Annu. Rev. Plant Biol. 2003. 54:547–74
doi: 10.1146/annurev.arplant.54.110901.160842

APOMIXIS: A Developmental Perspective

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Key Words asexual, embryo sac, embryo, endosperm, seed, sexual

■ **Abstract** The term apomixis encompasses a suite of processes whereby seeds form asexually in plants. In contrast to sexual reproduction, seedlings arising from apomixis retain the genotype of the maternal parent. The transfer of apomixis and its effective utilization in crop plants (where it is largely absent) has major advantages in agriculture. The hallmark components of apomixis include female gamete formation without meiosis (apomeiosis), fertilization-independent embryo development (parthenogenesis), and developmental adaptations to ensure functional endosperm formation. Understanding the molecular mechanisms underlying apomixis, a developmentally fascinating phenomenon in plants, is critical for the successful induction and utilization of apomixis in crop plants. This review draws together knowledge gained from analyzing ovule, embryo, and endosperm development in sexual and apomictic plants. It consolidates the view that apomixis and sexuality are closely interrelated developmental pathways where apomixis can be viewed as a deregulation of the sexual process in both time and space.

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INTRODUCTION

Apomixis is defined as asexual reproduction through seeds (85) and, thus, leads to the production of clonal progeny whose genotype is identical to that of the mother plant. Over the past few years, many reviews have focused on particular aspects of apomixis, including the different cytological mechanisms (27, 57, 85), its genetic control (34, 39, 85, 110), its relationship to the sexual pathway (36, 38, 57, 61, 120, 133), and its use in agriculture (10, 46, 47, 53, 59, 110, 112). However, apomixis is not only a useful agronomic trait with tremendous potential for plant breeding, it is also an interesting developmental phenomenon. Apomictic reproductive processes are diverse and result in the production of viable seeds, despite circumventing key steps critical for sexual seed production. The developmental and molecular analysis of apomixis provides an opportunity to understand how reproductive events in the ovule are linked, coordinated, and regulated. Apomixis is rarely considered from this perspective and the process, in this context, forms the focus of our chapter. Rather than provide a comprehensive review of the apomixis literature, we highlight specific developmental processes that are crucial for apomictic seed production.

OVULE, SEED, AND FRUIT DEVELOPMENT IN SEXUAL PLANTS

In sexual species, a strictly ordered and defined sequence of events is required to produce viable seeds (Figure 1) and associated fruit structures that facilitate seed dispersal. By contrast, apomixis results in seed and fruit formation despite the omission of meiosis and double fertilization. Analysis of apomictic development in plants requires a fundamental understanding of sexual reproduction. Before we examine how apomixis occurs and is accommodated within the ovule, we provide an overview of ovule morphogenesis and early seed development during sexual reproduction (53a, 91a).

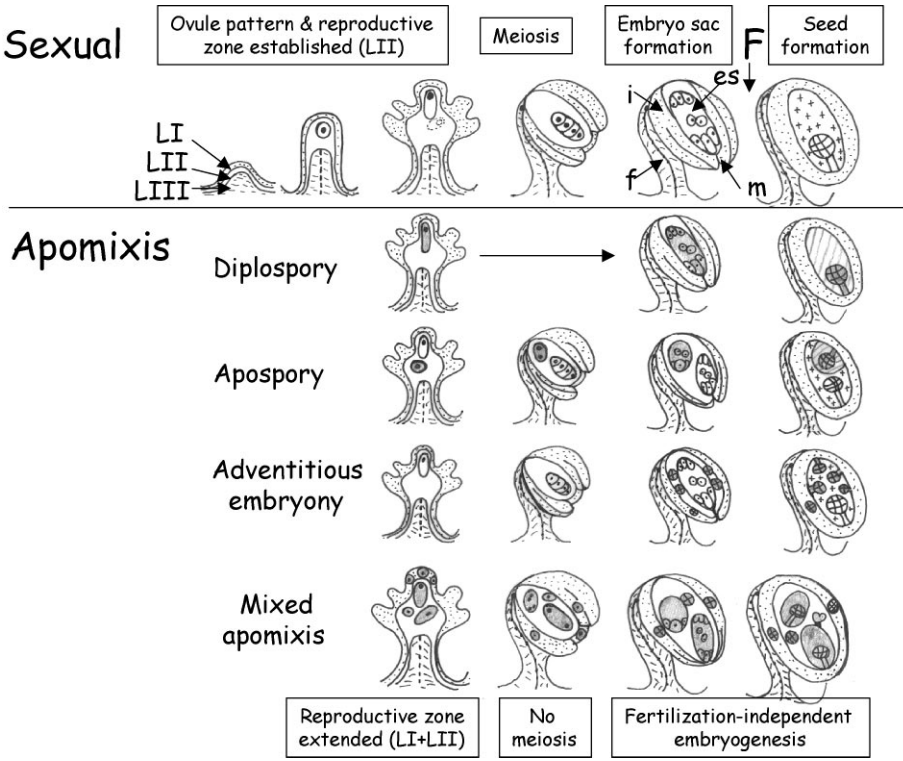


Figure 1 Sexual and apomictic reproduction in the ovule of flowering plants. The cartoon summarizes ovule development beginning with the formation of the primordium made up of the LI, LII, and LIII layers. The events of sexual reproduction are displayed in the top panel above the line. Apomictic processes are shown below the line and cells or structures involved in apomixis are shaded in grey while those pertaining to sexual reproduction remain white. Abbreviations: a, archesporial cell; e, embryo, en, endosperm; es, embryo sac; f, funiculus; F, double fertilization, i, integuments; m, micropyle; mmc, megaspore mother cell.

Ovule Development

Chronologically, the ovule is the last organ to form in the angiosperm flower. Since it is the progenitor of the seed, the ovule is the site of most processes relevant to sexual and apomictic reproduction. Ovules vary in shape, which is governed in part by the extent of their curvature and the elaboration of their epidermal cells (14), but all ovules share a number of structural elements (Figure 1). The embryo sac, or female gametophyte, is located in the core of the mature ovule, surrounded by one or two integuments that eventually form the seed coat. The integuments are not fused at the tip of the ovule where they form an opening called the micropyle, a canal that allows entry of the pollen tube during fertilization. In some species,

the endothelium, a single layer of cells, differentiates from the innermost cell layer of the integument. The endothelium forms a tube, open at both ends, which immediately adjoins the embryo sac. The ovule is attached to the placenta by a vascularized stalk called the funiculus (Figure 1). The development of the female gametophyte is coordinated with that of the sporophytic tissues of the ovule.

The Female Gametophyte Arises from Nucellar Tissue Derived from the LII Layer

Ovule development initiates with the formation of a primordium, comprising three layers, LI, LII, and LIII. Satina (107) used periclinal chimeras in *Datura stramonium* to examine the contribution of these three layers to the different structural elements of the ovule. The ovule primordium initiates as an outgrowth from the carpel placental tissue with cells dividing in the innermost LIII layer (Figure 1). Soon after initiation, LIII cell division slows and the cells of the sub-epidermal layer LII begin to multiply rapidly. These cells give rise to the nucellus, from which the sporogenous tissue differentiates. The ovule epidermis is derived from the outermost LI layer.

Megasporogenesis begins with the differentiation of an archesporial cell in the sub-epidermal layer of the ovule. Differentiation of more than one archesporial cell is not uncommon in angiosperms. Thus, reproductive potential is not confined to a single cell in the sub-epidermal layer but to several cells possibly derived from other cell layers of the nucellus. In general, only one cell develops into the megaspore mother cell (MMC) that undergoes meiotic reduction. However, differentiation of more than one MMC has been noted in sexual species such as *Arabidopsis* (40), *Datura* (107), and soybean (14). The infrequency of multiple MMC formation suggests the presence of a mechanism to restrict reproductive potential in the nucellus to a single cell.

Integument formation begins with the onset of archesporial cell differentiation in most plants. The integument(s) initiates just below the base of the archesporial cell and develops from the outermost layer, LI, and grows to envelope the differentiating MMC. The sequential growth of cells in the three layers means that the young linear ovule primordium can be divided roughly into three portions, with the funiculus derived from the LIII, and the nucellus and integuments derived from the LII and LI, respectively.

Genes Involved in Ovule Identity and Pattern Formation

Genetic and molecular analysis in *Arabidopsis*, petunia, maize, and other species has revealed the identity of a range of genes involved in ovule identity and pattern formation [reviewed in (24, 31)]. The genes essential for ovule identity include the petunia MADS (derived from *MCM1*, *AGAMOUS*, *DEFICIENS*, *SRF*) box genes, *FLORAL BINDING PROTEINS 7* and *11* (*FBP7*/*11*), and the *Arabidopsis* genes *AGAMOUS* (*AG*), *APETALA2* (*AP2*), and *BELL1* (*BEL1*). In addition, *LEUNIG* (*LUG*) and *AINTEGUMENTA* (*ANT*) in *Arabidopsis* are positive regulators of margin tissue and placenta and ovule formation (68). *HUELLENLOS* (*HLL*)

and *ANT* are involved in *Arabidopsis* ovule outgrowth and funiculus length. The molecular basis of early ovule pattern formation is not understood. However, in *Arabidopsis* ovules, which form two integuments, *ANT* and *INNER NO OUTER* (*INO*) are required for integument formation, with *INO* function essential for outer integument formation. *BEL1* is required for the development of the chalaza and the integuments. *NOZZLE* (*NZZ*), also known as *SPOROCYTELESS* (*SPL*), shares an overlapping function with *BEL1* in specifying chalazal identity, and *NZZ/SPL* is required for MMC formation (5).

The molecular mechanisms of MMC differentiation are not understood, but in maize *MAC1* is involved in limiting the number of MMCs to one, and is essential for MMC entry into meiosis (115). Four megaspores are the end products of meiotic reduction, and the number of cells that subsequently form the female gametophyte is variable in angiosperms (142). The Polygonum-type embryo sac, which is the most frequent in angiosperms, forms from a single megaspore; the remaining three degrade. The surviving megaspore initiates three rounds of mitosis, producing eight nuclei in a coenocytic sac. Each nucleus migrates to a specific position prior to cellularization, giving rise to a mature seven-celled female gamethophyte (28, 40, 145).

All cells that make up the Polygonum-type embryo sac originate from a single spore, and the lineage of each nucleus can be traced during the mitoitic divisions. However, it is not clear whether the differentiation events that specify their eventual function are influenced by changes in chromatin that follow each mitotic division, or whether differentiation occurs once mitosis and cellularization are complete. A series of mutants affecting Polygonum-type embryo sac development have been described (25, 26, 35, 76, J.M. Moore & U. Grossniklaus, unpublished data) and enhancer detection lines marking particular cells during embryo sac formation in *Arabidopsis* have been isolated (36, 37, 38, 134, 132). These lines are useful tools for analyzing the molecular events that control Polygonum-type embryo sac formation.

Seed and Fruit Initiation Can Be Uncoupled in Sexual Plants

Pollination and subsequent double fertilization in the ovule are normally required for fruit and seed development in sexually reproducing plants (16, 91a, 93, 129, 143). Parthenocarpy, or fertilization-independent fruit formation, is a heritable trait often selected for in horticultural crops because it gives rise to seedless fruit (127). Known loci involved in parthenocarpy include *pat-1* and *pat-2* in tomato (61) and *fruit without fertilization* (*fwf*) in *Arabidopsis* (138). Current genetic and molecular evidence in *Arabidopsis* suggests the presence of mechanisms that prevent the carpel from developing into a fruit without pollination and fertilization. These mechanisms involve, at least in part, *FWF* and inhibitory signals from floral whorls surrounding the carpel of the mature flower (138). The latter is consistent with observations that parthenocarpic mutants often have deformities in floral organs surrounding the carpel (81). These deformations might circumvent the floral whorl inhibitory mechanism in addition to inhibiting pollen formation and, thus, prevent seeded fruit development.

Double Fertilization and the Control of Embryo Initiation

The egg cell and the central cell of the embryo sac are the progenitors of the embryo and endosperm in the seed, respectively. Accessory cells in the embryo sac are crucial to the double fertilization process. The synergids, flanking the egg cell, produce a pollen tube attractant, which guides the pollen tube through the micropyle into the embryo sac (49). Generally only one of the synergids starts to degenerate prior to the arrival of the pollen tube, although the exact time-point, at which synergid degeneration starts, varies from species to species. The pollen tube penetrates the degenerating synergid and, upon entry, the pollen tube bursts and releases the two sperm cells (103, 104). Sperm cell release requires communication between the pollen tube and the synergid. If this signaling process is disrupted, as in the *feronia* mutant, the pollen tube does not burst and the sperm cells are not released (N. Huck, J.M. Moore & U. Grossniklaus, unpublished data). One sperm cell fuses with the egg cell to form the zygote, the other fuses with the central cell to initiate endosperm development. In the absence of fusion with a sperm cell, the egg and central cell remain in a quiescent state and eventually degrade as the flower undergoes senescence. This suggests the requirement for signaling processes to activate development of the fertilization products. The mechanisms that prevent the egg cell in plants from initiating embryogenesis without fertilization are unknown.

In animals, contact of sperm and egg cells triggers a calcium influx, which instigates a signaling cascade, initiating embryogenesis (126). In animals, parthenogenesis can be induced using calcium ionophores, which suggests that calcium influx is sufficient to trigger the entire signaling cascade (123, 130). 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 (1). Using pharmacological agents in plants to either enhance or block calcium influx can promote or inhibit certain aspects of egg activation, such as egg contraction and smoothening or cell wall formation, respectively (2). However, calcium influx alone does not lead to parthenogenetic embryo development in plants, suggesting that other activatory factors are required, which may be delivered by the sperm cell.

The Salmon system of wheat shows a high incidence (up to 90%) of parthenogenetic embryogenesis from the reduced egg cell (72). Culture of isolated reduced egg cells from Salmonwheat plants results in parthenogenesis in a cell-autonomous manner, a phenomenon which is not observed in egg cells isolated from other wheat lines (63). 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.

The Role of the *FIS* Genes in Endosperm Development

The formation of a viable embryo and the successful establishment of the seedling after germination require nutritional supplies from the endosperm, the second

fertilization product in the angiosperm seed (9, 91). Endosperm development is also triggered by fertilization and, as described above for the embryo, endosperm proliferation is actively repressed in its absence.

The isolation of mutants in *Arabidopsis* has shed light onto some aspects of endosperm initiation. Mutants of the *fertilization-independent seed* (*fis*) class, *medea* (*mea*), *fis2*, and *fertilization-independent endosperm* (*fie*), show endosperm proliferation without fertilization (22, 23, 42, 43, 89). *fis* class mutants form seed-like structures that eventually abort. However, concomitant fertilization-independent fruit development occurs, suggesting that events associated with endosperm formation are sufficient to trigger fruit initiation and maturation. Fertilized seeds derived from *fis* embryo sacs abort, showing defects in embryo and endosperm growth. This suggests that the *FIS* genes are essential in the control of cell proliferation [reviewed in (20, 21, 41)].

Analysis of cloned *FIS* class genes shows that they encode proteins of the *Polycomb* group (PcG) (43, 71, 90), which are thought to form multimeric protein complexes that repress gene expression through their effect on higher order chromatin structure. MEA and FIE interact physically in vitro as well as in the yeast two hybrid system, and the *FIS* class genes were expressed in partially overlapping domains during gametophyte and seed development (70, 119, 144). Thus, the FIS proteins may form complexes similar to their animal counterparts [reviewed in (32)]. However, the plant PcG complexes seem less stable during development and the repressive action of various complexes is relieved by specific stimuli, such as fertilization or vernalization (55, 99). It is likely that fertilization triggers the release of FIS-mediated repression and, thus, initiates endosperm formation in the wild-type situation [reviewed in (21, 41)].

MECHANISMS OF APOMIXIS

The apomictic mode of reproduction omits critical events observed in the sexual pathway. Meiosis is avoided prior to embryo sac formation (apomeiosis), embryo development is autonomous (parthenogenesis), and endosperm formation requires certain developmental adaptations. These three features are often referred to as components of apomixis. Sexuality and apomixis are not mutually exclusive modes of reproduction. With few exceptions, most apomicts retain the capacity for sexual reproduction and are, thus, facultative apomicts. Depending on the mode of apomixis, the events of sexual reproduction might occur in the same ovule or in different ovules of the same apomictic plant.

Apomixis Initiates from Cells Derived from both LI and LII Layers

Details of apomictic reproduction vary in terms of the origins of the cells initiating apomixis and whether their immediate fate is the formation of an embryo sac or an embryo. The means by which meiosis is avoided, the degree to which the sexual process continues in parallel, and the requirement of fertilization and specific

adaptations for endosperm formation are all factors contributing to the diversity of apomictic processes.

Collectively, apomixis can initiate at various stages during ovule development. The often concurrent events of sexual reproduction in the same ovule, or in adjacent ovules, are used as reference points for the timing of initation of apomixis and its progression. A survey of described apomictic processes (4, 27, 79, 85) indicates that cells initiating apomixis can arise from the nucellus, the integument, or epidermal cells in the ovule. Therefore, in contrast to sexual plants where the cells initiating reproductive events in the ovule originate from the LII layer, the zone of reproductive competency in ovules of apomicts has been significantly extended to include cells originating from both LI and LII layers (Figure 1).

Gametophytic Apomixis: Diplospory and Apospory

Cells initiating apomixis early in ovule development usually undergo gametophytic apomixis, which is the mitotic formation of unreduced embryo sacs. Gametophytic apomixis is subdivided into two forms depending on the origin of the cell that initiates unreduced embryo sac formation. If the progenitor cell differentiates in the position usually occupied by the MMC, the mode of embryo sac formation is called diplospory (Figure 1). Entry into meiosis is observed cytologically in some diplosporous species, but the process aborts and, after chromosome reorganization, the cell undergoes mitosis to form an embryo sac. Initiation of meiosis is not observed in all species classified as diplosporous.

The mitotic formation of an unreduced embryo sac from a cell in a position other than that occupied by a MMC is called apospory (Figure 1). Diplosporous and aposporous embryo sacs may or may not resemble the reduced embryo sacs observed in related sexual plants. Embryogenesis occurs autonomously in both diplosporous and aposporous embryo sacs, and endosperm formation may be autonomous or may, more commonly, require fertilization (referred to as pseudogamy).

Multiple aposporous initial cells can form in an individual ovule giving rise to multiple aposporous embryo sacs. In *Pennisetum* spp. (140) and *Hieracium* spp. (128) initiation of apospory leads to the abortion of the concurrent sexual process, whilst in *Brachiaria* spp. both sexual and aposporous embryo sacs coexist (3). Successfully initiating embryogenesis in embryo sacs derived from both pathways can lead to polyembryonic seed.

Adventitious Embryony and Mixed Allsorts of Apomixis

If the cell initiating apomixis directly develops into an embryo, this process is called adventitious embryony (Figure 1). This occurs late in ovule development from cells of either the integument or the nucellus. Usually embryo survival depends on successful fertilization of the adjacent meiotically derived embryo sac and on the ability of the adventitious embryo to grow sufficiently to gain access to the nutrient endosperm. More than one embryo can initiate and the zygotic embryo

may or may not survive. If fertilization does not occur, the adventitious embryo grows by obtaining nutrients from the degrading nucellar and integumentary cells. These embryos arrest early in development (79).

It is important to mention that diplospory, apospory, and adventitious embryony can and do exist in apomictic species of *Beta* (52) and in the Rosaceae (88). In some aposporous grasses, citrus, and certain *Hieracium* spp., apospory and adventious embryony coexist (79). For this reason such plants are not the most popular for apomictic studies.

Genetic Control of Apomixis

Apomixis is known in over 40 plant families (17). The pioneering studies of Nogler (86) and Savidan (109) proved that apomixis is under genetic control, although its expressivity may be affected by genetic modifiers or environmental conditions (85, 112). In genetic crosses the apomict is used as the pollen donor to a sexual female. Genetic analysis of apomixis is complicated by the polyploid nature of most apomicts, the suitable availability of compatible sexual plants, and difficulties in scoring the progeny, particularly as the components of apomixis may segregate.

In apomicitc *Panicum* spp., *Ranunculus* spp., and *Hieracium* spp. the dominant locus controlling apospory co-segregates with parthenogenesis, suggesting that a single locus, either simple or complex, controls apomixis [reviewed in (34, 39, 110)]. In other apomicts, the genetic loci controlling apomeiosis, parthenogenesis, and functional endosperm formation can be separated from each other [reviewed in (34, 39)]. Thus, in these species at least three distinct loci are involved in apomixis. Moreover, the genetic control of each apomixis component may be complex and involve more than one gene. Species exhibiting a mixed allsorts of apomixis may contain multiple loci conferring the different components in combination with associated modifiers.

Relationships between Apomixis and Sexual Reproduction

In the past, many apomixis researchers viewed sexual and apomictic reproduction as two distinct processes that have little in common. After Nogler (82) established that apospory in *Ranunculus auricomus* was under genetic control, he hypothesized that apospory might be viewed as a deregulation of the sexual process. Nogler (85) argued that this view was supported by the occurrence of some components of apomixis in sexual plants, such as multiple archesporial cells or parthenogenesis of reduced egg cells [reviewed in (36, 120)]. Nogler (85) referred to an "opening of the bonds" linking megasporogenesis and embryo sac initiation, clearly viewing this as the major modification of the sexual program and allowing for the expression of apospory in *Ranunculus*. This opening or breaking up of bonds that link consecutive steps in the sexual developmental program was not absolute because the sexual program still occurred in the plant (87).

The subsequent confirmation that relatively few loci control different modes of apomixis and that mutagenesis can induce apparent components of apomixis in

sexual plants has lent further support to a now widely held view that apomixis and sexual reproduction share gene expression pathways and regulatory components. From this point of view, apomixis can be viewed as a deregulation of the sexual developmental program in space and time, leading to putative cell fate changes and the omission of critical steps in the sexual process [reviewed in (36, 38, 57, 120)]. Depending on where and when this deregulation occurs, different types of apomixis may arise.

The expression patterns of molecular markers such as *SPL::GUS* and various *FIS-class::GUS* chimeric constructs with specific temporal and spatial expression patterns in *Arabidopsis* were recently examined in transgenic sexual and aposporous *Hieracium*. The results of these experiments confirmed that gene expression pathways and regulatory elements during sexual and apomictic reproduction in *Hieracium* are shared even though the aposporous embryo sacs in *Hieracium* do not always resemble those formed in the sexual plant (M. Tucker & A. Koltunow, unpublished data).

Genetic analysis in sexual plants has clearly shown that the events leading to female gametophyte formation and seed development are both independent of and interdependent on the events and signals from surrounding sporophytic ovule tissues (31). The importance of sporophytic ovule signals in governing the progression of the apomictic process is not entirely clear. Studies in *Hieracium* spp. with apospory show that alterations in ovule development correlate with changes in the apomictic process in terms of the frequency of initiating cells (58, 60). Continuation of these studies, targeting different cell types in the ovule, and similar examinations in other apomictic species should clarify further the role of sporophytic ovule tissues in apomictic development.

Overlaps Between Sexual and Apomictic Pathways

It is possible to find four different classes of progeny arising from self-fertile facultative apomicts. Rutishauser (105) first observed this in *Potentilla* spp., and it was later showed to be common both in apomicts requiring fertilization for the initiation of endosperm development (85, 109) and in those that form fertilization-independent endosperm (116). The first two progeny classes are self-explanatory: They are seedlings with a maternal genotype, arising from the apomictic mode of reproduction, and hybrid seedlings arising from sexual reproduction. The two less commonly occurring classes result from convergence of the sexual and apomictic pathways, due to their coexistence in an individual ovule. Class 3 seedlings have a higher ploidy level than the maternal parent because they are derived from the fertilization of an unreduced egg cell (B_{III} hybrid). Class 4 comprises seedlings with reduced ploidy (polyhaploids), compared to the maternal parent. It is derived from the autonomous development of a reduced egg cell and, thus, is similar to the parthenogenesis observed in the Salmon system of wheat.

Admittedly, the numbers of seedlings observed with increased and decreased ploidy are relatively small in facultative apomicts. However, these apparent overlaps or crossovers between sexual and apomictic pathways in the ovule are informative. The capacity to form B_{III}hybrids suggests that the unreduced embryo

sac is functionally comparable to one derived from meiotic reduction: It can attract the pollen tube and its egg cell can successfully participate in fertilization. The capacity to autonomously produce embryos from a reduced egg cell suggests that the locus controlling parthenogenesis is not as tightly linked to the genetic locus responsible for apomeiosis. Therefore, parthenogenesis can occur in an egg derived from a meiotically produced embryo sac. Alternatively, signals from maternal tissues of the apomict might activate the reduced egg to initiate embryonic development.

Cell Fate Decisions in Cells Initiating Diplospory and Apospory

The marker studies in *Hieracium* spp. confirm that apospory and the components of autonomous embryo and endosperm development share gene expression with sexual reproduction (M. Tucker & A. Koltunow, unpublished data). If apomixis represents a deregulation of the sexual pathway, the capability to initiate the individual components of apomixis might relate to switches in fate from a cell on the sexual pathway to apomixis. Alternatively, ectopic expression of a sequence of the sexual program in somatic cells might induce a particular apomictic component. Cell-type-specific markers make it possible to address these fundamental questions in apomictic and sexual plants.

Perhaps the clearest indication of a switch in fate from a sexual to an apomictic pathway is in diplospory where the MMC cytologically enters and then aborts meiosis prior to embryo sac formation. However, not all diplosporous apomicts make a clear entry into meiosis. In the absence of appropriate marker studies it is still unclear whether the cell occupying the position of the MMC in such cases originally has functional MMC identity.

Similar uncertainty concerns the identity of cells initiating apospory. A testable hypothesis is that apospory might involve a switch from a somatic cell to a reproductive program. If so, do these somatic cells adopt a MMC identity and then switch to a mitotic gametophyte-forming fate? Do they differentiate immediately into a megaspore or functional megaspore and then form an unreduced embryo sac? Marker analysis in aposporous and sexual *Hieracium* spp. has demonstrated that the early enlarging aposporous initial cell does not have the identity of an MMC, but attains embryo sac identity during the first nuclear division (M. Tucker & A. Koltunow, unpublished data).

Differentiation of the aposporous initial cell in apomictic *Hieracium* coincides with the demise of the sexual process in the ovule. This correlates with a change in the spatial pattern of marker expression in adjacent cells undergoing meiosis. The spatial change in marker expression identifies a group of cells that will cease further development and subsequently degrade. Cells undergoing apospory continue reproductive development and the subsequent expression of the introduced markers during aposporous embryo sac formation, and autonomous embryo and endosperm development is the same as that found in the sexual plant (M. Tucker & A. Koltunow, unpublished data). Thus, apomixis in *Hieracium* may reflect the induction of a short-circuited sexual pathway in aposporous initial cells. Other aposporous species form initials at different times of ovule development in

comparison to *Hieracium* spp. and in some of these, such as *Brachiaria* spp., the demise of the sexual pathway does not occur. Marker studies examining cell fates in other aposporous species should determine whether there are differences in the identity of cells initiating apospory. They should also lead to an understanding of the interrelationships between sexual and aposporous gametophyte development and to an understanding of the factors favoring the development of one type at the expense of the other, or enabling their coexistence.

The Autonomous Embryo Component: Clues from Somatic Embryogenesis?

The ability of a cell in the ovule to undergo fertilization-independent embryogenesis may share regulatory features with somatic embryogenesis, which is known to occur in vivo (97, 131) and also in vitro under appropriate conditions that often subject cells to stress (77).

Genetic and molecular analysis of embryogenesis in *Arabidopsis* and somatic embryogenesis in vitro reveals the identity of several genes influencing the potential of plant cells to undergo embryogenesis. The *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*), which is expressed in those cells of a carrot embryogenic culture that forms somatic embryos (113), may be a component of the hypothetical signaling cascade that initiates embryogenesis. Overexpression of *Arabidopsis SERK* (*AtSERK*) increases the embryogenic potential of cultured *Arabidopsis* cells. *AtSERK* is expressed in regions of the developing ovule, including the egg cell, where it may lead to the initiation of embryogenesis upon activation by the appropriate signal (48).

Several other genes that may be involved in embryo initiation have been described. The overexpression of the structurally unrelated transcription factors *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 1* and *2* (*LEC1*, *LEC2*), or *WUSCHEL* (*WUS*) leads to the formation of somatic embryos in transgenic plants (15, 69, 124, 146). However, it is unknown whether all of these genes are expressed in the egg cell or zygote during wild-type development. No expression was detected in the zygote for those genes analyzed at such early stages, (73; V. Gagliardini & U. Grossniklaus, unpublished data), suggesting that they may not be directly involved in embryo initiation. Their overexpression possibly causes some type of stress or activation response that leads to the de-differentiation of specific cells, and embryonic identity may be acquired as the default state.

This hypothesis is consistent with the finding that not all cells can respond to the overexpression of these transcription factors and that somatic embryo formation is restricted to young seedling stages (15, 124). Other factors may be required to allow a de-differentiated cell to acquire embryonic identity. These factors may only be present at early stages of development, allowing a stressed seedling to be rescued through somatic embryo formation, while stress at later stages leads to early flowering, which provides an opportunity for rescue through seeds. Similar stress responses may occur when cell cultures are treated with very high levels of auxin, leading to somatic embryogenesis, or upon heatshock of microspore cultures,

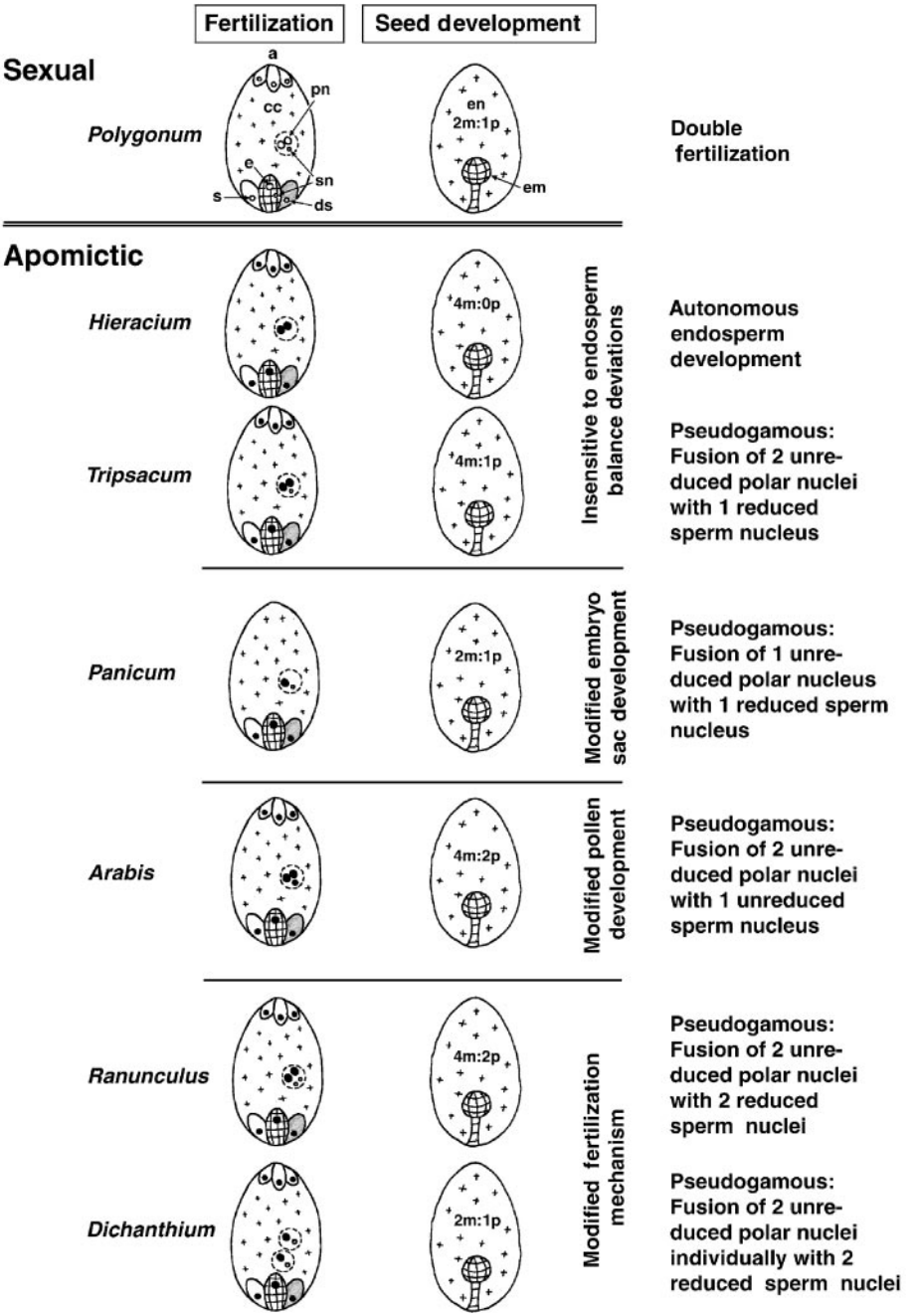
producing androgenetic embryos [reviewed in (77)]. In summary, although somatic embryo formation can be successfully induced in transgenic models, the molecular mechanisms that initiate embryogenesis upon fertilization, or in a parthenogenetic context, are not known. Examination of the expression of *SERK*, *LEC 1, LEC2*, *BBM*, and *WUS* markers or their homologues in apomictic species might be instructive in terms of their relevance in the induction of parthenogenesis and adventitious embryony.

FIS Gene Function in Apomicts

Fertilization-independent endosperm proliferation, as observed in the *fis* mutants, can be viewed as a component of apomixis. Autonomous endosperm development is not the rule in apomicts but does occur in a considerable number of apomictic species, particularly within the Compositae. Indeed, the *FIS* genes are required for aspects of seed development in *Hieracium* (M. Tucker & A. Koltunow, unpublished data). Because PcG repression is thought to be relieved by fertilization, the PcG complex is a likely target of the signaling cascade induced by fertilization in sexually reproducing plants, possibly through modification of the FIS proteins. The signaling cascade occurring in the central cell upon fertilization is not known, nor are all of its targets. In the absence of fertilization diploid endosperm proliferates in *fis* mutants. If pollinated, *fis* mutant central cells form triploid endosperm through fertilization (F. Matzk & U. Grossniklaus, unpublished data). Thus, once the mitotic divisions in the embryo sac are complete, *fis* central cells do not simply initiate endosperm proliferation; they halt at maturity, suggesting the existence of additional controls that monitor progression through this phase of reproductive development. In *fis* mutants, only the central cell proliferates, suggesting that the factors stimulating autonomous endosperm formation are not sufficient for parthenogenesis in *Arabidopsis*. Moreover, *fis* endosperm is not viable and usually arrests prior to cellularization. This may be because the *FIS* gene products are also required later during endosperm development, as suggested in studies by Sorensen et al (118), or because they represent only a subset of the targets that are induced or activated by fertilization. The lack of gene products, whose activity depends on fertilization but is not controlled by *FIS*-mediated repression, may lead to early endosperm arrest. This hypothesis is consistent with the finding that autonomous endosperm development proceeds much further if *fis* mutants are combined with a hypo-methylated paternal genome [i.e., by crossing them with a methyltransferase antisense (MET/as) plant (135)]. In MET/as plants hundreds, if not thousands, of genes are deregulated, some of which may depend on fertilization for normal activity or expression.

Genetic Constitution of Endosperm in Sexual and Apomictic Plants

Autonomous endosperm formation is rare in apomicts. Most apomicts require fertilization of the central cell to form functional endosperm. Adventitious embryos usually rely on endosperm that is produced by the sexual embryo sacs present in the



same ovule. In most gametophytic apomicts, the central cell requires fertilization (pseudogamy) while the egg develops parthenogenetically. In both autonomous and pseudogamous apomicts, the endosperm's genetic constitution differs from that of sexual plants, an issue that deserves consideration (Figure 2).

In embryo sacs of the Polygonum-type, the central cell contains two polar nuclei. These nuclei either fuse prior to fertilization, as in *Arabidopsis*, or fuse together with the sperm nucleus during fertilization, as in maize. Therefore, fertilization of the central cell produces triploid endosperm containing two maternal and one paternal genome (2m:1p). The ratio of paternal to maternal genomes is crucial for normal seed development because in many species interploidy crosses result in seed abortion due to endosperm failure [reviewed in (44)]. The genetic basis for seed abortion was investigated in detail for maize, where different m:p ratios could be generated in the endosperm without affecting the genome ratio of the embryo (66, 67). In maize, normal endosperm only develops with a 2m:1p ratio, whereas all other ratios lead to seed abortion. In *Arabidopsis*, the situation is less rigid with ratios other than 2m:1p affecting seed size and only extreme deviations leading to abortion (114). In *Arabidopsis*, the uncoupling of m:p ratios in the embryo from those of the endosperm is not possible and, therefore, the observed effects on seed size could not unambiguously be attributed to the endosperm (7).

The differences between maize and *Arabidopsis* may be due to different roles of the endosperm in seed development. The maize endosperm is permanent, constitutes about 80% of the seed weight, and is required for germination. In *Arabidopsis*, the endosperm is transient and is consumed during seed development. In other species, for instance among the Podostemonaceae and Orchidaceae, endosperm does not develop (8). Depending on the function of the endosperm in seed development, the importance of the m:p genome ratio may vary (74).

←

Figure 2 Adaptations to ensure functional endosperm development in apomicts. The cartoons show the embryo sac at fertilization on the left and the resulting products with the corresponding maternal:paternal (m:p) genome ratio on the right. The situation in sexual plants, where both the egg and central cell participate in double fertilization, is illustrated at the top. Reduced nuclei are indicated as circles, unreduced nuclei are filled. The degenerating synergid is shaded, egg cell and resulting embryo hatched, and central cell and resulting endosperm indicated by crosses. Nuclei that participate in endosperm formation are delineated by stippled circles. *Hieracium* and *Tripsacum* are insensitive to a deviation of the m:p ratio (unlike sexual plants), whereas in other apomicts specific developmental processes (embryo sac development, pollen development, fertilization) are modified to ensure a 2m:1p ratio required for functional endosperm development. For details see text. In contrast to the *Panicum* embryo sac shown, the embryo sac of sexual *Panicum* spp. is of the Polygonum-type. Abbreviations: a, antipodals; cc, central cell; ds, degenerating synergid; e, egg cell; em, embryo; en, endosperm; pn, polar nuclei; s, synergid; sn, sperm nuclei.

Although the effects of the m:p genome ratio on seed development are often interpreted to result from the action of genes that are regulated by genomic imprinting (38, 44, 136), such effects could be mediated through any mechanism that leads to unequal contributions of the two parental genomes. These include differential gene expression either pre- or postfertilization, i.e., the deposition of maternal products in the central cell and differential allele-specific expression dependent on parental origin (genomic imprinting), respectively, as well as other dosage effects (7, 12, 121).

Whatever the underlying mechanisms, endosperm balance is important for engineering apomixis in cereals, in which seed development is very sensitive to a deviation of the 2m:1p genome ratio. In apomicts, the 2m:1p ratio is not maintained because male meiosis is usually unaffected and a central cell, containing two unreduced polar nuclei, is fertilized by a reduced sperm cell, resulting in a 4m:1p ratio. This issue was discussed by Nogler (85) but has only recently attracted attention. The formation of unbalanced endosperm in apomictic hybrids may provide an explanation for the high frequency of seed abortion reported in introgression programs (38, 78). In contrast, natural apomicts are highly fertile, and to resolve this "endosperm problem," it seems appropriate to investigate endosperm development in apomictic species.

Modification of Developmental Programs to Maintain Endosperm Balance

In pseudegamous apomicts, fertilization of the unreduced central cell with a reduced sperm cell is expected to form endosperm with a 4m:1p genome ratio which, in maize and other sexual species, leads to seed abortion. Endosperm balance is also disturbed in autonomous apomicts, as there is no paternal genome contribution to endosperm formation (4m:0p). To overcome this problem, apomicts use several mechanisms that fall into two major groups (Figure 2). In the first group, endosperm development is insensitive to the m:p ratio. Autonomous apomicts and several pseudogamous species belong to this group. In *Tripsacum dactyloides*, a wide variety of m:p ratios support functional endosperm development (33). In *Paspalum notatum*, crosses between sexual diploid and tetraploid plants result in seed abortion, whereas apomictic plants tolerate a wide range of m:p ratios (94). This demonstrates that a tolerance for unbalanced endosperm does not occur in sexual relatives and is intimately related to apomictic reproduction.

In the second group, modifications of embryo sac development or the double fertilization process evolved, leading to a maintenance of the 2m:1p ratio (38, 41, 85, 110, 136). A wide-spread mechanism in the grasses is illustrated by *Panicum maximum*, where an eight-nucleate Polygonum-type embryo sac forms during sexual reproduction and a four-nucleate embryo sac of the Panicum-type results from aposporous apomixis (108, 139). Modifying the pattern of embryo sac development in the apomict usually results in a single polar nucleus and, thus, ensures a 2m:1p ratio when fertilized. Apomictic *Arabis holboellii*, a close relative of

Arabidopsis, exhibits an alteration in microsporogenesis that leads to the formation of unreduced sperm cells that fertilize the unreduced central cell, producing a 4m:2p ratio in the endosperm (13, 80). In other apomictic species, both sperm cells delivered by the pollen tube fuse with the central cell, indicating modifications in sperm release and/or transport compared with double fertilization in sexual species. Nuclear fusion occurs either with the fused, unreduced polar nuclei, as in *Ranunculus* spp. (106), or each sperm nucleus fuses separately with one of the unfused, unreduced polar nuclei, as in *Dichanthium annulatum* (98). In *Ranunculus auricomus*, the modification allowing both sperm cells to unite with the central cell, and to effect nuclear fusion with the polar nuclei, is genetically controlled and is not linked to the locus conferring apospory (83). Thus, in addition to factors controlling apomeiosis and parthenogenesis, developmental modifications allowing functional endosperm development in apomicts also need further consideration.

UPDATED MODELS FOR THE CONTROL OF APOMIXIS

We discussed developmental events and questions that are relevant to the major elements of apomixis focusing on apomeiosis, parthenogenesis, and functional endosperm formation. Now we must address what causes the developmental alterations observed in apomicts. Many models and hypotheses have been put forward over the last century. We do not attempt to comprehensively review all of these models. Instead, we present general concepts that might also explain the evolution of apomixis. Given the diversity of apomictic processes, and that apomicts have been reported in close to 40 plant families, the routes that led to the evolution of apomixis may be as diverse as the cytological mechanisms. It is quite likely that different concepts may play predominant roles in different apomicts, that they may not be mutually exclusive, and that they may occur in combination to create the developmental diversity and versatility observed in apomictic species.

Hybridization

At the beginning of the past century, Ernst postulated that apomixis might result from the hybridization of related species (30). This explanation took into account that virtually all natural apomicts are polyploid and highly heterozygous. The recovery of diploid apomicts (usually as polyhaploid progeny) in various species (11, 29, 56, 64, 84, 102, 137) has unequivocally shown that polyploidy is not a prerequisite for apomictic reproduction, but may be a consequence of asexual reproduction. The hybridization theory for the evolution of apomixis has received renewed interest through its extension by Carman (17, 18), who suggests that apomixis may result from hybridization of two ecotypes or related species with differences in reproductive characters. The hybrids are postulated to contain two sets of genes involved in female reproduction from the two genomes, whose asynchronous expression may lead to precocious embryo sac initiation and embryogenesis. Support for this hypothesis comes from hybrids of two sexual parents that display apomictic

traits and the occurrence of reproductive anomalies in allo-polyploids or paleo-polyploids [reviewed in (17)]. Importantly, the hybridization model relies only on the additive effect of native gene expression, rather than mutations in genes involved in sexual reproduction.

Mutation

Genetic analyses showing that apomixis is under the control of a few loci has led to the well established model that apomixis results from mutations at one or a few loci. For many years, researchers viewed apomixis and sexuality as two distinct and unrelated processes. Therefore, mutations causing apomixis were often thought of as neomorphic, providing novel functions. However, a developmental view stresses the inter-relatedness of the two processes, and the genes conferring apomixis are now viewed as mutated alleles of genes controlling sexuality. In this context, genes conferring apomixis may not actually code for novel or aberrant functions, but rather provide wild-type activity, albeit in the wrong cell type or at the wrong time. For instance, a wild-type gene promoting embryo sac initiation that is expressed in a nucellar cell could cause apospory (57). Studies using molecular markers in sexual and apomicitc *Hieracium* have shown that dividing aposporous initial cells express markers associated with the fate of the functional megaspore (M. Tucker & A. Koltunow, unpublished data).

Savidan (111) advocates that apomixis may be controlled by a master regulatory gene conferring apomeiosis and that the other elements of apomixis follow from this initial step as a pleiotropic effect in a cascade-like fashion. This hypothesis is attractive by virtue of its simplicity and many master regulators do lead to a gene regulatory cascade involving thousands of genes controlling the formation of entire organs [e.g., (45)]. While this may occur in some apomicts, in others, several distinct loci are involved in the genetic control of apomixis, each controlling an individual element (34, 39).

Epigenetic Gene Regulation

The genes controlling apomixis may not represent mutant alleles but could be a consequence of epigenetic changes in gene regulation (36, 120). For instance, stable and heritable epialleles can arise through changes in DNA methylation [e.g., (50, 51, 54, 117)] or chromatin structure (19, 122). This epigenetic model is attractive for several reasons.

First, 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 (65). Epigenetic changes may be a consequence of hybridization or result from ploidy changes in the hybrids [discussed in (34)]. Epigenetic changes observed after polyploidization in *Arabidopsis* can be stable and are maintained even after a reduction in ploidy (O. Mittelsten Scheid & J. Paszkowski, personal communication), as may have occurred in palaeo-polyploids.

Second, the epigenetic control model provides a solution to the enigma that several traits had to evolve coordinately to produce a functional apomict; for example, apomeiosis, parthenogenesis, and functional endosperm formation, which on their own are disadvantageous (75). As the simultaneous occurrence of several mutations is unlikely, models involving a single master regulatory gene were favored in the past. However, as outlined above, the components of apomixis are under independent genetic control in some species. If the changes leading to the formation of a functional apomict were epigenetic rather than genetic in nature, it is possible that these epimutations occurred in the same ancestral plant (120). Unlike mutations, epigenetic changes can occur rapidly and may be particularly frequent as a result of hybridization and polyploidization, providing the epigenetic raw material necessary to form an apomict. Strong support for this view comes from the fact that a simple chromosome doubling, not involving interspecific hybridization or mutation, can produce functional apomicts in *Paspalum* spp. (95, 96).

Third, the presumed polyphyletic origin of apomixis is often taken as an argument in favor of a simple control mechanism of apomixis as the occurrence of several mutations in one ancestor is so rare that it could hardly have occurred multiple times (111). As described above, if these changes are epimutations, rather than mutations, accumulation of the required apomixis components is far more likely. Alternatively, the polyphyletic origin can also be taken in favor of the view that the molecular mechanisms underlying apomixis are highly diverse and may be caused by mutations or epimutations in a large number of different genes.

Apomixis-Specific Factors

All three concepts described above are based on genes playing a crucial role in sexual development that are asynchronously expressed (under the wild-type control of one of the two genomes in an allo-polyploid), mutated, or misregulated through a genetic or epigenetic change in gene expression. In contrast, recent findings in *Pennisetum squamulatum* and *Cenchrus ciliaris* show that molecular makers, which are tightly linked to apospory, do not hybridize to DNA from sexual plants (92, 100). Thus, these sequences are either absent from the genome of sexual plants or highly divergent (39), suggesting a possible role of supernumerary, apomixis-specific chromatin in the control of apomixis (101). In all apomicts analyzed, the apomeiosis locus is located in a region of the genome where recombination is suppressed, often over dozens of centiMorgans [reviewed in (39)]. The resulting recombinational isolation of the apomeiosis locus may explain the divergence from the corresponding region in sexual species, where recombination is not suppressed. While the general region may be highly divergent, an ancestral sexual gene, whose activity has been altered in the apomict, may still reside in this region. Alternatively, the ancestral gene may be linked to the region, such that the inserted or highly rearranged region affects its activity.

Another explanation, which can reconcile 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 supernumerary chromatin acts as a sink for proteins that regulate a 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.

Although there is now some evidence that gene expression pathways are shared during sexual and apomicitc reproduction and that genes are differently regulated during apomixis (M. Tucker & A. Koltunow, unpublished data), it cannot yet be excluded that supernumerary chromatin, which has no counterpart in sexual plants, plays a crucial role in apomixis. This would be somewhat reminiscent of infectious parthenogenesis in insects, where endo-parasitic *Wolbachia* bacteria alter their host's mode of reproduction (6, 125, 141). Certain insects infected with *Wolbachia* reproduce parthenogenetically, producing exclusively female offspring, thus increasing the chance of spreading the bacteria, which are only transmitted through the egg. In a way, the *Wolbachia* endoparasites represent foreign DNA, determining the mode of reproduction not unlike the supernumerary chromatin in apomictic plants. The origin of apomixis-specific chromatin is unknown and open to speculation. It may be derived from sexual ancestors or, in analogy to *Wolbachia*, from another organism, may be an endo-symbiont or endo-parasite. Such gene transfer of DNA from the endo-parasite to the host genome was recently documented for *Wolbachia* (62). In any case, such foreign control would have to rely on pre-existing developmental programs, which it could act on to modify the mode of reproduction.

CONCLUSIONS

In this review, we focused on the developmental events of sexual reproduction that are altered or modified in apomixis. Whatever the genetic basis and underlying molecular mechanisms, it is clear that the sexual program served as the basic framework from which apomixis evolved. Without reference to the underlying causes, the major developmental hallmark of apomixis is a deregulation of sexual processes in time and space. In diplosporous species, embryo sac initiation occurs prior to the completion of meiosis or, in aposporous apomicts, in a nucellar cell other than the megaspore. Later, embryogenesis and sometimes endosperm development initiate without fertilization. Furthermore, functional endosperm formation in apomicts requires modifications in embryo sac development, or double fertilization, or both. Nogler (85) referred to an opening of the bonds linking megasporogenesis and embryo sac initiation in apomicts. He viewed this modification of the sexual program as the determining step. The developmental changes in apomicts have also been described as a short-circuiting of the sexual program (57, 133), but more generally, a deregulation occurs both at the spatial and temporal level (36). Indeed, the first comparative studies in sexual and apomictic *Hieracium*

using molecular markers have shown that apomictic and sexual processes do share the same components. Importantly, a cell fate change and the omission of sexual developmental steps could be documented in dividing aposporous initials.

The sexual processes that are deregulated occur in the sporophytic tissue of the ovule and in the embryo sac. Embryo sac initiation, embryo sac development, and double fertilization, which are all modified in apomicts, are largely gametophytic functions. However, the genetic control of these events is still poorly understood, even in sexual model systems. The elucidation of the fascinating developmental processes occurring in apomicts will require a multifaceted approach in both sexual and apomictic plants. The dissection of the genetic components involved in ovule and embryo sac development is underway in model systems. Their role in apomicts can now be characterized in comparative studies and has already provided some of the first important insights. With these new tools at hand, light will be shed on the molecular mechanisms underlying sexual and apomictic reproduction, which will eventually lead to the better understanding required for controlling and modifying seed production in crop plants. Further molecular analysis of apomixis, together with the events of sexual reproduction in the ovule, will provide information to control seed production in agriculture with much greater flexibility.

ACKNOWLEDGMENTS

We thank Gian Nogler and Sacco de Vries for helpful discussions. Nogler's 1984 review foresaw most of the issues we discussed in this review, and his work has been an inspiration for many scientists working on apomixis. We are grateful to Siân Curtis, Charlie Spillane, Mark Curtis, and Matthew Tucker for helpful comments on the manuscript and to Ortrun Mittelsten Scheid, Jurek Paszkowski, and our coworkers for allowing us to cite unpublished data. We thank Graziella Pulver for help with the bibliography and Jean-Jacques Pittet for help with Figure 2. Apomixis research in Anna Koltunow's laboratory is supported by the Australian Centre for International Agricultural Research and an Adelaide University Premiers Scholarship to Matt Tucker. Ueli Grossniklaus' research on apomixis is funded by the Kanton of Zürich, the Roche Research Foundation, the APOTOOL Project in Framework V of the European Union through a grant of the Schweizer Bundesamt für Forschung und Wissenschaft, and a Searle Scholarship.

The *Annual Review of Plant Biology* is online at http://plant.annualreviews.org

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Volume 54, 2003

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