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REVIEW

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Molecular characterization of the genomic region linked with apomixis in *Pennisetum/Cenchrus*

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Abstract Apomixis is defined as asexual reproduction through seeds, although this outcome can be achieved by multiple pathways. Since little is known about the molecular control of these pathways, how they might intersect is also a mystery. Two of these pathways in the grass family, diplospory and apospory, are receiving attention from molecular biologists. Apospory in *Pennisetum/Cenchrus*, two genera of panicoid grasses, results in the formation of four-nucleate embryo sacs that lack antipodals. Sexual reproduction frequently aborts so that the resulting seed is composed of (1) a parthenogenetically derived embryo that is genetically identical to the mother and (2) endosperm formed through pseudogamy. The transmission of apomixis is associated with the transfer of a linkage block on a single chromosome. This linkage block contains repetitive sequences as well as hemizygous, low-copy DNA sequences. Fluorescence in situ hybridization has demonstrated that these DNA regions occur on only a single chromosome, but not its homologs, in the polyploid apomicts studied. Features of the apomixis-associated region resemble those of other chromosomal segments isolated from recombination and replete with "selfish" DNAs.

Keywords Apomixis · Agamospermy · Apospory · *Pennisetum/Cenchrus*

Introduction

Apomixis is a term that indicates the developmental outcome of asexual reproduction through seeds. Although a seemingly simple definition, several pathways can be followed by flowering plants to achieve this outcome (Nogler 1984a; Crane 2001). Until we understand the molecular mechanisms underlying these pathways, we will not be able to determine to what extent the apparently independent pathways leading to apomixis may actually be integrated with one another through the developmental network of the ovule. Upon deeper reflection on the consequence of apomixis, one realizes that it poses not only a fascinating scientific challenge to explore the developmental adaptations of the ovule, but also a tremendous practical opportunity for plant breeding and hybrid production (Hanna 1995; Savidan 2000). The vigor of hybrids between divergent genotypes is often greater than that of inbreds as a result of heterosis. Hybrid breeding is the foundation for production of many crops in developed countries. Progeny from these hybrids are obviously not genetically uniform; therefore, crosses to produce hybrid seed must be made repeatedly. An apomictic hybrid could radically change this situation, since it could produce genetically identical offspring. Unfortunately, no major crops demonstrate apomictic reproduction.

The broad categories into which apomicts have been grouped include adventitious embryony, a sporophytic type of apomixis, and two gametophytic forms, diplospory and apospory (Koltunow 1993; Fig. 1). In sporophytic apomixis, there is no alternation of generations required for the development of embryos of the maternal genotype because one or more non-generative cells of the ovule derive the fate of embryo initials which directly form embryos. This type of apomixis is commonly associated with *Citrus* (Koltunow et al. 1995a). In gametophytic apomixis, the female gametophyte (embryo sac) develops from the megaspore mother cell after mitotic division (diplospory) or from a nearby nucellar cell (apospory). In both cases, the female gametophyte contains nuclei that are genetically identical to the maternal parent since they are products of mitosis, not meiosis. Furthermore, one of these nuclei becomes the egg cell which continues to undergo only mitotic division in the absence of fertilization. This parthenogenetic development of the egg cell must be accompanied by

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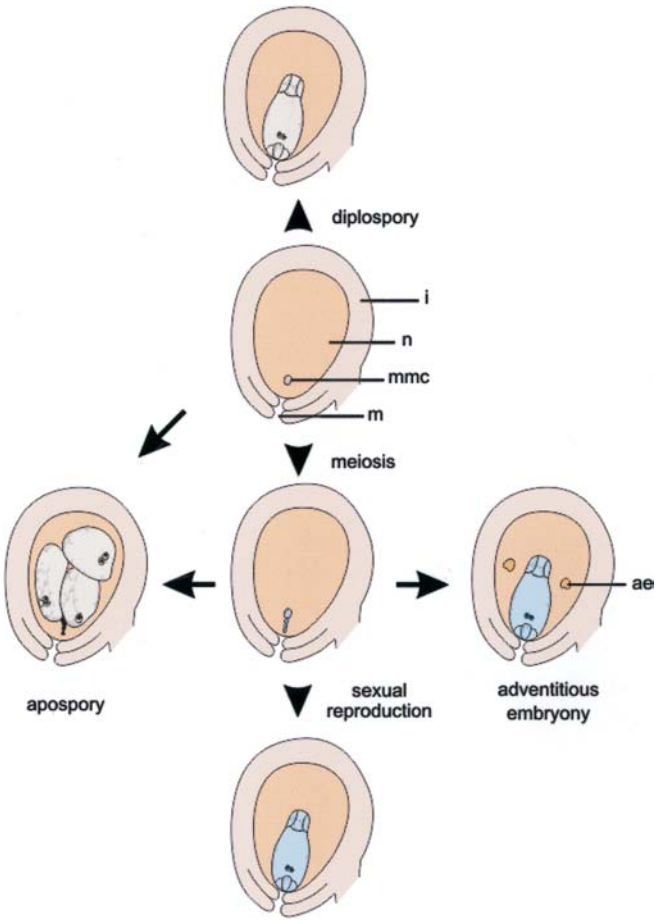

Fig. 1 Ovule development as governed by different types of apomixis. The nucellus (*n*) is the site of divergent developmental patterns. The labeled ovule shows the nucellus surrounded by two integuments (*i*) that remain unfused at the micropyle (*m*). In sexual reproduction, a single megaspore mother cell (*mmc*) develops at the micropylar end of the nucellus and undergoes meiosis to form a linear tetrad of four megaspores (*blue*). The three micropylar megaspores degenerate, leaving the chalazal megaspore to develop into an eight-nucleate, seven-celled embryo sac. Each nucleus of the embryo sac (*blue*) is haploid with respect to the maternal tissue (*peach*). In adventitious embryony, the same developmental processes occur as in sexual reproduction with a superimposed direct development of nucellar (adventitious) embryos (*ae*) surrounding the embryo sac. In diplospory, meiosis is either completely or partially bypassed so that the single embryo sac formed from the mmc contains unreduced nuclei. At anthesis, the structure of the ovule may be indistinguishable between diplospory and sexual reproduction. In apospory, sexual reproduction can sometimes proceed to completion, but is most often arrested in favor of one or more unreduced embryo sacs that develop from somatic cells of the nucellus

endosperm formation in order to produce viable seed. The endosperm develops autonomously from the central cell in some apomicts, but most require pollination and probably fertilization of the central cell (pseudogamy). Therefore, to achieve apomixis in crops, female meiosis must be subordinated or placed under inducible control, the embryo must develop from a maternal, genetically unaltered cell, and compatible endosperm will be required. Much has been learned about these "components" or "elements" of apomixis by the study of natural apomicts and mutants of sexual plants (Grossniklaus et al. 2001). This combined knowledge may eventually allow the "synthesis" or "transfer" of apomixis in non-apomictic plants.

Reviews with apomixis as the subject are numerous (Asker and Jerling 1992; Grimanelli et al. 2001; Grossniklaus et al. 2001; Koltunow et al. 1995b; Roche et al. 2001a; Savidan 2000; Spillane et al. 2001; van Dijk and van Damme 2000). This review will be more narrowly focused on a group of species in the grass family (Poaceae), subfamily Panicoideae, tribe Paniceae. These closely related species that display apomixis reproduce by the formation of aposporous embryo sacs within which parthenogenetic development of the egg cell and fertilization of the central cell complete the apomictic pathway. Both developmental and molecular research on this group of species will be discussed.

Phylogeny of *Pennisetum* and *Cenchrus*

The phylogeny of the subfamily Panicoideae has recently been investigated using the chloroplast gene *ndhF* (Giussani et al. 2001). The subfamily was concluded to be monophyletic and the tribe Paniceae (x=9 members) was one of three strongly supported clades within this subfamily. The other two clades were Andropogoneae and Paniceae (x=10 members). The x=9 Paniceae clade includes, among others, the genera *Pennisetum*, *Cenchrus*, *Setaria*, *Brachiaria*, and *Panicum* (some species), all of which contain apomictic species or cytotypes. Using another chloroplast gene, *rpoC2*, Duvall et al. (2001) confirmed the monophyly of the x=9 Paniceae clade, but *Cenchrus* (*C. ciliaris*, *C. agrimonioides*, *C. echinatus*) and *Pennisetum* (*Pennisetum* sp., *P. clandestinum* and *P. setaceum*, *P. purpureum*) formed paraphyletic groups within the monophyletic "bristle" clade. Similarly, Giussani et al. (2001) and Doust and Kellogg (2002) concluded that *Pennisetum* (*P. alopecuroides* and *P. setaceum*) falls in a monophyletic "bristle" clade with *Cenchrus*, although the *Cenchrus* clade (*C. ciliatus*, *C. echinatus*, *C. myosuroides*, *C. setigerus*) was derived from the paraphyletic *Pennisetum*, and indeed, *C. ciliaris* (buffelgrass) is often identified by its synonym *Pennisetum ciliare* (Hitchcock 1951). Clearly, only some species of the larger x=9 Paniceae clade contain apomicts, but these recent observations on the phylogenetic position of *Pennisetum* and *Cenchrus*, together with our previous demonstration of the conservation of molecular markers linked to apomixis (Lubbers et al. 1994; Roche et al. 1999), support our working hypothesis that apomixis may have evolved once within this group.

Apomixis in *Pennisetum* and *Cenchrus*

Within the genus *Pennisetum*, at least 17 species containing apomictic cytotypes have been described

(Table 1). Diploid members of a species are always sexual, but polyploid cytotypes typically are apomictic and reside in the *x*=8/9 group of species that comprise the tertiary gene pool of pearl millet [*P. glaucum* (L.) R. Br.; Harlan and deWet 1971]. Pearl millet is the major cultivated member of the genus and is grown in tropical and sub-tropical regions for forage and grain. This domesticated species has a basic chromosome number of *x*=7 which probably reflects a derived state from the predominant basic chromosome number of *x*=9 in the genus.

Table 1 Species in the genera *Pennisetum* and *Cenchrus* that contain apomictic cytotypes. Data and original references can be found in Dujardin and Hanna (1984a), Fisher et al. (1954), Hanna (1987), Jauhar (1981), and Schmelzer (1997). Morphological descriptions of the species shown in *bold* can be found in the World Grasses Database at the Kew Royal Botanic Gardens (<exref type="URL">www.rbgkew.org.uk/herbarium/gramineae/wrld-gr.htm</exref>)

Species	2*n*	*x*
C. ciliaris	36	9
C. setigerus	36	9
P. clandestinum	36	9
P. dubium	14–86	?
P. flaccidum	18, 36	9
P. frutescens	63	9
P. hordeoides	36, 54	9
P. latifolium	36	9
P. macrostachyum	54	9
P. macrourum	36	9
P. massaicum (syn. *P. mezianum*)	16, 32	8
P. orientale	18, 27, 36, 45, 54	9
P. pedicillatum	36, 45, 54	9
P. polystachion	18, 36, 45, 54	9
P. setaceum	27, 54	9
P. setosum	54	9
P. squamulatum	54	9
P. subangustum	18, 36, 54	9
P. villosum	18, 27, 36, 45, 54	9

Apomictic species/cytotypes of *Pennisetum*/*Cenchrus* display very similar ovule developmental patterns. Apomixis is of the gametophytic and aposporous type with a four-nucleate megagametophyte at maturity (Fig. 2). The progression of developmental events has been most thoroughly described for *C. ciliaris* (Fisher et al. 1954; Snyder et al. 1955; Sherwood 1995; Vielle et al. 1995; Peel et al. 1997), and *P. squamulatum* (Dujardin and Hanna 1984a; Chapman and Busri 1994; Peel et al. 1997; Wen et al. 1998), although data are available for other apomictic *Pennisetum* species (Simpson and Bashaw 1969; Jauhar 1981; Dujardin and Hanna 1984a). In both sexual and apomictic genotypes, an archesporial cell is specified and becomes the megaspore mother cell (MMC) whose wall contains callose (Peel et al. 1997). The MMC enters meiosis, but megaspore formation in apomicts typically is incomplete, its termination varying from the early initiation stage of meiosis to the triad or tetrad stage. Aposporous initials can be observed to develop from nucellar cells in proximity to the MMC or its products, and it is possible that their development may influence the events of meiosis by affecting the position of cell division plates (Peel et al. 1997). The aposporous initials continue to enlarge and the nuclei begin to divide, often in multiple initials. As many as eight aposporous embryo sacs have been observed in some genotypes of *C. ciliaris* and the number may be under quantitative genetic control given the variation observed among genotypes (Snyder et al. 1955). Aposporous embryo sacs often occupy most of the nucellar region, and those that reach maturity contain four nuclei in three to four cells. The aposporous embryo sac nearest the micropyle may develop faster than the others (Wen et al. 1998). The mature aposporous embryo sac has an egg, one or two synergids and one or two polar nuclei. Snyder et al. (1955) observed that the egg apparatus was positioned toward the nearest outer cell layer of the nucellus which often was distant from the micropyle. At anthesis, uninucleate central cells (Snyder et al. 1955; Chapman and Busri 1994; Vielle et al. 1995; Morgan et

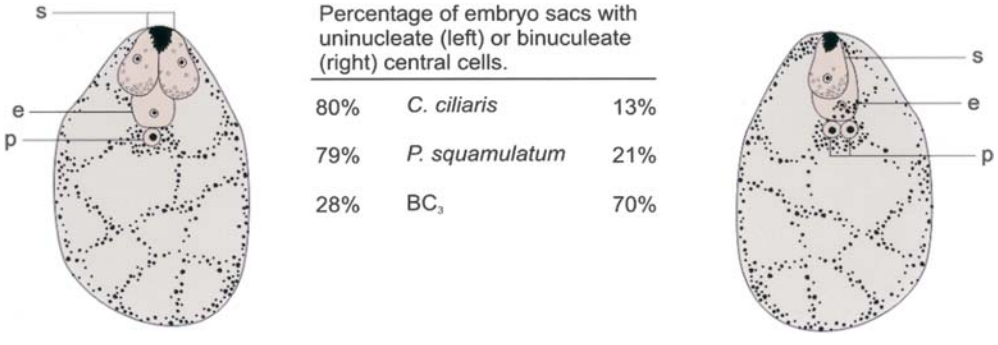

Fig. 2 *Panicum*-type embryo sac structure at maturity. The *Panicum*-type embryo sac contains only four unreduced nuclei at maturity. The natural apomicts, *Cenchrus ciliaris* and *P. squamulatum*, have predominantly uninucleate central cells, perhaps as a means to avoid deleterious maternal:paternal genome ratios in endosperm after fertilization of the central cell. An introgression line, BC_3, where apomixis was transferred from *P. squamulatum* to pearl millet, has predominantly binucleate central cells and displays low seed set due in part to endosperm abortion. The number of synergids can be one or two because they appear to compensate for the variable number of polar nuclei. The remaining nucleus is designated as the egg which develops without fertilization (after Vielle et al. 1995; Morgan et al. 1997). *e* Egg, *p* polar nucleus, *s* synergid

al. 1998) and binucleate central cells (Chapman and Busri 1994; Vielle et al. 1995; Morgan et al. 1998; Wen et al. 1998) have both been observed in mature four-nucleate aposporous embryo sacs. Wen et al. (1998) reported that the two central cell nuclei fuse to form a secondary nucleus prior to anthesis, although Sherwood (1995) reported that the polar nuclei had a 2C DNA content expected of a diploid (unreduced) cell in the G1 phase of the cell cycle. No seeds are formed in the absence of pollination (Snyder et al. 1955; Simpson and Bashaw 1969), but there is some disagreement regarding the need for fertilization of the central cell. Wen et al. (1998) reported that the secondary nucleus divides after stimulation by pollination. Fisher et al. (1954) could not observe pollen tubes entering ovules and presumed that pollination alone may be sufficient, although Snyder et al. (1955) did observe pollen tube entry into embryo sacs of similar materials, while Birari (1981) provided evidence for sperm entry into central cells. The observation of 3*n* chromosome numbers in endosperm of *C. ciliaris* (Snyder et al. 1955) and 3C DNA amounts in endosperm of an apomictic backcross (Morgan et al. 1998) is evidence that endosperm development requires not only pollination, but also fertilization. Synergid degeneration can be observed prior to pollination and/or fertilization (Chapman and Busri 1994; Vielle et al. 1995). The embryo develops parthenogenetically, i.e., without fertilization, and divisions occasionally are initiated prior to anthesis (Simpson and Bashaw 1969; Birari 1981; Vielle et al. 1995; Wen et al. 1998). For unreduced eggs which have not become activated prior to anthesis, the cell wall becomes complete shortly after pollination, contrary to no change in cell wall structure observed in the reduced egg (Vielle et al. 1995).

Two irradiation-induced reproductive mutants have been recovered from sexual *P. glaucum* whose phenotypes show components of apomixis (Hanna and Powell 1973, 1974). One of these, female sterile, is a simply inherited trait where the ovules are immature at anthesis and in which megasporogenesis aborts and multiple embryo sacs are formed (Hanna and Powell 1974; Arthur et al. 1993). The embryo sacs are largely non-functional, however, since no seed formation occurs. The second mutant, stubby head, segregates as a two-linked-gene trait and results in multiple ovules as well as multiple embryo sacs within an ovule (Hanna and Powell 1973; Morgan et al. 1997). Test crosses using stubby head as the female parent and pearl millet containing a dominant marker as the male parent have provided evidence for maternal reproduction in this mutant (no transmission of the dominant marker to test-cross progeny). Neither mutant has been studied at the molecular level because pleiotropic effects in each extend to gross morphological changes in inflorescence structure (Morgan et al. 1997). These complex phenotypes suggest that different or additional components of the network of reproductive gene expression may be affected compared with naturally occurring apomixis in the genus.

Inheritance of apomixis

Apomixis (apospory) in *Pennisetum/Cenchrus* behaves as a single dominant trait, but always exists in the heterozygous condition in an apomictic parent. All of the *Pennisetum/Cenchrus* apomicts are polyploid ranging from triploid to hexaploid, and rarely higher (Table 1). Polyploid genetics increases the complexity of segregation analysis, particularly when little is known about the pairing relationships of individual chromosomes. Nevertheless, because of the interfertility between several of the apomictic *Pennisetum* species and pearl millet, it has been possible to study the transmission of apomixis in interspecific crosses (Hanna 1987). The most successful crosses were conducted with diploid or induced tetraploid pearl millet as the female parent by *P. squamulatum*, *P. setaceum*, or *P. orientale* as pollen donors (Dujardin and Hanna 1989a; Marchais and Tostain 1997). Relatively few hybrids were obtained from crosses with the latter two species, although crosses between tetraploid pearl millet and *P. squamulatum* were highly successful. These F_1 hybrids segregated for mode of reproduction (apomixis vs sexuality; Dujardin and Hanna 1983) and have been useful for genetic mapping studies (Ozias-Akins et al. 1998). Such hybrids are often highly male fertile, but fertility declines dramatically in the first backcross to pearl millet, preventing their use in a backcross breeding program (Dujardin and Hanna 1985, 1989b). Male fertility could be maintained, however, when a bridging species (sexual *P. purpureum*) was introduced into the breeding scheme (Dujardin and Hanna 1984b, 1989b). The transmission of apomixis and linked molecular markers in these materials will be discussed in detail below.

For tetraploid *C. ciliaris*, intraspecific crosses between sexual and aposporous genotypes have shown that a single dominant allele appears to be required for the transmission of apomixis although it has been reported to exist in either the simplex or duplex condition (Sherwood et al. 1994). The gene for apomixis also can be hypostatic to a gene for sexuality (Taliaferro and Bashaw 1966); therefore, in this special situation, apomicts can segregate in the offspring of a sexual plant. The genotypes being used in the more recent molecular studies of apomixis in buffelgrass (Gustine et al. 1996, 1997; Roche et al. 1999; Jessup et al. 2002) do not show evidence for hypostasis of apomixis and may have lost any epistatic "suppressor" of apomixis.

Molecular markers for apomixis

The application of molecular markers to genetic studies of apomixis has grown considerably beyond the first such publication in 1993 (Ozias-Akins et al. 1993). Molecular markers, particularly those that are PCR-based, have been used for the analysis of genetic uniformity among offspring of a suspected apomict (Nybom 1996). Such markers also are proving invaluable for mapping regions

Table 2 Sequences isolated from or expressed in reproductive organs of aposporous apomicts. Table does not include the 950 EST sequences deposited from a *P. ciliare* apomictic pistil cDNA library (GenBank accession nos. BM083978–BM084927)

GenBank accession no./Author	Organism	Isolation method[a]	Size (bp)	Similarity to known genes[b]
AF325717/Li et al.	*P. ciliare*	cDNA	995	None
AF325718/Li et al.	*P. ciliare*	cDNA	822	None
AF325719/Li et al.	*P. ciliare*	cDNA	965	Calcium-binding protein
AF325720/Li et al.	*P. ciliare*	cDNA	851	Calreticulin
AF325721/Li et al.	*P. ciliare*	cDNA	1,216	Hypersensitive-induced response protein
AF325722/Li et al.	*P. ciliare*	cDNA	649	*Arabidopsis thaliana* hypothetical protein
AF325723/Li et al.	*P. ciliare*	cDNA	1,160	Phosphate-induced (phi1) protein
U65386/Hussey	*P. ciliare*	DD	242	None
U65387/Hussey	*P. ciliare*	DD	329	None
U65388/Hussey	*P. ciliare*	DD	263	None
U65389/Hussey	*P. ciliare*	DD	199	None
D37940[c]/Hulce et al.	*P. ciliare*	cDNA	486	None
D37938[c]/Hulce et al.	*P. ciliare*	cDNA	1,398	*Oryza sativa* unknown protein
D37939[c]/Hulce et al.	*P. ciliare*	cDNA	876	Ankyrin-repeat protein
U40219/Hulce et al.	*P. ciliare*	cDNA	941	GTP-binding protein (RAS-related Rab7)
AB000809/Chen et al.	*Panicum maximum*	cDNA	1,177	Dehydration-responsive protein (BURP domain-containing protein)
AF242537/Pessino et al.	*Paspalum notatum*	DD	396	Similar only to AF242538, AF242539
AF242538/Pessino et al.	*Paspalum notatum*	DD	398	Similar only to AF242537, AF242539
AF242539/Pessino et al.	*Paspalum notatum*	DD	417	Similar only to AF242537, AF242538
AJ271598/Tucker et al.	*Hieracium piloselloides*	cDNA	1,328	Beta-1,3-glucanase
AF180365/Guerin et al.	*Hieracium piloselloides*	cDNA, DD	687	DEFICIENS homolog DEF2
AF180364/Guerin et al.	*Hieracium piloselloides*	cDNA, DD	875	DEFICIENS homolog DEF1

[a] *cDNA* cDNA library, *DD* differential display
[b] Similarity based on E values <e-06
[c] Previous entries in GenBank with identical sequence (D37940 = U13147, Z36545; D37938 = U13148, Z36544; D37939 = U13149, Z36546)

of genomes associated with components of apomixis or the complete reproductive process (Grossniklaus et al. 2001). In aposporous grasses, there is little evidence that parthenogenesis can segregate independently of aposporous embryo sac development, except in the case of *Poa* (Albertini et al. 2001). In the Asteraceae, however, evidence for genetic separation of parthenogenesis and diplosporous embryo sac formation is more compelling (Noyes and Rieseberg 2000; van Dijk et al. 1999).

Molecular markers based on linkage with apomixis (Barcaccia et al. 1998; Grimanelli et al. 1998; Gustine et al. 1997; Labombarda et al. 2002; Leblanc et al. 1995; Noyes and Rieseberg 2000; Ozias-Akins et al. 1993, 1998; Pessino et al. 1997, 1998; Pupilli et al. 2001; Roche et al. 1999; Jessup et al. 2002) and on expression differences between sexual and apomictic genotypes (Chen et al. 1999; Leblanc et al.1997; Pessino et al. 2001; Vielle-Calzada et al. 1996) have been isolated. None of the differentially expressed sequences have yet been shown to be linked with apomixis. Expressed sequences isolated from pistils of aposporous apomicts that have been deposited in GenBank (as of April 2003), along with their putative functions, are listed in Table 2.

Markers linked with apomixis have largely been derived from random PCR-based markers, anonymous DNAs cloned from the species being mapped, or from cloned DNAs from other species. Many of the DNAs from other species such as maize and rice have had the advantage of being mapped in those and related species; therefore, the emergence of syntenic relationships among cereals might be expected. Although synteny does appear to exist between the apospory-associated region of *Brachiaria brizantha* and maize chromosome 5 (Pessino et al. 1997, 1998), of *Paspalum simplex* and rice chromosome 12 (Pupilli et al. 2001), and of buffelgrass and sorghum chromosome D (Burow et al. 2001), the syntenic regions on the grass circle (Gale and Devos 1998) are incongruent. These results could be explained by the polyphyletic origin of aposporous apomixis in the tribe or by genome rearrangements that have disturbed local synteny. In spite of segregation of apomixis as an apparently single locus, several mapping studies have observed a repression of recombination at the locus (see below), which suggests that the locus may be large, encompassing multiple genes, perhaps more than one of which may be required for apomixis.

Molecular markers in *Pennisetum/Cenchrus*

Molecular markers linked with apomixis were first isolated from an apomictic addition/substitution line of pearl millet referred to as BC_3 (Ozias-Akins et al. 1993). A single BC_3 plant was obtained through an introgression program intended to transfer apomixis to pearl millet (Dujardin and Hanna 1989b). It is a near-obligate apomict, thus its offspring are genetically identical. Since BC_3 was the product of a complex cross involving two inbred lines of tetraploid pearl millet, *P. purpureum* and *P. squamulatum*, molecular marker analysis was focused

on polymorphisms in BC_3 which were unique to the apomictic parent, *P. squamulatum*, and their transmission to the BC_4 generation. Out of seven polymorphic markers from *P. squamulatum*, only two were shown to be linked with apomixis providing evidence that more than one chromosome from *P. squamulatum* was present in BC_3, but that all may not be required for apomixis. Recombination was not observed between the two apomixis-linked markers, which might have been expected given the addition of an alien chromosome to a divergent genetic background. A genetic map, however, would require materials with meiotic recombination. This condition was subsequently met by mapping the male gamete contribution to an F_1 population of pearl millet × *P. squamulatum* (Ozias-Akins et al. 1998). Pools of DNA from individuals with discrete reproductive phenotypes were surveyed for DNA polymorphisms (bulked segregant analysis; Michelmore et al. 1991). Polymorphisms detected with this sampling method have a high probability of being linked to the trait of interest that was distinctly represented in each DNA pool. The polymorphisms consisted of randomly amplified polymorphic DNAs (Williams et al. 1990; Welsh and McClelland 1990) which were converted to sequence characterized amplified regions (SCARs; Paran and Michelmore 1993) prior to mapping. Unexpectedly, all 12 of these PCR-based markers strictly cosegregated with the capacity for aposporous embryo sac formation in a progeny size of 397 individuals. The lack of recombination in this case was not related to the interspecific hybrid nature of the mapping population because only the gametes from *P. squamulatum* provided information for mapping. Potential causes for low or nil recombination will be discussed below.

Interestingly, many of the apomixis-linked markers from *P. squamulatum* are conserved in their association with apomixis in other *Pennisetum* and *Cenchrus* species (Lubbers et al. 1994; Roche et al. 1999). Ten of the *P. squamulatum*-derived markers have been mapped in an intraspecific hybrid of buffelgrass (a total of 84 F_1 individuals segregating for mode of reproduction) but only one potential recombinant with one marker was observed (Roche et al. 1999). Other RAPD markers with linkage to the trait in this population also have been described (Gustine et al. 1997). Thus far, all PCR-based markers isolated have been linked in coupling with apomixis which could reflect the presence of a higher level of polymorphism in the chromosomal region required for apomixis vs the allelic regions of homo(eo)logous chromosomes, or it could reflect the low probability of detecting markers linked in repulsion in polyploids with random chromosome pairing (Wu et al. 1992) as has been suggested for *P. squamulatum* (Ozias-Akins et al. 1998) and buffelgrass (Sherwood et al. 1994). Through our work, evidence is mounting that the chromosomal region associated with the transmission of apomixis has been subject to evolutionary forces different from those affecting other regions of the genome, probably as a consequence of its low/nil recombination and maintenance through apomictic reproduction. Features of its behavior that are contrary to most genetic studies are (1) transmission of the apospory-specific genomic region (ASGR) as a tight linkage block of unknown, but probably large size (Roche et al. 2002) and (2) severe segregation distortion against transmission of the trait through rare female meioses and slight segregation distortion through the male (Roche et al. 2001b). Possible explanations for such behavior have been reviewed in Roche et al. (2001a), all of which are based on chromatin or chromosomal context. We recently have determined the chromosomal positions of the ASGR in *Pennisetum/Cenchrus* and have visualized extensive hemizygosity, both of which support our hypotheses for the underlying cause(s) of low/nil recombination.

Molecular cytogenetics

During the course of genetic mapping with PCR-based markers, the copy number of apomixis-linked markers was estimated by Southern blot hybridization with genomic DNAs. Six out of 12 of the *P. squamulatum*-derived markers were of high copy number in *P. squamulatum* whereas the other 6 were low copy. Of the 6 low-copy markers, 4 showed hybridization only to apomictic F_1 individuals meaning that no allelic fragment was transmitted to sexual F_1s (Ozias-Akins et al. 1998). These markers were considered to be hemizygous. Hemizygosity of an apomixis-linked marker also has been recently observed in *Paspalum simplex* (Labombarda et al. 2002). The 6 low-copy markers from *P. squamulatum* have been used to isolate BAC clones from a library constructed from an apomictic polyhaploid plant containing the complete ASGR from *P. squamulatum* (Roche et al. 2002). The markers that were mapped in buffelgrass were also used to extract BAC clones from a buffelgrass library. These BAC clones have been fingerprinted and analyzed by hybridization. Both methods of analysis indicate that some markers which are hemizygous are nevertheless duplicated within the ASGR and thus reside on the same chromosome (Roche et al. 2002).

Fluorescence in situ hybridization (FISH) has become an important tool in plant genetics for validation of genetic and physical maps (Cheng et al. 2001; Kim et al. 2002). In the application of FISH to our materials, we have not yet visualized the duplications mentioned above, although the hemizygosity has been confirmed (Fig. 3a). FISH also has become essential for determining the chromosomal location of the ASGR and for defining the physical distances between markers that do not genetically recombine or do so at frequencies below our current level of detection. Using BAC clones that contain ASGR-linked marker sequences, we have determined that: (1) all of the clones tested thus far hybridize to only a single chromosome in hexaploid *P. squamulatum* and in tetraploid *C. ciliaris*; (2) the chromosomal position of the ASGR differs between the two species—it is proximal to the centromere in *C. ciliaris* and near the telomere in *P. squamulatum*; and (3) the buffelgrass chromosome car-

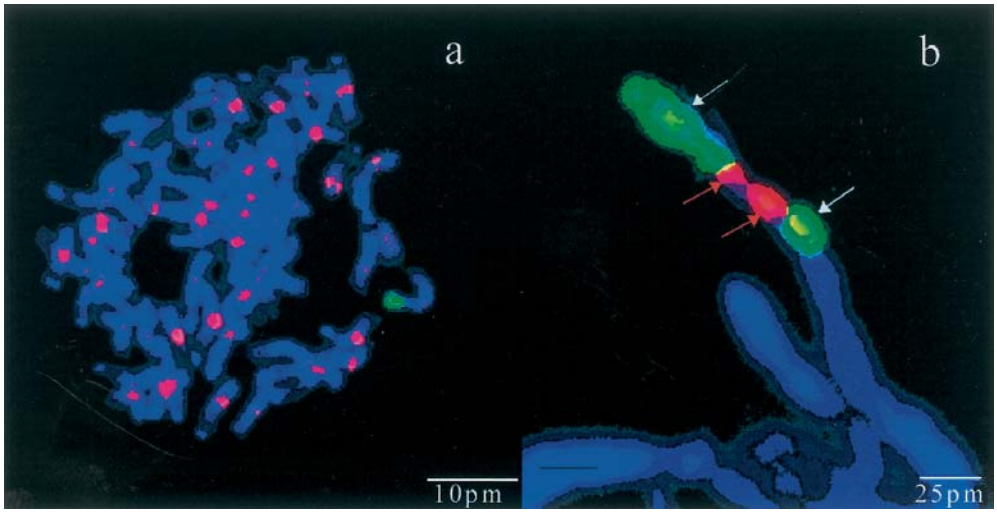

Fig. 3 a Fluorescence in situ hybridization (FISH) of BACs, containing apospory-specific genomic region- (ASGR) linked markers, to metaphase chromosomes of polyploid *Pennisetum squamulatum* reveals only a single chromosome with strong hybridization signal (*green*). *Red signal* indicates hybridization of a centromeric probe. The hemizygous nature of the ASGR has been observed with BACs containing repetitive DNA (*green signal*) and low-copy DNA sequences (Goel et al. 2003). **b** Pachytene chromosomes of *P. squamulatum* hybridized with three BACs: one containing repetitive sequences (*green signal*) and two containing low-copy sequences (*red signals* or *yellow* where red and green signals overlap). The region delineated by the signal from repeats (*green*) flanks a largely low-copy region and encompasses approximately half of a chromosome arm

rying the ASGR also has a ribosomal DNA locus (Goel et al. 2003). Although all of the signals from two-color FISH with different combinations of BAC clones appear to overlap on mitotic chromosomes, the signals can be separated on pachytene chromosomes (Fig. 3b). As BAC clones are isolated with additional molecular markers mapped to the ASGR, it will be possible to position these BACs relative to one another by pachytene FISH. Pachytene FISH also has provided us with the information for selection of BACs flanking the narrowest gap that would have a good chance of closure during a chromosomal walk.

FISH with BACs mapped to the ASGR but containing a considerable proportion of repetitive DNA has also been very informative for characterization of this genomic region. The first indication that repetitive sequences were clustered in the ASGR came from hybridization of a pool of repetitive SCAR markers to BC_3 and its derivatives to BC_7. In dual-color FISH, the pool of ASGR-linked markers was labeled with one fluorophore and total genomic DNA of *P. squamulatum* was labeled with a second fluorophore. Both were probed onto mitotic spreads along with blocking DNA from the recurrent backcross parent, pearl millet. In some apomictic backcross lines, only a single chromosome that hybridized with *P. squamulatum* DNA was present and the end of one chromosome arm was labeled with the ASGR-linked markers (Fig. 4; Goel et al. 2003). This signal from repetitive SCARs has been further investigated with BACs containing the repetitive SCARs. The BACs hybridize to pachytene chromosomes in two clusters near the end of the chromosome (Fig. 3b). At least one of the SCAR sequences, R13, that contributes to the distal hybridization signal has sequence similarity to a retrotransposon as determined by BLASTX (maize retrotransposon Opie-2, GenBank accession T04112, E-value of 5e-11; rice sequence from chromosome 1 PAC, GenBank accession BAB03384, E-value of 2e-20). Thus far, out of about 50 end-sequences from apomixis-linked *Pennisetum/Cenchrus* BACs, >30% show similarity to retrotransposon-like sequences. SCAR R13 hybridizes not only with multiple bands in BACs containing the R13 SCAR, but also with BACs isolated with four other repetitive SCARs (although not within the SCARs themselves). Using FISH, all of these BACs containing repetitive sequences show a similar pattern of hybridization as shown in Fig. 3b (green signal). The FISH signal is prominent on only a single chromosome of *P. squamulatum*, although Southern blot analysis indicates that sequences similar to R13 are also dispersed in the genome of pearl millet and *P. squamulatum*. More extensive sequence characterization will be necessary to verify the retrotransposon-like families and diversity within the ASGR; however, these preliminary results suggest that this region of the genome may be evolving in a similar manner as Y (Kuroda-Kawaguchi et al. 2001; Okada et al. 2001) and B chromosomes (Camacho et al. 2000; Langdon et al. 2000; Stitou et al. 2000), both of which are largely isolated from recombination and accumulate repeated sequences.

Features of the genomic region required for apomixis resemble selfish genetic elements—molecular parasite or mutualist?

Selfish DNA proliferates in a genome to ensure its own survival but confers no obvious benefit to the host and

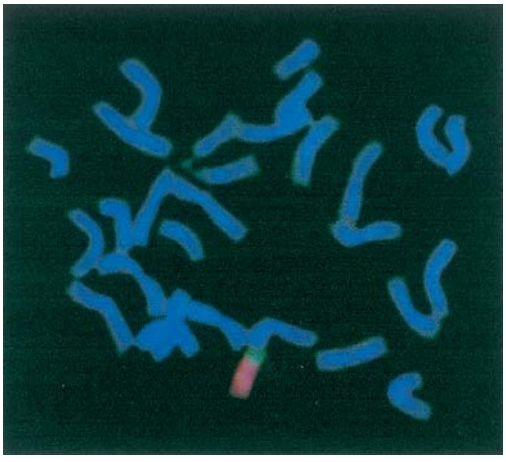

Fig. 4 Mitotic spread from an advanced backcross line with *Pennisetum squamulatum* as the apomictic parent and tetraploid pearl millet as the recurrent parent. Labeled total *P. squamulatum* DNA (*red*) hybridized to a single chromosome in this backcross line. *Blue signal* is from DAPI-stained chromosomes of pearl millet. The *green signal* on the end of the *red chromosome* was produced by the hybridization of a labeled mixture of ASGR-linked SCAR DNAs

may actually decrease fitness. Transposable elements have been described by some as selfish DNAs (Orgel and Crick 1980; Doolittle and Sapienza 1980), although the prevailing view is that such molecular parasites, which most often induce deleterious mutations in the genome, also can be co-opted for altering gene regulation and diversity in a continuum that leads to "domestication" of these elements over the long term (McDonald 1995; Zeyl and Bell 1996; Kidwell and Lisch 2001). Evolutionary theory predicts that such elements require sex to spread and after a period of accumulation, would be driven to extinction in asexual organisms (Charlesworth and Wright 2001).

Apomixis is an evolutionarily derived trait that can coexist with sex in many genotypes. In near-obligate apomicts the genome may be isolated from meiotic recombination for many generations because the mode of reproduction is strictly asexual and maternal. This reproductive isolation may allow many somatically derived mutations to accumulate as long as they are mildly or non-detrimental to the asexual lineage. Since most pseudogamous apomicts still require viable pollen, gene flow from apomicts through the male is possible, and these meiotic events may constrain any mutant alleles to those that are not deleterious in the haploid male gametophyte. Another mechanism for maintenance of mutant alleles is polyploidy. In fact, there is some evidence that homozygosity of the apomixis-associated region in a haploid male gametophyte may reduce its viability (Nogler 1982, 1984b). This effect is presumably due to linked recessive, deleterious alleles and may enforce polyploidy. Therefore the dynamics of transposable element evolution in a pseudogamous apomict may be balanced by the lack of maternal sex and the need for paternal sex.

Why does the apomixis-associated region in *Pennisetum/Cenchrus* appear to be evolving in a manner different from other regions of the genome that also are largely isolated from meiotic recombination because of the asexual mode of reproduction? Is a region of a single chromosome, presumably with pairing homologs in these polyploid genomes, maintaining and perhaps accumulating sequence differences? Or, alternatively, could the apparent hemizygosity be due to sequence elimination that occurred at the time of allopolyploidization (Eckardt 2001)? Perhaps this region of the genome is gene poor or harbors duplicate genes that are dispensable because of their redundancy. Such a situation might permit the accumulation of retrotransposon-like repetitive sequences through somatic transposition either because the transposition events may have been targeted to or better tolerated by this region of the genome. Transposable elements are known to accumulate in gene-poor, heterochromatic regions of a genome (McDonald 1998; Dmitri and Junakovic 1999) and their accumulation can even incite the formation of heterochromatin (Steinemann and Steinemann 1997). Our data (based on DAPI staining of pachytene chromosomes and contraction ratios between specific points on the chromosome) do not support the idea that the ASGR is a large, uniformly heterochromatic block, but the technique is insufficient in resolution to precisely define small heterochromatic regions. If the hybridization signals shown in Fig. 3b do represent clusters of retrotransposon-like sequences, is this pattern simply the consequence of recombinational isolation and low gene density or is there some functional significance? Could rearrangements such as inverted repeats, being accelerated by the presence of transposons, or the host response to transposon invasion of what is now the ASGR, underlie the evolution of apomixis? Or is the abundance of repeats in the ASGR of *P. squamulatum* simply a consequence of genome tolerance?

The genetic studies with apomicts in the grass family still have not resolved the question of whether one gene or multiple genes are required for expression of the phenotype. Are there transcribed genes interspersed among the repetitive sequences, similar to the pericentromeric regions of *Arabidopsis* (Lin et al. 1999), that may serve as regulatory genes or part of a signaling pathway required for the initiation of apomixis? It is conceivable that a single transcribed gene, such as the hypothetical embryo sac initiator proposed by Peacock (1992), is embedded amongst repeats or even encoded by a repeat. A Y chromosome-specific, male sex organ-expressed gene in the liverwort, *Marchantia polymorpha*, is embedded in repeat unit variants (Okada et al. 2001). Testis-specific transcription has been observed for multiple genes interspersed with massive repeats in the *AZfc* region of the Y chromosome of humans (Kuroda-Kawaguchi et al. 2001). Some of the transcription units in this region of the Y chromosome are noncoding transcripts. The potential role of noncoding regulatory RNAs in growth and development has only recently been recognized (Eddy 2001; Storz 2002; Voinnet 2002). Their function is likely

to be connected to RNA silencing mechanisms, which although first observed as epigenetic gene silencing (co-suppression) in transgenic plants, are now understood to be the result of a conserved, RNA-mediated genome surveillance system (Plasterk 2002). Gene silencing probably evolved as a response to viral and/or transposon invasion (Vance and Vaucheret 2001).

The unusual genomic architecture of the ASGR tempts us to consider models more complex than a single regulatory protein for the control of apomixis. Ozias-Akins et al. (1998) suggested that aspects of apomixis could be explained by the action of the apomixis-associated region to silence, in trans, genes expressed during sexual development. Given our rapidly advancing understanding of post-transcriptional gene silencing (PTGS) and RNA interference (Baulcombe 2002; Llave et al. 2002; Plasterk 2002), one could even speculate that noncoding RNAs from the ASGR might be trans-acting factor(s) responsible for the dominant, plastic, and incomplete penetrance of apomixis in most systems. A PTGS-related mechanism, originated in the hemizygous repeat cluster, potentially could silence meiotic genes involved in replication timing and pairing. Such genome-wide silencing of sequences that have high similarity to DNA that remains unpaired during meiosis has been reported in *Neurospora* (Shiu et al. 2001). The possible involvement of repeats and noncoding RNAs, in addition to protein coding genes, in the expression of apomixis should not be overlooked. Kashkush et al. (2003) recently observed that newly synthesized wheat amphiploids showed elevated transcript levels for one retrotransposon family. Some of these transcripts were chimeric and also contained adjacent protein-encoding gene sequences in sense or antisense orientation. A corresponding change in gene expression for these specific genes, some being silenced and others activated, was observed. This additional mechanism for gene regulation based on transcriptional activation of retrotransposons supports the "mutualist" viewpoint for retrotransposon evolution. The question of repetitive DNA in the ASGR as molecular parasite or mutualist can only be answered once we more narrowly delineate and thoroughly characterize the chromosomal segment essential for apomictic reproduction.

Conclusions

Apomixis in the *Pennisetum/Cenchrus* clade is restricted to pseudogamous apospory where the development of four-nucleate embryo sacs may vary in the allocation of nuclei to synergid and central cells. The egg develops parthenogenetically into an embryo, sometimes being activated prior to anthesis, while the nucleus/nuclei of the central cell only show activity after pollination and likely require fertilization for development into the endosperm. Molecular mapping in *P. squamulatum* and *C. ciliaris* has demonstrated that recombination is repressed in the region of the genome required for apomixis (ASGR) even though the chromosomal position of the ASGR varies between the two species. The ASGR in *P. squamulatum* contains abundant repeats and its evolution may parallel that of Y and B chromosomes. The genetic mechanism underlying apomixis remains to be elucidated but could be as simple as a single regulatory protein-encoding gene or as complex as the alteration (silencing) of gene expression due to the genome context of multiple gene sequences embedded in this region.

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