Chapter 9

Screening Procedures to Identify and Quantify Apomixis

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Introduction

Mendel was no doubt puzzled when he studied the progenies from crosses in the *Hieracium* species to confirm his *Pisum* experiments: F_1 families were highly variable and some F_1 hybrids produced homogeneous progenies (Mendel 1870). So what about the world famous laws of inheritance worked out on *Pisum*? In fact, the laws still held. What the great geneticist did not know was that he had made the first progeny tests with apomictic species.

The term "apomixis" initially covered all of the mechanisms of asexual reproduction (Winkler 1908), but today it is applied strictly to asexual reproduction through seeds (Nogler 1984). There are two main types of apomixis, based on the origin of the embryo: adventitious embryony, in which the embryo forms directly from the sporophyte (the gametophyte phase is bypassed), and gametophytic apomixis, in which the embryo develops parthenogenetically from an unreduced female gametophyte (Gustafsson 1947; Stebbins 1950). Apomictic developments bypass meiosis and fertilization, the bases of sexual reproduction and genetic recombination, and therefore, offspring are genetically identical to the mother plant.

Apomixis has been widely identified in the plant kingdom (Asker and Jerling 1992; Carman 1997), and occurs in families of economic importance (Rutaceae, Poaceae, Rosaceae). Moreover, it appears to be a very common mode of reproduction in the Panicoideae subfamily of Poaceae (Brown and Emery 1958), which includes several major grain crops. Apomictic processes are still poorly understood, but the potential impact of apomixis on agriculture appears great, provided that it is proven to be ecologically safe (Vielle-Calzada et al. 1996; van Dijk and van Damme 1999; Toenniessen, Chap. 1). Identifying sources of apomixis, understanding its inheritance, and breeding and manipulating apomictic species will require reliable and efficient procedures to screen for mode of reproduction.

This chapter concentrates on identifying and quantifying gametophytic apomixis, but for the most part, the procedures are the same for adventitious embryony. After presenting basic features of apomixis, screening procedures for the reproductive mode are described and the various challenges encountered by scientists working with apomixis are discussed.

Apomictic Mechanisms as Potential Screening Indicators

Seed production through gametophytic apomixis requires production of embryo sacs with unreduced nuclei unreduced female gamete (no reduction of chromosome number or apomeiosis), followed by embryogenesis without fusion of nuclei of the male and female gametes (parthenogenesis). The regulatory and quantitative aspects of parthenogenesis in unreduced egg cells have been poorly

documented (Asker 1980; Nogler 1984; Mogie 1988), but fertilization-independant mutants for both seed and endosperm development, recently described in *Arabidopsis thaliana* (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998), might provide new insights into embryogenesis in apomicts. Pseudogamy is the most common path of seed development, but autonomous apomixis occurs in some cases (Nogler 1984). By contrast, apomeiosis is well documented and may follow different pathways. Several reviews (Nogler 1984; Asker and Jerling 1992; Koltunow 1993; Crane, Chap. 3) provide detailed descriptions of most types of apomixis that occur in the wild.

The two types of apomeiosis—aposporY and diplospory—and their characteristics are briefly described in this chapter in order to highlight differences with sexual reproduction that are pertinent for the development of screening tools. In adventitious embryony, both megasporogenesis and megagametogenesis are bypassed; this type of apomixis has been extensively reviewed by Naumova (1992) and Koltunow et al. (1995a).

Types of Meiotic and Apomeiotic Embryo Sac Formation

Sexual reproduction starts with the differentiation of one hypodermal archesporial nucellar cell into a megaspore mother cell (MMC). See Figure 9.1. This MMC enlarges and produces a dyad of megaspores through the first meiotic division and a tetrad of megaspores through the second division. At least two biochemical pathways have been reported as critical to or associated with meiosis: callose synthesis throughout megasporogenesis in angiosperms that produce mono- and bisporic ESs (Rodkiewicz 1970), and altered expression of plasma membrane arabinogalactan protein (Pennell and Roberts 1990).

Mature meiotic ES structure varies among taxa, but it generally displays antipodal cells, two polar nuclei within the central cell,

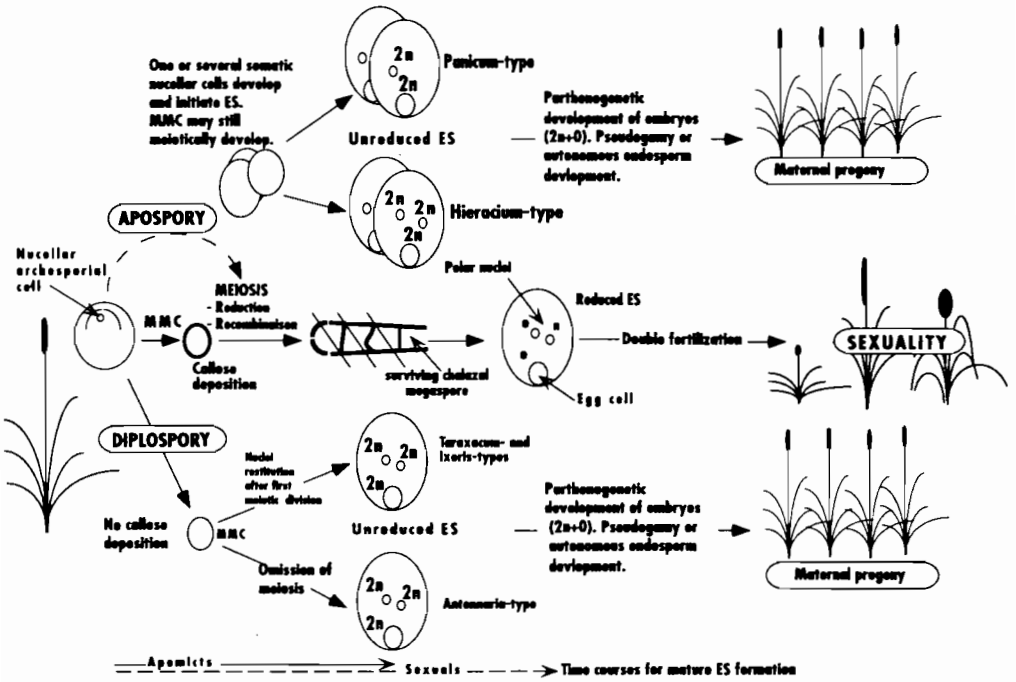

Figure 9.1 Mechanisms of pseudogamous gametophytic apomixis: consequences and comparison with sexual reproduction.

synergid(s) and one egg cell (Polygonum-type development). All nuclei from the meiotic ES are reduced (n chromosomes). Double fertilization is required for embryo and endosperm development to begin. Each of the sperm cells is involved in different fusion events: fusion with the egg restores the sporophytic chromosome number, and fusion with the central cell produces a nutritive tissue, the endosperm.

In diplospory, meiosis is totally omitted (e.g., Antennaria-type) or perturbed (e.g., Taraxacum-, Ixeris- or Allium-types). In both cases, ESs form through three or more mitoses: (*i*) from MMCs behaving as unreduced megaspores (Antennaria-type); (*ii*) from unreduced megaspores after restitution nucleus (Taraxacum- and Ixeris-types); or (*iii*) from 2n megaspores after premeiotic chromosome doubling (Allium-type). The characteristic meiotic sequence (MMC, dyad, tetrad) is absent and callose deposition does not occur (Naumova et al. 1993; Leblanc et al. 1995a) or is strongly disturbed in diplosporous pathways (Carman et al. 1991; Peel et al. 1997). Lack of callose deposition also has been reported in meiotic mutants of *Medicago sativa* that produce unreduced embryo sacs (Barcaccia et al. 1996) and in apomictic *Antennaria* hybrids obtained from parents displaying floral asynchrony (Carman 2000). In apospory, several ESs generally differentiate from nucellar (somatic) cells. In contrast to diplospory, which seems to result from genetic lesions directly affecting meiosis, some authors have stated that meiotic and apomeiotic developments are independent in apospory (Harlan et al. 1964; Nogler 1984). Both developments can theoretically occur at the same time within the same ovule, but usually the legitimate sexual line is eliminated in subsequent developmental stages. Abnormal patterns of callose deposition have been observed in various aposporous species (Naumova et al. 1993; Peel 1993; Peel et al. 1997), but little light was shed on their reproductive behavior. (Peel 1993; Peel et al. 1997). However, recent studies on callose deposition patterns and the dynamics of beta-1,3-glucanase (HpGluc) expression in aposporous *Hieracium* provide new insights into the role of callose (Tucker et al. 2000): both altered patterns and persistence of callose during megasporogenesis occur in apomictic plants when compared to sexual ones. In addition, the HpGluc enzyme might also play a role in promoting the aposporous pathway over megasporogenic callose dissolution.

Megagametogenesis in apomicts and in their sexual counterparts is usually similar. One exception is the aposporous ES structure in the Panicoideae and Andropogoneae subfamilies (Poaceae), which is a 4-nucleate ES (Panicum-type, after Warmke 1954), the sexual counterparts producing 8-nucleate ESs (Polygonum-type).

Embryo and Seed Formation

The formation of viable seeds usually requires endosperm differentiation. This is achieved in apomicts through (*i*) pseudogamy (single fertilization of polar nuclei) or (*ii*) autonomous endosperm development (central cell develops autonomously). Most apomicts are pseudogamous.

Megagametogenesis in both reproductive modes appears to take the same amount of time, but time periods for megasporogenesis differ. Such differences have been documented for several aposporous species (*Ranunculus auricomus*, Nogler 1984; *Panicum maximum*, Savidan 1982a; *Paspalum notatum*, Martínez et al. 1994) and two diplosporous species (*Tripsacum zopilotense* and *T. dactyloides*, Leblanc and Savidan 1994). The complete maturation of the apomeiotic ES before the meiotic ES may contribute to the failure of unreduced egg cell fertilization: by the time the pollen tube reaches

the ovule, unreduced egg cells may not be receptive. This loss of receptivity is not yet well understood, but several hypotheses have been proposed, including chemical or mechanical barriers (e.g., a complete cell wall around the egg) and a temporal window of receptivity, among others.

Consequences of Apomictic Seed Formation

In sexual reproduction, the two gametes that fuse are produced through meiosis. Sexual development allows genetic recombination and segregation, thereby enhancing genetic diversity. Aside from strict autogamy and from the very specific case of permanent translocation heterozygosity (Ellstrand and Levin 1982), offspring from sexual plants are new genotypes. Apomictic pathways are characterized by unreduced egg cell parthenogenesis, resulting in offspring that are exact genotypic replicas of the mother plant. However, genetic recombination may occur during apomictic reproduction in plants that show partially synaptic and restitutional meiosis or somatic DNA rearrangements (Richards 1997).

Complete (100%) maternal progenies are recovered when the mother plant reproduces through obligate apomixis. But generally, apomixis is facultative and progenies comprise various types, each resulting from a different combination of failure or success in meiosis and fertilization (Table 9.1). A fairly strict genetic control for both the formation of unreduced ES (reviewed by Sherwood, Chap. 5) and the degree of apomixis (Asker 1980) has been reported in most taxa studied.

Table 9.1 The four theoretical offspring classes in progenies from facultative pseudogamous apomicts. Note that apomeiotic mechanisms can induce chromosome losses and result in unbalanced unreduced female gametes.

	Egg cell development after	
Egg cell origin	Fusion with a sperm cell (+n)	Parthenogenesis (+0)
Reduced megaspore after meiosis : n gamete	Sexuality n+n offspring New genotypes	(Poly)haploid production n+0 offspring New genotypes
Apomeiosis (apospory or diplospory): 2n gamete	"Genomic accumulation" 2n+n offspring New genotypes	***Apomixis*** 2n+0 offspring Maternal genotypes

Levels of Screening and Related Tools

There are several indicators of apomixis, including high frequency of multiple seedlings, high seed fertility in plants expected to be sterile (e.g., wide hybrids, triploids, autopolyploids, and aneuploids), homogeneous progenies, etc. (Bashaw 1980; Hanna and Bashaw 1987; den Nijs and van Dijk 1993). They are sometimes difficult to use in the case of wild materials and, in all cases, further investigation is required to assess apomixis type and level of expression. The relative advantages or disadvantages of the screening procedures presented here are discussed further in "Choosing Suitable Procedures."

Analyses at the Plant Level

1. Molecular markers cosegregating with apomixis. To date, the identification of isozymic or molecular markers strongly linked with apomixis is the only procedure for detecting apomixis prior to flowering.

Molecular marker-based analyses in apomicts were conducted either to investigate the molecular basis of apomixis, to assist in transferring apomixis into crops, or to ultimately isolate the gene(s) responsible for its regulation. Segregating progenies or bulk segregant analyses were used after determining the reproductive behavior on the basis of cytoembryological observations or progeny testing. Because of conflicting results, debate continues over whether apomixis is controlled by a single locus or by multiple loci.

However, molecular markers (RFLP, SSR, and AFLP) for apomixis, apomeiosis, and parthenogenesis have been reported for several aposporous genera (*Pennisetum*: Ozias-Akins et al. 1993, 1998; *Cenchrus*: Gustine et al. 1997; Roche et al. 1999; *Brachiaria*: Miles et al. 1994; Pessino et al. 1997, 1998; *Poa*: Barcaccia et al. 1998; *Erygon*: Noyes and Rieseberg 2000), and in diplosporous *Tripsacum dactyloides* (Leblanc et al. 1995b; Kindiger et al. 1996; Blakey et al. 1997; Grimanelli et al. 1997a).

2. Cytoembryology. Cytoembryological differences between sexual and apomictic developments appear at different times. Observations to determine the origin of ESs are therefore based on differences in megasporogenesis (i.e., MMC behavior, occurrence of meiosis products, see Table 9.2) in the case of diplospory, on nucellar cell initiation in the case of apospory, and on differences in mature ESs for apospory of the Panicum-type. To standardize time of sampling, pistils can be classified according to pollen developmental stage if the flowers are hermaphrodite, or by size if they are monoecious. In pseudogamous species showing early embryo divisions before anthesis and endosperm formation (precocious embryony), cytoembryological observations within ESs can also help identify and quantify parthenogenesis (Kojima and Nagato 1992a; Naumova et al. 1993).

Table 9.2 Main characteristics of megasporogenesis and megagametogenesis during both sexual reproduction and gametophytic apomixis

	Meiosis	Megasporogenesis Callose	Cytoembryology	Megagametogenesis	References
SEXUALITY	Completed. The chalazal megaspore of the tetrad develops into an ES. Altered expression of arabinogalactan protein was shown to be associated with sexual development in *Pisum*. [1].	Callose deposition in Angiosperms producing mono- and bisporic embryo sacs [2].	Meiotic sequence (MMC, dyad, tetrad).	Mature 8-nucleate ES forms from chalazal reduced megaspore through 3 (Polygonum-type) or more mitoses. Mature ES are produced latter than in apomeiotic (meiosis delays megagametogenesis)	[1] Pennel and Roberts, 1990. [2] Rodkiewicz, 1970. -Herr, 1971. -Russel, 1978. -Dumas and Mogensen, 1993.
APOMIXIS					-Nogler, 1984. -Crane, chap. 3.
Apospory	Meiosis is initiated but generally fails soon or latter.	Yes (meiotic products). D Disturbed callose patterns may indicate apospory.	Concomitant development of the reproductive cell through meiosis (sexuality) and somatic cell(s) through mitoses after enlargement.	ES forms from somatic cells through mitoses. Polyembryony: Several somatic cells may develop. Reduced ES can be formed. Panicum-type: 4-nucleate ES. Hieracium-type: ES are similar to sexuals.	-Müntzig, 1940. -Warmke, 1954. -Burson and Bennett, 1970. -Young et al., 1979. -Savidan, 1982b. -Naumova et al., 1993. -Tucker et al., 2000
Diplospory	Antennaria-type: meiosis is totally over-passed. Taraxacum- and Ixeris-types: meiosis fails early producing a restitution nucleus. Allium-type: endomitosis	No callose deposition in megasporocyte cell walls.	MMC enlarges (*Tripsacum* spp., *Eragrostis curvula*) or elongates (*Elymus rectisetus*). Relation between enlargement/ Antennaria type and elongation/Taraxacum type?	ES forms from the reproductive cell. No polyembryony. Generally similar to sexuality. Binucleate ES shape can be characteristic (*Tripsacum*) Four nucleate ES described in *Eragrostis curvula*	-Voigt and Bashaw, 1972. -Crane and Carman, 1987. -Carman et al., 1991. -Kojima and Nagato, 1992b. -Peel et al., 1997

Paraffin sectioning methods (Figure 9.2) combined with staining (e.g., safranin-fast green stain, Johansen 1940; or aniline blue, Russel 1978) have been used over the last century for cytoembryological studies of reproductive development and in apomixis research (e.g., Snyder 1957; Voigt and Bashaw 1972; Burson et al. 1990). However, preparing paraffin sections is arduous and time consuming, and interpretations may be difficult. Clearing procedures (Figure 9.3) were described more than 90 years ago (Strasburger and Hillhouse 1900), but have been recently rediscovered and greatly improved (see Crane, appendix of Chap.3). They do not require sectioning or squashing and thus allow ovules to be observed in situ in three dimensions, making interpretations easier than from a series of sections. Squashing techniques, generally combined with staining, were developed for studies of megagametogenesis or megasporogenesis in various species (Hillary 1940; Bradley 1948; Saran and de Wet 1966; Darlington and La Cour 1966), but have proven only moderately successful. Nevertheless, improved squashing techniques combined with clearing procedures provide good results when analyzing female meiosis (Jongedijk 1987; Kojima et al. 1991a; Kojima and Nagato 1992b).

Clearing techniques using non-aqueous fluids (Herr 1971; Young et al.1979; Crane and Carman 1987) now represent the best tool for observing ovule details during both megasporogenesis and megagametogenesis in aposporous and diplosporous materials. Procedures combining Mayer's hemalum staining with methyl-salicilate clearing have been successfully used for observations within whole ovules of *Solanum* (Stelly et al. 1984) and *Medicago* (Tavoletti et al. 1991). These techniques are of great interest for embryological analyses in apomicts because they do not require the use of special optics. Clearing procedures combining aqueous solution (sucrose, KI) and aniline blue have recently been developed for observation of callose deposition during megasporogenesis (Carman et al. 1991; Leblanc et al. 1995b; Peel et al. 1997).

3. Egg cell parthenogenetic capacity. Egg cells produced through apospory or diplospory should be better able to differentiate parthenogenetically than those produced through sexual development, because of the apparent linkage between the two steps of apomictic development. Matzk (1991) recently proposed a new procedure to identify and quantify parthenogenesis for a wide range of cool season grasses. The technique, known as the auxin test, involves applying a synthetic auxin compound a few days before anthesis to induce parthenocarpic development in unpollinated ovaries. Auxin induced grains will lack endosperms, because the fusion of the sperm and polar nuclei is no longer possible, but egg cells with parthenogenetic capacities will develop into embryos. Studies in *Poa pratensis* using the auxin test to estimate the degree of parthenogenesis in various genotypes showed good reliability and low variation across years and environments (Mazzucato et al. 1996).

Progeny analysis

In classical progeny testing, one compares the mother plant with its offspring and/or evaluates heterogeneity within progeny. Offspring from apomictic plants are expected to be genetically identical to the mother plant; therefore phenotypic identity with the maternal type suggests apomictic reproduction, whereas variations indicate sexuality, recombination, and/or fertilization. Traditionally, progeny tests based on gross morphology have been used in apomixis research because they are easy to perform (e.g., Duich and Musser 1959; Burton et al. 1973; Gadella 1983), but many other descriptors may

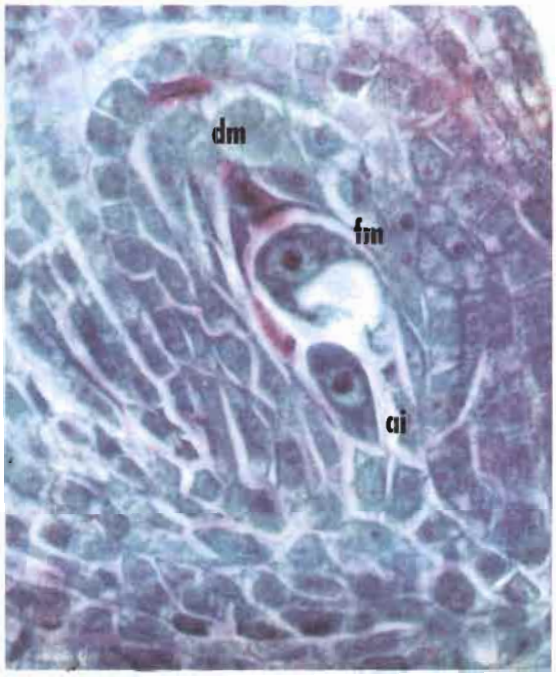

Figure 9.2 Aposporous development of Hieracium-type in *Poa pratensis* (sectioning and staining procedure). Macrospore degeneration on the micropylar side (dm), enlarged functional megaspore (fm), and aposporous initial in lateral-chalazal position (ai) (800x). (Mazzucato et al. 1995).

Figure 9.3 Clearing techniques in *Tripsacum* spp. a. Diplosporous enlarged megaspocyte observed under interference-phase contrast after a methyl benzoate-dibutyl phthalate clearing procedure (600x). (Leblanc et al. 1995a). b. Callose deposition during megasporogenesis in a sexual line after a sucrose-aniline blue clearing procedure (250x).

be useful for progeny tests and should therefore be considered. Progeny tests are usually performed on seedlings or fully-grown plants, but other tissues from earlier developmental stages, such as ovaries, endosperms or seeds, can also be used.

1. Analysis of pollinated ovaries or seeds. Determining ploidy levels in pollinated ovaries or seeds (albuminated) provides information on both reduction (meiosis) and fertilization events. Ratios between endosperm and embryos and between female and male contributions to the endosperm in apomicts often differs from those in sexual plants except for the Panicum-type aposporous development (Figure. 9.1). For many other apomictic pathways, these ratios differ. For example, endosperms found in tetraploid diplosporous apomicts are higher than in their sexual tetraploid counterparts for identical pollen donors (i.e., 10x versus 6x if the pollen is 2x); endosperm/embryo ratio for autonomous apomixis is 2:1 and 5:2 [(4x + 4x) + 2x / 4x + 0] for tetraploid pseudogamous apomicts (tetraploid pollen donor). Fertilization by unreduced pollen (Chao 1980; Huff and Bara 1993) and endopolyploidization, which sometimes occurs during endosperm development, is also possible and may further complicate analyses. However, endosperm ploidy level(s) may suggest apomictic reproduction or allow the quantification of facultative apomixis. Nevertheless, it cannot reveal the precise nature of the apomictic mechanisms involved.

Ploidy level in fertilized ovaries or immature seeds cannot easily be determined using classical chromosome counting methods, but flow cytometry now permits rapid measurement of DNA content in a variety of plant tissues, including single embryos, young endosperms, or seeds (Galbraith et al. 1983; Kowles et al. 1990; Hignight et al. 1991). Analyses in numerous apomictic species have proven flow cytometry to be a rapid and reliable procedure for determining the mode of reproduction (Mazzucato et al. 1994; Brautigam and Brautigam 1996; Grimanelli et al. 1997b; Gupta et al. 1998; Naumova et al. 1999; Matzk et al. 2000). Another option for DNA content estimation of the endosperm nuclei is to combine staining with 4'-6-diamidino-2-phenylindole (DAPI), fluoresence microscopy, and image analysis (Naumova et al. 1993; Sherwood 1995; Caceres et al. 1999).

2. Ovule regenerated plants. In tetraploid accessions of *Allium tuberosum*, Kojima and Kawaguchi (1989) reported a high frequency of tetraploid regenerated plants from unpollinated cultured ovules, suggesting apomixis expression. This indicator could be applied in screening because, in similar culture media, sexual plants would generate few (poly)haploids, whereas apomeiotic ovules would grow mostly into plantlets with the same number of chromosomes as the mother plant.

3. Analysis of progeny plants. Progeny tests must clearly identify either hybrid offspring (n + 0 types are generally poorly represented) or seed production in absence of pollination when pseudogamous apomixis or autonomous apomixis, respectively, are suspected. Hybrids can be identified using (*i*) morphological descriptors, (*ii*) cytological data, and/or (*iii*) marker analyses, if the origin of the progeny is appropriate.

Remarks on progeny size. The use of progenies from controlled crosses is recommended. Male parents bearing discriminating traits (dominant traits, different chromosome numbers, etc.) should be chosen when available, limiting possible confusions between selfed and hybrid progenies. However, open pollinated progenies can be used when mother plants are sufficiently heterozygous to detect

segregation after selfing and when there is significant diversity in the surrounding field collection, as is the case for most apomictic species, which are generally polyploid, polymorphic, and highly heterozygous.

Identifying or quantifying apomixis does not require the same number of progeny. To detect apomixis, a relatively small number of progeny (15–25) can be analyzed. Aberrant rates typically are a:n ratios with 'n' the progeny size and 'a' the number of aberrants observed in the progeny. Statistically, such samplings are binomial; 'p' (aberrant rate) is the ratio to be estimated for a given value of n (progeny size) on the basis of an observed value for a (number of aberrants detected within the progeny). Confidence limits for p in a binomial sampling are given in Figure 9.4 for various values of n (a = 0.025). Note that for n>30, confidence limits can be estimated using formulas for the normal distribution. Curves shown in Figure 9.4 clearly indicate that, up to n = 100, the information obtained is poorly significant regardless of the value of a. Finally, to obtain good estimations of aberrant rates (i.e., less than 10% confidence limits), it appears that a high number of individuals is required.

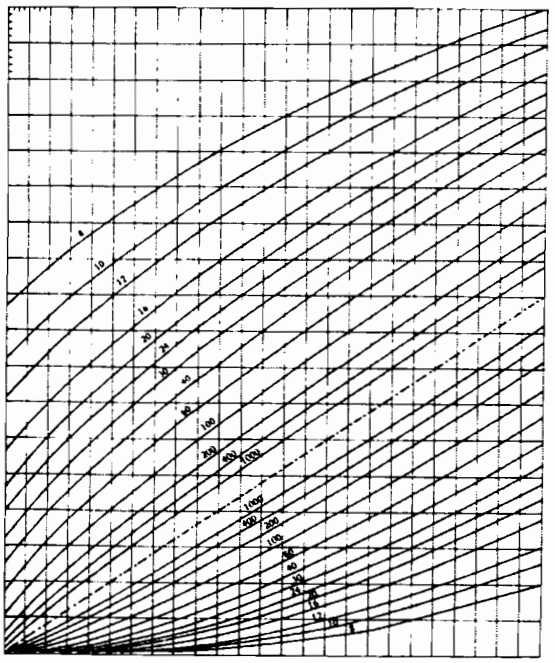

Figure 9.4 Confidence limits (α=0.025) for p in binomial sampling, given a sample fraction a/n. The numbers printed along the curves indicate the sample size, n. For a given value of a/n (abscissa), limits for p (p_A and p_B) are the ordinates read from the appropriate lower and upper curves ($Pr\{p_A \leq p \leq p_B\} \leq 1\text{-}2\alpha$).

Chromosome number determination within progenies. The sexual or asexual origin of offspring is not detectable from crosses made at the same level of ploidy, but 2n + n and n + 0 off-types are easily detected even at the seedling stage. Interploidy-level crosses could be used to detect all classes of offspring, but information can be biased by disturbances caused by unstable ploidy levels or ploidy barriers. Chromosome counts can be made from root tips, microspores, or any somatic tissue using flow cytometry (Hignight et al. 1991).

Detection of seed production in absence of pollination. Species carrying non-hermaphrodite flowers obviously represent the easiest situation, the only precaution required being to avoid pollen contamination. Contrarily, emasculation will be required unless an appropriate genetic system that ensures male sterility can be developed. Such systems require thorough knowledge of the genetics and genetic stocks of the material under study, making their application very limited in natural populations. They have been exclusively developed in experimental mutagenic populations of sexual model species (*Aradidopsis thaliana*, *Petunia hybrida*), with the aim of identifying mutants that reproduce through autonomous apomixis (Koltunow et al. 1995b; Chaudhury et al. 1997; Ramulu et al. 1997).

Markers for hybrid detection. Traits under simple genetic control are ideal for progeny testing by crossing recessive maternal genotypes with homozygous dominant testers (Hanna et al. 1970; Bashaw and Hanna 1990). Models for estimating levels of apomixis by following marker segregation have been developed (Marshall and Brown 1974), however, recombination can occur without fertilization, and the presence of dominant traits in progeny tells nothing about the origin of the off-types (n + n or 2n + n) in the absence of cytological data. Moreover, identification of such "ideal" markers in apomictic species or agamic complexes is not necessarily easy, because traits in polyploid apomicts are difficult to analyze genetically.

Morphological descriptors are the easiest means for conducting progeny tests. If the tester (pollen donor) differs significantly from the progeny-tested plant, hybrids will vary sufficiently from the maternal type to allow detection. In the case of selfing, because apomicts are generally highly heterozygous, offspring arising through sexuality will vary sufficiently from the mother plant to be scored as off-types. In most species, (poly)haploids are easily detected because of their particular phenotypes and the low vigor they exhibit (Asker and Jerling 1992). However, when using morphological descriptors, it is often not possible to distinguish between sexuality (n+n) and genomic accumulation (2n + n). But when morphological and cytological (chromosome number) data are combined, the identification of all classes is theoretically possible. Analysis of seedlings has the major advantages of timeliness and saving space, but the most informative descriptors for screening purposes are usually expressed at maturity. There are few reports of successful progeny testing for morphology on seedlings after interspecific crosses (Williamson 1981).

Isozymes or molecular markers can be used to assess variation in progenies (fingerprinting analyses; Nybom 1996). Finding good polymorphic isozyme systems, RFLP probes, or primers for PCR as candidates for fingerprint experiments is not a major obstacle. Although genetic analysis is still hindered by polyploidy, any variation in isozyme or DNA patterns indicates off-type production, provided that somatic recombination does not occur frequently in the material under study. Esterase and peroxydase were the first systems used to isolate sexual plants from *Panicum maximum* (Smith 1972). Apomixis expression was also confirmed or quantified using isozymes in *Taraxacum* (Ford and Richard 1985), *Arabis holboellii* (Roy and Rieseberg 1989), *Allium tuberosum* (Kojima et al. 1991b), *Poa pratensis* (Wu et al. 1984; Barcaccia et al. 1994), *Tripsacum* spp. (Leblanc 1995), and *Malus* sp. (Ur-Rahman et al. 1997).

Mazzucato et al. (1995) showed a slightly higher capacity of RAPD markers in discriminating off-types in progenies from the same species, when compared with three polymorphic isozyme systems or with analysis of traditional morphological traits. Although still seldom used, molecular markers have been successfully used for progeny fingerprinting (e.g., *Poa pratensis*: Huff and Bara 1993; Barcaccia et al. 1997; *Paspalum notatum*: Ortiz et al. 1997).

Choosing Suitable Procedures

Analyses at the Plant Level versus Progeny Tests

1. Nature of the information obtained. Apomixis results from apomeiotic processes (apospory or diplospory) that produce unreduced ESs, and parthenogenetic embryo development from unreduced eggs. Although nonreduction and parthenogenesis are thought to be closely linked in apomicts, observations and/or analyses of the plant itself

obviously provides insights only about apomeiotic or meiotic events, not about the complete process of apomixis. Data on the next generation (progeny test) must be collected to study fertilization and parthenogenesis events as well as the degree of apomixis. The choice of the level of analysis (apomeiosis / parthenogenesis / apomixis) depends on the objectives of the research, i.e., whether one wishes to determine only cytological processes, study parthenogenesis, or investigate apomixis in its entirety.

2. Comparing results. Limited information is available on diplosporous development, but cytological analyses of parent plants compared with progeny tests generally show good agreement between apomeiosis and apomixis screenings in *Eragrostis curvula* (Voigt and Burson 1981), *Allium tuberosum* (Kojima and Nagato 1992b), and *Tripsacum* spp. (Leblanc 1995). By contrast, the situation in aposporous species appears more complex: cytoembryological analyses generally revealed higher sexual potential than did morphology-based progeny tests in *Panicum maximum* (Savidan 1982b), *Poa pratensis* (Nygren 1951), and *Bothriochloa-Dichanthium* (Harlan et al. 1964). The same tendency was also observed by Mazzucato et al. (1996) in *Poa pratensis*, when auxin tests and field data were compared. However, using progeny tests on more than 100 *Brachiaria* F_1s, Miles and do Valle (1991) classified ten plants that were highly facultative apomicts as sexual, according to cytoembryological tests. Sexual potential in aposporous tropical grasses has generally been scored according to the formation of 8-nucleate ESs that may develop concomitantly with several apomeiotic (4-nucleate) ESs. The competition among ESs—more favorable to apomeiotics (Savidan 1982a)—and the possible weakness of certain hybrids that are eliminated early, may explain the overestimation of sexuality in facultative apospore as measured using cytoembryology (Clausen et al. 1947; Kojima and Nagato 1992b). This was confirmed by Savidan (1982a) in one *Panicum maximum* accession: sexual potential was estimated using a clearing procedure at 22.5%, but only 3% of the open pollinated adult progeny, were off-types. Elimination of hybrid offspring occurred at germination (-7%) or after transferring plants to the field (-12.5%), because of their inbred nature (resulting from selfing or hydridization with genetically close genotypes in the collection). On the other hand, after self- or sib-pollination, the lack of heterozygous loci in segregation may cause an overestimation of apomixis, with progeny tests showing the presence of "apparent apomixis" (Bayer et al. 1990).

Screening Procedures: Advantages and Constraints

Until recently, screening tools for mode of reproduction were limited to easy-but-late morphological progeny tests or skill-demanding and time-consuming cytological sectioning methods (see Table 9.3). During the past 15 years, new tools in molecular and cell biology have made screening for mode of reproduction more efficient, rapid, and reliable. These techniques include ovary progeny testing, flow cytometry for determining ploidy level, auxin test, and molecular markers that cosegregate with reproductive mode. The major disadvantage of the new methods is their expense. In addition, though the methods seem to agree with cytological and/or field observations, additional data are needed to confirm their reliability.

1. Apomixis identification and characterization. As mentioned, apomixis may be detected in various ways, but cytoembryological observations are ultimately needed to confirm the origin of the ES and to determine the type of apomixis. Clearing techniques are now quick and easy but require the use of phase-contrast or differential

interference contrast optics, both entailing considerable expense. Stain clearing techniques that allow observation of ovule details under traditional optics are less expensive. Molecular markers that cosegregate with apomixis, which enable analysis at earlier growth stages than cytoembryology, require the development of special plant materials and protocols, and the cost of associated supplies is often beyond the means of many research groups. Moreover, they may not be used with materials that differ in origin from the materials used to identify the markers, especially in the case of the highly cross-specific RAPDs (Williams et al. 1993). Morphological progeny tests are time- and space-consuming because good descriptors are usually expressed in adult plants and a minimum of 15 to 25 offspring are needed. However, these tests do not require much equipment or technical skill, and can thus be managed everywhere. Their main drawback is that they produce frequent errors because facultative apomixis occurs more often than previously thought. Moreover, progeny with sexual origin may resemble the mother plant in morphology, leading to misclassification and to an overestimation of the degree of apomixis. The existence of this gray area in progeny plant classification was reported by Williamson (1976), after extensive progeny testing in *Poa* sp. This makes morphological progeny tests unreliable when apomixis is highly facultative, but more efficient as apomixis expression increases. Early progeny tests using isozymic or molecular markers can be conducted for apomixis detection on 15–25 offspring. Only a few isozyme systems are

Table 9.3 Advantages and disadvantages of important procedures for the investigation of modes of reproduction at the plant and progeny levels. * See Ragot and Hoisington (1993) for RFLP and RAPD costs.

	Plant level analyses			Progeny tests			
Procedures	Cytoembryology (clearing procedures)	Molecular markers co-segregating with apomixis*	Auxin tests	Chr. counting in ovaries or seeds	Adult Plants Chr. counting*	Morphology	Fingerprinting
Information expected	Apomixis type determination and sexual potential estimation.	Depends on the nature of the marker(s) identified (to date linkage with apomeiosis).	Indication of apomixis expression; estimation of the degree of parthenogenesis	Indication of apomixis expression; estimation of the degree of apomixis.	Off-types of 2n+n and n+0 nature detection.	Apomixis identification and quantification; off-types nature if combined with chromosome counting.	
Plant materials required	15 to 100 flowers, depending on the objectives.	Already determined materials in segregation for marker identification.	100 flowers.	50 to 100 ovaries/seeds.	Apomixis identification: 15 to 25 offspring. Apomixis quantification: at least 100 offspring.		
Advantages	Easy and quick to perform after flowering.	Analyses can be performed early.	Easy and quick to perform after flowering	Easy and quick to perform after pollination.	Easy if flow cytometry (embryo, endosperm, plantlets).	Easy	Analyses on young offsprings possible.
Constraints	Expensive equipment for microscopy.	Preliminary work to determine materials. Use of the markers across accessions of different origins? Expensive.	The auxin test has been mainly used to date in cool-season grasses.	Expensive equipment for flow cytometry.	Time consuming (classical methods) or expensive if flow cytometry is used.	Time and space consumming. Morphological tests: unreliable if apomixis is highly facultative.	

required to indicate apomixis and determine the nature of the hybrids detected. RFLPs and RAPDs can also be used in the same way, but at greater expense.

2. Degree of apomixis expression. Many offspring are needed to obtain a good estimate of the degree of apomixis. Both auxin tests and flow cytometric analyses of pollinated ovaries or seeds provide good estimates of sexual potential, though distinguishing 2n + 0 from n + n offspring might be difficult in certain cases. In contrast, systematic chromosome counting within progenies is useful for detecting 2n + n and n + 0 off-types, but it does not separate 2n + 0 from n + n offspring, and without flow cytometry it becomes tremendously time consuming. Progeny tests combining cytology and marker analyses represent the best option for identifying the different classes of offspring within apomictic progenies. To limit cytology work (when flow cytometry is not available), markers can be applied first to separate maternal offspring from (poly)haploids or hybrids. The origin of the latter may be determined according to the patterns they produce (i.e., 2n + n off-types must carry all bands from the mother plant, plus extra bands from the pollen), and then cytologically confirmed.

Choosing a Procedure

There are four main areas of apomixis research, each with distinct constraints and objectives: (*i*) the search for apomixis or elements of apomixis in new taxa, coupled with genetic studies in wild populations, (*ii*) germplasm characterization of apomictic species, (*iii*) genetic and biological studies for further manipulation of apomixis, and (*iv*) breeding of apomicts and introduction of apomixis into sexual crops.

Since gametophytic apomixis is formidably limited to perennial, polyploid, and outcrossing species, the search for apomixis in additional species should begin with taxa presenting these traits. The very first screening can be based on the expression of the already mentioned "indicators of apomixis," while more discriminative procedures may be applied to promising specimens. For germplasm evaluation, a representative sample of the collection must be chosen on the basis of morphological and cytological data, and traits of agronomic value such as disease resistance. Chromosome number, repro-ductive development, and degree of apomixis are the primary factors for which basic data must be collected to develop strategies for further research. Genetic studies also may be attempted to genetically dissect apomictic mechanisms (number of genes involved and their effects). Following this preliminary work, appropriate tools for larger-scale screening should be developed or chosen according to the apomixis characteristics of the collection (e.g., callose patterns for diplospory, ES clearing for apospory of the Panicum-type, etc.).

Sexual parents involved in crosses for apomixis inheritance studies must be carefully chosen using cytoembryology. Highly facultative apomicts are easily misclassified as sexuals using progeny tests. This causes distortions of segregation ratios for mode of reproduction among progeny. In the same way, looking for differences between sexual and apomictic development at the molecular level requires the analysis of genotypes that are well characterized for mode of reproduction. This may allow the development of near isogenic lines, an important step in identifying the gene(s) controlling apomixis.

Before apomixis can be transferred into crops or used in breeding programs, researchers need procedures to identify apomictic genotypes (see do Valle and Miles, Chap. 10; Savidan, Chap. 11) and to quantify apomixis in genotypes selected for varietal release. Progeny

testing in such programs may help identify apomixis, because offspring are necessarily produced as part of breeding schemes, but an entire plant cycle must pass before data are obtained (a serious drawback in the case of annual plants). Notwithstanding, in some cases—especially when low female fertility is affecting the plants (e.g., interspecific or intergeneric hybrids)—this may be the best way to test for mode of reproduction. Because in most species apomixis and sexuality do not express at the same ploidy level, estimating chromosome number within progenies using flow cytometry allows easy identification of apomictic genotypes in early backcross generations, but becomes less effective when chromosome numbers close to that of the recurrent parent are recovered. Appropriate cytological procedures or marker-assisted selection may also be used to identify apomixis.

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