Chapter 11

Transfer of Apomixis through Wide Crosses

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Introduction

Interspecific hybridization has been used extensively to transfer agronomically important genes that control resistances to diseases and insect pests. Recent advances in tissue culture, especially in molecular biology, have further widened the scope of alien gene transfer—and the outlook for wide hybridization in crop improvement seems more promising than ever. But does that outlook also apply to the transfer of sequences involved in plant reproduction, especially those involved in apomixis?

The interface between conventional cytogenetical approaches and new molecular techniques makes the "conventional" wide cross approach very competitive when the trait is simply controlled and the gene(s) to be transferred is (are) available in a species that belongs to the secondary gene pool. The genetic analyses reviewed by Savidan (2000) and Sherwood (Chap. 5) suggest that apomixis a good candidate and offer support for the ongoing wide cross projects. Such projects have encountered unexpected difficulties, and several papers have questioned the ultimate likelihood of transferring apomixis to any crop. Nevertheless, knowledge gathered through the quest for wide crossing apomixis into useful crop species, which relates to the genetic control, transmission, and expression of the trait (Grimanelli et al., Chap. 6), has proven extremely valuable for those investigating other approaches. Accordingly, three paths are now being pursued in the effort to introduce apomixis into major crops: (*i*) the wide hybridization, (*ii*) the identification, isolation, and manipulation of sequences from wild apomicts, and (*iii*) the creation of an apomictic reproduction *de novo*, from individual mutations (Grossniklaus, Chap. 12; Praekelt and Scott, Chap.13). In this chapter, I review progress to date and the problems or questions that have emerged from work aimed at wide crossing of the apomictic trait.

Scientists have tried for decades to use wide crosses to transfer the apomixis trait into valuable food crops, including wheat, maize, and pearl millet. The first attempt involved maize and was initiated approximately forty years ago (Petrov et al. 1979, 1984). Crossing a tetraploid maize ($2n = 4x = 40$) with a tetraploid *Tripsacum dactyloides* ($2n = 4x = 72$), the Russian scientists successfully produced maize-*Tripsacum* F_1s and BC_1 hybrid derivatives that, according to progeny tests, reproduced apomictically. The BC_1 plants combined 20 maize chromosomes with one complete set (18) of *Tripsacum dactyloides* chromosomes. Efficient techniques for evaluating chromosome numbers, embryo-sac analysis, etc., were not available, making screening of large numbers of progenies for apomixis difficult. Consequently, little progress was made in this transfer effort. Recently, the Russian materials were transferred to the United States, and introgression efforts were reinitiated. An important piece of information generated by

these renewed efforts relates to the facultativeness of apomixis: because maize-*Tripsacum* hybrids and hybrid derivatives are completely male-sterile, progress in backcrossing is linked to the degree of facultativeness, and especially the presence of B_{II} or $n + n$ off-types as a requisite for a possible return to a normal maize chromosome number. According to Kindiger and Sokolov (1995) and Kindiger et al. (1996), the *Tripsacum dactyloides* lines they are using never produce such off-types, making the transfer impossible by conventional means only.

A program initiated in pearl millet at the end of the 1970s may still be considered the most advanced and possibly the most promising (Hanna et al. 1993). Early constraints to the program (the availability of genetic resources of the apomictic wild relatives and the limitations of the available screening tools in the 1970s) slowed progress in its early years. Recent molecular mapping activities, however, have provided intriguing new data (Ozias-Akins et al. 1993, 1998; Leblanc et al. 1995; Grimanelli et al. 1998a, and Chap. 6) and offered encouragement to scientists working in this area.

Efforts to wide cross apomixis to wheat have struggled to bypass crossing barriers and very low apomixis expression in the wheat genome background. Carman (1997, Chap. 7) now concludes that apomixis can only be expressed from interspecific hybridizations using progenitors with contrasting timings of megagametophyte development.

The maize-*Tripsacum* introgression project developed jointly by IRD (formerly ORSTOM) and CIMMYT, which serves as the focus of this chapter, has passed the BC_5 generation. Early steps and progress are reported in the following pages, as are several fundamental questions that must be addressed before apomixis can be successfully transferred to major crops.

Source of Apomixis and Choice of Parental Materials

Basic Traits to Consider

Identifying a material for molecular studies, especially for apomixis gene isolation, is discussed in other chapters (especially Bicknell, Chap. 8). Choosing a progenitor specifically as a source of apomixis for wide cross transfer, however, is a somewhat different venture. In this instance, such work should take into account the following:

1. Genetic resources available. With only a few exceptions (*Elymus rectisetus*, *Tripsacum* spp.), collection of the wild apomictic relatives of important food crops has been notably inadequate. Consequently, wide cross projects have been forced to rely on a limited number of introductions. This in turn means that the diversity present in the wild relatives may go undetected, and that scientists do not gain access to the components of this diversity that offer optimum crossability with the crop species. A preliminary effort to collect such genetic resources of interest is critically needed in most cases.

2. Chromosome number of the potential donor species. An important factor for wide crossing apomixis is whether the basic chromosome number is the same in the donor and crop species. Crosses between related species with different basic chromosome numbers are generally considered less likely to succeed. Ploidy level is another point to consider, since wild apomixis is found almost exclusively in polyploids (for exceptions, mostly in the dicots, see Asker and Jerling 1992).

3. Genome homoeology. Chromosomal exchanges are more likely to occur when the chromosomes of the two species show some degree of pairing. Molecular genetics, seldom available at the beginning of such projects, can likely provide more detailed information about chromosome homoeology than classical

cytogenetics, e.g., in *Tripsacum*, Galinat (Galinat et al. 1970; Galinat 1971) described four maize chromosomes that are capable of pairing with *Tripsacum* chromosomes. Meanwhile, mapping analyses (Grimanelli et al., Chap. 6) suggest a more widespread colinearity between the maize and *Tripsacum* genomes.

4. Pollen fertility. Except for the highly facultative apomicts, first generation hybridizations between the crop and donor species must use the latter as male. Several apomictic species have been described with greatly reduced male fertility (e.g., *Elymus rectisetus*, the apomictic wild relative of wheat); in such cases, a preliminary selection is needed.

5. Type of apomixis. Apospory has always been presented as an easier type of apomixis to work with, being associated with 4-nucleate embryo sacs in tropical and subtropical grasses and transmitted as a single dominant gene (Savidan 1982a; Nogler 1984; Asker and Jerling 1992; Savidan 2000). Recent studies on diplospory in *Tripsacum* strongly challenge this view, bolstered by flow cytometry, which can be used to analyze modes of reproduction (Grimanelli et al. 1997), and screens that use different types of molecular markers. Nevertheless, the type of apomixis must still be considered, as different types of screens may be applied to different types of apomixis. Whether one type of apomixis than another is more likely to be expressed in a particular crop background is still largely speculative.

6. Degree of apomixis (or degree of facultativeness). The degree of apomixis appears to be a major factor related to the feasibility of wide cross transfer of apomixis. An obligate apomixis cannot be used unless some degree of male fertility is recovered in the F_1s, which is seldom the case in interspecific hybrids; but to produce near obligate apomictic crops, facultativeness must be low and well controlled. This factor is addressed in more detail later in this chapter.

7. Agronomic characteristics. A species with poor agronomic traits will produce hybrids and hybrid derivatives that may conserve undesirable traits for several generations, slowing the progress of the transfer.

8. Previous knowledge. Previous knowledge concerning the interspecific or intergeneric hybridization under consideration is a definite advantage. For example, knowing the number of backcrosses needed to go from the maize-*Tripsacum* F_1s to a 20-chromosome recovered maize (Harlan and de Wet 1977) was important in developing the first work plan for the IRD-CIMMYT apomixis team and in maintaining its confidence about the feasibility of its approach.

Case History: *Pennisetum*

Pennisetum glaucum, a cultivated pearl millet, has a basic chromosome number of $x = 7$. The only known and widespread tetraploid wild species with the same basic chromosome number is *P. purpureum* ($2n = 4x = 28$). Though described as aposporic by Brown and Emery (1958), this species appears to be entirely sexual, as confirmed by a cyto-embryological survey made in morphologically uniform wild populations from West Africa (Y. Savidan, unpublished). Apomixis has been described in several other *Pennisetum* species, all of which belong to the secondary or tertiary gene pools and share a basic chromosome number of $x = 9$. Dujardin and Hanna (1989) demonstrated that three out of the seven apomictic species tested were capable of producing F_1 hybrids with pearl millet. The genus *Pennisetum*, however, is one of the most complex in the grass family. In addition, the number of species varies greatly according to the taxonomist, the most conservative estimates being approximately 100 different species (Purseglove 1972), most of which are perennial, polyploid, and likely apomictic. Because of a lack of available germplasm, no extensive

search for an opitimum apomictic donor for pearl millet has been conducted. Three species studied by Dujardin and Hanna (1989) that show crossability with the crop are *Pennisetum orientale*, a tetraploid with $2n = 36$; *P. setaceum*, a triploid with $2n = 27$; and *P. squamulatum*, a hexaploid with $2n = 54$. They all reproduce apomictically and their apomixis was described as obligate, which means that 100% of the observed progeny appeared to be maternal in field tests (Dujardin and Hanna 1984a, b).

Advantages of *P. squamulatum* as a donor species for apomixis include good pollen fertility, 4-nucleate embryo sacs, and a unique

Issue # 1. Obligate vs. Facultative Apomixis: An Artifact?

The facultativeness of apomixis has been considered to be a disadvantage (Bashaw et al. 1970; Bashaw 1975) because (*i*) it may result in uncontrolled variation in the progeny while farmers require homogeneous varieties and (*ii*) it is apparently quantitatively inherited, i.e., under a complex, yet unknown genetic control. Nevertheless, facultativeness may be needed in attempts to transfer apomixis to crops through wide hybridization. Wide crosses generally produce highly sterile hybrids that can only be backcrossed by using them as female. If these hybrids are obligate apomicts, the wide cross approach for transferring apomixis is a dead end. But is obligate apomixis ever totally obligate?

Asker (1979) assigned a question mark to obligate apomixis. The developmental process has been described at the ovule level, where meiosis succeeds or fails. At the plant level, obligate apomixis is already questionable. At the level of the population or species, obligate apomixis is likely an artifact of the screening tools (see Leblanc and Mazzucato, Chap. 9).

A large number of ovules in the case of *P. squamulatum* (Dujardin and Hanna 1984a) have been examined and 8% were classified as aborted based on the absence of a normally developed embryo sac. In *Panicum maximum*, another aposporous tropical forage grass, ovules with no sac could be either abortive, in which case they show enlarged nucellar cells with little or no cytoplasm in an overall shrivelled ovary, or in early meiotic development stages. Sexual embryo sacs (ES) were significantly late as compared to the nucellar unreduced ES (Savidan 1982a). Differences in timing of development between meiotic and apomeiotic embryo sacs should be considered in order to provide an accurate estimate of the degree of facultativeness. This difference has been found in several aposporous species aside from *Panicum*, e.g., *Ranunculus auricomus* (Nogler 1984), *Brachiaria* spp (Ndikumana 1985), *Paspalum notatum* (Martinez et al. 1994), and diplosporous *Tripsacum* species (Leblanc and Savidan 1994), among others. Dujardin (personal comm.) confirmed that the nucelli from ovules he classified as aborted were perfectly normal, hence apomixis in the *P. squamulatum* introduction was perhaps not as obligate as originally thought.

Recent data (Hanna et al. 1993) showing high degrees of facultativeness in later generation hybrid derivatives can possibly be reinterpreted in the light of this hypothesis. Modification of the genetic or epigenic background is known to affect facultative apomixis expression, with an extremely high rate of sexuality possibly being observed. This was seen in guineagrass (*Panicum maximum*), in one natural interspecific hybrid with *P. infestum* (see also Berthaud, Chap. 2).

Though apomixis is probably always facultative in the wild to some extent, the facultativeness of the donor species in a transfer attempt should be limited and/or controllable for apomixis to be properly manageable in agriculture. Therefore, a compromise must be found between the facultativeness required for male sterile hybrids to be backcrossed, and the final objective of relative homogeneity in the farmers' fields.

potential, among the few species tested, for giving some female *and male* fertility to the F_1s. Disadvantages include the requirement of a bridge species, *P. purpureum*, the different basic chromosome number ($x = 9$, as compared with $x = 7$ in pearl millet), and the hexaploid level of ploidy. Progress made on mapping apomixis in *Pennisetum* and its implications for our understanding of the genetic control are presented in Grimanelli et al. (Chap. 6).

Case History: *Tripsacum*

Numerous maize x *Tripsacum* hybrids have been produced since the pioneering research of Mangelsdorf and Reeves more than 70 years ago (Mangelsdorf and Reeves 1931). Extensive hybridization studies have been carried out by Galinat (1971), Harlan and de Wet (1977), James (1979), and Bernard and Jewell (1985), among others. The main objective of these studies was to evaluate the potential role of *Tripsacum* in maize evolution and/or the feasibility of gene transfer, though not necessarily for apomixis. Claims of introgression have been made (Simone and Hooker 1976; de Wet 1979; and Bergquist 1981), but the *Tripsacum* progenitors involved were not tested beforehand for the target traits; consequently, the same traits could presumably have been present in neighboring maize collections. However, all these studies showed that from a maize-*Tripsacum* F_1 hybrid it was possible, in a few generations, to recover a 20-chromosome maize with some morphological features that were not present in the original maize progenitor. Most of these studies were based on using a diploid sexual *Tripsacum*, and most concentrated on a single species, *T. dactyloides*. Between 1990 and 1992, maize was successfully crossed with 66 apomictic populations, representing eight different species and intermediate forms between species (Table 11.1); 895 F_1 hybrids with $2n = 46 = 10M + 36Tr$ were obtained from these crosses. Most of these (598, or 66.8%) involved *T. dactyloides* subspecies or interspecific-like accessions involving some form of *T. dactyloides*. This confirmed high crossability for *T. dactyloides*. The number of F_1 plants per number of pollinated ears, however, showed a higher crossability between maize and *T. zopilotense*, which has the smallest area of distribution in Mexico (being found only in the Cañon de Zopilote, between Mexico City and Acapulco).

Advantages of using *T. dactyloides* as the donor species include good pollen fertility and an apomixis characterized by an absence of callose around the megasporocyte and subsequent cells, which is easily detected in fluorescence microscopy (Leblanc et al. 1995b; Leblanc and Mazzucato, Chap. 9). Diplospory is further characterized by endosperms with a ploidy level different from that of sexual seeds, resulting from the fertilization of two

Table 11.1 Crossabilities between maize and wild *Tripsacum* species and presumed natural interspecific hybrids

code	nb.pop	ears	emb.	cult.	F1s	F1s/ear
ZP	2	41	860	573	118	**2.88**
DT	2	92	324	169	97	**1.05**
iMZ	2	23	1119	140	20	**0.87**
iIT	6	103	1143	427	83	**0.81**
iDH	7	132	1527	452	84	**0.64**
iPL	4	65	2169	257	33	**0.51**
DH	30	776	10816	2892	386	**0.50**
PL	1	4	10	-	1	**0.25**
IT	5	132	779	123	32	**0.24**
iDM	3	75	2513	444	14	**0.19**
DM	7	121	3655	813	17	**0.14**
LC	1	10	38	5	1	**0.10**
BV	5	96	2091	390	7	**0.07**
iBV	2	62	1847	352	2	**0.03**
PR	1	-	-	-	20	-
average		1732			895	**0.52**

nb.pop.= number of populations studied; ears= number of maize ears pollinated with the *Tripsacum* species; emb.= number of counted embryos, three weeks after pollination; cult.= number of embryos cultured; F_1s= number of F_1 hybrids grown to maturity. Species codes: ZP= T.zopilotense; DT= T.dactyloides dactyloides (US types); MZ= T.maizor; IT= T.intermedium; DH= T.dactyloides hirsutum; PL= T.pilosum; DM= T.dactyloides mexicanum; LC= T.lanceolatum; BV; T.bravum; PR= T.peruvianum; i= intermediate forms (presumes natural interspecific hybrids).

unreduced polar nuclei. This trait can also be used for screening modes of reproduction in segregating populations by means of flow cytometry (Grimanelli et al. 1997). Previous studies showing that 5–6 backcrosses are needed to produce introgressed 20-chromosome maize plants provided another advantage to using this species. Disadvantages include total male sterility, which is seemingly retained until reaching addition forms with very few *Tripsacum* chromosomes, and the difference in basic chromosome numbers (x = 18 compared to x = 10 in maize).

Production of Interspecific or Intergeneric F_1 Hybrids

Several procedures are available to produce hybrids between cultivated and distantly related wild species. Special techniques, including chromosome manipulation, bridging species, hormonal treatment, embryo rescue, ovary culture, and in vitro pollination, are available for overcoming the cross incompatibility and the sterility of the F_1s. The presence of apomixis makes the cross more difficult because it can only be performed in one direction, with the apomixis progenitor being used as pollinator. Therefore, the donor must exhibit good pollen fertility. Because most apomicts require fertilization with reduced pollen to produce endosperm, pollen quality is generally not affected by apomixis. An exception to this rule is *Elymus rectisetus*, in which male infertility is a problem with most accessions (J. G. Carman, personal comm.).

Crossing Techniques

Most of the crossing techniques are common to intra- and interspecific crosses. A prerequisite is good knowledge of the self-sterility or self-incompatibility systems existing within the crop. For most crops, however, hand emasculation is preferred.

Crossing species with different flower sizes and shapes may require special tricks, e.g., in the case of maize x *Tripsacum*, more hybrids are produced if the silks are shortened to about 2–3 cm. Most wide crosses require embryo rescue techniques, using classical media such as MS (Murashige and Skoog 1962) or N_6 (Chu et al. 1975). Small embryos from maize x *Tripsacum* F_1s grew better on 50 g/l sucrose as compared with standard embryo culture medium containing 30 g/l sucrose. Several environmental factors can further affect the production and culture of hybrid embryos. As a result, the production of hybrids may be good one year, but poor the next.

When apomixis is not found in wild relatives, transfer may be attempted from a more distant apomictic species by using protoplast fusion. Such a transfer was started for sorghum using apomixis from *Cenchrus ciliaris* (Bharathi et al. 1991). However, no reports of plant regeneration have surfaced to date, apomictic or not, from such protoplast fusions. A more recent approach, developed by Ramulu et al. (1996), explores the production of microprotoplasts containing only one or two alien chromosomes and the direct production of monosomic addition lines after fusion with protoplasts from the receptor species.

Sterility of the F_1s

Sterility in interspecific and intergeneric F_1s and subsequent backcross generations is a characteristic of wide crosses. Restoring fertility of the F_1 hybrids through chromosome doubling is the most common approach. In both pearl millet and maize transfer attempts, however, F_1s from some wild species accessions were totally sterile, while those obtained from other accessions showed some degree of fertility, making the chromosome doubling unnecessary.

The transfer programs in pearl millet and wheat have produced F_1 hybrids with some

degree of male fertility. However, as described below for *Tripsacum*, this is not an absolute requirement. Nevertheless, it obviously helps, because the F_1s generally have morphological features close to that of the wild progenitor, e.g., a limited number of fertile flowers to pollinate. In maize, the F_1s have less than 20 flowers per inflorescence, while the recurrent maize parent, if it could be used as female (i.e., if the F_1 hybrid had some male fertility), would offer hundreds.

Pennisetum setaceum ($2n = 3x = 27$) was the first apomictic species crossed with pearl millet. F_1 hybrids had $2n = 25$ chromosomes, were male sterile, but reproduced apomictically (Hanna 1979). This interspecific cross was abandoned because of male sterility. *Pennisetum orientale* ($2n = 4x = 36$) was then crossed with pearl millet. F_1 hybrids had $2n = 25 = 18$ *P. orientale* (Or) + 7 pearl millet (Pm) chromosomes (Hanna and Dujardin 1982). They were male sterile, but backcrossing was attempted using pearl millet as the pollinator.

Pennisetum squamulatum ($2n = 6x = 54$) was successfully used to pollinate tetraploid pearl millet. Crosses with diploid pearl millet failed (Dujardin and Hanna 1989). Of 20 F_1 hybrids, 15 were facultative apomicts, based on embryo-sac analyses. One F_1 was classified as an obligate apomict, although 35% of the ovules were considered aborted. This may possibly be interpreted in another way if the timing of sexual and aposporic pathways of development is different (see *Issue # 1*). Pollen fertility of this hybrid was surprisingly high (66%) and therefore it was used to pollinate tetraploid pearl millet to produce a BC_1 progeny. The BC_1 plants were totally male sterile. The breakthrough was found in making a tri-specific hybrid. The pearl millet x *P. squamulatum* male fertile F_1 (classified as an obligate apomict) was used to pollinate a pearl millet x napier (*P. purpureum*) F_1, and 1,730 hybrids were produced. A sample of 64 segregated 31 apomictic (30 classified as obligate) and 30 sexual, which suggests dominance of apomixis over sexuality.

Relative crossabilities in maize x *Tripsacum* and pearl millet x wild species of *Pennisetum* are shown in Tables 11.1 and 11.2, respectively. According to J. G. Carman (personal comm.), the crossability between wheat and apomictic *Elymus rectisetus* as measured by the same F_1s/ear ratio was less than 1%. Differences in crossability may possibly be due to relative differences in genetic distance between the crop and its wild relatives or to genetic effects.

Production of Apomictic Progenies through Backcrossing

Facultativeness becomes especially important when interspecific or intergeneric hybrids are totally male sterile. Dujardin and Hanna (1989) considered male sterility as an impediment to the transfer of apomixis because their progenitors were apparently obligate apomicts. This was certainly reasonable based on the available techniques and limited number of plants used for analysis at the beginning of their project in the early 1980s. In the progenies of the maize x *Tripsacum* BC_3

Table 11.2 Crossabilities between pearl millet and three apomictic wild *Pennisetum* species

Cross combination	ears	F1s	F1s/ear
pearl millet ($2n = 14$) x P.orientale ($2n = 36$)	88	20	**0.23**
pearl millet ($2n = 28$) x P.orientale ($2n = 36$)	70	2	**0.03**
pearl millet ($2n = 14$) x P.setaceum ($2n = 27$)	7	28	**4.00**
pearl millet ($2n = 28$) x P.squamulatum ($2n = 54$)	59	337	**5.71**
average	224	387	**1.73**

ears = number of pearl millet inflorescences pollinated with the Pennisetum wild species; F_1s= number of F_1 hybrids grown to maturity.

Issue # 2. Is facultativeness controllable?

Bashaw et al. (1970) and Bashaw (1975) presented facultative apomixis as a difficult trait to manipulate in breeding because of uncontrolled variation (off-type frequency) that may result from crossing such apomicts with sexual plants. Our experience with aposporous *Panicum maximum* suggested that facultative apomixis, when the rate of facultativeness was low (1–5%), could be maintained with the same or even lower rate of sexuality through consecutive generations of hybridization. In such cases, the F_1 and BC_1 hybrids between sexual and apomictic guineagrass accessions had the same degree of facultativeness as their apomictic progenitor (Savidan 1982b). On the other hand, crossing a highly facultative apomict with sexual guineagrass accessions produced a large variation for the rate of facultativeness among the apomictic hybrids (Savidan 1982b). Whatever the complexity of the genetic control of facultativeness, it seemed to be transmitted as a cluster along with the control of apomeiosis (Savidan, 1982). *Tripsacum* diversity was not screened for facultativeness. Whether sexual x apomictic *Tripsacum* intra- or interspecific crosses may result in a similar conservation of the degree of facultativeness is therefore unknown. Maize x *Tripsacum* hybrid derivatives could exhibit contrasting rates of facultativeness, despite having originated from the same apomictic F_1 hybrid. Given our current state of knowledge, this may be either a characteristic of *Tripsacum* apomixis or only a consequence of the intergeneric, genetic, and / or epigenetic backgrounds.

hybrid derivatives, only 0.9% of the plants apparently resulted from fertilization of a reduced egg cell, i.e., the rate of diplospory in BC_3s was 99.1%, which would probably not be detectable if only 30 or 40 plants were analyzed in a progeny test.

The obligate nature of apomixis may be overestimated because of the population size, e.g., Burton et al. (1973) classified approximately 80% of their *Panicum maximum* accessions as obligate apomicts based on 10-plant progeny tests. Savidan (1982b), however, found only 20% of such obligate apomicts using a 100-ovary embryological analysis for each accession. Therefore, the male-sterile apomictic interspecific F_1 hybrid may probably always be used as female in the backcross, provided progenies of sufficient size can be screened. One can expect that a few off-types will be produced from sexual reproduction ($n + n$ combinations) to help bypass the sterility barrier. Some may reproduce apomictically, assuming the apomixis "allele" is dominant and simplex, as observed in all sexual x apomictic hybrids produced so far in the grass family (see Nogler 1984 for review; Sherwood, Chap. 5). In *Pennisetum*, male sterile apomictic hybrids could have been a good starting point for the transfer of apomixis if flow cytometry had been available for screening of large progenies, but the technology only became available to plant scientists several years after the project began (Galbraith et al. 1983).

The BC_1 plants from pearl millet x *Pennisetum orientale* hybrids had 23, 27, or 32 chromosomes. The latter were $2n + n$ off-types with 25 + 7 Pm, as pearl millet was used as pollinator. The 23-chromosome plants were described as facultative apomicts, with a low rate (or expression) of apomixis.

From the crosses with *P. setaceum*, a $2n = 27$ BC_1 plant appeared to be totally male sterile, but could be pollinated by pearl millet or *P. setaceum*. Pollination with pearl millet produced no seed, while pollination with *P. setaceum* produced four plants, three maternal and one $2n + n$. The *P. orientale* pathway was considered unsuitable for apomixis transfer because of the low expression of apomixis or complete male sterility in the BC_1 derivatives.

Hybrids that are totally male sterile and obligately apomictic are indeed dead ends: pollinating such hybrids with the crop pollen will produce only maternal offspring, i.e., perfect copies of the sterile F_1. However, if apomixis is slightly facultative, off-types can be produced, some of which may be $n + n$ and still apomictic, representing progress toward a return to the chromosome number of the crop. The rate of facultativeness has to be low, however, if one expects the backcross procedure to eventually produce an apomictic crop germplasm with a high degree of apomixis. Analyses made on *Panicum maximum* (Savidan 1982a,b) show that the rate of facultativeness, and more precisely of $n + n$ off-types, may remain relatively conserved through generations of hybridization. It was therefore suggested that a limited range of variation could possibly allow selection back to obligate apomixis. In the intergeneric background of maize x *Tripsacum* hybrid derivatives, the variation observed (Table 11.3) appeared less stable, possibly because the apomictic *Tripsacum* progenitor was already much more facultative than the guineagrass accessions used by Savidan (1982). By selecting among *Tripsacum* accessions for their ability to produce hybrid derivatives in backcrossing F_1s with maize, the team possibly selected one of the most facultative of the apomictic tripsacums.

Table 11.4 shows the cumulative result of the analysis of approximately 6,000 progenies produced from maize x *Tripsacum* BC_1s with

Issue # 3. Can apomixis be expressed at the diploid level?

In the wild, apomixis is found only among polyploids (although a few, questionable exceptions have been cited, see Asker and Jerling 1992). Population geneticists have suggested that sexuality would be eliminated if apomixis could be expressed at the diploid level (Pernès 1972; Marshall and Brown 1981). Nogler (1984) claimed, with little evidence to support it, that apomixis is probably linked to a lethal factor expressed at the haploid (gamete) level only. After obtaining 23-chromosome pearl millet x *P. orientale* BC_1 plants, Hanna et al. (1993) stated that polyploidy is probably not needed for the expression of gene(s) controlling apomixis, because these 23-chromosome plants had only one (simplex) set of nine *P. orientale* chromosomes. The genomic structure of these plants is likely 14 Pm + 9 Or however, suggesting that the locus involved could possibly be present in triplicate. Another such case of apomictic expression in a nonpolyploid form was previously reported (Dujardin and Hanna 1986), which related to a polyhaploid plant from a pearl millet x *P. squamulatum* F_1 hybrid which had $2n = 41 =$ 14Pm + 27Sq. This haploid had $2n = 21$ chromosomes. Again, as the $2n = 21$-chromosome plant likely had seven chromosomes from pearl millet and 14 from the wild species that had a basic chromosome number of nine, the locus involved was possibly in triplicate and not in duplicate.

In the *Tripsacum* project, a few polyhaploids were obtained in the progeny of $2n = 56 =$ 20m + 36tr BC_1s (Leblanc et al. 1996). These plants have one set of maize and one set of *Tripsacum* chromosomes, as confirmed by in situ hybridization (Leblanc et al. 1996), and some of them could express apomixis. Whether they represent exceptional cases of recombination between apomixis and a lethal system linked to it is open to speculation (see Grimanelli et al. 1998b). Grimanelli et al. (1998b) suggest, however, that apomixis can be expressed even when the allele(s) involved are in a duplex situation, a position that rejects the hypotheses of dosage effect presented earlier by Mogie (1988) and Noirot (1993), and suggests that the transmission barrier, whatever its nature, may be overcome through haploidization to produce functional diploid apomicts.

$2n$ = 56 chromosomes, i.e., 20 maize + 36 *Tripsacum* chromosomes. Note that the average rate of facultativeness at that level was very close to that of the *Tripsacum* progenitor, although variation was important.

A few dihaploids have been obtained from the progeny of $2n = 56$ BC_1s, as $n + 0$ off-types (Table 4). They grew well, flowered, and produced a good seed set. Their progeny were 80% maternal and 20% $2n + n$ hybrids with $2n$ = 38 chromosomes.

The backcross series was continued in an attempt to recover apomictic maize plants with only a few *Tripsacum* chromosomes. At each generation, plants were screened for apomixis and chromosome number. Embryo-sac analyses, which have been used extensively in several genetic analyses (Sherwood, Chap. 5), cannot be applied to intergeneric hybrids or hybrid derivatives in which inflorescences are too precious to be destroyed. Modes of reproduction are therefore estimated using progeny tests, e.g., a $2n$ = 38 maize x *Tripsacum* BC hybrid that produces mostly $2n$ = 38 progenies is likely to be apomictic, while a $2n$ = 38 maize x *Tripsacum* BC hybrid that produces progeny ranging from $2n$ = 22 to $2n$ = 32 is sexual. An alternative can be offered by using markers linked with apomixis, provided that apomixis is indeed controlled by one gene or small segment of DNA, and that such markers are closely linked.

A 1:1 segregation for apomixis and sexuality was observed among maize x *Tripsacum* F_1s, as 31 hybrids were classified as apomictic and 30 as sexual, based on embryological analyses. These plants were used for a bulk segregant analysis (see Grimanelli et al., Chap. 6) aimed at identifying molecular markers that cosegregate with apomixis. Three RFLP markers were first identified as linked with apomixis; these belong to the same linkage group in maize and are located on maize chromosome-6 long arm (Leblanc et al. 1995). Other markers were subsequently added (Grimanelli et al. 1998a, and Chap. 6).

Using both flow cytometry and marker-assisted screening for apomixis, rare but useful apomictic plants can be selected among many at each generation. A source population must be grown to constantly produce new progeny until the next generation population is large enough to enable progress to be achieved in the backcross program. With a rate of only 3% useful plants, we decided to raise the BC_1 population to 3,500 plants. After about 6,000 progeny had been analyzed, we substituted this BC_1 nursery with a BC_3 nursery obtained from in vitro multiplication of the $2n$ = 38 apomictic off-types produced by the BC_2 polyhaploids ($2n$ = 28). More than 2,500 BC_3s were established in the field. The analysis of a 125,000-

Table 11.3 Facultativeness of apomixis and diplospory rate in the *Tripsacum* accession used in the backcross transfer of apomixis into maize and three BC_1 progenies, showing variation for this rate. D: diplospory rate.

	No. of progenies	2n+0 maternal	2n+n off-types	n+n off-types	others	D %
T.dactyloides						
#65-1234	98	69	26	3	0	96.9
BC_1-6-82	55	40	15	0	0	100
BC_1-6-52	98	73	22	1	2	99.0
BC_1-5-45	78	63	6	8	1	89.7

Table 11.4 Chromosome numbers of BC_1 ($2n = 56$) progenies as estimated by flow cytometry

Progenies total no.	maternal 2n+0=56	off-types 2n+n=66	off-types n+n=38	off-types n+0
6259	5006	1024	218	11
%	80.0	16.4	3.5	0.2

plant progeny is shown in Table 11.5, in which the rate of $n + n$ off-types was below 0.2%. Almost 200 hybrid derivatives have been produced and classified as BC_4, with chromosome numbers ranging from $2n = 20$ to $2n = 36$. Modes of reproduction could be being determined for some of them by RFLP markers linked with apomixis, by progeny-tests, or by ploidy of the endosperms evaluated through flow cytometry (Table 11.6). The progeny size was recently increased further.

Screening the modes of reproduction through flow cytometry is a unique opportunity offered by diplosporous species such as *Tripsacum dactyloides*. In sexual plants, triploid endosperms result from the fertilization, by a reduced pollen, of two reduced polar nuclei. Diplosporous plants form endosperm as a result of the fertilization of two unreduced polar nuclei by a reduced pollen. The difference is shown in Figure 11.1. Diploid sexual plants have triploid endosperms (peak 2 in Figure 11.1a), while tetraploid apomictics produced endosperms (peak 2 in Figure 11.1b), with a DNA content 2.5 times that of the embryos (Grimanelli et al. 1997).

Preliminary data indicated apomixis could be transmitted to the BC_4 generation, although no fertile apomictic BC_4 had been confirmed as combining 20 maize chromosomes with less than 16 *Tripsacum* chromosomes (Table 11.6). Increasing the progeny size did not change the trend, an observation suggesting that the original transfer scheme (Figure 11.2) had to be reconsidered, especially since its 38-chromosome plant step could not produce the addition lines that were expected.

Table 11.5 Maize x *Tripsacum* BC_3 progenies, in which the BC_4s are the $n + n$ category

Progenies total no.	maternal 2n+0=38	off-types 2n+n=48	off-types n+n=20-36	off-types n+0=10, 28	others*
125916	114602	10778	158	78	300
(%)	91.01	8.56	0.12	0.06	0.24
reproduction	apomictic	apomictic	segregating	sexual, apo	apomictic

* mostly 4n (restitution nuclei)

Table 11.6 Maize x *Tripsacum* BC_4 with known mode of reproduction

Plant	2n	ISH*	RFLP	Endo	PGT	plant	2n	ISH	RFLP	Endo	PGT
1496	20		Sex			1457	27	13M+14Tr	Sex		
1500	20		Sex			1476	27		Apo		
1502	20	20M	Sex			1460	28	20M+8Tr?	Sex		Sex
1503	20		Sex			1484	28	20M+8Tr?	Sex		Sex
1516	20		Sex			1348	30		Apo		
1529	20		Sex			1346	31		Apo		
1454	21		Sex		Sex	1347	31		Apo		
1482	21		Sex		Sex	1439	31				Apo
1489	21		Sex			1453	31		Apo		
1492	21		Sex		Sex	1479	31	17M+14Tr	Apo		
1535	21		Sex		Sex	1276	32		Apo		
1275	22				Sex	1339	32		Sex		
1338	22		Sex		Sex	1426	32		Apo		
1345	22		Sex			1306	33		Sex		
1422	22		Sex			1349	33	18M+15Tr	Apo	Apo	Apo
1499	22	20M+2Tr			Sex	1493	33		Apo		Apo
1534	22		Sex			1532	33		Apo		
1393	23	20M+3Tr	Sex		Sex	1313	34				Sex
1515	23		Sex			1394	34	16M+18Tr	Sex?		Apo
1229	24		Sex			1494	34	16M+18Tr	Apo	Apo	Apo
1425	24		Sex		Sex	1517	34		Apo		Apo
1481	24		Sex			1521	34		Sex		Apo
1526	24	20M+4Tr	Apo	Apo	Sex?	1522	34		Apo		Apo
1528	24	20M+4Tr	Sex		Sex	1523	34		Apo		
1471	25	20M+5Tr	Sex		Sex	1544	35				Apo
1501	25	20M+5Tr			Sex	1308	36	20M+16Tr	Apo		Apo

*ISH: in situ hybridization data; RFLP: use of markers linked to apomixis; Endo: flow cytometry analysis of the ploidy of the endosperms; PGT= progeny-test (chromosome counts).

Transfer of Gene(s) for Apomixis from an Alien Chromosome to the Crop Genome

Possibilities of recombination between maize and *Tripsacum* chromosomes are extremely limited before the BC_3 generation. As shown in the scheme presented in Figure 11.2, the only meiotic event prior to this level occurs with BC_1 plants. However, pairing is preferentially maize-maize (M-M) or *Tripsacum-Tripsacum* (Tr-Tr) (Engle et al. 1974), although trivalent and tetravalent associations have been infrequently reported (Engle et al. 1973). In the BC_3s, 20 chromosomes of maize are associated with one haploid set of *Tripsacum* chromosomes, and some M-M-Tr pairing may occur. The same may happen in later generations with less *Tripsacum* chromosomes. Associations between maize and *Tripsacum* chromosomes have been reported to increase with each BC generation (Engle et al. 1973), however, they seem to involve a limited number of maize chromosomes.

Addition lines with $2n = 21$ to 24, whenever and whatever way they are produced, are expected to show some degree of male fertility, as observed in all previous studies. Levels of fertility may vary according to the number and quality of these alien chromosomes. Most of their progeny, using them as male, will likely be $2n = 20$ because of chromosome elimination and pollen competition.

The next step in transferring apomixis to maize is still to produce fertile addition lines with one to three *Tripsacum* chromosomes. This on its own remains a large challenge, although several indirect avenues are presently under investigation. Pairing and recombination

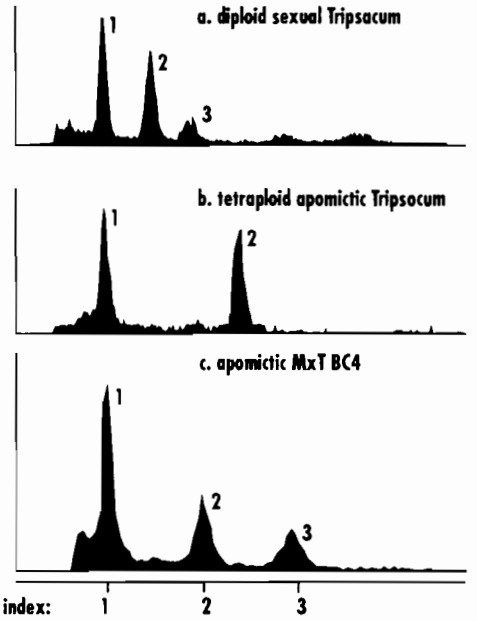

Figure 11.1 Flow-cytometric analyses on entire seeds. a. $2n = 36$ diploid sexual *Tripsacum*; peak 1: embryo ($2n = 36$), peak 2: endosperm ($2n = 54$), peak 3: duplicated cells from the embryo (G2 stage of cell cycle); b. $2n = 72$ tetraploid apomictic *Tripsacum*; peak 1: embryo ($2n = 72$), peak 2: endosperm (relative DNA content suggests $2n = 10x = 180$); c. $2n = 24$ BC4 maize-*Tripsacum* hybrid; peak 1: embryo ($2n = 24$), peak 2: duplicated cells from the embryo (G2 stage of cell cycle), peak 3: endosperm (relative DNA content suggests $2n = 2x + 2x + x + x = 68$)

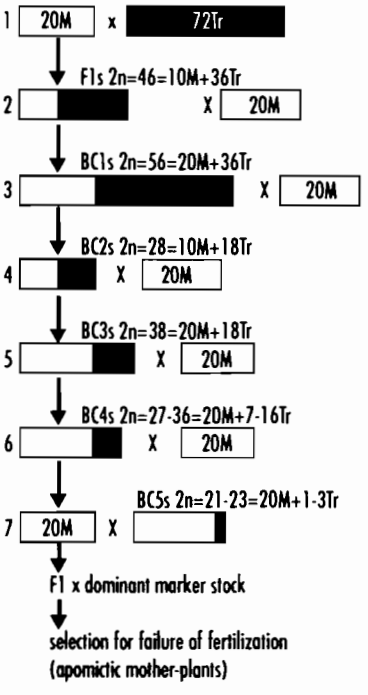

Figure 11.2 Backcross scheme for the transfer of apomixis from *Tripsacum* into maize.

between the *Tripsacum* chromosome-arm controlling apomixis and its homologous segment in maize may not occur spontaneously if recombination around apomixis is limited or impossible. Apomixis would then require the artificial induction of such a recombination. Several agents are available to induce artificial translocations. Final screening will have to be made on very large-scale progenies, and flow cytometry will be of little value because of the small differences in chromosome numbers and the small size of *Tripsacum* chromosomes. Dominant marker systems would be useful in screening between plants derived from fertilized eggs and those derived from parthenogenetic and unreduced eggs. Adequate stocks can be built up, taking advantage of available maize collections.

Is the transfer of apomixis to maize through wide hybridization feasible? Could it possibly result in a functional diploid apomictic germplasm? When? These questions remain

Issue # 4. Can a diploid apomict produce a normal seed?

Most apomicts require fertilization of the polar nuclei to produce a viable endosperm, a process known as pseudogamy. Few others, mostly Asteraceae, do produce endosperm in the absence of fertilization, a process known as autonomous apomixis. The latter, to our knowledge, is not found in the grass family, where fertilization of the endosperm is an absolute requirement related to dosage effects between alleles of maternal (m) vs. paternal (p) origin (2m:1p ratio) (for review, see Birchler 1993).

Experiments on wild apomictic grasses have revealed several types of endosperm formation. Interestingly, apomixis is especially frequent in the Panicoideae subfamily, in which embryo sacs are 4-nucleate, a single polar nucleus is fertilized, and the 2m:1p ratio is conserved. Other cases reported in the literature (Nogler 1984; Savidan 2000) in which the 2m:1p ratio is recovered, found that even in the presence of two unreduced polar nuclei (*i*) they remain unfused and are fertilized by one male nuclei each or (*ii*) the fused polar nuclei are fertilized by two male nuclei (double-fertilization). In both cases, an interesting consequence is that the most frequent off-types, the B_{III} or $2n + n$ hybrids, are eliminated from the progenies with the two male nuclei being used for endosperm formation.

Wild apomictic *Tripsacum* form 8-nucleate embryo sacs and the endosperm results from a simple (single) fertilization, making the ratio between maternal and paternal component either 8x:2x (or 4m:1p) when the tetraploid apomict is fertilized by the pollen of another tetraploid or its own pollen, or 8x:1x when the pollinator is a neighboring diploid. A complex series of this ratio can be found in a *Tripsacum* nursery where levels of ploidy from 2x to 6x are mixed together without significantly affecting the seed set and germination. Therefore, *Tripsacum* seems unaffected by an abnormal dosage effect, contrary to most other grasses (Grimanelli et al. 1997).

A few BC_4 plants with high seed set were analyzed. Preliminary data suggest that endosperms could result from a double-fertilization of the polar nuclei (Figure 1c). Part of the BC_3 seed already exhibited this endosperm structure. An attempt to correlate presence/absence of such an endosperm structure with grain gross morphology, however, proved disappointing.

If creating an autonomous apomixis *de novo* is possible, which is still in question given the apparent complexity of the control in wild apomicts (as suggested by molecular analyses) (Grimanelli et al., Chap. 6), it is also questionable whether it could be satisfactorily expressed in any grass species, especially the grain crop species, because of their imprinting requirements.

unanswered. However, more progress has been achieved toward producing an apomictic grain during the last ten years than ever before, mostly because of the development and application of new techniques. As molecular dissecting tools continue to improve, we will see great progress in our understanding of how apomixis is controlled and the isolation and manipulation of its components. Another promising avenue, approaches based on mutagenesis, is discussed in the following two chapters. These approaches will undoubtedly better our understanding of the regulation of reproduction as a whole. In the end, apomixis certainly cannot be manipulated without a thorough understanding of how it is controlled in the wild.

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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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