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REVIEW

Altering Sexual Development in *Arabidopsis*

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The reproductive system determines the way in which gametes develop and interact to form a new organism. Therefore, it exerts the primary level of control of genotypic frequencies in plant populations, and plays a fundamental role in plant breeding. A basic understanding of plant reproductive development will completely transform current breeding strategies used for seed production. Apomixis is an asexual form of reproduction in which embryogenesis occurs in a cell lineage lacking both meiosis and fertilization, and that culminates in the formation of viable progeny genetically identical to the mother plant. The transfer of apomixis into sexual crops will allow the production of self-perpetuating improved hybrids, and the fixation of any desired heterozygous genotype. The initiation of apomictic development invariably takes place at early stages of ovule ontogeny, before the establishment of the megagametophytic phase. The developmental versatility associated with megagametophyte formation suggests that the genetic and molecular regulation of apomixis is intimately related to the regulation of sexuality. Differences between the initiation of sexual and apomictic development may be determined by regulatory genes that act during megasporogenesis, and that control events leading to the formation of unreduced female gametophytes. To test this hypothesis, we are isolating and characterizing genes that act during megasporogenesis in *Arabidopsis thaliana* and investigating their potential role in the induction of apomixis. We are using a recently established transposon-based enhancer detection and gene trap insertional mutagenesis system that allows the identification of genes based on their expression patterns. An initial screen of transposants has yielded over 20 lines conferring restricted GUS expression during early ovule development. We have obtained the sequence of genomic fragments flanking the transposon insertion. Several have homology to genes playing important roles in plant and animal development. They include cell cycle regulators, enzymes involved in callose hydrolysis, leucine-rich repeat protein kinase receptors, and expressed sequence tags (ESTs) of unknown function. Independently, a genetic screen allows the identification of female sterile mutants defective in megasporogenesis. Results from these experiments will improve our basic understanding of reproductive development in plants, and will set the basis for a sustained effort in plant germ line biotechnology, a first step toward a flexible transfer of apomixis into a large variety of sexual crops.

Keywords: *apomixis*, *megasporogenesis*, *Arabidopsis*, *enhancer detection*, reproduction

Introduction

In recent years, the utilization of genetic and molecular approaches has contributed to major advances in our understanding of floral organogenesis (Coen and Meyerowitz, 1991), pollen formation (Mascarenhas, 1992) and embryo development (Jurgens *et al.*, 1997). Pollen development has provided a well defined and accessible system in which to study the control of gene expression during male gametogenesis (Curie and McCormick, 1997). It has been shown that microsporogenesis requires many discrete differentiation events controlled by a large number of genes, and a multitude of anther and pollen specific genes have been identified and cloned (McCormick, 1993; Twell, 1994).

Despite these rapid but limited advances in the

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understanding of plant reproductive development, little is known about the genetic regulation of megagametophyte (female gametophyte or embryo sac) formation. There is now overwhelming evidence that pollen development requires the strict control of gametophytic gene expression (McCormick, 1993; Twell and Howden, 1998). In contrast, only a few female gametophytic mutations have been described in detail (Huang and Sheridan, 1996; Christensen *et al.* 1997; Moore *et al.*, 1997), and genes expressed exclusively in the megagametophyte have yet to be cloned (Drews *et al.*, 1998; Grossniklaus and Schneitz, 1998). Even if megagametophytic development represents a key process of special scientific and commercial interest, reproductive research has been limited by the location and the small number of nucellar cells involved. Numerous studies have focused on cytological descriptions of the developing megagametophyte (Cass and Jensen 1970; Russell 1985; Mogensen, 1982; Mogensen 1988; Huang and Russell 1992), but little emphasis has been given to its molecular and genetic regulation (Vollbrecht and Hake, 1995; Nadeau *et al.*, 1996; Moore *et al.*, 1997).

Sexual and Apomictic Development

The life cycle of sexual higher plants consists of a dominant sporophytic generation and two morphologically different gametophytic phases taking place in specialized reproductive structures (Greyson, 1994). In male and female reproductive organs, the establishment of a gametophytic phase requires that germ line cells are differentiated from sporophytic cells. Flowering plants produce two types of meiotically derived cells (spores) that give rise to the gametophytic phase. In the anther many microsporocytes develop into pollen grains, which represent the male gametophyte. During megasporogenesis usually a single megaspore mother cell (MMC) in the ovule undergoes meiosis and gives rise to four haploid cells (megaspores); this process is called megasporogenesis. In the majority of higher plants, including *Arabidopsis* (Grossniklaus and Schneitz, 1998) a single megaspore gives rise to the female gametophyte, as the three other megaspores degenerate (Willemse and Van Went, 1984). The functional megaspore enlarges, and its nucleus divides mitotically three times to form the megagametophyte. A mature megagametophyte of the Polygonum type consists of seven cells: three antipodals, two synergids, the egg cell, and a binucleate central cell whose nuclei fuse prior to fertilization. After fertilization of both the egg cell and the central cell, the ovule develops into a seed.

In the angiosperms there are numerous examples of developmental abnormalities that suggest a flexible regulatory control of the mechanisms that lead to megagametophyte formation. In many plants sexual reproduction can be associated or replaced by apomixis, a method of asexual reproduction that leads to the formation of clonal seeds (Gustafsson, 1947; Asker and Jerling, 1992). Apomixis has been reported in more than 400 species belonging to 35 different families. The benefits of apomixis for agriculture have been extensively reviewed elsewhere (Savidan, 1992; Vielle-Calzada *et al.*, 1996a). Apomixis provides a system for crop improvement that will allow the fixation of any desired genotype. The genetic transfer of apomixis to cultivated crops is perceived as one of the most important technological and commercial challenges faced by agriculture.

The initiation of apomictic development invariably takes place during early ovule ontogeny. In apomictic plants the embryo can be formed directly from a somatic cell in the ovule (adventive embryony) or from an unreduced megagametophyte whose egg cell develops parthenogenetically (gametophytic apomixis). In the latter case, the megagametophyte is formed from an aberrant meiotic cycle that prevents reduction and recombination (diplospory) or from direct differentiation of somatic nucellar cells in the ovule (apospory). Apomictically derived egg cells are unreduced, and give rise to viable embryos without fertilization (Vielle *et al.*, 1995). Cytological analysis indicates that the developmental regulation of many aspects of sexual reproduction is preserved during apomixis; these aspects include postmeiotic development of the megagametophyte, embryogenesis, endosperm formation, and maternal seed coat development. A comparison of mRNA subpopulations in *Pennisetum ciliare*, an aposporous forage grass, identified only a few cDNAs corresponding to genes differentially expressed in sexual and apomictic ovaries (Vielle-Calzada *et al.*, 1996b). Several cytological studies have described the close spatial and temporal association that usually exists between dying megaspores and active somatic cells initiating aposporous differentiation (Naumova and Willemse, 1995; Naumova and Vielle-Calzada, 1998). In addition, all forms of diplospory are the consequence of alternative meiotic aberrations that affect megasporogenesis. In diplosporous species, the lack of callose deposition around the megaspore mother cell

has been shown to be indicative of apomictic development (Leblanc *et al.* 1995: Peel *et al.*, 1997). In species reproducing by adventitious embryony, the first morphological evidence of embryocyte differentiation is usually observed during early megagametophyte formation (Koltunow *et al.*, 1997; Naumova and Vielle-Calzada, 1998), suggesting that the determination of embryonic initials takes place during late megasporogenesis. Whereas autonomous egg cell development (parthenogenesis) is necessary for apomictic seed formation, fertilization-independent endosperm development is a rare event in apomictic species, and only a few cases have been reported to date. In most cases, endosperm formation and viable seed set depends on fertilization of the central cell (pseudogamy).

The Genetic Basis of Apomixis

Apomixis is known to be genetically controlled; however, its genetic regulation remains poorly understood (for a detailed review see Nogler, 1984a). In the absence of sexually functional megagametophytes apomictic plants can only be used as male parents and cannot be selfed to create segregating populations. Many individuals resulting from the cross of sexual and apomictic genotypes are apomicts that retain the capacity of both sexual and apomictic reproduction, and have a level of apomictic expression that is influenced by environmental conditions (Knox, 1967). In many agamic complexes composed of plants with several ploidy levels, diploid individuals are obligately sexual and polyploids are apomictic (Asker and Jerling, 1992). In several species, autoploidization of sexual diploid individuals has resulted in apomictic tetraploid plants (Burton, 1992). Despite these inherent experimental limitations, several genetic studies have shown that apospory, understood as the initiation of somatically derived non-reduced embryo sacs within the ovule, is controlled by a single dominant Mendelian trait. This simple mode of inheritance has been demonstrated in phylogenetically distant genera such as *Ranunculus* (Nogler, 1984b), *Panicum* (Savidan, 1980) and *Pennisetum* (Sherwood *et al.*, 1994). The recovery of diploids in several apomictic species strongly suggests that polyploidy is not an obligatory requirement for functional apomixis (Nogler, 1984; Bicknell, 1997; Kojima and Nagato, 1998).

The Establishment of the Gametophytic Phase in the Ovule

Despite its importance for seed production, our understanding of the mechanisms that direct the transition from the sporophytic to the gametophytic phase is very limited. Intensive efforts using methods of increased sophistication have generated very few sequences expressed specifically in plant meiocytes (Dickinson, 1995). Most of these sequences are expressed during both male and female meiosis, or are specific to particular stages of microsporogenesis (Bouchard, 1990). A better understanding of megasporogenesis will not only improve our basic understanding of reproductive development in plants, but will also set the basis for a sustained effort in plant germ line biotechnology, the first step towards the flexible introduction of apomixis into a wide variety of sexual crops.

Since little is known about the nature and function of genes expressed during male meiosis, it is not surprising that genes specifically expressed in megasporogenesis are yet to be reported. Megasporogenesis occurs deep within the nucellar tissue and usually involves only one MMC per ovule. Early studies in *Lilium* revealed that the levels of cytoplasmic RNA present in the MMC decrease during pre-prophase and reach their minimal level during leptotene/zygotene (Porter *et al.*, 1984). As in the male cells, both mitochondria and plastids dedifferentiate during early prophase I. A thin layer of callose is usually deposited around the MMC. In *Arabidopsis*, differentiation of two sister MMC occurs at a relatively low frequency (Grossniklaus and Schneitz, 1998); however, only a single megasporocyte initiates meiosis as the other one dies (J-Ph. Vielle-Calzada and U. Grossniklaus, unpubl. data). After completion of meiosis II, callose deposition around the three dying megaspores partially isolates these cells from each other and from the functional megaspore. In most species analyzed to date, megaspores emerge from meiosis in a highly dedifferentiated state and remain so until early megagametogenesis (Willemse and Van Went, 1984). In most species the functional megaspore is located at the chalazal region of the developing nucellus. In *Arabidopsis thaliana* as well as in other species, dying megaspores have far fewer organelles than the functional megaspore, suggesting that an organellar gradient must be established in the syncytial tetrad of megaspore haploid nuclei prior to cytokinesis.

Apomixis and Unreduced Gamete Formation

Even if no fully apomictic mutants have been

recovered in sexual species, several mutants display individual components of apomixis, such as the formation of unreduced female gametes (Rhoades and Dempsey, 1966), the formation of parthenogenetic haploids (Kimber and Riley, 1963), and the autonomous development of the endosperm (Ohad *et al.*, 1996; Chaudhury *et al.*, 1997). A number of well-studied mutations cause aberrations that mirror some of the most early events occurring in gametophytic apomixis. Mutant, affecting various meiotic events have been described in large number of species (Baker *et al.*, 1976; Jackson and Casey, 1980; Kaul and Murthy, 1985). Most have been shown to be inherited as monogenic recessive traits causing partial or total sterility (Koduru and Rao, 1981; Kaul and Murthy, 1985). Many others result in the formation of unreduced gametes (Hermsen, 1984; Veilleux, 1985; Parrott and Smith, 1986; D'Amato, 1989; Ramanna, 1992; Bretagnolle and Thompson, 1995). The mechanisms governing unreduced gamete formation operate during the meiotic phase of megasporogenesis or in sporophytic cells of the ovule prior to meiosis. Many of these mutants have been found to exhibit a high sex specificity, suggesting that little genetic correlation may be found between unreduced pollen and unreduced egg formation (Veronesi *et al.*, 1986; Werner and Peloquin, 1987; De Haan *et al.* 1992). Although the genetic determination of unreduced pollen formation has been studied in some detail (Maizonnier, 1976; Parrot and Smith, 1986), the genetic basis of unreduced egg formation remains poorly understood. Gametes with the sporophytic chromosome number have proven to represent unique tools for plant breeding, particularly when they provide an effective method of transmitting heterozygosity from the diploid (2x) to the tetraploid (4x) level (Watanabe and Peloquin, 1989). Taken together, these considerations suggest that by manipulating the reproductive system of sexual plants it may be possible to induce developmental events that mirror the initial stages of apomixis.

Genetic and Molecular Dissection of Megasporogenesis in Arabidopsis

To identify genes expressed in megasporogenesis we are using an insertional mutagenesis strategy based on gene trapping and enhancer detection, (O' Kane and Gehring, 1987; Bellen *et al.*, 1989; Wilson *et al.*, 1989; Bier *et al.*, 1989), which allows the identification of developmentally regulated genes based on their pattern of expression (Sundaresan *et al.*, 1995). Prompted by their spectacular success in *Drosophila*, similar techniques have been developed for *Arabidopsis* (Kertbundit *et al.*, 1991; Fobert *et al.*, 1991; Topping *et al.*, 1991; Springer *et al.*, 1995; Sundaresan *et al.*, 1995; Smith and Fedoroff, 1995). Enhancer detection relies on a mobile genetic element carrying a reporter gene under the control of a constitutive promoter. If this promoter comes under the control of a genomic cis-regulatory element, the reporter gene is expressed in a specific temporal and spatial pattern. Gene traps are a modification of this approach involving the generation of transcriptional fusions to the reporter gene (Skarnes *et al.*, 1990). Both technologies have been especially useful in studying developmental processes that occur late in development (i.e. after the effective lethal phase of a corresponding mutation). They also allow the identification of genes whose function is redundantly specified and cannot be identified in classical phenotypic screens (Wilson *et al.*, 1989; Grossniklaus *et al.*, 1989). Additionally, remobilization of the enhancer/gene trap element allows the recovery of derivative alleles extremely useful in genetic studies (Bellen *et al.*, 1989; Grossniklaus *et al.*, 1992). Over the last three years we have generated more than 4300 transposant lines carrying single randomly distributed enhancer detectors or gene trap elements that serve as the basis for large scale genetic screens (U. Grossniklaus, J. Moore, J-Ph. Vielle-Calzada, and W. Gagliano, unpubl. results).

Enhancer Detection in Ovules Undergoing Megasporogenesis.

Taking advantage of the *Ac/Ds* transposon system of maize, a *Ds* element has been engineered to act as an enhancer detector or gene trap by carrying the *uidA* gene encoding β-glucuronidase (GUS).

A whole-mount staining and clearing procedure allows screening for GUS expression at different developmental stages encompassing megasporogenesis, from the time when the ovule primordia has just started its elongation (before MMC differentiation) to stages where the viable megaspore is already differentiated. To date, we have analyzed expression patterns in more than a 1000 enhancer/gene trap lines at stages encompassing megasporogenesis. We have identified 25 lines that show expression in specific regions of the developing ovule during stages of megasporogenesis (Fig. 1). For most of these lines (18/25) the initiation of reporter gene expression can be traced back to ovules at the onset of

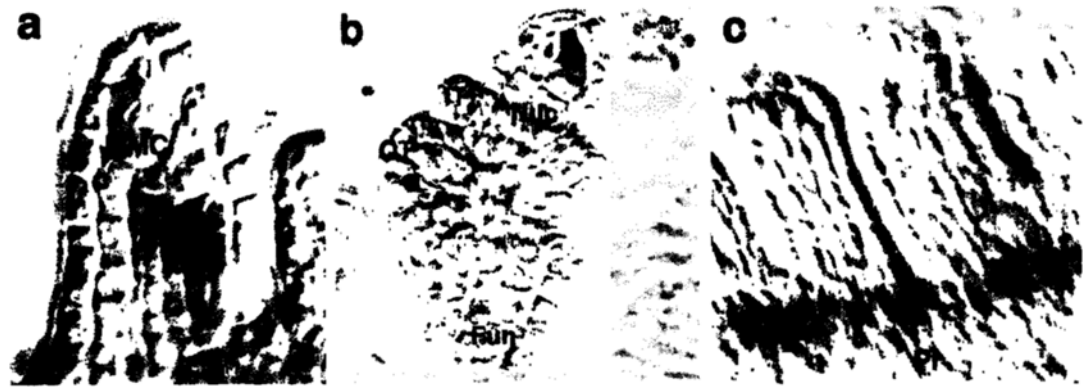

Fig. 1. Enhancer detection during early ovule development in *Arabidopsis*. Reporter gene expression can be detected in specific regions of the ovule. Examples include lines staining the middle region of the primordia at the onset of meiosis (a), degenerating megaspores following meiosis (b), or restricted proximal regions of the young ovule primordium (c). Abbreviations: Fun, funiculus; IT, inner integument; MMC, megaspore mother cell; Nuc, nucellus; OT, outer integument; Pl, placenta.

megasporogenesis, either before or during MMC differentiation. Specific lines show initial GUS expression restricted to either the proximal, middle, or distal portion of the ovule primordium. We used a PCR-based method, thermal asymmetric interlaced PCR (TAIL-PCR; Liu *et al.*, 1995) to isolate genomic regions flanking the transposon insertion as described in Grossniklaus *et al.* (1998c). For 96% of the lines attempted, at least one border fragment has been isolated (Grossniklaus *et al.*, 1998a and 1998b). Several sequences have homology to genes playing important roles in plant and animal development. These include cell cycle regulators, enzymes involved in callose hydrolysis, leucine-rich repeat protein kinase receptors, and genes encoding for proteins involved in organelle differentiation. Several others have homology to *Arabidopsis* expressed sequence tags (ESTs) of unknown function.

Identification of Mutants Defective in Megasporogenesis

Simultaneously, we are conducting an independent screen to identify transposant lines defective in megasporogenesis. In *Arabidopsis*, cytokinesis usually occurs only after the completion of meiosis II, and cell walls are formed simultaneously around all four megaspores. Since the haploid phase of the life cycle only starts after specification of the functional megaspore, we expect that mutations affecting megasporogenesis will segregate as sporophytic traits acting at the diploid level. Seeds derived from enhancer detector and gene trap lines are planted to identify families segregating one quarter sterile plants. To determine whether sterility is due to male or female defects, sterile individuals are reciprocally crossed to wild-type plants. As shown in previous screens of this type (Pruitt *et al.* 1994), a large majority of these mutants is male sterile. To date, we have screened more than 3200 transposants and have identified lines with ovule morphological defects (Table 1) that are associated with restricted patterns of GUS expression (see above), suggesting that the gene responsible for the corresponding mutation has been tagged. We have also identified lines that appear to be defective in both male and female reproductive development, and might be associated with mutations affecting meiosis. All female sterile mutations identified to date appear to segregate as recessive (loss-of-function) sporophytic traits.

Altering the Sporophytic-Gametophytic Transition

Genetic analysis suggests that differences between sexual development and some forms of apomixis

Table 1. Searching for Female Sterile Mutations in *Arabidopsis*.

Number of lines screened		3200
Sterile Lines		39
Floral morphological defects		5
Male sterile		20
Male and female sterile		3
Female Sterile		6
Ovule morphological defects	5	
Defective in megasporogenesis	1	
Lines in which the defect is not yet determined		5

may be initially determined by a regulatory switch that directs individual somatic cells to form an embryo sac without undergoing meiosis. It is possible that a gene inducing apomixis encodes a protein that normally functions during megasporogenesis in sexual species, but may have an altered temporal and/or spatial pattern of expression in apomictic ovules (Koltunow, 1993). A major switch controlling the transition between sexual and apomictic development is likely to be regulated by a small number of genes responsible for activating a cascade of regulatory events that direct megagametophyte formation. The fact that most of the sporophytic cells do not spontaneously develop into megagametophytes (in the case of apospory) or embryocytes (in the case of adventitious embryony) indicates that developmental changes associated with apomictic initiation only occur after specific cells have acquired some form of competence. Numerous studies showing that modifications of plant growth conditions can alter the sporophyte to gametophyte transition (Bell, 1992) and even the whole plant reproductive outcome (Knox, 1966) indicate that the presence of molecular signals which determine the fate of competent cells is fundamental for switching from a sporophytic into a gametophytic developmental program. Taken together, this considerations suggest that by mutagenizing a sexually reproducing plant it may be possible to generate mutants defective in megasporogenesis, and to genetically engineer developmental events that mirror the initial stages of apomixis.

Understanding the fundamental mechanisms that control the transition from sporophyte to gametophyte in flowering plants will have strong implications for the manipulation of the reproductive system and its role in seed production. It is becoming apparent that the genetic control of meiosis and female gametophyte development is directed by molecular regulators that can be used to modify the reproductive pathway. The long-term objective of this project is to understand the molecular genetic mechanisms that control female meiosis and megasporogenesis in *Arabidopsis*, and to explore the possibilities of inducing apomixis as an initial step for transferring this highly desirable trait into economically important crops.

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