Chapter 14

Genetic Engineering of Apomixis in Sexual Crops: A Critical Assessment of the Apomixis Technology

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Introduction

According to projections, world population will increase from six billion people today to eight billion in 2020, stabilizing at 9–11 billion people around the middle of the 21st century (Lutz et al. 1997; Evans 1998; Toenniessen, Chap. 1). Profuse quantities of high quality and safe food products will be required to feed this growing population. At the same time, strong pressures are at work demanding that this food be produced in an environmentally friendly manner, e.g., using less agrochemicals. In Europe, agricultural production has steadily increased while population has begun to decrease, resulting in an overproduction of food products. By contrast, the developing world will need to produce two or three times as much food as it does today (Toenniessen, Chap. 1). By 2020, cereal production, for example, will need to increase by 41%, and root and tuber production by 40% (Spillane 1999). To meet this dramatically increasing demand, new plant varieties are needed that are both higher yielding and better adapted to specific climatic conditions. Essentially, this challenge must be met without a significant expansion of agricultural area.

Although less agricultural production will be needed in the developed world, new products, so-called 'novel foods,' 'functional foods,' 'designer foods,' as well as renewable raw materials will soon gain more agricultural market share. It is expected that most of these new products will be produced through biotechnology. Therefore, it is not surprising that the global market for agricultural biotechnology products is expected to increase from US$500 million in 1996 to US$20 billion within the next 15 years (James 1997).

One biological process in particular—apomixis—could revolutionize 21st century agriculture in both developed and developing countries. The harnessing of apomixis is expected to launch a new era for plant breeding and seed production. Mastering apomixis would allow (*i*) immediate fixation of any desired genetic combination (genotypes, F_{1s} included); (*ii*) propagation of crops through seed that are currently propagated vegetatively (seed is easier to transport and to sow); (*iii*) faster and less expensive plant breeding and seed production (e.g., hybrid seeds could be easily produced); (*iv*) a larger pool of germplasm to be used to create more locally adapted varieties (once apomixis is integrated into breeding schemes); and (*v*) a carryover of beneficial phytosanitary side effects through seed propagation, because very few pathogens are transferred through seeds (Grossniklaus et al. 1998a; Bicknell and Bicknell 1999). Furthermore, exploiting apomixis would allow breeding with obligate apomictic species (e.g., *Pennisetum spec.*), where introgression of new traits is currently very limited (do Valle and Miles, Chap. 10), and the use of male sterile plants for seed production. In turn, this would prevent the migration of transgenes from crop plants to wild relatives.

All these advantages taken together undoubtedly would lead to large increases in agricultural production and prompted Vielle-Calzada et al. (1996a) to coin the term "Asexual Revolution" to describe the potential impact of the technology.

The possible economic benefits of the technology are also considerable. In rice, added productivity would total more than US$2.5 billion per year (McMeniman and Lubulwa 1997). It is projected that the heterosis effect alone would result in yield increases of more than 30% (Yuan 1993; Toennissen, Chap. 1). Of today's US$15 billion global market in commercial seed, hybrid seed accounts for 40% of sales (Rabobank 1994), a further indication of the enormous economic potential of apomixis for agricultural enterprises.

Unfortunately, scientific and economic potential shed little light on the actual intricacies of how the genes involved in apomictic reproduction work. Many have concluded that the genes that control apomixis are also crucial for sexual development, indicating that apomixis is a short-circuited sexual pathway (Koltunow et al. 1995; Grossniklaus, Chap. 12). The genetic engineering of apomixis, therefore, requires a better understanding of both apomictic and sexual pathways of reproduction.

In general, apomixis is thought to occur in polyploid species (Asker and Jerling 1992), especially in the Rosaceae, Asteraceae, and in the Poaceae (for review see Berthaud, Chap. 2). For most species in which apomixis has been described, diploids reproduce sexually, while polyploids of the same species are apomictic. Most natural apomicts reproduce through facultative apomixis (Asker and Jerling 1992; Berthaud, Chap. 2). The degree of apomictic reproduction is influenced by the genetic background, ploidy level, modifier genes, and the environment. There is also a great diversity of apomictic behavior: nine types of gametophytic apomixis have been described in addition to sporophytic apomixis (adventitious embryony) (Crane, Chap. 3).

Unfortunately, apomixis is not found in the most important cultivated crops, which could be a result of crop domestication, selection, and segregation analysis (Grossniklaus, Chap. 12). There are three main options for the engineering of apomixis into sexual crops: (*i*) transfer the trait into crops from wild, naturally apomictic relatives through numerous backcrossings, (*ii*) screen sexual crops for apomictic mutants, and (*iii*) *de novo* synthesize the apomictic trait directly into crops. These approaches will be discussed in the following pages.

Transfer of the Apomixis Trait to Sexual Crops

Breeding and Introgression from Wild Relatives

Generally, breeding apomictic species is very difficult, consequently, there have been only a few breeding programs, and these focused on a very limited number of tropical grass species. The basic structure of such breeding programs is described in this book, using *Brachiaria* as an example, an important forage grass in South America, (do Valle and Miles, Chap. 10). Obligate apomicts cannot serve as maternal plants and breeding of such species is therefore impossible. The polyploid and highly heterozygous nature of most apomictic plants further complicates genetic analysis. In addition, controlled pollination is needed to analyze reproductive behavior (methods are described by Sherwood, Chap. 5). Additional techniques are needed to monitor reproduction behavior in progeny plants of new varieties. Such techniques are described in this book by Berthaud (Chap. 2), Crane (Chap. 3), and Leblanc and Mazzucato (Chap. 9). The techniques described include chromosome counting, flow cytometry, clearing and

squashing techniques, sectioning, molecular markers, and the "auxin test." Ultrastructural studies using electron microscopy (Naumova and Vielle-Calzada, Chap. 4) reveal even more information, but are very laborious, time-consuming, and poorly suited to large-scale progeny analysis. Flow cytometry analysis of seeds is a fast and easy tool and thus probably the method of choice for first progeny testings. This is because large numbers of progeny populations have to be produced and investigated at each generation in order to analyze reproductive behavior (Matzk et al. 2000; Savidan, Chap. 11).

Several sexual crop plants are closely related to wild apomicts, and introgression of the apomixis trait through wide crosses has successfully been performed with wheat, maize, and pearl millet (reviewed by Bicknell, Chap. 8; Savidan, Chap. 11). Nevertheless, there are some limitations: total male sterility was observed frequently in F_1 hybrids of wide crosses, representing a dead end once the apomixis trait is obligate. In wide crosses between *Tripsacum* and maize, fertile apomictic BC_4 with less than 11 *Tripsacum* chromosomes could not be identified (Savidan, Chap. 11), resulting in maize lines devoid of agronomic value. Another disadvantage of this approach is that transfer of natural apomixis genes from wild species into related sexual crops by introgression is likely to remain limited to those crops that have apomictic relatives and so will not be applicable to other species.

Mutagenesis Approaches

Mutagenesis approaches have been described in great detail earlier in this book by Grossniklaus (Chap. 12) and Praekelt and Scott (Chap. 13). Therefore, we will discuss only the main conclusions here.

The basis for all mutagenesis approaches is the assumption that apomictic reproduction pathways are developmental variations of the sexual pathway, thus a short-circuited sexual pathway. Mutant screens have therefore been designed to induce sexuality in apomicts and apomictic mutants in sexual plants by the inactivation of genes. Many mutants were identified as being defective in meiosis, megasporogenesis, and gametogenesis (for review, see Yang and Sundaresan 2000; Grossniklaus, Chap. 12). Mutant analysis of megagametogenesis, for example, suggests that a large number of loci are essential for embryo-sac development. Other mutants are described as displaying autonomous embryo and/or endosperm development. The corresponding genes have been recently cloned. *Mea/fis1* (*medea/fertilization independent seed 1*) is a gametophyte maternal effect gene probably involved in regulating cell proliferation in the endosperm and also partially in the embryo (Grossniklaus et al. 1998b; Luo et al. 1999). *Fis2* shows a similar mutant phenotype and encodes a putative zinc-finger transcription factor (Luo et al. 1999). Autonomous endosperm development was observed in the *fie (fertilization independent endosperm/fis3)* mutant. *Mea/fis1* and *fie/fis3* display homology to Polycomb proteins (Grossniklaus et al. 1998b; Ohad et al. 1999), which are involved in long-term repression of homeotic genes in *Drosophila* and mammalian embryo development (Pirrotta 1998).

The most important conclusion derived from the description of these mutants is that all the elements of apomixis can indeed be induced by mutations in sexual plants. In addition, it is obvious that more than one mutation will be necessary to obtain vital apomictic seeds in sexual crops. Nevertheless, a combination of such isolated genes could be used for known gene approaches, but additional genes will be needed to obtain fully developed seeds. Until now, most mutagenesis screens have concentrated on the partial or complete inactivation of the genes that are needed for

progression or inhibition of development. Future screens will also include activation tagging in order to induce genes under a spatial, temporal, or developmental regime that differs from that in the sexual wild type plants.

Known Gene Approaches

Known genes used for genetically engineering the apomixis trait should lead to the following biological processes:

(1) avoidance and bypassing of meiosis (apomeiosis);
(2) formation, ideally, of one functional unreduced embryo sac within each ovule;
(3) autonomous development of the unreduced egg cell by parthenogenesis;
(4) development of a functional endosperm—this could be autonomous or pseudogamous after fertilization of the central cell; and
(5) an inducible/repressible system that is necessary to switch between apomictic and sexual reproduction pathways, because sexuality and recombination will be required for the introduction of new traits into crops, which will result in new and improved plant varieties.

Based on analyses of mutants in apomictic and sexual plant species, it is unlikely that the apomixis trait can be engineered using a single gene. This is supported by the fact that in most cases apomixis is facultative and that the proportion of apomictic progeny can be influenced by different factors, e.g., by environmental factors. Variability within the different apomictic reproduction pathways further indicates that asexual seed development cannot be explained on the basis of a single gene.

One possibility for engineering apomixis is based on isolating the apomixis gene(s) from natural apomicts and inserting them into sexual crops. Molecular mapping of apomixis genes and gene isolation by map-based cloning or transposon tagging (described by Grimanelli et al., Chap. 6) are performed in various laboratories, but until now no apomixis genes could be isolated and markers still lie within cM distance. One major problem with several apomicts is suppression of recombination around the apomixis loci (e.g., *Pennisetum* and *Tripsacum;* Grimanelli et al., Chap. 6). In addition, apomictic species do not belong to the classical model plant species, and therefore positional cloning is difficult because of the relatively low number of available markers, which are needed to "walk" to the apomixis gene(s). Transposon tagging is not possible for most apomicts (*Tripsacum* is an exception because it can easily be crossed with maize lines carrying active transposon elements), and for the near future, T-DNA tagging will remain restricted to dicotyledonous apomicts such as *Hieracium*, which are accessible to *Agrobacterium tumefaciens* transformation (Bicknell, Chap. 8). Moreover, it is also possible that because of the polyploid nature of natural apomicts, no such phenotype exists.

Known genes/promoters from sexual species that could be used for genetic engineering include those involved with (*i*) ovule development, (*ii*) initiation of meiosis, (*iii*) female gametophyte development, (*iv*) parthenogenesis, and thus autonomous embryo development, and (*v*) initiation of endosperm development. Grossniklaus (Chap. 12) speculates that the genes controlling apomixis are under relaxed or aberrant temporal and/or spatial control, thus developmental checkpoints and feedback mechanisms may be ignored or altered, leading to precocious development of the megaspore mother cell and/or the unreduced egg cell.

Ovule- and nucellus-specific genes/promoters are now available as tools (see Tables 14.1 and 14.2). The molecular control of meiosis is well characterized in yeast (Vershon and Pierce 2000) and some animal systems, e.g.,

Caenorhabditis elegans (Zetka and Rose 1995), and many genes have been isolated and characterized during the last few years. Much less is known about the genes involved in plant meiosis. However; the first homologs to yeast meiosis genes were recently isolated (reviewed by Grossniklaus, Chap. 12), and many meiosis mutants remain available for further characterization (e.g., in maize and *Arabidopsis*; Neuffer et al. 1997; Yang and Sundaresan 2000). Genes that are expressed during the induction of meiosis have been identified in lily (Kobayashi et al. 1994). Most work on meiosis in plants has been accomplished through investigating male meiosis, but for genetic engineering, female meiosis genes will be of particular interest. Some genes involved with female gametophyte development have been identified, of which some are specifically expressed in different cells of the female

Table 14.1 Examples of isolated genes and their promoters that might be useful as tools for *de novo* synthesis of the apomixis trait in sexual crops

Process to be manipulated *Gene* (expression/function)	(Origin)	Reference
'Apomixis genes'		
not isolated yet (?)		
Ovule and nucellus-specific target gene expression		
FBP7 promoter (ovule-specific)	(*Petunia*)	Colombo et al., 1997
DEFH9 promoter (ovule-specific)	(*Anthirrhinum*)	Rotino et al., 1997
WM403 promoter (nucellus-specific)	(water-melon)	Shen et al., unpublished
Nucellin cDNA (nucellus-specific)	(barley)	Chen and Foolad, 1997
Prevention of meiosis/apomeiosis		
diverse cDNAs (early meiosis-specific)	(lily)	Kobayashi et al., 1994
pAWJL3 cDNA (early meiosis-specific)	(wheat)	Ji and Langridge, 1994
DMC1 gene (MMC*-specific)	(*Arabidopsis*)	Klimyuk and Jones, 1997
SYN1 gene (chrom. condensation/pairing)	(*Arabidopsis*)	Bai et al., 1999
Parthenogenesis (autonomous embryo development)		
SERK gene (competence to form embryos)	(carrot, *Arabidopsis*)	Schmidt et al., 1997
LEC1 gene (competence to form embryos)	(*Arabidopsis*)	Lotan et al., 1998
BBM1 gene (competence to form embryos)	(*Brassica, Arabidopsis*)	Boutilier et al., unpublished
ZmES1-4 promoter (embryo sac-specific)	(maize)	Amien and Dresselhaus, unpublished
(Autonomous) endosperm development		
MEA/FIS1 gene (suppressor)	(*Arabidopsis*)	Grossniklaus et al., 1998b Luo et al., 1999
FIS2 gene (suppressor)	(*Arabidopsis*)	Luo et al., 1999
FIE/FIS3 gene (suppressor)	(*Arabidopsis*)	Ohad et al., 1999
ZmES1-4 promoter (embryo sac-specific)	(maize)	Amien and Dresselhaus, unpublished
Imprinting		
MET1 a/s (hypomethylation)	(*Arabidopsis*)	Adams et al., 2000 Vinkenoog et al., 2000
Inducible/repressable systems		
Steroid-inducible promoter	(mammals)	Schena et al., 1991
Copper-inducible promoter	(yeast)	Mett et al., 1993
Tetracycline-inducible/-inactivatable promoter	(bacterium)	Weinmann et al., 1994
Ethanol-inducibele promoter	(fungus)	Caddick et al., 1998

*MMC: Mega- and Microspore mother cells.

Table 14.2 Examples of patents linked with the engineering of the apomixis trait in sexual crops.
Sources: Intellectual Property Network (*http://www.delphion.com*), European Patent Office (*http://ep.dips.org/dips*), and Bicknell and Bicknell (1999).

Apomixis technology Patent number* (Publication date)	Title (and content)	Applicant(s)
Breeding strategies		
WO8900810 (Feb. 9, 1989)	Asexual induction of heritable male sterility and apomixis in plants (use of male sterility factors).	Maxell Hybrids INC.
CN1124564 (June 19, 1996)	Hybrid vigor fixing breeding process for rice apomixis (breeding and selection strategy).	Chen J.
US5710367 (Jan. 20, 1998)	Apomictic maize (introgression of apomixis from *Tripsacum* to maize).	USDA
WO9710704 (Sep. 22, 1998)	Apomixis for producing true-breeding plant progenies (introgression of apomixis from *Pennisetum squamulatum* to cultivars).	USDA
WO9833374 (Aug. 6, 1998)	Methods for producing apomicitic plants (breeding program).	University of Utah State
WO0007434 (Feb. 17, 2000)	Novel genetic material for transmission into maize (introgression of apomixis from *Tripsacum*).	Eubanks M.W.
Stimulation of apomictic reproduction		
EP0127313 (Dec. 5, 1984)	The production of haploid seed, of doubled haploids and of homozygous plant lines therefrom (causing apomixis by applying an apomixic agent).	Rohm & Haas
SU1323048 (July 15, 1987)	Stimulator of floral apomixis (no file available).	Poltav Selskokhoz IG Nikitskij
US4818693 (April 4, 1989)	Methods and materials for enhanced somatic embryo regeneration in the presence of auxin.	PGS
US5840567 (Nov. 24, 1998)	Simplified hybrid seed production by latent diploid porthenogenesis and parthenote cleavage (induced by controlled environmental conditions).	University of California
De novo synthesis of apomixis (genes and promoters)		
WO9743427 (Nov. 11, 1997)	Production of apomictic seed (using a SERK gene for embryogenic potential).	Novartis and inventors
WO9808961 (March 5, 1998)	Endosperm and nucellus specific genes, promoters and uses thereof.	Doan, D.N.P., Olsen, O.-A. and Linnestad, C.
WO9828431 (July 2, 1998)	Transcriptional regulation in plants (using a meiosis specific promoter).	John Innes Centre Innov. LTD and inventors
US5792929 (Aug. 11, 1998)	Plants with modified flowers (modifying flower cells after transformation with foreign DNA).	PGS
WO9836090 (Aug. 20, 1998)	Means for identifying nucleotide sequences involved in apomixis (isolation and modification of sexual genes for the expression of apomixis in *Gramineae*).	IRD and CIMMYT-ABC
WO9837184 (Aug. 27, 1998)	*Leafy cotyledon1* genes and their use (using embryo specific genes and their promoters).	University of California
US5907082 (May 25, 1999)	Ovule-specific gene expression (using ovule-specific genes).	University of California
WO9935258 (July 15, 1999)	Nucleic acid markers for apospory-specific genomic region (from the genus *Paspalum*).	University of Georgia Reseorch Found. INC.
WO9953083 (Oct. 21, 1999)	Seed specific polycomb group gene and methods of use for same (using repressors of embryo and endosperm development).	Cold Spring Harbor Lab.
WO0024914 (May 4, 2000)	Apomixis conferred by expression of SERK interacting proteins (see above WO9743427).	Novartis

* WO, US, EP, CN and SU refer to World patents, US-, European, Chinese and former Sowjet Union patents.

gametophyte (Grossniklaus, Chap. 12; Cordts and Dresselhaus, unpublished results). Through the use of mutant approaches (Vollbrecht and Hake 1995; Drews et al. 1998; Yang and Sundaresan 2000; Grossniklaus, Chap. 12; Praekelt and Scott, Chap. 13), we can anticipate that many more genes involved in female gametophyte development will soon be isolated. Gene trap screens such as T-DNA insertional mutagenesis, transposon mutagenesis, and enhancer detection (Grossniklaus, Chap. 12) are very powerful molecular tools for isolating the corresponding genes and/or their promoters from sexual model plants like maize and *Arabidopsis*. Further tissue/cell-specific genes and their promoters will be isolated by transcript profiling methods (e.g., Liang and Pardee 1992; Welford et al. 1998; Matsumura et al. 1999) and from tissue/cell-specific cDNA libraries (e.g., Dresselhaus et al. 1994; Diatchenko et al. 1996). Initial attempts have been made to compare gene expression profiles between sexual and apomictic lines within the same species. A few genes that are specifically expressed in the ovules of either sexual or apomictic lines were isolated (Vielle-Calzada et al. 1996b). These genes may eventually be useful tools for inducing apomictic development in sexual lines or sexual development in apomictic lines.

Parthenogenetic embryogenesis from unreduced eggs is the next required step for successfully engineering the apomixis trait. Whether this will occur spontaneously once the egg is diploid has yet to be shown. Quarin and Hanna (1980) found that doubling a sexual diploid *Paspalum* line generated a tetraploid that was facultative aposporous, thus unreduced egg cells developed parthenogenetically into embryos. Spontaneous parthenogenetic development was observed at a low frequency in maize (Chase 1969; Bantin and Dresselhaus, unpublished results). Wheat lines have been described that produced up to 90% parthenogenetic haploids (Matzk et al. 1995). Very little molecular data concerning parthenogenesis are available for higher plants. One protein (a-tubulin) was identified whose expression is associated with the initiation of parthenogenesis in wheat (Matzk et al. 1997). And auxin (2,4 D) treated sexual eggs from maize can be triggered to initiate embryo development at a low frequency (Kranz et al. 1995), however, the molecular mechanism is not understood. Three genes were used to successfully initiate the formation of embryo-like structures on vegetative tissue (*lec1*: *leafy cotyledon1*, Lotan et al. 1998; and *bbm1*: *baby boom1*, Boutilier et al., unpublished results) or to enhance the rate of somatic embryos in culture (*SERK1*: *somatic embryogenesis receptor-like kinase 1*, Hecht et al., unpublished results), respectively. It remains to be demonstrated whether these genes are also useful for inducing embryo development in reproductive cells.

Parthenogenesis may also arise as a function of timing, taking into account that parthenogenetic embryogenesis is usually initiated before anthesis. In contrast to sexual eggs, parthenogenetic eggs (e.g., *Pennisetum ciliare* and wheat) contain ample amounts of ribosomes and polysomes and a large number of cristae in mitochondria, thus suggesting a highly active metabolic status prior to pollination (Naumova and Vielle-Calzada, Chap. 4; Naumova and Matzk 1998). In contrast to sexual eggs, degeneration of synergids in aposporous *Pennisetum ciliare* female gametophyte was precocious and rapid. In addition, a complete cell wall around the eggs was already generated before the arrival of the pollen tube (Vielle et al. 1995). In maize, zygotic gene activation (ZGA), the switch from maternal to embryonic control of development, occurs soon after fertilization (Sauter et al. 1998; Dresselhaus et al. 1999; Bantin and Dresselhaus, unpublished). Precocious expression of zygotic genes before pollination/fertilization could thus eventually

be used as a tool to induce parthenogenetic development of sexual eggs, and perhaps those same genes might be useful for inducing endosperm development. Although the existence of repressor molecules that prevent unfertilized eggs from initiating embryo development has not been proven, it is reasonable to postulate their reality. Once isolated, they might be a useful tool for engineering parthenogenetic embryo development as a component of apomixis.

Induction of endosperm development will probably be the biggest obstacle to the utilizing apomixis in sexual crop species (discussed further under "Main Limitations"). Nevertheless, an in vitro system for endosperm development in maize was reported recently (Kranz et al. 1998), providing impetus to molecular investigations about gene expression and regulation during the earliest steps of endosperm development.

Transformation and Inducible Promoter Systems

Tremendous progress has been made in plant genetic engineering since the first reports of successful plant transformation appeared in the early 1980s, and many commercially relevant genes have been transferred to crop plants (Christou 1996). *Agrobacterium*-mediated transformation has been the method of choice for introducing exogenous DNA into dicotyledonous plants. *Agrobacterium* transformation has proven difficult with cereals, and consequently, alternative methods such as particle bombardment have been employed. Nevertheless, because *Agrobacterium*-mediated gene delivery offers many advantages (easy protocols, often low- or even single-copy integrations, mostly full-length integration of transgenes, short or no tissue culture period), considerable effort has been dedicated to establishing this method for cereals (Komari et al. 1998). *Agrobacterium* transformation of rice is now routine, while successful transformation of maize and wheat has also been reported (Ishida et al. 1996; Cheng et al. 1997). Even so, particle bombardment of wheat and maize immature scutellum tissue remains the most widely used method in most public laboratories. Relatively efficient transformation systems are now available for all major crops as well as some forage grasses (Spangenberg et al. 1998). Development of transformation systems for apomictic species is in progress, and transformation protocols for pearl millet will be established once interesting apomixis genes become available (P. Ozias-Akins, personal comm.). Transformation of *Brachiaria* and *Tripsacum* are foci of apomixis programs at the International Center for Tropical Agriculture (CIAT) and the International Maize and Wheat Improvement Center (CIMMYT), respectively.

A major problem related to transgene activity is the instability of expression (Jorgensen 1995; Matzke and Matzke 1995). Often inactivation of transgene expression is accompanied by an increase in DNA methylation (Meyer 1995). In addition, transgenes may be integrated in hypermethylated chromosomal regions displaying a spatial and temporal change of methylation during plant growth and development (position effect). Transgenes with homologous sequences to endogenous genes may be silenced through the cosuppression effect (Jorgensen 1995; Matzke and Matzke 1995). All the same, plants stably expressing the transgenes can be selected over generations, although this is time-consuming and expensive. Suggestions have been made as to how vectors used for genetic transformation can be optimized in order to minimize the cosuppression effect (Meyer 1995). Single-copy integration of transgenes will be enabled by the deployment of *Agrobacterium*-mediated gene delivery. This in turn will increase the rate of plants that stably express the transgenes. Gene targeting by homologous recombination, i.e., the

generation of null mutants, is probably the ideal way to stably silence genes. The deployment of this approach, however, is still relatively limited for higher plants (Puchta 1998). An alternative is homology-dependent gene silencing (HDGS; for review, see Kooter et al. 1999), especially through the use of double-stranded RNA (RNAi: RNA interference technology) as a template for gene silencing (Bass 2000). Gene silencing at rates up to 100% was reported with transgenic plants using the latter approach.

Inducible/repressible systems are necessary to engineer the apomixis trait, because genetic recombination through sexual crossing will always be required for the introduction of new traits into crops. In a panel discussion with industrial representatives during the Third European Apomixis Workshop (April 21–24, 1999, Gargnano, Italy), it became very clear that inducible systems for engineering the apomictic trait are highly desired (http://www.apomixis.de; see workshops), mainly because they serve as a natural means of protecting intellectual property rights (see "Intellectual Property Rights," this chapter). The question is whether such systems are practically possible, given the problems encountered with the application of gametocides. Various chemical inducible systems have been reported, e.g., the tetracycline inducible/inactivatable promoter system, and steroid-, copper- and ethanol inducible promoter systems (for review, see Gatz and Lenk 1998). Whether these systems are applicable and acceptable for use under field conditions is doubtful; spraying antibiotics, steroids, and heavy metals is environmentally unacceptable. Ethanol systems might offer an alternative. Most of these systems, however, are leaky and have some background activity, or they may be too sensitive. In addition, there is the question of how homogeneously the induction works in different organs, especially in embedded cells like megaspore mother cells and the cells of the embryo sac, which are the main target cells for the genetic engineering of different apomixis components. Seed producers anticipate efficiency rates as high as 99% for such systems (http://www.apomixis.de; see panel discussion during the Third European Apomixis Workshop). Existing systems, therefore, must be optimized, or preferably, new systems using natural, easily biodegradable, and harmless chemicals as inducers must be developed to satisfy seed producer demands and environmental necessities.

Main Limitations

Perhaps the biggest obstacle to genetically engineering apomictic grain crops is that fertilization of the central cell is likely to be required because of dosage effects (Birchler 1993; Savidan, Chap. 11) and because autonomous endosperm development occurs at low frequencies in cereals. A balanced maternal:paternal genome ratio (2m:1p) is an absolute requirement for endosperm development in cereals (Birchler 1993). In most cases, deviation from this ratio leads to embryo abortion or seeds with diminished fertility (Birchler 1993; Praekelt and Scott, Chap. 13). In contrast to cereals, Scott et al. (1998) have shown that in *Arabidopsis*, 2m:2p, 4m:1p and 4m:2p ratios are allowed. Also observed in most pseudogamous apomicts are ratios of 4m:1p and 4m:2p. In apomictic lines of the maize relative *Tripsacum*, Grimanelli et al. (1997) identified 2m:2p, 4m:1p, and 8m:1p ratios. Imprinting of gametic nuclei is the genetic reason behind this phenomenon: one set of alleles is silenced on the chromosomes contributed by the mother, while another set is silenced on the paternal chromosomes. Each genome thus contributes a different set of active alleles (Vinkenoog et al. 2000; Alleman and Doctor 2000). A few imprinted loci have

been investigated in plants (e.g., Kinoshita et al. 1999; Vielle-Calzada et al. 2000; Alleman and Doctor 2000; Crane, Chap. 3), but we are just beginning to understand the molecular mechanisms underlying these processes. Nevertheless, the combination of maternal hypomethylation in combination with a loss of *fie* function was recently shown to enable the formation of differentiated endosperm without fertilization in *Arabidopsis* (Vinkenoog et al. 2000). It remains to be demonstrated whether this approach is also feasible for crops, especially cereals, but it represents a promising step in assembling the many components needed to engineer apomixis into sexual crops.

Another obstacle that needs to be overcome is the relatively high number of genes/promoters that are required; in addition to inducible/repressible systems, it is likely that the precise and controlled interaction of many genes will have to be engineered. In natural apomicts, genes from different chromosomes are required for the expression of apomictic reproduction pathways. Blakey et al. (1997) have shown that in apomictic *Tripsacum*, genes required for seed set are located on at least five *Tripsacum* linkage groups, which are syntenic to four maize chromosome arms. Sherwood (Chap. 5) observes that the expression of apospory requires the dominant allele of a major gene or linkat and that the degree of apomixis may be further influenced by many other genes (e.g., modifiers). Fewer data are available for diplospory, but in this case as well, a single master gene or a number of genes that behave as a single locus may be required for the expression of apomixis. The technical difficulties of introducing multiple genes within a single transformation event were successfully resolved recently using *Agrobacterium*-transformation with rice (Ye et al. 2000). Four genes were integrated on one construct; by crossing transgenic lines carrying other transgenes, a whole biosynthetic pathway was engineered into rice endosperm (Ye et al. 2000).

To sum up, our understanding of the molecular regulation of apomictic and amphimictic reproduction pathways in crops, especially cereals, is still in its infancy, and thus, due to the complexity of these biological processes, modifying or controlling the pathways will probably not be achieved within the next five years.

Intellectual Property Rights

Intellectual property rights (IPR) are a means of promoting commercially relevant innovation and for sharing resources. The IPR owner obtains the right to use the intellectual property (IP) exclusively, license it, or not use it at all for a limited period (e.g., 20 years). In agricultural biotechnology and plant breeding, both scientific knowledge and its commercial applications are increasingly being claimed by companies, but also by public institutions such as universities and research centers (Spillane 1999). With hundreds of millions of dollars invested every year in plant biotechnology and breeding research, companies need effective IP protection to provide an incentive for making large research investments. These research results offer enormous benefits for agrochemical and seed companies, farmers, and the society as a whole. In the United States, IPR include (*i*) general utility patents, (*ii*) Plant Variety Protection (UPOV), and (*iii*) plant patents for asexually reproduced plants (Jondle 1999).

Given this context, it is not surprising that IPR for methods and genes/promoters that are useful for the genetic engineering of apomixis have been claimed (Table 14.2). Most of the patents were filed during the last five years, probably because of improvements in plant gene technology and in recognition of the enormous economic potential of utilizing

apomixis for crop improvement. These apomixis patents raised concerns about the use of apomixis technology. The Rural Advancement Foundation International (RAFI), a nongovernmental organization, recently expressed the concern that apomixis IPR could wind up in the hands of only a few dominate global agrobusiness players, and that farmers in both developed and developing countries might become totally dependent on their seed products. Other concerns are that genetic diversity could significantly decline and that developing countries will not have access to this technology because they will be unable to afford the required rights and licenses (RAFI 1998). The latter concern is shared by leading apomixis researchers and was formalized in 1998 in the Bellagio Apomixis Declaration (for full text, see http://billie.harvard.edu/apomixis). Signatories to the declaration were interested in how to develop novel approaches for generating the enabling technology, and how to patent and license it. Currently, patents related to apomixis enabling technology are dispersed among many parties (Table 14.2). Furthermore, it is expected that the number of patents will greatly swell as numerous public and private research institutions continue investigating different aspects of apomictic and amphimictic reproduction pathways using different species and approaches (see e.g., Bicknell and Bicknell 1999).

Another negative impact stemming from apomixis patents is that communication of research results to the scientific community is either delayed until patents have been filed or they are simply not communicated at all. A widespread phenomenon in today's biomedical research is that while IPR is growing rapidly, scarce resources are poorly utilized because too many patent owners are blocking one another. Paradoxically, more IPR may lead to fewer useful products for the improvement of human health (Heller and Eisenberg 1998). In regards to apomixis, it is unlikely that the situation will change in the near future because it is still possible to file very broad apomixis patents.

The question of whether farmers in developing countries will get access to disclosed apomixis technology remains unanswered. One can hope that many of the relevant patents will be secured by public organizations such as the Consultative Group on International Agricultural Research (CGIAR) and other public institutions (see Hoisington et al. 1999), thus giving interested parties in developing countries the possibility of acquiring free access to this powerful technology. Certainly, the public image of the big agrobusiness players would benefit from freely licensing the technology to CGIAR institutions or directly helping farmers in developing countries use this technology. The bulk of profits, after all, will be earned in the more developed countries. Introducing the apomixis trait into local varieties would give farmers in developing countries access to powerful and productive hybrid technology (Hoisington et al. 1999). To some extent, these farmers should have the right to save seed for subsequent replanting, thus allowing them to significantly increase their crop yield and personal income.

Risk Assessment Studies

Risk assessment research and studies relate to the use and or release of genetically modified organisms (GMOs) into the environment. Since the first release of genetically modified plants (GMPs) some twelve years ago, many short-term studies have been conducted (de Vries 1998). Short- and long-term risk assessment studies are also needed to evaluate the environmental implications of novel apomictic crops. One key issue for investigation is whether the apomixis trait can move to the landraces and wild ancestors of food crop

plants, and if so, what would be the impact. This issue is especially important in the centers of origin for the crop plants. Furthermore, the issue of how apomixis might affect genetic diversity, and whether it would increase or decrease monoculture farming needs to be explored. Based on field studies on herbicide and/or insecticide resistant plants, we can probably expect engineered apomixis genes to move through vertical gene transfer (transfer of a gene from plant to plant via sexual reproduction/pollen) (Lutman 1999). The rate of horizontal gene transfer (asexual gene flow between organisms) is relatively low and the risk negligible, however, microbiological risk assessment studies in this area could be useful (Syvanen 1994). Given our current knowledge, it appears unlikely that microorganisms could gain some advantage over wild relatives after uptake of apomixis genes.

If apomixis is controlled by multiple genes, the probability of diffusing this trait to wild relatives is extremely low. The transfer of several genes to a wild plant should lower its fitness to a level unacceptable for survival in the wild (Berthaud, Chap. 2). If apomixis is controlled by a single gene, which would result in obligate apomictic wild races, these races would lose their potential to evolve. If dominant, an apomixis gene could rapidly become fixed in an outcrossing sexual population. Therefore, in theory, apomixis transgenes could possess advantages that might result in the uncontrollable spread of the transgenes (van Dijk and van Damme 2000). Inducible apomictic systems and male sterility might circumvent these problems. Nevertheless, the described possibilities indicate that risk assessment studies and research to investigate the ecological implications of novel apomictic crops (once available) to the environment are an absolute necessity. In addition, socioeconomic studies on the positive and negative implications of this technology for breeders, seed companies, and farmers in both developing and developed countries (see also IPR) will be required, and the research results should be communicated to all potential users.

Summary

The extensive introduction of apomixis into sexual crops will undoubtedly rely on genetic engineering, as we anticipate that more candidate genes (especially regulatory genes and tissue/cell-specific promoters) and enabling techniques will be identified and developed in the near future. Transformation technology for all major crops is now available and inducible systems are currently being developed and optimized, allowing the control of transgene expression and activity even under field conditions. Adventious apomixis using already described or novel genes under the control of ovule-, nucellus- or archespore-specific promoters is probably the easiest way to engineer the apomixis trait. Plant breeders and seed producers would like to generate inducible obligate mitotic diplospory in combination with autonomous endosperm development. The latter is probably the most difficult aspect of engineering apomixis, especially for cereals such as wheat, rice, and maize, because of dosage and imprinting effects.

Although apomixis is a hot topic in plant research, our current understanding of both apomictic and amphimictic reproduction pathways in higher plants is still extremely limited. The economic potential of apomixis might provide the impetus to bring apomictic crops to the marketplace, and in the process it may well contribute significantly to our future understanding of the molecular regulation of the many different sexual and apomictic plant reproduction pathways.

International and interdisciplinary approaches and efforts are now needed to study and manipulate seed reproduction. It will be

necessary (*i*) to characterize the genetic regulation of apomixis and isolate the responsible genes, (*ii*) to analyze the genetic and molecular bases of sexual reproduction and to isolate the corresponding genes, and (*iii*) to produce the tissue/cell-specific and inducible/repressible promoters that will be needed to control the expression of the target genes. Concerted international research efforts have been made in Europe aimed at understanding apomictic and sexual reproduction pathways in order to develop tools for the manipulation of the apomictic trait (e.g., an E.U. Research Technology and Development (RTD) project entitled "The manipulation of apomixis for the improvement of tropical forages," coordinated by M. D. Hayward; a RTD project entitled "Apomixis in agriculture: a molecular approach," coordinated by M. van Lookeren Campagne; and a Concerted Action Project entitled "Introducing and controlling asexual reproduction through seeds in apomictic systems and sexual crops," coordinated by T. Dresselhaus). In 1999, a transatlantic consortium was initiated between two public institutions (CIMMYT and IRD) and three private companies (Pioneer Hi-Bred, Novartis, and Group Limagrain). This is just a beginning and more concerted projects are needed in order to reach the ambitious aim of manipulating the apomixis trait in crops.

Apomixis technology will offer many exciting opportunities for the agriculture of the 21st century, and indeed many patents already have been filed with many more yet to come. It is critically important that these patents be held and used for the good of all. Public institutions in particular must safeguard the access of developing countries to these enabling technologies. In all likelihood, constraints to the broad and generous use of apomixis technology will be political and economic rather than technical in the future.

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The Flowering of APOMIXIS:

From Mechanisms to Genetic Engineering

Y. Savidan, J. G. Carman, and T. Dresselhaus, Editors

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