sup> (Figure 1). These developmental differences occur in commonly identifiable combinations, such that apomictic processes have often been divided into three mechanisms – diplospory, apospory and adventive embryony.

These apomictic mechanisms differ from one another in the time at which the process is initiated during ovule development, relative to the normal sexual pathway. Diplospory and apospory result in the formation of a megagametophytic structure without meiotic reduction, and the embryo develops from a cell in unreduced megagametophyte. Diplospory and apospory are, therefore, commonly referred to as gametophytic apomictic processes<sup>6,7</sup>. By contrast, adventive embryony is initiated late in ovule development and usually occurs in mature ovules. Embryos are initiated directly from individual cells in the tissues of the ovule that are external to the megagametophyte. Therefore, adventive embryony has been described as sporophytic apomixis<sup>6,7</sup>. Most plants with gametophytic apomixis are polyploids and genera with adventive embryony are generally diploids<sup>7</sup>.

## Gametophytic apomixis

In this type of apomixis, embryo sacs are produced from unreduced initial cells. The egg cell develops parthenogenetically into an embryo that gives rise to a plant true to the maternal parent. Gametophytic apomixis may be either obligate or facultative (plants containing both apomictic

\*For correspondence. (e-mail: bhatv64@rediffmail.com)

















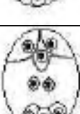
































































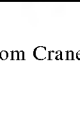
Types of Embryo sac									Arrangement of female gametophyte at maturity
		Mei I	Mei II		Mit I	Mit II	Mit III		
<i>Polygonum</i>									
<i>Allium</i>									
<i>Taraxacum</i>									
<i>Ixeris</i>									
<i>Blumea</i>									
<i>Elymus</i>									
<i>Antennaria</i>									
<i>Hieracium</i>									
<i>Eragrostis</i>									
<i>Panicum</i>									

Figure 1. Schematic diagram of apomictic embryo sacs in comparison to *Polygonum* type (modified from Crane<sup>8</sup>).

and sexual flowers). Gametophytic apomixis is further divided into two categories – diplospory and apospory<sup>8</sup> (Figure 1).

*Diplospory:* In diplospory, unreduced embryo sacs are formed from megaspore mother cells (MMCs) by the cir-

cumvention of meiosis and the embryo develops parthenogenetically from the unreduced egg, whereas the endosperm develops either autonomously (without fertilization) from the unreduced polar nuclei or pseudogamously after fertilization of the unreduced polar nuclei with the reduced male gamete. It is further categorized into two types, meiotic and mitotic diplospory. Seven types of diplosporic embryo

sac development have been described<sup>8</sup>. These have been named after the genera in which they were first described.

i) *Taraxacum* type (meiotic diplospory): The MMC initially enters the meiotic prophase but normal chromosome pairing does not take place due to asynapsis. The univalents are scattered over the spindle at metaphase I. A restitution nucleus is formed after the first meiotic division, which subsequently divides mitotically to form a dyad with somatic ( $2n$ ) chromosome number. Further mitotic divisions of the chalazal cell result in the formation of an 8-nucleate embryo sac<sup>1,9</sup>. This type of apomixis occurs in some of the genera of Compositae and in *Arabis* and *Paspalum* species<sup>6</sup>.

ii) *Ixeris* type (meiotic diplospory): The MMC undergoes asyndetic meiotic prophase resulting in a restitution nucleus. This is followed by a division similar to the second meiotic division except that it is not accompanied by cytokinesis. Two further mitotic divisions of the unreduced nuclei result in an 8-nucleate embryo sac<sup>6</sup>.

iii) *Antennaria* type (mitotic diplospory): MMC does not enter into meiosis and functions as a megaspore. After a long interphase, growth and pronounced vacuolation, it begins to divide mitotically which results in the formation of a typical 8-nucleate embryo sac. This type of diplospory has a wider taxonomic distribution<sup>6</sup>.

iv) *Allium* type: In *Allium* type of diplospory, megasporocyte undergoes endomitosis and  $2n$  number of bivalents is observed at meiotic metaphase I. The first meiotic division produces a dyad whose chalazal nucleus develops into a mature unreduced embryo sac after three rounds of mitosis<sup>8</sup>.

v) *Blumea* type: MMC undergoes mitotic division to form a dyad of  $2n$  megaspores. The chalazal megaspore gives rise to the mature, 8-nucleate embryo sac<sup>8</sup>.

vi) *Elymus* type: In *Elymus rectisetus*, the megasporocyte nucleus enlarges and becomes deformed. The first prophase follows the nuclear deformation leading to a mitotic division resulting into a dyad of completely separated cells. The chalazal member of the dyad undergoes three rounds of mitosis to form an 8-nucleate embryo sac<sup>8</sup>.

vii) *Eragrostis* type: In this type, there are no meiotic stages and MMC undergoes only two rounds of mitotic division, leading to a 4-nucleate embryo sac with an egg, two synergids and one polar nucleus<sup>8</sup>.

**Apospory:** In apospory, unreduced embryo sacs develop from nucellar cells in the ovule. Several cells of the nucellus may start aposporous development but usually only one of them gives rise to a mature embryo sac. Apospory is initiated after MMC differentiation. The megaspore degen-

erates and the aposporous embryo sac occupies the position near the micropylar end of the ovule. The embryo develops parthenogenetically from the unreduced egg, but pollination and fertilization are required for the development of endosperm. Apospory is of common occurrence in a large number of apomicts of the grass family (*Pennisetum*, *Cenchrus*, *Poa*) and it is of two types:

i) *Hieracium* type: One or several nucellar cells enlarge, become vacuolated, and go through three rounds of mitosis, resulting in 8-nucleate embryo sacs. The MMC may undergo meiosis in some species forming sexual embryo sac as well. In some species, both reduced and unreduced embryo sacs can coexist in the same ovule<sup>8</sup>.

ii) *Panicum* type: In this type, a 4-nucleate monopolar unreduced embryo sac is formed<sup>10,11</sup>. Compared to the *Hieracium* type, the *Panicum* type is characterized by the absence of the initial polarization in the progenitor cell of the embryo sac and by the vacuolation of the chalazal end of the cell. The spindle of the first mitotic division lies crosswise at the micropylar end, and a second mitosis leads to the formation of four free nuclei. Later, these nuclei organize into the female gametophyte consisting of a three-celled egg apparatus and a single polar nucleus; the antipodal cells are absent.

#### *Sporophytic apomixis: Adventive embryony*

Adventive embryos arise from individual cells of two different somatic tissues, nucellus or integument<sup>12</sup>. Adventive embryony is purely a sporophytic form of agamospermy. It usually occurs in the presence of normal sexual reproduction and results in polyembryony. It is initiated late in ovule development and usually occurs in mature ovules. Embryos are initiated directly from the individual cells and are not surrounded by megagametophytic structure or embryo sac. This is in contrast to sexual, aposporous and diplosporous reproduction, in which the cell that develops into the embryo is part of a megagametophyte-like structure<sup>10</sup>. Adventive embryony commonly occurs in diploid species. The common examples are *Citrus* sp. and mango.

#### **Mechanisms of apomixis**

During gametophytic apomictic reproduction, three major developmental components are observed, such as the generation of a cell capable of forming an embryo sac without prior meiosis (apomeiosis), fertilization-independent development of the embryo (parthenogenesis) and autonomous development of the endosperm or an endosperm derived from fertilization<sup>10,13</sup>. In sporophytic apomixis, embryos arise spontaneously from cells of the ovule late in the temporal sequence of ovule maturation. Thus, these two methods differ mainly in the mediation of embryo

sac development and the type of cell that develops into an embryo. The gametophytic apomicts share some common characteristics such as (i) most of them are polyploids, (ii) apomixis affects only the female reproductive pathway and male gametes are still produced normally, (iii) most apomicts are facultative, in the sense that a proportion of the progeny still results from sexual reproduction. Thus, apomixis may not replace sexuality and more often, it coexists with sexual development within the same plant<sup>14</sup>. Therefore, apomixis can be viewed as a result of a relaxation of temporal and spatial constraints on sexual developmental processes<sup>15</sup> and asexual pathways are built by reassembling, in space and time, the elements of 'normal' sexual reproductive pathways.

### Genetics of apomixis

The genetics of apomictic species has not been studied critically as the data from most crosses between apomictic and sexual individuals have not been conclusive. Some of the difficulties are due to complex polyploidy, lack of recombination and irregular segregation pattern of the apomictic species. Various models have been proposed to explain the regulation of apomixis. Assuming that apomicts contain an allele which is absent in sexual plants, apomixis would have arisen as a result of mutations at one or several loci having a role in reproductive process. Matzk *et al.*<sup>16</sup> proposed a new five locus model with differences in gene expressivity and penetrance in *Poa pratensis*, based on flow cytometric seed screen analysis (FCSS)<sup>17</sup> of segregating progenies originated from intercrossing and selfing of obligate sexual and facultative apomictic parents. Differences in expressivity and interactions of these genes are responsible for the wide variation in the mode of reproduction. It was observed that apospory and parthenogenesis as well as the initiator and preventer genes of these components segregated independently.

The inheritance of gametophytic apomixis has long been reported to be associated with the transfer of either single locus or a small number of loci in most of the systems studied to date. In the aposporous grasses *Pennisetum*<sup>18</sup>, *Panicum*<sup>19</sup> and *Brachiaria*<sup>20</sup>, apomixis is known to be simply inherited with the trait conferred by the transfer of a single dominant factor. Simple dominant inheritance also has been reported for apospory in the dicotyledonous genera *Ranunculus*<sup>21</sup> and *Hieracium*<sup>22</sup>. Among the diplosporous apomicts, independent inheritance of diplospory and parthenogenesis has been observed in dandelion (*Taraxacum*)<sup>23</sup> and in *Erigeron*<sup>24,25</sup>, whereas *Eragrostis curvula*<sup>26</sup> showed a simple dominant inheritance of diplospory<sup>26</sup>. Similarly, the inheritance of diplospory in Eastern gamagrass (*Tripsacum dactyloides*) is reported to be simple and dominant<sup>27</sup>. There is evidence for segregation ratio distortion in some of the above taxa, may be because the dominant factor(s) associated with apomixis

also appear to confer gamete lethality, restricting its transfer to some gamete genotypes<sup>21, 28–30</sup>.

Using more suitable apomictic species and focusing on one element of apomixis, apomeiosis, the pioneering studies made by Nogler in *Ranunculus auricomus* and by Savidan in *Panicum maximum* indicated that apospory in these two species segregated as a single dominant Mendelian factor<sup>21,31</sup>, which can represent any genetic constitution from a single gene to an entire chromosome (e.g., mammalian sex determination). According to this model, apomictic plants possess the simplex genotype *Aaaa*, carrying in addition to the dominant apomeiosis allele *A*, several recessive alleles for sexual reproduction. Apomictic plants thus carry the potential for sexual reproduction, but in a repressed state, because of the presence of the dominant apomixis factor. Limited penetrance of apomixis can be explained by the occurrence of facultative apomixis. The presence of recessive sexual alleles explains how a cross between two facultative apomicts can generate abundant purely sexual offspring. Although the occurrence of apomixis fits this model, the degree of apomixis often is dependent on environmental conditions<sup>6</sup> and/or on modifier genes<sup>22</sup>.

### Is apomeiosis locus a recombinationally suppressed region?

Based on the segregation analysis of molecular markers that are linked to the putative apomixis loci in several species, a strong suppression of recombination around the apomeiosis locus was indicated. Many markers were found co-segregating with apomeiosis in aposporous *Pennisetum squamulatum*<sup>32</sup> and diplosporous *Erigeron annuus*<sup>25</sup>. In *Brachiaria decumbens*<sup>33</sup>, *Tripsacum dactyloides*<sup>28</sup> and *Paspalum simplex*<sup>34</sup>, comparative mapping with maize or rice markers showed a lack of recombination in the region associated with the apospory locus. Markers that were spread over a region ranging from 15 to 40 centimorgans in the sexual relatives strictly co-segregated in these apomicts. Repression of recombination could slow down map-based cloning efforts as closely linked markers were at great physical distances from the apomixis loci<sup>35</sup>. Evidences also pointed to conservation of sequences between apospory-specific genomic regions (ASGR) of *P. squamulatum* and *C. ciliaris*<sup>36</sup>. Fluorescence *in situ* hybridization (FISH) on mitotic chromosomes with the bacterial artificial chromosomes (BACs) that contained primarily low-copy sequences demonstrated that the ASGR in both these species was hemizygous<sup>37</sup>. Further analysis indicated that the ASGR was positioned near the telomere in *P. squamulatum*, while in *C. ciliaris* it was located close to the centromere of a chromosome that also contained the 18S rDNA locus. High resolution physical mapping using FISH indicated that the ASGR in *C. ciliaris* is located on a heterochromatic and hemizygous region of a

single chromosome<sup>38</sup>. The entire hemizygous region in *P. squamulatum* was estimated to be approximately 50 Mbp. The repetitive elements in the high copy BACs have shown similarity with the Opie-2 retro-transposon family from maize<sup>39</sup>.

Suppressed recombination occurs in both dicot and monocot species indicating that it may be a general characteristic of apomeiosis locus. Otherwise, it could be an evolutionary by-product of long-term asexual reproduction<sup>40,41</sup>. In *Pennisetum* species, markers that are linked to apospory in the apomicts could not be detected by hybridization in sexual relatives<sup>32,42</sup>, indicating that the apomicts were either hemizygous for the apomixis locus (*A*---) or that the alleles were highly divergent (*A a' a' a'*), as was observed for the *Brassica* *S* locus<sup>43,44</sup>.

### Segregation distortion of apomixis loci

Is gametophytic apomixis incompatible with diploidy? Nogler showed that diploid offspring that developed parthenogenetically from reduced diploid egg cells of tetraploid apomicts or diploids produced through anther culture were able to reproduce apomictically<sup>45</sup>. This showed that apomixis and diploidy are not incompatible as has been confirmed in several other species<sup>46,47</sup>. However, what matters is the origin of the diploid offspring, because zygotic diploids derived from the fusion of haploid egg cells and haploid sperm never reproduced apomictically in *Ranunculus*. Nogler hypothesized that the apospory (*A*) locus was recessive lethal in the gametes. Consequently, the *A* locus could be transmitted via diploid gametes to generate polyploid apomicts but not via haploid gametes to generate diploid apomicts. It is also possible that mutations closely linked to the *A* locus cause haploid gamete nonfunctionality. The net result is that haploid gametes carrying the *A* locus do not contribute to offspring production, resulting in segregation distortion of the *A* locus<sup>35</sup>.

Additional evidence for segregation distortion of apomixis loci came from other plant species, such as *Trip-sacum dactyloides*<sup>48</sup>, *Pennisetum squamulatum*<sup>32</sup> and *E. annuus*<sup>25</sup>. Transmission studies of markers linked to apomixis loci in *E. annuus* indicated different causes of non-transmission. The parthenogenesis locus *P* in *E. annuus* was not transmitted because of selection against haploid gametes, as was observed for the *A* locus in *R. auricomus*. The diplospory locus *A*, in contrast, was not transmitted because of meiotic drive. In these triploid apomicts, the nondiplospory chromosomes seem to pair preferentially, leaving the diplospory chromosome as a univalent that always ends up in a diploid pollen grain<sup>25</sup>. In *Hieracium piloselloides*, different crossing schemes indicated that apomixis can be transmitted via both haploid and diploid gametes, but post-zygotic lethality rather than segregation distortion causes the absence of apomixis in diploids<sup>22</sup>.

### Apomixis and polyploidy

Polyploidization had a major impact on the increase in genome size during evolution. A few significant correlations have been found between genome size and reproductive processes such as microsporogenesis, megasporogenesis and endosperm development by comparing 107 families of angiosperms, but any significance disappeared when members of monocots and dicots were analysed separately<sup>49</sup>. In *P. notatum*, the expression of apomictic trait was found to be ploidy dependent as the results indicated that an unexpressed gene for apomixis exists at the diploid level. A rise in ploidy level induced the expression of apomixis which was ascribed to either the influence of ploidy on the locus controlling apomixis through some transcription factors or via a secondary locus which requires a higher allele dosage to affect the expression of the main locus<sup>50</sup>. Trends concerning coevolution of mode of reproduction and genome size were studied by screening 71 species/subspecies of the genus *Hypericum*<sup>51</sup>. Two independent agamic complexes were identified such as *Ascyreia* with ten, and *Hypericum* with five apomictic species. The apomicts of the evolutionarily older section *Ascyreia* have significantly larger genomes than all other species due to polyploidization and higher DNA content per chromosome.

Two hypotheses<sup>52</sup> were enumerated to explain the prevalence of apomixis among polyploids. According to Carman's hybridization-derived floral asynchrony theory, apomixis arose from asynchronous expression of duplicate sexual reproductive gene sets in hybrid or polyploid genomes. Nogler<sup>6</sup>, and Naumova and Vielle-Calzada<sup>52</sup> postulated that the alleles responsible for apomixis may act as, or are linked to, recessive lethal factors, thus they can only be transferred by a diploid or polyploid gamete.

Polyploid crops must pair and segregate closely related chromosomes correctly during meiosis in order to maintain genetic stability and fertility. Homologous chromosomes in polyploids, must accurately distinguish each other<sup>53</sup>. Additionally, facultative apomixis is associated with a high and irregular ploidy level or a high and variable level of heterozygosity<sup>54</sup>. Thus in polyploids, apomixis is a difficult and precise process.

Irrespective of the mechanism they use, gametophytic apomicts are almost invariably polyploids, yet sexual members of the same or closely related species are very commonly diploids<sup>7</sup>. Three main theories have been forwarded to explain this situation. Some have proposed that the optimum expression of apomixis may be achieved only in conjunction with a polyploid genome<sup>50</sup>. Because rare diploid gametophytic apomicts have been reported<sup>7,46,47,55,56</sup>, polyploidy need not be absolutely required for the expression of apomixis. However, in these examples, asexual seed formation often was poor, so polyploidy may enhance the expression of apomixis in many systems rather than ensuring its expression per se.

Indications as to how this might operate come from yeast<sup>57</sup> and *Arabidopsis*<sup>58</sup>, in which alterations in ploidy status are known to affect methylation and the expression of different alleles. Conversely, there are intriguing cases of apomixis being expressed in previously sexual plants after chromosome duplication<sup>50,59</sup>. However, there is some debate in these examples about the possibility of innate predisposition, because the plants used were sexual members of groups containing apomicts<sup>50</sup>. On the contrary, sexual plant was recovered after the doubling of an apomictic biotype of *Potentilla argentea*<sup>60</sup>. Finally, polyploidy has been induced in a large number of plants, but apomixis is reported very seldom in the products<sup>61</sup>.

An apparent interspecific hybrid origin also is a common feature among apomicts, and the combination of polyploidy and hybridity is believed to have resulted in allopolyploidy in many gametophytic apomicts<sup>13,29,62,63</sup>. The action of tetrasomic inheritance in many systems, however, also indicated the presence of autopolyploidy, or possibly segmental allopolyploidy in these plants<sup>13,63,64</sup>, which indicated that a combination of hybridity and polyploidy can lead to disjunction of key regulatory events during critical stages of megasporogenesis, megagametogenesis and fertilization. Disjunction in turn may lead not only to apomixis but also to other unusual developmental events, such as polyspory and polyembryony. Based on his own work and through a comprehensive survey of the botanical literature, Carman observed that apomictic polyploids could contain interracial or interspecific genomes polygenically co-adapted to divergent environmental conditions. According to his hybridization derived floral asynchrony theory, apomixis occurs when the hybrids are produced from ecotypes that are distinctly divergent with respect to their start times and rates of MMC formation, meiosis, embryo sac formation, and embryogenesis relative to gross ovule development. Different types of apomixis and related phenomena are all expected outcomes of a theoretical model based on the disjunction of a relatively small number of key regulatory events. Whether this is universally true, and whether it can be used to harness apomixis in crop species, are questions remaining to be answered.

Carman's hypothesis was further refined<sup>65</sup> by proposing that supernumerary chromatin may be the principal driver in this process. A hybrid origin, segmental allopolyploidy and the activity of reproductive drivers are all reported characteristics of supernumerary chromatin biology<sup>66</sup>. Another explanation, which can link the presence of apomixis-specific chromatin with the modified function of a gene involved in sexuality, relies on a *cis*-acting effect of this region. It is possible that the supernumerary chromatin acts as a sink for proteins that regulate gene(s) involved in megasporogenesis, for example by sequestering DNA-binding proteins or chromatin components. The reduced level of these regulators could cause the proposed mis-expression that leads to apomeiosis. In this

scenario, apomeiosis would co-segregate with the supernumerary chromatin, although the gene causing the effect, which is identical in sexual and apomictic plants, could be located elsewhere in the genome. A similar mechanism could affect the expression of genes involved in the other components of apomixis<sup>56</sup>.

## Meiotic mutants

Meiosis is a fascinating process with tremendous practical potential. The production of gametes with unreduced chromosome number and diplosporic pathway of apomixis hold much promise for genetic improvement. Many meiotic genes have been cloned from yeast, *Drosophila*, *Caenorhabditis elegans* and humans<sup>67,68</sup>. In contrast, relatively little knowledge is available in higher plants. Several genes and mutations that affect male meiosis have been reported recently<sup>69,70</sup>. It was evident that different genes are employed during male and female meiosis in plants<sup>71</sup>. Compared to genes that specifically control male meiosis, few genes or mutations have so far been identified that specifically affect female meiosis. Transfer of genes regulating synapsis and recombination, nuclear restitution, and cytokinesis in a single genotype may induce synthetic diplosporic apomixis in a sexual crop species<sup>72</sup>. Recently, an *Arabidopsis* mutant called *DYAD* with female specific arrest of meiosis at the dyad stage, that is after meiosis I, has shown defective synapsis and absence of bivalent formation, in centromere organization and cohesion<sup>73</sup>. The *dyad* mutant showed increased and persistent expression of a meiosis-specific marker, *pAtDMC1::GUS* during female meiosis, indicative of defective meiotic progression. The *DYAD* gene does not show significant homology to any known gene and may encode a novel protein involved in meiotic chromosome organization and female meiosis progression.

## Fertilization and parthenogenesis

The egg cell and the central cell of the embryo sac are the progenitors of the embryo and endosperm in the seed, respectively. In apomicts, embryogenesis can occur completely without the contribution of the paternal genome. The mechanisms that prevent the egg cell in plants from initiating embryogenesis without fertilization are unknown.

In animals, contact of sperm and egg cell triggers a calcium influx, which in turn starts a signalling cascade, initiating embryogenesis<sup>74</sup>. In animals, parthenogenesis can be induced using calcium ionophores, which suggests that calcium influx is sufficient to trigger the entire signalling cascade<sup>75,76</sup>. A calcium influx also occurs during *in vitro* fertilization in plants, initiating at the site of sperm cell fusion and then propagating as a wave across the egg, as observed in animal systems<sup>77</sup>. The use of pharmacological agents in plants to either enhance or block calcium in-



flux can promote or inhibit certain aspects of egg activation, such as egg contraction and smoothening of cell wall formation, respectively<sup>78</sup>. However, calcium influx alone does not lead to parthenogenetic embryo development in plants, suggesting that other activating factors are required, which may be delivered by the sperm cell.

To study individual components of apomixis, Matzk developed the 'Salmon' (after the well-known fish prized for its pink flesh) system in wheat consisting of three isogenic homozygous but alloplasmic lines. An important advantage of this system is that two isogenic male sterile lines with high parthenogenetic capacity (90%) can be compared with one isogenic completely sexual line. This system of wheat showed a high incidence (up to 90%) of parthenogenetic embryogenesis from the reduced egg cell<sup>79</sup>. Culture of isolated reduced egg cells from salmon wheat plants results in parthenogenesis in a cell-autonomous manner, a phenomenon which is not observed in egg cells isolated from other wheat lines<sup>80</sup>. This suggests that the elements required to initiate embryogenesis are already in place in quiescent salmon egg cells. The precise sequence of molecular events stimulating either fertilization-dependent embryogenesis or parthenogenesis from reduced salmon egg cells is not known.

## Endosperm development

Endosperm development in apomicts can be autonomous or it may require pollination and fertilization (pseudogamy). Pseudogamy is typical in members of families such as Rosaceae and Gramineae and is the most common mode of the endosperm development in the apomictic plants. Crosses between plants with different ploidy levels cause abnormal endosperm development, affecting successful hybridization. The maternal genome content of pseudogamous endosperm depends on the number of unreduced central cell nuclei fusing with the unreduced/reduced sperm cell nucleus. Autonomous endosperm production is rare in apomicts and is more common in the Compositae<sup>81</sup>.

The situation is more complex for autonomous apomixis, where the initiation of endosperm development has to rely on alternative pathways. Screens for mutants that allow fertilization-independent seed development in *Arabidopsis thaliana* have identified a class of mutations that partially allow autonomous endosperm development<sup>82</sup>. The three genes of the *FIS* (*fertilization-independent seed*) class, *FIS2*<sup>83</sup>, *FIE* (*fertilization-independent endosperm*)<sup>84</sup> and *MEA* (*medea*)<sup>85</sup>, repress endosperm formation in the absence of fertilization. These gene products share structural and functional similarities with factors thought to control a higher-order chromatin structure in animals: both *MEA* and *FIE* encode polycomb group (PcG) proteins, and *FIS2* encodes a zinc finger protein, some of which are involved in PcG complex formation. Endosperm development in autonomous apomicts probably requires the

specific inactivation of the repressive PcG complexes. Recent results show that autonomous endosperm development progresses further if *fie* is combined with genome-wide hypomethylation<sup>86</sup>, indicating that the actual mechanisms operating in apomicts rely on the deregulation of a larger number of genes<sup>14</sup>. Recently, a rice cDNA representing a polycomb group gene, *OsiEZI*, encoding SET domain protein, was isolated and characterized<sup>87</sup> and its transcript levels were significantly high in flower in comparison to other plant parts. Strikingly, the transcript levels were almost undetectable in developing seeds 1–2 days post-fertilization but increased 3–5 days post-fertilization, indicating a predominant role for *OsiEZI* in embryogenesis. This *OsiEZI* gene could complement and restore telomere-based positional effect in a yeast mutant, reflecting functional conservation in gene repression mechanisms in eukaryotes<sup>87</sup>.

In *Citrus*, an adventive embryonic type, a reduced sexual embryo sac develops in the same ovule as the sporophytically derived parthenogenetic embryo, and fertilization is required for apomictic seed development. The development of sexual embryo may be arrested but the sexual endosperm is required to nourish the asexual embryo<sup>10</sup> and the seed contains an endosperm with the normal 2m : 1p genomic ratio, which poses no imprinting-related problems. As it normally happens in interploidy crosses involving gametophytic apomicts, the endosperm balance number (EBN) would be 4m : 1p, resulting in the abortion or poor development of endosperm. Various modifications that are employed to overcome this condition in nature include *Panicum* type of embryo sac with four nuclei that includes only one polar nucleus so that fertilization of central nucleus maintains balance of 2m : 1p ratio<sup>6</sup>. This balance is required since imprinting silences some alleles in the maternally contributed genome and others in the paternally contributed genome; each parent transmits a different set of potentially active alleles to the endosperm. In apomictic species such as *Tripsacum dactyloides*, a single sperm fuses with an unreduced central cell to produce a 4m : 1p endosperm<sup>88</sup>. Normal endosperm development was observed during controlled pollination experiments<sup>89</sup> in the progenies having 8 : 1 or 8 : 2 EBN ratios, indicating less stringent imprinting-mediated dosage requirement. Thus, it appears that increasing the relative dose of paternal genomes is correlated with the increase in endosperm proliferation and seed size, while increasing the relative maternal dose has the opposite effect.

How do autonomous apomicts develop both endosperm and embryo circumventing the requirement for 2 : 1 EBN? Probably, the imprints from the maternally derived endosperm genome are removed leading to expression of maternal genes that are normally silenced, and effectively supplies the missing paternal genome<sup>90</sup>. In *Arabidopsis*, it is proposed that a combination of the *fie* mutation and hypomethylation of the genome creates such a situation in the endosperm genome resulting in autonomous development in the absence of fertilization<sup>90</sup>.

## Prevalence of apomixis

Apomixis has been described in >400 flowering plant taxa, that represent >40 families<sup>13</sup>. It is well represented among the monocotyledonous and eudicotyledonous plants. Among the plants that show gametophytic apomixis, 75% belong to three families, the Asteraceae, Rosaceae and Poaceae, which constitute only 10% of the flowering plant species. According to another estimate<sup>91</sup>, about 80 families and over 300 genera reproduce through apomixis. The possibility that the occurrence of polyembryony may also include simultaneous presence of apomixis in the species needs to be examined<sup>13</sup>. Although apomixis is known among the Orchidaceae, it appears to be relatively uncommon<sup>61</sup>. Some authors have postulated that the current patterns of distribution may reflect the predisposition of certain plant groups to the unique developmental and genetic changes that characterize apomixis<sup>92</sup>. This hypothesis appears intuitively attractive, but like many issues associated with apomixis, it remains a conjecture until it is tested experimentally. Some of this bias also might relate to the ease of embryological examination in some plant groups or to data accumulated from embryological investigations associated with activities in crop improvement. With the exception of apple and *Citrus*, apomixis is not very common in agriculturally important crops<sup>61</sup>. Among trees, there seemed to be some association between dioecy and agamospermy<sup>93</sup> and it can be best exemplified by *Garcinia*<sup>94</sup> and *Commiphora*<sup>95</sup>.

## Model systems available

For identifying different elements of apomixis, it is appropriate to select a few model systems so that experimental approaches can be applied to unravel the specific mechanisms involved. Biological attributes such as easy to cultivate *in vivo* and *in vitro*, short life cycle, perennial and vegetatively propagated types, small and compact plant stature to facilitate maintain large populations in mutagenesis experiments are desirable<sup>96</sup>. The model system should also represent different types of apomixis including the facultative types. The formation of endosperm is also an important consideration for model experimental systems. Genetic attributes should consider the amenability of the model system to genetic transformation<sup>97,98</sup>, its suitability to genetic linkage mapping<sup>99</sup> and having a small genome size to facilitate identification of critical loci. So far, two approaches have been considered in developing model systems such as the modification of an established model plant species and the experimental manipulation of a known apomict. Many agamic complexes have been studied to either screen for the sexual species/genotypes in the natural germplasm<sup>100,101</sup> or to establish the phylogenetic relationships among closely related apomictic taxa<sup>102</sup>. Different species have distinct advantages, for example,

ferns present some unusual opportunities to study apomixis<sup>103</sup>. Study of the individual component processes during megasporogenesis and megagametogenesis is possible since these two events are physically separated. In some ferns, parthenogenetic development of the sporophyte can be influenced *in vitro*<sup>104</sup>. The sporogenic tissue is relatively exposed simplifying the study on avoidance of meiosis. Similarly, for molecular studies, it is also important to consider apomicts which have larger multiple ovules to get sufficient tissues. Since crop plants occur among both monocotyledons and dicotyledons, it is necessary to include one natural apomict from each.

## Experimental approaches

Various techniques involving cytological, genetic and histochemical examinations are used to screen for apomixis and to identify the apomictic mechanism. Some of the commonly used techniques are reviewed below:

### Morphology

Uniformity of progeny from heterozygous or cross-pollinated parents is the best indication of apomixis. Occurrence of maternal phenotypes in crosses is another indication of apomixis. Screening for apomixis can be expedited if pollen from a dominant marker stock is used. Production of maternal progeny with recessive phenotype would indicate an apomictic mode of reproduction. The technique would be more efficient if the dominant marker is identifiable at seedling stage. High seed set in the progenies of aneuploid plants or wide-cross derivatives is another indication of apomixis. Occurrence of multiple stigma and multiple ovules per floret may be due to apomictic mode of reproduction<sup>105,106</sup>. Consistently high frequency of twin seedlings in the progenies is another indication of apomixis. Multiple seedlings per seed can be due to development of multiple aposporous embryos in an ovule or facultative apomixis in which embryos develop in both sexual and apomictic embryo sacs, and, development of adventive embryos in addition to the sexual embryo.

### Cytological techniques

Embryo sac analysis is one of the commonly used techniques for studying apomixis. Cytological analysis of developing embryo sac is made at different stages from initiation of MMC to the formation of mature embryo sac. In apospory, the embryo sac develops from a somatic cell. Multiple aposporous embryo sac initials are observed, which may be clustered around the megaspore. One of the aposporous cells matures into an embryo sac that has four nuclei, three of which form the egg-apparatus and the fourth serves as a polar nucleus. Thus, lack of antipodals in the embryo sac is a diagnostic feature of apospory.



The pistil-clearing technique<sup>107</sup> is widely used for examination of the embryo sacs. The pistil clearing methods using aromatic esters greatly reduce the time needed to prepare the samples for examination. The method consists of fixing pistils at the time of anthesis in formalin-acetic acid-alcohol (FAA) consisting of 70% ethanol, glacial-acetic acid, 37% formaldehyde (18 : 1 : 1) and passing pistils through a graded series of alcohol and clearing with methyl salicylate and examining under the phase contrast microscope. Using this technique two sexually reproducing species of *Cenchrus* such as *C. prieurii* and *C. echinatus* were reported<sup>100</sup>; normally, the *Cenchrus* species exhibit high incidence of apomixis.

In sexually reproducing plants, the walls of the MMC (or megasporocyte), the tetrad of megaspore and the degenerating megaspore are marked by the temporary accumulation of callose. The deposition of callose is dissolved from the walls of the selected megaspore during its expansion and the initiation of mitotic events of embryo sac development. Apomictic embryo sacs are devoid of a callose layer. In case of *Tripsacum*, presence or absence of callose has been used for screening apomicts<sup>108</sup>. Callose fluorescence is used in combination with pistil clearing to detect diplosporous embryo sac development<sup>109</sup>. A sucrose clearing solution (2.46 M sucrose, 1.36 mM aniline blue, 50 mM K<sub>2</sub>HPO<sub>4</sub>, pH 9.5) has been shown to induce excellent callose fluorescence of embryo sac walls. This technique is being used at the International Maize and Wheat Improvement Center (CIMMYT) to screen *Tripsacum* germplasm and the breeding materials from Maize-*Tripsacum* crosses. Callose was completely absent from aposporous initial cells in *Poa pratensis*, *Pennisetum*<sup>110</sup>, *Brachiaria*<sup>111</sup> and *Hieracium*<sup>112</sup>.

### Histological techniques

These are employed for studying embryo sac development by examining histological serial sections of ovules. Female florets at different stages of maturity are collected and fixed in FAA for 24 h and are then transferred to 70% ethanol. Pistils are dissected and dehydrated using the ovule clearing method<sup>107</sup>. The pistils are then embedded in parafilm, sectioned at 10 µm and stained with safranin O-fast green. Embryo sac development was analysed by examining histological sections of ovules of *Tripsacum dactyloides*<sup>113</sup> to detect failure of meiosis.

### Biochemical techniques

Based on the criteria that apomictic progenies will be genetically uniform and will be identical to the mother plant, various biochemical techniques can be employed to distinguish apomictic and sexual progenies. Isozyme markers can be used to study genetic variation and to detect the presence of apomixis. The apomictic breeding behaviour

was detected in *Arabidopsis holboellii*<sup>114</sup> and *Allium tuberosum*<sup>115</sup> through enzyme electrophoresis, whereas structural organization of the agamic complex of the Maximae (*Panicum maximum*, *P. infestum* and *P. trichocladum*)<sup>116</sup> was elucidated using zymogram analysis.

### Molecular techniques

With the advent of newer molecular biology techniques, a variety of approaches have become available to study the genetic variation among individuals at the DNA level. These methods offer advantages as they are quick, easy and precise. If the genes for apomixis can be tagged with molecular markers such as RAPD, RFLP or AFLPs, breeding material can be screened for apomixis. Two molecular markers<sup>117</sup> (UGT 197 and OPC-04) that co-segregated with apomictic mode of reproduction, in the cross between apomictic aneuploid plant derived from a trihybrid of two wild species of *Pennisetum* and sexual pearl millet, were identified. These markers were further<sup>118</sup> used to screen BC4 progenies derived from the cross of apomictic BC3 plants and sexual pearl millet. The value of molecular markers, tightly linked to the apomictic genes, in breeding programs is thus obvious.

### Epigenetics and genomic imprinting problems

The unsuccessful attempts to transfer apomixis to an experimental or crop species through conventional breeding procedures could be ascribed to a phenomenon called genomic imprinting, which refers to parent-of-origin specific gene expression, and it renders maternal and paternal genomes functionally different to each other<sup>119,120</sup>. This is an epigenetic system of transcriptional regulation by which some genes are expressed only from the maternally or paternally contributed allele<sup>121</sup>. In mammals, genomic imprinting renders the maternal and paternal genomes complementary for genes that are essential to embryo development<sup>120,122</sup>, and thus it ensures that both genomes are present in the zygote. The relatively frequent occurrence of apomixis indicates that embryo development in plants must be governed by radically different rules. In particular, it suggests that the presence of a paternal genome might not be an absolute requirement for embryo development. Imprinting in angiosperms operates mainly in endosperm as its development requires a specific ratio of maternal to paternal genomes<sup>123</sup> as mentioned earlier in this article. A possible explanation for this observation has been offered<sup>124</sup>.

In a survey of 20 genes in *Arabidopsis thaliana*, no paternally derived transcripts were detectable in the developing seed (embryo or endosperm) for the first few days after fertilization. Their observation suggested that this early phase of development could be largely under maternal control, through a combination of maternal products stored in the gametes before fertilization and uniparental expression of

some genes after fertilization; that is, genomic imprinting. This indicates that, in apomicts, the fundamental developmental processes of early embryo growth are essentially unchanged from those in sexual plants; in both instances, to a large extent, only the female genome is involved. Hence, the difference between apomictic and sexual seed development should lie in the mechanisms that regulate the activation of the corresponding programme, not the programme itself. This would support a simple inheritance model for parthenogenesis: although the programme is necessarily complex, its activation might rely on a limited number of regulatory factors. This resembles the hypothesis put forward earlier for gametogenesis, and is equally hypothetical. Nevertheless, several regulatory factors have been identified in plants, which, when mutated or expressed ectopically, induce partial embryo development or embryo-specific gene expression<sup>125,126</sup>. The identification of proteins that regulate or are targets of such genes might shed light on the mechanisms of embryo initiation, and therefore on the mechanisms that are altered in apomictic plants.

The epigenetic model of gene regulation during apomixis has become important because (i) it unites the mutation and hybridization theories because epialleles can behave genetically like mutations and epigenetic changes in gene expression have been documented after hybridization<sup>58</sup>, (ii) it provides a solution to the enigma that several traits had to evolve coordinately to produce a functional apomict<sup>127</sup> and (iii) the presumed polyphyletic origin of apomixis is taken as an argument in favour of a simple control mechanism of apomixis and the molecular mechanisms underlying apomixis are highly diverse and may be caused by mutations or epimutations in a large number of different genes<sup>56</sup>.

### Molecular relationships between sexual and apomictic pathways

Apomixis can occur due to heterochronicity or heterotopicity of the sexual developmental process of reproduction. Heterochronicity refers to mistakes in the timing of developmental processes, for example, megagametogenesis occurs before megasporogenesis is completed or embryogenesis starts prior to fertilization<sup>128</sup>. Causes of the heterochronic development can be perturbation of developmental checkpoints or changes in gene expression due to polyploidization<sup>15</sup>. Heterotopicity is related to mistakes in the location where developmental events occur. It may be due to deregulation of cell fate specification<sup>15</sup>. The formation of the embryo initials from integumentary or nucellar cells (adventive embryony) and the formation of a gametophyte from nucellar cells (apospory) are examples of heterotopicity effects in apomicts<sup>128</sup>. Heterochronicity in *Tripsacum dactyloides* and maize-*Tripsacum* hybrids was characterized by altering the developmental timing of sporogenesis and early embryo development. Initiation of

embryo development was precocious regardless of meiotic reduction and hence was not a direct consequence of absence of meiosis. Thus, it was shown that apomixis in *Tripsacum* causes a highly plastic heterochronic phenotype resulting from deregulation of the developmental timing of sporogenesis and early embryogenesis<sup>129</sup>.

The molecular relationships between sexual and apomictic pathways<sup>130</sup> were examined using fusion constructs of  $\beta$ -glucuronidase (GUS) with a variety of *Arabidopsis* genes (promoter:GUS or chimeric protein fusion constructs) that are associated with different aspects of sexual reproduction in this species. These markers were introduced into sexual and apomictic *Hieracium* plants, and their expression patterns were monitored during ovule and seed development. The same constructs also were introduced into *Arabidopsis* for a comparison with sexual *Hieracium*. The *Arabidopsis* marker sequences used were from *SPOROCTELESS (SPL)/NOZZLE*, which is required for male and female sporogenesis in *Arabidopsis*<sup>131,132</sup>, *SOMATIC EMBRYO RECEPTOR KINASE1 (SERK1)*, which is thought to play a role in embryogenesis<sup>133</sup>, and three *FIS*-class genes *MEDEA (MEA)/FIS1*, *FIS2*, and *FERTILIZATION-INDEPENDENT ENDOSPERM (FIE)/FIS3*, mutations in any one of which result in the fertilization-independent initiation of endosperm development<sup>134,135</sup>. Overall, the results showed a remarkable conservation of expression patterns of reproductive marker genes in apomictic compared with sexual *Hieracium* plants during embryo and endosperm development. The major differences that were observed occurred early in ovule development, close to the point of divergence between sexual and apomictic processes.

### Identification and cloning of transcripts related to apomixis

Differentially expressed genes have been isolated from a wide range of experimental systems by using comparative gene expression studies in *Panicum maximum*<sup>136</sup>, *Brachiaria* sp.<sup>137–139</sup>, *Pennisetum* sp.<sup>140</sup>, *Paspalum* sp.<sup>141</sup> and *Cenchrus ciliaris*<sup>142</sup> using either differential display or subtractive hybridization techniques. Using cDNA-AFLP transcriptional profiling technique<sup>143</sup>, nearly 179 differentially expressed transcripts were isolated out of which two genes, namely SERK (somatic embryogenesis receptor kinase) and APOSTART were characterized<sup>144</sup> in detail from *Poa pratensis*. These two genes are reported to be involved in cell-to-cell interaction of both the signalling pathway and hormone stimulation. They proposed that PpSERK gene activation in nucellar cells of apomictic genotypes is the switch that channels embryo sac development and 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. The impli-

cation of APOSTART in meiosis and programmed cell death has also been suggested. Recently, partial cDNA fragments showing homology to SERK and LRR-kinase genes of *Arabidopsis* have also been isolated in *Cenchrus ciliaris* by subtractive hybridization<sup>142</sup>.

### Potential value of apomixis in agriculture

Apomixis is an attractive trait for the enhancement of crop species because it mediates the formation of large genetically uniform populations and perpetuates hybrid vigour through successive seed generations. Many agronomic advantages of apomixis can be envisioned: the rapid generation and multiplication of superior forms through seed from novel, currently underused germplasms; the reduction in cost and time of breeding; the avoidance of complications associated with sexual reproduction, such as pollinators and cross-compatibility; and the avoidance of viral transfer in plants that are typically propagated vegetatively, such as potatoes<sup>145–149</sup>. The value of these opportunities will vary between crops and between production systems. For farmers in the developed world, the greatest benefit is expected in the economic production of new, advanced, high-yielding varieties for use in mechanized agricultural systems. Conversely, for farmers in the developing world, the greatest benefits are expected in the release of robust, high-yielding varieties for specific environments, and improvements in the security of the food supply, and greater autonomy over variety ownership<sup>150,151</sup>.

However, apomixis is very poorly represented among crop species. The main exceptions to this appear to be tropical and subtropical fruits, such as mango, mangosteen and citrus, and tropical forage grasses such as *Panicum*, *Brachiaria*, *Dicathanthium* and *Pennisetum*. It is possible that the low representation of apomixis among crops arose unintentionally from a protracted human history of selecting superior plants for future cultivation. Selection for change over a parental type would work against a mechanism such as apomixis that acts to maintain uniformity. The presence of the trait among tropical fruits and grass crops may be a reflection of this effect, because focused efforts to improve these crops are comparatively recent events<sup>61</sup>.

There are also few apomictic species of significant relatedness available for use in introgression programmes, which may explain at least some of the difficulties experienced when attempts have been made to introduce apomixis into crops through hybridization. For example, major programmes aimed at introducing apomixis into maize<sup>148,152</sup> from the wild relative *Tripsacum dactyloides* have been under way now for decades, yet they have proven unsuccessful in terms of generating apomictic plants with agronomically acceptable levels of seed set. Difficulties also have been encountered in efforts to pro-

duce apomictic lines of hybrid millet<sup>153,154</sup>. Even if successful, it seems likely that introgression lines would provide limited flexibility in terms of practical capacity to manipulate apomixis in agricultural breeding systems. Current breeding efforts with apomictic crop species, such as the forage grasses *Brachiaria* and *Panicum* are frustrated by the need to use complex breeding strategies to accommodate the inaccessibility of the female gamete to generate hybrid progeny<sup>16</sup>. We believe therefore that the best solution would be the introduction of apomixis into crops in an inducible format, permitting its use during seed increase but allowing for its silencing during hybridization. To achieve this, information will be required concerning the genes that control the trait, their interrelationship with sexual processes and the impact the trait might have on seed yield, viability and quality for a given plant<sup>61</sup>.

### Ecological risks

The biosafety issues regarding *de novo* engineering of apomixis have become recent concerns. A dominant apomixis transgene may spread through pollen across populations to a related outcrossing species resulting in rapid fixation of genotypes and displacement of sexual siblings<sup>155</sup>. This kind of infectious apomixis could lead to a reduction in the genetic diversity both within a crop and among its close relatives. Since these apomicts get the full benefit of asexuality at no cost, they should be able to predominate in a sexual environment which is not generally observed in nature<sup>156</sup>.

Another possible risk is in apomicts becoming invasive when they are introduced in some ecological niches. Generally, apomicts seem to have potential to be good colonizers<sup>157</sup> and there are some examples of apomictic weeds and their invasiveness<sup>158</sup>. Another apprehension of apomixis technology is that it may entrench the trend of monocultures in agriculture<sup>159</sup> thus reducing biodiversity. But, unlike self-pollinators, apomicts tend to maintain high levels of heterozygosity which will be available to other sexually reproducing plants.

### Conclusion

In recent years, there has been increased interest in understanding the mechanisms of apomixis. The genetics of apomixis is appearing to be more complex than was understood earlier. With the availability of genome sequences of *Arabidopsis* and rice, novel strategies encompassing functional genomics could be designed for understanding the role of key regulatory elements during meiosis, endosperm development and parthenogenetic embryo development. Another way of inducing apomixis could be through genetically engineering the elements of apomixis *de novo* by deregulating the expression of key developmental

genes. Various molecular biological tools such as gene and enhancer traps can be used for misexpression of genes conferring the elements of apomixis.

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