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

Maricel Podio¹², Maria P. Rodríguez¹, Silvina Felitti¹, Juliana Stein¹, Eric J. Martínez², Lorena A. Siena¹, Camilo L. Quarin², Silvina C. Pessino¹ and Juan Pablo A. Ortiz¹²

¹Laboratorio de Biología Molecular, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Zavalla, Santa Fe, Argentina.
²Instituto de Botánica del Nordeste, Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Corrientes, Argentina.

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 (Vieille-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...
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 F1, F2 and BC1 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, 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 SPNA2) 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 (Pupilli et al., 2004) and C545, C996A and C1069 (rice chromosome 12) rice showed that clones C560 and C932 (rice chromosomes 2 and 12) are 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 cytogenetical and molecular analyses (Stein et al., 2004).

**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 F1 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 F1 individuals (55 sexual and 10 aposporous) were used to corroborate the linkage between molecular markers and apospory. The F1 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 cytogenetical and molecular analyses (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, E33M33c, E33M33a, E33M33b, E33M33f, E33M42e, E33M42g, E35M33p, E35M33n, E36M37c, E36M37d, E36M38a, E35M43e and E35M43g) (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 F1 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). Recombinants of insert containing 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 MgCl2 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 F1 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 (*EcoRI, HindIII* and *BamHI* or *PstI*). 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) F1 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 *Mspl* and *HpaII* in combination with the apospory-specific clones *PnMAM3* and C1069. *Mspl* and *HpaII* 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 F1 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 F1 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 F1 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).

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

<table>
<thead>
<tr>
<th>Marker type</th>
<th>Marker linked to apospory</th>
<th>Sequence identity</th>
<th>Length (bp)</th>
<th>Expected value</th>
<th>Best Blastn/Blastx alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD</td>
<td>BCU243-377&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>PnMA243</em></td>
<td>351</td>
<td>5.0e^-50</td>
<td>gb FL982880.1 <em>Panicum virgatum</em> cDNA clone. Similar to hypothetical protein DNA binding protein</td>
</tr>
<tr>
<td>AFLP</td>
<td>E32M33&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>PnMAC5</em></td>
<td>96</td>
<td>-</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>E33M32&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>PnMAI3</em></td>
<td>279</td>
<td>-</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>E33M42&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>PnMAJ5</em></td>
<td>207</td>
<td>2.0e^-07</td>
<td>gb AC243221.1 <em>Panicum virgatum</em> clone PV_ABu006-D05</td>
</tr>
<tr>
<td></td>
<td>E33M42&lt;sup&gt;g&lt;/sup&gt;</td>
<td><em>PnMAJ7</em></td>
<td>115</td>
<td>-</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>E36M37&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>PnMAM3</em></td>
<td>87</td>
<td>7.3e^-06</td>
<td>Os07g22800.1 retrotransposon protein putative Ty1-copia subclass</td>
</tr>
<tr>
<td></td>
<td>E36M38&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>PnMAN1</em></td>
<td>42</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E35M43&lt;sup&gt;n&lt;/sup&gt;</td>
<td><em>PnMAU14</em></td>
<td>91</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E35M43&lt;sup&gt;p&lt;/sup&gt;</td>
<td><em>PnMAU16</em></td>
<td>176</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>RFLP</td>
<td>C560&lt;sup&gt;c&lt;/sup&gt;</td>
<td>D15383</td>
<td>421</td>
<td>2.7e^-53</td>
<td>LOC_Os04g39700 60S ribosomal protein L6 putative expressed</td>
</tr>
<tr>
<td></td>
<td>C932&lt;sup&gt;c&lt;/sup&gt;</td>
<td>D22694</td>
<td>329</td>
<td>5.0e^-152</td>
<td>LOC_Os02g52290.1 peptidyl-prolyl cis-trans isomerase FKBP-type</td>
</tr>
<tr>
<td></td>
<td>C454&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C98049</td>
<td>725</td>
<td>2.7e^-113</td>
<td>LOC_Os12g40560.1 KH domain-containing protein</td>
</tr>
<tr>
<td></td>
<td>C996A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>C98189</td>
<td>271</td>
<td>1.2e^-49</td>
<td>LOC_Os12g42180 50S ribosomal protein L14</td>
</tr>
<tr>
<td></td>
<td>C1069&lt;sup&gt;g&lt;/sup&gt;&lt;sup&gt;r&lt;/sup&gt;</td>
<td>D15675</td>
<td>400</td>
<td>1.8e^-228</td>
<td>LOC_Os12g40530.1 transposon protein putative mutator sub-class</td>
</tr>
</tbody>
</table>

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 EcoRI consisted of bands with different migration rates in Q4188 and Q4117, while the polymorphism produced by PstI 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

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

<table>
<thead>
<tr>
<th>Extension direction</th>
<th>Contig name</th>
<th>Length (bp)</th>
<th>E value</th>
<th>Best alignment (Blastn)</th>
<th>Annotation1</th>
<th>In silico mapping onto rice and maize genomes2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′</td>
<td>PnGSA1</td>
<td>350</td>
<td>3e-08</td>
<td>gbFE614154.1 CBYX11857.b1 CBYX Panicum virgatum callus cDNA</td>
<td>No significant hits in the rice genome</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PnGSA2</td>
<td>384</td>
<td>2e-05</td>
<td>gbJG806067.1 CFNU4264.b1 CFNU Panicum virgatum shoot cDNA</td>
<td>No significant hits in the rice genome</td>
<td></td>
</tr>
<tr>
<td>3′</td>
<td>PnGSA3</td>
<td>689</td>
<td>2e-09</td>
<td>gbCD433587.1 Endosperm_5 Zea mays cDNA LOC100281493 N6-adenosine-methyltransferase MT-A70-like protein</td>
<td>OS 2. Position: 27358073 - 27358338 (E-val: 5.1 e-42). In the proximity of several apospory-linked markers</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.1e-42</td>
<td>LOC_Os02q45110.1 Transcript. MT-A70 domain containing protein</td>
<td>ZM 5. Position: 194 339 512 - 194 343 577 (E-val: 4.2e-28). In the proximity of several apospory-linked markers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PnGSA4</td>
<td>1077</td>
<td>8e-54</td>
<td>gb CD433356.1 EL01N0307F02.b Endosperm_3 Zea mays cDNA</td>
<td>Multiple alignments (lowest E-val: 1.9 e-25)</td>
<td></td>
</tr>
</tbody>
</table>

1Determined 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 F1 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) F1 individuals. PCR amplifications showed that most fragments were unlinked to apospory. However, amplification of sequence PnGSA1 with primers V13R and V135BF1 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 F1 progenies (Figure S2). This fragment (GenBank accession number: JN25001) showed sequence similarity to a maize transposable element (GRMZM5G80837_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.
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 HpaII and MspI. The methylation-insensitive enzymes EcoRI and HindIII 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 EcoRI and HindIII indicating genetic differences among genotypes at the specific loci. Hybridization of samples digested with HpaII and MspI 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 EcoRI and HindIII produced the expected polymorphic markers associated with apospory reported by Martínez et al. (2003) (not shown).

The hybridization patterns of samples digested with HpaII and MspI 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 Q4106 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

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

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Rice chromosome (Os): bp</th>
<th>E value</th>
<th>%ID</th>
<th>Maize chromosome (Zm): bp</th>
<th>E value</th>
<th>%ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>PnMA243</td>
<td>Os: 2: 22 235 716 - 22 235 909</td>
<td>0.003</td>
<td>65.2</td>
<td>Zm: 4: 94 738 078 - 94 738 380</td>
<td>1.2e-11</td>
<td>65.9</td>
</tr>
<tr>
<td>PnMAJ5</td>
<td>n.s.</td>
<td>n.s</td>
<td>n.s</td>
<td>Zm: 4: 181 677 342 - 181 677 356</td>
<td>5.6e-07</td>
<td>70.3</td>
</tr>
<tr>
<td>PnMAM3</td>
<td>Os: multiple alignments</td>
<td>7.6e-05</td>
<td>86.6</td>
<td>Zm: multiple alignments</td>
<td>7.2e-05</td>
<td>84.4</td>
</tr>
<tr>
<td>PnMA764</td>
<td>Os: 12: 18 536 262 - 18 536 706</td>
<td>4.8e-07</td>
<td>68.2</td>
<td>Zm: multiple alignments</td>
<td>4.0e-05</td>
<td>67.6</td>
</tr>
<tr>
<td>C560</td>
<td>Os: 2: 22 876 845 - 22 878 075</td>
<td>3.7e-03</td>
<td>100.0</td>
<td>Zm: 5: 179 392 786 - 179 393 814</td>
<td>2.9e-07</td>
<td>87.2</td>
</tr>
<tr>
<td>C932</td>
<td>Os: 2: 32 011 864 - 32 014 026</td>
<td>5e-12</td>
<td>100.0</td>
<td>Zm: 5: 209 262 954 - 209 264 371</td>
<td>1.3e-03</td>
<td>87.0</td>
</tr>
<tr>
<td>C454</td>
<td>Os: 12: 25 058 216 - 25 065 293</td>
<td>0.0</td>
<td>100.0</td>
<td>Zm: 1: 167 869 383 - 167 870 468</td>
<td>4.2e-08</td>
<td>75.1</td>
</tr>
<tr>
<td>C996A</td>
<td>Os: 12: 26 106 507 - 26 108 035</td>
<td>4.4e-06</td>
<td>100.0</td>
<td>Zm: 3: 86 923 502 - 86 928 449</td>
<td>1.0e-02</td>
<td>78.1</td>
</tr>
<tr>
<td>C1069</td>
<td>Os: 12: 25 047 073 - 25 047 471</td>
<td>1.8e-28</td>
<td>100.0</td>
<td>Zm: 1: 167 565 640 - 167 566 038</td>
<td>1.0e-04</td>
<td>86.0</td>
</tr>
<tr>
<td>PnGSA3</td>
<td>Os: 2: 27 358 073 - 27 358 338</td>
<td>5.1e-22</td>
<td>69.8</td>
<td>Zm: 3: 115 792 597 - 115 792 988</td>
<td>1.4e-05</td>
<td>85.5</td>
</tr>
<tr>
<td>PnGSA4</td>
<td>Os: multiple alignments</td>
<td>1.9e-23</td>
<td>66.3</td>
<td>Zm: multiple alignments</td>
<td>8e-54</td>
<td>68.0</td>
</tr>
</tbody>
</table>

1Orthologous 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. 2In cases of multiple alignments, the lowest E-value and the highest %ID are indicated (corresponding to the best match).

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 HpaII (H) and MspI (M). B: Hybridization in ten sexual (F1s) and ten apomictic (F1a) F1 hybrids digested with HpaII (H) and MspI (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 F1 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 squamulatum* (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-recombining 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 (*PnMAM423*) 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 sequences 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 aposporo-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* apomixy-specific sequences in the rice and maize genomes identified several orthologous sequences in segments of rice chromosomes 2 and 12, previously associated with apomixy 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 apomixy-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 apomixy in *Brachiaria* hybrids (Pessino et al., 1997, 1998). Accordingly, these chromosomal segments may contain apomixy-related sequences.

Several *P. notatum* apomixy-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 (Ochogavia et al., 2011).

Methylation-sensitive RFLP experiments indicated that, in leaves, repetitive elements located at the aposporous 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.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.

References


Akiyama Y, Hanna WW and Ozias-Akins P (2005) High-resolution physical mapping reveals that the aposporic-specific genomic region (ASGR) in *Cenchrus ciliaris* is located on a heterochromatic and hemizygous region of a single chromosome. Theor Appl Genet 111:1042-1051.


Quarin CL, Urbani MH, Blount AR, Martinez EJ, Hack CM, Burton GW and Quesenberry KH (2003) Registration of Q4188
Characterization of apospory markers


**Internet Resources**


**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

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
**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 VI3F - 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 F₁ 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 *EcoRI*, *HindIII*, *HpaII* (H) and *MspI* (M). Arrows indicate methylation-sensitive markers.
**Table S1** - Primers used to extend the apospory-specific AFLP-derived sequence *PnMAI3*.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I3NP31(^1)</td>
<td>AAATTCGGCTTCACGGCATTGGTCATT</td>
</tr>
<tr>
<td>I3NP32(^2)</td>
<td>GATG CCTTG GGTCTTCTTATACGTT</td>
</tr>
<tr>
<td>I3NP51(^1)</td>
<td>CAGGCGTCGGACGTCCAGTGAATGCA</td>
</tr>
<tr>
<td>I3NP52(^2)</td>
<td>CTA GGGTCCGAT TGGTGCTTGAA TT</td>
</tr>
</tbody>
</table>

\(^1\)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.

<table>
<thead>
<tr>
<th><em>PnMAI3</em> anchor primer (5´-3´)</th>
<th>Contig sequence</th>
<th>Specific-primer of the extended sequence (5´-3´)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI3R: CAATGACCAATGCCGTGAAGC</td>
<td><em>PnGSA1</em></td>
<td>VI35BF1: GGCTGGTATCTAGCAGCTCAA</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI35BF2: GCATGTTCAAGCACACATCTATC</td>
<td>279</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI354BF2: CAGAAGGCCAGAAGAACTCA</td>
<td>291</td>
</tr>
<tr>
<td>VI3F: CCCAGACCGTTTCGATATGTGTAAT</td>
<td><em>PsGSA3</em></td>
<td>VI338AR1: GGAATCCATCCTGAAGTTTCG</td>
<td>538</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI338AR2: GCTCGTAGTGACGACATATTTGC</td>
<td>417</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI3310AR1: TAGTCATTTGCGGTGGTGGAT</td>
<td>342</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI3310AR2: GTGAGTGCCAGAAGACTCTTCT</td>
<td>774</td>
</tr>
</tbody>
</table>