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Transmission of the apomictic mode of reproduction in *Pennisetum*: co-inheritance of the trait and molecular markers

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Summary. Apomixis, asexual reproduction through seed, is an obligate mode of reproduction in several species from the genus *Pennisetum*. Transfer of apomixis to sexual, cultivated pearl millet (*P. glaucum*) from a wild species *P. squamulatum* has resulted in an obligate apomictic backcross line with a low, but unknown number, of chromosomes from the wild species. Molecular markers (restriction fragment length polymorphisms and random amplified polymorphic DNAs) have been identified that unequivocally demonstrate the presence of *P. squamulatum* DNA in BC_3. Three of the informative RFLP clones have been sequenced and converted to sequence-tagged sites that can be amplified by the polymerase chain reaction. Molecular genetic analysis of more advanced backcross individuals, using the two types of polymerase chain reaction-based molecular markers, has demonstrated co-inheritance of apomictic reproduction and two of the molecular markers. The remaining five molecular markers generally co-segregate with each other but are not linked with the mode of reproduction. These results suggest that genes for apomixis apparently can be transmitted by a single chromosome. Chromosome-specific markers will provide a starting point for the mapping of this genetically intractable reproductive trait.

Key words: Apomixis – Agamospermy – Pearl millet – Interspecific hybrids

Introduction

Apomixis, also termed agamospermy, is defined as asexual reproduction through seed (Winkler 1908; Nogler 1984; Asker and Jerling 1992). It is a reproductive process that bypasses female meiosis and syngamy to produce embryos genetically identical to the maternal parent. With apomictic reproduction, progeny of either specially-adapted or hybrid genotypes would maintain their genetic fidelity throughout repeated life cycles. This trait would revolutionize agriculture, particularly in countries dependent on low-input cropping methods. Obligate apomictic reproduction has not been achieved in any major crop species, but its potential impact on crop production would have global consequences.

In the grass family, *Poaceae*, tribe *Paniceae*, the predominant form of apomixis is apospory (Brown and Emery 1958). Aposporous apomicts are characterized by the participation of one or more nucellar cells in the direct formation of one or more embryo sacs. Each nucleus of the aposporous embryo sac has the somatic chromosome number and genotype of the maternal plant. Some aposporous species require pollination and fertilization of polar nuclei for the induction of endosperm (pseudogamy), but the unreduced aposporous egg develops without fertilization (parthenogenetically). Female meiosis is usually terminated in aposporous apomicts which form all of their seed asexually (obligate apomicts) so that no functional megaspore continues development beyond the first mitotic division. Facultative apomicts exist in which meiosis and aposporous development occur simultaneously and both reduced and unreduced embryo sacs ultimately reside in the same individual and/or the same ovule. Thus the two modes of reproduction (sexual and asexual) can coexist or one can be dominant over the other. During obligate apospory, several events must be coordinately regulated, including development of aposporous embryo sacs, termination of meiosis, and parthenogenesis; nevertheless, only one or a few genes may be responsible for initiation of this cascade of

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events. Scant genetic studies, although open to interpretation, suggest that aposporous apomixis is simply inherited (Nogler 1984; Asker and Jerling 1992). Results to be presented in this paper support the limited evidence that only one or a few tightly linked genes, residing on a single chromosome, may be required for the genetic transmission of apomixis in *Pennisetum*.

The genus *Pennisetum*, in which the major cultivated taxon is sexual pearl millet, *P. glaucum* (L.) R. Br., contains an abundance of non-domesticated apomictic species (Hanna 1986). Transfer of gene(s) controlling apomixis from wild to cultivated species of *Pennisetum* through crossing has proceeded empirically over the last decade (Hanna 1986). Interspecific hybrids with pearl millet have generally been highly male-sterile. However, normal male meiosis in an apomictic hybrid, resulting in fertile pollen, is a prerequisite for continued crossing since an inherent property of apomixis is the lack of meiotically-reduced (i.e., recombinant) female gametes. Progress with introgression has been achieved by elevating male fertility in complex hybrids produced between induced tetraploid pearl millet ($2n=4x=28$), the wild apomictic species, *P. squamulatum* Fresen ($2n=6x=54$), and a bridging species, *P. purpureum* Schum. ($2n=4x=28$) (Dujardin and Hanna 1984). Backcrosses of this trispecific (double-cross) hybrid to 28-chromosome, tetraploid pearl millet have progressed through four generations during which continual selection for apomictic reproduction has been imposed. Wild-species chromosomes were progressively eliminated at each backcross generation up to backcross 3 (BC_3), from which a single obligate apomictic, 29-chromosome plant was selected (Dujardin and Hanna 1989). The BC_3 clonal line, and the four cultivars or accessions appearing in its pedigree, were the genotypes used to initiate a search for molecular markers potentially linked with apomixis. Two types of molecular markers dependent on DNA sequence or arrangement have been applied in our linkage study: namely, restriction fragment length polymorphisms (RFLP) (Botstein et al. 1980; Soller and Beckman 1983; Tanksley et al. 1989) and random amplified polymorphic DNAs (RAPD) (Welsh and McClelland 1990; Williams et al. 1990).

Materials and methods

Plant material

All genotypes contributing to the pedigree of BC_3 and the BC_3 clonal line were used for DNA isolation. These genotypes included pearl millet inbred lines '23BE' and '239DB', *P. purpureum* accession N39-2, and apomictic *P. squamulatum* accession PS26. BC_4 plants were raised from seed produced by crossing male-sterile tetraploid pearl millet with BC_3 (Dujardin and Hanna 1989). The male-sterile female parent insured that backcrosses, and not selfed progeny, were obtained. Sixty-six male-sterile BC_4s were screened for embryo sac development by clearing ovules in methyl salicylate (Young et al. 1979). To supplement the number of apomicts, ten apomictic BC_4S_1 (S_1 = first selfed generation from BC_4) progeny from four previously classified male-fertile apomictic BC_4 plants, one BC_4S_1 male-sterile plant, and 33 segregating progeny (14 sexual, 4 obligate apomicts, 15 facultative apomicts) from one previously identified male-fertile BC_4 facultative apomict (designated A1), were included in the analysis. Tender furled leaves were harvested from the above plant materials, then frozen and stored at −80 °C.

DNA isolation, blotting, and hybridization

A method modified from Tai and Tanksley (1990) was used for DNA extraction. Approximately 10 g of frozen tissue was ground to a fine powder with liquid nitrogen then added to 75 ml of extraction buffer, consisting of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA; 500 mM NaCl, 1.25% SDS, and 0.38% sodium bisulfite added just before use. The homogenate was incubated at 65 °C for 20–60 min and subsequently processed according to the published protocol. Genomic DNA was digested with *Dra*I, *Hin*dIII, *Eco*RI, *Eco*RV, or *Bam*HI, electrophoresed in an 0.8% agarose gel in Tris-borate-EDTA buffer, and transferred to nylon membranes (Magnagraph, Genescreen Plus) by the capillary method of Southern (1975). Blots were prehybridized and hybridized according to the respective manufacturers' instructions using aqueous hybridization solutions at 65 °C. PCR-amplified or gel-purified plasmid inserts were labelled with ^{32}P by the random hexamer method. Hybridized blots were washed at a final stringency of 0.1 × SCC, 65 °C.

Library construction

DNA from BC_3 was digested with *Pst*I and ligated with *Pst*I-cut pUC19. Plasmid DNA was transformed into host strain DH5α and recombinant clones were selected for ampicillin resistance and inactivation of *lac*Z by white colony formation on media containing bluo-gal. Approximately 90% of the *Pst*I clones were single to low copy number as judged by hybridization with ^{32}P-labelled total genomic DNA from pearl millet.

DNA sequencing

Plasmid inserts were amplified with M13 forward and reverse primers from New England Biolabs and purified by HPLC prior to sequencing. Purified insert DNA was sequenced using the DyeDeoxy Terminator Sequencing kit and automated sequencing equipment from Applied Biosystems. Sequencing primers were nested M13 forward and reverse sequences. Sufficient sequence was generated to allow the design of oligonucleotide primers that would amplify sequence-tagged sites (STS) from genomic DNA.

DNA amplification

Forward and reverse oligonucleotide primers for sequence-tagged sites were synthesized by the Molecular Genetics Facility, University of Georgia as follows: UGT184f 5′ CTGCAGAAGTGCAGATCCAA 3′; UGT184r 5′ CTGCAGCATATGGGCTCCTC 3′; UGT197f 5′ CTGCAGACCTCCAAACAG 3′; UGT197r 5′ CTGCAGCATGTGAACCAT 3′; UGT1f1 5′ CTGCAGAACGAAACAAGTGTG 3′; UGT1r2 5′ GTGTGTCTCTGAATCTGGAG 3′. Amplification conditions in a Perkin Elmer/Cetus thermal cycler were denaturation for three cycles of 1 min at 97 °C and 32 cycles of 1 min at 94 °C, annealing for 1 min at 45 °C (UGT197) or 55 °C (UGT184, UGT1), and extension for 2 min at 72 °C with a 3 s auto-segment extension of each cycle. RAPD primers were obtained as kits of

20 10-base primers from Operon Technologies, Alameda, Calif. Primer kits B, C, D, E, and F were surveyed. Amplification conditions were essentially according to Williams et al. (1990) except that primer concentration was increased to 0.5 µM. The PCR reaction mix (25 µl) contained 10 mM of Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM of $MgCl_2$, 0.1% Triton X-100, 100 µm each of dATP, dCTP, dGTP, dTTP, 0.5 µM of primer, 25 ng genomic DNA, and 0.5 U of Taq DNA polymerase (Promega). Amplification conditions consisted of three cycles of 1 min at 97 °C, 1 min at 36 °C, 2 min at 72 °C followed by 32 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C with a 3 s auto-segment extension of each cycle.

Results and discussion

Identification of sequence-tagged sites and RAPD markers

Over 90% of the 48 RFLP probes were single to low copy number as judged by hybridization with genomic DNAs. Approximately 94% of the probes were polymorphic between *P. glaucum* and *P. squamulatum*. Seven out of forty-eight RFLP probes hybridized to a restriction fragment in BC_3 that was shared only with the apomictic parental accession of *P. squamulatum* (Fig. 1). One of the informative probes (UGT197) was particularly interesting because it did not hybridize at all with the non-apomictic genotypes in the pedigree of BC_3 (Fig. 1 B). Three of the probes (UGT197, UGT184, UGT1) were converted to sequence-tagged sites by amplification of the expected fragments of 144 bp, 181 bp, and 1 kb, respectively, from BC_3 genomic DNA. Two unpredicted results were also obtained from DNA amplified with the UGT197 and UGT184 oligonucleotide primers. First, a RAPD pattern was observed when three of the four primers were used individually in an amplification reaction (Fig. 1 C), and second, UGT184 forward + reverse primers amplified a band from *P. purpureum* that appeared to be indistinguishable from the band amplified in *P. squamulatum* and BC_3. Even though UGT184 was amplified in parental *P. purpureum*, the UGT184-probed genomic Southern indicated that only the *P. squamulatum* marker was present in BC_3. One-hundred RAPD primers were surveyed for their ability to amplify informative fragments. Twenty-six of the primers resulted in essentially no amplification. Of the remaining 74 primers, all amplified one or more polymorphic bands between *P. glaucum* and *P. squamulatum*. Only four primers (OPC-04 5′CCGCATCTAC 3′; OPE-11 5′GAGTCTCAGG 3′; OPE-14 5′TGCGGCTGAG 3′; OPF-05 5′CCGAATTCCC3′) amplified clearly distinguishable and reproducible fragments that were shared solely by BC_3 and *P. squamulatum*. Several additional primers amplified fragments shared by *P. purpureum*, *P. squamulatum*, and BC_3. This result might indicate a closer relationship between *P. purpureum* and *P. squamulatum* than had previously been suggested. It also complicated our molecular analysis by reducing the number of potentially informative markers.

Segregation of markers and apomixis in BC_4 progeny

Mendelian segregation could not be established from any of our backcross populations because the chromosome bearing the gene(s) for apomixis is from an alien genome and presumably does not have a homolog with which to pair regularly in BC_3 (Dujardin and Hanna 1989). The

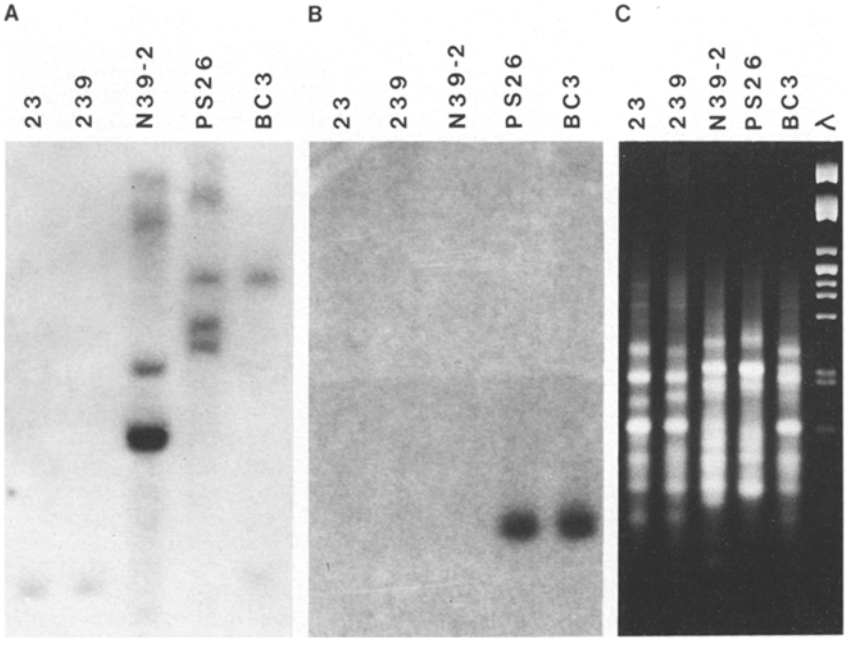

Fig. 1 A–C. A Southern blot of *Dra*I-digested genomic DNA from BC_3 and its pedigree lines hybridized with radiolabelled plasmid insert of UGT184 (**A**) or UGT197 (**B**). A band shared only by PS26 and BC_3 can be observed in both **A** and **B**. **C** A RAPD pattern produced with all genomic DNAs and the single 18-base primer UGT197f. Multiple bands also were observed with UGT197r and UGT184f. Abbreviations: *P. glaucum* '23BE' (*23*) and '239DB' (*239*); *P. purpureum* (*N39-2*); *P. squamulatum* (*PS26*); backcross 3 (*BC3*). Marker lane is lambda DNA digested with *Pst*I

frequency of transmission of apomixis in these progeny is known to be low (Dujardin and Hanna 1989) and the possibility for recombination is unknown. Because of these limitations, we could not rely on the usual statistical analysis of the molecular data in the backcross population but instead assumed linkage if a molecular marker phenotype and reproductive phenotype corresponded at least 95% of the time.

Segregation of seven informative molecular markers (four RAPD and three STS markers) was followed in the male-sterile BC_4 population. We chose to use a male-sterile female parent to generate the BC_4 population for molecular analysis so that we would be assured of the hybrid nature of all backcross progeny and would eliminate the possibility of selfs. This strategy is not useful, however, for perpetuating a backcrossing program since all BC_4 individuals would be male-sterile and could not be used as male parents to advance to the BC_5 generation. In an obligate apomict, recombination occurs only during male gametogenesis; thus transfer of apomixis must occur through the pollen and not through the unreduced, maternally-derived egg. Sixty-six male-sterile BC_4 progeny were screened for mode of reproduction by examining cleared ovules and scoring embryo-sac development. Sixty-one segregated as sexual individuals, two were obligate apomicts, and the remaining three showed some ambiguity in embryo-sac development and so were classified as facultative apomicts. Based on the segregation of the seven PCR-based markers, it appears that there are at least two independently assorting linkage groups in BC_3 that were derived from *P. squamulatum* (Table 1). One of the linkage groups, identified by cosegregation of five of the molecular markers (OPE-11, OPE-14, OPF-05, UGT184, UGT1), was transmitted to 43% of the male-sterile BC_4 progeny regardless of reproductive mode (Table 1, Fig. 2). One individual (BC_4-9) lacked three of five markers (OPF-05, UGT184, UGT1) and one (BC_4-109) lacked the other two of five markers (OPE-11, OPE-14) indicating loss through recombination or chromosome breakage. Strict cosegregation of the remaining two markers (UGT197, OPC-04) did occur (Table 1, Fig. 3). These markers were tightly linked with apomixis and were always present in obligate apomicts. Only one (BC_4-109) out of 61 sexual, male-sterile BC_4 progeny had both markers for apomixis. Three explanations could account for this observation: the plant collected was mislabelled or misclassified, expression of the gene(s) for apomixis was somehow attenuated in this individual, or chromosome breakage separated the markers from the gene(s) for the trait.

None of the 14 sexual male-fertile A1 (BC_4S_1) individuals had the markers for apomixis, while the four obligate apomicts from this population all carried the markers. The markers for apomixis were present in 72% of the individuals from two populations classified as facultative apomicts. Although this result might indicate that apomixis occurs in individuals lacking the markers, we would reserve this conclusion until a better method for categorizing facultative apomicts is developed.

Implications of marker-based selection for apomixis

One objective of our work has been to apply marker-based selection of apomictic backcross progeny to the breeding program. Currently, discrimination between apomictic and sexually reproducing plants involves embryo-sac histology, which requires either that the plants reach flowering, or else that progeny tests be carried out in the next generation, before identification can be completed (Bashaw 1980). In an annual plant such as pearl millet, flowering followed by senescence can occur rapidly. When short generation time is compounded with the necessary screening of thousands of progeny, because of infrequent transmission through the pollen of the chromosome(s) with a gene(s) controlling apomixis, early identification of phenotype becomes extremely valuable. Of the two types of molecular markers used in our study, the PCR-based RAPD technique is much quicker to perform and requires much less plant material than RFLP screening. We have also succeeded in converting some of

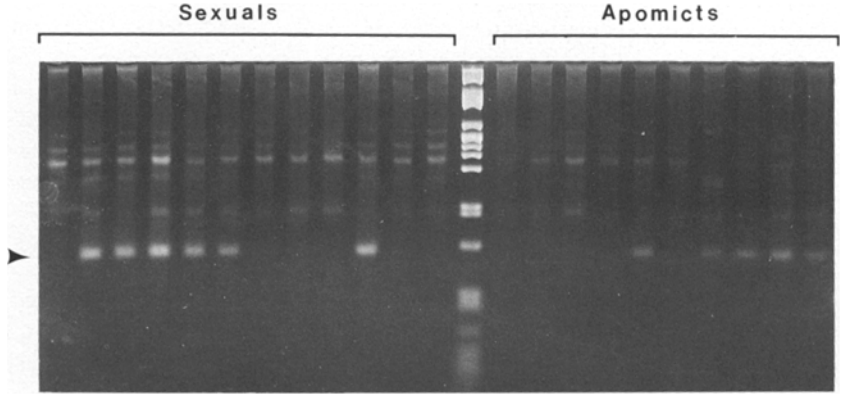

Fig. 2. The *P. squamulatum* linkage group that was transmitted most frequently to BC_4 progeny is represented by one fragment amplified by OPE-11 (*arrow*). The same individuals show expected fragments amplified by OPE-14 and UGT184. Marker lane is lambda DNA digested with *Pst*I

Table 1. Amplification of the five informative markers in *Pennisetum* parental genotypes and BC_4 and BC_4S_1 progeny. Sexual (S) plants comprise four possible genotypes, two of which are rare and occur in only one plant each. Facultative (F) apomicts do not display a consistent genotype, but the ambiguity in classification prevents any definite conclusions. Obligate apomicts (A) always have the linkage group represented by OPC-04 and UGT197, and are variable for presence of the second linkage group

Individuals	Repro-duction	Marker				
		OPE-11 OPE-14	UGT1	OPF-05	UGT184	UGT197 OPC-04
Parental genotypes						
23BE	S	−	−	−	−	−
239DB	S	−	−	−	−	−
N39-2	S	−	−	−	+	−
PS26	A	+	+	+	+	+
BC_3	A	+	+	+	+	+
Male-sterile BC_4 progeny						
1, 2, 7, 8, 12, 13, 25, 26, 29, 30, 32, 35–37, 39, 44, 54, 56, 65, 79, 80, 83, 86, 88, 90, 92–94, 101, 102, 114, 119	S	−	−	−	−	−
3, 41, 48	S	−	+	−	−	−
5, 10, 11, 14, 19–22, 28, 38, 40, 43, 45, 47, 50, 52, 58, 61, 97, 100, 110	S	+	+	+	+	−
49, 51, 91	S	+	−	+	+	−
9	S	+	−	−	−	−
109	S	−	+	+	+	+
42	F	−	−	−	−	+
59, 118	F	+	+	+	+	−
53	A	+	+	+	+	+
84	A	−	−	−	−	+
BC_4S_1 apomictic progeny						
A1-4, 8, 11, 14	A	−	−	−	−	+
A4-3, 4, 9	A	−	−	−	−	+
A10-1	A	+	+	+	+	+
A21-7, 11, 28	A	−	−	−	−	+
A25-1, 8, 10, 12	A	−	−	−	−	+

the informative RFLP markers to sequence-tagged sites that can be amplified using PCR (Olson et al. 1989; Williams et al. 1991).

Application of our marker-based selection strategy will initially proceed cautiously since we do not know the potential for chromosome breakage, rearrangement, or deletion. At worst, however, selecting for the two markers cosegregating with apomixis might bias our selection towards plants with significant linkage drag if there is considerable genetic distance between the markers and the trait. Because of the lack of detectable recombination between the alien chromosomes and the pearl millet genome, we are not able to achieve mapping resolution such as has been accomplished with disease resistance loci in tomato (Young et al. 1988; Klein-Lankhorst et al. 1991; Messeguer et al. 1991) and barley (Hinze et al. 1991), for example. Until the genetic distance between our markers and the gene(s) for apomixis is known, we risk eliminating desirable translocations between a portion of the alien chromosome and a chromosome of the cultigen, or a rare recombination event. A similar interspecific situation in *Brassica* has shown that C genome-specific markers were transmitted as an intact group approximately 50% of the time in two different chromosome addition lines (Hu and Quiros 1991). Based on karyotype analysis, disappearance of one or more markers from the group was interpreted to be the result of large internal and terminal deletions. In *Pennisetum* interspecific hybrids, it is presently very difficult to cytologically distinguish the smallest pearl millet chromosomes from the alien chromosomes.

Few apomictic plants have been used for genetic studies, and the nature and inheritance of the reproductive trait varies between genera (Nogler 1984; Asker and Jerling 1992). *P. squamulatum* is a hexaploid perennial species with at least two dissimilar genomes (Jauhar 1981)

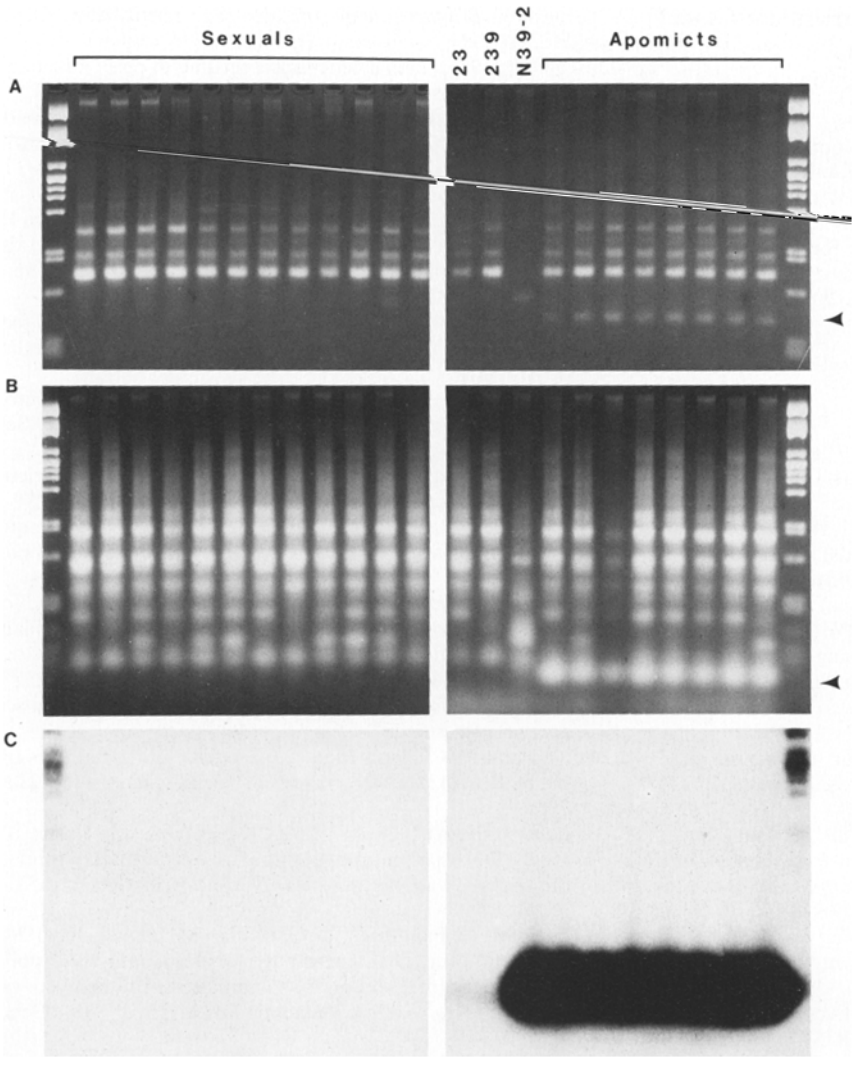

Fig. 3A–C. Molecular markers co-segregating with the apomictic mode of reproduction in *Pennisetum*. **A** The linkage group that almost invariably segregates with apomixis is represented by an OPC-04-amplified band (*arrow*). **B** UGT197 (*arrow*) shows the same pattern of segregation as OPC-04. **C** To confirm that the expected UGT197 fragment was amplified by UGT197f+r primers, a blot of the PCR gel was hybridized with a UGT197 plasmid insert. Only one fragment showed homology. The other amplified bands, presumably RAPD bands, served as useful internal controls to judge the success of amplification. The apomictic donor parent, PS26, (not shown) has the expected OPC-04- and UGT197-amplified bands. Abbreviations: *P. glaucum* '23BE' (*23*) and '239DB' (*239*); *P. purpureum* (*N39-2*). Marker lane is lambda DNA digested with *Pst*I

any one of which might be the source of the gene(s) for apomixis. No sexual plants of *P. squamulatum* are available for inheritance studies. Nevertheless, we have chosen *Pennisetum* as a model system because strong expression of the apomictic trait persists in hybrids, and genetic material suitable for molecular studies are available. *P. squamulatum* is heterozygous for apomictic reproduction since both sexual and apomictic reproductive phenotypes occur among interspecific F_1 hybrids between pearl millet and *P. squamulatum*. Our evidence indicates that the gene(s) for apomixis is(are) located on a single chromosome. Presently, the only possible route for mapping markers associated with apomixis in *Pennisetum* is through an interspecific cross. Two interspecific F_1 hybrids between tetraploid pearl millet and *P. squamulatum*, one obligate sexual individual and one obligate apomict, were maintained from the inception of the gene-transfer project (Dujardin and Hanna 1983). We have observed segregation of the UGT197 sequence-tagged site which was amplified in the apomictic F_1 but not in the sexual F_1 (data not shown). If other markers also show a polymorphism that indicates heterozygosity between the chromosomal homologs that harbor the apomixis locus (loci), we will proceed to map genetic and physical distances between the trait and the markers in an interspecific F_1 population segregating for the mode of reproduction.

In conclusion, we believe that obligate apomixis would be a highly desirable mode of reproduction in crop plants if transfer of the trait outside its naturally occurring range could deliver true-breeding progeny. Obligate apomixis in crop plants could greatly reduce the efforts required for hybrid seed production (Hanna and Bashaw 1987). Serious attempts to transfer the gene(s) for

apomixis beyond natural crossing barriers must await further elucidation of the inheritance and genetic control of this understudied mode of reproduction.

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