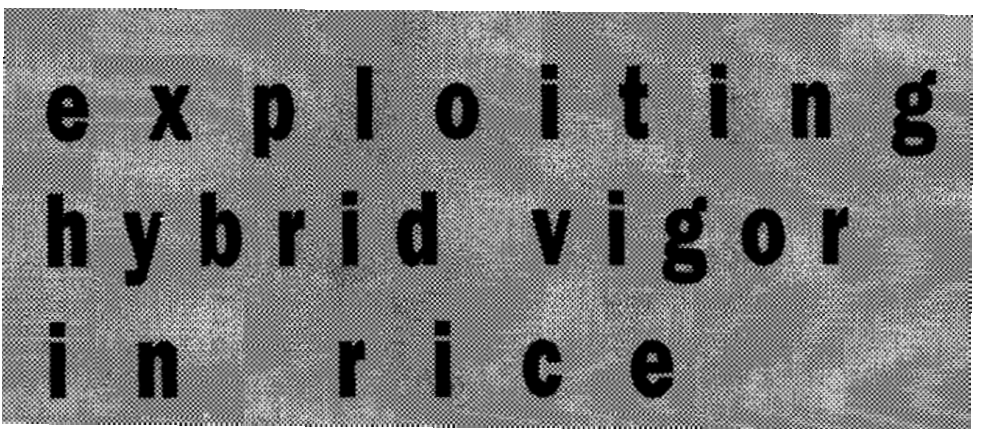
APOMIXIS:

exploiting hybrid vigor in rice

IRRI
INTERNATIONAL RICE RESEARCH INSTITUTE

APOMIXIS:



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Contents

Foreword

By the year 2025 rice farmers must produce 70% more rice. They will have less land. less water and labor will be more expensive. Clearly there is a need to increase the land productivity of rice to meet this need.

Indeed the potential yield of rice in the tropics must increase from the present 10 tons/ha (per crop) to 15 tons/ha. This will translate into increases in yield from 4.9 to 9.3 tons/ha in farmers' fields in the irrigated ecosystems. Similarly, yields in the rainfed areas must increase from less than 2 tons/ha to 4.5 tons/ha to meet the demands of the estimated 4.3 billion rice consumers by the year 2025.

The process of plant breeding takes approximately 10 years from the initial crossing to release of a new cultivar in farmers' fields. Thus, we have three 'generations' to meet these targets.

What are the 'generations' of plant types that need to be developed to meet the I5 tons/ha yield target? The initial increase in yield to around 12.5 tons/ha will use two sources. The first, tropical hybrids based on Indica × Indica crosses. These are already being grown by farmers in India and are being tested in other countries. They follow upon the success of hybrid rice in the temperature regions of China. Not all farmers will benefit from these hybrids, because new seed must be purchased for each sowing. The hybrids will he grown in those areas with a favorable land - labor relationship and where a capacity for hybrid seed production exists.

The second approach is a "New Plant Type" with a yield of 12-13 tons/ha. IRRI scientists started developing the new plant type in 1989. It is based on tropical japonica germplasm.

The third generation of rice cultivars needed to attain the 15 tons/ha yield will be based on hybrids between the tropical indica × japonica. Already such crosses exhibit a 25% heterotic effect due to the diversity of the parental lines.

Thus hybrid rice production is an integrated strategy for ensuring food at reasonable prices for the 4.3 billion rice consumers by the year 2025.

But what of the rice farmer? Will the small-scale rice grower be able to use the hybrid rice technology that currently requires the purchase of new and more expensive seed for each sowing? Can hybrids, which also exhibit superior performance under rainfed conditions, also benefit the resource-poor farmers in these ecosystems?

Exploiting apomixis - asexual seed production that occurs naturally - would be of enormous benefit in helping poor farmers adopt high yielding hybrid rice technology. They could surmount the barrier of hybrid seed costs and reap the benefit of hybrid vigor. Apomictic hybrids breed true. Farmers would be able to use seed from one harvest to plant their next crop, year after year.

Apomixis occurs in more than 300 plant species. Genetic studies of some of those species could show us how to transfer this mechanism to rice. This publication contains information on efforts to transfer the apomixis traits into various crops, and the recommendations it contains will help guide further work in this area, particularly on rice.

K. S. Fischer
Deputy Director General for Research

Apomixis for rice improvement

G.S. Khush, D.S. Brar, J. Bennett, and S.S. Virmani

Major increases in rice production have occurred during the last 25 years due to large-scale adoption of high-yielding, semidwarf varieties and associated improved technology. World rice production doubled in a 25-year period from 257 million tons in 1965 to 518 million tons in 1990. During this period, rice production increased at a slightly higher rate than population. However, the rate of increase of rice production is slowing down and if the trend is not reversed severe food shortages can occur. The present world population of 5.5 billion is likely to reach 6 billion in 2000, 7 billion in 2010 and 8 billion in 2020. The population of rice-consuming countries is increasing faster than that of the rest of the world, and the number of rice eaters will probably double in the next 30 years. Thus, rice production will have to double by 2025 to feed the rice consumers at the present level of consumption. It is estimated that the demand for rice will exceed production by the end of this century (IFPRI 1977).

Major increases in the area planted to rice are unlikely to occur. In fact there has been no increase in the area planted to rice during the last 15 years. Further increases in rice production will have to come from increased yields per unit land area. Therefore, we need rice varieties with higher yield potential and better management practices. In its strategy document, IRRI (1989) accorded the highest priority to increasing yield potential of rice.

Two strategies have been adopted to develop rice germplasm with higher yield potential. One strategy aims at further modifying the existing high-yielding plant type to increase the harvest index to 0.6 instead of 0.5 (Khush 1993). Such plant types are expected to have a yield potential of 12.0-12.5 t/ha under tropical conditions instead of 10 t with the existing high-yielding varieties. The second strategy aims at developing hybrid rices using the new plant types. Such hybrids are expected to have 20-25% heterosis or a yield potential of 15 t/ha (Khush and Aquino 1994).

Hybrid rices have been successfully developed and used in China, with approximately 50% of the rice area now planted to hybrid varieties (Yuan et a1 1994). Hybrid rice research at IRRI was initiated in 1979 (Virmani et al 1981). Several other countries in Asia, such as India, Indonesia, Philippines, and Vietnam, are also working on hybrid rice (IRRI 1994). Some of the IRRI and locally bred hybrids have been released in India, Vietnam and Philippines.

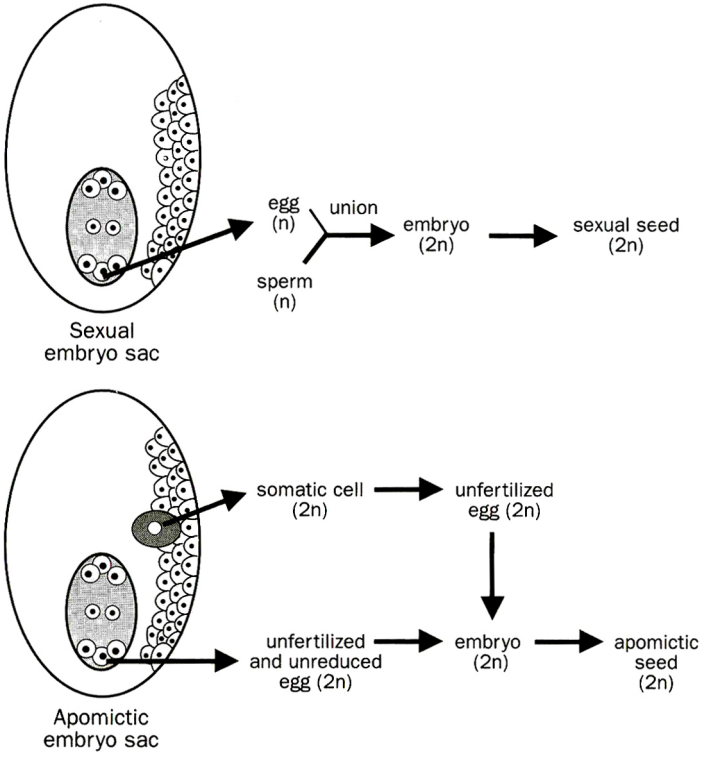
The limiting factor for wide-scale adoption of hybrid rice in the tropics and subtropics is the complexity of seed production based on cytoplasmic male sterility (Lin and Yuan 1980; Yuan and Virmani 1988). A simpler two-line method of hybrid breeding using thermosensitive genic male sterility (TGMS) or photosensitive genic male sterility (PGMS) is also being explored in China (Lu and Wang 1988), Japan (Maruyama et al 1991) and at IRRI (Virmani and Voc 1991). However, rice hybrids produced through cytoplasmic male sterility or genic male sterility systems do not breed true, and lose the yield advantage in subsequent generations. Therefore, farmers have to buy seed for each crop. The cost of hybrid seeds is generally 10-15 times higher than that of ordinary seed, thus lowering the expected profitability of the farmers from the use of hybrids. Moreover, resource-poor farmers are unable to purchase the costly seeds and cannot take advantage of hybrid technology.

The development of apomixis in rice hybrids will enable rice farmers to grow their crops from seeds produced on their commercial hybrid crop year after year, and even resource-poor farmers will be able to benefit from growing hybrid rice. Apomixis will also increase the efficiency of hybrid rice breeders in producing many new true breeding hybrids compared with those produced by using three-line or two-line hybrid breeding systems. The availability of large numbers of hybrids will help increase genetic diversity and reduce genetic vulnerability.

Apomixis is derived from two words: *Apo* and *mixis.* Apo means “away from” and mixis is the “act of mixing or mingling”. It refers to asexual reproduction through seed. It is a method of reproduction in which the embryo (seed) develops without the union of egg and sperm (Fig. 1). It is a reproductive process that bypasses female meiosis and syngamy to produce embryos genetically identical to the maternal parent.

Distribution of apomixis

Apomixis is widely distributed amongst higher plants. More than 300 species belonging to 35 families are apomictic. It is most common in Gramineae, Compositae, Rosaceae and Rutaceae (Bashaw 1980, Hanna and Bashaw 1987). Among the major cereals, maize, wheat and pearl millet have apomictic relatives. As a rule, apomixis is more prevalent in the polyploid species. For example, seven wild species of *Pennisetum* are apomictic and all are polyploid. *Tripsacurn dactyloides,* an apomictic relative of maize, is also polyploid. Apomixis has also been reported in *Elymus rectisetus,* a polyploid relative of wheat. It is also widespread in several forage grasses such as *Cenchrus, Eragrostis, Paspalum, Poa, Bothriochloa, Capillipedium* and *Dichanthium,* and most of them are polyploids.



1. Development of seed through sexual reproduction and through apomixis.

Types of apomixis

A defined sequence of events occurs before seed is formed through sexual reproduction in angiosperms. These sequences comprise differentiation of megaspore mother cell (MMC) from nucellus, megaspore formation through meiosis (megasporogenesis), megaspore selection, embryo sac development by mitotic processes (megagametogenesis), embryo sac maturation which results in a reproductive structure with eight haploid nuclei; an egg, two synergids, two polar nuclei and three antipodals. Double fertilization results in a diploid sexual embryo and a triploid endosperm. In apomictic reproduction, some of these steps are interrupted and still a viable embryo is formed within the confines of the ovule. Generally, apomictic processes deviate from sexual reproduction in more than one respect (Nogler 1984, Asker and Jerling 1992).

Apomixis is broadly classified into two types: (1) gametophytic apomixis and (2) adventitious embryony.

Gametophytic apomixis

In this type of apomixis embryo sacs are produced from unreduced initial cells. The egg cell develops parthenogenetically, giving rise to embryos and plants resembling the

maternal type. Gametophytic apomixis may be obligatory (predominantly apomictic) or to some extent combined with sexuality (facultative). It is strongly connected with polyploidy and is rare in diploids. Gametophytic apomixis is further divided into two categories: diplospory and apospory.

Diplospory. In diplospory unreduced embryo sacs are formed from MMCs by circumvention of meiosis. The embryo develops parthenogenetically from the unreduced egg, and the endosperm develops autonomously (without fertilization) from the unreduced polar nuclei. Thus, pollination in the diplosporous apomicts is not necessary. Koltunow (1993) classified diplospory into two categories: meiotic and mitotic diplospory.

As early as 1898, Juel demonstrated diplospory where MMC does not enter meiosis but proceeds directly to first mitosis and develops into an unreduced embryo sac. Since then diplospory has been reported in several species, including *Eragrostis, Rubus, Tripsacum, Parthenium,* and *Potentilla* (Brown and Emery 1958, Asker and Jerling 1992). Four types of diplosporic embryo sac development have been described (Nogler 1984). These have been named after the genera in which they were first described.

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

(ii) *Ixerix:* The MMC undergoes asyndetic meiotic prophase resulting in restitution nucleus. This is followed by a division similar to the second meiotic division except that it is not associated with cytokinesis. Two mitotic divisions of the unreduced nuclei result in eight-nucleate embryo sac.

(iii) *Antennaria* (Mitotic diplospory): MMC does not go through meiosis and functions as an unreduced megaspore. After a long interphase, it begins to divide mitotically and results in the formation of a typical eight-nucleate embryo sac. This type of diplospory has a wider taxonomic distribution.

(iv) *Allium:* In *Allium* type of diplospory, premeiotic chromosome doubling is the cause of unreduced embryo sac formation (Hakansson and Levan 1957). Chromosome number is doubled by a premeiotic endomitosis and a normal meiosis results in a tetrad of unreduced nuclei. Two subsequent mitoses in the chalazal dyad result in eight-nucleate embryo sac. Among diplosporous species, *Allium nutans* and *A. odorum* have a disporic embryo sac development of *Allium* type. Kojima and Nagato (1992) determined 98% diplospory in *A. tuberosum* based on the percentage of reduplicated embryo sac mother cells.

Apospory. In apospory, unreduced embryo sacs arise from somatic cells in the ovule. The somatic cells of the ovule from which embryo sacs develop belong to the nucellus. Several cells of the nucellus may start aposporous development but usually only one of them gives rise to mature embryo sac. Apospory is initiated after MMC differentiation. The megaspore degenerates and the aposporous embryo sac occupies

the position near the micropylar end of the ovule. The embryo develops parthenogenetically from the unreduced egg, but pollination is required for the development of endosperm. Apospory is common in a large number of apomicts in the grass family *(Pennisetum, Cenchrus, Poa)*. In Poaceae, apospory is the predominant form of apomixis. Apospory is of two types:

(i) *Hieracium:* In this type, eight-nucleate bipolar embryo sac is formed. The unreduced embryo sac has its origin in a somatic cell confined to the center of the nucellus adjoining the chalazal pole of the MMC. Aposporous initials are recognized by the growth and enlargement of the nucleus and nucleolus and by vacuolation causing compression and degeneration of sexual megaspore (Nogler 1984).

(ii) *Panicum:* In this type, four-nucleate monopolar embryo sac is formed. Compared to the *Hieracium* type, the *Panicum* type is characterized by the absence of the initial polarization in the progenitor cell of the embryo sac and by the vacuolation of the chalazal end of this cell. The spindle of the first mitosis division lies crosswise at the micropylar end, and a second mitosis leads to the formation of four free nuclei. Later, these nuclei organize into the female gametophyte consisting of a three-celled egg apparatus and a single polar nucleus; antipodals are absent. Thus the four-nucleate monopolar embryo sacs formed are unreduced. Although the four-nucleate embryo sac is the rule in the *Panicum* type apospory, some exceptions have been reported. In certain cases, unreduced embryo sacs are bipolar and eight-nucleate.

Adventitious embryony

In adventitious embryony, embryos develop from cells in tissues external to a sexual embryo sac. Adventitious embryos arise from individual cells of the two different somatic tissues, nucellus or integument (Lakshmanan and Ambegaokar 1984). Adventitious embryony is purely a sporophytic form of agamospermy. It usually occurs in the presence of normal sexual reproduction and results in polyembryony. It is initiated late in ovule development and usually occurs in mature ovules. Embryos are initiated directly from the individual cells and are not surrounded by megagametophytic structure or embryo sac. This is in contrast to sexual, aposporous and diplosporous reproduction, in which the cell that develops into the embryo is part of a megagametophyte-like structure (Koltunow 1993). Adventitious embryony commonly occurs in diploid species. It is common in citrus and mango.

Inheritance of apomixis

Apomictic species are poor subjects for genetic study. The data from most crosses between apomictic and sexual individuals have not been conclusive. Some of the difficulties are due to the complex polyploid nature of the apomictic species. Powers (1945) investigated inheritance of apomixis in *Parthenium argentatum* and postulated that two genes are responsible for apomictic mode of reproduction. Taliaferro and Bashaw (1966) reported that aposporous apomixis in buffelgrass is controlled by two genes with epistasis. Somewhat similar results were obtained from studies with bahiagrass by Burton and Forbes (1960) who suggested recessive genes for apomixis.

Sax (1959) reported apomixis to be dominant over sexual reproduction in apple. Funk and Han (1967) suggested apomixis may be controlled by two or more dominant genes in *Poa pratensis.* Hanna et a1 (1 973) found sexuality to be dominant in crosses of sexual x apomictic *Panicum maximum.* Sexuality was controlled by at least two loci and conditioned by a dosage effect of two or more dominant alleles. Savidan (1980, 1981) reported that apospory in guinea grass *(Panicum maximum)* is controlled by adominant gene. Dujardin and Hanna (1983) suggested apospory in *Pennisetum squamulatum* is under dominant gene control. Asker (1970) reported apospory in *Potentilla* to be recessive. Nogler (1975) found apospory in *Ranunculus* to be controlled by a single dominant gene. Polyembryony in citrus was reported to be under single dominant gene control (Parlevliet and Cameron 1959). Similarly, genetic control of female meiosis in diplosporous *Taraxacum* appears to be monogenic (Mogie 1988). Thus, the available data suggest that only a few genes control apomixis with profound effects on the mechanism of sexual reproduction.

Screening techniques

Various techniques involving cytological, genetic and histological examinations are used to screen for apomixis and to identify the apomictic mechanisms. Some of the commonly used techniques are described below.

Cytological techniques

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

The pistil-clearing technique (Young et a1 1979, Crane and Carman 1987) is widely used for examination of the embryo sacs. Pistil clearing methods using aromatic esters greatly reduce the time needed to prepare the samples for examination. The method consists of fixing pistils at the time of anthesis in formalin-acetic acid-alcohol (FAA) consisting of 70% ethanol, glacial acetic acid, 37% formaldehyde (18:1:1), and passing pistil through alcohol series and clearing with methyl salicylate and examining under phase contrast microscopy. The contents of the entire ovule are examined by changing the focal level.

Callose fluorescence is used incombination with pistil clearing to detect diplosporous embryo sac development. Callose is deposited in cell walls of MMC during megasporogenesis in sexual species, but such deposition is nearly absent in the cell walls of apomictic embryo sacs. Carman et a1 (1991) compared the embryo sacs of sexual *Elymus scabrus* and diplosporous *E. rectisetus.* Callose accumulated in and around the cell walls of embryo sacs of *E. scabrus* but was absent in those of *E. rectisetus.* Peel and Carman (1 992) combined the pistil clearing technique with callose fluores-

cence to screen rapidly for apomixis. A sucrose clearing solution (2.46M sucrose, 1.36 mM aniline blue, 50 mM K_2HPO_4, pH9.5) induced excellent callose fluorescence of embryo sac walls. This technique is being used at the International Maize and Wheat Improvement Center (CIMMYT) to screen *Tripsacum* germplasm and the breeding materials from maize-*Tripsacum* crosses.

Histological technique

This technique is employed to study the embryo sac development by examining histological serial sections of ovules. Female florets at different stages of maturity are collected and fixed in FAA for 24 hours and are then transferred to 70% ethanol. Pistils are dissected and dehydrated using the method of Young et al (1979). The pistils are then embedded in paraffin, sectioned at 10 µm and stained with safranin O-fast green. Sherman et al (1991) analyzed embryo sac development by examining histological sections of ovules of *Tripsacum* dactyloides to detect failure of meiosis. In buffelgrass, Bashaw and Hignight (1990) found proembryos in 15% of the ovules collected before pollination, but none of them contained endosperm. Apparently pollination is necessary for endosperm development, and probably also precedes the initiation of embryo development in most ovules. Adventitious embryony can be identified by the presence of proembryos that develop directly into embryos from somatic cells in the ovule. Since apomictic embryo sacs are not formed, there are no polar nuclei or endosperm associated with these embryos.

Biochemical techniques

Isozyme markers can be used to study genetic variation and to detect the presence of apomixis. Roy and Rieseberg (1989) detected the apomictic breeding behavior of *Arabis holboellii* through enzyme electrophoresis. Progeny tests revealed no genetic variation within families, yet interfamily diversity was quite high. Kojima et al (1991) estimated the degree of apomixis by electrophoretic analysis of leaf esterase in progenies obtained from crosses of six cultivars of *Allium tuberosum.* More than 90% of seedlings showed the same zymogram as their pistillate parent, the remaining seedlings showing hybrid zymograms indicating 90% apomixis in each of the cultivars.

Molecular techniques

If the genes for apomixis can be tagged with molecular markers such as RFLPs, breeding materials can be screened for apomixis through linked molecular markers. Ozias-Atkins et al (1993) found two molecular markers (UGT 197 and OPC-04) that cosegregate with the apomictic mode of reproduction, in the cross between apomictic aneuploid plant derived from a trihybrid of two wild species of *Pennisetum* and sexual pearl millet. Hanna et al (1993) used these linked molecular markers to screen BC_4 progenies derived from the cross of apomictic BC_3 plants and sexual pearl millet. In a random sample of eight BC_4 progenies examined through molecular markers, five were apomictic and three were sexual. Embryo sac analysis of the eight plants confirmed the results of molecular analysis. The value of molecular markers, tightly linked to the apomictic genes, in a breeding program is thus obvious.

Progeny tests

Uniformity of progenies from heterozygous or cross-pollinated parents is the best indication of apomixis. Occurrence of maternal phenotypes in crosses is another indication of apomixis. Screening for apomixis can be expedited if pollen from a dominant marker stock is used. Production of maternal progeny with recessive phenotype would indicate an apomictic mode of reproduction. The technique would be more efficient if the dominant marker is identifiable at seedling stage. High seed set in the progenies of aneuploid plants or wide-cross derivatives is another indication of apomixis.

Occurrence of multiple stigmas and multiple ovules per floret may be due to apomictic mode of reproduction (Hanna et a1 1970, Hanna 1991). Consistently high frequency of twin seedlings in the progenies is another indication of apomixis. Multiple seedlings per seed can be due to: (1) development of multiple aposporous embryos in an ovule, (2) facultative apomixis where embryos develop in both sexual and apomictic embryo sacs, and (3) development of adventitious embryos in addition to the sexual embryo.

Apomixis in varietal improvement

The use of apomixis in plant breeding was articulated by Hanna and Bashaw (1987). Apomixis increases the opportunity for developing superior gene combinations and facilitates the rapid incorporation of desirable traits. Progeny testing for genetic stability is not needed for obligate apomixis, since any superior apomictic plant is ready for immediate performance evaluation. The greatest application of apomixis, however, is in hybrid development for commercial production. It simplifies seed production because isolation is not necessary and there is no need to maintain and increase parental lines. Chances of contamination by outcrossing are eliminated in obligate apomicts. Commercial F_1 hybrids can be produced through apomixis even if the sources of cytoplasmic male sterility and or restorer systems are not available. It allows the breeders to produce many new true breeding hybrids in comparison to those produced by using three-line or two-line hybrid breeding systems. Most importantly farmers can grow their crops from seeds produced on their commerical hybrid crop year after year, thus increasing the profitability of crop production through hybrids.

To date only an apomictic for age grass species has been improved through breeding. Bashaw (1980) developed three improved cultivars of apomictic buffelgrass through hybridization between sexual and apomictic clones. Efforts are under way to transfer genes for apomixis from wildrelatives to cultivated species with the ultimate objective of developing apomictic hybrids. However, the progress has been hindered by the higher ploidy levels of the apomictic species, and restricted recombination between the chromosomes of wild and cultivated species. Most advanced programs are with pearl millet at the University of Georgia and USDA-ARS, Tifton, Georgia, and with maize at CIMMYT in Mexico.

The genus *Pennisetum,* in which the major cultivated species is the sexual pearl millet *P. glaucum,* contains several wild apomictic species (Hanna 1979, Dujardin and

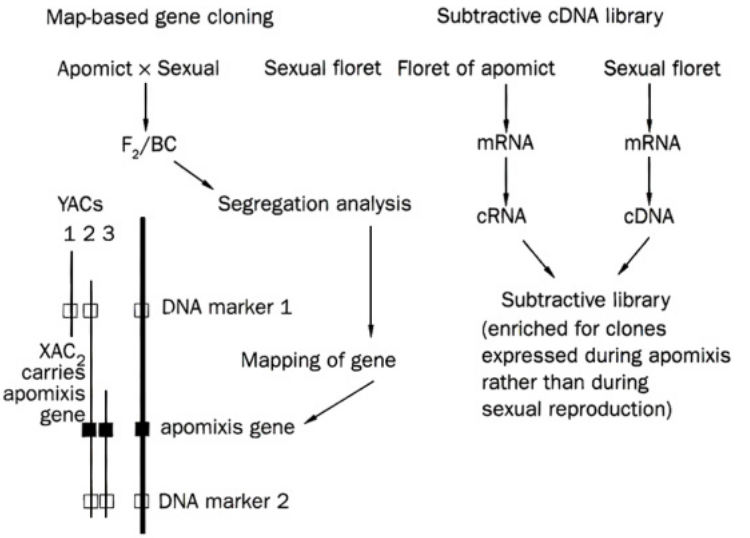
Hanna, This Volume). The interspecific hybrids with pearl millet are highly sterile. In order to introgress the apomictic trait from the wild species, Dujardin and Hanna (1984) produced a complex hybrid between induced tetraploid pearl millet (2n=4X=28), wild apomictic species, *P. squamulatum* (2n=6X=54) and abridging species, *P. purpureum* (2n=4X=28). This trispecific hybrid was backcrossed to tetraploid pearl millet three times, and backcross progenies were selected for apomictic mode of reproduction at each backcross generation. The chromosomes of the wild species were progressively eliminated and at BC_3 generation an obligate apomictic individual with 29 chromosomes was selected (Dujardin and Hanna 1989). The alien chromosome from *P. squamulatum* with gene(s) for apomixis was thus identified. The apomictic trait has been tagged with closely linked molecular markers (Ozias- Akins et al 1993) and efforts are under way to transfer the gene(s) for apomixis from the alien chromosome to the pearl millet genome.

The ORSTOM-CIMMYT project isattempting to transfer apomixis from *Tripsacum dactyloides* to maize. F_1 progenies from the wide crosses of these two species have been backcrossed twice to maize, and apomictic backcross progenies with 20 chromosomes of maize and 2-6 chromosomes of *Tripsacum* have been selected. Further backcrosses are under way (Savidan et al, This Volume). A similar program is under way at USDA-ARS, Woodward, Oklahoma (Dewald et al 1992).

Techniques for cloning genes for apomixis

Cloning of genes for apomixis from apomictic species and their introduction into sexual species through transformation is an exciting possibility. It is possible that genes controlling apomixis will be isolated within the next decade from several apomictic grass species. Two methods have been proposed for the isolation of such genes: map-based gene cloning and screening of subtractive cDNA libraries (Fig. 2).

Map-based gene cloning involves mapping the apomixis gene relative to DNA markers and then walking along the chromosome from the markers to the gene. Mapping of apomixis genes has been initiated from several apomictic species such as *Pennisetum* (Ozias-Akins et al 1993), *Brachiaria* (J. Tohme, pers. commun.) and *Hieracium* (A.W. Koltunow, pers. commun.). The method requires that a cross be made between apomictic and sexual species, and that the apomixis character segregate in the F_2 population. A comparison of the segregation behavior of the apomixis character and several hundred mapped DNA markers will lead to the identification of the pair of DNA markers most closely flanking the apomixis gene (see Fig. 2). For chromosome walking, libraries of large-fragment clones (such as yeast artificial chromosomes or YACs) are prepared from the genome of the apomict and then screened using the flanking markers as probes. Once a YAC carrying both flanking markers has been identified (Fig. 2), it can be assumed that the apomixis gene lies between them on the same YAC. However, because the distance between the markers may be large enough to span dozens or hundreds of genes, further criteria will probably be necessary to identify the gene specifying apomixis. The fact that the gene for



2. Methods for isolating apomixis genes.

apomixis is likely to be expressed (perhaps exclusively) in the nucellus/embryo sac should help in its identification at this point.

An allied approach (Fig. 2) involves the preparation of subtractive cDNA libraries using messenger RNA extracted from florets of apomictic and sexual species harvested around the time of meiosis (Gustine and Sherwood 1992). In principle, this approach will permit the isolation of cDNA clones for genes expressed as mRNA only in the apomictic florets at the time of the molecular switch. In practice, it may be difficult to identify which clone in such a library represents the gene responsible for the switch. However, if the two approaches depicted in Fig. 2 were combined, the likelihood of isolating and identifying the apomixis gene would be increased. The clones of the subtractive library could be tested for their ability to hybridize to the YAC in question; any clone that hybridized would be highly likely to be the desired gene. Similarly, any clone in the library that mapped near the apomixis character would also be likely to be the desired gene. Ultimately, however, any candidate gene could only be proven to be the apomixis gene by its ability to convert the apomixis character on the sexual species into the apomict in a transformation experiment.

Strategies for developing apomictic rice

Apomixis has not been reported in cultivated rice and chances of finding apomictic rice are remote. To produce apomictic rice, three approaches have been proposed:

1) Search for apomixis in the wild germplasm of *Oryza.* If successful, transfer the apomixis character to cultivated rice through wide hybridization.
2) Use mutagenesis to induce apomixis in rice.
3) Use molecular approaches to engineer apomixis in rice.

Table 1. Chromosome number and genomic constitution of Cultivated and wild species of *Oryza*.

Species	2n	Genome	Distribution
O. sativa L. (cultivated)	24	AA	Worldwide
O. nivara Sharma et Shastry	24	AA	India
O. perennis Moench			
Asiatic (subsp. *rufipogon)*	24	AA	Asia
American (subsp. *cubensis* Ekman)	24	$A^{cu}A^{cu}$	West Indies
African (subsp. *barthii* A. Chev.)	24	A^{b} A^{b}	Africa
O. glaberrima Steud. (cultivated)	24	A^{g} A^{g}	Tropical West Africa
O. breviligulata A. Chev. et Roehr.	24	A^{g} A^{g}	Tropical West Africa to Sudan
O. longistaminata A. Chev. et Roehr.	24	A^{g} A^{g}	Tropical West Africa to Madagascar
O. punctata Kotschy ex Steud.	24, 48	BB,BBCC	Tropical Northeast Africa, Madagascar
O. minuta J. S. Presl. ex C. E. Presl.	48	BBCC	Malaysia, Philippines, Indonesia
O. officinalis Well ex Watt	24	CC	India, Bangladesh, Burma, Thailand
O. eichengeri A. Peter	24	CC	Tanzania, Uganda
O. latifolia Desv.	48	CCDD	Central and South America, West Indies
O. alta Swallen	48	CCDD	Honduras, Brazil, Paraguay, South America
O. grandiglumis (Doell) Prod.	48	CCDD	Brazil, South America
O. australiensis Domin.	24	EE	Western and Northern Australia
O. brachyantha A. Chev. et Roehr.	24	FF	West Tropical and Central Africa
O. schlechteri Pilger		unknown	Papua New Guinea
O. meyeriana (Zoll. et Mor. ex Steud.) Baill.	24	unknown	Indonesia, Philippines, Thailand
O. granulata Nees et Arn. ex Watt	24	unknown	India, Bangladesh. Ceylon, Burma, Thailand, Indonesia
O. ridleyi Hook. f.	48	unknown	Malaysia, Thailand, Papua New Guinea
O. longiglumis Jansen	48	unknown	Papua New Guinea

Search for apomixis in wild *Oryza* species

As discussed earlier, apomixis has been reported in the wild relatives of several crop species. Besides the two cultivated species, there are 18 wild species of the genus *Oryza* (Table 1). Rutger (1992) screened 547 accessions of closely related wild species of rice with AA genomes through the pistil clearing technique. Results were negative. Apomixis is rare in diploid species but commonly occurs in polyploid relatives of crop plants. Therefore, we have started to screen germplasm of tetraploid wild species of *Oryza* using three techniques: (i) pistil clearing, (ii) callose detection using fluorescence microscopy, and (iii) histological sections.

As shown in Table 1, seven species of *Oryza* are polyploid. Over 180 accessions of these species are available in the IRRI germplasm center. We have screened 95 of them

for apospory through pistil clearing technique which is simple and fast (Young et al 1979). None of them showed any evidence of apospory.

We have now started to screen polyploid *Oryza* germplasm for diplospory through callose detection using fluorescence microscopy. The technique is used at CIMMYT to screen diplosporous embryo sac development in backcross progenies of maize x *Tripsacum* (Y. Savidan, pers. commun.). We are also examining the histological sections of ovules of tetraploid species through the standard paraffin method to detect apomixis.

Once apomictic germplasm is identified, gene(s) for apomixis will be transferred to cultivated rice through the standard backcrossing procedures outlined by Savidan et al (This Volume) for the maize-*Tripsacum* program.

The feasibility of producing wide cross hybrids of rice through embryo rescue (Jena and Khush 1984) and monosomic alien addition lines of rice (Jena and Khush 1989, Multani et al 1994) has already been demonstrated. Moreover, gene transfers from wild species into cultivated rice have been obtained in the wide cross progenies (Jena and Khush 1990, Ishii et al 1994).

Mutagenesis to induce apomixis

A defined sequence of events in the ovules leads to the development of haploid eggs which when fertilized by male gametes result in sexual embryos. These steps involve: (1) differentiation of a megaspore mother cell, (2) megasporogenesis through meiosis leading to development of a haploid megaspore, and (3) gametogenesis resulting in embryo sac with egg apparatus, polar nuclei and antipodal cells. In meiotic diplospory, the meiosis fails resulting in unreduced megaspore. In mitotic diplospory the megaspore mother cell does not enter meiosis and directly undergoes gametogenesis. The end result in both cases is the production of embryo sacs with 2n eggs. Further these eggs are prevented from fertilization and develop into embryos with maternal chromosome number.

In apospory, nucellar cells adjacent to the megaspore mother cell differentiate and give rise to unreduced embryo sac by mitotic process. The aposporous embryo sacs develop faster than the sexual embryo sacs because they do not have to go through meiosis. The development of the sexual embryo sac is terminated and the aposporous embryo sacs take their place at the chalazal end. Like the diplosporous embryo sacs, the unreduced egg starts to develop into embryo without fertilization.

Thus it appears that switchover from sexual to apomictic reproduction involves simple steps. Rather simple inheritance of apomixis as discussed earlier supports these conclusions. Occurrence of apomixis in more than 300 species belonging to 35 families of higher plants suggests that such mutations must have occurred frequently in nature. Appearance of sexual plants in species that are predominantly obligate apomicts suggests that even the reverse mutation at the apomixis locus can occur. For example, a seed grower in Texas spotted a rare plant in the field of obligate apomictic buffelgrass that was different and was found to be sexual (Bashaw 1962).

If mutations for apomictic mode of reproduction can occur in nature, it should also be possible to induce such mutations through mutagenesis. Many physical and

chemical mutagens are known to induce mutations for various plant characteristics and processes. Attempts to induce mutations for apomixis in higher plants have been only halfhearted. One of the first apomictic strains of grain sorghum was selected from irradiated progenies (Hanna et al 1970). Hanna and Powell (19 73) obtained facultative apomicts of pearl millet following treatment of seed with thermal neutrons and diethyl sulfate.

Treatment of fertilized egg cells with N-methyl-N-nitrosourea (MNU) has been found to be effective for inducing mutations in rice (Satoh and Omura 1986, Kitano et al 1993). We have started a program of mutagenesis with MNU to explore the possibility of inducing mutations for apomixis in rice. Identification of mutants with asexual mode of reproduction in a large population of mutagenized individuals is not easy. We are employing a genetic male sterile line (msms) of rice variety IR36 for induction of mutations. Male sterile plants are pollinated with pollen from fertile (MsMs) plants. Fertilized egg cells (Msms), 16-18 hours after pollination, are treated with 1.5 mM of MNU for 1 hour. We are in the process of screening mutagenized progenies. M_1 progenies (Msms) breeding true for fertility in the M, would be further examined as a possible source of dominant mutation for apomixis. Recessive mutations can be identified in the M_3 generation.

We have also selected a dominant purple leaf mutant of rice for identifying the apomictic mutants following mutagenesis. Emasculated panicles of IR36 will be pollinated with the purple leaf mutant and the fertilized eggs will be mutagenized with MNU treatment. M_2 progenies breeding true for purple leaf would be further examined for dominant mutation for apomixis.

Molecular approaches to apomixis

As noted above, apomixis and sexual reproduction follow the same fundamental pathway from floral induction to seed maturation. They differ principally in the route by which a single nucellar cell in the ovule gives rise to an embryo (Asker and Jerling 1992). Like the mutational approach, the genetic engineering approach is inspired by the notion that only a small number of genes (and possibly only a single gene) may determine which route is taken (Gustine and Sherwood 1992; Ozias-Akins et al 1993). Although the mutational approach relies on chance disruptions of the rice genome to throw the switch from the sexual pathway to apomixis, the genetic engineering approach starts from the supposition that the key genes of this switch can be isolated, identified, and inserted into rice by transformation in a modified state such that reproduction will be switched permanently, or in a regulatable manner, in the direction of apomixis.

Agenda for molecular research on apomixis in rice. Once available, apomixis genes from apomictic grasses will be engineered into rice and the behavior of the ovule prior to pollination will be monitored for signs of apomixis. The gene will also be used as a probe to determine whether rice contains homologous genes and, if so, why the rice genes are apparently inactive in switching the pathway of reproduction from sexuality to apomixis.

In the interim, molecular biologists can contribute to a program on apomixis in rice in two ways:

(1) by improving the transformation procedures for rice, and

(2) by enhancing our understanding of gene expression in the developing ovary and embryo.

Transformation of rice. Foreign genes were first introduced into rice by protoplast-based methods (Ou-Lee et al 1986, Uchimiya et al 1986). The same methods were used to produce the first fertile transformed japonica rice (Shimamoto et al 1989) and indica rice (Datta et al 1990, 1992, Peng et al 1992). Later the biolistic method was used to produce japonica and indica transformants (Christou et al 1991, Cao et al 1992, Li et al 1993). In spite of the success of protoplast-based methods, the biolistic method is of interest because it involves less tissue culture, and may for that reason yield transformants of greater fertility and uniformity from a greater range of varieties, IRRI has an active program dedicated to applying and improving protocols for rice transformation, IRRI began its rice transformation program in 1991 with the protoplast technique, and added the biolistic technique in 1993. Fertile transgenic plants have been produced by both methods. Protoplasts were transformed by the polyethylene glycol (PEG)-mediated method, and the helium-driven microprojectile delivery system was used for biolistic transformation. Two or more genes were transferred simultaneously in these experiments, including the *gus* reporter gene, which encodes the enzyme b-glucuronidase (GUS), and the *hph* selectable marker gene, which encodes hygromycin B phosphotransferase. The soybean trypsin inhibitor *(SBTI)* gene has also been introduced into rice at IRRI as a potential anti-feedant mechanism against insects such as the yellow stem borer.

A choice of routes to genetically engineered apomixis. The three major types of apomixis (diplospory, apospory and adventitious embryony) alter different stages of sexual embryo sac formation (Nogler 1984, Koltunow 1993). We may therefore choose the type of apomixis that we attempt to introduce into rice through genetic engineering.

If we attempt to introduce diplospory into rice, the megaspore mother cell would still have to form the embryo sac but it would do so without undergoing meiosis. As a result, the egg cell would be diploid and could conceivably be induced to initiate embryogenesis without fertilization.

In engineered apospory, the megaspore mother cell might form but it would have to degenerate. A nucellar cell would replace it as the origin of the diploid embryo sac. We can conceptualize the induction of apospory in terms of triggering nucellar cells to develop into embryo sacs. Two mitotic divisions would have to be induced to give the four-nucleate embryo sac as found in *Panicum,* whereas three mitotic divisions would be needed to give the eight-nucleate embryo sac as found in *Hieracium,* and, again, it would be necessary to trigger embryogenesis in the diploid egg cell of the embryo sac.

In engineered adventitious embryony, nucellar cells adjacent to the normal embryo sac would become embryogenic directly without forming embryo sacs, again much like those cell types in other rice tissues that form somatic embryos in culture.

Thus, the engineering of apomixis requires activation of the switch in the megaspore mother cell or the ground cells of the nucellus. In principle, apomixis genes isolated from apomictic grasses would be expected to contain promoters that are active at the correct time in the correct cell type. However, it is not necessarily true that these promoters would be regulated correctly in an alien background such as rice. Ideally, we should be able to combine an apomixis gene with an appropriate rice promoter to ensure appropriate expression of the gene in rice.

Markers for female gametogenesis. We need to isolate rice genes that can act as markers for the various developmental stages and cell types involved in female organogenesis and embryogenesis. Once the promoters of these genes have been fused with reporter genes such as b-glucuronidase or luciferase, they can be reintroduced into rice and serve as reporters for the molecular switches underlying reproduction.

The basic pattern of floral development in angiosperms is highly conserved and consists of the ordered production of sepals, petals, stamens and carpels. In rice, the glumes correspond to sepals and the palea and lemma are modified petals. The stamens and carpels are the sites of male and female organogenesis, respectively. The isolation of marker genes for female organogenesis in rice will benefit directly from similar studies already underway for other plants such as *Arabidopsis, Antirrhinum,* tomato and maize. The advantage of *Arabidopsis* lies both in its suitability for molecular genetics and the existence of an international network of scientists using *Arabidopsis* as a model plant for molecular analysis. However, two factors render studies on maize particularly interesting. Firstly, the close evolutionary relationship between maize and rice facilitates the search for homologous genes. Secondly, the development of separate male and female flowers in maize permits identification of female-specific gene expression without the need for dissection of stamens and carpels from each spikelet.

We propose to use two methods to isolate rice genes expressed specifically during female organogenesis.

(1) From other laboratories we will acquire genes found to be specific for female organogenesis in plants such as *Arabidopsis,* maize and tomato. We will then use these probes to isolate the corresponding rice genes from an IR36 genomic library.

(2) We will construct a cDNA library from the mRNA extracted from rice spikelets just prior to anthesis. The library will be screened with cDNA reverse-transcribed from mRNA of male and female inflorescences of maize. Female-specific cDNA clones of rice identified by this procedure will then be used to isolate genomic clones from the IR36 library.

The genomic clones identified by these hybridization procedures will then be characterized by DNA sequencing. Putative promoters from the genes will be fused to reporter genes and tested for their capacity to act as stage-specific markers in female organogenesis and possibly as promoters for appropriate expression of apomixis genes in rice.

Several stage-specific genes have already been isolated (Table 2). The tomato cDNA clone pMON9608 is pistil-specific and was localized by in situ hybridization to the integument of the ovule (Gasser et a1 1989). The *Arabidopsis* genes *AGAMOUS*

Table 2. Selected genes or clones of genes expressed during specific stages of plant reproduction.

Plant	Gene/Clone	Expression
Arabidopsis	*AGAMOUS (AG)*	endothelium of ovule
	APETALA 3	Integument of ovule
	AGL 1	ovules
	AGL 2	ovules
Maize	*ZAG 1*	primordia of pistil and stamen
	ZAG 2	pistil primordia
Tomato	pMON9608	Integument of ovule
Carrot	EP2	protodermis of globular stage of embryogenesis

(AG) and *APETALA3* are floral homeotic genes transcribed in the endothelium and the integuments, respectively (Bowman et al 1991, Jack et al 1992). Two AG-like genes (AGL1 and AGL2) are transcribed exclusively in the ovules of *Arabidopsis* (Ma et al 1991). The maize genes *ZAG1* and *ZAG2* are flower-specific (Schmidt et a1 1993); *ZAG1* is related to AG and is expressed in both the pistil primordia and the stamen primordia, whereas *ZAG2* is expressed exclusively in the female flower (pisti1primordia). A lipid transfer protein *EP2* is a marker for protodermal cells in the globular stage of embryogenesis in carrot (Sterk et al 1991). Many interesting female-sterile mutants have recently been isolated from *Arabidopsis* (Gasser and Robinson-Beers 1993), including several tagged with transposons (Reiser and Fischer 1993). When the tagged genes are eventually isolated, they will also be suitable as probes for the isolation of homologous rice genes.

Purely biochemical methods of studying gene expression in the ovule are rendered difficult by the minute quantities of tissue available for analysis. Some progress has been made, however, in tomato and maize, such as the isolation of pistil-specific genes from tomato. A more powerful approach may lie in molecular genetic studies on induced female-sterile mutants, especially in the case of *Arabidopsis,* where the molecular genetics of reproduction is most advanced and the isolation of mutated genes is most likely to be successful. Once ovule-specific genes have been isolated from *Arabidopsis* or other plants, they could be used as probes to isolate the corresponding genes from rice by the screening of a genomic library. This is not to say that female-sterile mutants could not be identified in a population of mutagenized rice plants. Since mutagenesis of rice is under way at IRRI for induction of apomixis, it would be possible to use the same M_2 families to screen for ovule mutants.

The female-sterile mutants detected in *Arabidopsis* include short integument *(sin)* mutants, bell-shaped *(bel)* mutants and ovule mutants *(ovm)* (Robinson-Beers et al 1992, Gasser and Robinson-Beers 1993, Modrusan et al 1993, Reiser and Fischer 1993). The *ovm2* mutant (Reiser and Fischer 1993) is particularly interesting in the present context because it contains an ovule with inner and outer integuments and forms megaspore and binucleate megagametophytes, however the space normally

occupied by the embryo sac appears to be filled with nucellar cells. This mutant may be defective in the conversion of megaspores into embryo sacs, but whether the defect lies in gene expression in the megaspore or in gene expression in ovule tissue interacting with the developing embryo is not clear. Among the interesting reproductive mutants of maize are the meiosis (*mei*), mutants that affect meiosis in male and female sporogenesis, disrupting such processes as initiation of meiosis, pairing of homologous chromosomes, and mitotic divisions within the gametophytes, yet permitting otherwise normal ovule development (Golubov skaya 1989). The isolation of rice genes homologous to the genes mutated in *ovm2* and *mei* would be a big step forward in the study of gene expression in female organogenesis in rice.

We should acknowledge the existence of two problems that must be overcome. Firstly, engineered apomictic mechanism should ideally not have to compete with sexual reproduction. The apomictic rice plants should be obligate apomicts rather than facultative. This problem is likely to be more severe when attempting to mimic apospory or adventitious embryony rather than when attempting to mimic diplospory. In the former cases, nucellar cells have to initiate apomictic development in competition with the normal megaspore mother cell.

The second problem is that it may be difficult totally to dispense with pollination. Sperm nuclei of pollen may be required to initiate endosperm formation in the apomictic seed. In apospory and adventitious embryony the endosperm is derived from fusion of sperm nucleus and an unreduced polar nucleus, whereas in diplospory it develops autonomously. One possibility is to develop in rice a form of pollen-dependent apomixis in which the sperm nucleus of the pollen takes part in endosperm formation.

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Tranferring apomixis to maize

Y. Savidan, O. Leblanc, and J. Berthaud

Three possible pathways have so far been envisioned for the transfer of apomixis to any given crop: 1) the conventional wide-cross transfer from an apomictic relative; 2) induced mutation: and 3) genetic transformation using construct of genes isolated from *Arabidopsis* that are associated with reproduction. A fourth pathway should now be added: gene isolation from an apomictic crop. We anticipate obtaining the first such crop by 1995-96.

In previous discussions of apomixis sources, we generally concentrated on aposporous materials since apospory is widespread among tropical and subtropical grasses, is subject to simple genetic control, and offers the advantage of the four-nucleate embryo sac structure for screening purposes. Besides, apospory and diplospory have long been considered variations of the same phenomenon, not two unrelated events.

Advantage of using diplospory

Recent findings suggest that apospory and diplospory are related to totally different mechanisms. Apospory is "functionally independent of sexuality and superimposed upon the normal process of sexual reproduction" (Harlan et al 1964), whereas diplospory looks like a meiotic mutant. Recent data on callose patterns during meiosis and embryo sac development in a series of diplosporous and aposporous species show that megaspore mother cells and subsequent structures lack callose isolation from the maternal tissue when the plant is diplosporous, whereas aposporous species follow the same pattern as sexual plants. The absence of callose at the beginning of meiosis does not follow a process of early degradation as occurs in male sterile transgenic tobacco (Worral et al 1992). Callose is never observed, even in the very early stages of development, suggesting that diplospory relates to a breakage in callose synthesis. Absence of callose is related to the failure of meiosis, not with apomixis as a whole (as was thought before).

Though we do not have any reliable information on the genetic control ofdiplospory at this time, the breakage of callose synthesis is likely to be a simple event. The only segregation observed so far, in *Eragrostis curvula,* suggested a simple inheritance. Tetraploid sexual *Tripsacum* plants are being regenerated from colchicine-treated calli of diploid embryos. We therefore expect to have new data available in 1995 from crossing 4× sexual × 4× diplosporous *Tripsacum* plants.

Apomixis transfer to maize

Tripsacum vs Pennisetum

If an apomixis gene, or genes, are to be isolated from an apomictic crop, which material would be the most likely donor, and when? Advantage of maize × *Tripsacum* materials over the otherwise most advanced, the *Pennisetum* materials. are presented in Table I.

The most important point seems to be our Knowledge of the transfer pathway in maize, studied by many since Mangelsdorf and Reeves' first report (I931), as opposed to a complete ignorance of how many more backcrosses will be needed in the *Pennisetum* project. Secondly, genomic structures of' the addition form in the case of maize × *Tripsacum* hybrid derivatives can be controlled using in situ hybridization. The apparent addition forms in *Pennisetum* (Hanna et al 1993) appear to be morpho-

Table 7. Comparativeadvantageof the Pennisetumand Tripsacurn hybrid derivativesfor apomixis study, transfer, and gene isolation.

	Pennisetum	*Tripsacum*
Receptor crop	**Pearl millet**	**Maize**
donor species	*P. squamulatum* no sexual form known	11 species all wlth sexual diploid forms
apomixis	apospory: 4-nucleate embryo sacs indirect effect on melosis control?	diplospory: callose deficiency melotic mutant control study in progress
transfer pathway	original 60-year experlence, >7labs how many backcrosses? male fertility	BC is the 20-recovered maize F_1s^5 BC_1 s, BC_2S ms
cytological manipulations	homoeologies?	homoeologies B-A translocations transfers shown through in situ hybridization
molecular tools	molecular markers cosegregating RFLP map of pearl millet	BSA in progress RFLP map of maize RFLP map of *Tripsacum* (partial) in situ hybridization
expression of apomixis in 2x	transfer attempted on 4x pearl millet	transfer on 2x and 4x malze is malze a diploid? 2n=28 haplold is apomictic

logically and functionally more like substitutions, which are less suited for the purpose. Progeny in size is important in backcross transfer attempts. Success is regarded as being a function of our ability to produce and rapidly screen large numbers of progenies in each backcross generation. Recent papers suggest the BC_4 *Pennisetum* hybrid generation first reported in 1988 by Dujardin and Hanna is still the most advanced one under study, which also suggests getting to more advanced generations is neither fast nor easy with those materials (Hanna et al 1993). Other advantages of the maize x *Tripsacum* approach include the great potential for manipulation of recombination between alien and crop genomes, through use of B-A translocation lines (Kindiger and Beckett 1990) and other techniques, and the growing experience in gene tagging and isolation in maize.

The tetraploid-like structure of the maize genome revealed by recent cytology research (Molina and Naranjo 1987, and following papers in Maize Genetics Cooperation Newsletter), and confirmed by RFLP analyses, seems to be more favorable for apomixis gene expression in a 20-chromosome maize plant than in any other "true" diploid crop. Our lack of information about the possible expression of apomixis in a diploid crop (apomixis is only expressed in polyploids in the wild) means that we will make addition lines that will be crossed to both diploid and tetraploid maize in the last step of our transfer attempt.

From a maize x Tripsacum F_1 to BC_3

The transfer pathway is presented in Figure 1. The first three steps have been completed. BC, plants with 2n=38 are being multiplied and backcrossed at the same time. In vitro culture is being used to produce 1,000 BC_2 plants. We have screened BC, plants from over 3,000 BC_1 plants using flow cytometry (Figs. 2-4). The results for the first 3,600 progenies analyzed are given in Table 2. Little variation was observed in the rate of reduction in apomictic hybrid derivatives, i.e., diplospory remains nearly obligate, as in the donor accession, which produces 97% of the unreduced gametes. The first BC_3 plant from an apomictic BC_2 is also reported (Fig. 5). Given that BC_2 plants are male sterile, large-scale production of BC_3 plants will not start until we have established our 1,000-BC_2 plant nursery in the field.

Future research

We can plan the last steps of the apomixis transfer in Figure 1 using our knowledge of the introgression pathway in maize.

- 22-26 addition forms were produced during the first semester of 1994 and backcrossed using them as pollinators. These materials were the first in which partial male fertility was expected (from previous experience). Monosomic addition lines will be isolated from the backcross progenies.
- A bulk segregant analysis was started in late 1993, after a sufficient number of BC_2s had been screened for mode of reproduction. We used both RAPD and RFLP markers, and information from *Tripsacum* and maize maps (for choosing the set of RFLP probes). Results obtained at Tifton and at CIAT with *Brachiaria* (Miles,

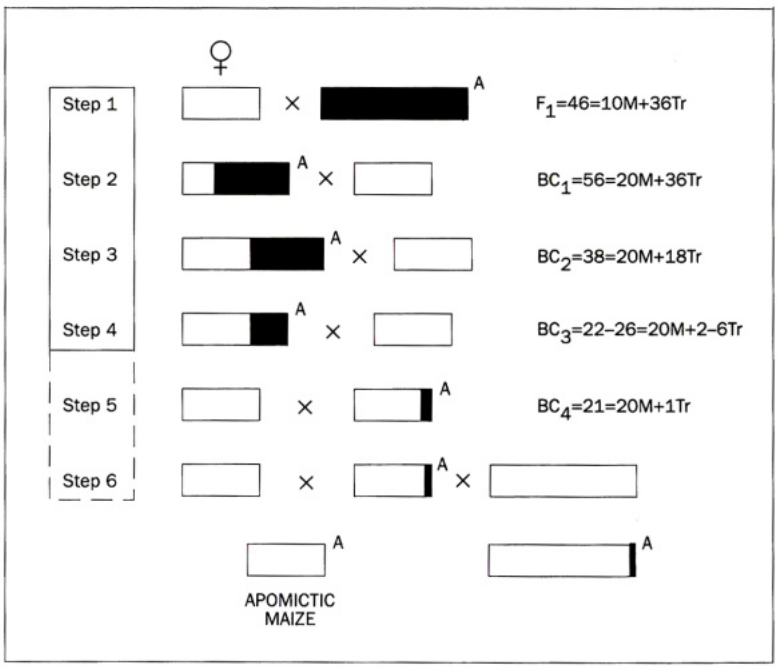
Table 2. Progenies of 2n=56-chromosome BC_1 plants from *T. dactyloides* #651-234. Ploidy levels are estimated through flow cytometry. A The average rate of diplospory (D%) is equivalent to that of the *Tripsacum* progenitor. B Some families, however, show it is possible to slightly increase (a and b) or decrease (c), the rate of diplospory, if necessary.

A

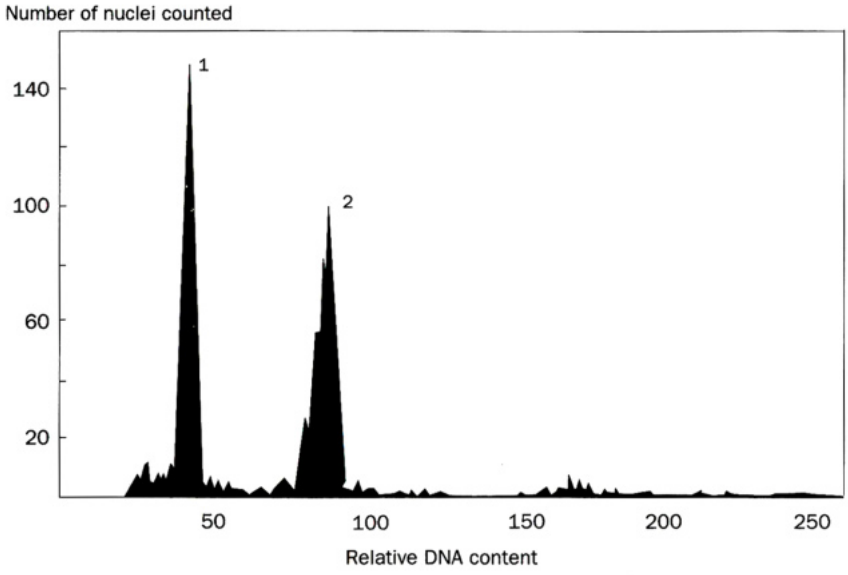
	total progenies	2n+0 maternal	2n+n	n+n	other types	D%
	3600	2874	556	76	94	97.9

B.

	no. progenies	2n+0 maternal	2n+n	n+n	other types	D%
a	55	40	15			100
b	98	73	22	1	2	99.9
c	78	63	6	8	1	89.7

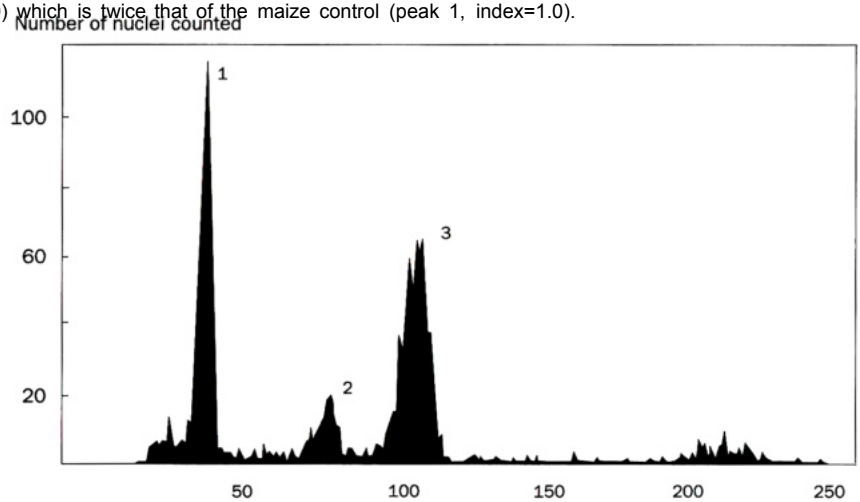


1. Apomixis transfer pathway.



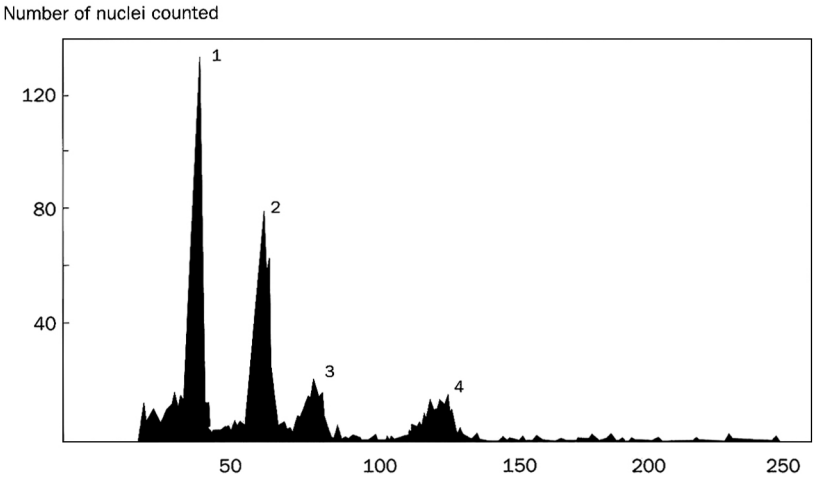
Peak	Index	Mode	Mean	Area #	Area %	CV %
1	1.00	42	42	648	40.30	2.38
2	2.04	86	85	773	48.07	2.94

2. 2n=56 progeny from 2n=56 BC_1. The genome structure is 20M+36Tr, with a DNA content (peak 2, index=2.0) which is twice that of the maize control (peak 1, index=1.0).



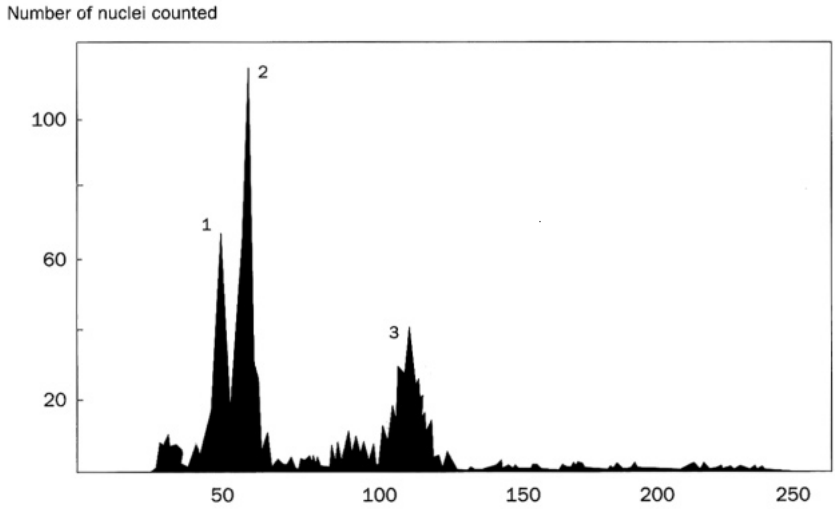
Peak	Index	Mode	Mean	Area #	Area %	CV %
1	0.97	41	41	524	33.38	3.66
2	1.95	82	80	149	9.49	3.75
3	2.59	109	109	649	41.34	3.21

3. 2n=66 progeny from a 2n=56 BC_1. The genome structure is 30 M+36Tr, with a DNA content (peak 3, index=2.5) which is 2.5 times higher than that of the maize control (peak 1, index=1.0). Peak 2 corresponds to maize cells that duplicated their DNA content before dividing.



Peak	Index	Mode	Mean	Area #	Area %	CV %
1	0.97	43	42	641	40.62	2.38
2	1.52	67	66	512	32.45	3.79
3	1.93	85	85	153	9.70	3.53
4	3.00	132	129	164	10.39	3.49

4. 2n=38 BC_2 from a 2n=56 BC_1. The genome structure is 20M+18Tr, with a DNA content (peak 2, index=1.5) which is 1.5 times higher than that of the maize control (peak 1, index=1.0). Peak 2 corresponds to maize cells that duplicated their DNA content before dividing.



Peak	Index	Mode	Mean	Area #	Area %	CV %
1	1.00	48	47	267	17.52	2.13
2	1.16	56	57	660	43.31	2.63
3	2.31	111	112	379	24.87	3.57

5. First BC_3 observed from an apomictic BC_2 with 2n=38. From the index of 1.16, a chromosome number of 2n=26 can be estimated, with a genome structure of 20M+6Tr. Peak 3 corresponds to the BC, plant cells that duplicated their DNA content before dividing.

pers. commun.) leave little doubt about the likelihood of success in searching for markers cosegregating with apomixis.

- Mapping of the *Tripsacum* chromosome(s) involved in apomixis expression started in early 1994. The objective was to get precise homoeology patterns between *Tripsacum* chromosome(s) and the maize map, to direct the choice of B-A translocation lines to be used in the last step of the project.
- The last step, i.e., crossing a monosomic addition line with B-A translocation line(s) would be carried out during 1995.

Conclusions

Advances in the speed of screening backcross generations through cytofluometry and marker-assisted selection of apomictic hybrid derivatives makes it likely that we will obtain the first apomictic maize by 1995 or 1996. Our most recent data have convinced us that efforts to transfer the gene(s) controlling apomixis to a crop have progressed further in the maize × *Tripsacum* materials than in any other genus. An apomictic maize will make apomixis gene(s) readily available for transfer to species such as rice or wheat long before any other pathway, whether mutagenesis or synthesis from isolated genes becomes totally functional.

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Transfer of alien chromosome-carrying gene for apomixis to cultivated *Pennisetum*

M. Dujardin and W.W. Hanna

Apomixis is a genetically controlled reproductive process that bypasses female meiosis and syngamy to produce embryos genetically identical to the maternal parent. This natural method for cloning plants through seed offers a unique system for cultivar development.

A major objective at our laboratories since the early 1970s has been to produce true-breeding hybrids of pearl millet, *Pennisetum glaucum,* an important grain and forage crop. Pearl millet is an annual diploid (2n = 14) species. A promising way to reach this goal is to transfer apomixis from related species.

Searching for apomixis

Apomixis has not been observed, or is not present, in *P. glaucum.* However, the genus *Pennisetum* contains numerous apomictic species. Seven apomictic species have been identified by cytological observation of megasporogenesis and embryo sac development, along with progeny tests (Dujardin and Hanna 1984b). All belong to the tertiary gene pool according to the classification of Harlan and de Wet (1971). These include *P. mezianum* (2n = 32), *P. macrourum* (2n = 36), *P.pedicellatum* (2n = 54), *P.polystachion* (2n = 54), *P. setaceum* (2n = 27), *P. orientale* (2n = 36) and *P. squamulatum* (2n = 54). These species reproduce through obligate nucellar apospory with pseudogamy. *P. setaceum, P. orientale,* and *P. squamulatum* gave rise to interspecific hybrids when used as pollen donors for diploid or tetraploid pearl millet.

Producing apomictic interspecific hybrids

Diploid (2n = 14) pearl millet pollinated with triploid (2n = 27) *P. setaceum* pollen resulted in hybrids with 2n = 25 chromosomes. All hybrids were male sterile and formed only

aposporous embryo sacs in a limited number of ovules (Hanna 1979). No hybrids were obtained from crosses with tetraploid (2n = 28) pearl millet (Dujardin and Hanna 1989a). Complete male sterility and poor female fertility with obligate apomixis did not allow any further progress in transferring apomixis from *P. setaceum* to pearl millet.

Interspecific hybrids between diploid (2n = 14) pearl millet and tetraploid (2n = 36) *P. orientale* were produced. These hybrids had 2n = 25 chromosomes. All were male sterile with high female sterility. Female fertile hybrids reproduced either sexually or by facultative or obligate apomixis (Hanna and Dujardin 1982). They gave rise to 23-, 27- and 32-chromosome backcross (BC) plants when pollinated with diploid pearl millet.

The 23- and 32-chromosome BC derivatives showed low levels of apomictic reproduction, whereas the 27-chromosome plants were obligately apomictic. Only 32-chromosome cytotypes were partially male fertile, but these were of little value for further backcrosses because of the low amount of apomixis.

P. orientale did not appear to be the best species for apomixis transfer. However, we learned from backcross derivatives that polyploidy was not necessary for apomixis expression, since apomixis was observed even when *P. orientale* chromosomes were in simplex condition.

In 1983, we discovered that hexaploid (2n = 54) *P. squamulatum* readily crosses with tetraploid (2n = 28) pearl millet. These interspecific hybrids (2n = 41) were either partially male fertile or completely male sterile (Dujardin and Hanna 1983, 1989a). They segregated for sexual or apomictic reproduction, indicating that *P. squamulatum* was heterozygous for apomictic reproduction. Male fertile apomictic hybrids were used as pollen parents in crosses with tetraploid pearl millet. Numerous sexual and apomictic 35-chromosome progeny were produced (Dujardin and Hanna 1985a), but most were semi-sterile and not useful for further backcross.

Creating a bridging hybrid

Obligate apomixis and male fertility were enhanced by developing complex hybrids (2n = 42) between tetraploid (2n = 28) pearl millet, the wild apomict species *P. squamulutum* (2n = 54), and a bridging species *P. purpureum* (2n = 28) (Dujardin and Hanna 1985b). The latter is an allotetraploid perennial species belonging to the secondary gene pool, and possesses one genome homologous to pearl millet genome. *P. purpureum* reproduces sexually. In 1983 we crossed sexual hexaploid pearl millet x *P. purpureum* hybrids (2n = 42) with male-fertile apomictic pearl millet x *P. squamulatum* hybrids (2n = 42). We obtained over 2,000 double cross hybrids, among which some were obligate apomicts with up to 94% stainable pollen (Dujardin and Hanna 1984a).

Eliminating wild genomes while maintaining apomixis

Tetraploid pearl millet was pollinated with pollen from apomictic double cross hybrids to produce an apomictic BC_1 plant with 45% stainable pollen. Backcrosses of this

double cross hybrid to tetraploid pearl millet have progressed through three generations, during which continual selection for obligately apomictic reproduction and male fertility have been imposed.

We found an obligately apomictic 29-chromosome BC_3 plant with 37% pollen fertility (Dujardin and Hanna 1989b). The number of chromosomes from the wild species in the apomictic BC_3 line is unknown, but the presence of *P. squamulatum* DNA in this line has been detected by molecular markers (Ozias-Akins and Hanna 1991). An additional backcross to pearl millet has produced 27-, 28- and 29-chromosome apomictic BC_4 (unpublished). Plants from advanced backcrosses closely resemble pearl millet morphologically.

Conclusion

The transfer of apomixis from *P. squamulatum* to pearl millet appears to be feasible and has progressed significantly.

The transfer process involves: 1) manipulating ploidy level of *P. glaucum* to successfully produce interspecific hybrids; 2) enhancing male fertility by introducing *P. purpureum* as a bridging species in the backcrosses; 3) eliminating the wild genomes from interspecific derivatives by recurrent backcrosses: 4) selecting hybrid derivatives that are obligately apomictic and male sterile from each generation; and 5) studying large backcross populations because of lower transmission frequency of the extra chromosome(s) with gene(s) controlling apomixis.

Further progress towards developing true-breeding apomictic pearl millet hybrids for grain and forage production will depend on natural recombination between the pearl millet and *P. squamulatum* chromosomes, or chromosome substitution with tetraploid pearl millet.

The identification of molecular markers linked with gene(s) controlling apomixis may help to identify apomictic plants in advanced backcrosses.

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Cytological mechanisms of apospory and diplospory

B.L. Burson

The cytological mechanisms of apomixis refer to the origin of the tissue within an ovule from which the embryo develops. Three different apomictic mechanisms are generally recognized in higher plants: adventitious embryony, apospory, and diplospory. In adventitious embryony the embryo develops directly from cells of the nucellus or integuments without forming an embryo sac. This mechanism is common in citrus but has not been reported as a regular occurrence in members of the Gramineae family. Therefore, this paper addresses only apospory and diplospory. To identify each mechanism or distinguish between them, it is necessary to microscopically observe the development inside the ovules from initiation of the megaspore mother cell to formation of the mature embryo sac.

Before discussing the apomictic mechanisms, a brief review is given of megasporogenesis and the embryo sac development in sexual species. A single cell, known as the megaspore mother cell or archesporial cell, in the hypodermal layer of a young ovule, enlarges and undergoes meiosis to produce a linear tetrad of megaspores. Because these four cells are the products of meiosis, their chromosome number is haploid or half that of the adjacent somatic nucellar cells of the ovule. The three megaspores nearest to the micropyle degenerate while the remaining chalazal megaspore enlarges and undergoes three mitotic divisions to produce an eight-nucleate embryo sac (female gametophyte) of the *Polygonum* type. The mature embryo sac consists of an egg cell, two synergid cells, two polar nuclei, and three antipodal cells. In mature embryo sacs of many grasses the synergids have disintegrated or are not readily visible, and often the antipodals continue to divide to produce a cluster of cells in the chalazal end of the sac.

Apospory

Apospory is the most common apomictic mechanism in grasses. Normally the megaspore mother cell enlarges and undergoes meiosis to produce a linear tetrad of megaspores. At this stage or shortly thereafter, one or more adjacent nucellar cells in the ovule become meristematic, enlarge and their nuclei stain more densely. In obligate apospory the functional megaspore or developing sexual embryo sac aborts and the enlarging nucellar cells occupy the center area of the ovule. These aposporous cells enlarge, become vacuolated and their nuclei divide mitotically to produce sacs usually with one to five nuclei. The number of aposporous sacs in an ovule varies from one to many depending on the species. Mature aposporous embryo sacs with five nuclei usually differentiate as an egg cell, two polar nuclei, and two additional cells which resemble synergids. Those with three nuclei usually differentiate as an egg and two polar nuclei. Some aposporous grasses typically have embryo sacs with four nuclei, which often differentiate as an egg cell, one polar nucleus and two synergids. This arrangement is commonly called the *"Panicum* type" (Warmke 1954); however, it is not typical of all Panicoideae grasses with four nuclei (Bashaw and Holt 1958; Bashaw 1962; Burson et al 1991). However, some aposporous species, such as *Poa pratensis,* have fully differentiated embryo sacs of the *Polygonum* type (Grazi et al 1961). Since most aposporous sacs do not have antipodal cells, the absence of antipodals is a major criterion for distinguishing between mature aposporous sacs and meiotically derived sacs. Facultative apomixis involving simultaneous development of a sexual embryo sac along with one or more aposporous sacs is common in grasses.

Diplospory

In diplospory the megaspore mother cell differentiates similar to that in sexual ovules, but instead of undergoing meiosis and producing a linear tetrad of megaspores, one of two events usually occurs. Either (1) meiosis does not occur or (2) meiosis is initiated in the megaspore mother cell but the meiotic process is not completed and the chromosomes are usually enclosed in a common restitution nucleus with an unreduced chromosome number. The former is known as the *Antennaria* type of diplospory and the latter as the *Taraxacum* type. In the *Antennaria* type the megaspore mother cell does not divide meiotically but it elongates and becomes vacuolated. Eventually, the nucleus divides mitotically, producing a two-nucleate embryo sac. The number of additional mitotic divisions varies for different species and even within species. *Eragrostis curvula, E. lehmanniana* and *Tripsacum dactyloides* are diplosporous apomictics and the number of nuclei in their mature sacs varies. *Eragrostis curvula* produces mature diplosporous sacs with 4, 6, and 8 nuclei (Voigt and Bashaw 1972). However, only four-nucleate sacs were observed in *E. lehmanniana,* and the nuclei remained in the micropylar end of the sac and differentiated as an egg, two synergids and one polar nucleus (Voigt et al 1992). At maturity the synergids disintegrated quickly and two-nucleate sacs were frequently observed. In *T. dactyloides* the diplosporous sacs have eight nuclei (an egg, two synergids, two polars and three

antipodals) and appear similar to the *Polygonum* type (Burson et al 1990). A variation of the *Anntenaria* type occurs in *Elymus rectisetus.* In addition to the typical *Antennaria* development, the megaspore mother cell often divided mitotically to produce a 2n megaspore dyad. The nucleus of the chalazal member of the unreduced dyad underwent three additional mitotic divisions to produce an eight-nucleate sac (Crane and Carman 1987). Reports of the *Taraxacum* type of development are rare in grasses; however, it has been reported in four *Paspalum* species (Chao 1964, 1974, 1980). In each of the apomictic plants the megaspore mother cell initiated meiosis but the chromosomes were asynaptic and present as univalents. An unreduced dyad resulted and the chalazal member divided mitotically to produce an eight-nucleate sac of the *Polygonum* type. To distinguish between eight-nucleate diplosporous sacs and meiotically derived sacs, the early developmental stages in the ovule need to be carefully observed. The best evidence of diplospory is the absence of meiosis, the lack of a linear tetrad of megaspores, or any remains of a linear tetrad of megaspores, or any remains of a linear tetrad. The presence of a single elongated megaspore mother cell extending from near the micropyle to near the center of the ovule indicates possible diplosporous development.

Pseudogamy

Pseudogamy is a term associated with apomixis and refers to the endosperm development in an apomictic sac. It has been demonstrated in some grasses that union of the polar nuclei and a sperm nucleus is necessary for endosperm development. However, in other apomictic grasses it is not known if fertilization is necessary or if only pollination is required for the endosperm to develop and perhaps for the maturation of a precocious proembryo.

Parthenogenesis

Parthenogenesis describes the autonomous development of an embryo without pollination. When mature ovules of apomictic plants are examined prior to anthesis, a young embryo is often observed instead of the expected egg cell indicating that the embryo developed without pollination.

Additional information regarding the different apomictic mechanisms and other aspects of apomixis is presented in the reviews by Gustafson (1947), Nogler (1983) and Asker and Jerling (1992).

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Genetics of apomixis mechanisms

S. Asker

Since the early part of the 20th century, scientists have attempted to analyze the genetic background of apomixis in several plant genera. The usual approach has been crosses between natural apomicts and related sexuals, followed by studying the mode of reproduction of the progeny. Unfortunately, as stated by Savidan (1990, 1992), at least 95% of the data from such crosses were inconclusive or negative, for various reasons. In the early 1970s, the genetic theories of apomixis were still mainly speculation.

However, a monogenic basis for apospory was demonstrated by Nogler (1975) in *Ranunculus,* and by Savidan (1975) in *Panicum.* There is also some evidence of a simple genetic background for the other main types of apomixis, diplospory and adventitious embryony.

Apomixis is well distributed among different angiosperm families, and must have originated independently on several occasions. It is probable that apomixis in related genera within a family has a common origin. However, we cannot exclude the possibility that similar types of apomixis have different genetic regulation in different plant groups, and that cases of more complicated genetic regulation occur.

Of course, a simple mode of inheritance for apomixis would make it easier to use in breeding. But even if there is one apomixis gene of the all-or-nothing type, apomixis in nature is accompanied by secondary changes that concern endosperm formation, timing of embryo sac development, and meiotic changes, and such traits may have separate regulation. Besides, a fine tuning of the mode of reproduction, of the balance between apomixis and sexuality, may be accomplished by a proper collection of "minor genes." These problems should not be underestimated, but important progress in transfer of apomixis to sexual crops has already been made, in spite of our incomplete knowledge about apomixis gene expression and regulation.

The important thing to study is the regulation of "established apomixis," the mechanisms that occur in natural plant populations where apomictic reproduction

prevails or regularly alternates with sexual reproduction. Its antithesis is "occasional apomixis" in plants where sexual reproduction is close to 100%. Occasional apomixis depends on factors such as distant or delayed pollination, and chemical treatments. Many reports of "apomixis" in sexual cultivars deal with haplo-parthenogenesis and other phenomena not related to established apomixis.

The sexual cycle of higher plants includes two essential processes, meiosis and fertilization, separated by the growth and development of the gametophyte. Meiosis and fertilization were thought to be influenced by different genetic and environmental factors. Until recently, it was assumed that formation of unreduced embryo sacs and the capacity of their egg cells for parthenogenetic development had independent regulation.

Under appropriate conditions, unreduced egg cells function in sexual plants, and reduced egg cells may develop parthenogenetically leading to formation of haploids. Sometimes such cases have been spoken of as "elements of apomixis." But it cannot be said too often that occasional function of unreduced embryo sacs and egg cells may not have anything to do with apomixis. The same is true of occasional or in some way induced formation of haploids by parthenogenetic development of reduced egg cells. Mutations leading to increased production of unreduced embryo sacs, or increased frequency of haplo-parthenogenesis, need not be looked upon as "apomixis mutations."

In well-studied genera, such as *Panicum* and *Ranunculus,* the genes controlling unreduced embryo sac formation and parthenogenesis appeared to be closely linked. They were thought to form a "super gene," possibly together with other genes influencing apomictic reproduction. But trials to analyze the background of parthenogenesis have not given any clearcut results. Hence here, and in certain other genera as well, we need not postulate any special genes for parthenogenesis. The egg in an aposporous sac already has the capacity for parthenogenetic development.

The origin of apomixis in populations where unreduced embryo sac formation and parthenogenesis have independent genetic regulation is difficult to understand. Plants with either character alone would have fitness zero. The occurrence of a monogenic common regulation means that the origin of apomixis is reduced to the spreading and assumption of power of one successful allele in a population.

The apomixis gene may be looked upon as a homeotic gene, profoundly influencing differentiation in the same way that genes with mutant alleles cause formation of monstrous animals in *Drosophila.* Heberle-Bors (1992) calls it an ectopic gene—a regulator gene that initiates embryo sac and embryo formation in a tissue where it would not normally occur. Formation of somatic and zygotic embryos is controlled by the same genes. If this is true, synthetic apomicts could be produced by introducing the proper regulator gene to the nucellus of an amphimict.

According to Savidan (1990), "one single dominant trigger induces a cascade of events which result in maternal-type progeny." If apomixis is controlled by one dominant gene, A, then early A expression leads to apospory. and late A expression to diplospory. However, intermediate situations exist, like in *Beta,* where apospory and diplospory are observed together.

Peacock (1992) suggests an *Esi* (embryo sac induction) gene, which is normally switched on in the megaspore after meiosis is completed. In an aposporous plant, the *Esi* gene is switched on early in a group of nucellar cells. before the onset of meiosis. Each of these cells responds to the *Esi* transcription factor to produce an embryo sac.

Environmental factors influence the mode of reproduction, particularly in facultative apomicts. Changes in temperature and light regimes sometimes influence reproductive behavior. The state of the plant also plays a role. Factors such as plant age, nutritional supply, and problems of competition may be important. Certain chemical treatments are known to influence reproduction. Finally, pollination conditions such as choice of pollinator, state of pollen applied, and time of pollination, decide the type of seed formation.

Apomixis is strongly connected to polyploidy and hybridization, although these factors are not looked on as the cause of apomictic reproduction. On the other hand, apomixis sometimes breaks down aftercrosses between related (facultative) apomicts, and the offspring is quite sexual. Similarly, chromosome doubling and other changes in the level of ploidy may lead to an increased level of sexual seed formation, even in obligate apomicts. The causes of such events are largely unknown.

Do true diploid apomicts exist in nature? Among the few cases of diploids with apomixis (gametophytic) reported, some are doubtful, some have chromosome numbers probably derived from tetraploids, and in the well studied *Potentilla argentea* (Asker 1991), results indicate that within pure diploid populations reproduction is largely sexual.

In *Ranunculus auricomus,* the tetraploid apomicts are Aaaa, where A is the apospory factor. Diploid Aa gametes function, whereas haploid gametes do not transfer apospory — the presence of A makes haploid gametes nonfunctional. Thus apomictic diploids can only be produced as dihaploids. In *Panicum maximum,* apomictic diploids do not exist. Dihaploids produced from apomictic tetraploids are sexual or sterile. Similar conditions seem to prevail among other members of the Panicoideae. According to Quarin (1986), genes for apomixis may occur at the diploid level without expression.

Can apomixis be induced at the diploid level? Is it possible to transfer apomixis to diploid crops like rice, maize and sorghum? Or should work be directed at production of apomictic (auto) tetraploids? These are important questions that cannot be ignored. Theoretically, of course, induced apomixis could be different from natural apomixis, but so far we have not seen such a synthetic apomixis function.

More studies of the regulation of apomixis are needed. The existence of monogenic inheritance is hopeful for breeding purposes. Apomixis can be transferred by crosses or by genetic engineering. The main problem is the isolation of the apomixis gene. Strategies for this enterprise have been proposed.

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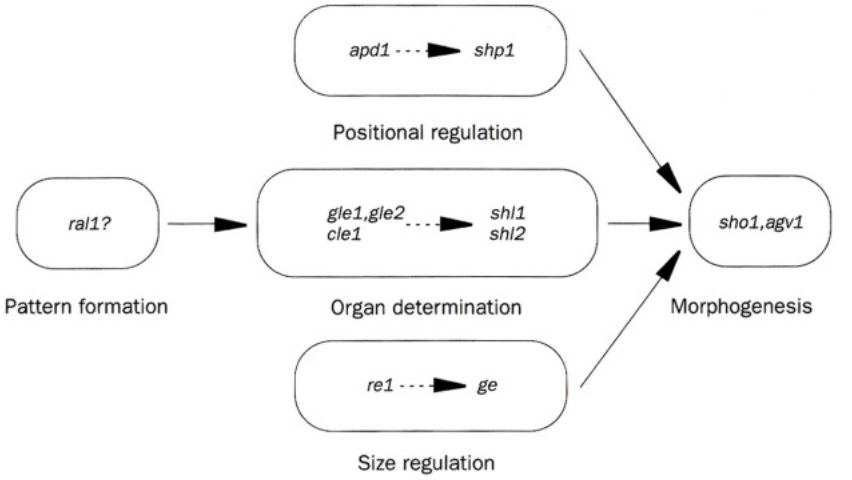
Embryo and flower development in rice and apomixis in Chinese chive (*Allium fuberosum*)

Y.Nagato

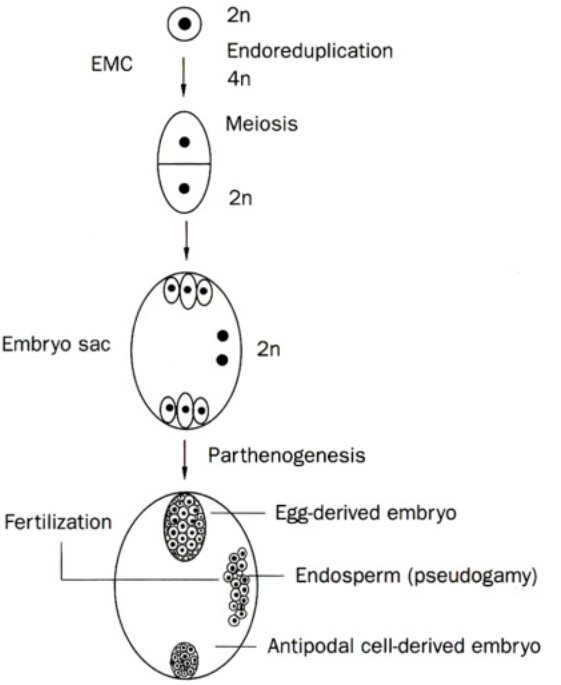
Genetic regulation of embryo and flower development in rice is poorly understood, but may lead to a new approach in rice breeding. We have identified many mutants that may help understand the regulatory mechanisms of embryogenesis and flower development (Nagato et al 1989, Kitano et al 1993).

We have detected 150 single-gene recessive embryo mutants with wide phenotypic diversity, suggesting that complicated regulations are operating in embryogenesis. The phenotypes of the mutants are categorized into eight classes (Table 1). Embryoless mutants are lethal at the very early globular stage so will not be related to development. Organless, shootless, and radicleless mutants are all associated with the determination of embryonic organs. In organless mutants, embryos become large but fail to form any organ, and the wild-type genes are considered to participate in the determination of all embryonic differentiation. Shootless and radicleless mutants are concerned with the determination of only one organ. Similarly, modified organ position and modified embryo size mutants indicate that two types of genes are functioning, one affecting the whole embryo (affecting the positions or sizes of all embryonic organs) and the other affecting only one organ. Many other mutants are associated with morphogenesis of embryonic organs, including those showing fundamentally abnormal shoot organization (Tamura et al 1992) or defective in gravitropism. These embryo mutants suggest that during embryogenesis, several regulatory processes are functioning in which many genes are hierarchically expressed (Fig. 1).

Although we have screened up to 5000 M_2 lines, several interesting mutants are still to be identified, such as one in which the zygote develops into an unorganized cell mass (callus-like) instead of into an embryo. This wild-type gene would function in the switching of developmental pathways. Unfortunately, we could not find any apomictic (polyembryonic) mutant (apospory or adventitious embryony).



1. Schematic representation of regulatory processes operating during embryogenesis in rice.



2. Apomixis in *Allium tuberosum*.

Table 1. Categorization of embryo mutants in rice.

Phenotype	No. of mutants	Gene
Embryoless	4	*eml1*
Organless	21	*gle1, gle2, orl1, cle1*
Shootless	11	*shl1, shl2*
Radicleless	3	*ral1*
Modified organ position	5	*apd1, shp1*
Modified embryo size	12	*re1, ge-2, ge-3*
Abnormal organ morphology	68	*sho1, agr1*
Variable abnormalities	26	*vap1, vap2*

Table 2. Floral mutants in rice.

Process	No. of mutants
Spikelet initiation	2
Flower organization	9
Organ number	4
Organ identity	2
Morphogenesis	22

Extensive studies are being done on flower development in two dicot species, *Arabidopsis thaliana* and *Antirrhinum majus.* However, monocot flowers are poorly understood. We have identified nearly 39 mutants of rice (Table 2). Spikelet initiation mutants include those in which the inflorescence branches reiteratively to several orders but no flower primordia are formed. This wild type gene will be related to the switching of inflorescence meristem to floral meristem. Flower organization mutants are those in which organ number, identity, and morphology are simultaneously affected. Mutants that affect only the number of organs, for instance, more pistils and stamens resulting in double seeds in one spikelet, have also been detected. Homeotic mutants are those in which pistils are converted into stamens, or stamens and lodicules are replaced by pistils and glumes respectively. In addition, many morphological mutants of each organ have also been detected.

An interesting mutant is that where a cell mass derived from the proliferation of integumentary cells forms on the style. This mutant suggests that integument cells retain the ability to divide even after differentiation as integument is completed.

Apomixis in Chinese chive

Chinese chive is a facultative, diplosporous apomict, including two tetraploid morphospecies (2n=4x=32), *Allium tuberosum* (Fig. 2) and *A. ramosum.*

We estimated the degree of displospory (frequency of 4n EMCs), the degree of parthenogenesis (frequency of parthenogenetic egg cells), and the degree of apomixis (frequency of apomixis-derived progenies) for six accessions. Frequency of normal,

2n EMCs was very low, with 32 chromosomes associated in univalents, bivalents, and multivalents. In most EMCs of the six accessions, 2n number of bivalents (32II) were observed. Chromosome separation in 4n EMC is quite regular at both first and second anaphases, so meiosis of 4n EMC resulted in the formation of 2n unreduced megaspores. The degree of diplospory ranged from 76 to 98% (Kojima and Nagato 1992a).

In Chinese chive, parthenogenesis of the egg takes place autonomously, without pollination or fertilization of the polar nuclei. The endosperm develops only after the polar nuclei are fertilized. The parthenogenetic embryo develops autonomously into the globular stage, but further development requires the presence of endosperm. We estimated the degree of parthenogenesis by observing the ovules of unpollinated flowers on the fifth day after anthesis. The degree of parthenogenesis was 62-94% in the six accessions (Kojima and Nagato 1992b). The degree of apomixis, estimated by progeny tests using esterase isozymes as markers, was higher than 90% in all the accessions (Kojima et al 1991). Two out of the six accessions were nearly obligate apomicts, and may be useful as genetic resources for the transfer of apomixis into amphimictic monocot species.

We examined the degree of parthenogenesis in 32 accessions and 16 hybrids of Chinese chive. All the accessions and most hybrids were highly parthenogenetic. Two hybrids, however, showed zero parthenogenesis, but their embryo sacs were highly diplosporous. These two diplosporous, nonparthenogenetic hybrids produced hexaploid (2n=6x=48) progenies after fertilization. This indicates that two essential components of gametophytic apomixis, apomeiosis and parthenogenesis, are genetically and developmentally separable in Chinese chive, suggesting that at least two genes are required for expression of apomixis in this crop.

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Modifying embryo sac development and strategies for cloning the apomixis gene from buffelgrass

R.T. Sherwood and D.L. Gustine

Pennisetum ciliare (Gramineae - Panicoideae) is a drought-tolerant, warm-season, perennial species adapted to southern Africa, India, Australia, and the southwestern USA. It exists mostly as tetraploids (2n = 36), and as pentaploids, hexaploids, and aneuploids. Florets are hermaphroditic, protogynous, and cross- and self-fertile. The species reproduces by pseudogamous apospory. Apomixis is often facultative, but highly sexual plants are rare. Sexual plants form single reduced embryo sacs of the eight-nucleate *Polygonum* type. Apospory is of the monopolar *(Panicum)* type, based on a four-nucleate scheme lacking antipodals. Multiple unreduced aposporous sacs often form in the nucellus. In aposporous lines, the single archespore regularly undergoes meiosis and forms tetrads, but further sexual development is usually prevented. Pistils may form mature aposporous or sexual sacs only, or both types together. Segregations of progeny from selfing and crossing a highly sexual plant lead Taliaferro and Bashaw (1966) to postulate that apomixis is regulated by twoindependent, disomic genes: the dominant allele of gene A is required for apospory, and the dominant allele of gene B is epistatic to gene A and restores sexuality.

Research on regulation of apomixis in buffelgrass at the USDA Laboratory in Pennsylvania utilized materials provided by Dr. E.C. Bashaw, USDA, College Station, Texas. Megasporogenesis, megagametogenesis, and embryo sac morphology are easily viewed in whole pistils cleared in methyl salicylate (Young et al 1979). Observation of large samples revealed facultativeness in lines previously believed to be obligate (Sherwood et al 1980). Frequency of sexual sacs varied seasonally. Environmental conditions responsible for variation in facultative expression were not found in experiments testing daylength (Hussey et al 1991), night temperature, or drought stress (Gustine et al 1989).

To test the possible role of plant growth regulators, DeGroote and Sherwood (1984) developed an in vitro procedure to culture spikelets (including attached involucres)

aseptically on modified Murashige and Skoog medium. Spikelets placed in culture at the archesporial stage completed normal megasporogenesis and megagametogenesis. Myo-inositol seemed necessary for maturation of sexual embryo sacs, but not for aposporous sacs. Effects of abscisic acid (ABA), indole acetic acid (IAA), gibberellic acid (GA), and zeatin were tested in different combinations. No treatment altered the kind of embryo sac formed by highly sexual or apomictic lines. For facultative line 18-35, media that included GA, and omitted IAA, supported increased frequency of sexual sacs. Gounaris and Sherwood (unpubl. data) screened about 50 plant growth regulators (PGR), anti-PGRs, and inhibitors of translation, transcription or metabolism applied to whole plants. No effect on reproductive type was seen.

Salt stress was found to influence embryo sac type and development. Sexual plants bearing premeiotic inflorescences were watered with 1 M $(NH_4)_2SO^4$ solution every second day for 1-2 wk. Among 493 pistils examined, 145 formed normal *Polygonum* type sacs, 190 formed sacs that protruded through the micropyle, 72 were sterile, 27 pistils formed multiple embryo sacs and 59 were unclassifiable (Gounaris et al 1990). Multiple sexual embryo sacs were not formed in controls. We tested 1 M solutions of $CaCl_2$, NaCl, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, and Na_2SO_4 on several sexual and aposporous lines (Gounaris et al 1991a). Sexual lines responded to all salts as described above. In aposporous lines that showed no sexuality in untreated controls, *Polygonum* type sacs formed in 4-13% of the salt-stressed pistils. We postulated that salt stress suppressed the competitive advantage of aposporous embryo sacs over reduced cells in the apomictic lines. To account for multiple sacs in sexual lines, we suggest that salt stress abolished the normal developmental priority of the chalazal megaspore over other megaspores of the tetrad.

A highly sexual plant from Dr. E.C. Bashaw (USDA, College, Station, Texas) was used in an attempt to breed lines that were homozygous recessive or dominant for putative genes A and B or both. Segregation ratios from selfs, crosses, and backcrosses could not be fitted to any one- or two-disomic gene models, but were compatible with a tetrasomic gene model, with the dominant allele conferring apospory. It was necessary to assume random assortment of chromatids, or to assume that the A allele acted as a recessive lethal in the gametophyte as in Nogler's (1984) model for *Ranunculus.* Sexuals were assigned genotype aaaa, and apomicts were Aaaa or AAaa. Except for the molecular markers developed at Tifton (see Ozias-Akins et al, This Volume), no traits are known that are linked with apomixis genes. Our initial attempt to locate the apomixis gene in buffelgrass lines having the A/a system was based on the conjecture that the gene might code for a translational (protein) product. Total proteins from premeiotic and postmeiotic pistils and anthers of several sexual and aposporous lines were resolved by two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis. Spot patterns were highly homogeneous over genotypes, plant parts, and stages. More than 90% of the 300 proteins detected were expressed by all lines. Four proteins appeared exclusively in stamens; two were stage specific (Gounaris et al 1991b). No protein cosegregated with the A gene. Gene products labeled in vivo with ^{35}S-methionine showed no proteins associated with apospory. Isozyme polymorphisms examined for 12 enzymes showed no association with apomixis. We consider it

unlikely that gene A acts as a simple structural gene; it probably has a major regulatory role.

Accordingly. two other molecular approaches were begun: 1) construction of subtracted cDNA libraries, and 2) location of cosegregating sequences using RAPD techniques. Subtractive library strategy was based on the hypothesis that the dominant A allele would be exclusively expressed as unique mRNA in ovules undergoing aposporic sac initiation (Hulce et al 1992, Gustine et al 1993). Poly A + RNA was isolated from florets of apomictic line Higgins (putative AAaa) and sexual line B-11–7. cDNAs of each were cloned into lambda gt23A. cDNA sequences common to both lines were subtracted using Klickstein's (1987) procedure as described below.

Insert cDNA sequences of Higgins and B-11-7 were released with *Not*I and *Sal*I. Single-stranded ends of B-11-7 inserts were removed with S1-nuclease to produce blunt-ended cDNA fragments, and the insert cDNAs were digested with *Alu*I and *Rsa*I. To remove sequences common to both plants, denatured Higgins cDNA was hybridized with a 50- to 100-fold excess of denatured B-11-7 cDNA fragments. Double-stranded, blunt-ended sequences were not clonable. The double-stranded (nonhomologous) sequences with sticky ends were cloned into lambda gt22A to produce a subtracted library. Clones of the subtracted library enriched for Higgins sequences were passed through four rounds of plaque lift screening against biotinylated poly A + RNA from pools of aposporous or sexual lines. At each cycle, plaques were selected that apparently hybridized with the pooled aposporous poly A + RNA probe but not the pooled sexual probe. Several clones selected through four rounds of screening were subcloned into a pBluescript-II phagemid vector system, and they are being screened for tissue specificity. Additional screening of the subtraction libraries is being conducted to seek cDNA sequences that can serve as markers or probes.

RAPD techniques were applied to genomic DNA from four aposporous and six sexual lines. In a survey of 15 oligonucleotide primers, 79 RAPD bands were formed. but no band was uniquely associated with mode of reproduction. Immediate future work will center on finding RAPD or RFLP markers associated with apomictic mode of reproduction. Screening will be conducted among sibling progeny and their backcrosses, and on a population derived from wide crosses. Markers will be used to establish a low-level map of the buffelgrass genome, with emphasis on obtaining markers closely linked to the A gene sequence.

A possible route to cloning the apomixis gene will be identification of tightly linked flanking markers followed by walking to the gene. Buffelgrass is one of very few species that appear to have a naturally occurring, highly expressed A/a gene system within one ploidy level. It may have advantages compared with species that have received the gene by introgressive breeding from distant genomes. The functional gene is already integrated in proper sequence with all flanking sequences, including those supporting expression and those which may be used for chromosome walking. The target for insertion and transformation is genetically compatible. All the supportive genetic elements needed for expression should be in place.

Apomixis is most successful in heterozygous polyploids. It seems to be absent from true euploid diploids. The wild type allele a may play a vital function in cell cycles

(evidence from Nogler 1984 and Mogie 1988 indicated that A seems to act as a recessive lethal in gametophytes). Lessons in molecular transfer of apomixis may best be learned using genetically compatible heterozygous tetraploid targets.

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Molecular markers for apomixis in *Pennisetum*

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Apomixis (agamospermy) is a mode of reproduction expressed in a number of species of *pennisetum* (family Poaceae). *Pennisetum* is a diverse genus containing species with basic chromosome numbers of 5, 7, 8, and 9. Most of the apomictic species have basic chromosome number 9, and are either tetraploid or hexaploid. *Pennisetum glaucum* (pearl millet) is the primary cultivated species (2n=2x=14). Heterosis in pearl millet results in increases in grain yield even among crosses between African landraces (Ouendeba et a1 1993). Fixation of hybrid vigor through apomixis would be particularly beneficial to pearl millet production in developing countries, where hybrid seed production and distribution is too costly. Broader applications involving other grain crops could be envisioned if the gene(s) for apomixis could be cloned and transferred to other plants. We have begun a program at the Coastal Plain Experiment Station that uses molecular techniques to isolate markers linked with gene(s) for apomixis. These markers will be useful for genetic mapping of the trait in *Pennisetum,* marker-assisted selection of apomictic genotypes, and perhaps eventually map-based cloning of the gene(s).

The search for molecular markers was begun with an obligately apomictic BC_3 individual derived from a complex crossing and backcrossing strategy (Dujardin and Hanna 1989). Genotypes that contributed to the pedigree of BC_3 included two pearl millet inbreds, Tift 23 and Tift 239. one *P. purpureum* accession. and one apomictic *P. squamulatum* accession (PS26). DNA was isolated from each of these genotypes, digested with restriction enzymes (*Eco* RI, *Eco* RV. *Hin* dIII. *Dra* I). subjected to electrophoresis and blotting, and probed with clones randomly selected from a *Pst* I genomic library of BC_3. Approximately 90% of the *Pst* I clones were single to low copy. A restriction fragment that was shared by both BC, and the apomictic parent, PS26, but not present in any of the sexual parents, was considered informative. Using these criteria, approximately 13% of the clones have been informative. In addition to

restriction fragment length polymorphisms (RFLPs), RAPDs (random amplified polymorphic DNA) have also been useful for identifying markers in BC_3 inherited from PS26, although the frequency for obtaining a useful RAPD band has been considerably less than for RFLPs.

Some of the informative RFLP clones have been sequenced and converted to sequence-tagged sites (STSs) to develop a polymerase chain reaction (PCR)-based technique for marker-assisted selection at the seedling stage. A BC_4 population known to segregate for mode of reproduction has been analyzed with four RAPD and three STS markers. We observed that two groups of markers assorted independently. Two markers, the RAPD OPC-04 600 bp and the STS UGT197, cosegregated with each other and with apomixis (Ozias-Akins et al. 1993). The linkage group bearing these markers and the gene(s) for apomixis was transmitted at a low frequency (2-5%) during backcrossing. There was no evidence that recombination between this chromosome and pearl millet chromosomes occurred during backcrossing. With low or no recombination of the desirable linkage group during backcrossing, genetic mapping using this population will be impossible. Other strategies for estimating the degree of linkage between molecular markers and apomixis have been devised.

The presence of UGT197 (RFLP and STS) has been surveyed in a selection of apomictic and sexual *Pennisetum* species. None of the sexual species *(P. glaucum, P. purpureum, P. ramosum, P. alopecuroides, P. basedowii, P. hohenackeri, P. nervosum, P. schweinfurthii),* nor the apomicts in the section *Brevivalvula (P. subangustum, P. polystachyon, P. pedicellatum)* hybridized with UGTl97, nor was the STS amplified. All of the remaining apomicts *(P. squamulatum, P. setaceum,, P. villosum, P. orientale, P. flaccidum, P. massaicum, P. macrourum, P. ciliare)* did hybridize with UGT197 and the STS was amplified in the PCR. The only obvious RFLP with respect to UGT197 among these species was in *P. ciliare* (syn. *Cenchrus ci1iaris*). Fragments homologous with the RAPD marker, OPC-04 600 bp, were amplified only in a subset of apomictic species, *P. squamulatum, P. massaicum,* and *P. ciliare.* It was concluded that the RFLP/STS UGT 197 should be linked more closely to apomixis than OPC-04 600 bp, because it was conserved more during evolution among apomictic species.

Our immediate objective is to produce a genetic map of the chromosome in *P. squamulatum* on which the gene(s) for apomixis reside. *Pennisetum squamulatum* is heterozygous for mode of reproduction. Interspecific hybrids between *P. squamulatum* and tetraploid pearl millet segregate for apomixis and sexuality (Dujardin and Hanna 1983). *Pennisetum squamulatum* is also heterozygous for at least one of the apomixis-associated markers, UGTl97 STS. This marker is present in one apomictic individual maintained from the initial interspecific hybrid population, but is absent from the sexual individual that has been maintained. We are currently producing another interspecific hybrid population that will be screened for mode of reproduction, while simultaneously isolating additional markers that cosegregate with apomixis. Recombination between these markers and apomixis during male meiosis in the *P. squamulatum* parent will allow genetic mapping of the single chromosome of interest.

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Using *Arabidopsis* in the analysis of megaspore development

A. Ray

Genetic mechanisms that control development of the ovule and the haploid female gametophyte (megagametophyte) in flowering plants are not understood. How is ovule morphogenesis related to the differentiation of the megagametophyte? What leads to commitment for meiosis in a vegetative cell within the ovule? What controls the programmed death of three of four haploid megaspores? What is the mechanism underlying the precise lineage of haploid cellular differentiation and morphogenesis? These are some of the general questions we would like to explore. We have chosen *Arabidopsis thaliana* as a model organism for reasons well-argued elsewhere. In principle, we are mutating *Arabidopsis* to female sterility and/or abnormal ovule development, and are analyzing the phenotypes by a combination of classical and molecular genetics.

Genetic approaches to the study of embryo sac development

A class of mutants defective in embryo sac development are expected to be female sterile. However, female sterile mutations, in contrast to male sterile mutations, are rare in plants. Only two sporophytic female sterile mutations are known in *Arabidopsis.* These are *bell* and *sin1* (Robinson-Beers et al 1992).

Why are female sterile mutants so rare? There may be several trivial reasons. A relatively few genes may be important for female fertility. Given the complexity of female gametophytic generation compared to the male, the number of genes involved in the former is unlikely to be significantly less than the latter. Alternatively, genes needed for megagametogenesis may be present in multiple copies, as opposed to single copies of the genes required for male gametogenesis. While possible, there is no a priori reason for that assumption. A third possibility is that most genes for megagametogenesis are important for sporophyte viability. Thus, most female sterile mutations will be

lethal. While some genes may be of this nature, there should be others unique to the female gametophyte. A more plausible reason behind the paucity of female steriles is that most genes for female fertility could be expressed in the gametophyte. Since it is not possible to obtain homozygous gametophytic mutants, these will be preferentially underrepresented in the M_2 generation, when screening for sterility is traditionally enforced.

We are trying to recover female gametophytic mutations in *Arabidopsis* by novel means. Approximately 12,000 M_2 plants, obtained from selfing several thousand EMS-treated seeds at 14°C, were screened for temperature-sensitive female sterility at 25°C. Seven candidate mutant lines have been chosen for further studies. In addition, we have screened approximately 2,000 T-DNA mutagenized plant lines and isolated approximately 10 mutant lines that segregate 50% or less fertile ovules. Properties of a few mutants are described below, including that of *bell* (Robinson-Beers et al 1992).

bel1 mutation transforms ovules to carpels

Homozygous *bell* mutant plants are morphologically normal but are female sterile and flower indeterminately. The megasporocyte enlarges and divides to form a tetrad, but does not differentiate into the gametophyte. Although chromatins condense, it is not confirmed whether the division is meiotic. In early ovules, the stalk is well formed but integument initiation is delayed. The integuments do not differentiate normally and fail to enclose the nucellus. Instead, they form a disorganized mass of cells at the base of the elongated nucellus. The megaspore is not recognizable in later ovules. Fluorescence microscopy of Hoechst 33258-stained preparations indicate that hypodermal cells within the ovules lose their nuclei in mature flowers. In 6% of *bell* ovules, some cells within the projecting nucellus differentiate into a specialized vascular structure that fluoresces orange upon staining with ethidium bromide, which indicates callose deposition on the walls of these cells. This single vascular structure resembles the termini of extensive vascular elements seen within the style immediately below the stigmatic tissue of the gynaecium. A few of the ovules (<1-3%) show transformation of the ovule into a carpel-like structure, with stigmatic papillae at the micropylar end of the ovule.

Since *AGAMOUS* expression triggers carpel development in early flower buds, and *AG* expression within the ovule is restricted to the endothelial cells, we asked whether *BEL1* function provides the tissue-specific restriction of *AG* expression in mature ovules. This implies that *AG* would be expressed aberrantly in certain *BEL1* ovules.

In situ hybridization of sections of wild-type and *bell* ovules to a ^{35}S-labeled anti-*AGAMOUS* probe indicated that, whereas *AG* expression in mature wild-type ovules is restricted to the endothelial cell layer, *AG* message was found distributed uniformly within the *BEL1* ovules. In addition, there was a marginal increase in the levels of *AG* expression in *BEL1* ovules compared to that in the wild type. We suggest that *BEL1* gene product may restrict *AG* expression to specific tissues in the wild-type ovule, either directly or indirectly.

fms1 mutation affects the nucellus and the inner integument

fms1 is an EMS-induced, nonconditional female sterile mutation that specifically affects the ovule. The megasporocyte appears to initiate, but does not complete, meiosis. In 74% of mature, *fms1* ovules, the outer integument does not expand fully. The inner integument extends outwards to cover a highly elongated nucellus. In 24% of ovules, the outer integument expands to the wild-type level, the inner integument enlarges massively to differentiate into a seed coat-like structure, but has no embryo or endosperm inside. In 2% of *fms1* ovules, the terminal phenotype appears morphologically similar to mature wild-type ovules, but they are much larger (approximately five times). The size of these latter ovules is similar to that of fertilized seeds of the same chronological age. This is in contrast to the wild-type ovules that occasionally fail to fertilize, and remain small. We asked whether the production of enlarged ovules in *fms1* mutants is triggered by pollination by manually emasculating the *fms1* flowers. Occurrence of enlarged ovules has been drastically reduced in emasculated flowers. but was restored when these were artificially pollinated. Thus, pollination induced ovule enlargement in *fms1* flowers.

EXC1 mutation produces extra cells in the nucellus

This mutant line has been isolated from the T-DNA-induced mutant collection generated by Dr. Ken Feldmann (Univ. of Arizona. pers. commun.). The homozygous mutant is partially female sterile, due to abortion of approximately 70% of its ovules. The defect seems to be due to abnormal positioning of the embryo sac within the ovule. In the worst-affected ovules, some embryo sac cells are virtually pushed out of the ovule through the micropyle. In the least-affected ovules, the embryo sac is almost normally positioned. A band of cells at the chalazal end of the embryo sac seems to proliferate abnormally in mutant ovules to a varying degree, producing a range of severity of the defect, displacing the embryo to sac from its normal location.

Overview

Positional information, short-range intercellular interaction, and epigenetic imprinting are three important mechanisms by which a developmental program may unfold. Megagametophyte development in *Arabidopsis* offers the opportunity to study all three mechanisms in a model flowering plant. Positional information may be an important determinant in the programming of ovule development - within the ovule, both the embryo sac and the embryo are highly polar structures. The precise pattern of lineage of the haploid nuclei is reminiscent of epigenetic programming. Within the megagametophyte, four cell types are in intimate and precise contact with one another. but the nature of their interactions is not understood. Detailed examination of mutants that are affected in ovule development will address these fundamental questions for female gametogenesis. Cloning of the genes identified by genetic studies, and analysis of their expression patterns, should generate new insights that may eventually lead to progress in the experimental induction of apomixis in crop plants.

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Developmental genetics of floral patterning in *Arabidopsis thaliana*

J.L. Bowman

Flowering in most angiosperm species is induced by a combination of both environmental and internal cues. Once floral induction occurs, the shoot meristem switches from producing leaf meristems to producing flower meristems. Each flower meristem produces a defined number of floral organ primordia in a precise pattern. The identity of each floral organ primordium is specified according to its position in the flower. The relative positions of the four basic organ types (sepals, petals, stamens, and carpels) are remarkably constant throughout the flowering plants. Once specified, each floral organ primordia then undergoes a specific developmental program during which both common and organ-specific cell types differentiate. Thus, the flowering process can be thought of as a series of developmental stages. Consistent with this, mutations have been isolated in a number of flowering plant species, which suggests that a genetic hierarchy controls the stages of flower development (Coen and Meyerowitz 1991).

The mechanisms that control flower cell differentiation can be investigated by isolating mutants in which cells appear to have differentiated into inappropriate cell types. The corresponding wild-type products of these genes are likely to play a central role in the specification of cell fate. Significant progress has been made in identifying some of the genes involved in the specification of both flower meristem and floral organ identity in the small Crucifer *Arabidopsis thaliana.* Recent progress on how these two sets of genes specify flower meristem and flower organ identity is discussed.

Specification of flower meristem identity

In *Arabidopsis,* flower meristems are derived from the flanks of indeterminate inflorescence meristems (Smyth et al 1990). Three of the factors that specify their identity are the products of the *LEAFY, APETALA1,* and *CAULIFLOWER* genes (Irish and Sussex 1990, Schultz and Haughn 1991, Weigel et al 1992, Mandel et al 1992b, Huala

and Sussex 1992, Bowman et al 1993). When either the *LEAFY* or *APETALA1* gene is inactivated by mutation, structures that have properties of both flowers and inflorescenccs develop instead of flowers (i.e., flowers are partially converted into inflorescences). However, the partial transformations in *leafy* and *apetala1* mutants are strikingly different. For example, *apetala1* mutants have determinate branched structures consisting of several individual flowers in the positions normally occupied by flowers (Irish and Sussex 1990, Bowman et al 1993). In contrast, leafy mutants produce structures consisting of a number (sometimes indeterminate) of sepaloid and carpelloid organs initiated in a spiral, in contrast to the whorled pattern seen in wild-type flowers (Weigel et al 1992). In addition, *leafy* "flowers" are subtended by bract-like organs; wild-type flowers are bractless. More complete flower to intlorescence transformations are observed in *leafy apetala1* double mutants, indicating that the two genes function in concert to specify flower meristem identity (Weigel et al 1992). The phenotypes of both *leafy* and *apetala1* mutants are sensitive to the same environmental conditions that affect the transition to flowering. Thus, both *LEAFY* and *APETALA1* may be responsive to (i.e., activated by) the factors that mediate floral induction. This could also help explain the acropetal variation in phenotype observed in both *leafy* and *apetala1* mutants.

The *LEAFY* gene encodes a novel protein with some characteristics of transcription factors (Weigel et al 1992), and the *APETALA1* gene is a member of the *MADS* box gene family (Mandel et al 1992b), the putative products of which show significant similarity to known transcription factors from yeast and vertebrates. Both *LEAFY* and *APETALA1* are expressed in young flower meristems with *LEAFY* being expressed slightly earlier in the floral anlagen (Weigel et al 1992, Mandel et al 1992b). Thus, the expression pattern of these genes is consistent with their proposed roles in specifying floral meristem identity.

Mutations in the *CAULIFLOWER* locus enhance the phenotype of *apetala1* mutants. *Apetala1 cauliflower* double mutants exhibit complete rather than partial flower to inflorescence transformations (Bowman et al 1993). However, plants homozygous for *cauliflower* mutations alone exhibit no phenotypic differences from wild type. The *CAULIFLOWER* gene product appears to enhance *APETALA1* by acting as a redundant positive regulator of the accumulation or both *LEAFY* and *APETALA1* mRNA in flower primordia. Consistent with this, *leafy apetala1 cauliflower* triple mutants are indistinguishable from *leafy apetala1* double mutants.

Thus, the specification of flower meristem identity seems to involve the action of the products of two primary genes (*LEAFY* and *APETALA1*), whose functions are partially redundant (Weigel et al 1992, Bowman et al 1993). However, *LEAFY* and *APETALA1* also have distinct functions as well. For example, *LEAFY* suppresses bract formation, and *APETALA1* is necessary for proper pedicel development. Other factors, such as the products of the *CAULIFLOWER* and *APETALA2* genes, act in secondary or redundant roles to ensure the specification of flower meristem identity. Finally, each of these genes may be activated at least in part by the factors that mediate floral induction, suggesting a direct link to the preceding developmental step.

Specification of floral organ identity

Arabidopsis flowers consist of four concentric whorls of organs, with each whorl being composed of a different organ type — sepals, petals, stamens, and carpels from the outermost whorl to the innermost. The specification of floral origin identity seems to depend on the products of three classes of homeotic genes acting alone and in combination in three overlapping fields (Bowman et al 1991 b). Field A (whorls I and 2) is represented by *APETALA2* and *APETALA1,* field B (whorls 7 and 3) is represented by *APETALA3* and *PISTILLATA,* and field C (whorls 3 and 1) is represented by *AGAMOUS.* The combination of homeotic gene products present in each whorl specifies the development of the organ primordia in that whorl. In the first whorl, the A function genes act alone to specify sepals. If the B function genes are active as well as the A function genes, as in the second whorl, petals are specified. Stamens are specified in the third whorl by B function genes acting with the C function genes. In the fourth whorl, C function genes act alone to specify carpels. In addition, it is proposed that the A and C functions arc mutually antagonistic. In other words, in a C function mutant (e.g.. *agamous* mutants), A function expands into all four whorls. and conversely, mutations in A function, or specifically *APETALA2* (but not *APETALA1),* lead to an expansion of C function into all four whorls. The model is based on the phenotypes of single, double, and triple mutants. and is largely supported by the molecular data on the expression patterns of the floral homeotic genes acquired subsequently.

Mutations in the class A genes, *APETALA1* and *APETALA2,* both alter the identity of the outer two whorls of floral organs, although their phenotypes are quite different (Bowman et al 1989, Irish and Sussex 1990, Bowman et al 1991 b, Bowman et al 1993). In *apetala1* mutants, bract-like organs replace the sepals and the second whorl organs are mostly absent or are stamen rather than the wild-type petals. In contrast, the outer whorl of *apetala2* mutant flowers is occupied by carpels, and the second whorl organs are either absent or staminoid. Although the identities of the third and fourth whorl organs are not affected in *apetala1* or *apetala2* mutants, their numbers and positions may be altered. This is particularly evident in *apetala2* mutants, where the number of third whorl stamens is markedly reduced.

Mutations in the class B genes, *APETALA3* and *PISTILLATA,* cause floral organ identity alterations in the second and third whorls (Bowman et al 1989, Hill and Lord 1989, Bowman et al 1991 b, Jack et al 1992). The phenotypes of *apetala3* and *pistillata* mutant flowers are indistinguishable — the second whorl organs develop as sepals rather than petals as in wild type and the third whorl organs are transformed from stamens into carpels. These third whorl carpels may congenitally fuse to those of the fourth whorl, forming a single gynaecium.

Mutations in the only class C gene identified, *AGAMOUS,* affect the development of the third and fourth whorls (Bowman et al 1989, Bowman et al 1991 b). In *agamous* mutants, the third whorl organs develop as petals, indistinguishable from those in the second whorl. The cells that normally give rise to the gynaecium in wild-type flowers behave as another flower meristem in *agamous* flowers. This internal flower meristem

reiterates the developmental program such that agamous flowers consist of an indeterminate number of nested flowers in which the floral organs are in the repeating pattern (sepals, petals, petals)$_n$.

Certain predictions about the phenotypes of doubly mutant flowers can be made, and these are largely confirmed (Bowman et al 1991b). For example, *apetala3 agamous* double mutants would have A function only, since both B and C functions are mutant. Additionally, A function activity would be in all four whorls since the C function gene is inactive. Since A function alone leads to the specification of sepals, *apetala3 agamous* flowers would be expected to consist only of sepals. Indeed, *apetala3 agamous* (and *pistillata agamous*) flowers consist of an indeterminate number of whorls of sepals. Similar arguments lead to the prediction that *apetala2 apetala3,* and *apetala2 pistillata* flowers consist entirely of carpels. The prediction for *apetala2 agamous* double mutants is more difficult because if both A and C functions are disrupted, only the B function remains, resulting in a distribution of homeotic gene products that is not found in any of the whorls of wild-type flowers. The formation of both petals and stamens depends on B function in conjunction with A (petal) or C (stamen). Thus, the second and third whorl organs of *apetala2 agamous* flowers might be expected to be neither wild-type petals nor wild-type stamens, but to have characteristics of both. This is indeed the case. Homeotic gene activities are not present in the first and fourth whorls of *apetala2 agamous* double mutants, thus the organs in these positions should be of a type not normally found in flowers. In actuality, the organs occupying these positions are leaf-like. However, these leaf-like organs retain some carpelloid characteristics, suggesting that other genes in addition to *AGAMOUS* are involved in specifying carpels. Thus, *agamous* mutations have phenotypic effects in the outer whorls of *apetala2* mutant flowers, but not in otherwise wild-type flowers. Conversely, *apetala2* mutations have phenotypic effects on organ identity in the inner two whorls in an *agamous* mutant flower, but not in an otherwise wild-type flower. This strongly suggests that the two genes are mutually antagonistic. Finally, *apetala2 pistillata agamous* triple mutant flowers, in which members of all three classes of homeotic genes are inactivated, consist of an indeterminate number of carpelloid leaves.

Several of these genes have been cloned and four of them *(APETALA1, APETALA3, AGAMOUS, PISTILLATA)* are members of the *MADS* box gene family encoding putative transcription factors (Yanofsky et al 1990, Jack et al 1992, Mandel et al 1992b. In support of the model, the patterns of expression of the floral homeotic genes correlate largely with their respective mutant phenotypes. For example, *PISTILLATA* and *APETALA3* expression is restricted to the second and third whorls of wild-type flowers (Jack et al 1992, Goto and Meyerowitz, unpubl. data), and *AGAMOUS* expression is restricted to the third and fourth whorls of wild-type flowers (Drews et al 1991). Although *APETALA1* is expressed uniformly throughout the flower meristem prior to the formation of any organ primordia, its expression becomes restricted to the outer two whorls at about the time the first whorl organ primordia arise (Mandel et al 1992b). Further molecular support for the combinatorial field model of specification of floral organ identity has come from the demonstration that *APETALA2* is a negative regulator

of *AGAMOUS* mRNA accumulation in the outer two whorls of the flower (Drews et al 1991). Thus, part of the mutual antagonism between *APETALA2* and *AGAMOUS* occurs at the transcriptional level. The initial activation of each of these genes precedes the formation of the organ primordia they specify. The precise initial spatial boundaries of expression of the homeotic genes appear to be defined by interactions both with earlier signals and between the homeotic genes themselves. For example, the boundary between the second and third whorl is defined by the mutual antagonism of the A and C function genes. Negative regulation also seems to be involved in defining the inner boundary of the B function genes. In this case, *SUPERMAN* mutations, which cause the production of extra stamens at the expense of the carpels, prevent the accumulation of B function genes in the fourth whorl (Bowman et al 1992). Finally, floral homeotic gene expression is dependent, either directly or indirectly, on the activity of those genes that specify floral meristem identity (Weigel and Meyerowitz 1993).

Cell specification within the floral organs

How regional positional information encoded by the homeotic gene products is translated into organ-specific cellular differentiation is still a mystery. However, it is of interest that some homeotic genes are expressed in well-defined spatial patterns late in flower development. For example, although *AGAMOUS* is expressed uniformly throughout the third and fourth whorl floral organ primordia, it later becomes restricted to certain cell types within differentiating stamens and carpels (Bowman et al 199la). *AGAMOUS* expression is high in the stigmatic papillae and the integumentary tapetum in the carpels, and in the endothecium and connective tissue of the stamens. In contrast *AGAMOUS* mRNA is not detected in pollen grains or in the sporogenous tissue that gives rise to the pollen grains. Additionally, *AGAMOUS* mRNA is excluded from the cell layers of the integuments and from the embryo sac throughout its development. The function of this dynamic expression pattern is not clear, but two types of experiments have suggested that there may be some functional significance. Firstly, although no stigmatic tissue is ever observed in *agamous* mutant flowers, *apetala2 agamous* flowers have stigmatic tissue at the tips of their first and fourth whorl organs. This indicates that *AGAMOUS* activity is not absolutely required for the development of stigmatic papillae. Secondly, transgenic tobacco plants constitutively expressing *AGAMOUS* produce flowers that are completely male sterile and have reduced female fertility (Mandel et al 1992a). The male sterility is due to nondifferentiation of pollen grains, whereas reduced female fertility is due to aberrant growth of ovules into carpelloid spaghetti-like structures with stigmatic papillae at their tips. This suggests that for proper cell differentiation of stamens and carpels, the exclusion of *AGAMOUS* expression from specific cell types may be more important than its expression in other cell types. In the case of ovule development, the ectopic expression of *AGAMOUS* in the embryo sac and integuments of developing ovules of the transgenic plants may cause the abnormal growth of ovules into carpelloid spaghetti-like structures (Mandel et al 1992a). In this regard, Evans and Malmberg (1989) have shown that tobacco ovules from placental tissue cultured in vitro pass through an initial transient stage,

where they retain the ability to develop into carpels. One possible explanation is that *AGAMOUS* is negatively regulated in certain cells of the ovule to prevent their developing into carpel-like structures. A possible candidate for mediating this negative regulation is *APETALA2.* However, the late expression pattern of *AGAMOUS* is not altered in *apetala2* mutants, suggesting that *APETALA2* is not involved in this cell-specific regulation of *AGAMOUS* (Bowman et al 1991a). On the other hand, since *apetala2* mutants have a seed coat phenotype, it is likely that *APETALA2* plays some role in cellular differentiation late in flower development. Another possible candidate for a negative regulator of late *AGAMOUS* expression is *BELLI.* In *bell1* mutants, the cells that would normally give rise to the integuments develop into abnormal structures (Robinson-Beers et al 1992). Further studies and comparisons of transgenic and mutant ovules may provide insights into how the fates of cells within the ovule are specified.

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Isolating apomictic mutants in *Arabidopsis thaliana* — prospects and progress

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Apomixis is the process by which sexual reproduction (amphimixis) is substituted by asexual reproduction to produce seeds (Gustafsson 1946, Asker and Jerling 1992). While sexually reproducing organisms contain recombined and independently assorted genes from both parents, apomictic organisms are genetically exact copies of the mother.

Apomixis has been divided into two fundamentally different types, based on the origin of the apomictic embryo (Gustafsson 1946, Asker and Jerling 1992). In the first type, described as adventitious embryony (or sporophytic apomixis), the embryo arises directly from the nucellus or the integument ofthe ovule. The second type, gametophytic apomixis, includes two classes known as apospory and diplospory. In apospory, unreduced embryo sacs originate from nucellar cells. In diplospory, the embryo sacs originate from premeiotic female archesporial cells by mitosis (*Antennaria* type), or by modified meiosis *(Taraxacum* type, *Ixeris* type).

In pseudogamous apomixis, seed formation depends on the fertilization of the polar nuclei for endosperm development. In autonomous apomixis, endosperm development is independent of fertilization of the polar nuclei.

Distribution of apomixis among higher plants

Each of the above types of apomixis have been described in higher plants (Gustafsson 1946, Asker and Jerling 1992). Adventitious embryony is quite common among citrus species and orchids (Bashaw 1980). Apospory has been described, among other plants, in the grasses *Poa* and *Pennisetum* (Gustafsson 1946, Hanna and Bashaw 1987). Diplospory has been found in, among other plants, *Taraxacum, Ixeris,* as well as in *Arabis holboellii,* a crucifer (Bocher 1951).

Genetic control of apomixis

Apomixis is under genetic control. By studying hybrids of sexual plants and their apomictic derivatives, examples of both dominant and recessive control have been obtained (Asker 1980). In *Pennisetum,* the genetic trait for apospory has been mapped to a chromosomal region, and coinheritance of apomictic reproduction and molecular markers has been demonstrated (Ozias-Akins et al 1993).

Although the genetic characteristics of the cell that becomes an apomictic embryo are different from those of zygotic embryo, the apomictic embryo is formed and nurtured by processes that mirror normal postmeiotic embryo sac formation. The subsequent development of the embryo, endosperm, and seed are also comparable to the processes that occur in sexual reproduction. An attractive possibility is that apomictic reproduction is caused by the premature activation of embryo sac development, catalyzing a cascade of gene actions in one or more nucellar cells, resulting in embryo sac formation that bypasses meiosis.

If this view of gene regulation of apomixis is correct, we might be able to find mutations that convert a sexually reproducing plant into an apomictic one. Such mutations would presumably inactivate a transcriptional repressor that normally suppresses an apomictic pathway, or might give rise to a transcription factor with a novel specificity, capable of heterochronic induction of an apomictic pathway of reproduction.

To mutagenize a sexually reproducing plant into apomixis is not easy. The major obstacle is the sexual mode of reproduction, which would mask the formation of apomictic progeny. It is thus important that sexual seed formation is completely abolished by appropriate mutations before apomictic seed formation is sought. There are several ways of achieving this end. For instance, a synthetic lethal system could be constructed whereby sexual zygotes would be nonviable. Alternatively, one could diagnose apomixis by having a system by which a sexual zygote could be readily distinguishable from the matroclinous progeny. Although these detection systems are useful for identifying apomixis, a truly powerful system would be one that dispenses completely with the sexual pathway by eliminating the male sexual organs. When these feminized plants are mutagenized, seed production itself becomes indicative of a nonsexual, or apomictic, mode of reproduction.

Arabidopsis thaliana, a convenient plant in which to screen for apomictic mutants

Arabidopsis thaliana has emerged as an ideal experimental angiosperm for the isolation of genes required for diverse plant processes (Meyerowitz 1987). For the isolation of genes important for apomixis, *A. thaliana* provides several advantages over plants in which apomixis has been studied previously. The relatively small size and fast life cycle of *Arabidopsis* means that a very large number of plants can be screened after mutagenesis to detect potential mutants. The small genome size, and the limited repeated DNA, combined with the development of all the necessary techniques of plant

genetic engineering in *A. thaliana,* mean that once such a gene is identified it can be cloned relatively easily. The occurrence of apomixis in *Arabis holboellii,* a crucifer closely related to *Arabidopsis,* suggests that apomixis could occur in *Arabidopsis* as well. Most importantly, reproductive mutants of *Arabidopsis* provide an attractive opportunity to detect apomixis without the laborious progeny testing that would be required in other plants. Our genetic screen is based on exploiting these advantages of *Arabidopsis.*

Reproductive organs of *Arabidopsis thaliana*

Arabidopsis is a self-fertilizing plant. The female sexual organ consists of the future seed-pod or silique. made of two fused carpels containing the ovules and topped by the stigma. During anthesis, the stigma is close to six stamens topped by anthers that release pollen. The stamens, in turn, are surrounded by four petals and four sepals. In self-fertilizing plants, anthesis and pollination are completed even before the flowers have properly opened. As fertilization takes place and the seeds are formed, the siliques elongate about five-fold, giving rise to full length seed pods. In male-sterile mutants, self pollination does not take place and the seed pods remain short.

Structural male sterile mutants of *Arabidopsis*

Arabidopsis mutants are available in which the stamens are absent but female fertility is unimpaired. These mutants include *ap3* and *pistillata,* in both of which the third whorl stamens are missing (reviewed in Coen and Meyerowitz 1991), and *antherless,* in which the anthers are either missing or are transformed into sepals (Chaudhury et al 1992). The absence of male organs and unimpaired female fertility in structural male sterile mutants make them attractive genetic backgrounds in which to screen for apomictic mutants.

Pollen donor to detect pseudogamy

To detect pseudogamy, male sterile plants containing presumptive apomictic mutations will have to be pollinated with pollen containing one or more dominant mutations. After such a pollination, most of the progeny plants will be sexual heterozygotes, while the rare apomicts would have the maternal phenotype. For instance, in the *pistillata* screen, the sexually fertilized plant would have a *pi/PI* genotype (phenotype: self-fertile, normal flowers), and the apomict would have the *pi/pi apo/apo* genotype (phenotype: stamenless, but with long siliques). Since the stamens of wild type plants are surrounded by petals, they are not ideal pollen donors for pseudogamy. By combining a floral meristem identity gene *apl* (Meyerowitz 1987), a cadastral gene *superman* (Schultz et al 1991), and an uncharacterized cryptic modifier gene segregating in one of the parental strains, we have been able to construct a plant in which flowers are made entirely of stamens with functional pollen (the "pollen-brush" phenotype). Such a donor plant would be ideal for facilitating cross-pollination.

Mutagenesis and screening for putative apomictic mutants

pi/PI heterozygous seeds were made by pollinating female *pi/pi* homozygotes with pollen from *PI/PI* plants. Several thousand *pi/PI* heterozygous seeds were mutagenized with ethylmethanesulfonate (EMS), and the M_1 plants selfed. M_2 plants were screened for the presumptive apomictic phenotype. Pollination was performed to isolate pseudogamous plants.

In separate experiments, *pi/pi* M_1 plants were screened for dominant apomixis by looking for mitotic sectors of inflorescence (in which seed is produced without a reversion of the stamens phenotype). In the dominant screen as well, plants were pollinated to detect pseudogamous apomixis. Analyses of presumptive mutants are in progress.

Future directions

We have devised a simple visual genetic screen to detect apomixis in a higher plant, and have isolated some candidates that are under further investigation. The ease of the screen and the unambiguous nature of the subsequent analyses make this screen useful for large-scale screening to detect apomixis. We feel confident that, if apomixis is controlled by a single gene so that a sexual plant like *Arabidopsis* can be mutated to apomixis, our screen can detect it relatively easily. The visual screen devised by us could also be used to test heterologous genes for their ability to convert a sexual plant into an apomictic one.

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Summary of discussions and recommendations

During the last 4-5 years, major advances have been made in tissue culture and molecular biology of rice. Some of the exciting developments include construction of a comprehensive molecular genetic map of rice consisting of more than 1000 DNA (RFLP) markers, successful regeneration of plants from protoplasts of both indica and japonica rices, efficient protocols for DNA transformation, and production of transgenic rices. These advances offer great potential to obtain apomictic rice.

Isolation of genes for apomixis

Several laboratories are engaged in the isolation of genes for apomixis. Molecular markers have been used to establish linkage with apomictic reproduction in *Pennisetum.* Mapping populations are being developed in *Tripsacum* and *Tripsacum* /maize hybrids to identify apomictic gene(s). Significant progress has been made in *Pennisetum, Cenchrus* (buffelgrass), and *Tripsacum* to identify useful apomictic genes for transfer into cultivated rice by transformation. Genetic stocks have also been developed for *Tripsacum* to transfer apomictic genes into maize through conventional methods. Future efforts should focus on tagging genes for apomixis. Tight linkage with molecular markers provides opportunity to isolate such genes through chromosome walking. Maize transposable elements or transposons from heterologous systems possibly could also be used to isolate gene(s) for apomixis.

Isolation of homologous genes in rice may be undertaken when such genes become available in rice or after such genes were isolated from other apomictic species.

Rice transformation. Transformation systems (protoplast mediated and by biolistic methods) are becoming increasingly well defined in rice. Given the availability of crucial genes from apomictic species, these systems could be used to produce apomictic rice.

Synthetic approach: Since the genetic control of basic components of gametogenesis and apomixis is not well understood (see next section), it may be premature to recommend the synthetic approach to obtain apomictic rice.

Knowledge base: There is a need to better understand the megasporogenesis and the development of female gametophyte. Various mechanisms triggering apomixis in the female gametophyte need to be critically examined. The knowledge from *Arabidopsis* mutants affecting female gamete formation *(bel-1*,*fms1* and others likely to be isolated in the future) would be valuable to understand the process of megasporogenesis and female gametophyte development in apomictic species and in rice. The mutant genes cloned from *Arabidopsis* might be useful for detecting differences between apomictic and sexual development. The breakdown of apomictic process such as disruption of meiosis in the female and activation of nucellar cells need to be carried out. Studies on ultra structure of apomictic embryo sac at various stages of development and the time factor in the precocious development of embryo should be examined. Molecular probes to detect such apomictic events, when they become available in *Arabidopsis,* should be used in rice. Embryo sac-specific cDNA libraries should be used to develop from appropriate species in which the stages of female gamete formation are readily studied. Promoters from these gametophyte-specific genes should be used to explore the pathway of gametogenesis in *Arabidopsis* and other plants.

More basic research is needed to increase knowledge on molecular genetics of apomixis.

Mutational approaches to induce apomixis

Apomictic reproduction is believed to be caused by the premature activation of embryo sac development, catalyzing a cascade of gene action in one or more nucellar cells, or an unreduced megaspore resulting in embryo sac formation that bypasses meiosis. Therefore, it should be possible to find mutations that convert a sexually reproducing plant into an apomictic one. Such mutations would presumably inactivate a transcriptional repressor that normally suppresses an apomictic pathway, or might give rise to a transcription factor with a novel specificity, capable of heterochronic induction of an apomictic pathway of reproduction.

The participants held comprehensive discussions to identify appropriate germplasm, mutagens and stage of application of the mutagen to induce apomictic mutations in rice. Guidelines to screen for apomictic mutants in M_2 generation were also discussed.

The following recommendations were made:

1) Stamenless structural male sterile (pistillate type) mutant and genic male sterile mutants of rice should be appropriate for inducing apomictic mutations. The protocol to develop apomictic mutants in *Arabidopsis thaliana* being used at CSIRO Australia may be used for the purpose, keeping in view both the dominant and recessive nature of the trait, and pseudogamous or autonomous pathway of the endosperm development.

2) Mutagens ethyl methane sulphonate (EMS), sodium azide and NMU (nitroso methyl urea) were considered suitable. Mutagens causing chromosomal deletions are less useful for the purpose. Dosage of the mutagen should be adjusted such that it should not have more than 50% lethality in M_1 plants; a higher dosage may cause excessive sterility in the population.
3) Treatment of seeds with mutagens was considered suitable to induce apomictic mutants.
4) In order to identify apomictic mutants in M_2 generation, an appropriate screen, which is effective as well as practical, needs to be developed. Although no specific screen was suggested, use of pollen from an alien species for mass-pollination of suspected apomictic mutants or auxins for inducing parthenogenesis were recommended.
5) To better understand the phenomenon of apomixis in rice, meiotic and embryo sac mutants should be induced and studied.

Screening techniques and germplasm resources for apomixis

Screening techniques

1) Among the available techniques, pistil clearing technique is the most suitable since it is rapid and reliable. Pistil clearing should be done at a very early stage of development (from premeiosis to shortly after meiosis), enabling detection of apospory and diplospory types of apomixis.
2) During initial or mass screening of genotypes, examination of 20-25 ovules per accession is adequate. An IRRI staff member should spend 3-4 weeks at CIMMYT to become familiar with the Pistil Clearing technique being used for screening maize-*Tripsacum* hybrid progenies.
3) If any abnormality is detected during mass screening, more ovules of that accession should be examined. If apomixis is present, the mechanism, whether facultative or obligate, should be investigated. Progeny tests are also essential. Isozyme markers may be used for progeny testing.

Germplasm for screening

1) Large numbers of *O. sativa* accessions have been screened in China, USA and at IRRI. No real case of apomixis has been detected so far.
2) Chances of finding apomixis are greater in polyploids. Therefore, evaluation of tetraploid *Oryza* germplasm (secondary as well as tertiary gene pools) should be emphasized.
3) IRRI currently has over 180 accessions of tetraploid species. Tetraploids with highest number of accessions should be evaluated first.
4) Additional germplasm of wild tetraploid *Oryza* species should be collected on a priority basis.

Transferring apomixis to cultivated rice

1) Upon detection of apomixis, a hybridization program using cultivated rice as female parent and wild apomictic types as male parent, should be initiated.
2) F_1 hybrids should be screened for mode of reproduction, and apomictic hybrids should be selected for further breeding.
3) A backcross program to transfer genes of apomixis into cultivated rice should then be initiated.

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