Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 5127–5132, April 1998
Genetics

Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes

PEGGY OZIAS-AKINS*†, DOMINIQUE ROCHE*, AND WAYNE W. HANNA‡

*Department of Horticulture and ‡U.S. Department of Agriculture–Agricultural Research Service, University of Georgia Coastal Plain Experiment Station, Tifton, GA 31793-0748

Communicated by Glenn W. Burton, U.S. Department of Agriculture, Tifton, GA, February 23, 1998 (received for review November 18, 1997)

ABSTRACT **Apomixis is a naturally occurring mode of reproduction that results in embryo formation without the involvement of meiosis or fertilization of the egg. Seed-derived progeny of an apomictic plant are genetically identical to the maternal parent. We are studying a form of apomixis called apospory that occurs in the genus *Pennisetum*, a taxon in the grass family. A cultivated member of this genus, pearl millet (*Pennisetum glaucum*), reproduces sexually. A wild relative of pearl millet, *Pennisetum squamulatum*, that is an obligate aposporous species, is cross-compatible with pearl millet when used as a pollen donor in the interspecific cross. We present herein the genetic mapping of 13 molecular markers in an interspecific hybrid population of 397 individuals that segregates for apomixis and sexuality. Surprisingly, 12 of the 13 markers strictly cosegregated with aposporous embryo sac development, clearly defining a contiguous apospory-specific genomic region in which no genetic recombination was detected. Lack of or suppression of recombination may be coincidentally associated with the chromosomal context of the apomixis locus or it may be a consequence of its evolution that is essential for preservation of gene function as has been previously shown in studies of complex loci in both plant and animal species.**

Plant reproduction recently has become an intensively studied field, in part because the determination of cell fate in plants is intrinsically different from animals, and the alternation of sporophytic and gametophytic generations is uniquely invoked in seed plants. Most research on plant reproduction in angiosperms has focused on floral development in the sporophytic phase because of its size and accessibility. Microsporogenesis and male gametophyte development also have received considerable attention; the microsporangium is readily accessible and development within it is relatively synchronous. In contrast, study of megasporogenesis and the development of the female gametophyte has been confined largely to classical morphological/cytological approaches due to an embedded location within the ovule. Molecular techniques only recently have emerged that allow development within a single cell of a multicellular organism to be probed (1, 2).

During sexual reproduction, the female gametophyte typically develops from one of the four products of meiosis, or megaspores, and at maturity consists of seven cells: the egg, two synergids, three antipodals, and one binucleate central cell. Fusion of the egg cell with a sperm completes the sexual cycle and generates a new sporophytic phase. Gametophytic apomixis in angiosperms is a modified mode of reproduction in which an alternation of sporophytic and gametophytic generations persists but without the genetic consequences of meiosis (3). In gametophytic apomicts, the female gametophyte develops from a cell that is mitotically derived and whose products do not participate in gametic fusion, thus the genetic constitution of the maternal parent is passed on to the progeny, and the progeny are effectively clones of the parent. Gametophytic apomixis is further classified into two categories, diplospory and apospory (4). Both types of apomixis circumvent meiosis, the former by the development of the megaspore mother cell itself into an embryo sac and the latter by the participation of other nucellar cells in embryo sac formation. The form of apomixis we are mapping is apospory, and it occurs in *Pennisetum squamulatum* Fresen, a member of the grass family and relative to the domesticated species *Pennisetum glaucum* (L.) R. Br. (pearl millet). Aposporous embryo sacs can easily be distinguished from meiotically derived embryo sacs in this material by the absence of antipodal cells in the former (5). This article describes the use of molecular markers to characterize a genomic region containing the apospory locus.

MATERIALS AND METHODS

Plant Material and Trait Screening. A population for mapping of the trait apospory was produced from a cross of *P. glaucum* (induced tetraploid; $2n = 4x = 28$) × *P. squamulatum* ($2n = 6x = 54$). The apomictic parent *P. squamulatum* is obligate and could only be used as the male parent because chromosomally reduced gametes that would carry potentially recombinant products of meiosis are produced only during male gametogenesis. Inflorescences of pearl millet (an induced tetraploid from "Tift 8677") were bagged prior to stigma exsertion. Pollen was collected from *P. squamulatum* (PS26) and dusted onto the stigmas at anthesis. Because pearl millet is protogynous, there is a gap of 2 or 3 days between the beginning of stigma exsertion and anther exsertion that allows cross-pollination without emasculation.

For mapping purposes, only segregating alleles from heterozygous *P. squamulatum* that were contributed to the hybrid were considered, and the pearl millet alleles were ignored. The capacity to form aposporous embryo sacs was determined from microscopic examination of 20 cleared ovules per individual (6). Three hundred ninety-seven F_1 individuals formed the mapping population and each individual was categorized as sexual (no evidence of apospory) or aposporous (included both of the following phenotypes: obligate, where only aposporous sacs were observed, or facultative, where both aposporous and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ASGR, apospory-specific genomic region; RAPD, random amplified polymorphic DNA; SCAR, sequence-characterized amplified region.

†To whom reprint requests should be addressed. e-mail: ozias@tifton.cpes.peachnet.edu.

sexual embryo sacs were observed at any ratio). With sampling of only 20 ovules, a low frequency of aposporous embryo sacs potentially could result in misclassification of an aposporous individual as sexual but not the converse. Mode of reproduction was confirmed in more than 100 F_1 individuals by examining progeny produced after pollination of the F_1 with pollen from tetraploid pearl millet homozygous for a dominant "red" marker gene (7). The presence of red progeny indicated that syngamy occurred, most probably in meiotically reduced embryo sacs through sexual reproduction. Exclusive development of green progeny from the test cross was a strong indication that parthenogenesis of an unreduced egg, and thus apomictic reproduction, had occurred.

Isolation of Molecular Markers. Except for two restriction fragment length polymorphism markers isolated in a previously published work (8), all markers were derived from random amplified polymorphic DNAs (RAPDs; ref. 9). Genomic DNA was isolated from young leaves according to Ozias-Akins *et al.* (8). Bulked-segregant analysis (10) was used to homogenize the sequence heterozygosity within two pools of DNA except around the apospory locus. Equal amounts of DNA from 16 F_1 individuals from each phenotype, aposporous (A) and sexual (S), were pooled and used as templates for PCR. PCR mixtures (25 μl) contained 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, all four dNTPs (each at 100 μM), 0.5 μM of each primer, 25 ng of genomic DNA, and 0.5 unit of *Taq* DNA polymerase. Cycling was performed on a forced-air thermal cycler (Biotherm, Fairfax, VA) programmed for six cycles of 15 s at 93°C, 30 s at 36°C, and 1 min at 72°C; followed by 36 cycles of 15 s at 93°C, 30 s at 40°C, and 1 min at 72°C. Primers were obtained as kits of 20 decamers per kit (Operon Technologies, Alameda, CA). After all primers had been screened on A and S genomic DNA pools, a search for additional polymorphisms was initiated by using the same sets of primers but in reactions containing pooled template DNA digested with *Alu*I, *Hae*III, *Msp*I, *Rsa*I, or *Taq*I. Presumably, the primary effect of predigestion would be to eliminate a band if a restriction site fell between two priming sites, although this explanation of mechanism may be too simplistic (11).

Sequence-Characterized Amplified Region (SCAR) Development. Each RAPD reaction that initially indicated a polymorphism between the A and S pools was repeated on four subsets of each large bulk that contained DNA pooled from only four individuals. Reproducible polymorphic fragments were separated on agarose gels and excised, and DNA was purified by using GeneClean (Bio 101). Fragments were cloned into pGEM-T (Promega) or T/A (Invitrogen) cloning vectors. The correct clone was confirmed by hybridizing radiolabeled insert with a Southern blot of RAPD fragments amplified from all subpools (A and S) and parents (pearl millet and *P. squamulatum*). Clones were end-sequenced and primers were designed to each end of the sequence. Where possible, the sequence of the decamer was included in a longer (18–24 nucleotides) sequence-specific primer (Table 1). Optimal amplification for each SCAR (12) was empirically determined by varying annealing temperature (Table 1) before screening of the entire population of 397 individuals.

Restriction Fragment Length Polymorphism Analysis. DNA (15 μg) was digested overnight with 50 units of *Dra*I (New England Biolabs) and separated by electrophoresis for 16 hours in 1% GTG agarose (FMC) in 1× TBE buffer. DNA was transferred to Genescreen*Plus* nylon membrane (NEN) and hybridized with labeled insert DNA generated by PCR amplification from RAPD clones using SCAR-specific primers. Hybridized blots were washed at a final stringency of 0.1× SSPE (0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA) containing 0.1% SDS at 65°C for 30 min.

Data Analysis. A χ^2 analysis was carried out to test various models for inheritance. To detect linkage between two loci, the numbers within each gamete class (AB, A, B, and null) were determined and $\chi^2 = (a - b - c + d)^2/(a + b + c + d)$, where *a, b, c,* and *d* represented the four gamete classes. The recombination fraction was calculated as the frequency of recombinant gametes, $r = (b + c)/(a + b + c + d)$, as described by Wu *et al.* (13) for single-dose alleles linked in coupling.

RESULTS

Segregation of Trait. The F_1 population segregated for mode of reproduction, apospory vs. sexuality, which indicated that it was heterozygous for the dominant allele. Individuals were designated as aposporous regardless of the frequency of ovules containing aposporous embryo sacs; i.e., facultative and obligate individuals were both scored as aposporous. The entire population segregated for 162 aposporous and 235 sexual phenotypes, a ratio that does not fit the expected 1:1 of a single-dose allele. The χ^2 analysis was used to test for tetrasomic inheritance and tetrasomic inheritance with a linked homozygous-gamete-lethal allele (Table 2). The segregation ratio best fit the model of tetrasomic inheritance with a linked gametic lethality.

PCR-Based Marker Development. Twenty-two RAPD fragments were isolated from the apospory pool that were absent from the sexual pool. The approach to identify additional RAPDs using the same primers on digested template DNA was successfully applied and allowed the isolation of 19 of the 22 RAPD markers. The distribution of markers with respect to

Table 1. Primer pairs and amplification conditions for markers tightly linked with apomixis

Marker	Primer 1 (5′ → 3′)	Primer 2 (5′ → 3′)	Annealing temperature, °C	Fragment size, bp
A10H	AAACCTTAATCATGCAACCTCGGA	ATGTCACCCGCCTTCTTTGATGCT	65	300
A14M	TTGATTGAGTTTATTCCTATTTGG	**GTGCTGG**TACAAGAAGAACTGGTG	55	200
C4	**CCGCATCTAC**AATAATCA	GAAATAAAGGCACTGGGA	54	500
O7M	**CAGCACTGAC**ATCAACTAGGACGA	**AGCACTGAC**CAACTTTACTGAATC	63	550
P16R	**CCAAGCTGCC**ATATCTCCATGCTC	ATCCGGGACATGCTGTGCGATTTC	65	950
Q8M	GAGCTTGNCCAATCGGGAAA	**A**TGGTGATGGATCTTTTGGAC	60	800
R13	**GGACGACAAG**AACAAGAAGGACGA	GAATAGCACCCTCAGACAGCACTC	65	200
U12H	**TCACCAGCCA**GTTCAACCC	ATTGTTTACATACCATCACCAGAA	60	650
V4	TCGGATAAGCTGTAGGAGTCT	CACATCCATTNTCTCTTCCAG	55	1,600
W10M	CATGTATTTCTCTGTCGTACTTGGTC	AGCCCATAAAACAGCTCCTAAA	60	600
X18R	AGTTGGGAAGAAAGCCGAGTTGTT	CAATCTTGGAAGTGCGTCGAAAAT	60	550
ugt197	GGATGAATAAAACGGTGTTGGGAG	AGAACAACCGCACAAGTGAGAGAA	62	850

Bold-face type designates the nucleotides in common with the decamer primer from Operon.
Marker name consists of the Operon primer designation plus a terminal letter indicating whether the RAPD fragment was amplified from digested template (H, *Hae*III; M, *Msp*I; R, *Rsa*I).

Table 2. χ^2 analysis of models of inheritance for apomixis in a population of 397 individuals and the only segregating marker (ugt204) screened in a population of 80 individuals

Locus	Model for inheritance	Expected			Observed		χ^2	$P_{df=1}$
		Ratio	N_i +	N_i −	N_i +	N_i −		
Apomixis	Disomic or tetrasomic, random chromosome	1:1	198.5	198.5	162	235	13.42	<0.01
	Tetrasomic, random chromatid	13:15	184	213	162	235	4.90	0.03
	Tetrasomic, random chromatid, gamete lethal	12:15	176	221	162	235	2.00	0.16
ugt204	Disomic or tetrasomic, random chromosome	1:1	40	40	29	51	6.05	0.01
	Tetrasomic, random chromatid	13:15	37	43	29	51	3.22	0.07
	Tetrasomic, random chromatid, gamete lethal	12:15	35.5	44.5	29	51	2.14	0.14

N_i, number of individuals; + and −, presence and absence of the trait, respectively.

restriction enzyme tended to favor some enzymes over others. The largest number of markers (8 markers) was recovered from *Msp*I-digested DNA, *Hae*III and *Rsa*I revealed similar numbers (4 and 5 markers, respectively), and *Alu*I and *Taq*I were the least useful (2 and 0 markers, respectively). Of these 22 markers, 15 have been cloned and end-sequenced. Sequence-specific primers have allowed 11 of the 15 to be developed into SCARs (Table 1 and Fig. 1).

Twelve Molecular Markers Are Totally Linked to the Locus Conferring Apospory. From screening of the entire mapping population (397 individuals), 12 PCR-based markers [11 SCARs and 1 sequence-tagged site, ugt197, from a previous study (8, 14)] were always present in 162 aposporous individuals and absent from all 235 sexual individuals. Thus, these markers must belong to the same linkage group and are linked in coupling with the trait. No reproducible markers linked in repulsion were found. Although the 12 molecular markers initially detected polymorphism between relatively small pooled samples of sexual and apomictic genotypes, it was unexpected that they would define a genomic region with no recombination recorded in a progeny size of 397 individuals. These data were accepted only after extensive resampling of ambiguous individuals for classification of embryo sac development and for DNA analysis. One F_1 individual originally was categorized as sexual but contained all of the apomixis-linked markers. Upon reexamination, this plant was indeed highly sexual with only 7% (6 of 78) of the ovules showing aposporous embryo sacs. Some form of altered expression/penetrance of apomixis may be functioning in this individual.

Only a single marker, ugt204, has thus far shown recombination with the trait for apospory. This marker represents a single-dose restriction fragment length polymorphism that has only been scored in 80 individuals. Although it may be linked, it mapped at a considerable genetic distance from the apospory linkage block ($\chi^2 = 22.05$; $P_{df=1} < 0.01$; recombination fraction = 0.2375) and is likely to be at a large physical distance from the remaining markers.

Hemizygous Regions of DNA Are Linked to the Trait for Apospory. Five of the 11 cloned RAPD fragments and ugt197 hybridized as low-copy-number DNAs on genomic Southern blots. When eight sexual and eight apomictic F_1 individuals were probed with these six clones, four proved to be hemizygous for these sequences; i.e., hybridization signal was observed only with the apomictic individuals and not with the sexual individuals (Fig. 2). The remaining two probes did not show a hemizygous pattern, but rather an additional polymorphic fragment was observed in the apomicts that was not represented in the sexuals. Partial hemizygosity near the apomixis locus led us to designate this region, whose physical size remains unknown, as an apospory-specific genomic region (ASGR).

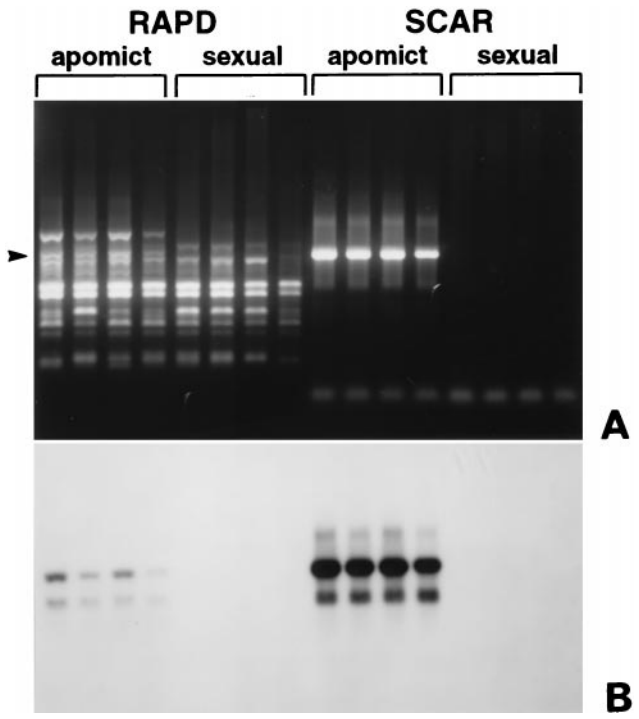

FIG. 1. (*A*) RAPD pattern (lanes 1–8) with primer P16 and pooled DNA from apomictic and sexual individuals and the 950-bp SCAR (lanes 9–16). Each lane represents four genotypes bulked by phenotype (apomictic or sexual). (*B*) A blot of the gel shown in *A* probed with the cloned P16 RAPD fragment.

DISCUSSION

Molecular Markers Linked to the Trait for Apospory Belong to the Same Linkage Group and Define an ASGR. It is unlikely that false or pseudolinkage would have resulted in such an extreme pattern of nonrecombination in a population of 397 individuals and in a high ratio of apomictic to sexual individuals. During chromosomal reassortment, the probability of joint segregation, $P(j.s)$, of n subsets of linked markers with either disomic or tetrasomic inheritance is given by the following formula, $P(j.s) = 0.5^n$. Thus the probability of independent segregation, $P(i.s) = 1 - 0.5^n$, increases with n (n being the number of linkage groups). If our markers resided on two linkage groups, the probability of independent segregation for two subsets of markers is $P(i.s) = 0.75$. Out of 397 meiotic products tested from *P. squamulatum*, 298 would have shown independent segregation for two subsets of markers unless a mechanism driving extreme distorted segregation were operating. Therefore, we can assume that all 12 molecular markers

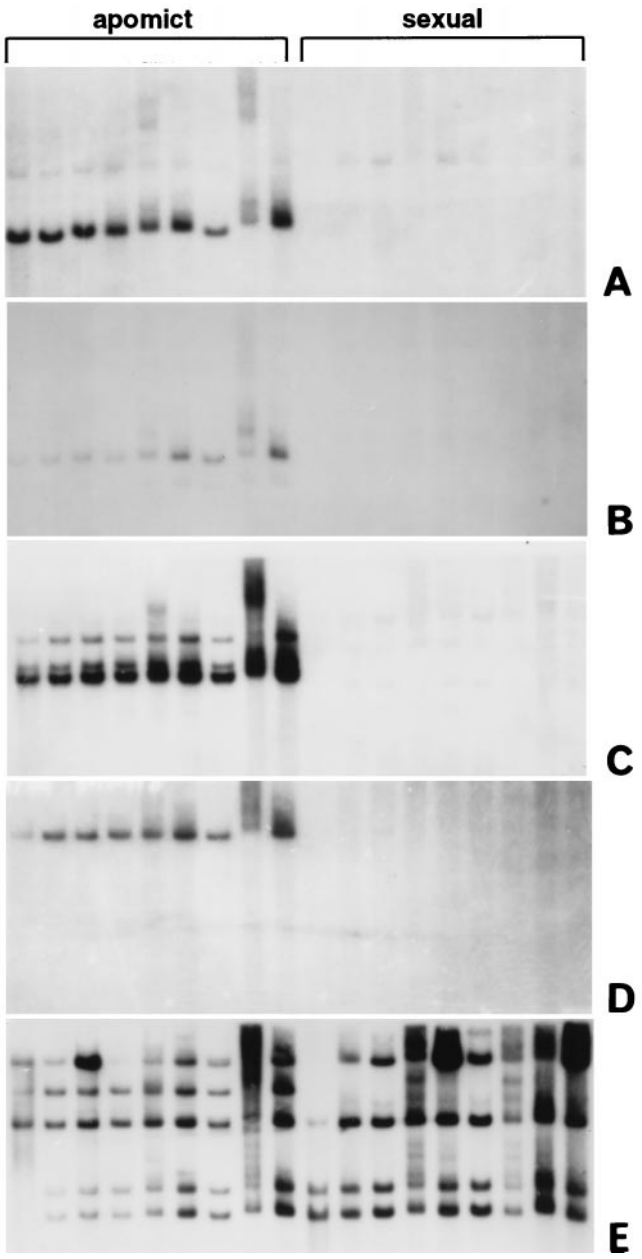

FIG. 2. A genomic DNA digest (*Dra*I) of *P. squamulatum* (first lane of apomicts), *P. glaucum* (first lane of sexuals), and eight F_1 individuals of each reproductive phenotype probed with ugt197 (*A*), C4–600 (*B*), Q8M-800 (*C*), A14M-200 (*D*), and O7M-600 (*E*). The same blot was stripped and reprobed with each sequence. All probes detected either hemizygous (*A–D*) or polymorphic (*E*) DNA sequences in the apomictic individuals. None of the probes hybridized with bands of the same molecular weight.

belong to the same linkage group because any interchromosomal recombination would have significantly altered this tight linkage in a progeny size of 397 individuals.

It also is unlikely that we have saturated a small region of a linkage group with molecular markers even though we used a targeting strategy of bulked segregant analysis with 16 individuals in each bulk. A similar study on another heterozygous plant (potato) using a targeting strategy (four genotypic pools of 14 individuals each) with amplified fragment length polymorphism (AFLP) markers found that 29 of 3,200 AFLP markers displayed linkage to the disease-resistance locus (*Phytophthora infestans* in potato) and eight mapped within the targeted 6 cM genetic interval (15). Only two of the AFLP markers cosegregated with the trait in a population size of 461 individuals. The population size was designed to allow high-resolution mapping within the 6-centimorgan interval. Given our population size of 397 individuals and no verifiable recombination among 12 apomixis-linked markers out of approximately 4,000 total, we have concluded that our gene(s) lies in a region of the genome where recombination is repressed, and this region, herein defined as an ASGR, probably contains a disproportionately high number of single-dose alleles.

Harlan *et al.* (16) made the observation that "apomixis and sexual reproduction are not alternative modes of reproduction, either genetically or operationally, but are simultaneous and independent phenomena. . . . The genes controlling normal sexual reproduction are not allelic to those controlling apomixis in the conventional sense." The evolution of apomixis in natural populations does not appear to have occurred as a simple mutation of a single gene in the sexual reproductive pathway, and evidence for an ASGR lends support to the hypothesis that apomixis and sexuality are not allelic alternatives. Therefore, efforts to induce apomixis in sexual diploids, although certain to generate mutants useful for understanding reproductive biology, are likely to encounter obstacles in rapidly synthesizing a viable apomictic system.

Nothing presently is known of the molecular basis for apomixis, although there has been much speculation and discourse (17–21). Evidence from our study of a large segregating population that apospory is controlled by an ASGR is not compatible with a recently postulated model that the mechanism for apomixis, including apospory, is based on asynchronous expression of many duplicate genes in polyploid angiosperms (21). From this hypothesis, we would not have predicted the segregation of a trait with a significant associated region of hemizygosity in the *P. squamulatum* genome. Furthermore, it is noteworthy that this ASGR may be partially conserved in several other species of *Pennisetum* where at least two of the same molecular markers are tightly linked to apospory (14). The level of hemizygosity that we have observed around the apomixis locus suggests that at least a portion of the chromosomal homolog bearing this locus in *P. squamulatum* has been isolated by the lack of recombination for a considerable period of time. The ASGR could be a unique region that underwent extensive sequence divergence as a consequence of its recombinational isolation from the remainder of a progenitor genome and that could have radiated largely intact during hybridization and speciation within the genus.

Tight Clustering of Molecular Markers Linked to Apospory May Indicate the Presence of a Complex Locus. Solid evidence for the lack of recombination near the apomixis locus has been found in our study on apospory and to a lesser extent in a preliminary study on diplospory in *Tripsacum* (22). Two other species where apospory has recently been mapped are *Brachiaria brizantha* (23) and *Pennisetum ciliare* (24). Interestingly, two of the markers most closely linked to the trait in these studies were identical to two we previously isolated, OPC4 and ugt197 (8). The small genetic distance between apospory and ugt197 published by Gustine *et al.* (24) likely was due to a PCR or classification error because we have not been able to confirm recombination in the same population (unpublished results). The partial map of the apomixis linkage group in *Brachiaria* is colinear with the end of the short arm of maize chromosome 5, although only OPC4, and no maize probes, mapped distal to the apospory locus. No genetic distance between OPC4 and the apomixis locus was given in this study, although genetic recombinants apparently were observed in this small population of 43 individuals. Further evaluation of the *Brachiaria* population for the apomixis-linked markers from *P. squamulatum* would provide useful data to test the generality of nonrecombination around the apomixis locus.

Several mechanisms could be postulated as the genetic basis for nonrecombination in our population. (*i*) The apomixis locus could be located in a centromeric or other heterochromatic region of a chromosome or perhaps on an unpaired minichromosome. Recombination is known to be reduced in centromeric regions of the genome (25, 26). The centromeric location of the apomixis locus remains a viable hypothesis in the absence of cytological evidence to the contrary, and extreme linkage disequilibrium of surrounding sequences previously observed (14) would have been promoted by the lack of recombination. The evidence from comparative mapping in *Brachiaria*, however, suggests a telomeric location of the apospory locus. (*ii*) The apospory locus could be located in a heterozygous inversion or in a region of DNA introgressed from another species. Inversions can cause localized asynapsis and nonhomologous pairing during meiosis or when paired with homologous segments, form loop bivalents (27). A single crossover event in a paracentric inversion can result in dicentric and acentric chromatids that carry genetic duplications and deficiencies, respectively. Pollen grains carrying these aberrant chromatids usually abort and would preclude the recovery of recombinants in this region. A large inversion would not seem to be the most likely explanation for nonrecombination because pollen viability in *P. squamulatum* is relatively high (82%) and comparable to that observed in a sexual hexaploid species, *Pennisetum basedowii* (80%; ref. 5). (*iii*) It is also possible that the ASGR represents a complex locus through which apospory is controlled not by a single gene but rather by two or more genes that could be maintained as an intact genetic unit. Mechanisms to prevent recombination within this unit would be required (28) and might involve those described above. Well-documented examples of complex loci in eukaryotes are meiotic drive systems in animals (29, 30), the human major histocompatibility complex (31), the *Chlamydomonas* mating-type locus (32), and the self-incompatibility locus in *Brassica* (33). Significantly there is no recombination observed between the critical genes within the complex loci, and most systems are characterized by rearrangements of regions of low-copy-number and interspersed repetitive DNA and polymorphisms of chromosome structure (linked inversions, deletions, duplications, and translocations).

The existence of a complex locus also might raise the possibility for the involvement of gene silencing as part of the apomixis mechanism. Silencing or repression of genes required for normal sexual reproduction could occur through several mechanisms, either transcriptional or posttranscriptional (34, 35). If the apospory locus were a silencing locus or could act in trans to repress other genes, the dominance of the trait in many species, its incomplete penetrance in facultative apomicts, and its potential for suppression by other loci could be accommodated. Evidence for suppression of the apomixis gene has been observed in *Cenchrus ciliaris* (36) and in one of our F_1 hybrids.

The Mode of Inheritance of the Apomixis Linkage Block Suggests Tetrasomy with a Linked Gametic Lethality. When apomixis is dominant, the inheritance of the trait can only be studied by using the apomict as the male parent. Although considerable literature exists on genetic analysis of apomicts that presents evidence for recessive and dominant control of single and multiple gene action (3), the most recent data on well characterized apomicts within the grass family, *Tripsacum dactyloides*, *C. ciliaris* (*P. ciliare*), and *P. squamulatum*, strongly support dominant inheritance of a single-gene trait. All of these species are polyploid (tetraploid or hexaploid) and thus either disomic or polysomic inheritance could be functioning. None of these species displayed strict bivalent pairing of chromosomes at meiosis (5, 37, 38), but the extent of homologous or homeologous pairing of the chromosome bearing the apomixis locus is not known. A common segregation pattern for all three species [*T. dactyloides* (39); *C. ciliaris* (40); *P. squamulatum*, present data], however, was often a ratio less than the predicted 1 apomict to 1 sexual for a single dose of the apomixis allele (although not always a statistically significant difference based on the small numbers in some populations). Sherwood *et al.* (40) proposed tetrasomic inheritance of the trait in *C. ciliaris* and lethality of the homozygous apomixis locus in male gametes as the model that best fit their data. Nogler (41) concluded that gametes homozygous for the apomixis allele were not capable of transmitting the trait in *Ranunculus auricomus*. Our data support the hypothesis for tetrasomic inheritance of the trait with a gametic lethal factor linked to the apomixis locus. Furthermore, additional evidence for tetrasomy is that none of the markers we isolated were linked in repulsion with the trait. If the apospory locus were following a tetrasomic inheritance as suggested above, the maximum detectable recombination fraction ($max r_2$) for linkage in repulsion in a progeny of 32 individuals (two bulked DNA samples, each from 16 plants) is zero (13) and repulsion linkages would not be observed.

In one type of complex locus, meiotic drive, the homozygous state is correlated with lethality, male sterility, or reduced fertility (30); therefore, heterozygosity is selectively maintained. In *P. squamulatum*, the apomixis linkage block is extremely heterozygous, and some of our apomixis-linked clones even appear to be hemizygous; i.e., there is no detectable allele other than the one associated with the apomixis linkage group. As far as is known, homozygous aposporous apomicts do not occur in nature (4), even though they theoretically could be produced through rare chromosomal reassortment events in facultative apomicts. If, as our data suggest, the apomixis allele itself or an allele held in association by linkage disequilibrium were a recessive lethal in homozygous or haploid gametes (refs. 4 and 42, see also http://192.100.189.39/CIMMYT/Biotechnology/Apomixis), survival of the male gametophyte could only occur if the gamete-lethal gene were compensated for by a normal allele in unreduced gametes. Thus polyploidy, due to occasional fusion of unreduced male gametes (which would be the only functional male gametes carrying the apomixis trait) with unreduced female gametes (43), should be a natural outcome and could explain the predominance of polyploidy in apomicts. In addition, if the apomixis locus were actually a strong silencing locus (44, 45), the penetrance of the silencing effect might even be enhanced in polyploids (46, 47).

Implications of Low Recombination for Map-Based Cloning. Crucial to the success of map-based cloning is the ability to position the trait phenotype with respect to molecular markers based on recombination distance. In the absence of genetic recombination, physical mapping becomes a tedious but essential process for ordering molecular markers associated with the trait. Only a deletion or insertional mutagenesis approach would allow positioning of the apomixis gene(s) with respect to the linked molecular markers.

We thank Peggy Jo Bledsoe and Zhenbang Chen for technical assistance. This work was supported by National Research Initiative Competitive Grants Program Grants 91–37304-6577 and 93–37304-9363 and the Rockefeller Foundation.

1. Karrer, E. E., Lincoln, J. E., Hogenhout, S. Bennett, A. B., Bostock, R. M., Martineau, B. Lucas, W. J., Gilchrist, D. G. & Alexander, D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3814–3818.
2. Sundaresan, V., Springer, P., Volpe, T., Hayward, S., Jones, J. D. G., Dean, C., Ma, H. & Martienssen, R. (1995) *Genes Dev.* **9,** 1797–1810.
3. Asker, S. & Jerling, L. (1992) *Apomixis in Plants* (CRC, Boca Raton, FL).
4. Nogler, G. A. (1984) in *Embryology of Angiosperms*, ed. Johri, B. M. (Springer, Heidelberg), pp. 475–518.
5. Dujardin, M. & Hanna, W. W. (1984) *Theor. Appl. Genet.* **67,** 197–201.

6. Young, B. A., Sherwood, R. T. & Bashaw, E. C. (1979) *Can. J. Bot.* **57,** 1668–1672.
7. Hanna, W. W. & Burton, G. W. (1992) *J. Hered.* **83,** 386–388.
8. Ozias-Akins, P., Lubbers, E. L., Hanna, W. W. & McNay, J. W. (1993) *Theor. Appl. Genet.* **85,** 632–638.
9. Williams, J. G. K., Kubelik. A. R., Livak, K. J., Rafalski, J. A. & Tingey, S. V. (1990) *Nucleic Acids Res.* **18,** 6531–6535.
10. Michelmore, R. W., Paran, I. & Kesseli, R. V. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9828–9832.
11. Koebner, R. M. D. (1995) *Genet. Anal. Biomol. Eng.* **12,** 63–67.
12. Paran, I. & Michelmore, R. W. (1993) *Theor. Appl. Genet.* **85,** 985–993.
13. Wu, K. K., Burnquist, W., Sorrells, M. E., Tew, T. L., Moore, P. H. & Tanksley, SD (1992) *Theor. Appl. Genet.* **83,** 294–300.
14. Lubbers, E. L., Arthur, L., Hanna, W. W. & Ozias-Akins, P. (1994) *Theor. Appl. Genet.* **89,** 636–642.
15. Meksem, K., Leister, D., Peleman, J., Zabeau, M., Salamini, F. & Gebhardt, C. (1995) *Mol. Gen. Genet.* **249,** 74–81.
16. Harlan, J. R., Brooks, M. H., Borgaonkar, D. S. & DeWet, J. M. J. (1964) *Bot. Gaz.* **125,** 41–46.
17. Koltunow, A. M., Bicknell, R. A., Chaudhury, A. M. (1995) *Plant Physiol.* **108,** 1345–1352.
18. Jefferson, R. A. (1993) in *Proceedings of the International Workshop on Apomixis in Rice*, ed. Wilson, K. J. (CAMBIA, Canberra, Australia), pp. 207–217.
19. Peacock, J. (1993) in *Proceedings of the International Workshop on Apomixis in Rice*, ed. Wilson, K. J. (CAMBIA, Canberra, Australia), pp. 11–21.
20. Calzada, J. P. V., Crane, C. F., Stelly, D. M. (1996) *Science* **274,** 1322–1323.
21. Carman, J. G. (1997) *Biol. J. Linn. Soc.* **61,** 51–94.
22. Grimanelli, D., Leblanc, O., Gonzalez de Leon, D. & Savidan, Y. (1995) *Apomixis Newsletter* **8,** 37–39.
23. Pessino, S. C., Ortiz, J. P. A., Leblanc, O., do Valle, C. B., Evans, C. & Hayward, M. D. (1997) *Theor. Appl. Genet.* **94,** 439–444.
24. Gustine, D. L., Sherwood, R. T. & Huff, D. R. (1997) *Crop Sci.* **37,** 947–951.
25. Wu, K. S. & Tanksley, SD (1993) *Plant Mol. Biol.* **23,** 243–254.
26. Sherman, J. D. & Stack, S. M. (1995) *Genetics* **141,** 683–708.
27. Schulz-Schaefer, J. (1980) *Cytogenetics: Plant, Animal and Human* (Springer, New York).
28. Charlesworth, B. (1994) *Curr. Biol.* **4,** 739–741.
29. Lyttle, T. W. (1991) *Annu. Rev. Genet.* **25,** 511–557.
30. Lyttle, T. W. (1993) *Trends Genet.* **9,** 205–210.
31. Trowsdale, J. (1993) *Trends Genet.* **9,** 117–122.
32. Ferris, P. J. & Goodenough, U. W. (1994) *Cell* **76,** 1135–1145.
33. Boyes, D. C., Nasrallah, M. E., Vrebalov, J. & Nasrallah, J. B. (1997) *Plant Cell* **9,** 237–247.
34. Matzke, M. A. & Matzke, A. J. M. (1995) *Trends Genet.* **11,** 1–3.
35. Meyer, P. (1996) *Biol. Chem. Hoppe-Seyler* **377,** 87–95.
36. Taliaferro, C. M. & Bashaw, E. C. (1966) *Crop Sci.* **6,** 473–476.
37. Anand, S. C. & Leng, E. R. (1964) *Cytologia* **29,** 324–329.
38. Hignight, K. W., Bashaw, E. C. & Hussey, M. A. (1991) *Bot. Gaz.* **152,** 214–218.
39. Leblanc, O., Grimanelli, D., Gonzalez-de-Leon, D. & Savidan, Y. (1995) *Theor. Appl. Genet.* **90,** 1198–1203.
40. Sherwood, R. T., Berg, C. C. & Young, B. A. (1994) *Crop Sci.* **34,** 1490–1494.
41. Nogler, G. A. (1982) *Bot. Helv.* **92,** 13–22.
42. Richards, A. J. (1996) *Apomixis Newsletter* **9,** 1–3.
43. Harlan, J. R. & DeWet, J. M. J. (1975) *Bot. Rev.* **41,** 361–390.
44. Matzke, M. A. & Matzke, A. J. M. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 53–76.
45. Jorgensen, R. (1994) *Dev. Genet.* **15,** 523–532.
46. Scheid, O. M., Jakovleva, L., Afsar, K., Maluszynska, J. & Paszkowski, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7114–7119.
47. Guo, M., Davis, D. & Birchler, J. A. (1996) *Genetics* **142,** 1349–1355.