Title

Asexual reproduction through seeds: the complex case of diplosporous apomixis

Short running title

Diplospory review

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21 November 2022

The main text of the manuscript contains Tables 1-2 and Figures 1-2.

Word count from introduction to start of acknowledgements is 6468 words.

Highlight

In this review, we retrace the history of diplospory, from the first cytological observations to the latest findings regarding its genetic control and potential exploitation for crop improvement.

Abstract

Apomixis is considered a potentially revolutionary tool to generate high-quality food at a lower cost and shorter developmental time due to clonal seed production through apomeiosis and parthenogenesis. In the diplosporous type of apomixis, meiotic recombination and reduction are circumvented either by avoiding or failing meiosis or by a mitotic-like division.

Here, we review the literature about diplospory, from early cytological studies dating back to the late 19th century to recent genetic findings. We discuss diplosporous developmental mechanisms, including their inheritance. Furthermore, we compare the strategies adopted to isolate the genes controlling diplospory with those to produce mutants forming unreduced gametes.

Nowadays, the dramatically improved technologies of long-read sequencing and targeted CRISPR-Cas mutagenesis justify the expectation that natural diplospory genes will soon be identified. Their identification will answer questions such as how the apomictic phenotype can be superimposed upon the sexual pathway and how diplospory genes have evolved. This knowledge will contribute to the application of apomixis in agriculture.

Keywords

Apomixis, breeding, clonal progeny, diplospory, flowering plants, gametophyte, meiosis, reproduction, sporogenesis, unreduced gametes

Abbreviations

DIP = *DIPLOSPOROUS*, ES = embryo sac, FDR = first division restitution, FM = functional megaspore, MMC = megaspore mother cell, NOR = nucleolar organizer region, *PAR* = *PARTHENOGENESIS*, SDR = second division restitution

General overview

The fascinating reproductive diversity of flowering plants has attracted the interest of researchers for centuries, since the time of Darwin (Darwin, 1876; 1877; Holsinger, 2000). Sexually reproducing plants undergo an alternation of generations involving a haploid gametophyte and a diploid sporophyte. The two generations are respectively formed by meiosis and syngamy (Fig. 1A), distinctive processes of sexual reproduction. The meiotic process generates haploid spores from diploid precursors, whereas syngamy restores the diploid generation by fusing the female and

the male gametes. Angiosperms produce two types of spores through a process named sporogenesis. These spores are formed in the post-embryonic developmental phase from sub-epidermal cells within the primordia of ovules and anthers. Ovules usually contain a single megaspore mother cell (MMC), which pass into meiosis, and only one megaspore from the tetrad survives (Fig. 1B). Instead, anthers contain multiple pollen mother cells that undergo meiosis synchronously to form microspores. Therefore, the regulatory mechanisms of sporogenesis present both shared and distinct aspects between females and males. The spores eventually undergo a series of mitotic divisions in a process named gametogenesis, generating the female embryo sacs and male pollen grains (Fig. 1B; Ma, 2005; Yang *et al.*, 2010; Drews and Koltunow, 2011).

Some angiosperms can also reproduce asexually by seed through apomixis (from the Greek *apo* "away from" and *mixis* "mixing"). This mode of reproduction is based on apomeiosis, i.e., lack of a proper meiotic process, that eventually results in the generation of an unreduced egg cell which autonomously develops into an embryo by parthenogenesis (Fig. 1C). Hence, the absence of syngamy leads to a clonal progeny genetically identical to the mother plant. Apomeiosis predominantly affects female gamete formation, whereas male gametes are mainly produced through the genetic reduction mode (Savidan *et al.*, 2001). It has been suggested that this reproductive system has originated multiple times during evolution and is polyphyletic (Carman, 1997; Hand and Koltunow, 2014). Indeed, it is widespread throughout 78 plant families and approximately 300 genera, although it corresponds to only 2.2% of angiosperm genera and is missing from major crops such as maize, wheat, and rice (Hojsgaard *et al.*, 2014; Albertini *et al.*, 2019). A more recent hypothesis considers apomixis and sexuality to be ancient polyphenisms, and the switch between them might be triggered by external signals that regulate downstream the reproductive pathways (Carman *et al.*, 2011; Gao, 2018).

Flowering plants show different forms of apomixis, which involve the development of maternal embryos directly from somatic cells of the nucellus or from unreduced gametophytes. Respectively, these forms are indicated as sporophytic and gametophytic apomixis. Sporophytic apomixis, also known as adventitious embryony, is an asexual reproductive mode found in mango and *Citrus* spp., among others. In this form of apomixis, somatic nucellar embryos develop by mitosis from sporophytic cells of the ovule alongside the sexually derived embryo and endosperm (Xu *et al.*, 2021). Gametophytic apomixis is subdivided into two types, i.e., diplospory and apospory, whether the gametophyte develops from the MMC or an aposporous initial, respectively. Edman (1931) first introduced the terms diplospory and apospory. Both types involve (1) the formation of an unreduced embryo sac (ES) by apomeiosis, and (2) the development of the embryo without fertilization by parthenogenesis. In apospory, the meiotically-produced spores usually degenerate, and an unreduced gametophyte forms from a somatic cell of the ovule. Occasionally, both the

sexual and the apomictic gametophytes survive, leading to the presence of multiple ESs (Asker, 1980; Nogler, 1984). Apospory was first discovered by Rosenberg (1907) in the genus *Pilosella* (formerly *Hieracium* subgenus *Pilosella*). Detailed information about the cytogenetic and embryological aspects of sporophytic apomixis and apospory have already been comprehensively discussed in preceding reviews (Nogler, 1984; Koltunow, 1993; Savidan *et al.*, 2001). This review will focus on the diplosporous type of apomixis and the state of the art regarding its genetic control.

History of diplospory research

What would later become known as mitotic diplospory was initially investigated in detail in *Antennaria alpina*, a member of the Asteraceae family (Juel, 1900). This dioecious species had been suspected of parthenogenesis for years since Kerner (1876) had observed seed setting in female plants in the absence of male plants. Indeed, Juel (1898) demonstrated by cytological analysis that the embryo of *A. alpina* developed without fertilization. This was the first documented case of parthenogenesis in flowering plants. Two years later, Juel published his comparative study of the megasporogenesis in *A. alpina* and the sexually related species *Antennaria dioica*. Interestingly, he concluded that, in *A. alpina*, the female meiosis was replaced by a mitotic division, resulting in an unreduced ES by further mitotic divisions. Thanks to these analyses, this type of diplospory was named *Antennaria*-type or mitotic diplospory (Fig. 2E).

Shortly afterwards, Raunkiaer (1903) suggested the occurrence of apomixis in *Taraxacum*, observing seed formation after inflorescence emasculation. Indeed, in contrast to *Antennaria*, this is a hermaphrodite plant species, and self-pollination is, in principle, possible. Further cytological examination of the *Taraxacum* megasporogenesis by Murbeck (1904) and Juel (1905) showed that the first meiotic division proceeded abnormally. A diploid restitution nucleus was formed similarly to the "contraction nucleus" described by Rosenberg in *Hieracium* pollen (1927). The second meiotic division proceeded normally, leading to the formation of two unreduced megaspores. This meiotic form of diplospory was named the *Taraxacum*-type (Fig. 2B). A similar form of this type of diplospory has been described in *Ixeris dentata* and named after it the *Ixeris*-type (Fig. 2C; Okabe, 1932).

In 1951, Böcher published an extensive study on the genus *Boechera* (previously *Arabis*), describing diplospory in diploid, triploid, and aneuploid accessions. He showed that ES development could occur through the *Taraxacum*-type and, rarely, through the mitotic *Antennaria*-type. Later, other forms of diplospory were described, among those the different *Allium*-type (Fig. 2G; Håkansson and Levan, 1957). The diverse diplospory types combined with the diverse types of ES development (e.g., bisporic) can generate a great variability in unreduced gametophytic formation.

Cytological characterization of diplospory

In diplosporous plants, the chromosome number across generations is maintained through different routes (Fig. 2B-G; Table 1):

1) Meiotic diplospory: the MMC undergoes an aberrant meiotic division generating a dyad of unreduced megaspores (*Taraxacum*-, *Ixeris*-, and *Elymus*-type; Fig. 2B-D).
2) Mitotic diplospory: the MMC entirely omits the meiotic process and directly enters into gametogenesis by mitotic division (*Antennaria*-, and *Eragrostis*-type; Fig. 2E-F).
3) Endoreduplication: before meiosis, the MMC undergoes an extra chromosome endoduplication followed by reductional meiosis, resulting in the formation of unreduced megaspores (*Allium*-type; Fig. 2G).

As a consequence of these apomeiotic processes, the resulting unreduced ES contains an unreduced egg cell that develops into an embryo by parthenogenesis; additionally, some species are characterized by precocious embryony before anthesis to avoid possible pollination. Endosperm formation can be autonomous or pseudogamous, whether the central cell develops autonomously or needs to be fertilized by a sperm cell (Fig. 1C). Some plants are obligate apomicts, whereas others are facultative apomicts, i.e., they retain some degree of sexuality. Sporadic cases of unreduced pollen grains formation have been described in some species; however, diplospory concerns only the female part (Böcher, 1951; Asker, 1980; Nogler, 1984; Savidan *et al.*, 2001; Mau *et al.*, 2013; 2021).

The study of diplospory has been hampered mainly because the MMC is not easily accessible but deeply buried inside the ovary. Nowadays, the development of new technologies such as confocal laser scanning microscopy has allowed the achievement of increased resolutions, leading to a better understanding of the dynamics of diplospory progression (e.g., chromosome patterns, nuclear envelope dynamics, spindle formation, and vacuolization).

Plant species exhibiting meiotic diplospory (Fig. 2B-D; Table 1) are characterized by an aberrant meiotic process in which the first reductional division is omitted by nuclear restitution (Juel, 1905). During meiosis I, the homologous chromosomes hardly synapse, mainly leading to the formation of univalents. At metaphase I, univalent chromosomes remain scattered along the spindle instead of aligning at the equatorial plate, and they are progressively surrounded by a single nuclear envelope without any segregation. This restitution nucleus has an elongated dumbbell shape and sometimes contains multiple nucleoli, residual of the aborted first meiotic division. Later, meiosis II occurs properly, generating a dyad of unreduced megaspores. The plants exhibiting meiotic diplospory are mostly 3x, in that they avoid random chromosome segregation by skipping the first reductional step of the meiotic process (Okabe, 1932; Hair, 1956; Savidan *et al.*, 2001).

Recently, meiotic diplospory of the *Taraxacum*-type (Fig. 2B; Table 1) has been characterized in detail in some apomictic *Taraxacum* spp. (van Baarlen *et al.*, 2000; Musiał *et al.*, 2013; Janas *et al.*, 2016) and *Chondrilla* spp. (Musiał and Kościńska-Pająk, 2017; 2019). Furthermore, this type is also distinctive of dioecious *Antennaria carpatica* along with apospory, even though in this species parthenogenesis seems to be absent because nearly all the embryos degenerate (Bergman, 1951). Albeit most of the known *Paspalum* spp. are aposporous, a few species exhibit meiotic diplospory of the *Taraxacum*-type. Diplosporous *Paspalum* spp. feature a restitution nucleus also during male meiosis and a pseudogamous endosperm (Pi and Chao, 1974; Bonilla and Quarin, 1997; Ortiz *et al.*, 2013). This type of diplospory also occurs in *Boechera*, one of the few genera in which apomixis appears in the diploid cytotype. Here, male meiosis can also be affected in some apomictic accessions, producing reduced, unreduced, or aneuploid pollen grains (Böcher, 1951; Mau *et al.*, 2013). Recently, the co-occurrence of diplospory and apospory has been described for some *Boechera* accessions (Brukhin *et al.*, 2019; Carman *et al.*, 2019).

A similar meiotic diplospory type was observed in *Ixeris dentata*, characterized by the presence of univalents at metaphase I and the formation of a restitution nucleus at the end of meiosis I. During meiosis II, a thin cell plate between the two newly formed nuclei is also detected; nevertheless, it disappears soon after. At the end of the meiotic process, a single coenospore (i.e., an unreduced megaspore with two genetically identical nuclei) is formed. This cell directly functions as a binucleate ES, requiring only two rounds of mitosis to generate the mature ES (Fig. 2C; Okabe, 1932). However, the distinction between the *Taraxacum*- and *Ixeris*-type is mainly in the type of embryo sac (i.e., monosporic and bisporic, respectively), not in the mechanism of diplospory itself (i.e., the formation of a restitution nucleus during meiosis I) (Nogler, 1984). The *Ixeris*-type has also been identified in other species, such as *Erigeron* spp. and *Rudbeckia* spp. (see Table 1; Battaglia, 1945; 1946; Fagerlind, 1947).

Meiotic diplospory has also been described in apomictic *Elymus recticetus* (Fig. 2D), a member of the Poaceae family. Female meiosis is delayed because the MMC undergoes a long period of vacuolization and nuclear elongation before nuclear division. This nuclear division could work as a second meiotic division, as prophase I and metaphase I were not observed in this species; later, an unreduced dyad of megaspores is formed (Hair, 1956). The chalazal member of the dyad is usually selected for the generation of the ES. Even though meiotic diplospory occurs in *E. recticetus*, more often this species develops through mitotic diplospory of the *Antennaria*-type (Hair, 1956; Crane and Carman, 1987; Carman *et al.*, 1991). A similar type of meiotic diplospory was originally described by Chennaveeriah and Patil (1971) in *Blumea eriantha* and *Blumea oxydonta*, members of the Asteraceae, but was controversially classified as a mitotic-diplospory type (Bhat *et al.*, 2005; Noyes, 2007; Table 1).

In mitotic diplospory, the MMC undergoes extended interphase and vacuolization period, typical features of the functional megaspore (FM). After this prolonged phase, it entirely skips the meiotic process and directly divides by mitosis to generate a mature ES (Fig. 2E-F; Nogler, 1984).

Mitotic diplospory of the *Antennaria*-type has a wider taxonomic distribution compared with other types of diplospory (Fig. 2E; Table 1). Species exhibiting this type are mostly characterized by polyploidy and autonomous endosperm formation. Pollen is often not viable, and some species develop gynoecious flowers only (e.g., *Antennaria parlinii*, *Boehmeria tricuspis*) (Juel, 1900; Bayer and Stebbins, 1983; Peel *et al.*, 1997; Tang *et al.*, 2016).

The mitotic diplospory of the *Eragrostis*-type derives its name from morphological observations performed in *Eragrostis curvula*, a member of the Poaceae. In this pseudogamous species of high polyploidy (4x to 8x), the MMC omits meiosis and divides mitotically, resembling the *Antennaria*-type of diplospory (Voigt and Bashaw, 1972). The peculiarity of this species is found during megagametogenesis, where the FM undergoes only two rounds of mitosis, leading to the generation of a 4-celled ES (Fig. 2F). The mature gametophyte contains one egg cell, two synergid cells, and only one mononucleate central cell; hence, it is possible to maintain the 2:1 maternal:paternal genome contribution to the endosperm, to which cereals are very sensitive (Meier *et al.*, 2011; Carballo *et al.*, 2021) (Table 1).

In some species, the meiotic and mitotic routes can occur in different ovules of the same plant. This co-occurrence suggests that meiotic and mitotic diplospory might not be two distinct types but two temporal variations of the onset of apomeiosis. Moreover, some plants can even exhibit diplospory and apospory in the same individual, suggesting that the different forms of apomixis are possibly controlled by a change in regulation rather than by distinct genes (Asker, 1971; Carman, 1997).

Endoreduplication is typical of plants of the *Allium*-type, and at least four diplosporous *Allium* spp. have been described (Håkansson and Levan, 1957; Kojima and Nagato, 1992; Yamashita *et al.*, 2012) (Table 1). This type of diplospory is characterized by an extra chromosome duplication before meiosis, allowing each univalent to become an "*autobivalent*". The first reductional meiosis occurs, generating a dyad of 2n spores. Usually, the micropylar spore has an abnormal-shaped nucleus and degenerates, whereas the chalazal one undergoes meiosis II. This meiotic process results in a 2n functional megaspore. A bisporic ES characterizes the *Allium* spp.; therefore, the development of a mature ES requires only two rounds of mitosis (Fig. 2G). Endoreduplication before male meiosis is rare; however, multivalents to univalents are formed at metaphase I, resulting in microspores that can be reduced, unreduced, or aneuploid. This causes the formation of different types of pollen grains needed for endosperm formation (Håkansson and Levan, 1957;

Kojima and Nagato, 1992). Similar endoreduplication examples are found in ferns (Manton, 1950), earthworms, planarians, and others outside the plant kingdom (Mogie, 2013).

A possible role of callose in apomeiosis

Callose deposition pattern has been studied in a few species showing diplosporous apomixis (Table 1), and it has been suggested to have a role during asexual reproduction. Callose, a 1,3-β-glucan homopolymer with some β-1,6-branches, is one of the plant cell wall components and has a crucial role in several biological processes throughout plant growth and development, including the reproductive phase (Chen and Kim, 2009). In female and male sporogenesis, a precise pattern of callose deposition and degradation accompanies the transition from somatic to germline identity and the subsequent steps of meiosis until the formation of functional spores. During megasporogenesis, callose is initially deposited in the cell wall surrounding the MMC, and it accumulates in the cell plates that divide the newly formed spores after each meiotic division. At the end of megasporogenesis, callose persists around the three degenerating megaspores, while it is degraded from the FM as it enters into megagametogenesis (Rodkiewicz, 1970; Ünal *et al.*, 2013). The importance of the crosstalk between the differentiated MMC and the surrounding sporophytic tissue has already been demonstrated in both sexual (Bencivenga *et al.*, 2011) and asexual reproduction (Tucker *et al.*, 2012); therefore, it has been suggested that callose can function as a temporary barrier that selectively regulates this cell-to-cell communication program, stimulating the switch from sporophytic to gametophytic gene expression.

In some apomictic accessions, the callose deposition pattern during the first steps of megasporogenesis differs from that of their sexual counterparts (Peel *et al.*, 1997; Musiał and Kościńska-Pająk, 2017). For instance, data on non-obligate sexual *Boechera stricta* individuals with a tendency to apomictic development showed irregularity of callose deposition during megasporogenesis (Rojek *et al.*, 2018). Whether these alterations during megasporogenesis are a cause or a consequence of the apomictic mode of reproduction is still under investigation. (Peel *et al.*, 1997; Musiał and Kościńska-Pająk, 2017).

Meiotic diplosporous *Taraxacum atricapillum* (Musiał *et al.*, 2015), *Chondrilla juncea*, and *Chondrilla brevirostris* (Musiał and Kościńska-Pająk, 2017; 2019) share a similar pattern of callose deposition during megasporogenesis. The MMC is not surrounded by callose but shows an accumulation only at the micropylar side of the cell wall. Following the first nuclear restitution, callose is also accumulated at the chalazal side, featuring a bipolar pattern. After the second meiotic division, a thick deposition of callose is detected in the wall separating the dyad of megaspores. As in sexual plants, callose is then degraded from the selected FM. Carman *et al.* (1991) described a similar pattern for *Elymus recticetus*, in which callose is mainly deposited at the micropylar side of

the MMC. Additional studies on species from different families are needed to verify whether this pattern is characteristic of all the meiotic diplosporous species. For example, in *Paspalum minus* (Bonilla and Quarin, 1997), a thick deposition of callose was found around the MMC.

On the contrary, callose is completely lacking during megasporogenesis in mitotic diplosporous species of the *Antennaria*-type (Table 1), whereas its deposition around the apomictic MMC of *Eragrostis curvula* is controversial (Peel *et al.*, 1997; Meier *et al.*, 2011). One hypothesis is that, in mitotic diplospory, the MMC directly functions as an FM; therefore, callose degradation is not needed for its selection.

Variations in the callose deposition pattern in diplosporous MMC might be caused by heterochronic expression of genes usually acting during sexual reproduction in different ways, either through a general deregulation of the sexual program (e.g., lack of callose deposition by callose synthases), or early superimposition of the ES development program (e.g., degradation of callose by glucanases) on the meiotic program (Peel *et al.*, 1997).

Genetic approaches to study diplospory

As previously described, diplospory leads to the formation of unreduced female gametophytes through three different pathways affecting the legitimate MMC, i.e., meiotic restitution, mitotic-like division, or endoreduplicational meiosis. However, the molecular mechanisms underlying these processes are still under investigation. Powers (1945) was the first to postulate a genetic model for the inheritance of apomixis. For diplosporous guayule (*Parthenium argentatum*), he proposed three recessive genes: one for apomeiosis (*a*), one for not being fertilizable (*b*), and one for being parthenogenic (*c*). The apomictic genotype would be *aabbcc*. However, this was hard to reconcile with the high heterozygosity of apomicts. This heterozygosity became evident when the apomictic plants were used as pollen donors, generating a very diverse progeny, as was already experienced by Gregor Mendel in *Hieracium* (van Dijk and Ellis, 2016).

The first clue to the genetic control of diplospory came from Sørensen (1958), who found that loss of a satellite Nucleolar Organizer Region (NOR) chromosome in a hypo-triploid offspring of an apomictic *Taraxacum* lineage led to the loss of diplospory. Based on this, Richards (1970; 1973) postulated an apomixis model with two dominant genes, one for diplospory and one for parthenogenesis. Crosses of diploid sexual plants with polyploid apomictic pollen donors, performed by van Dijk and coworkers (2020), showed that the offspring could exhibit only diplospory or parthenogenesis, suggesting that two unlinked loci control these elements. Diplospory was determined by test crosses employing diploid testers and then evaluating the ploidy level of the progeny with flow cytometry. Analysis of the crosses with codominant SSR markers showed that diplospory (*D*) was dominant over sexuality (*d*), and apomicts had the *Ddd* simplex genotype.

Chromosomal fluorescent *in situ* hybridization with repetitive BAC DNA additionally validated *DIPLOSPOROUS* (*DIP*) locus localization on the NOR chromosome (Vašut *et al.*, 2015). The codominant SSR markers linked to the dominant *DIP* allele could not be transmitted to the diploid offspring, suggesting that haploid pollen carrying the *DIP* allele is lethal (van Dijk *et al.*, 2009). This fact could explain the absence of diploid apomictic *Taraxacum*, which is formed from haploid pollen. On the contrary, diploid pollen carrying the *DIP*-linked SSR markers gave rise to viable triploid apomicts. More detailed genetic AFLP mapping suggested the absence of suppression of recombination in the *DIP* locus (Vijverberg *et al.*, 2003), in contrast to diplospory-linked loci in *Erigeron annuus* (Noyes and Rieseberg, 2000) and *Tripsacum dactyloides* (Grimanelli *et al.*, 1998 a) in which recombination is suppressed. Thanks to the screening of mutant populations, seven *loss-of-diplospory* individual lines were selected (van Dijk *et al.*, 2017). The analysis of these mutants indicated *Vacuolar protein-sorting13* (*VPS13*) as one of the putative genes controlling diplospory in *Taraxacum officinale* (van Dijk *et al.*, 2020). *VPS13* is conserved in all eukaryotes and encodes a protein involved in the tethering between different organelles to transfer lipids within the cell (Dziurdzik and Conibear, 2021). However, the molecular mechanism of *VPS13* in diplospory has not been unravelled yet. In parallel, the same experimental approach used to identify *DIP* led to the successful isolation of the *PARTHENOGENESIS* (*PAR*) gene. Sexual *PAR* alleles are expressed only in pollen, whereas the dominant apomictic allele is expressed in the egg cell, triggering embryogenesis (Underwood *et al.*, 2022).

In a series of elegant crossing studies, Noyes and collaborators showed that the elements of apomixis, i.e., diplospory and parthenogenesis, are under independent genetic control in *Erigeron annuus* (Noyes and Rieseberg, 2000; Noyes *et al.*, 2007). Crosses between sexual seed plants and apomictic pollen donors yielded recombinant offspring that displayed only one of these apomixis elements. The segregation ratios fit an inheritance model with 3x simplex genotypes for two unlinked dominant loci: *Ddd* for diplospory and *Fff* for both parthenogenetic embryo and endosperm development. Clustering of dominant AFLP markers in a genetic map based on 2x × 3x crosses indicated suppression of recombination in the *D* locus, whereas suppression was absent in the *F* locus (Noyes, 2006).

In the genus *Boechera*, Mau *et al.* (2021) performed crossing experiments between diploid sexuals and diploid apomicts that produced either reduced or unreduced pollen grains. In the F1, recombination between diplospory and other apomixis elements (i.e., parthenogenesis and pseudogamy) was occasionally observed, arguing for their independent genetic control. In addition, they monitored the transmission of two apomixis-associated markers for female (*APOLLO*, apomictic allele; Corral *et al.*, 2013) and male apomeiosis (*UPGRADE2*; Mau *et al.*, 2013). *APOLLO*, encoding a Glu-Asp-Asp-His exonuclease, was initially isolated as a gene differentially

expressed between sexual and apomictic premeiotic ovules. *UPGRADE2* is a chimeric long non-coding RNA that originated from the duplication of gene fragments followed by exonization, with no homologous in sexual accessions. Interestingly, *UPGRADE2* has been recently shown to be located on a heterochromatic chromosome, *Boe1*, which is associated with apomixis (Mau *et al.*, 2022). *APOLLO* and *UPGRADE2* were also strongly associated with each other in triploid progeny derived from diploid pollen grains, whereas this association was much weaker in diploid progeny derived from haploid pollen grains. This is expected if the markers and diplospory segregate in the male meiosis leading to haploid pollen. The diploid F1, derived from reduced haploid pollen, showed considerable variation in the penetrance of apomixis. Mau *et al.* (2021) favoured a gene-based causal model of apomixis in *Boechera* above a causal model based on interspecific hybridization *per se*.

The genetic inheritance of apomixis elements in *Tripsacum dactyloides* has been studied by means of maize RFLP markers (Leblanc *et al.*, 1995 a). Co-segregation of three non-recombining markers suggested a dominant simplex *Dddd* genotype for diplospory. Moreover, the mapping of the three maize RFLP markers in sexual *Tripsacum* showed considerable recombination between them, suggesting suppression of recombination in the apomictic haplotype of the *DIP* locus. The markers fitted a tetrasomic mode of inheritance, implying that the apomict was an autotetraploid rather than an allopolyploid, arguing against the idea that apomixis was a consequence of interspecific hybridization (Grimanelli *et al.*, 1998 b). The single dominant locus control of apomixis would not necessarily imply a single master regulator but could involve several genetically linked genes, each controlling an element of apomixis (Blakey *et al.*, 2001).

In the grass *Eragrostis curvula*, crosses between sexuals and diplosporous apomicts at the tetraploid level fit a model of dominant monogenic inheritance of diplospory, although the observed apomictic embryo sacs in the F1 varied from 3 to 100%. Sixty-seven F1 individuals were genotyped by sequencing, leading to the identification of four SNPs fully co-segregating with diplospory. The four GBS-SNP markers were located on a single contig spanning a non-recombinant region of approximately 10.5 Mb (Zappacosta *et al.*, 2019; Carballo *et al.*, 2021).

Lastly, preliminary analysis of segregating populations of *Allium ramosum*, generated by back-crossing sexual and apomictic parental lines, revealed that diplospory and parthenogenesis are controlled by a single (or very few) dominant genes and that these genes are unlinked. Furthermore, the presence of the diplospory gene seems to be epistatic for the expression of the parthenogenesis gene (Yamashita *et al.*, 2012).

The outcome of these genetic experiments revealed that apomictic plants are typically heterozygous or hemizygous for the loci regulating the apomixis components, and they carry a single apomictic allele with a dominant effect, regardless of the ploidy level. Apomixis-related loci

are usually characterized by large- and small-scale rearrangements and enriched with transposable elements and repeat sequences, likely a consequence of long-term asexual reproduction (Ozias-Akins and van Dijk, 2007; Underwood *et al*., 2022).

Meiotic mutants producing unreduced gametes in sexual species

Sexually reproducing plants retain the ability to form restitution nuclei by first and second division restitution (FDR and SDR, respectively), causing the formation of unreduced gametes as occurs in apomictic plants. For instance, meiotic diplospory involves the formation of FDR spores without recombination to preserve the maternal genotype, whereas other types of division restitution (e.g., FDR with recombination or SDR) result in loss of heterozygosity. Occasional events in sexual species, such as unreduced egg cell formation and parthenogenesis, led Petrov (1976) to believe that "*the different elements of apomixis lie within the reproductive potentiality of sexual plants*" (cited in Asker, 1980). However, Nogler (1984) was critical of Petrov's opinion. According to Nogler, the crucial part of apomixis compared to sexual reproduction is the breaking of the bonds between megasporogenesis and megagametogenesis, especially concerning apospory and mitotic diplospory.

In Arabidopsis (*Arabidopsis thaliana*), at least 23 genes have been identified to have a role in the correct meiotic divisions (Brownfield and Köhler, 2011; De Storme and Geelen, 2013; Liu *et al*., 2021). Initially identified by transcription profiling, *Omission of second division1* (*OSD1*) has a role in both female and male sporogenesis (Table 2; d'Erfurth *et al*., 2009). The Arabidopsis mutant *osd1*, as its name suggests, omits the second meiotic division; hence the chromatids do not segregate. However, reductional meiosis I is normal, causing segregation and recombination of the maternal genotype.

So far, the only single meiotic mutation causing FDR has been identified in Arabidopsis *SWITCH1/DYAD* (Table 2; Mercier *et al*., 2001; Agashe *et al*., 2002; Ravi *et al*., 2008). The SWI1 protein is essential for sister chromatid cohesion and bivalent formation at meiosis I. Knock-out mutations cause severe female and male sterility, but the *dyad* allele, forming a truncated protein, is of medium strength (Ravi *et al*., 2008). In this mutant, the first reductional meiotic division is skipped, directly separating the sister chromatid. Therefore, megasporogenesis produces a dyad of unreduced megaspores, mimicking meiotic diplospory. However, only a very low frequency of these megaspores progresses into functional unreduced embryo sacs. Polymorphic codominant microsatellite markers demonstrated that unreduced egg cells transmitted all heterozygosity to the offspring, suggesting functional apomeiosis.

Natural mutants producing a considerable frequency of unreduced egg cells are found in other sexual species (Table 2). Hermsen (1980) considered the possibility of bringing mutants of

apomixis-like elements together to synthesize apomictic potato. In *Solanum tuberosum*, *desynapsis* (*ds-1*) is a natural mutant that produces unreduced female and male gametes (Jongedijk *et al.*, 1991). *Ds-1* was cytologically characterized as a desynaptic mutant with reduced crossing-over. The penetrance of non-reduction was variable, and the inheritance of markers indicated a mixture of FDR and SDR gametes, depending on the precociousness and timing of meiosis.

In alfalfa (*Medicago sativa* subsp. *falcata*), plants that produce unreduced female and male gametes were used for sexual polyploidization and breeding (Veronesi *et al.*, 1986). The locus controlling 2n egg cell formation in *two-n egg* (*tne*), a spontaneous alfalfa mutant, has been identified; however, the *TNE* gene has not been cloned yet (Palumbo *et al.*, 2021).

Rhoades and Dempsey (1966) described a maize natural recessive mutant named *elongate* (*el*) because of the elongated appearance of the chromosomes at both meiotic anaphases, which produced unreduced embryo sacs, but reduced functional pollen grains. They concluded that *el* might have resulted from an SDR, later confirmed by Barrell and Grossniklaus (2005) using confocal laser scanning microscopy. A genetic consequence of SDR is the loss of heterozygosity of the maternal genotype; therefore, the *el* cytological phenotype differs fundamentally from diplospory, which derives from an FDR without recombination.

Screening of mutant populations for diplospory

Using a forward genetic screening of an active-*Mu*-transposon population, Singh *et al.* (2011) isolated a dominant sporophytic mutation in maize, causing unreduced female and male gametes. The mutant, named *Dominant non-reduction4* (*Dnr4*), showed a cytological phenotype similar to that of meiotic diplospory (Table 2). The mutated gene was isolated by *Mu* tagging and found to be *AGO104*, a member of the *ARGONAUTE* family. Other four *ago104* mutant alleles were isolated with reverse genetics, all showing the same diplosporic phenotypic effect. Chromatin condensation, spindle formation, and chromosome segregation were strongly impaired in mutant pollen compared to wild-type pollen; additionally, MMC meiotic divisions were also affected. Cellular localization studies indicated that *AGO104* is expressed in the nucellar somatic tissues but not in the MMC. AGO104 controls female and male spore formation in a cell-non-autonomous way by chromatin modification through the RNA-dependent DNA methylation pathway. Interestingly, the Arabidopsis ortholog AGO9 has also been suggested to control spore development in a similar cell-non-autonomous way. AGO9 inhibits the somatic cells in the nucellus from acquiring MMC-like features; indeed, premeiotic ovules of Arabidopsis *ago9* mutants show multiple enlarged cells in the subepidermal layer of the nucellus (Olmedo-Monfil *et al.*, 2010; Mendes *et al.*, 2020).

Fox *et al*. (2016) set up a similar mutant screening for dominant diplospory mutants in sexual maize. This screening led to the identification of the *non-reduction in female4* (*nrf4*) mutant (Table

2; Grossniklaus, 2019). The gene was cloned, but no functional annotation existed. Orthologous of *nrf4* were found in several grass species. In contrast to *AGO104*, *nrf4* is female-specific but recessively inherited. However, the penetrance of unreduced egg cells ranged between 85–100%, higher than that of *AGO104* (20–80%; Singh *et al.*, 2011). Genetic markers indicated that normal meiosis was replaced by a mixture of mitosis, FDR, and SDR, depending on the genetic background of the mother plant. Up to 30% of the egg cells maintained maternal heterozygosity.

Comparative gene expression studies

Over the last decades, comparative transcriptomic analyses between apomictic and sexual accessions have been carried out for a variety of diplosporous species. A heterochronic shift in gene expression has been documented in *Tripsacum dactyloides* (Grimanelli *et al.*, 2003; Bradley *et al.*, 2007). In this species, the onset of apomictic development seems to be anticipated compared to the sexual pathway, leading to a precocious induction of embryogenesis over the meiotic program. Data collected from maize-*Tripsacum* hybrids suggest that the apomeiotic switch that reverts meiosis into mitosis-like can occur between MMC differentiation and pachytene. After this stage, the commitment to meiosis seems irreversible (Grimanelli *et al.*, 2003). Similar findings in yeast support this hypothesis (Honigberg and Esposito, 1994).

Differences at the transcriptomic level between sexual and apomictic accessions of *Boechera* spp. have revealed a general downregulation of gene expression in apomictic early-developing ovules at the MMC stage. Notable processes resulting from the comparison of genes differentially expressed between sexual and apomictic accessions are hormonal pathways, epigenetics, cell cycle control, protein degradation, and post-transcriptional regulation (Sharbel *et al.*, 2009; 2010; Amiteye *et al.*, 2011; Zühl *et al.*, 2019).

Differences in gene expression, small-RNA presence, and methylation patterns have also been shown between sexual, facultative, and full apomictic accessions of *Eragrostis curvula*. For instance, *EcAGO104* and *EcDMT102*, genes implicated in the RNA-dependent DNA methylation, were found to be differentially expressed in apomictic vs sexual genotypes, even though functional studies on mutants have not been carried out yet (Selva *et al.*, 2017). Moreover, the latest findings support the idea of a micro-RNA involvement in the regulation of apomixis in *E. curvula* (Garbus *et al.*, 2019; Pasten *et al.*, 2022).

The outcome of these studies suggested that, at least in some species, apomixis could have originated from the spatiotemporal deregulation or silencing of the sexual pathway, not from its disruption. The heterochronic expression of some genes may cause a change in cell fate. In diplosporous species of the *Antennaria*-type, for example, the precocious induction of embryo sac development might superimpose on the MMC program, leading to a total avoidance of meiosis.

This hypothesis may also explain the existence of facultative apomicts, which retain the machinery of sexual reproduction, as well as the presence of different types of apomixis in ovules of the same plant (e.g., in *Boechera* spp., *Antennaria carpatica*, *Paspalum minus*) (Bergman, 1951; Bonilla and Quarin, 1997; Carman *et al.*, 2019).

Potential application of diplospory in agriculture

It is widely recognized that apomixis has the potential to drastically revolutionize agriculture, providing a tool to produce high-quality food at lower costs (Spillane *et al.*, 2004; Barcaccia and Albertini, 2013). The global food security issue is currently one of the main concerns of our society. Feeding the increasing population in a changing environment without increasing land resources is one of the 17 goals set by the United Nations to be achieved by 2030 (United Nations, 2015). In sexually reproducing plants, desired trait combinations stacked in hybrid varieties are lost in the offspring due to genetic recombination during meiosis. In contrast, the fixation of a given genotype occurs naturally in species exhibiting apomixis through the generation of a clonal progeny. Although apomixis has evolved multiple times in different plant orders, none of the major crops is known to reproduce in this way (Savidan *et al.*, 2001; Hojsgaard *et al.*, 2014); therefore, transferring this clonal reproduction system to sexual species of agronomic interest still represents a major challenge in plant breeding. One advantage of apomixis is the one-step fixation of any important trait across multiple generations and full exploitation of heterosis, simplifying hybrid and cultivar production (Wang, 2019). Moreover, it could reduce sexual or vegetative propagation complications, like incompatibility barriers, complex pollination strategies, and disease transmission (Barcaccia and Albertini, 2013). In this perspective, a deep knowledge of the mechanisms regulating gametophytic development in both sexual and apomictic reproduction is of pivotal importance to the future of agriculture.

Recently, many groups have worked on the isolation of genes controlling critical steps of the apomictic pathway, and mutants exhibiting apomictic-like phenotypes have been obtained in sexual model plants. Remarkably, d'Erfurth *et al.* (2009) showed that the combination of a triple knock-out of meiotic genes led to the replacement of meiosis with mitosis, aptly named *MiMe* (*mitosis instead of meiosis*) mutant. These three mutations are *spo11*, *rec8*, and *osd1*. SPO11 is necessary for recombination and crossing over; the null mutation produces randomly segregating univalent. REC8 is a protein ensuring sister chromatid cohesion during meiosis I. The *osd1* mutation omits meiosis II, as previously discussed. The *MiMe* triple mutant was shown to transmit full maternal heterozygosity to the offspring. Knock-out of the three orthologous in rice also resulted in the replacement of meiosis by mitosis (Mieulet *et al.*, 2016). Later, *MiMe* plants were combined with *GEM* (Genome Elimination induced by a Mix of CENH3 variants) lines in Arabidopsis (Marimuthu

et al., 2011) and with *mtl* (*MATRILINEAL*) mutants in rice (Wang *et al.*, 2019) to produce clonal offspring. Furthermore, Khanday *et al.* (2019) produced hybrid clonal seeds in rice by combining the mutant for *MiMe* orthologous with the egg-cell-specific expression of the *BABY BOOM* transcription factor (Boutilier *et al.*, 2002). Recently, a significant increase in the frequency of these clonal seeds has been achieved, rising from 10–30% to more than 95% across multiple generations (Vernet *et al.*, 2022), suggesting that this approach of synthetic apomixis is applicable in agriculture. Autonomous endosperm development would also be needed to exploit all the benefits of apomixis, making seed set independent of unreliable pollination.

Conclusion

Over the last decades, multiple studies have shown that diplospory in natural apomicts is dominant and female-specific. Therefore, apomixis can be introgressed into a sexual gene pool without the need for inbreeding to become expressed, as would be needed for recessive traits. The introgression of natural diplospory into a sexual background would result in a large array of new clonal lineages from which the best could be selected. Synthetic apomixis, at least in its present form, demands *de novo* engineering in sexual F1 hybrids which have already been developed and it is rather a seed production system for the maintenance of hybrid vigour than a plant breeding tool. The introgression of apomixis genes would become a form of active breeding and fixation of new gene combinations in F1 hybrids. The identification of natural apomixis genes will enable the introduction of apomixis into sexual crops via reverse engineering.

There are still outstanding questions concerning the functioning of diplospory genes, such as:

- It has been observed that different types of diplospory can occur in the same plant. Could this indicate that a diplospory gene(s) may express different cytological phenotypes?
- Conversely, are similar types of diplospory in different species, e.g., *Taraxacum* and *Chondrilla*, controlled by the same gene(s)?
- If so, is the functional polymorphism the same coming from a common ancestor, or is this parallel independent evolution of different functional polymorphisms in the same gene?
- What are the diplospory master regulators and their downstream targets?

The cloned diplospory genes must be able to explain the dominance of diplospory over sexuality and the female-specificity, e.g., by RNA silencing and interference, but also truncated inactive proteins in complexes.

The identification of natural diplospory-controlling genes by map-based cloning has been severely hindered by polyploidy, recombination suppression, segregation distortion, and variable penetrance (Ozias-Akins and van Dijk, 2007). Nevertheless, the first parthenogenesis genes have been cloned in natural apomicts, i.e., *Pennisetum squamulatum PsASGR-BBML* (Conner *et al.*,

2015), *Taraxacum officinale ToPAR*, and *Pilosella piloselloides PpPAR* (Table 1; Underwood *et al.*, 2022). Interestingly, these genes are not completely new genes, but modifications of conserved sexual genes, suggesting that a "copy nature" strategy for the introduction of apomixis in sexual crops could be successful.

The diplospory genes can be identified by following the same strategies as in these parthenogenesis studies, by combining a small number of positional candidates with expression studies between sexual vs apomicts or targeted CRISPR-Cas mutagenesis. With these developments, the cloning of the first natural diplospory genes can be expected in the near future. This will throw a light on the molecular function of these enigmatic genes and will bring the application of apomixis in agriculture a significant step closer.

Acknowledgements

The authors would like to thank the reviewers for their constructive suggestions that helped improve the quality of the manuscript. We would also like to thank Arjen van Tunen, Rik Op den Camp, Diana Rigola, and Tanya Radoeva for stimulating discussions on the topics of this review and Rosanna Petrella for providing us with parts of Figure 1.

Conflict of interest

P.J.V.D. is employed at KeyGene N.V. No conflict of interest declared.

Funding

This work was supported by the European Union's Horizon 2020 MSCA-RISE-2020 POLYPLOID [grant agreement 101007438]; KeyGene N.V. [to Le.C., C.B., M.C., P.J.V.D.]; Università degli Studi di Milano [PSR2021–Linea 2 to M.C.]; and Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) [to Le.C. and C.B.]

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Tables

Table 1. General information about ploidy level, type of diplospory, pollen formation, genetic control of diplospory, and callose deposition pattern in diplosporous apomictic species. Abbreviations: *DIP*, diplospory; *PAR*, parthenogenesis.

Table 2. List of mutants generating unreduced female gametes in different plant species. Abbreviations: FDR, first division restitution; SDR, second division restitution.

Figure legends

Fig. 1. Main steps of sexual reproduction in flowering plants and developmental variations in diplosporous apomixis. (A) Schematic representation of the sexual life cycle, highlighting the alternation of generations. Meiosis of female and male spore mother cells (MMC and PMC, respectively) represents the onset of the gametophytic generation (n), whereas syngamy restores the ploidy of the sporophyte (2n). (B) Inside the ovule of sexual plants, the MMC (2n) undergoes meiosis to produce a haploid FM (n), which eventually generates an 8-celled ES. Here we show the Polygonum-type ES, which is the most common type in angiosperms. In anthers, each PMC (2n) generates four microspores by meiosis, which all develop in male gametophytes. Double fertilization consists of syngamy and central cell fertilization, which trigger embryogenesis and endosperm development, respectively. (C) In diplosporous species, the FM derived from apomeiosis is unreduced (2n), thus forming an unreduced ES. The embryo develops by parthenogenesis, whereas the endosperm can be autonomous (*) or pseudogamous (**), whether the central cell develops autonomously or needs to be fertilized. Colours represent the ploidy level (pink, n; green, 2n; yellow, 2n + n; blue, ≥2n). Abbreviations: ES, embryo sac; FM, functional megaspore; MMC, megaspore mother cell; PMC, pollen mother cell. Adapted from Kawashima and Berger (2014) and Petrella *et al.* (2021).

Fig. 2. Schematic representation of megasporogenesis and megagametogenesis in sexual and diplosporous plants. Different diplosporous apomixis (B-G) types are compared to the sexual *Polygonum*-type (A). (B-D) Meiotic diplospory of the *Taraxacum*-type (B), *Ixeris*-type (C), and *Elymus*-type (D) produce an unreduced FM through the alteration of the normal meiotic progression. (E, F) In mitotic diplospory, the meiotic step is bypassed, as shown in *Antennaria*-type (E) and *Eragrostis*-type (F). (G) Endoreduplication of the *Allium*-type is characterized by an extra chromosomal duplication of the MMC, followed by reductional meiosis. FM ploidy level remains equal to that of the sporophytic tissues in any diplosporous type. The colours of the nuclei represent the ploidy level (red, n; blue, 2n; yellow, 4n). Abbreviations: ES, embryo sac; FM, functional megaspore; MMC, megaspore mother cell. Adapted from Crane (2001).

Table 1. General information about ploidy level, type of diplospory, pollen formation, genetic control of diplospory, and callose deposition pattern in diplosporous apomictic species. Abbreviations: *DIP*, diplospory; *PAR*, parthenogenesis.

Species	Ploidy (2n)	Diplospory type	Male meiosis	Genetic control	Callose deposition	References
Meiotic diplospory						
Antennaria carpatica (Asteraceae)	3x=40-42	*Taraxacum*-type, Facultative	Unbalanced meiosis (triploidy)			Bergman (1951)
Blumea eryantha (Asteraceae)	3x-4x	*Blumea*-type, Autonomous endosperm	Unbalanced meiosis (triploidy)			Chennaveeriah and Patil (1971), Bhat *et al*. (2005)
Boehmeria silvestrii (Urticaceae)		*Taraxacum*-type				Zang *et al*. (1997)
Chondrilla brevirostris (Asteraceae)	3x=15	*Taraxacum*-type, Autonomous endosperm, Obligate			Callose is deposited at the MMC micropylar wall, and at both poles after restitution nucleus	Musiał and Kościńska-Pajak (2019)
Chondrilla juncea (Asteraceae)	3x=15	*Taraxacum*-type, Autonomous endosperm, Obligate	Reduced and unreduced pollen grains		Callose is deposited at the MMC micropylar wall	Bergman (1950); Musiał and Kościńska-Pajak (2017)
Erigeron annuus (Asteraceae)	3x=27	*Ixeris*-type, Autonomous endosperm	Unbalanced meiosis (triploidy)	*DIP* and *PAR* are controlled by two independent loci; *DIP* locus is characterized by suppression of recombination		Fagerlind (1947); Noyes and Reisenberg (2000); Noyes *et al*. (2007)
Erigeron karwinskianus var. *mucronatus* (Asteraceae)	4x=32-36	*Ixeris*-type, Autonomous endosperm, Facultative	Normal meiosis			Fagerlind (1947); Battaglia (1950)
Ixeris dentata (Asteraceae)	3x=21	*Ixeris*-type, Autonomous endosperm	Unbalanced meiosis (triploidy)			Okabe (1932), Pak and Kawano (1990), Noyes

						(2007)
Paspalum minus (Poaceae)	5x=50	*Taraxacum*-type, Pseudogamous endosperm, Obligate	Restitution nucleus?		Thick deposition of callose around the MMC	Bonilla and Quarin (1997)
Paspalum conjugatum (Poaceae)	4x=40	*Taraxacum*-type, Pseudogamous endosperm	Restitution nucleus			Chao (1980)
Rudbeckia speciosa, ———————— *Rudbeckia laciniata* (Asteraceae)	4x=76	*Ixeris*-type, Pseudogamous endosperm	Normal male meiosis Restitution nucleus			Battaglia (1945; 1946), Kościńska-Pajak *et al.* (2014)
Statice oleaefolia var. *confusa* (Plumbaginaceae)	3x=27	*Ixeris*-type, Autonomous endosperm, Facultative	Normal male meiosis			D'Amato (1949)
Taraxacum atricapillum (Asteraceae)	3x=24	*Taraxacum*-type, Autonomous endosperm, Obligate			Callose is deposited at the MMC micropylar wall	Musiał *et al.* (2015)
Taraxacum officinale (Asteraceae)	3x-4x=24-32	*Taraxacum*-type, Autonomous endosperm, Obligate	Unbalanced meiosis (triploidy)	*DIP* and *PAR* are controlled by two independent loci; in *DIP* locus, recombination occurs; the gene controlling *PAR* has been identified (*ToPAR*)		van Baarlen *et al.* (2000); Vijverberg *et al.* (2010); van Dijk *et al.* (2020); Underwood et al., 2022
Mitotic diplospory						
Antennaria parlinii (Asteraceae)	4x-8x =56-112	*Antennaria*-type, Autonomous endosperm, Facultative				Bayer and Stebbins (1983)
Boehmeria tricuspis (Urticaceae)	3x-4x =42-56	*Antennaria*-type, Autonomous endosperm, Obligate	Male flowers not present			Tang *et al.* (2016, 2017)
Calamagrostis	15x-17x	*Antennaria*-				Greene (1984)

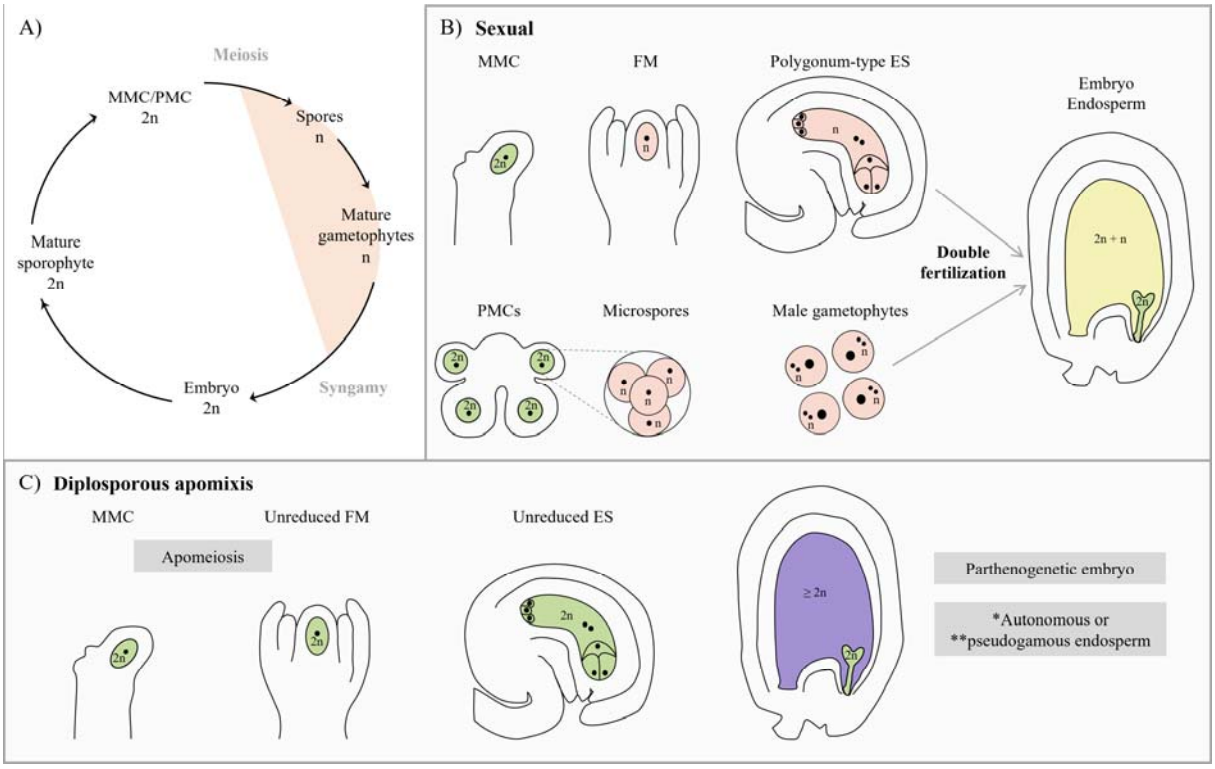
stricta subsp. *inexpansa* (Poaceae)	=104-123 ca.	type, Autonomous endosperm, Obligate				
Eragrostis curvula (Poaceae)	4x-8x =40-80	*Eragrostis*-type, Pseudogamous endosperm, Facultative or obligate	Normal male meiosis	A single 10.5 Mb non-recombinant genomic region associated with diplospory	Callose deposition around the apomictic MMC is controversial	Peel *et al.* (1997); Meier *et al.* (2011); Carballo *et al.* (2021)
Eupatorium laevigatum (Asteraceae)	6x=60	*Antennaria*-type, Autonomous endosperm	Aberrant synapsis at meiosis I			Bertasso-Borges and Coleman (2005)
Eupatorium tanacetifolium (Asteraceae)	3x=30	*Antennaria*-type, Autonomous endosperm, Obligate	Aberrant meiosis, no viable pollen			Rozenblum *et al.* (1988)
Hieracium (Asteraceae)	3x-4x	*Antennaria*-type, Autonomous endosperm, Facultative or obligate	Unbalanced meiosis (triploidy)			Hand *et al.* (2015)
Medicago sativa subsp. *falcata* (Fabaceae)	2x=16	*Antennaria*-type, Facultative			Lack of callose deposition during sporogenesis	Barcaccia *et al.* (1996)
Poa nemoralis and *Poa palustris* (Poaceae)	x=7 Autopolyploid	*Antennaria*-type, Pseudogamous endosperm, Facultative	Aberrant synapsis at meiosis I		No callose detected around the MMC	Naumova *et al.* (1993); Naumova *et al.* (1999)
Potentilla spp. (Rosaceae)	2x-9x=14-63	Pseudogamous endosperm, Facultative or obligate	Unbalanced meiosis (odd-ploidy)	*DIP* and *PAR* are controlled by two independent loci		Asker (1971); Dobeš *et al.* (2013)
Praxelis clematidea (Asteraceae)	3x=30	*Antennaria*-type, Autonomous endosperm, Obligate	Unbalanced meiosis (triploidy)		No callose detected around the MMC	Zhang *et al.* (2021)
Trypsacum dactyloides (Poaceae)	3x-6x	*Antennaria*-type, Pseudogamous endosperm,	Normal male meiosis	Apomixis seems to be controlled by more than one	Lack of callose around the MMC cell	Leblanc *et al.* (1995 a, b); Peel *et al.* (1997);

		Facultative or obligate		gene; *DIP* locus is characterized by suppression of recombinatio n	wall (thin)	Blakey *et al.* (2001)
Mitotic and meiotic diplospory						
Boechera spp**.** (Brassicaceae)	2x-6x =14-42	*Taraxacum*- or *Antennaria*-type, Pseudogamou s or autonomous endosperm, Facultative or obligate	Normal male meiosis, unbalanced meiosis (triploidy), or restitution nucleus			Böcher (1951); Mau *et al.* (2013); Brukhin *et al.* (2019); Carman *et al.* (2019); Mau *et al.* (2021)
Elymus recticetus (Poaceae)	6x=42	*Elymus*-type or *Antennaria*-type, Pseudogamou s endosperm, Facultative or obligate	Normal male meiosis		Callose deposition at the micropylar MMC cell wall and at random locations in the nucellus	Hair (1956); Crane and Carman (1987); Carman *et al.* (1991)
Endoreduplication						
Allium odorum, Allium tuberosum (Alliaceae)	4x=32	*Allium*-type, Pseudogamou s endosperm, Facultative	Reduced, unreduced, and aneuploid pollen grains			Håkansson and Levan (1957); Kojima and Nagato (1992)
Allium ramosum (Alliaceae)	4x=32	*Allium*-type, Pseudogamou s endosperm, Facultative		*DIP* and *PAR* are controlled by two unlinked dominant genes		Yamashita *et al.* (2012)

Table 2. List of mutants generating unreduced female gametes in different plant species. Abbreviations: FDR, first division restitution; SDR, second division restitution.

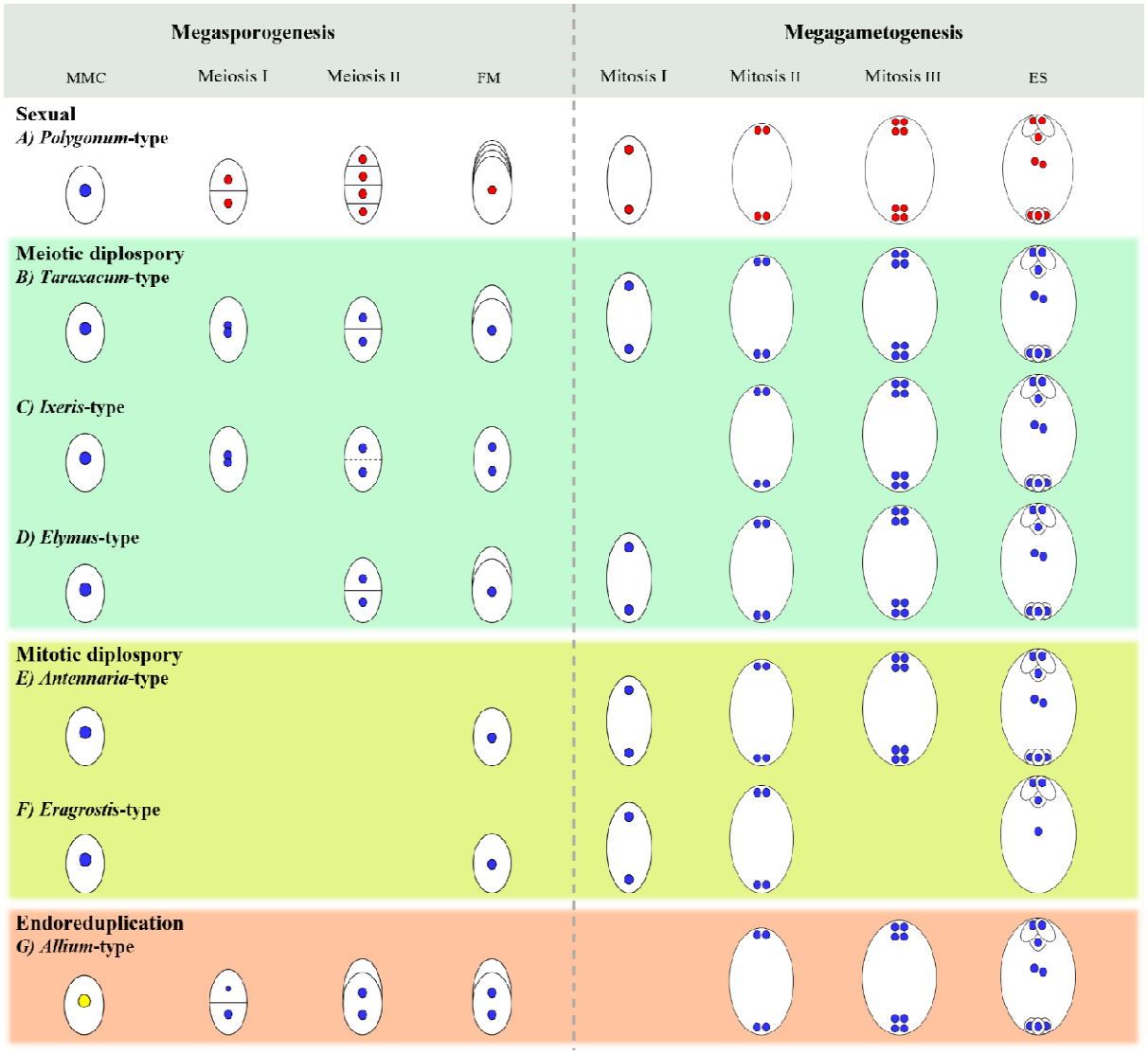
Mutant		Species	FDR or SDR	Phenotype	References
desynapsis	*ds-1*	*Solanum tuberosum*	FDR and SDR	Female and male unreduced gametes	Jongedijk *et al.* (1991)
dominant non-reduction4	*dnr4*	*Zea mays*		Female and male unreduced gametes	Singh *et al.* (2011)
dyad	*dyad*	*Arabidopsis thaliana*	FDR	Female unreduced gametes	Mercier *et al*. (2001); Agashe *et al*. (2002); Ravi *et al.* (2008)
elongate	*el*	*Zea mays*	SDR	Female unreduced gametes	Rhoades and Dempsey (1966); Barrell and Grossniklaus (2005)
non-reduction in female4	*nrf4*	*Zea mays*	FDR and SDR	Female unreduced gametes	Fox *et al.* (2016); Grossniklaus (2019)
omission of second division1	*osd1*	*Arabidopsis thaliana*	SDR	Female and male unreduced gametes	d'Erfurth *et al.* (2009)
two-n egg	*tne*	*Medicago sativa* subsp. *falcata*		Female and male unreduced gametes	Palumbo *et al.* (2021)

Fig. 1.



Main steps of sexual reproduction in flowering plants and developmental variations in diplosporous apomixis. (A) Schematic representation of the sexual life cycle, highlighting the alternation of generations. Meiosis of female and male spore mother cells (MMC and PMC, respectively) represents the onset of the gametophytic generation (n), whereas syngamy restores the ploidy of the sporophyte (2n). (B) Inside the ovule of sexual plants, the MMC (2n) undergoes meiosis to produce a haploid FM (n), which eventually generates an 8-celled ES. Here we show the *Polygonum*-type ES, which is the most common type in angiosperms. In anthers, each PMC (2n) generates four microspores by meiosis, which all develop in male gametophytes. Double fertilization consists of syngamy and central cell fertilization, which trigger embryogenesis and endosperm development, respectively. (C) In diplosporous species, the FM derived from apomeiosis is unreduced (2n), thus forming an unreduced ES. The embryo develops by parthenogenesis, whereas the endosperm can be autonomous or pseudogamous (**), whether the central cell develops autonomously or needs to be fertilized. Colours represent the ploidy level (pink, n; green, 2n; yellow, 2n + n; blue, ≥2n). Abbreviations: ES, embryo sac; FM, functional megaspore; MMC, megaspore mother cell; PMC, pollen mother cell. Adapted from Kawashima and Berger (2014) and Petrella *et al.* (2021).

Fig. 2.



Schematic representation of megasporogenesis and megagametogenesis in sexual and diplosporous plants. Different diplosporous apomixis (B-G) types are compared to the sexual *Polygonum*-type (A). (B-D) Meiotic diplospory of the *Taraxacum*-type (B), *Ixeris*-type (C), and *Elymus*-type (D) produce an unreduced FM through the alteration of the normal meiotic progression. (E, F) In mitotic diplospory, the meiotic step is bypassed, as shown in *Antennaria*-type (E) and *Eragrostis*-type (F). (G) Endoreduplication of the *Allium*-type is characterized by an extra chromosomal duplication of the MMC, followed by reductional meiosis. FM ploidy level remains equal to that of the sporophytic tissues in any diplosporous type. The colours of the nuclei represent the ploidy level (red, n; blue, 2n; yellow, 4n). Abbreviations: ES, embryo sac; FM, functional megaspore; MMC, megaspore mother cell. Adapted from Crane (2001).