Genetics and Molecular Biology, Online Ahead of Print

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Sequence characterization, *in silico* mapping and cytosine methylation analysis of markers linked to apospory in Paspalum notatum

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Abstract

In previous studies we reported the identification of several AFLP, RAPD and RFLP molecular markers linked to apospory in Paspalum notatum. The objective of this work was to sequence these markers, obtain their flanking regions by chromosome walking and perform an *in silico* mapping analysis in rice and maize. The methylation status of two apospory-related sequences was also assessed using methylation-sensitive RFLP experiments. Fourteen molecular markers were analyzed and several protein-coding sequences were identified. Copy number estimates and RFLP linkage analysis showed that the sequence PnMAI3 displayed 2-4 copies per genome and linkage to apospory. Extension of this marker by chromosome walking revealed an additional protein-coding sequence mapping *in silico* in the apospory-syntenic regions of rice and maize. Approximately 5 kb corresponding to different markers were characterized through the global sequencing procedure. A more refined analysis based on sequence information indicated synteny with segments of chromosomes 2 and 12 of rice and chromosomes 3 and 5 of maize. Two loci associated with apomixis locus were tested in methylation-sensitive RFLP experiments using genomic DNA extracted from leaves. Although both target sequences were methylated no methylation polymorphisms associated with the mode of reproduction were detected.

Keywords: apomixis, chromosome walking, gene mapping, molecular markers.

Received: February 27, 2012; Accepted: July 1, 2012.

Introduction

Apomixis is a route of asexual reproduction through seeds (Nogler, 1984). This mode of reproduction has been described in about 400 genera from 40 plant families and seems to have arisen multiple times during evolution (Carman, 1997). Apomixis avoids meiosis and fertilization of the egg cell to generate progeny that are clones of the mother plant (Savidan, 2000). In gametophytic apomixis, the type of apomixis found in the Poaceae, embryo sacs bearing non-reduced nuclei are formed from the megaspore mother cell itself (diplospory) or from nucellar cells (apospory), followed by development of the embryo through parthenogenesis from unreduced (2n) egg cells. Depending on the species, the endosperm develops after fertilization of the polar nuclei (pseudogamy) or autonomously (Koltunow, 1993). Gametophytic apomixis tends to occur in polyploids, most often at the tetraploid stage or higher levels (Asker and Jerling, 1992). Despite the widespread occurrence of apomixis in angiosperms, the trait is rare in crop gene pools, although apomictic wild relatives have been identified for important cereals, including maize and wheat (Spillane *et al.*, 2004).

Several studies have examined the inheritance of apomixis in tropical and subtropical forage grasses (reviewed by Ozias-Akins and van Dijk, 2007). These species are usually polyploid, highly heterozygous and genetically poorly-characterized. Transferring apomixis to sexually reproducing crops could have an enormous impact in agriculture. The most important potential benefits derived from harnessing apomixis would be the fixation and cloning of elite genotypes and hybrids through seeds, as well as the multiplication of vegetatively-reproducing crops via seeds (Vielle-Calzada *et al.*, 1996; Savidan, 2000; Toenniessen, 2001).

Paspalum notatum Flügge (bahiagrass) is a polymorphic forage grass native to South America. Tetraploid cytotypes (common bahiagrass) are important natural forage resources in tropical and subtropical areas of southern

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Brazil, Paraguay and northeastern Argentina; their reproduction is by nearly-obligate aposporous apomixis (Gates *et al.*, 2004). Completely sexual tetraploid plants have never been collected from nature, although several individuals have been artificially created by colchicine treatment of diploids or by crossing highly sexual facultative tetraploid genotypes (Quarin *et al.*, 2003). Experimentally-generated tetraploid sexual plants have allowed the development of intraspecific populations that segregate according to their mode of reproduction; these populations have been used for genetic and molecular studies of apomixis in the species.

Inheritance analyses done on F_1, F_2 and BC_1 families derived from sexual x apomictic crosses indicated that apospory in tetraploid *P. notatum* is controlled by a single locus with a distorted segregation ratio, probably due to a pleiotropic lethal effect with incomplete penetrance of the allele controlling apospory or, alternatively, to a partially lethal linked factor (Martínez *et al.*, 2001). A group of RAPD, RFLP and AFLP molecular markers linked to the apospory locus has been identified (Martínez *et al.*, 2003; Pupilli *et al.*, 2004; Stein *et al.*, 2004, 2007; Rebozzio *et al.*, 2012). These markers define a chromosome block characterized by recombination restriction and preferential chromosome pairing (Martínez *et al.*, 2003; Stein *et al.*, 2004). Several AFLP and two SCAR markers (*SPNA1* and *SPNA3*) linked to the trait are consistently present in a group of apomictic accessions from diverse geographic origins (Rebozzio *et al.*, 2012). These findings suggest that the structure of the chromosome segment carrying apospory is highly conserved in apomictic races of this species.

Based on the mapping data reported by Stein *et al.* (2007), the apospory region of tetraploid *P. notatum* may consist of a large chromosomal segment. A comparative analysis done with RFLP markers previously mapped in rice showed that clones C560 and C932 (rice chromosomes 2) and C545, C996A and C1069 (rice chromosome 12) mapped completely linked to apospory in this species (Pupilli *et al.*, 2004). Synteny between the apospory locus and rice chromosome 12 was conserved in at least other three *Paspalum* species (*P. mallacophyllum*, *P. simplex* and *P. procurrens*) (Pupilli *et al.*, 2004; Hojsgaard *et al.*, 2011). Information derived from comparative mapping analyses in apomixis research is extremely valuable since this trait is currently considered to represent a reproductive deviation caused by mutations and/or epimutations involving canonical sexual pathway genes, rather than the development of a new function. Consequently, gene order comparisons with sexual model grass species for which the genomes have been fully sequenced could allow rapid, exhaustive identification of candidate genes whose structure and/or expression may be affected in polyploid, highly heterozygous and poorly characterized apomictic species.

Although the structure of the chromosomal segment carrying apospory in *P. notatum* has been partially characterized by using molecular markers (Martínez *et al.*, 2003; Pupilli *et al.*, 2004; Stein *et al.*, 2007) there is currently no sequence information for this region. The sequencing of markers fully linked to apospory would allow a detailed analysis of synteny involving model grasses such as rice and maize, and facilitate the identification of candidate genes physically associated with apospory. We are aware that characterizing the locus that controls apospory in *P. notatum* is a difficult task since polyploidy, heterozygosity, a lack of recombination and the presence of retrotransposons can complicate strategies used to recover specific sequence and provide unequivocal positional validation. However, since no sequence information is available for this species, data derived from markers linked to apospory would help to refine the study of synteny and accelerate the identification of candidate genes related to apospory.

The objective of this work was to analyze the sequences of molecular markers linked to apospory in *P. notatum*, extend them by chromosome walking and perform an *in silico* analysis of synteny in maize and rice. The cytosine methylation of markers linked to apospory were assessed by methylation-sensitive RFLP.

Materials and Methods

Plant material

The plant material used in this work consisted of the tetraploid (2n = 4x = 40) genotypes Q4188 and Q4117 and an F_1 mapping population derived from them. Q4188 is a completely sexual experimental hybrid derived from a cross between a highly sexual genotype (Q3664) and a natural apomictic plant (Quarin *et al.*, 2003) and Q4117 is an obligate apomictic tetraploid accession collected from southern Brazil (Ortiz *et al.*, 1997). Along with the parental genotypes, 65 F_1 individuals (55 sexual and 10 aposporous) were used to corroborate the linkage between molecular markers and apospory. The F_1 hybrids were the remaining part of a larger mapping population of 113 individuals that segregated according to the mode of reproduction developed by Stein *et al.* (2004). This population was used to identify molecular markers linked to apospory, to determine the type of inheritance in tetraploid races and to construct a genetic linkage map of the species (Stein *et al.*, 2004, 2007). All of the hybrids were initially classified according to their mode of reproduction based on cytoembryological observations and molecular analysis (Stein *et al.*, 2004).

Molecular markers linked to apospory

Two RAPD markers (BCU243-377 and BCU259-1157) (Martínez *et al.*, 2003), 14 AFLP markers (E32M33e, E33M32c, E33M33a, E33M33b, E33M33f, E33M42e, E33M42g, E35M33p, E35M33n, E36M37c, E36M37d, E36M38a, E35M43n and E35M43p) (Stein *et al.*, 2004, 2007) and five cDNA RFLP clones (C560, C932,

C454, C996A and C1069, from the New Landmarker set, Rice Genome Research Program, Japan), previously reported as completely linked to apospory in tetraploid *P. notatum* (Martínez *et al.*, 2003; Pupilli *et al.*, 2004) were analyzed. The RAPD and AFLP markers were re-amplified as described by Martínez *et al.* (2003) and Stein *et al.* (2007), respectively, from genomic DNA of Q4188, Q4117 and all F_1 progenies. Markers linked to apospory were identified on agarose or polyacrylamide gels based on their molecular weight and cosegregation with the mode of reproduction. Target bands from Q4117 were excised from the gels and eluted in buffer containing 0.5 M ammonium acetate and 1 mM EDTA, pH 8. DNA fragments were precipitated with absolute ethanol, dried at room temperature, dissolved in 20 µL of distilled water, re-amplified using the corresponding RAPD or AFLP primers and purified with the DNA Wizard SV Gel and PCR Clean-up system (Promega). Clean fragments were cloned with the pGEM-T Easy Vector system (Promega). Inserts of recombinant plasmids were sequenced by Macrogen Inc. (Korea). The sequences of RFLP clones were retrieved from the GRAMENE web page (www.gramene.org).

Chromosome walking procedure

Genomic sequences flanking the AFLP-derived fragment *PnMAI3* were recovered from genomic DNA of Q4117 by using the Genome Walker™ universal kit protocol (Clontech Laboratories, Inc.). The outer adaptor primer AP1 (see GenomeWalker universal kit user manual) and an outer sequence-specific primer were used (Table S1) for the first amplification. The PCR products were used as templates for a second PCR with the nested adaptor primer AP2 (GenomeWalker) in combination with nested sequence-specific primers (Table S1). The PCR products were electrophoresed on 2% agarose gels and the bands of interest were cut out, cloned and sequenced as described above. The extended fragments were validated by searching for the corresponding upper and lower primers and aligning the overlapping segments with the original *PnMAI3* sequence. Alignments were done with Sequencher 4.10.1 Demo Version (Gene Codes Corporation). Contigs between *PnMAI3* and the flanking sequences were assembled with MegAlign v.4.03 (DNASTAR Inc.).

Amplification of apospory-specific contigs from genomic DNA

Apospory-specific contigs were amplified from Q4188 and Q4117 genomic DNA by PCR using internal primers designed within each extended sequence in combination with anchored primers aligned with *PnMAI3* (Table S2). The PCR mixtures used 100 ng of DNA, 30 ng of forward and reverse primers, 1X *Taq* buffer (Promega), 200 mM of each dNTP, 1 mM $MgCl_2$ and 1 U of *Taq* polymerase (Promega). The amplification reactions included an initial step of 2 min at 94 °C followed by seven cycles of 1 min at 94 °C, 1 min at 62 °C and 1 min at 72 °C with decreases in the annealing temperature of 1 °C per cycle, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C with a final incubation at 72 °C for 5 min. The PCR products were separated by electrophoresis on 6% polyacrylamide gels at 60 W for 1 h and silver-stained. The linkage between amplicons and apospory was tested in 10 sexual and 10 aposporous F_1 individuals of the mapping population.

In silico mapping analysis of Paspalum notatum apospory-specific sequences

Sequences of molecular markers linked to apospory in *P. notatum* were located in the rice and maize genomes by using the BLAST (Altschul *et al.*, 1990) tool through the GRAMENE and MaizeSequence web pages, respectively. Putative orthologous sequences were determined based on the procedure described by Salse *et al.* (2004) by applying the criterion of at least 65% identity over at least 60% of the length of the sequences at E-values < 0.005.

RFLP analyses of Paspalum notatum apospory-specific sequences

Genomic DNA was extracted from leaf tissue as described by Martínez *et al.* (2003). Three clones (*PnMAC5*, *PnMAI3* and *PnAM3*) derived from AFLP markers linked to apospory were used as RFLP probes. Hybridizations were done using a non-radioactive procedure (Ortiz *et al.*, 2001) on Nylon Hybond N membranes (Amersham-Pharmacia) containing 20 µg of DNA alternatively digested with three restriction enzymes (*Eco*RI, *Hind*III and *Bam*HI or *Pst*I). Putative genetic linkage between markers and apospory was assessed by bulk segregant analysis (BSA) (Michelmore *et al.*, 1991) in which 10 sexual (BS) and 10 aposporous (BA) F_1 progenies were used to construct each group.

Methylation-sensitive RFLP analysis of markers linked to apospory

Methylation-sensitive RFLP experiments were done using the isoschizomers *Msp*I and *Hpa*II in combination with the apospory-specific clones *PnMAM3* and C1069. *Msp*I and *Hpa*II recognize the same target sequence (CCGG) but exhibit different sensitivities to the cytosine methylation status (McClelland *et al.*, 1994; Tardy-Planechaud *et al.*, 1997). Genomic DNA was extracted as described above from leaf tissue of plants grown in a greenhouse. The methylation conditions of the target sequences were determined by comparing the hybridization patterns generated by the isoschizomers from a given sample. Linkage between markers and the mode of reproduction was estimated by BSA analysis and the corresponding de-bulk assay that included 10 sexual and 10 aposporous F_1 progenies, as described by Martínez *et al.* (2003).

Results

Sequence analysis of molecular markers completely linked to apospory

Two RAPD and 14 AFLP molecular markers previously reported to be completely linked to apospory in tetraploid *P. notatum* were re-amplified using the corresponding primers from genotypes Q4188 (sexual), Q4117 (aposporous) and their F_1 hybrids (55 sexual and 10 aposporous). The amplification products were electrophoresed and markers linked to apospory were identified based on their molecular weight and strict cosegregation with the mode of reproduction of the F_1 plants. The bands of interest were isolated from Q4117, cloned and sequenced. Of the 16 markers tested (two RAPDs and 14 AFLPs), only nine (one RAPD and eight AFLPs) were successfully recovered and cloned (Table 1). The rest of the PCR-based markers were lost during the cloning step or could not be confirmed because they lacked specific primers. A consensus was built for each marker by assembling sequences from at least three clones of the same fragment, with a minimum homology of 95%. The sequences of the cloned fragments ranged in length from 42 bp to 351 bp (Table 1). BLAST analysis revealed that only three of the sequences (33.3% of the total sequences characterized) shared significant homology with sequences in the databases; the others probably represented poorly-conserved intergenic sequences. Fragment *PnMA243* was similar to a cDNA clone of *Panicum virgatum* and sequence *PnMAJ5* aligned with a *P. virgatum* genomic clone. Sequence *PnMAM3* shared similarity with rice locus LOC_Os07g22800.1 that encodes a putative Ty1-copia subclass retrotransposon protein (Table 1).

RFLP markers from rice cDNA clones that mapped at the *P. notatum* apospory locus encoded for L6 and L14 ribosomal proteins (C560 and C996, respectively), a peptidyl-prolyl cis-trans isomerase protein (C932), a KH domain-containing protein (C454) and a mutator subclass transposon protein (C1069) (Table 1).

Copy number estimation of apospory-associated sequences

The AFLP-derived clones *PnMAC5*, *PnMAI3* and *PnMAM3* were used as probes in Southern blot experiments with genomic DNA from the Q4188 and Q4117 genotypes in order to determine the copy number of these clones in the *P. notatum* genome. Clone *PnMAM3* was included as a high-copy number control since it showed similarity with retrotransposons in the BLAST searches (Table 1). Hybridization with the control sequence (*PnMAM3*) resulted in a smeared pattern with some discrete bands, a

Table 1 - Sequences identified by RAPD, AFLP and RFLP molecular markers linked to apospory in *P. notatum*.

Marker type	Marker linked to apospory	Sequence identity	Length (bp)	Expected value	Best Blastn/Blastx alignment[1]
RAPD	BCU243-377[a]	*PnMA243*	351	$5.0e^{-50}$	gb FL982880.1 *Panicum virgatum* cDNA clone. Similar to hypothetical protein DNA binding protein
AFLP	E32M33e[b]	*PnMAC5*	96	-	n.s.
	E33M32c[b]	*PnMAI3*	279	-	n.s.
	E33M42e[b]	*PnMAJ5*	207	$2.0e^{-07}$	gb AC243221.1 *Panicum virgatum* clone PV_ABa006-D05
	E33M42g[b]	*PnMAJ7*	115	-	n.s.
	E36M37c[b]	*PnMAM3*	87	$7.3e^{-06}$	Os07g22800.1 retrotransposon protein putative Ty1-copia subclass
	E36M38a[b]	*PnMAN1*	42		n.s.
	E35M43n[b]	*PnMAU14*	91		n.s.
	E35M43p[b]	*PnMAU16*	176		n.s.
RFLP	C560[c]	*D15383*	421	$2.7e^{-53}$	LOC_Os04g39700 60S ribosomal protein L6 putative expressed
	C932[c]	*D22694*	329	$5.0e^{-152}$	LOC_Os02g52290.1 peptidyl-prolyl cis-trans isomerase FKBP-type
	C454[c]	*C98049*	725	$2.7e^{-113}$	LOC_Os12g40560.1 KH domain-containing protein
	C996A[c]	*C98189*	271	$1.2e^{-49}$	LOC_Os12g42180 50S ribosomal protein L14
	C1069[a b c]	*D15675*	400	$1.8e^{-228}$	LOC_Os12g40530.1 transposon protein putative mutator sub-class

[a]Martínez *et al.*(2003); [b]Stein *et al.*(2004, 2007); [c] Pupilli *et al.*(2004). [1]Best alignment in the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), TIGR Rice Genome Annotation (http://blast.jcvi.org/euk-blast/index.cgi?project=osa1), GRAMENE (http://www.gramene.org/) and MaizeSequence database (http://www.maizesequence.org/index.html). n.s. = no significant similarity found. GenBank accession numbers for nucleotide sequences: *PnMA243*= JN250998, *PnMAI3*= JN250999 and *PnMAJ5*= JN251000.

characteristic of highly repetitive sequences (Table 1, Figure 1A). Assays with *PnMAC5* showed only one hybridization band with the three restriction enzymes tested and no polymorphisms between parental plants (Figure 1B). Clone *PnMAI3* showed 2-4 hybridization bands with polymorphic fragments between Q4188 and Q4117 for two restriction enzymes (Figure 1C). The polymorphism revealed by *EcoR*1 consisted of bands with different migration rates in Q4188 and Q4117, while the polymorphism produced by *Pst*1 consisted of a band present in Q4117 and absent in Q4188 (Figure 1C). To determine the association between polymorphisms and the mode of reproduction, bulked segregant analysis (BSA) was done by hybridizing *PnMAI3* on both sexual (BS) and apomictic (BA) bulks (Figure 1D). The hybridization pattern showed that the polymorphisms detected between parental plants were also observed between sexual and apomictic bulks (Figure 1C and D), indicating genetic linkage between the target sequence and apospory.

Characterization of PnMAI3-flanking sequences by chromosome walking

Based on results described above, we initiated chromosome walking around *PnMAI3* since this clone had a low-copy number and showed linkage to apospory. Successive rounds of amplification yielded four consensus sequences (%ID > 80), two of them extending towards the 5 flanking region and two towards the 3 flanking region, respectively (Table 2). Only fragments that could be validated by identifying the primers used during the second round of amplification and by assembling the corresponding contig with the original sequence were considered for analysis. The length of the extended fragments ranged from 54 bp to 133 bp for the 5' flanks and 102-826 bp for the 3' flanks, respectively. These results agreed with the copy number (2-4) estimated for this marker, indicating that the

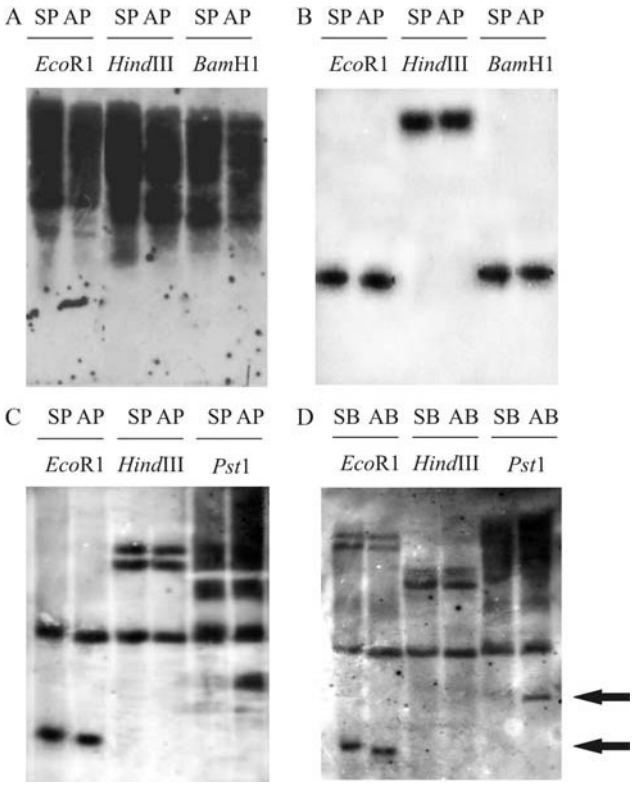

Figure 1 - Southern blot of apospory-specific AFLP-derived sequences in DNA from sexual and apomictic *Paspalum notatum*. A, B and C: Hybridization of DNA from genotypes Q4188 (SP) and Q4117 (AP) with clones *PnMAM3*, *PnMAC5* and *PnMAI3*, respectively, after digestion with three restriction enzymes. D: Hybridization of *PnMAI3* against sexual (SB) and apomictic (AB) bulks obtained from ten F_1 sexual and apomictic progenies, respectively. Arrows indicate polymorphic bands between parental plants and sexual and apomictic bulks.

Table 2 - Characterization of *P. notatum* genomic contigs derived from extension of the apospory-specific marker *PnMAI3*.

Extension direction	Contig name	Length (bp)	E value	Best alignment (Blastn) /Annotation[1]	*In silico* mapping onto rice and maize genomes[1]
5'	*PnGSA1*	350	3e-^{08}	gbFE614154.1 CBYX11857.b1 CBYX *Panicum virgatum* callus cDNA	No significant hits in the rice genome
	PnGSA2	384	2e-^{05}	gbJG806067.1 CFNU4264.b1 CFNU *Panicum virgatum* shoot cDNA	No significant hits in the rice genome
3'	*PnGSA3*	689	2e-^{59}	gbCD438587.1 Endosperm_5 *Zea mays* cDNA LOC100281493 N6-adenosine-methyltransferase MT-A70-like protein	*OS*2. Position: 27358073 - 27358338 (E-val: 5.1 e^{-42}). In the proximity of several apospory-linked markers
			5.1e-^{42}	LOC_Os02g45110.1 Transcript. MT-A70 domain containing protein	*ZM* 5. Position: 194 339 512 - 194 343 577 (E-val: 4.2e^{28}). In the proximity of several apospory-linked markers
	PnGSA4	1077	8e-^{54}	gb CD433356.1 EL01N0307F02.b Endosperm_3 *Zea mays* cDNA	Multiple alignments (lowest E-val: 1.9 e^{-25})
			1.9e-^{25}	LOC_Os03g45030.1 Retrotransposon protein putative Ty3-gypsy subclass	

[1]Determined by BLAST analysis (Altschul *et al.*, 1990) via the GRAMENE and MaizeSequence web pages. *OS*: rice chromosome, ZM: maize chromosome.

fragment was located in at least two *loci*, probably surrounded by different flanking sequences. The sequences of contigs *PnGSA1* and *PnGSA2* (5' amplifications) were similar to cDNA from *P. virgatum* calli and shoots, respectively, while the sequence of *PnGSA3* was highly similar to maize endosperm cDNA (gb CD438587.1) that encodes a N6-adenosine-methyltransferase MT-A70-like protein. The *PnGSA3* sequence also shared high similarity with LOC_Os02g45110.1 that encodes an MT-A70 domain-containing protein of rice. Contig *PnGSA4* was similar to a maize EST expressed in endosperm and to locus LOC_Os03g45030.1 of rice that encodes a putative retrotransposon protein (Table 2).

PCR amplifications based on primers that covered the four extended sequences and the original marker (Table S2) were used to amplify the whole contig in order to verify the presence of contigs derived from extension of the apospory-specific sequence *PnMAI3* in the *P. notatum* genome (thereby excluding possible contamination or chimeric assemblies) and to determine which of the two sequences isolated at each flank mapped to the apospory locus. Amplifications done on genomic DNA from both parental plants showed fragments of the expected size, confirming correct assembly, in addition to other fragments. However, the expected bands showed no polymorphisms between parental genotypes. This outcome prevented mapping the fragments using the F_1 hybrids. To overcome this difficulty, we undertook an *in silico* mapping analysis with the contig sequence on rice to distinguish which one was located in the region previously associated with apospory reported by Pupilli *et al.* (2004). Table 2 summarizes the *in silico* mapping results. The two 5'-flanking regions showed no significant match with the rice genome in GRAMENE BLAST searches, even though they showed homology to two *Panicum virgatum* cDNAs. These sequences may represent novel sequences present only in a group of related grasses. Of the two 3'-flanking regions, *PnGSA3* mapped to rice chromosome 2, close to RFLP probes previously associated with apomixis (see below). The other 3'-flanking region (*PnGSA4*) showed multiple alignments and probably represented a copy of a repetitive sequence located adjacent to a second *PnMAI3* fragment but at a different genomic location.

Interestingly, during contig validation, several other fragments of unexpected size were amplified. Some of these were polymorphic between Q4188 and Q4117 (Figure S1). Additional linkage analyses to determine the association between polymorphic bands and the mode of reproduction were done using the corresponding primers for each contig on DNA from both parental plants and a sample of 20 (10 sexual and 10 aposporous) F_1 individuals. PCR amplifications showed that most fragments were unlinked to apospory. However, amplification of sequence *PnGSA1* with primers VI3R and VI35BF1 generated a band of 764 bp (*PnMA764*) that was present in Q4117 and in all aposporous individuals but was absent in Q4188 and in all sexual F_1 progenies (Figure S2). This fragment (GenBank accession number: JN25001) showed sequence similarity to a maize transposable element (GRMZM5G800837_T01). Likewise, amplification of *PnGSA4* with primers VI3F and VI3310AR2 showed a high molecular weight band (*PnMA1200*) that also cosegregated with apospory (not shown). However, all attempts to clone this marker failed and their sequence could not be analyzed.

In silico mapping analysis of the whole set of apospory-associated sequences

All sequences derived from the *P. notatum* apospory-specific markers were mapped *in silico* onto the rice and maize genomes in order to determine the location of orthologous sequences in both model species. Rice RFLP clones previously associated with apospory were also included as reference points (Table 3). Putative orthologues to *PnMA243* and *PnMAM3* were found located on rice chromosome 2, close to the apospory-related markers C560 and C932. *PnMA764* aligned with a genomic sequence of rice chromosome 12 and maize chromosome 5, near to the apospory-related rice clones C1069 and C932, respectively. Apospory-related sequences mapping onto rice chromosomes 2 and 12 and maize chromosomes 3 and 5 covered approximately 10 Mbp and 30 Mbp, respectively (Figure 2). The rest of the sequences analyzed mapped on maize chromosomes 1 (C454), 4 (*PnMA243*, *PnMAJ5* and *C560*) and 6 (*PnMAJ5*) (Table 3).

Methylation-sensitive RFLP analysis of the apospory-related sequences

Since the activity of repetitive elements is known to be controlled through specific cytosine methylation pat-

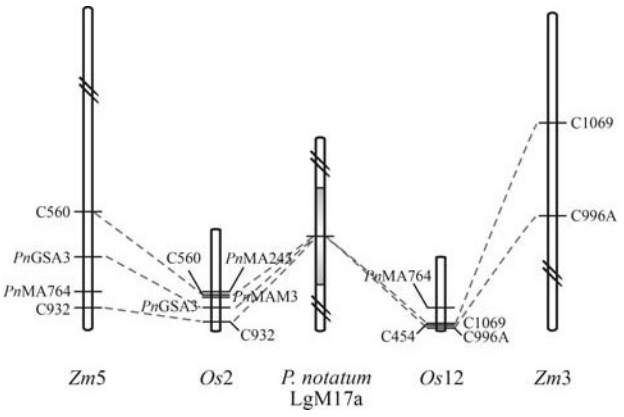

Figure 2 - *In silico* mapping of apospory-specific sequences in the rice and maize genomes. *Paspalum notatum* and rice sequences were located on rice (*Os*) and maize (*Zm*) chromosomes by using the BLASTn tool available at GRAMENE. The relative position of each marker was determined based on the physical position of the orthologous sequences listed in Table 3. Rice RFLP clones from rice chromosomes 2 and 12 that generated markers completely linked to apospory in the species were included as reference points.

Table 3 - *In silico* mapping of *P. notatum* apospory-specific sequences in rice and maize genomes.

Sequence	Rice chromosome (Os): bp[1]	E value[2]	%ID	Maize chromosome (Zm): bp[1]	E value[2]	%ID
PnMA243	*Os*2: 22 235 716 - 22 235 909	0.003	65.2	*Zm*4: 94 738 078 - 94 738 380	$1.2e^{-11}$	65.9
PnMAJ5	n.s.	n.s	n.s	*Zm*4: 181 677 342 - 181 677 525	$5.6e^{-07}$	70.3
				*Zm*6: 14 579 479 - 14 579 638	$6.6e^{-09}$	71.1
PnMAM3	*Os*: multiple alignments	$7.6e^{-05}$	86.6	*Zm*: multiple alignments	$7.2e^{-05}$	84.4
PnMA764	*Os*12: 18 536 262 - 18 536 706	$4.8e^{-7}$	68.2	*Zm*: multiple alignments	$4.0e^{-05}$	67.6
C560	*Os*2: 22 876 845 - 22 878 075	$2.3e^{-147}$	100.0	*Zm*4: 128 263 137 - 128 264 614	$2.5e^{-79}$	86.9
	*Os*4: 23 476 213 - 23 477 653	$3.7e^{-213}$		*Zm*5: 179 392 786 - 179 393 814	$2.9e^{-79}$	87.2
C932	*Os*2: 32 011 864 - 32 014 026	$5e^{-152}$	100.0	*Zm*5: 209 262 954 - 209 264 371	$1.3e^{-53}$	87.0
C454	*Os*12: 25 058 216 - 25 065 293	0.0	100.0	*Zm*1: 167 869 383 - 167 870 468	$4.2e^{-98}$	75.1
C996A	*Os*12: 26 106 507 - 26 108 035	$4.4e^{-160}$	100.0	*Zm*3: 86 923 502 - 86 928 449	$1.0e^{-32}$	78.1
C1069	*Os*12: 25 047 073 - 25 047 471	$1.8e^{-228}$	100.0	*Zm*1: 167 565 640 - 167 566 038	$1.0e^{-8}$	86.0
				*Zm*3: 115 792 597 - 115 792 988	$1.4e^{-75}$	85.5
PnGSA3	*Os*2: 27 358 073 - 27 358 338	$5.1e^{-42}$	69.8	*Zm*5: 194 339 512 - 194 343 577	$4.2e^{-28}$	79.6
PnGSA4	*Os*: multiple alignments	$1.9e^{-25}$	66.3	*Zm*: multiple alignments	$8e^{-54}$	68.0

[1]Orthologous sequences were assigned based on the criteria of 65% identity over at least 60% of the length of the sequences at E-values < 0.005. This assignment was done using the Blastn tool available from GRAMENE and MaizeSequence.[2]In cases of multiple alignments, the lowest E-value and the highest %ID are indicated (corresponding to the best match).

terns, methylation-sensitive RFLP experiments were done by using the apospory-associated clones *PnMAM3* (retrotransposon protein Ty1-copia subclass) and C1069 (transposon protein, mutator subclass) in combination with the restriction enzymes *Hpa*II and *Msp*I. The methylation insensitive enzymes *Eco*RI and *Hind*III were included as controls since they generate markers completely linked to apospory when clone C1069 is used as a probe (Martínez *et al.*, 2003). Analyses were initially done on DNA from the sexual (Q4188) and apomictic (Q4117) parents of the mapping cross. Hybridization with *PnMAM3* showed a smeared pattern with some individual bands for the four enzymes tested. Polymorphisms between genotypes were detected on samples digested with both *Eco*RI and *Hind*III indicating genetic differences among genotypes at the specific loci. Hybridization of samples digested with *Hpa*II and *Msp*I revealed differences between enzymes in both plants, indicating the existence of cytosine methylation, although a similar pattern was observed for the sexual and apomictic genotypes (Figure S3). Assays with probe C1069 showed only discrete fragments. Samples digested with *Eco*RI and *Hind*III produced the expected polymorphic markers associated with apospory reported by Martínez *et al.* (2003) (not shown).

The hybridization patterns of samples digested with *Hpa*II and *Msp*I confirmed methylation of the target sequence and also revealed differences between sexual and apomictic genotypes (Figure 3A). Genotype Q4188 showed three methylation-sensitive hybridization fragments (patterns 01 or 10); one of these (~ 0.9 kb) was specific for the sexual plant while the other two (~3.5 kb and 0.5 kb) were also present in Q4117 (Figure 3A). Genotype Q4117 showed two methylation-insensitive hybridization bands (pattern 11) of ~2.0 kb and ~0.7 kb, respectively, that were absent in Q4188. Hybridization of probe C1069 on DNA from sexual (BS) and apomictic (BA) bulks showed that the three methylation-sensitive markers were monomorphic between groups. The corresponding de-bulked

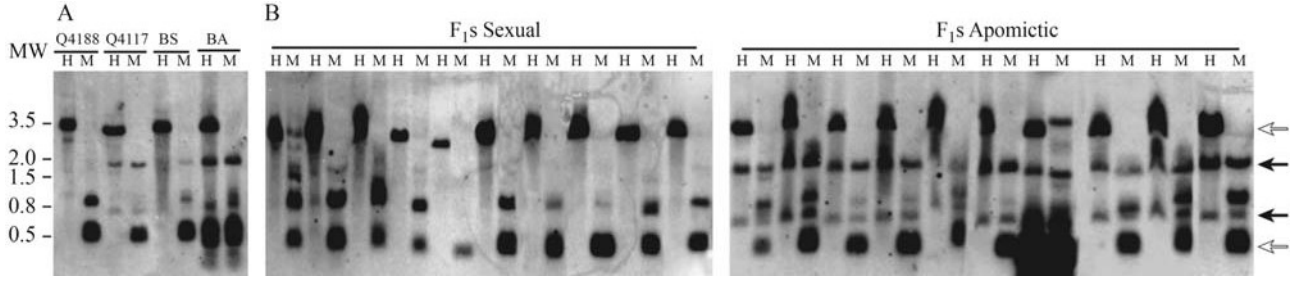

Figure 3 - Methylation-sensitive RFLP experiments with apospory-specific clone C1069. A: Hybridization done with DNA from parental genotypes Q4188 and Q4117 and sexual (BS) and apomictic (BA) bulks after digestion with *Hpa*II (H) and *Msp*I (M) restriction enzymes. B: Hybridization in ten sexual (F_1s) and ten aposporous (F_1a) F_1 hybrids digested with *Hpa*II (H) and *Msp*I (M). White arrows show methylation-sensitive markers and black arrows show methylation-insensitive markers.

analysis confirmed that methylation-sensitive markers showed similar patterns in sexual and aposporous F_1 hybrids such that no association with the reproductive mode could be detected. On the other hand, methylation-insensitive markers were specific for aposporous plants and therefore linked to apospory (Figure 3B). These polymorphisms could be attributed to: 1) an absence of the restriction site in Q4188 because of genetic variation between genotypes or 2) full methylation of the CCGG sequence. These two possibilities could not be distinguished by the approach used here.

Discussion

The availability of experimentally-generated tetraploid sexual genotypes of *P. notatum* that can be used as female parents in crosses with natural apomictic tetraploid plants has allowed the generation of populations that segregate according to the mode of reproduction without the need to use inter-specific or inter-ploid crosses. This type of segregating population has revealed some of the genetic and molecular features of apomictic reproduction and the extensive genetic variability for traits of agronomic importance in this species (Acuña *et al.*, 2009).

The chromosome segment coding for apospory in *P. notatum* resembles the apospory-specific genomic region (ASGR) found in other grasses such as *Pennisetum squamulantum* (Ozias-Akins *et al.*, 1998; Roche *et al.*, 2001), *Cenchrus ciliaris* (Ozias-Akins *et al.*, 2003; Roche *et al.*, 1999), *Paspalum simplex* (Labombarda *et al.*, 2002; Pupilli *et al.*, 2004) and *Panicum maximum* (Ebina *et al.*, 2005). In all of these species the ASGR is characterized by a lack of recombination. In *Pennisetum*, *Cenchrus* and *Paspalum*, there is also a strong distortion of segregation (Ozias-Akins *et al.*, 1998, 2003; Roche *et al.*, 1999, 2001; Labombarda *et al.*, 2002; Pupilli *et al.*, 2004) that is absent in *P. maximum* (Ebina *et al.*, 2005). Deciphering the genetic structure of these complex non-recombinant chromosome blocks would provide key information about genes governing apospory. In *P. squamulatum* (Akiyama *et al.*, 2004), *C. ciliaris* (Conner *et al.*, 2008) and *P. simplex* (Calderini *et al.*, 2006, 2010) this task has been attempted by sequencing BAC clones carrying molecular markers completely linked to apospory. In these three species, putative protein-coding regions as well as a large number of highly repetitive sequences have been identified (Akiyama *et al.*, 2004; Calderini *et al.*, 2006, 2008). Recently, several ovule transcripts originating from the ASGR-carrier chromosome were identified in *Pennisetum* by using a novel approach based on the comparison of two transcriptomes derived from microdissected ovules (Zeng *et al.*, 2011).

In the present work, we used a previously classified mapping population that segregated according to mode of reproduction and a group of molecular markers linked to apospory in order to characterize sequences present in the ASGR of *P. notatum* and identify possible candidate genes. Southern blot experiments confirmed the presence of low and high copy-number sequences in the ASGR. Hybridization bands were detected in sexual (Q4188) and apomictic (Q4117) parents of all the clones tested, indicating that the target sequences were present in both genotypes. Likewise, all contigs derived from extension of the apospory-specific marker *PnMAI3* were amplified from both parental plants, with no differentiation between maternal- and paternal-specific sequences. However, since only a small part (~5 kb) of the large ASGR (estimated size: 36 Mbp) was characterized, hemizygosity in other regions cannot be discarded.

Only two sequences that originated from RAPD and AFLP markers aligned with cDNA clones encoding a hypothetical protein (*PnMA243*) and a retrotransposon element of the Ty1-copia subclass (*PnMAM3*), respectively. The remaining AFLP-derived sequences showed no homologies in BLAST searches, probably because of the short length of the fragments analyzed and the fact that AFLP markers often target centromeric or non-coding regions (Castiglioni *et al.*, 1999). Rice cDNA RFLP clones mapping at the apospory locus encoded for ribosomal proteins (C996 and C569), a peptidyl-prolyl cis-trans-isomerase protein (PPIase) (C932), a KH domain containing protein (C454) and a putative mutator sub-class transposon protein (C1069). Among all these candidates, peptidyl-prolyl cis-trans isomerases (PPIases) deserve particular consideration because of their possible involvement in developmental processes (Dobson, 2004; Shaw, 2007). PPIases of the FKBP type are associated with cell division and cell elongation mediated by cytokinins and brassinosteroids in *Arabidopsis* (Harrar *et al.*, 2001). Other interesting sequence corresponded to KH domain-containing proteins, which are RNA-binding proteins involved in mRNA stability and gene expression regulation at the posttranscriptional level (Burd and Dreyfuss, 1994; Lorkovic and Barta, 2002). In maize, KH proteins have been associated with the maintenance of an inactive chromatin state in *knox* genes within the peripheral zone of the shoot apical meristem required for proper leaf development (Buckner *et al.*, 2008).

The extension of clone *PnMAI3* by chromosome walking allowed the assembly of four contigs. *PnGSA3*, which mapped at the apospory-syntenic region of rice and maize, aligned with a cDNA clone of maize expressed in the endosperm and was similar to an N6-adenosine-methyltransferase MT-A70-like protein. MT-A70 proteins are mRNA methyltransferases associated with dividing tissues, particularly reproductive organs, shoot meristems and emerging lateral roots (Clancy *et al.*, 2002; Zhong *et al.*, 2008). Inactivation of the *Arabidopsis* ortholog of yeast and human mRNA adenosine methylase (MT-A) results in failure of the developing embryo to progress to the globular stage (Zhong *et al.*, 2008). Further investigations aimed at isolating the complete sequences of the candidate genes from sexual and apomictic genotypes, and the detailed

analysis of their expression in reproductive tissues by *in situ* hybridization and qRT-PCR should be done to determine possible functional associations between them and the mode of reproduction.

An *in silico* mapping analysis of *P. notatum* apospory-specific sequences in the rice and maize genomes identified several orthologous sequences in segments of rice chromosomes 2 and 12, previously associated with apospory in this species (Pupilli *et al.*, 2004), and maize chromosomes 3 and 5. A recent study based on a comparative RFLP mapping strategy showed that several markers of rice chromosome 12 (including C996 and C1069 analyzed here) bracketed the chromosomal region responsible for apomixis in four *Paspalum* species of two taxonomic groups (Hojsgaard *et al.*, 2011). Interestingly, the range of the ASGR estimated for *P. squamulatum*, *C. ciliaris* and *P. notatum* (Akiyama *et al.*, 2004, 2005; Stein *et al.*, 2007) agreed with the physical distances covered by apospory-specific markers in rice chromosomes 2 and 12 and maize chromosomes 3 and 5. Considering that apomixis may have arisen from the deregulation of genes involved in sexual reproduction, the identification of coding sequences within the ASGR syntenic regions and a comparison with information derived from expression analyses could help to identify genes physically and functionally related to the trait. In another study, several transcripts differentially expressed in reproductive tissues of sexual and aposporous *P. notatum* were found to map in the same region of rice chromosome 2 (Laspina *et al.*, 2008). Moreover, chromosome 2 of rice and chromosome 5 of maize are associated with apospory in *Brachiaria* hybrids (Pessino *et al.*, 1997, 1998). Accordingly, these chromosomal segments may contain apospory-related sequences.

Several *P. notatum* apospory-specific sequences appeared to be related to repetitive elements such as mutator sub-class transposons and Ty1-copia and Ty3-gypsy sub-class retrotransposons. The presence of these elements agrees with the occurrence of repetitive sequences in the ASGR of other grass species (Akiyama *et al.*, 2004; Calderini *et al.*, 2006; Conner *et al.*, 2008). Moreover, null-mutants defective for the expression of a PAZ-PIWI AGO9 protein involved in the processing of transcribed retrotransposons were reported to produce non-reduced gametes in *Arabidopsis thaliana*, thereby mimicking the first step of aposporous development (Olmedo-Monfil *et al.*, 2010). In a recent study of *P. notatum*, retrotransposon elements carrying transduplicated gene segments were shown to be differentially expressed in inflorescences of sexual and apomictic genotypes and a possible regulatory function of these elements in gene expression was proposed (Ochogavía *et al.*, 2011).

Methylation-sensitive RFLP experiments indicated that, in leaves, repetitive elements located at the apospory locus were methylated, but there was no difference in the methylation pattern of apomictic and sexual genotypes. However, Rodriguez *et al.* (2012) reported variation in the methylation patterns of diploid (sexual) and tetraploid (sexual and apomicts) races of *P. notatum* based on MSAP (methylation-sensitive amplification polymorphism) markers. Moreover, the inactivation of a DNA methylation pathway in maize reproductive organs resulted in apomixis-like phenotypes (Garcia-Aguilar *et al.*, 2010). Since DNA methylation is involved in numerous biological processes, including embryogenesis, genomic imprinting, silencing of transposable elements and regulation of gene transcription (Zilberman *et al.*, 2007) further experiments should be done on DNA extracted from inflorescences in order to better characterize the cytosine methylation of these apospory-related sequences.

Acknowledgments

The authors thank Florencia Galdeano for technical assistance. This study was financed by ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica; PICT 2007 No. 00476 and PME 2006 No. 03083), Argentina, CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, PIP 112-200801-01378), Argentina. M.P., M.P.R. and L.A.S. were supported by fellowships from CONICET. S.F., E.J.M., C.L.Q., S.C.P. and J.P.A.O. are Career Members of CONICET.

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Internet Resources

Rice Genome Research Program, Japan, http://rgp.dna.affrc.go.jp/E/Publicdata.html (November 15, 2011).

GRAMENE, http://www.gramene.org (November 15, 2011).

MaizeSequence, http://www.maizesequence.org (November 15, 2011).

TIGR Rice Genome Annotation, http://blast.jcvi.org/eukblast/index.cgi?project=osa1 (November 15, 2011).

Supplementary Material

The following online material is available for this article:

Table S1 - Primers used for extending *PnMAI3* marker.

Table S2 - Primers for *PnMAI3* contigs amplification from genomic DNA.

Figure S1 - Amplification of contigs *PnGSA1-4* from genomic DNA.

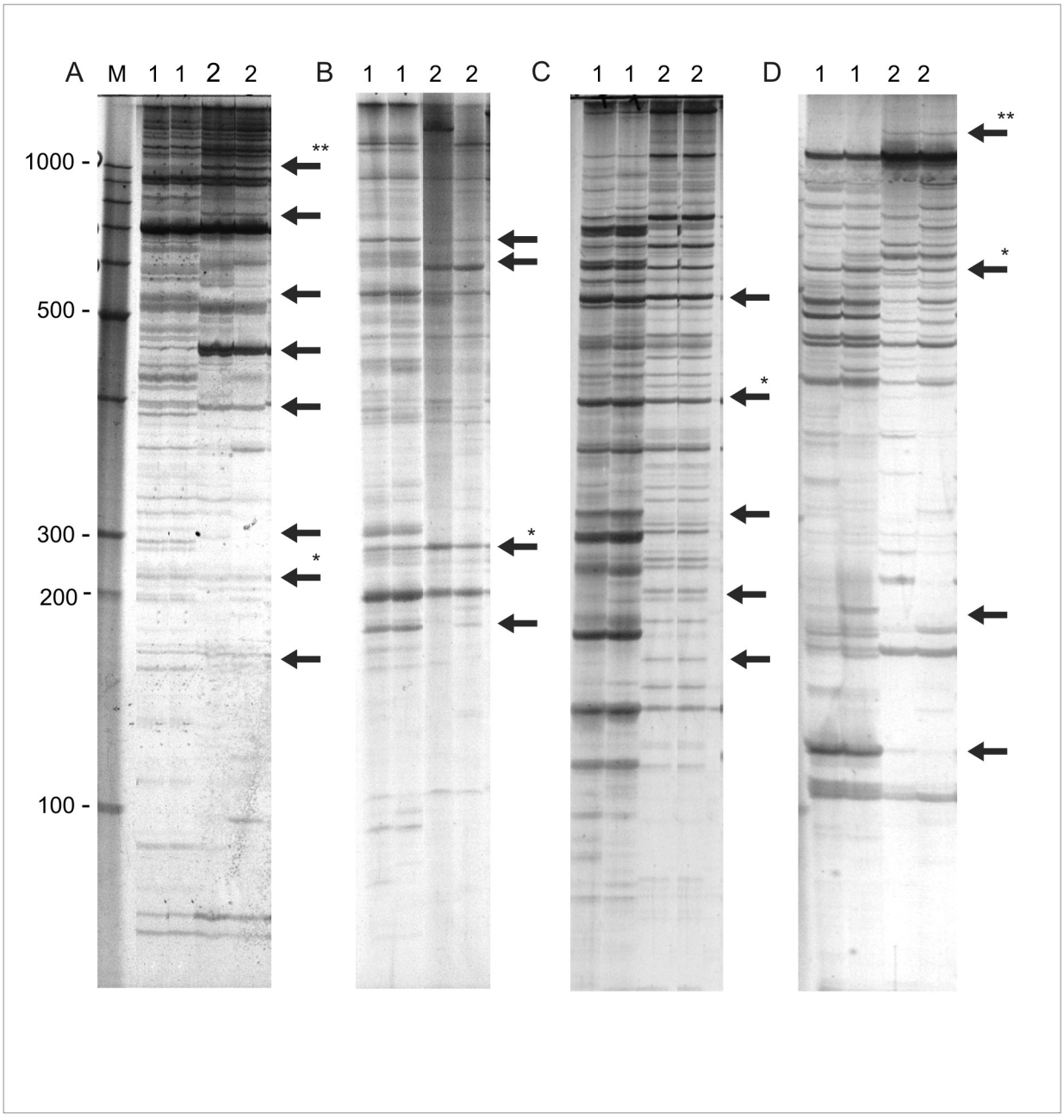
Figure S2 - Mapping *PnGSA1* in *Paspalum notatum*.

Figure S3 - Methylation-sensitive RFLP analysis carried out with clone *PnMAM3*.

This material is available as part of the online article from http://www.scielo.br/gmb.

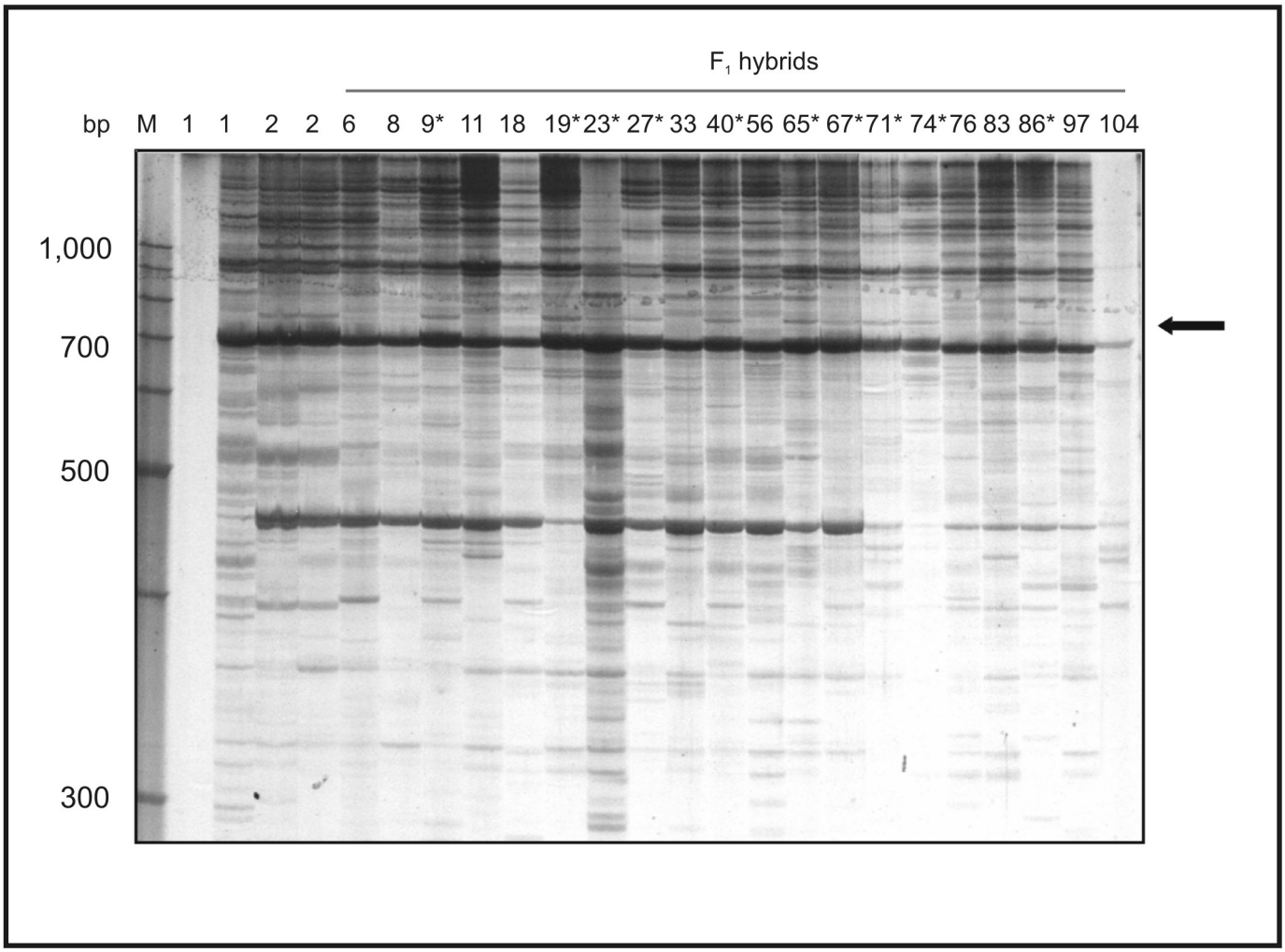
Associate Editor: Marcia Pinheiro Margis

Figure S1: PCR amplification of contigs *PnGSA1-4* from genomic DNA



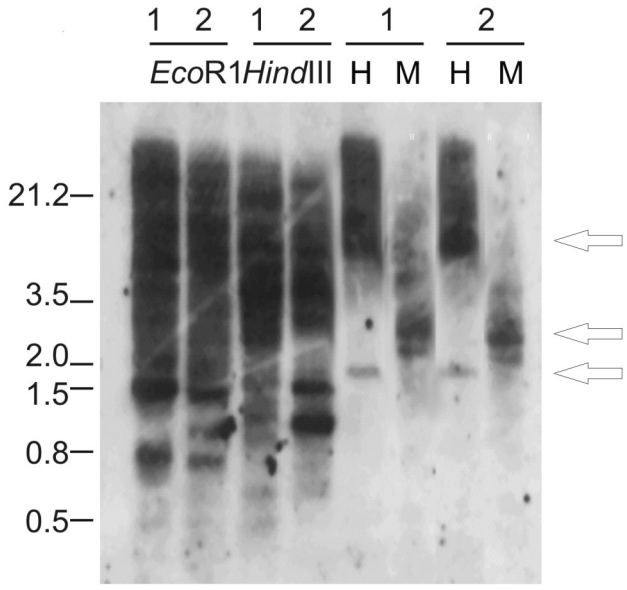
A, **B**, **C** and **D**: Acrylamide gels showing amplification products derived from *PnGSA1* (with primers VI3R - VI35BF1), PnGSA2 (with primers VI3R - VI354BF2), *PnGSA3* (VI3F-VI338AR1) and *PnGSA4* (with primers VI3F- VI3310AR2) (Table S2). **M**: molecular weight marker. Arrows indicated polymorphic fragments between parental genotypes. *indicates fragments of the expected size according to the original sequence. ** indicate markers linked to apospory.

Figure S2: PCR amplification of contig *PnGSA1* in a sample of *Paspalum notatum* mapping population



Acrylamide gels showing amplification products derived from the *PnGSA1* with primers VI3R and VI35BF1 (Table S2). M: molecular weight marker. **1** and **2**: DNA samples from Q4188 and Q4117 loaded by duplicate (the amplification in the first lane of Q4188 failed) . 6-104: experimental number of F_1 hybrids of the mapping population. *aposporous plants. Arrow indicated the band. cosegregating with apospory.

Figure S3: Methylation-sensitive RFLP analysis carried out with clone *PnMAM3*



Methylation-sensitive RFLP experiments with apospory-specific probe *PnMAM3*. Q4188 (1) and Q4117 (2) DNA samples were digested with *Eco*RI, *Hind*III, *Hpa*II (H) and *Msp*I (M). Arrows indicate methylation-sensitive markers.

Table S1 - Primers used to extend the apospory-specific AFLP-derived sequence *PnMAI3*.

Primer name	Sequence
I3NP31[1]	AAATTCGGCTTCACGGCATTGGTCATT
I3NP32[2]	GATGCCTTGGGTCTTCTCTTATACGTT
I3NP51[1]	CAGGCGTCGGACGTTCCAGTGAATGCA
I3NP52[2]	CTAGGGGTCCGATTGGTTGCTTGAATT

[1]Primers specific for the outer sequence; [2]Nested primers.

Table S2 - Primers used to amplify contigs derived from extension of the apospory-specific sequence *PnMAI3* from *Paspalum notatum* genomic DNA.

PnMAI3 anchor primer (5´-3´)	Contig sequence	Specific-primer of the extended sequence (5´-3´)	Length (bp)
VI3*R*: CAATGACCAATGCCGTGAAGC	*PnGSA1*	VI35BF1: GGCTGGTATCTAGCAGCTCAA	309
		VI35BF2: GCATGTTCAAGCACATCTATC	279
	PnGSA2	VI354BF2: CAGAAGGCCAGAAGAACTCA	291
VI3*F*: CCCAGACCGTTCGATATGTGTAAT	*PsGSA3*	VI338AR1: GGAATCCATCCTGAAGTTTCG	538
		VI338AR2: GCTCGTAGTGGACATATTTGC	417
	PnGSA4	VI3310AR1: TAGTCATTGGCGGTGGTGGAT	342
		VI3310AR2: GTGAGTGCCAGGAACTCTTCT	774