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Understanding Apomixis: Recent Advances and Remaining Conundrums

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INTRODUCTION

It has been 10 years since the last review on apomixis, or asexual seed formation, in this journal (Koltunow, 1993). In that article, emphasis was given to the commonalties known among apomictic processes relative to the events of sexual reproduction. The inheritance of apomixis had been established in some species, and molecular mapping studies had been initiated. The molecular relationships between apomictic and sexual reproduction, however, were completely unknown. With research progress in both sexual and apomictic systems in the intervening years, subsequent reviews on apomixis in the literature have considered the economic advantages of providing apomixis to developing and developed agricultural economies (Hanna, 1995; Savidan, 2000a) and strategies to gain an understanding of apomixis by comparison with sexual systems (Koltunow et al., 1995a; Grimanelli et al., 2001a). The potential to ''synthesize apomixis'' in agricultural crops in which it is currently absent by modifying steps in sexual reproduction and the possible ecological consequences of the release of ''synthesized apomicts'' in nature also have been discussed (van Dijk and van Damme, 2000; Grossniklaus, 2001; Spillane et al., 2001). Recently, comparative developmental features of apomixis have been considered in light of the now considerable knowledge accumulated about ovule and female gametophyte development, and seed formation in sexual plants (Koltunow and Grossniklaus, 2003). In this review, we focus on the initiation and progression of apomixis in plants that naturally express the trait. Since 1993, there has been a growing understanding of the complexity that underlies apomixis; some contentious issues have been resolved and others raised. There also have been significant advances in terms of new model systems and approaches being used to study apomixis. We structure the wider discussion around the knowledge of apomixis we have accumulated from our study of *Hieracium* species, or hawkweeds, a model system we established, and consider additional factors that should be taken into account to induce apomixis in crops. The continued comparative analyses of apomictic and sexual reproduction at the fundamental level in appropriate model systems remains essential for the development of successful strategies for the greater application and manipulation of apomixis in agriculture.

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WHAT IS APOMIXIS?

Apomixis in flowering plants is defined as the asexual formation of a seed from the maternal tissues of the ovule, avoiding the processes of meiosis and fertilization, leading to embryo development. The initial discovery of apomixis in higher plants is attributed to the observation that a solitary female plant of *Alchornea ilicifolia* (syn. *Caelebogyne ilicifolia*) from Australia continued to form seeds when planted at Kew Gardens in England (Smith, 1841). Winkler (1908) introduced the term apomixis to mean ''substitution of sexual reproduction by an asexual multiplication process without nucleus and cell fusion.'' Therefore, some authors have chosen to use apomixis to describe all forms of asexual reproduction in plants, but this wider interpretation is no longer generally accepted. The current usage of apomixis is synonymous with the term ''agamospermous'' (Richards, 1997). Because seeds are found only among angiosperm and gymnosperm taxa, this definition of apomixis limits its use to those groups. In lower plants, phenomena similar to apomixis are known, but discussion remains about the use of this term in cases in which the reproductive structures involved are different yet are considered analogous (Asker and Jerling, 1992).

PREVALENCE OF APOMIXIS

Although it is sometimes referred to as a botanical curiosity, apomixis is far from rare, being relatively prevalent among angiosperms, with a pattern of distribution that suggests that it has evolved many times. It has been described in >400 flowering plant taxa, including representatives of >40 families (Carman, 1997), and it is well represented among both monocotyledonous and eudicotyledonous plants; curiously, though, it appears to be absent among the gymnosperms. These estimates are almost certainly very conservative. Unequivocal confirmation of apomixis requires the simultaneous examination of both genetic and cytological evidence (Nogler, 1984a). Embryological examination of plant taxa for apomixis has not been exhaustive, and supporting genetic evidence is uncommon even when

apomixis has been declared for a given plant. It seems likely that as our understanding of this phenomenon grows and methods to determine its presence improve, many more angiosperm taxa will be found to include apomictic representatives and some suspected cases will be revised. A recent study by Plitman (2002) supports this prediction.

Several authors have noted a marked bias in the distribution of apomixis among angiosperms (Asker and Jerling, 1992; Mogie, 1992; Carman, 1997; Richards, 1997). Of the plants known to use gametophytic apomixis (Figure 1), 75% of confirmed examples belong to three families, the Asteraceae, Rosaceae, and Poaceae, which collectively constitute only 10% of flowering plant species. Conversely, although apomixis is known among the Orchidaceae, the largest flowering plant family, it appears to be comparatively uncommon among these plants. Some authors have postulated that the current patterns of distribution may reflect the predisposition of certain plant groups to the unique developmental and genetic changes that characterize apomixis (Grimanelli et al., 2001b). This hypothesis appears intuitively attractive, but like many issues associated with apomixis, it remains conjecture until it is tested experimentally. Some of this bias also might relate to the ease of embryological examination in some plant groups or to data accumulated from embryological investigations associated with activities in crop improvement.

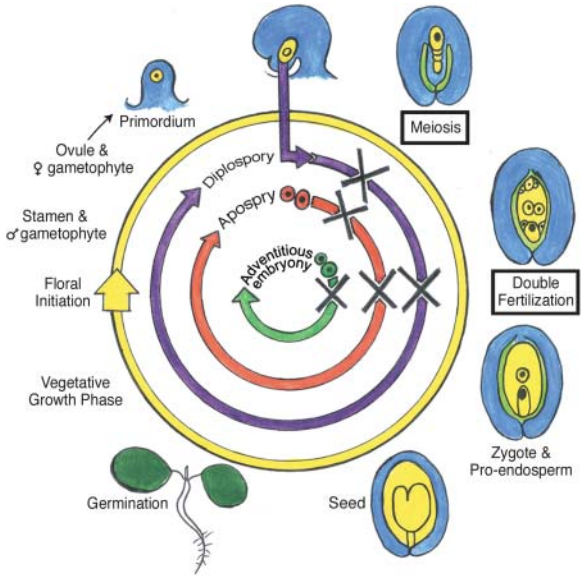

Figure 1. Initiation and Progression of Apomictic Mechanisms Relative to Events in the Sexual Life Cycle of Angiosperms.

The normally dominant vegetative phase of the life cycle is curtailed in this figure to emphasize the events of gametophyte formation, particularly the events in the ovule leading to sexually derived seeds (pathway colored in yellow). Diplospory (purple) and apospory (red) are termed gametophytic mechanisms because they initiate from a cell in the position of the MMC or from other ovule cells, respectively, that bypasses the events of meiosis and divides to mitotically to form an unreduced embryo sac. Embryogenesis occurs autonomously from a cell(s) in these unreduced embryo sacs. Endosperm formation may require fertilization, or in a minority of apomicts it may be fertilization independent. Adventitious embryony (green) is termed sporophytic apomixis because embryos form directly from nucellar or integument cells adjacent to a reduced embryo sac. The maturation and survival of adventitious embryos is dependent on the endosperm derived from double fertilization in the reduced embryo sac.

There are other noted associations between apomixis and various plant life history traits that provide insight into the nature and possible ecological role of this phenomenon. Apomixis frequently is associated with the expression of mechanisms that limit self-fertilization (autogamy). Many apomictic plants belong to genera in which sexual members predominantly exhibit physiological self-incompatibility, dioecy, or heterostyly (Asker and Jerling, 1992). In some cases, it is clear that the apomicts themselves have retained such mechanisms. Dioecy is known in a number of apomicts, such as *Antennaria* (O'Connell and Eckert, 1999), *Cortaderia* (Philipson, 1978), and *Coprosma* (Heenan et al., 2002). Similarly, self-incompatibility, a known characteristic of the sexual biotypes of *Hieracium* subgenus *Pilosella* (Gadella, 1984, 1991; Krahulcovà et al., 1999), was demonstrated recently in the apomictic species *H. aurantiacum* and *H. piloselloides* (Bicknell et al., 2003).

Apomictic species also are almost invariably perennials, and they often use a vegetative mechanism of asexual reproduction, such as stolon or rhizome growth. Thus, in the field, through a combination of apomixis and vegetative division, apomicts can form large clonal stands, and these may persist through long periods of time. Apomixis also frequently leads to the formation and maintenance of numerous morphologically distinct, yet interfertile, varieties growing true to type from seed. The taxonomy of such agamic complexes can be a difficult and contentious task (Dickinson, 1998; Horandl, 1998). Examples of genera in which the apomictic mode of reproduction is strongly combined with morphological polymorphism include *Alchemilla*, *Hieracium*, *Poa*, *Potentilla*, *Ranunculus*, *Rubus*, and *Taraxacum* (Czapik, 1994). Apomicts are found more commonly in habitats that are frequently disturbed and/or either where the growing season is short, such as arctic and alpine sites, or where other barriers operate to inhibit the successful crossing of compatible individuals, such as among widely dispersed individuals within a tropical rain forest (Asker and Jerling, 1992). There also are clear indications that glaciation events defined the distribution patterns of many agamic complexes (Asker and Jerling, 1992). Gametophytic apomixis is known among herbaceous and tree species, but it is considerably more common among the former. This may simply be a reflection of the predominance of the trait in the Poaceae and the Asteraceae, both of which are composed largely of herbaceous species. Similarly, gametophytic apomixis (Figure 1) is common among plants that have dehiscent fruits, as seen in the Poaceae, Asteraceae, and Ranunculaceae, but again, this may be more coincidence than causally linked.

MECHANISMS OF APOMIXIS

All known mechanisms of apomixis share three developmental components: the generation of a cell capable of forming an embryo without prior meiosis (apomeiosis); the spontaneous, fertilization-independent development of the embryo (parthenogenesis); and the capacity to either produce endosperm

autonomously or to use an endosperm derived from fertilization (Koltunow, 1993, Carman, 1997). That said, although broad similarities can be seen at the level of the key events of apomixis, it is probably true that there are as many mechanisms of apomixis as there are plant taxa that express the trait. Some clear commonalities have been identified, however, and these have been used to categorize apomictic mechanisms into broad groupings. Figure 1 portrays these mechanisms in relation to the temporal sequence of events in the reproductive cycle of sexual plants. Two main subgroups of mechanisms are apparent. In sporophytic apomixis, or adventitious embryony, embryos arise spontaneously from ovule cells late in the temporal sequence of ovule maturation (Figure 1). Gametophytic apomixis operates through the mediation of an unreduced embryo sac. Endosperm development in these plants may be either spontaneous (autonomous) or fertilization induced (pseudogamous) (Koltunow, 1993).

Gametophytic mechanisms are further subdivided based on the cell type that gives rise to the unreduced embryo sac. In diplosporous types, the megaspore mother cell (MMC) or a cell with apomictic potential occupying its position is the progenitor cell for the unreduced embryo sac. That cell may enter meiosis but this aborts, and development proceeds by mitotic division to achieve embryo sac formation (meiotic diplospory). Alternatively, that cell might undergo direct mitosis to form an unreduced embryo sac (mitotic diplospory). In aposporous apomicts, one or more somatic cells of the ovule, called aposporous initials, give rise to an unreduced embryo sac. Aposporous initials can differentiate at various times during ovule development. Meiotically reduced and aposporous embryo sacs might coexist in the ovule, or the aposporous embryo sac might continue development while the reduced sexual embryo sac degenerates (Figures 1 and 2). The further subdivision of gametophytic mechanisms (not shown in Figure 1) has been based on characteristics related to the involvement, or avoidance, of the different phases of meiosis, the number of mitotic divisions, and the eventual form of the embryo sac (Crane, 2001).

The existence of apomixis in 40 plant families and the diversity of apomictic processes suggests that the routes that led to the evolution of apomixis may be as diverse as the known cytological mechanisms. Morphological comparisons of structures formed during sexual and apomictic reproduction provide hints that the two reproductive pathways may share common elements, but there also are numerous abnormalities. In the case of diplosporous and aposporous embryo sac formation, a meiotic tetrad is not observed. Positional differentiation of aposporous initials (and adventitious embryos) occurs most frequently adjacent to cells undergoing sexual reproduction, but they do not necessarily arise from the nucellus, because epidermal and integumentary origins have been described (Koltunow and Grossniklaus, 2003). Irregularities also occur in the development of diplosporous and aposporous embryo sacs, with structural abnormalities more prevalent in apospory. Common abnormalities include a lack of polarization of nuclei during early mitotic divisions, odd or excessive numbers of nuclei varying in size, monopolar embryo sacs, embryo sacs with inverted polarity or an abnormal situation of the egg apparatus, and increased numbers of apparent polar nuclei. In some cases, the final embryo sac structure is quite stably different and used as a diagnostic tool, the best example being the four-nucleus *Panicum*-type embryo sac (Koltunow, 1993; Czapik, 1994).

Most apomicts produce viable pollen. Even in diplosporous apomicts, defects in the meiotic events of female gametophyte development are not automatically extended to male gametophyte formation or function. The presence of viable pollen provides the possibility of fertilization of unreduced eggs. In apomicts such as *Taraxacum* (Cooper and Brink, 1949; Richards, 1997), *Poa*, *Parthenium*, *Tripsacum* (Asker and Jerling, 1992), and *Hieracium piloselloides* (Koltunow et al., 1998), embryo formation can be precocious, initiating before anthesis or even before the opening of the flower, limiting the possibility of unreduced egg cell fertilization. In many sexual species, the egg cell has an incomplete cell wall, and synthesis of a complete cell wall occurs only in the zygote after fertilization. In the aposporous apomict *Pennisetum ciliare*, however, a complete cell wall forms around the unreduced egg cell before the arrival of the pollen tube containing the two sperm cells (Vielle et al., 1995). *P. ciliare* is a pseudogamous species, requiring fertilization to initiate endosperm development. The early development of a complete cell wall around the unreduced egg of this species appears to serve as a means to avoid fusion of the second sperm cell with the unreduced egg cell at the time of fertilization-induced endosperm formation. Naumova and Vielle-Calzada (2001) further noted that polysomes, endoplasmic reticulum, Golgi bodies, and mitochondrial cristae were more abundant and more developed in unreduced egg cells found in mature *P. ciliare* aposporous embryo sacs than in the meiotically derived egg cells of this species. These observations are consistent with precocious egg cell maturation and suggestive of a loss or truncation of the quiescent phase of egg cell development, a common feature of sexually reproducing plants.

In apomicts, embryo and endosperm pattern formation may or may not be conserved relative to that observed in related sexual plants, although the latter has been least studied (Koltunow, 1993; Czapik, 1994). Apomicts also appear to tolerate imbalances in parental gene dosage in their endosperm and still form viable seeds (Grimanelli et al., 1997; Koltunow and Grossniklaus, 2003). Understanding how this occurs and its impact on seed quality is particularly relevant for the installation of apomixis in cereals in which imbalances in parental gene dosage in endosperm are not tolerated.

THE POTENTIAL VALUE OF APOMIXIS IN AGRICULTURE

Apomixis is an attractive trait for the enhancement of crop species because it mediates the formation of large genetically uniform populations and perpetuates hybrid vigor through successive seed generations. Many agronomic advantages of apomixis can be envisioned: the rapid generation and multiplication of superior forms through seed from novel, currently underused germplasms; the reduction in cost and time of breeding; the avoidance of complications associated with sexual reproduction, such as pollinators and cross-compatibility; and the avoidance of viral transfer in plants that are typically propagated vegetatively, such as potatoes (Hanna, 1995; Jefferson and Bicknell, 1995; Koltunow et al., 1995a, Savidan,

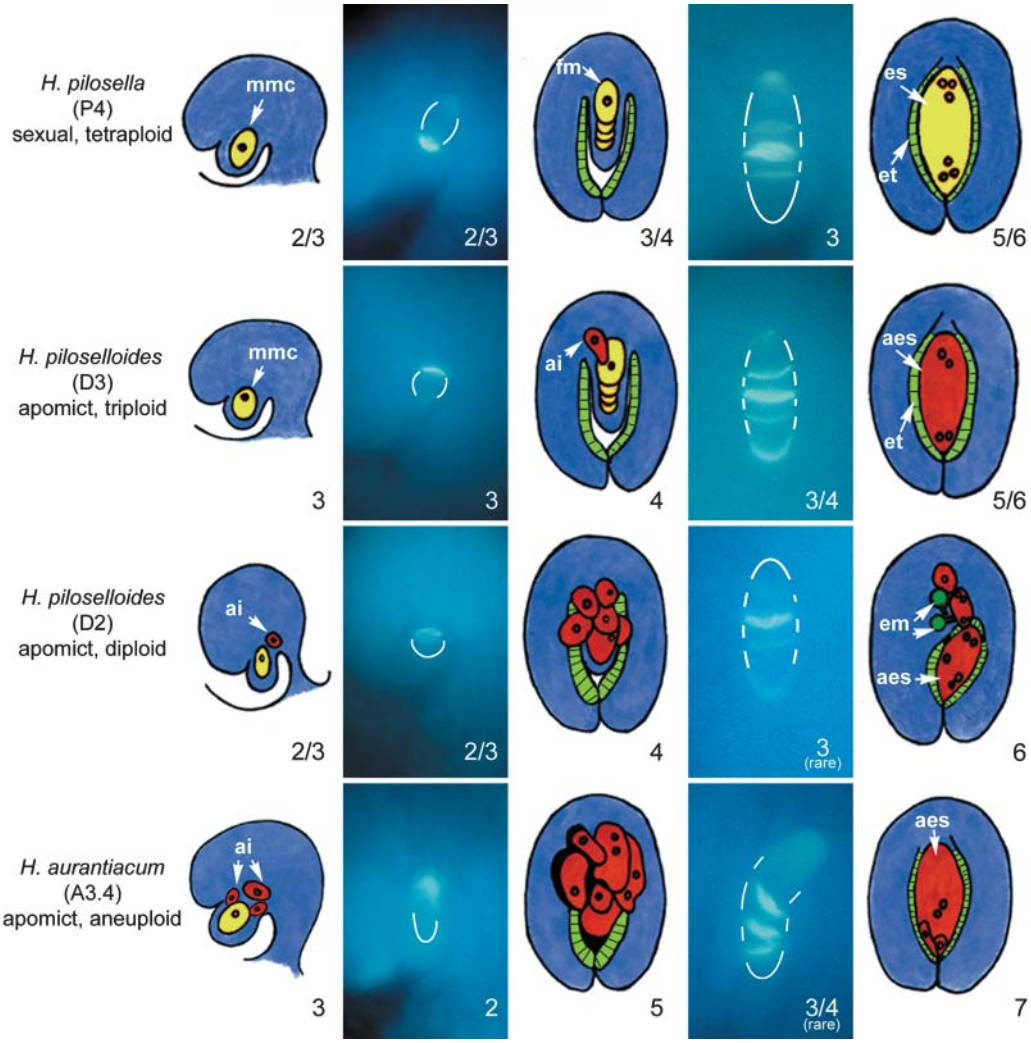

Figure 2. Early Events of Embryo Sac Formation in Ovules of Sexual and Apomictic *Hieracium* Plants.

The early events of reduced embryo sac formation in sexual plants and in apomicts are colored yellow, and aposporous embryo sac formation is colored red. The numbers represent morphological stages of capitulum development as defined by Koltunow et al. (1998), and mature embryo sac structures are not shown for stages P4 and D3. In stage A3.4, multiple embryo sacs form with few nuclei, and these coalesce to form a single embryo sac (Koltunow et al., 1998). The presence of callose in the walls of MMCs in ovules at stages 2 or 3 and in meiotic tetrads in ovules at stages 3 or 3/4 is shown by the fluorescence of trapped aniline blue dye after exposure to UV light (Tucker et al., 2001). Meiotic tetrads in stages A3.4 and D2 are rare because of the presence of multiple aposporous initials and embryo sacs that physically distort and/or crush the structure. Aposporous initial cells do not contain callose in their cell walls (Tucker et al., 2001). Stage D2 also forms isolated embryos external to an embryo sac (Koltunow et al., 2000). aes, aposporous embryo sac; ai, aposporous initial; em, embryo; es embryo sac; et, endothelium; fm, functional megaspore; mmc, megaspore mother cell.

2000a, 2000b). The value of these opportunities will vary between crops and between production systems. For farmers in the developed world, the greatest benefit is expected to be the economic production of new, advanced, high-yielding varieties for use in mechanized agricultural systems. Conversely, for farmers in the developing world, the greatest benefits are expected to relate to the breeding of robust, high-yielding varieties for specific environments, improvements in the security of the food supply, and greater autonomy over variety ownership (Bicknell and Bicknell, 1999; Toenniessen, 2001).

However, apomixis is very poorly represented among crop species. The main exceptions to this appear to be tropical and subtropical fruit trees, such as mango, mangosteen, and *Citrus*, and tropical forage grasses, such as *Panicum*, *Brachiaria*, *Dichanthium*, and *Pennisetum*. It is possible that the low representation of apomixis among crops arose unintentionally from a protracted human history of selecting superior plants for future cultivation. Selection for change over a parental type would work against a mechanism such as apomixis that acts to maintain uniformity. The presence of the trait among tropical fruit and

grass crops may be a reflection of this effect, because focused efforts to improve these crops are comparatively recent events.

There also are few apomictic species of significant relatedness available for use in introgression programs, which may explain at least some of the difficulties experienced when attempts have been made to introduce apomixis into crops through hybridization. For example, major programs aimed at introducing apomixis into maize (Sokolov et al., 1998; Savidan, 2000a, 2001) from the wild relative *Tripsacum dactyloides* have been under way now for decades, yet they have proven unsuccessful in terms of generating apomictic plants with agronomically acceptable levels of seed set. Difficulties also have been encountered in efforts to produce apomictic lines of hybrid millet (Morgan et al., 1998; Savidan, 2001). Even if successful, it seems likely that introgression lines would provide limited flexibility in terms of practical capacity to manipulate apomixis in agricultural breeding systems. Current breeding efforts with apomictic crop species, such as the forage grasses *Brachiaria* and *Panicum*, are frustrated by the need to use complex breeding strategies to accommodate the inaccessibility of the female gamete to generate hybrid progeny (Valle and Miles, 2001). We believe, therefore, that the best solution would be the introduction of apomixis into crops in an inducible format, permitting its use during seed increase but allowing for its silencing during hybridization. To achieve this, information will be required concerning the genes that control the trait, their interrelationship with sexual processes, and the impact the trait might have on seed yield, viability, and quality for a given plant.

EXPERIMENTAL APPROACHES AND MODEL SYSTEMS IN APOMIXIS RESEARCH

Current research on apomixis generally is divided into two complementary approaches: the evaluation of the trait in natural apomictic systems, and the ''synthesis'' of the trait through the directed modification of reproductive events in a sexual species. Of the native monocot apomictic systems under study, most work focuses on aposporous plus pseudogamous species in the genera *Panicum* (Savidan, 1980; Chen et al., 1999), *Pennisetum* and *Cenchrus* (Roche et al., 1999), *Brachiaria* (Pessino et al., 1997, 1998), *Paspalum* (Martinez et al., 2001), and *Poa* (Naumova et al., 1999; Albertini et al., 2001a, 2001b) and the diplosporous plus pseudogamous genus *Tripsacum* (Grimanelli et al., 1998a, 1998b; Sokolov et al., 1998). The eudicot genera under study include the daisy genera *Taraxacum* and *Erigeron* (diplosporous plus autonomous) (Noyes and Rieseberg, 2000; van Dijk et al., 2003) and *Hieracium* (aposporous plus autonomous) (Koltunow et al., 1998, 2000). *Hypericum perforatum* (aposporous plus pseudogamous), a member of the St. John's wort family, has been well studied (Matzk et al., 2001, 2003), and *Boechera holboellii* (syn. *Arabis holboellii*; diplosporous plus pseudogamous) (Naumova et al., 2001; Sharbel and Mitchell-Olds, 2001), a relative of Arabidopsis, also is receiving attention for its potential to exploit the vast genetic and molecular resources developed in Arabidopsis. We suspect that these efforts may become more focused in coming years as the relative advantages of one or a small number of these systems leads to their predominant use (Bicknell, 2001). Among the sexual species under study for the synthesis of apomixis, there is little doubt that Arabidopsis, rice, and maize will remain the systems of choice because of the availability of genetic and molecular tools and, for the latter crops, the strong economic and social drivers seeking the installation of the trait (Khush, 1994; Savidan, 2000a).

HIERACIUM SUBGENUS *PILOSELLA*, A MODEL SYSTEM FOR APOSPOROUS PLUS AUTONOMOUS GAMETOPHYTIC APOMIXIS

During the last 10 years, *Hieracium* subgenus *Pilosella* has been developed into a model system for the genetic and molecular study of gametophytic apomixis (Bicknell, 1994a, 2001; Koltunow et al., 1998; Koltunow, 2000). These plants are highly suited for molecular studies because they are small, herbaceous perennials with a rapid generation time (3 to 6 months) and a long-day photoperiodic response that allows them to be flowered on demand at any time of the year (Yeung, 1989). Some species include both sexual and apomictic biotypes, and hybrids formed between most of the species have proven to be fertile. This allows the inheritance of apomixis to be studied using intraspecific hybridization, in contrast to many other apomictic plant systems. Methods for the micropropagation (Bicknell, 1994b), anther culture (Bicknell and Borst, 1996), genetic transformation (Bicknell and Borst, 1994), and progeny class estimation (Bicknell et al., 2003) of *Hieracium* have been developed to further facilitate its use in the molecular study of apomixis. The plants also are very amenable to vegetative propagation, which is of particular value when genetic or molecular manipulations affect flower form and/or seed set. Vegetative propagation also is the preferred mode of stock maintenance, because a fraction of the progeny is not true to type (see below).

In all of the wild-type apomicts of *Hieracium* studied to date, both the embryo and endosperm arise spontaneously inside an unreduced aposporous embryo sac (Figure 2). The initiation of apospory is stochastic in these plants, but in each form, the majority of aposporous initial cells tend to differentiate at a particular time relative to the concurrent sexual process in the ovule. This makes the plants valuable for examining factors that influence the timing of aposporous initial formation. In most of the apomictic *Hieracium* plants characterized to date, the sexual process usually ceases soon after apospory initiates (Koltunow et al., 1998, 2000; Bicknell et al., 2003). This provides an opportunity to examine signaling between apomictic and sexual pathways and to examine factors that mediate the survival of one mode of embryo sac formation over the other (Figure 2). Whether one or many aposporous embryo sacs initiate, almost always a single one survives; nevertheless, in some species, multiple embryos can form in an individual aposporous embryo sac. Thus, the mechanisms that limit aposporous embryo sac and embryo frequency can be examined.

Endosperm development was examined recently in the autonomous apomict *H. piloselloides* (tall hawkweed) and compared with that in a sexual biotype, *H. pilosella* (mouse ear hawkweed). In the apomict, autonomous endosperm development initiates primarily, but not exclusively, from fused polar nuclei. In contrast to the fertilization-dependent endosperm development in the

sexual plant, migrating clumps of nuclei form during early endosperm development in the apomict, but as nuclei numbers increase, this is rectified so that the cellularization and maturation events are cytologically indistinguishable in the two types (M. Tucker and A.M. Koltunow, unpublished results). Apomictic *Hieracium* plants exhibit developmental abnormalities reminiscent of epigenetic phenomena. For example, there are disturbances in the pattern of embryo formation and phyllotaxis during early seedling growth in apomictic *Hieracium* that are reset eventually to a normal pattern with subsequent plant growth (Koltunow et al., 1998, 2000).

Sexual reproduction, or components of it, are not excluded completely in apomictic *Hieracium*. In some ovules, either apospory does not initiate or it fails and the meiotic process proceeds to completion. In addition, fertilization may or may not occur in these plants irrespective of the reduced or unreduced nature of the egg. Thus, *Hieracium* is facultative for apomixis, because a fraction of the seedlings are nonmaternal in genotype.

DIFFERENT PROGENY TYPES FOUND IN FACULTATIVE APOMICTS

The coexistence of apomixis and sexuality is certainly not unique to *Hieracium*. For all of the mechanisms of apomixis known, and for almost all of the genotypes studied in depth, it has been reported that some degree of sexuality remains possible in apomictic plants; thus, most are regarded as facultative. True obligate apomicts, those able to form seeds by apomixis alone, are very uncommon (Asker and Jerling, 1992). Facultative, gametophytic apomicts are capable of hybridization using either a reduced or an unreduced egg cell. The previously described mechanisms of early egg cell wall completion and precocious embryogenesis of unreduced eggs are by no means fully penetrant. This can lead to the formation of two different types of hybrid progeny. Reduced hybrids result from the fusion of a reduced egg cell with a reduced sperm cell, whereas unreduced hybrids typically result from the mediation of an unreduced egg cell. In the nomenclatural system of Rutishauser (1948), these are referred to as BII and BIII hybrids, respectively. Harlan and De Wet (1975) termed them n+n and 2n+n hybrids, respectively, whereas Asker (1977) used the terms R- and U-hybrids. Unfortunately, all of these terms are still used in the literature, but it does appear that the system of Harlan and De Wet (1975) is now the most popular choice, and we will continue with its use in this article. A further progeny class of note in these plants are n+0 seedlings, or ''polyhaploids.'' These plants result from the parthenogenetic development of a reduced egg cell, leading to the formation of a plant with a ploidy state less than that of the parent. Although male meiosis and male gamete function are typically normal in gametophytic apomicts, the formation and use of unreduced pollen also has been observed (Matzk et al., 2001). Both the formation of unreduced gametes and the parthenogenetic development of unfertilized egg cells are widely recorded phenomena in sexual species (Grossniklaus, 2001); however, they are typically much more frequent events among apomicts. In the case of *H. aurantiacum* and *H. piloselloides*, the rates of parthenogenesis (which includes 2n+0 and n+0 progeny) were 97.6 and 98.0%, respectively (Bicknell et al., 2003) (Figure 3) compared with reported examples of 0.11% for maize and 0.018% for *Datura* (Kimber and Riley, 1963).

An important practical outcome of the diversity of progeny types produced by apomictic *Hieracium*, and for that matter by most other apomicts, is that the progeny of these plants cannot be considered to be uniformly of clonal, maternal origin. In particular, because different progeny classes result from the different actions of segregation (meiosis) and recombination (fertilization), different inheritance ratios are expected in each progeny class, and this fact must be considered in any analysis of trait transmission. Even in sporophytic apomicts such as *Citrus*, in which spontaneous embryogenesis adjacent to a reduced embryo sac is the defining manifestation of this form of apomixis (Koltunow et al., 1995b), some authors have noted that unreduced embryo sacs do also form (Naumova, 1992). Plants with unexpectedly high ploidy levels are frequently observed in breeding populations of Citrus, and it seems likely that such individuals arise following the fertilization of an unreduced egg cell, in a manner similar to that seen in gametophytic apomicts.

Given the importance of understanding the relative levels of different progeny types among the seedlings produced by

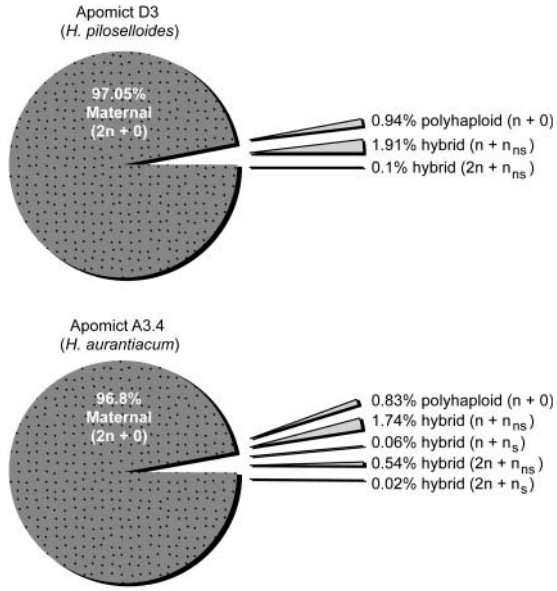

Figure 3. Progeny Types Formed in Crossed *Hieracium* Species Facultative for Aposporous Plus Autonomous Apomixis.

Most of the progeny are maternal (2n+0), because there is no genomic contribution from a male gametophyte. Nonmaternal seedlings form a smaller subset that can be subdivided into a number of types. Seedlings also are derived from the use of a reduced egg that undergoes autonomous embryogenesis (n+0); alternatively, the egg can be fertilized with a sperm cell from self (s) or nonself (ns) pollen to give rise to hybrids that arise from sexual reproduction ($n+n_s$ or $n+n_{ns}$, respectively). Unreduced egg cells also can be fertilized with sperm cells from self or nonself pollen to give rise to hybrid progeny ($2n+n_s$ or $2n+n_{ns}$, respectively) that have increased ploidy relative to the maternal parent. D3 ovules appear to be strictly self-incompatible, whereas in A3.4, progeny arising from self-pollination are evident (data are from Bicknell et al., 2003).

a facultative apomict, there has been a strong need for robust methodologies to determine these levels and to isolate representative subpopulations for separate analysis. For many years, the nonmaternal (''aberrant'' or ''off-type'') progeny of apomicts were identified through morphological scoring, chromosome counting, or progeny cross methods, which were too inaccurate and too expensive to warrant their use on any scale. Recently, methods based on flow cytometry and transgenesis have been published that address these limitations. The method of Matzk et al. (2000), which applies a flow cytometric test to individual seeds, is proving to be particularly versatile in that it permits the rapid screening of previously uncharacterized material for signature characteristics of apomixis (Matzk et al., 2001, 2003). In *Hieracium*, a method based on the inheritance of positive and negative selectable transgenic markers has been described (Bicknell et al., 2003). This method, which is based on the survival of different progeny types when placed on selective media, provides the added versatility of being nondestructive and less expensive to apply. It also provides a mechanism for the direct isolation of aberrant progeny individuals. Its use, however, is limited to plants that can be transformed genetically, and it is less suited for survey studies that assess and compare a large number of genotypes.

The facultative expression of apomixis, therefore, is an experimental complexity in the study of these plants. However, it has proven to be a valuable tool for exploring the unique biology of apomixis, particularly in the analysis of interactions between apomixis and sexuality. For example, polyhaploidy (n+0) was used to isolate diploid apomictic plants of the species *H. aurantiacum* and *H. piloselloides* (Bicknell, 1997; Koltunow et al., 2000; Bicknell et al., 2003), demonstrating that apomixis can occur in both diploid and polyploid members of this species. Similarly, the quantification of hybrid (2n+n and n+n) progeny in both *H. aurantiacum* and *H. piloselloides* indicated that a self-incompatibility mechanism was acting to influence progeny formation in these plants despite the autonomous nature of apomixis in *Hieracium* (Bicknell et al., 2003) (Figure 3).

Another area of study has been the origin of multiple embryos, a common occurrence in the seeds of many apomicts. The formation of multiple viable embryos, or polyembryony, is evident in up to 10% of *H. aurantiacum* seeds and 17% of *H. piloselloides* seeds (Koltunow et al., 2000; Bicknell et al., 2003). The origin of the additional embryos remains a mystery, because in the majority of ovules the sexual process terminates and a single aposporous embryo sac is present at the initiation of embryogenesis. We previously hypothesized that the additional embryos might arise from trapped aposporous initials or perhaps endosperm cells that had altered in fate (Koltunow et al., 1998). From a study quantifying progeny types (Bicknell et al., 2003), it was found that meiotically derived seedlings were seven times more likely to arise alongside viable, asexually derived embryo(s) in a polyembryonic seed than from a single embryo seed. The reason why a partnering asexual embryo is present so often remains unclear. One possibility is the formation of adventitious embryos either from reprogrammed aposporous initials or from other somatic cells. This is not completely speculative, given that an experimentally derived diploid form of *H. piloselloides* readily forms adventitious embryos (Koltunow et al., 2000) (Figure 2). Alternatively, embryogenic development may proceed from both coexisting aposporous and reduced sexual embryo sacs, an event that might have proven too difficult to detect cytologically because of its rarity.

The interplay between sexuality and apomixis in facultative plants provides an opportunity to consider the evolutionary and ecological implications of these different modes of reproduction (Krahulcovà et al., 2000; Houliston and Chapman, 2001). For example, Bicknell et al. (2003) noted that in *H. aurantiacum* and *H. piloselloides*, the rates of seedlings derived from meiotic egg usage (n+n and n+0) were very similar, 2.60 and 2.85%, respectively (Figure 3), yet the modes of aposporous embryo sac development are very different in these plants (Koltunow et al., 1998). This result indicates that the rate of meiotic egg use giving rise to viable progeny in these plants is unlikely to be a chance event; rather, it is a characteristic optimzed under selection to reflect the relative costs and opportunities of sexual and asexual reproduction in these plants. This proposal is supported by evidence for the operation of a self-incompatibility mechanism in these plants (Bicknell et al., 2003). Field evidence further indicates that sexuality is an important driver in the maintenance of diversity in these plants. Chapman et al. (2000) noted that the patterns of genetic diversity seen among *Pilosella officinarum* (syn. *Hieracium pilosella*) clones at five dispersed sites in New Zealand suggested the frequent action of hybridization, despite the relatively recent introduction of this weed into that country and the predominantly apomictic nature of the clones studied (Houliston and Chapman, 2001). Similar findings have been reported for the related daisy genus *Taraxacum* (van der Hulst et al., 2000).

CALLOSE DEPOSITION: EARLY CLUES TO APOMIXIS CELL TYPE IDENTITY?

One means of assessing the relationships between sexual and apomictic pathways is to use developmental markers that identify specific cells or structures in sexual systems and to examine their expression in apomictic plants. One of the earliest used was the biochemical detection of callose, a β-1,3-linked glucan that accumulates in a distinctive manner during female gametophyte development in sexual plants. The walls of the MMC (or megasporocyte), the tetrad of megaspores (Figure 2), and degenerating megaspores are marked by the temporary accumulation of callose. It is lost from the walls of the selected megaspore during its expansion and is absent once the mitotic events of embryo sac development initiate.

Differences in callose deposition during gametophyte development in sexual and apomictic plants have been noted, and in the case of *Tripsacum*, this has been used as a screening tool to identify apomicts (Leblanc and Mazzucato, 2001). In both diplosporous *Tripsacum* and *Elymus*, which exhibit diplospory (Carman et al., 1991), callose is absent from the walls of the cell that initiates diplospory, which is otherwise identified by position, size, and appearance as the MMC. Given that residual sexuality was low in the apomicts examined, it was not possible to directly compare meiotic and diplosporous processes in the same material; however, callose was present in the MMC of sexual

accessions. In aposporous species in which the initiation of both sexual and apomictic processes can be observed simultaneously, callose was completely absent from aposporous initial cells in *Poa pratensis*, *Pennisetum* (Peel et al., 1997), *Brachiaria* (Dusi and Willemse, 1999), and *Hieracium* (Tucker et al., 2001) but evident in the MMC (Figure 2). Studies in aposporous *Poa* and *Pennisetum* also have noted differences in callose distribution in the adjacent MMC. This was not obvious in aposporous *Hieracium*, but an early appearance of initials correlated with the persistence of callose (Tucker et al., 2001). Furthermore, changes in β-1,3-glucanase expression postulated by Peel et al. (1997) to coincide with aposporous initial formation were not evident in *Hieracium* with respect to mRNA distribution in the plants examined, although changes in protein activity cannot be discounted (Tucker et al., 2001). Assuming that apomixis is an aberrant form of sexual reproduction in all of the species mentioned above, then the lack of callose in cells that initiate diplospory and apospory implies that they do not share identity with functional MMCs. Peel et al. (1997) have proposed that the MMC of diplosporous plants undergoes ''precocious gametophytization,'' which could be interpreted as a change in identity, but other markers are required to clarify this possibility.

MOLECULAR RELATIONSHIPS BETWEEN SEXUAL AND APOMICTIC PATHWAYS

Considerable information has now been accumulated concerning the regulation of ovule and female gametophyte development in sexual plants, and this provides molecular tools for the comparative analysis of sexual and apomictic reproduction (Koltunow and Grossniklaus, 2003). Tucker et al. (2003) provided strong evidence that sexual and apomictic reproduction in *Hieracium* are related developmental pathways that share common regulatory programs (Eckardt, 2003). They examined the expression patterns of several reproductive marker genes from Arabidopsis in sexual and apomictic *Hieracium*. These markers were fusion constructs of β-glucuronidase (GUS) and a variety of Arabidopsis genes as promoter:GUS or chimeric protein fusions. The markers included *SPOROCYTELESS* (*SPL/NOZZLE*), which is required for male and female sporogenesis in Arabidopsis (Schiefthaler et al., 1999; Yang et al., 1999), *SOMATIC EMBRYO RECEPTOR KINASE1* (*SERK1*), which is thought to play a role in embryogenesis (Hecht et al., 2001), and three independent *FIS* class genes, mutations in any one of which result in fertilization-independent endosperm development (Luo et al., 2000). An astonishing conservation of expression pattern was observed in apomictic and sexual *Hieracium* plants using these chimeric genes that for the most part also reflected patterns observed in Arabidopsis (Tucker et al., 2003). *AtSPL*:*GUS* was expressed in MMCs but not in aposporous initials, providing the first molecular evidence that these cells develop from somatic cells that do not share identity with MMCs. *SERK1*:*GUS* expression was conserved in pattern in sexual and apomictic plants, and the three FIS chimeric genes were expressed coordinately during the early events of seed development in *Hieracium*.

The main differences in spatial and temporal expression pattern occurred early in ovule development and, in marked contrast to Arabidopsis *AtFIS2*:*GUS*, was expressed at the completion of meiosis in both sexual and apomictic *Hieracium* (Figure 4). The spatial pattern of expression, however, differed in sexual and apomictic plants. In sexual *Hieracium*, expression of *AtFIS2*:*GUS* was observed in the three megaspores destined to degenerate, was absent from the single cell layer enveloping them (nucellar epidermis) destined for degeneration, and was absent from the selected megaspore until the first nuclear division of embryo sac formation. By contrast, in two apomictic *Hieracium* species with differing modes of aposporous embryo sac formation, *AtFIS2*:*GUS* expression was observed in all four megaspores and the enveloping nucellar epidermis, all of which were destined for degeneration. Expression of *AtFIS2*:*GUS* was absent from aposporous initials until their first nuclear division, similar to the expression profile observed for the functional megaspore in sexual plants (Figure 4). We speculate that the spatial differences in *AtFIS2*:*GUS* expression in sexual and apomictic plants might reflect gene expression shifts associated with aposporous initial cell commitment to the events of embryo sac initiation and the concomitant displacement of the sexual pathway. Therefore, *FIS* genes might play a different role in *Hieracium* relative to that in Arabidopsis that could be related to the capacity for apomixis and autonomous seed development (Eckardt, 2003). The isolation and characterization of endogenous *Hieracium FIS* genes is under way (Koltunow and Tucker, 2003).

The findings of Tucker et al. (2003) indicate that sexual and apomictic pathways in *Hieracium* share common gene expression profiles and thus common molecular regulatory features, indicating that they are not distinct pathways (Figure 4). The form of apomixis in *Hieracium* (apospory coupled with autonomous embryo and endosperm development) appears to differ from sexual reproduction in a very limited number of ways. Specifically, it is comparable to sexual development except for two specific switch points: meiosis and fertilization. For apomixis to occur in *Hieracium*, the developmental program of one or at most a few cells is altered, and this appears to be sufficient to enable them to bypass meiosis yet mimic the normal program seen in the descendants of a selected megaspore. Later development bypasses fertilization but still moves through the normal embryo and endosperm developmental programs (Figure 5). These data suggest that apomixis is a deregulation of the sexual reproductive program in space and time, leading to cell fate changes and the omission of steps critical to sexual progression. It is conceivable that different types of apomixis, as seen in other species, might arise depending on when and where this deregulation occurs (Koltunow and Grossniklaus, 2003). Figure 1 also portrays this concept in the sense that it depicts the three broad classes of apomictic mechanisms as operating within the framework of the sexual reproductive cycle. Differential and subtractive hybridization have been attempted to isolate genes relevant to apomictic reproduction (Vielle-Calzada et al., 1996; Guerin et al., 2000; Pessino et al., 2001). Although not exhaustive, these have failed to find genes specific to structures arising from apomictic development when the expression of these genes has been examined (Guerin et al., 2000). Although negative in context, this observation further supports the proposal that sexual and apomictic developmental pathways differ primarily at the level of regulation of common elements.

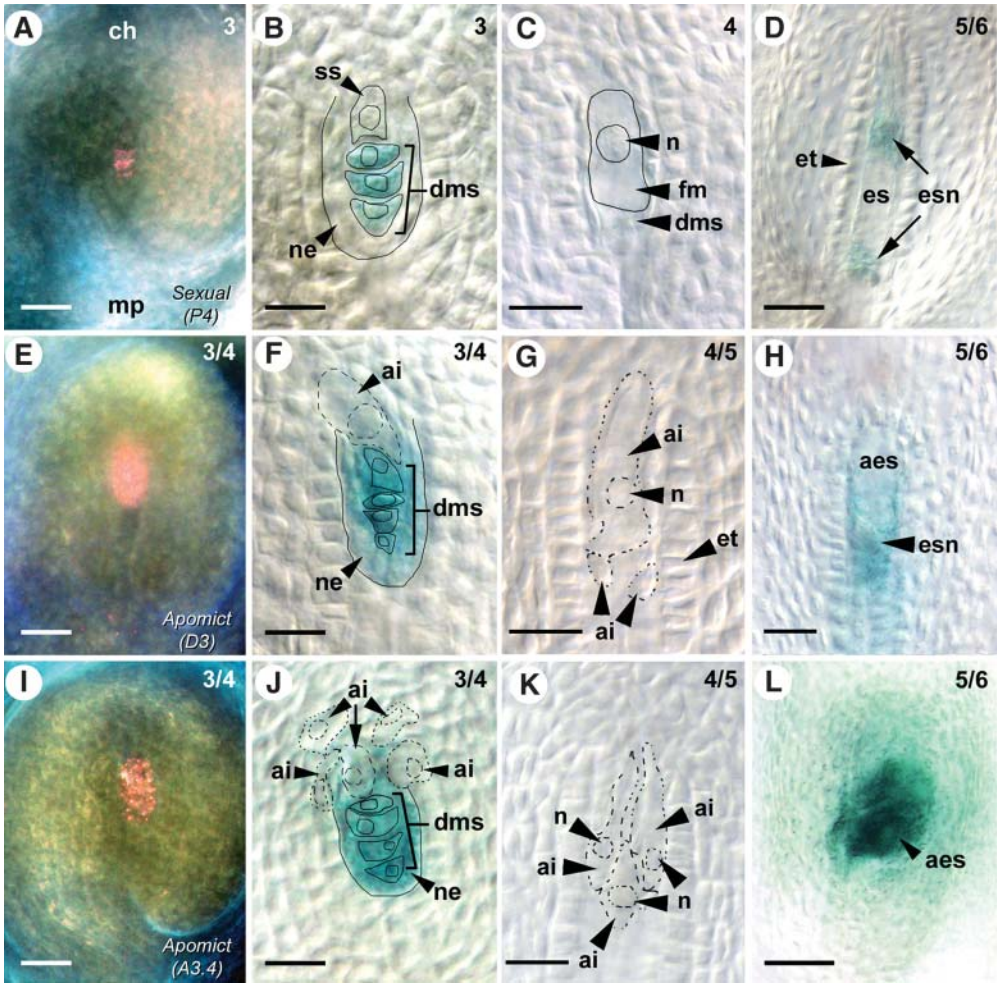

Figure 4. *AtFIS2:GUS* Expression during Early Ovule Development in *Hieracium*.

Ovules from sexual *Hieracium* P4 (**[A]** to **[D]**), apomictic *Hieracium* D3 (**[E]** to **[H]**), and apomictic *Hieracium* A3.4 (**[I]** to **[L]**) were stained with GUS and viewed whole mount using dark-field microscopy (**[A]**, **[E]**, and **[I]**) or Nomarski differential interference contrast microscopy. The numbers at the top right indicate the ovary stage (Koltunow et al., 1998). Bars = 50 μM in **(A)**, **(E)**, **(I)**, and **(L)**, and bars = 25 μM in **(B)** to **(D)**, **(F)** to **(H)**, **(J)**, and **(K)**.
(A) P4 ovule in the chalazal (ch) to micropylar (mp) orientation showing GUS stain in pink.
(B) Enlarging selected spore (ss) and blue GUS-stained degenerating megaspores (dms) surrounded by the nucellar epidermis. Indicated structures are outlined with a black line.
(C) Enlarging functional megaspore (fm) with a large nucleus (n) chalazal to degenerated megaspores. Indicated structures are outlined with a black line.
(D) Ovule containing an early embryo sac (es) containing dividing embryo sac nuclei (esn) and surrounded by the endothelium (et).
(E) D3 ovule showing the corresponding stage of apomictic development to **(A)**.
(F) Enlarging aposporous initial (ai) at the corresponding stage of apomictic development to **(B)**. The aposporous initial, outlined with a broken line, is forming in a slightly different plane to the other structures, which are outlined with an unbroken line.
(G) Enlarging aposporous initial cell above two smaller initials.
(H) D3 ovule containing a dividing aposporous embryo sac (aes) with embryo sac nuclei (esn) at the corresponding stage of apomictic development to **(D)**.
(I) A3.4 ovule showing the corresponding stage of apomictic development to **(A)** and **(E)**.
(J) Enlarging aposporous initials at the corresponding stage of apomictic development to **(B)** and **(F)**. Multiple aposporous initial cells are indicated with broken lines, and some form in slightly different planes compared with the sexual structures; these are outlined with an unbroken line.
(K) Enlarging aposporous initial cells.
(L) Aposporous embryo sac structures at the corresponding stage of apomictic development to **(D)** and **(H)**.
Figure reprinted from Tucker et al. (2003) with permission.

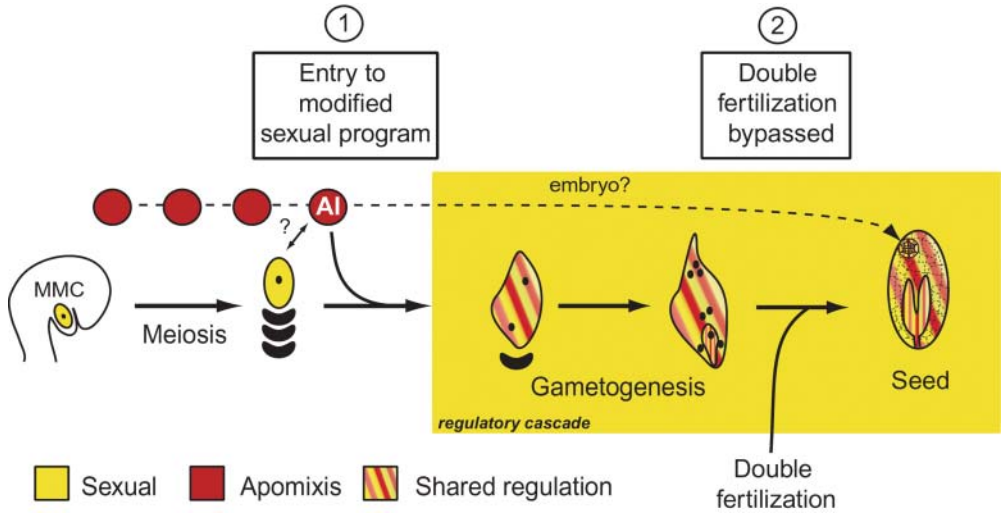

Figure 5. A Model for Apomixis in *Hieracium* (Modified from Tucker et al., 2003).

SIGNALS FROM OTHER OVULE TISSUES?

In sexual species such as Arabidopsis, the development of both the embryo sac and the embryo is influenced by signals from surrounding sporophytic ovule tissues (Ray et al., 1996; Gasser et al., 1998; Schneitz, 1999). Some preliminary lines of evidence suggest that the sequence of apomictic events in *Hieracium* is relatively flexible, influenced by information from surrounding ovule tissues. Ovule identity and form were altered significantly in flowers that develop from ectopic meristems in *H. piloselloides* plants expressing the *rolB* oncogene from *Agrobacterium rhizogenes*. This gene is known to alter plant growth morphogenesis and cellular sensitivity to auxin. In structurally deformed ovules characterized by elongated and distorted funiculi and aberrant integuments, the sexual process ceased earlier, before meiosis, and a higher frequency of aposporous initials was observed. Surprisingly, however, embryos and endosperm continued to develop (Koltunow et al., 2001). A diploid apomict, D2 (n+0 progeny from D3; Figure 3), was found to exhibit similar but less severe defects in funiculus and integument formation than those observed in ovules from *rolB* plants. The developmental events of apomixis in D2 ovules phenocopied those found in *rolB* ovules in terms of the increased frequency of apomictic initiation and continuation of the process in malformed ovules (Koltunow et al., 2000). However, the D2 plant also is capable of forming isolated embryos that are found external to an embryo sac structure, mechanistically resembling the process of adventitious embryony (Koltunow et al., 2000) (Figure 2). These embryos might arise from aposporous initial cells diverted to a different fate as a result of alterations in ovule structure (Figure 5). Alternatively, signaling changes resulting from ovule malformation may stimulate a parthenogenetic program in a set of somatic cells in a manner possibly reminiscent of stress-induced somatic embryogenesis (Mordhorst et al., 1997). All of these possibilities, including the role of auxin in apomictic events in *Hieracium*, require further investigation.

THE GENETIC BASIS OF APOMIXIS

The first known study of inheritance in an apomictic plant was unknowingly conducted by Gregor Mendel (1869) on *Hieracium*, ironically selected to assist in corroborating his laws of inheritance (reviewed by Correns, 1905; Nogler, 1994). It is not known how many crosses Mendel conducted on *Hieracium* because most of the data have been lost, but extrapolations from the information that is available indicates that he performed many thousands of crosses over a period of >10 years. By direct contrast to his observations in pea, the *Hieracium* F1 hybrids showed extensive segregation, whereas the F2 ''hybrids'' did not segregate and uniform progeny were obtained consistently. In correspondence with Nägeli, a *Hieracium* specialist (July 1870), Mendel noted the ''almost opposed behavior'' in the two systems ''both [of which represented] the emanation of a higher universal law.'' By the turn of the 20th century, apomixis was a known phenomenon in plants, and Ostenfeld (1904, 1906, 1910) returned to the study of inheritance in *Hieracium*. He conducted several cross combinations, including repetitions of Mendel's work, and, along with Rosenberg (1906, 1907), correctly noted the expression of apomixis in this genus. It had taken almost 40 years to explain Mendel's data.

The inheritance of gametophytic apomixis has since been reported to be associated with the transfer of either a single locus or a small number of loci in most of the systems studied to date. In the aposporous grasses *Pennisetum* (Sherwood et al., 1994), *Panicum* (Savidan, 1983), and *Brachiaria* (Valle et al., 1994), apomixis is reported to be simply inherited, with the trait conferred by the transfer of a single dominant factor. Simple dominant inheritance also has been reported for apospory in the dicotyledonous genera *Ranunculus* (Nogler, 1984b) and *Hieracium* (Bicknell et al., 2000). Among the diplosporous apomicts, independent inheritance of diplospory and parthenogenesis have been observed in the dandelion *Taraxacum* (van Dijk et al., 1999) and in *Erigeron* (Noyes, 2000; Noyes and Rieseberg, 2000),

whereas Voigt and Burson (1983) reported the simple dominant inheritance of diplospory in *Eragrostis curvula*, the weeping lovegrass. Similarly, the inheritance of diplospory in Eastern gamagrass (*Tripsacum dactyloides*) is reported to be simple and dominant (Leblanc et al., 1995). There is evidence of segregation ratio distortion in some of these systems, often because the dominant factor(s) associated with apomixis also appears to confer gamete lethality, restricting its transfer to some gamete genotypes (Nogler, 1984b; Grimanelli et al., 1998a; Roche et al., 2001a; Jessup et al., 2002).

From these and other older studies, it is widely generalized that there is either one locus, or only a small number of loci, involved in the inheritance of apomixis in native systems. There are, however, some important caveats associated with the interpretation of these data. In almost all of the cases mentioned, the inheritance of apomixis was recorded after crosses between closely related sexual and apomictic species. Very often in these studies, the expression of apomixis restricts the use of the apomict to the pollen parent. Reciprocal crosses are seldom reported, and the influence of maternal effects remains largely untested. Additionally, particular care must be exercised in scoring progeny during these studies. Apomixis is a complex trait. Ideally, it should be measured strictly through the production of genetically identical seedlings, coupled with an embryological study to confirm the reproductive mode of each progeny plant and an assessment of ploidy in all individuals (reviewed by Nogler, 1984a). This is often so time consuming that correlated characteristics are used as a measure of apomixis (for review, see Bicknell, 2001; Leblanc and Mazzucato, 2001). Examples include the development of tetranucleate embryo sacs in *Panicum* (Savidan, 1980) and the formation of globular embryos after emasculation in *Hieracium* (Bicknell et al., 2000). In all such cases, there are opportunities for inaccuracies in the estimation of intact, functional apomixis. For example, in *Panicum*, an overestimate may result if <100% of the embryo sacs observed form functional unreduced embryos, whereas in *Hieracium*, the embryos formed are clearly the result of parthenogenesis but it is not clear if they are derived from unreduced eggs. Indeed, from a reexamination of the data collected during earlier studies of *Hieracium*, including our own (Bicknell et al., 2000), together with a new analysis of inheritance in this system, we now believe that the number of controlling loci has been underestimated. Rather than a single locus being involved, it appears that as many as three unlinked dominant factors may need to be inherited to ensure the transfer of autonomous aposporous apomixis as an intact trait in *Hieracium* (our unpublished data). Recent studies also suggest that three unlinked loci are required for the inheritance of autonomous diplosporous apomixis in *Taraxacum officinale* (van Dijk et al., 2003).

Another difficulty with the interpretation of some of the published inheritance data for apomixis is that it often treats apomixis as a qualitative trait, one that is either inherited or not. As mentioned above, apomixis is expressed facultatively in most plants, and the level of viable asexual seed formation can vary considerably between individuals. A common observation from inheritance studies is that the level of apomixis expressed by many of the F1 progeny is well below that of the apomictic parent (Nogler, 1984b; Koltunow et al., 2000). This effect can be quite profound. Most of the apomictic F1 progeny observed in an inheritance study conducted by the authors on *Hieracium* expressed the trait at <5% of the total seed set, whereas apomixis was rated at 98% in the apomictic parent (Bicknell et al., 2000; Koltunow et al., 2000; R. Bicknell, unpublished results). Attempts to introgress apomixis from wild relatives to crops through serial backcrossing also provide an indication of this phenomenon. *Tripsacum dactyloides* is an apomictic relative of maize. Savidan and co-workers have been involved in an effort to develop apomictic maize for many years through the introgression of *Tripsacum* DNA into *Zea mays*. Their efforts are particularly impressive for the significant amount of effort involved and for the intelligent use of advanced technologies for the rapid screening of large hybrid populations. Despite their dedication and the apparently ''simple'' inheritance of the trait, however, although significant progress has been made to bring their materials to the BC4 generation, the integration of intact, functional apomixis into a genotype with just the 20 chromosomes of maize remains an intractable goal. Among the BC4 plants reported to date, no fertile apomictic individuals have been confirmed with fewer than 16 complete *Tripsacum* chromosomes (Savidan, 2001). Similar outcomes have been reported for the introgression of apomixis into pearl millet (*Pennisetum glaucum*) from related apomicts (also reviewed by Savidan, 2001).

These data and experiences have led some in the field of apomixis to speculate that the trait may not be as simply inherited or as simply controlled in natural systems as is often reported. Rather, some believe that individuals within a typical agamic complex, both sexual and apomictic, may share a predisposition to express this trait (Grimanelli et al., 2001b; Sharbel and Mitchell-Olds, 2001). The nature of this predisposition may be a developmental characteristic, such as the possible presence of a large, nutritive nucellus in *Citrus* (Koltunow et al., 1995b) or a nutritive integument in *Hieracium* (Koltunow et al., 1998) and *Taraxacum* (Cooper and Brink, 1949), or it may be genetic, such as the apparent linkage grouping in *Tripsacum* necessary for interspecific transfer (Savidan, 2001).

WHY ARE GAMETOPHYTIC APOMICTS POLYPLOIDS?

Gametophytic apomicts, irrespective of the mechanism they use, are almost invariably polyploids, yet sexual members of the same or closely related species are very commonly diploids (Asker and Jerling, 1992). The reason(s) for this association remains unclear. It is, however, potentially a critical issue, because a frequently stated aim of current research is the installation of apomixis into diploid crop species. Three main theories have been forwarded. Some have proposed that the optimum expression of apomixis may be achieved only in conjunction with a polyploid genome (Quarin et al., 2001). Because rare diploid gametophytic apomicts have been reported (Asker and Jerling, 1992; Bicknell, 1997; Kojima and Nagato, 1997; Naumova et al., 1999; Koltunow et al., 2000), polyploidy does not appear to be absolutely required for the expression of the apomixis. In these examples, however, asexual seed formation often was poor, so polyploidy may enhance the expression of apomixis in many systems rather than ensuring its presence per se. Some

indications of how this might operate come from yeast (Galitski et al., 1999) and Arabidopsis (Lee and Chen, 2001), in which alterations in ploidy status are known to affect methylation and the expression of different alleles. Conversely, there are intriguing examples of apomixis being expressed in previously sexual plants after chromosome duplication (Nygren, 1948; Quarin et al., 2001). However, there is some debate in these cases about the possibility of innate predisposition, because the plants used were sexual members of groups containing apomicts (Quarin et al., 2001). Furthermore, the reverse has been described by Asker (1967): a sexual plant was recovered after the doubling of an apomictic biotype of *Potentilla argentea*. Finally, polyploidy has been induced in a large number of plants, and apomixis is reported very seldom in the products.

An apparent interspecific hybrid origin also is a common feature among apomicts, and the combination of polyploidy and hybridity is believed to have resulted in allopolyploidy in many gametophytic apomicts (Ellerstrom and Zagorcheva, 1977; Carman, 1997, 2001; Roche et al., 2001a). The action of tetrasomic inheritance in many systems, however, also indicates the presence of autopolyploidy, or possibly segmental allopolyploidy, in these plants (Pessino et al., 1999). Carman (1997, 2001) postulated that a combination of hybridity and polyploidy can lead to the disjunction of key regulatory events during critical stages of megasporogenesis, megagametogenesis, and fertilization. This in turn may lead not only to apomixis but also to other unusual developmental events, such as polyspory and polyembryony. Through a comprehensive survey of the botanical literature, together with his own experimentation, Carman presents compelling evidence that there are associations between apomixis and these other phenomena, that hybridity between related species has been a key factor in the formation of many apomictic complexes, and that different types of apomixis and related phenomena are all expected outcomes of a theoretical model based on the disjunction of a relatively small number of key regulatory events. Whether this is universally true, and whether it can be used to harness apomixis in crop species, are questions remaining to be answered.

Roche et al. (2001b) provided a refinement on Carman's hypothesis, suggesting that supernumerary chromatin may be the principal driver in this process. A hybrid origin, segmental allopolyploidy, and the activity of reproductive drivers all are reported characteristics of supernumerary chromatin biology (McVean, 1995). There is growing evidence for the presence of supernumerary chromatin in several apomictic species, and it is clearly involved in the inheritance of apomixis in the grasses *Pennisetum squamulatum* and *Cenchrus ciliaris* (Roche et al., 2001a, 2001b, and references therein).

Matzk et al. (2003) recently combined a flow cytometric seed screen for reproductive mode with chromosome counts and found that apomictic *Hypericum* and *Ascyreia* plants had a higher DNA content per chromosome than related sexual species. The same appears to be true for apomictic *Hieracium* (M. Tucker, F. Matzk, and A.M. Koltunow, unpublished results). An increased genetic load mediated by transposon replication was postulated by Matzk et al. (2003) as a mechanism by which the increased DNA content of apomictic *Hypericum* and *Ascyreia* might arise. We have evidence that at least four classes of transposons are present in *Hieracium* species, but the relative content of each in sexual and apomictic genomes has not been established (M. Tucker, T. Tsuchiya, R. Bicknell, and A.M. Koltunow, unpublished results). Therefore, the enlarged genomes of apomicts might be more the consequence of asexual seed formation than its cause, and this may have contributed to the apparent involvement of supernumerary chromatin. This observation and conclusion, however, contradict the hypothesis that sexual species should have larger genomes than related asexual species (Wright and Finnegan, 2001), because sexual reproduction is thought to favor the spread of mobile elements between individuals of a population and asexuality is thought to prevent interindividual transfer (Hickey, 1982; Matzk et al., 2003).

It also has been proposed that the inheritance of apomixis may be favored by the mediation of a diploid or polyploid gamete (Nogler, 1984b, 1986). This would lead to the rapid establishment of polyploid agamic complexes. Under this proposal, the creation of apomixis may occur in a diploid plant, but any further dispersion of the trait throughout a species would require the mediation of an unreduced gamete. As described above, unreduced gametes, particularly unreduced eggs, are observed commonly in most gametophytic apomicts, providing an opportunity for this mechanism to play a role in the evolution of agamic complexes. Nogler (1984b) noted that in *Ranunculus auricomus*, a dominant allele conferring apomixis could be transferred only through a diploid gamete. Haploid gametes were produced by these plants, but their products all were sexual. This observation led Nogler to speculate that the dominant allele may play a gamete-lethal role when present in homozygous form in the pollen. Similar observations have been made for *Tripsacum* (Grimanelli et al., 1998a) and *Pennisetum* (Roche et al., 2001b; Jessup et al., 2002). There are cases, however, in which there does not appear to be a gamete-lethal effect associated with the transfer of apomixis. In *Hieracium*, haploid gametes do transfer competence for parthenogenesis, a component of apomixis (Bicknell et al., 2000). Intriguingly, in this case, diploid apomictic progeny could not be recovered, but selection against this class appeared to be acting at the level of the zygote, not the gamete. As mentioned above, there is growing evidence that natural apomicts frequently carry a high genetic load of deleterious alleles. This is expected to result in a ratchet effect, encouraging the formation of polyploids and reducing the viability of any diploid derivatives they may produce. Apomictic polyploids often are capable of producing diploid progeny, typically through the operation of polyhaploidy, a natural mechanism analogous to haploid parthenogenesis that often is observed in apomicts (Asker and Jerling, 1992). Diploids have been recovered among the progeny of several apomictic species. However, they are invariably considerably weaker than their polyploid parent, often to the point of being barely viable, which we believe to be a reflection of the inherited genetic load.

Similarly, we recently isolated a small number of genomic sequences of putative reproductive importance from both sexual and apomictic accessions of *Hieracium*. Sequence comparisons indicated that the genes from the apomict typically contained a higher frequency of transposon insertions and gene rearrangements (A.M. Koltunow and M. Tucker, unpublished results). We

hypothesize, therefore, that in *Hieracium* at least, once a polyploid apomict is formed, mutation rapidly increases its genetic load and limits the viability of any future diploid derivatives. That could clearly result in the haploid gamete lethality observed in *Ranunculus* and also may explain the diploid zygote lethality seen in *Hieracium*.

Finally, where apomixis requires the simultaneous inheritance of critical alleles at several unlinked loci, as seen in *Erigeron* (Noyes and Rieseberg, 2000) and *Taraxacum* (van Dijk and Bakx-Schotman, in press), unreduced gametes would ensure the intact transfer of the trait, essentially by acting as a single whole-genome linkage group. In many cases, this would provide a clear selective advantage because, typically, the inheritance of only part of a mechanism of apomixis, such as parthenogenesis without apomeiosis, leads to the production of disadvantaged progeny (n+0 progeny in this example; Figure 3). The complete inheritance of apomixis through the mediation of an unreduced gamete would clearly avoid this issue, but it also would lead to an increase in ploidy over the parental state after fertilization. As mentioned above, unreduced gametes, particularly unreduced eggs, are relatively common in these plants, and frequently they are involved in the formation of hybrid progeny (Figure 3) (Chapman and Bicknell, 2000; Bicknell et al., 2003). Therefore, in a manner similar to the role of genetic load, this effect can be expected to stimulate the formation of polyploid agamic complexes and to repress any reverse process by biasing against diploid apomict survival.

GENE IDENTIFICATION IN APOMICTS

The identification of the genes involved in apomixis appears to be tractable, because in most of the native apomictic systems under study, only a small number of loci have been determined to be critical for the inheritance of the trait. In most cases, a map-based approach has been taken to attempt the cloning of the sequences involved. The first and most comprehensive mapping efforts in apomicts have been reported from groups working in grass species, most of which are relatives of important cereal crops. The dominant factor associated with the inheritance of apomixis in the aposporous plus pseudogamous grass species *Pennisetum squamulatum* appears to be hemizygous, because no equivalent region has been found in sexual biotypes (Ozias-Akins et al., 1993, 1998; Roche et al., 2001a). Intriguingly, the same region was found in the apomictic relative *Cenchrus ciliaris*, also as a hemizygous region (Roche et al., 1999, 2001a). Efforts to isolate this region have revealed its complex nature. Twelve molecular markers were reported to cosegregate with the apospory-specific genomic region without separation, an early indication that this region may be associated with a localized suppression of recombination. After the isolation of BACs corresponding to these markers, it became apparent that this region of suppressed recombination spanned at least 50 Mbp in *P. squamulatum* (Roche et al., 2002) and may be considerably larger. Using the corresponding BAC clones as probes, Goel et al. (2003) demonstrated that the apospory-specific genomic region localizes to a single short arm of a *P. squamulatum* chromosome.

A similar effort is under way with the maize relative *Tripsacum dactyloides*, an apomict that uses diplospory plus pseudogamy. Again, markers were reported in linkage with the region associated with the inheritance of diplospory in this plant (Grimanelli et al., 1998b; Blakey et al., 2001). Suppressed recombination clearly is also a feature of this region, frustrating efforts to clone the critical genes involved in apomixis. Estimates of the size of this region are less accurate than with *Pennisetum*, but it appears to correspond to a 40-cM region of the sexual *Tripsacum* map (Grimanelli et al., 2001a). *Paspalum*, another grass genus, also is being used in a map-based cloning strategy. The apospory plus pseudogamy mechanism of apomixis in this plant is inherited as a simple dominant factor (Martinez et al., 2001). Grasses are known for their high synteny. Pupilli et al. (2001) noted the coinheritance of five rice markers with apospory in *Paspalum* and are attempting to clone the locus through their use as probes. Again, nonrecombination and hemizygosity were detected in association with the locus (Labombarda et al., 2002). Intriguingly, although all of the grasses mentioned above show considerable synteny with the rice genome, in each case the region associated with the inheritance of apomixis aligns to a different region of that genome (Grimanelli et al., 2001a). Albertini et al. (2001a, 2001b) also reported the isolation of markers linked to apospory in Kentucky bluegrass (*Poa pratensis*), and Pessino et al. (1997) noted similar findings for *Brachiaria*. Among the eudicotyledonous systems under study, progress is being made toward the mapping of ''apomixis'' genes in *Taraxacum* (van Dijk et al., 2003; Vijverberg et al., 2004) and *Erigeron* (Noyes and Rieseberg, 2000). There is some evidence for nonrecombinant sector(s) associated with apomixis genes in *Erigeron* and for gamete selection in *Taraxacum*, indicating that similar difficulties with cloning also may arise in these plants.

Critically considered, the map-based cloning strategy described above is clearly a valid and promising line of inquiry, but success is certainly not proving to be either easy or assured. The commonly observed suppression of recombination about loci associated with apomixis is particularly problematic, because it markedly reduces the power of the approach. Fine mapping usually is attempted in this method to reduce the distance that must be spanned by a genomic contig, but suppressed crossover frequencies result in a paucity of recombinants and little opportunity to refine the basic mapping data. It also should be remembered that these plants are unlike familiar sexual models such as Arabidopsis and rice. They typically have large, polyploid genomes, and almost certainly they have high representations of highly repetitive sequences, particularly those of transposons. In most cases, mapping is proceeding in a triploid or tetraploid highly heterozygous background. Fortuitously, the alleles associated with apomixis typically are simplex and dominant, simplifying phenotypic analysis of segregating progeny. However, once a dominant allele is mapped, contigs must be formed, taking care to ensure that the aligned genomic fragments are from the same homolog. At this time, the hemizygosity seen in *Pennisetum* and *Paspalum* may be an advantage, but it is probably also associated with the suppression of recombination mentioned above. Distances of 50 to 100 Mbp appear to be commonly involved in these mapping efforts. Matzk et al. (2003) have suggested that large, hemizygous arrays of DNA linked to apomixis may be attributable to the segregation of markers with

a retrotransposon(s) accumulated after the establishment of apomixis. The presence of highly repetitive sequences clustered about the locus of interest will further frustrate efforts in walking and cloning. Additionally, so little is known of the molecular basis of apomixis that the essential elements may not even be recognized even if they were sequenced. Given the regulatory nature of the trait, *cis*-acting factors, including chromatin-modeling factors, are likely to be as critical as structural sequences (Koltunow and Grossniklaus, 2003). Finally, many of these plants cannot be transformed genetically, so functionality testing of putative control elements is not yet possible. Efforts to address this limitation apparently are under way (Dresselhaus et al., 2001).

MUTAGENESIS STRATEGIES AND RECOVERED MUTANTS

In light of the difficulties described above, alternative strategies to identify genes involved in apomixis are being tried based on targeted mutagenesis. *Tripsacum dactyloides*, a diplosporous plus pseudogamous apomict, can be crossed to maize and apomictic progeny isolated from the hybrids formed. This feature has been used to introduce *Mutator* transposable elements from maize into an apomictic genotype, the progeny of which will then be scored for insertional mutagenesis (Grimanelli et al., 2001b). A transposon tagging strategy, based on the *Ac/Ds* elements of *Zea mays*, also was implemented in *Hieracium* through the introduction of engineered elements (Bicknell et al., 2001; Weld et al., 2002). Unfortunately, although the elements were shown to be operational in this plant, the tagging system is proving to be too slow and too complex to be practical, so it has been discontinued.

During the course of the *Ac/Ds* work in *Hieracium*, two mutants were identified, and these have been partially described (Bicknell et al., 2001). In both mutants, apomixis was lost, yet the plants remained able to form seed sexually. In one line, the plant was no longer able to form aposporous initials, whereas the other formed initials but these did not progress to form unreduced embryo sacs. Intriguingly, in the former case, the rate of sexuality remained at the wild-type level of 2%, whereas in the latter case, sexuality increased to essentially 100% of the viable reproductive effort of the capitulum. Together with evidence from inheritance (Bicknell et al., 2000) and recent comparative marker gene expression studies (Tucker et al., 2003), we interpret this result to indicate that one component of apomixis in this plant entails the formation of aposporous initials and another leads to the suppression of sexual reproduction. The two mutants cross-complement, permitting the recovery of apomixis, which indicates that they carry lesions in different genes. Another independent mutant has since been obtained that has lost the capacity both to form initial cells and to progress in the sexual process (A.M. Koltunow and S.D. Johnson, unpublished results). All three mutants were obtained as somaclonal variants, each after a transformation experiment. However, there is no evidence for insertional mutagenesis in any of these cases. Unfortunately, although these mutants are providing valuable insight into the cellular and genetic processes underlying the expression of apomixis in *Hieracium*, without clear molecular tags they do not provide a simple route to the identification of the sequences involved. A γ-induced mutation screen aimed at identifying loss-of-function apomictic mutants is providing promising results (Bicknell et al., 2001). More than 70 mutants have been isolated to date in which apomixis is lost yet sexuality is retained to varying degrees. Apomictic mutants reflecting all of the phenotypes described above have been obtained, together with others indicating that several factors associated with the expression of apomixis have been affected. Amplified fragment length polymorphism now is being used to identify chromosomal regions commonly lost in these plants and to provide probes to those regions.

THE PROSPECT OF INSTALLING APOMIXIS IN CROPS

During the 10 years since the last *Plant Cell* review on apomixis, many of the critical issues identified at the time have been addressed, together with a consolidation of the approaches being taken and a refinement of the methods and systems being used to study functional apomixis. We now believe that researchers in this field are in a much stronger position to predict the successful installation of this characteristic into diploid crop species. Some issues, such as the role of polyploidy, remain incompletely resolved, but they are now framed by clear hypotheses and these are being tested. One of the most important conclusions during this 10-year period has been the understanding that apomixis results from changes in a small number of key events typical of sexual reproduction. This clearly points the way toward identifying and manipulating those elements in a ''synthetic'' approach to engineering asexual seed formation. As many of the other articles in this special issue demonstrate, advances in our understanding of the regulation of sexual reproduction have been profound in recent years and are providing critical insights for this line of inquiry.

Significant effort still is required on a number of fronts before we can hope to successfully engineer a controlled, commercially viable form of apomixis in a wide range of target crops. We have no knowledge of the cues and genes that enable cells in the ovule to switch to an apomictic pathway. The interaction between embryo and endosperm development in apomicts is poorly understood, as is the capability of apomicts to tolerate parental genome imbalances during endosperm development that result in seed sterility in sexual plants. The role of epigenetic factors in the control of apomixis is unknown, yet they are widely implicated in the control of sexual reproduction. Similarly, molecular signaling events have been implicated in the development of both sexual and asexual structures within the plant ovule, yet the nature and role of these events in apomixis are largely unstudied to date. We believe that the continued investigation of both native apomicts and sexual systems will provide the best opportunity to elucidate the genetic and physiological factors that contribute to the control of apomixis and that this ultimately will permit the controlled formation of maternally derived, genetically identical seeds in flowering plants.

In conclusion and upon reflection, it is intriguing that Mendel selected two plant taxa to examine the nature of inheritance between generations. The study of *Pisum*, his first choice, established the particulate nature of inheritance, demonstrated trait segregation, confirmed the role of gametes as the couriers

of genetic determinants, and demonstrated the combined consequences of segregation and recombination. In choosing pea, Mendel is rightfully credited with the establishment of the field of genetics as it is known today. Mendel's second choice was *Hieracium*, a plant that proved unsuitable for its original intended purpose of verifying the findings made in pea. It is probably for this reason, and because the results Mendel obtained in *Hieracium* were reported only in abbreviated form, that this part of his work has remained largely unnoticed to this day. Therefore, there is a certain irony in noting that *Hieracium* is becoming such a useful tool in the study of a very different form of reproduction, that of facultative apomixis, >130 years after Mendel's first crosses with this plant were made.

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