

Gene expression analysis at the onset of aposporous apomixis in *Paspalum notatum*

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Abstract Apomixis is a route of asexual reproduction through seeds, that progresses in the absence of meiosis and fertilization to generate maternal clonal progenies. Gametophytic apomicts are usually polyploid and probably arose from sexual ancestors through a limited number of mutations in the female reproductive pathway. A differential display analysis was carried out on immature inflorescences of sexual and apomictic tetraploid genotypes of *Paspalum notatum*, in order to identify genes associated with the emergence of apospory. Analysis of ~10,000 transcripts led to the identification of 94 high-quality differentially expressed sequences. Assembling analysis, plus validation, rendered 65 candidate unigenes, organized as 14 contigs and 51 singletons. Thirty-four unigenes were isolated from apomictic plants and 31 from sexual ones. A total of 45 (69.2%) unigenes were functionally categorized.

While several of the differentially expressed sequences appeared to be components of an extracellular receptor kinase (ERK) signal transduction cascade, others seemed to participate in a variety of central cellular processes like cell-cycle control, protein turnover, intercellular signalling, transposon activity, transcriptional regulation and endoplasmic reticulum-mediated biosynthesis. In silico mapping revealed that a particular group of five genes silenced in apomictic plants clustered in a rice genomic area syntenic with the region governing apospory in *Paspalum notatum* and *Brachiaria brizantha*. Two of these genes mapped within the set of apo-homologues in *P. notatum*. Four genes previously reported to be controlled by ploidy were identified among those expressed differentially between apomictic and sexual plants. In situ hybridization experiments were performed for selected clones.

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Abbreviations

Apo Apomictic
Chr Chromosome
DD Differential display
ER Endoplasmic reticulum
Sex Sexual
SSCP Single-strand conformation polymorphisms

Introduction

Two asexual reproductive strategies are commonly found in angiosperms: vegetative propagation (where plants

reproduce via stolons, rhizomes, bulbs, bulbils or root sprouts) and apomixis (where seeds are produced without previous meiotic reduction and fusion of gametes). To date, agriculturalists have focused almost exclusively on the benefits of vegetative propagation for the production of such crops as potatoes, cassava, yams, banana, sugarcane, strawberries, apples and even some forestry species (Spillane et al. 2004). However, the harnessing of apomixis in crop plants would also provide major benefits to agriculture, such as for the widespread use and fixation of hybrid vigour, survival and fixation of combined genetic resources (including wide-cross progeny), true seed production from crops currently propagated vegetatively and the development of more rapid breeding programs (Vielle Calzada et al. 1996a).

Apomixis most probably evolved as an alternative reproductive system, through a rearrangement of the developmental programs that constitute the normal sexual pathway (Savidan 2000). A well-defined sequence of events must be completed successfully to result in the sexual production of a fertile and unique seed: megaspore mother cell differentiation from the nucellus, megaspore generation by meiosis (megasporogenesis), megaspore selection, embryo sac development by mitoses (megagametogenesis), embryo sac maturation, double fertilization and finally endosperm and embryo formation. In sexually reproducing plants, the absence or disruption of any one of these steps usually results in a premature termination of the developmental program and viable seeds are not produced. In contrast, apomicts can completely omit or misplace some of the events in this sequence, and still generate a fully formed, viable embryo within the limits of the ovule (Koltunow 1993).

In gametophytic apomixis of the aposporous type, the megaspore mother cell (the megasporocyte) often undergoes meiosis although all the four megaspores degenerate immediately; otherwise, the megasporocyte degenerates even before entering meiosis. Meanwhile, one or several cells from the nucellus differentiate and go through a series of mitoses leading to unreduced embryo sacs that produce embryos by parthenogenesis. Consequently, lack of meiotic reduction is compensated by a lack of fertilization to generate a clonal progeny at the original ploidy level. Plants presenting apospory are consistently polyploids. The generation of the endosperm is achieved by means of different strategies: either from fertilization of the unreduced polar nuclei (pseudogamy) or autonomously, depending on the species (Koltunow 1993).

Paspalum notatum Flüge, a perennial rhizomatous grass species native to South America, is widely distributed in the warm and humid regions of the western hemisphere (Gates et al. 2004). Diploid biotypes are sexual and self-incompatible while polyploids are self-fertile pseudogamous

aposporous apomicts. Cytogenetic observations and genetic analyses based on AFLP (Amplification fragment length polymorphisms) (Vos et al. 1995) segregation indicated that tetraploid races have a general tetrasomic inheritance, and an autopoloid origin (Forbes and Burton 1961; Quarin et al. 1984; Stein et al. 2004). Differential display analysis allowed the identification of 48 candidate genes whose expression is controlled by changes of the ploidy level in immature inflorescences of the species (Martelotto et al. 2005). Full genetic maps have been established at the diploid and tetraploid levels, in the latter case using a population segregating for apomixis (Ortiz et al. 2001; Stein et al. 2004, 2007). Apospory in *Paspalum notatum* has been shown to be controlled by a single dominant locus with a distorted segregation ratio, located in a large genomic region where recombination is severely restricted (Martínez et al. 2001, 2003; Stein et al. 2004, 2007). This vast non-recombinant genomic region was estimated to encompass approximately 36 Mbp (Juan Pablo Ortiz, unpublished data). The adjacent chromosomal territories present low recombination rates and preferential chromosome pairing with only one of the homologues (Stein et al. 2004, 2007). Comparative analyses revealed that the region presents partial conservation of synteny with two other species of the genus (*P. simplex* and *P. malacophyllum*) (Pupilli et al. 2004). While the region controlling apospory in *Paspalum notatum* demonstrates synteny to specific regions of rice chromosomes 2 and 12 (Martínez et al. 2003; Pupilli et al. 2004; Stein et al. 2007), the apo locus of *P. simplex* and *P. malacophyllum* shows synteny to chromosome 12 only (Pupilli et al. 2004). Clones mapping onto rice chromosome 2 had previously been reported to be linked to apospory in *Brachiaria* hybrids by Pessino et al. (1998).

Lack of recombination around the apo locus severely compromises forward genetic strategies to isolate the trigger of apospory by map-based cloning, imposing a need for massive sequencing and discrimination among hundreds of possible candidates. In this context, characterization of gene expression at the initial steps of aposporous development would greatly contribute to the discovery of genes that could be used for future manipulation of the trait. Moreover, mapping of sequences derived from expression experiments would help to detect candidate genes located within the apo genomic region, therefore assisting in the recognition of the natural trigger of the trait. The isolation of a few sequences differentially expressed in inflorescences/ovaries of sexual and aposporous plants, has been reported for *Pennisetum ciliare* (Vielle-Calzada et al. 1996a), *Panicum maximum* (Chen et al. 1999), *Paspalum notatum* (Pessino et al. 2001) and *Brachiaria brizantha* (Rodrigues et al. 2003). Later, the full-transcriptome characterization was completed for

inflorescences of aposporous and sexual *Poa pratensis* (Albertini et al. 2004), allowing association of several candidate genes with the expression of the trait.

The objective of the present experiment was to carry out a comprehensive transcriptome survey to identify genes differentially expressed in immature inflorescences of aposporous and non-aposporous tetraploid *Paspalum notatum* genotypes. Our results offer a list of candidate genes for the manipulation of apomixis, provide some information about possible molecular routes involved and suggest that a silencing mechanism might be implicated in the triggering of the trait.

Materials and methods

Plant material

The plant material used for differential display and mapping experiments consisted of a fully sexual genotype of experimental origin (Q4188, $2n = 4 \times = 40$) (Quarin et al. 2003), a natural aposporous plant (Q4117, $2n = 4 \times = 40$) (Ortiz et al. 1997) and a pseudo-test cross progeny of 113 hybrids segregating for apomixis derived from a cross of these two genotypes (Q4188 \times Q4117). The same family was employed by Stein et al. (2004, 2007) [k1] to estimate the mode of inheritance and to produce a full genetic map of *Paspalum notatum* at the tetraploid level.

Reproductive calendar construction

Inflorescences from genotypes Q4117 and Q4188 were collected at several developmental stages, fixed in FAA (18 parts 70% ethanol: 1 part 37% formaldehyde: 1 part glacial acetic acid) during 24 h and placed in ethanol 70% v/v at 4°C. Anthers and pistils were dissected from the spikelets under a magnifier, by using forceps and a scalpel. The length of anthers and pistils (ovary + style + stigma) was determined on a graduated slide. Anthers were covered with a drop of acetocarmine 2% p/v, squashed making pressure with a cover slip, and examined with a light microscope to determine the developmental stage of pollen. A few selected ovaries, at different stages of development, were fixed in 70% ethanol, acetic acid, formaldehyde (18:1:1), dehydrated in a tertiary butyl alcohol series, embedded in paraffin, sectioned at 10–12 μm , and stained in the safranin-fast green series.

Differential display analysis

For RNA extraction immature inflorescences were collected from the parental plants (Q4188 and Q4117). Both growth conditions and collection time (9:00 am) were

carefully checked to avoid expression variations caused by environmental factors or endogenous circadian rhythms. The central flower from one of the rows (there are two rows of spikelets in a raceme) was dissected and analysed to determine its developmental stage. When a stage I was detected (see Results, *Reproductive calendar* section), a few spikelets (8–10 flowers) around the central flower were cut apart and used to obtain RNA (the edges of the inflorescence are usually slightly less developed). Total RNA was obtained by using the SV Total RNA Isolation kit (Promega). Reverse transcription was performed using *Superscript II* reverse transcriptase (Gibco-Life Technologies) as indicated by the manufacturers. Differential display experiments were conducted under the general protocol reported by Liang and Pardee (1992) with minor modifications (Pessino et al. 2001; Martelotto et al. 2005). The anchored oligonucleotides were named DDT1, DDT2, DDT3 and DDT4 and corresponded to sequences 5' T₍₁₂₎(ACg)X 3', where X was A, C, G or T, respectively. Decamers from the British Columbia University RAPD Primer Synthesis Project (set 3) were used in combination with the anchored oligonucleotides to produce a total of 87 informative primer combinations (21 from DDT1, 24 from DDT2, 20 from DDT3 and 22 from DDT4). PCR reactions were prepared in a final volume of 25 μl containing 1 \times *Taq* activity buffer (Promega), 1.5 mM MgCl₂, 200 μM dNTPs, 0.70 μM arbitrary primer, 2.5 μM anchored primer, 2 U of *GoTaq* DNA polymerase enzyme (Promega) and 2.5 μl of the reverse transcription reaction (previously diluted 1/20). All samples (including controls, i.e.: RT (-) and non-template) were processed in duplicate. The cycle program consisted of an initial step of 3 min at 94°C, 40 cycles of 20 s at 94°C, 20 s at 38°C and 30 s at 72°C, followed by a final step of 5 min at 72°C. Samples were mixed with denaturing loading buffer, treated for 3 min at 95°C and separated in a 5% (w/v) denaturing polyacrylamide gel. Amplification products were silver-stained following the DNA Silver Staining System procedure (Promega). Differential fragments were excised, eluted in a buffer of 0.5 M ammonium acetate and 1 mM EDTA pH 8, precipitated in ethanol, and re-amplified using the same PCR conditions described above. Fragments were cloned with the aid of the pGEM[®]-T Easy Vector System (Promega). Sequencing of the differential display cDNA clones was performed by Macrogen Inc (Korea).

Validation of differential expression

Validation of genes differentially expressed between aposporous and sexual plants was conducted using real-time PCR amplification on bulked cDNA samples obtained from eight F1 progenies (4 apomicts and 4 sexuals). RNA samples were extracted from inflorescences at stage I (as

indicated at the *Differential Display Analysis* section). Equal amounts of RNA were bulked into apomictic and sexual groups. Real-time PCR reactions were prepared in a final volume of 25 μ l containing 500 nM gene specific primers, 1X SYBR Green Master Mix (PE Biosystems) and 40 ng of reverse-transcribed RNA (prepared by using *Superscript II*, Invitrogen). Gene specific primers were designed to amplify fragments 90–150 bp long. Standard samples containing 53 and 53,000 copies of the target gene were prepared by using the original plasmids including the cloned inserts, and used for quantification. Amplification efficiency was controlled to be equivalent for samples and the corresponding standards. RT (-) and non-template controls were incorporated in the assays. Two endogenous reference genes equally expressed in apomictic and sexual plants (clones 25 and 34, reported in Martelotto et al. 2005, whose sequences correspond to anthranilate synthase and a histone H1-like protein respectively) were used as endogenous controls to check identical amplification between samples. All apomictic and sexual bulks, standard samples, controls and endogenous references were amplified in duplicate. Amplifications were performed in an Eppendorf Mastercycler Realplex² thermocycler programmed as follows: 2 min at 95°C, 45 cycles of 15 s at 95°C, 30 s at 50°C and 20 s at 72°C, followed by 20 min 60°C for dissociation. Data were recorded and quantified using the program Eppendorf Realplex Mastercycler.

Sequencing and data analysis

Analysis of DNA similarity was carried out using the BLASTn and BLASTx packages on the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov/BLAST/>). Rice in silico mapping was done by using the tools provided at the Gramene webpage (<http://www.gramene.org/>).

Molecular marker generation

DNA extractions were carried out using the CTAB method according to Martínez et al. (2003). Single strand conformation polymorphisms (SSCP) were assayed on a sample of 88 individuals from the F1 progeny (the 15 aposporous and 73 randomly chosen non-aposporous plants). Amplifications were performed in 25 μ l reactions containing 90 ng of the genomic DNA, 1 \times *Taq* polymerase reaction buffer (Promega), 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 mM of each primer and 1 U *GoTaq* DNA polymerase (Promega). The PCR consisted of an initial denaturation step of 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 45°C for 1 min, 72°C for 1:30 min. A final extension of 72°C for 10 min was included. Amplifications reactions were carried out using a MJ Research, Inc. PTC-100

Programmable Thermal Controller thermocycler. PCR products were supplemented with 12.5 μ l of loading buffer (95% w/v formamide, 10 mM NaOH and bromophenol blue) denatured at 95°C for 5 min, immediately placed on ice and loaded onto 0.5 \times MDE gels (MDE Gel Solution, Cambrex Bio Science Rockland, Inc). Electrophoresis was carried out in a sequencing gel electrophoresis tank (Hoefer SQ3 Sequencer) at 15 W for 4 h using 0.6 \times TBE buffer (100 mM Tris-HCl, 90 mM boric acid, 1 mM EDTA, pH 8.0). Running temperature was maintained around 15°C. Gels were stained by using the DNA Silver Staining System (Promega). RFLP markers were assayed on a sample of 30 individuals from the F1 progeny (15 aposporous and 15 randomly chosen sexual plants), as described by Ortiz et al. (2001).

Mapping procedure

The presence/absence of each polymorphic fragment between Q4188 and Q4117 genotypes was visually determined and recorded for each progeny. Segregation data were analysed as described by Stein et al. (2007) by identifying single-dose markers (SDMs) segregating from the aposporous or the non-aposporous parent, depending on the case. A χ^2 test was used to determine the goodness of fit (at $P \leq 0.01$) between the observed and the expected number of genotypes for each class of segregation ratio. Single dose markers were incorporated to matrixes of the available genetic maps of each species (Stein et al. 2007). Map units in centimorgan (cM) were derived from the Kosambi (1944) mapping function. Linkage analysis was performed with MAPMAKER/EXP 3.0 (Lander et al. 1987) at a minimum LOD threshold of 3.0 and a recombination frequency $\theta = 0.39$ (the maximum detectable recombination fraction for a population of 113 individuals from an autotetraploid) (Wu et al. 1992).

Tissue in situ hybridization

In situ hybridization was performed following the protocol described by Dusi (2001) with minor modifications. Inflorescences in stage I were fixed in 4% paraformaldehyde/0.25% glutaraldehyde in 0.01 M phosphate buffer pH 7.2, dehydrated in an ethanol series and embedded in paraffin. Specimens were cut into 7 μ m thin sections and placed onto slides treated with poly-L-lysine 100 μ g/ml. Paraffin was removed using a xylol series. Plasmids containing the selected clones were linearized using restriction enzymes *NcoI* or *SalI* (Promega). Probes were labelled by using the Roche Dig RNA Labelling kit, following the manufacturers' instructions. Probes were hydrolysed to 150–200 bp fragments. Prehybridization was carried out in a buffer of 0.05 M Tris-HCl pH 7.5 containing 1 μ g/ml

Fig. 1 *Paspalum notatum* reproductive calendar. U: Undifferentiated, Pmc: Pollen mother cells, Tet: Tetrads, Eu: Early uninucleated, Mu: Intermediate uninucleated, Lu: Late uninucleated, Bn: Binucleated, An: Anthesis, Py: Pale yellow, Y: Yellow, Po: Pale orange, O: orange, MMC: megaspore mother cell, AI: apospory initials, W: White. Stage I was chosen for molecular analysis, since it immediately precedes the emergence of apospory initials. A variation of 10% in anther and pistil length was allowed for classification within a particular stage

Stage	0	I	II	III	IV	V	VI	VII
Pollen developmental stage	U	Pmc	Tet	Eu	Mu	Lu	Bn	An
Pollen morphology	-							
Anther length (mm)	0.2	0.2	1.0	1.3	1.7	1.8	1.8	-
Anther colour	Py	Py	Y	Y	Po	O	O	-
Megagametophyte morphology Apo	-	-	MMC AI					
Megagametophyte morphology Sex	-	-	MMC					
Pistil morphology								
Pistil length (mm)	0.3	0.4	1.0	1.0	1.7	2.0	2.5	4.0
Pistil colour	W	Py	Y	Po	O	O	O	O

proteinase K in a humid chamber at 37°C for 10 min. Hybridization was carried out overnight in a humid chamber at 42°C, in buffer containing 10 mM Tris-HCl pH 7.5, 300 mM NaCl, 50% formamide (deionized), 1 mM EDTA pH 8, 1× Denhart, 10% dextranulphate, 600 ng/ml total RNA and 60 ng of the corresponding probe. Detection was performed following the instructions of the Roche Dig Detection kit, using anti DIG AP and NBT/BCIP as substrates.

Results

Reproductive calendar

In order to standardize sample collections, a reproductive calendar was constructed for the genotypes used in the candidates screening. Inflorescences at different developmental stages were collected and examined for general exomorphological features. Reproductive organs were dissected and classified according to anther and pistil length and colour, general pistil morphology, megagametophyte and pollen developmental stage. Eight steps of development (0, I, II, III, IV, V, VI and VII) were arbitrarily established based on clear reference features. A schematic representation of the different stages considered is shown in Fig. 1. Microscopic analysis of ovaries and anthers at stage II showed that the coexistence of megaspore mother cells with aposporous initials was

synchronous with the formation of microspore tetrads (Fig. 2). Since we were interested in isolating candidates that trigger apospory, stage I (the step immediately prior to the emergence of apospory initials and/or the megaspore mother cell) was selected to perform the molecular studies.

Differential display experiments

Flowers at developmental stage I were collected from the apomictic and sexual parental genotypes (Q4117 and Q4188) and processed to obtain the differential display amplicons. Eighty-seven (87) primer combinations were selected for analysis based on the amplification profiles they generated. Bands were scored only in the middle portion of the gel, where resolution was maximal and fully reproducible. Candidate transcripts were distinguished by the clear differential signal between samples, while the intensity of the surrounding bands was roughly equivalent. Figure 3 shows an example of the banding patterns detected in flowers of the sexual and apomictic plants.

A total of 120 bands, showing a presence-absence pattern, were cut, eluted from the gels, re-amplified and sequenced. Only in a few particular cases bands presenting unambiguous quantitative differences were chosen. The molecular weight of the segments obtained ranged from 100 bp to 600 bp. High-quality sequence data was obtained for 94 of them. Initial clustering and assembly rendered 72 unigenes, organized as 21 contigs and 51 singletons.

Fig. 2 Histological section of genotype Q4117 ovary and anthers at developmental stage II. Panel (a) Pollen tetrads can be seen in the anther. Panel (b), left: magnification of panel (a) allows observation of the megaspore mother cell (MMC). Panel (b), right: using a different focal point apospory initials (AI) can be noticed surrounding the megaspore mother cell. The bar at the top references the scale (33 μ m)

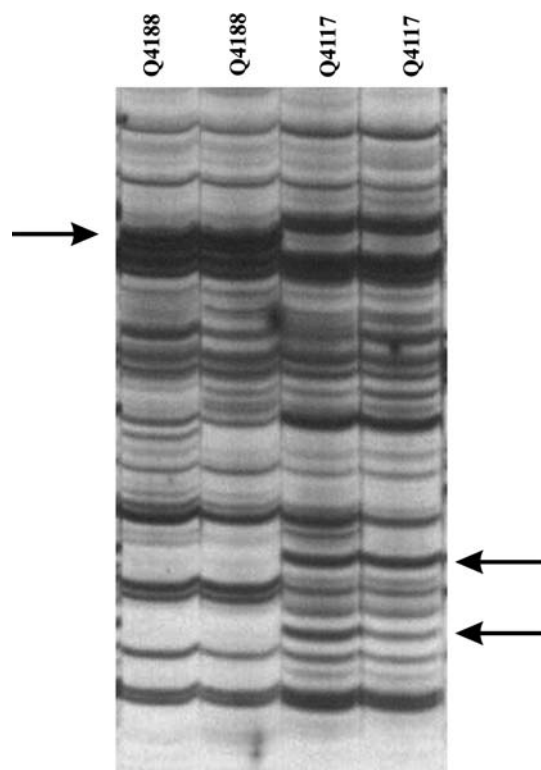
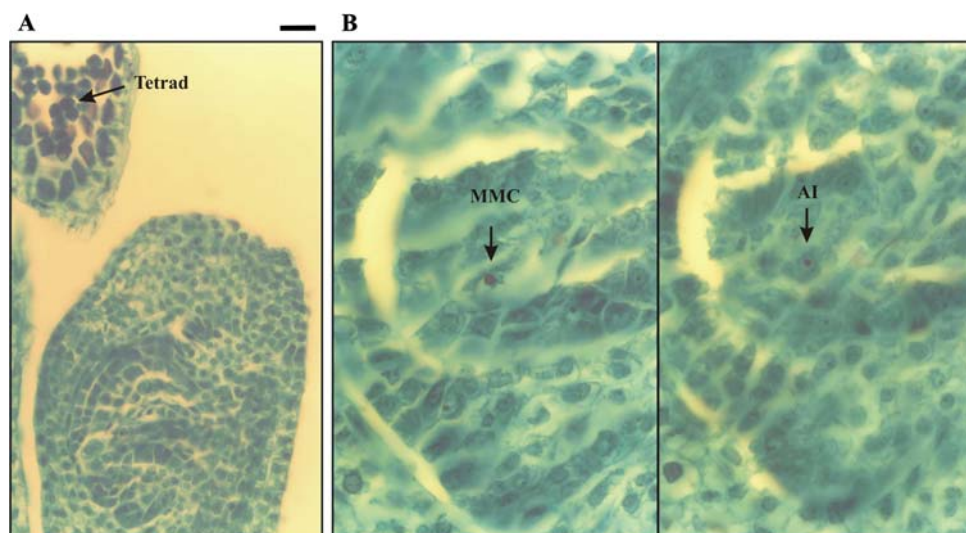


Fig. 3 Differential display banding patterns for genotypes Q4188 and Q4117. Samples were processed in duplicate, using the same oligonucleotide combination. Arrows point to some of the bands differentially expressed in both duplicates, which were isolated for identification of candidate genes

False positive detection and real time-PCR validation

Differential display polymorphic bands could originate either from unequal expression or eventually from allelic variation between Q4188 and Q4117. Sequence

polymorphisms occurring within the amplified fragment (internal polymorphisms) are expected to be relatively more frequent and would give rise to co-dominant markers with different migration distances that can be isolated from both lanes (apo and sex). Sequence polymorphisms at the primers' annealing sites (flanking polymorphisms) are relatively less frequent and will generate dominant markers present in only one lane (apo or sex).

Two different strategies were used to eliminate false positives due to allelic variation. The first was aimed at the most abundant artifacts originated from internal polymorphisms. It consisted of detecting unigenes, organized as assorted contigs, containing homologous sequences isolated from both the apo and sexual genotypes. Once detected, these assorted contigs were eliminated from the list of candidates. Seven unigenes (9.72% of the 72 initial unigenes detected by differential display) were organized as assorted contigs, and were therefore removed from the list. The remaining 65 unigenes were conserved for further analysis.

The second strategy was oriented to the elimination of the remaining false positives (due to residual internal polymorphisms or less abundant flanking polymorphisms). It consisted of performing real time PCR using specific primers on samples from 4 apomictic and 4 sexual progeny plants, for a significant number of randomly-chosen candidates.

Real time PCR reactions were carried out on 4 bulked apomictic or sexual samples to amplify 11 randomly chosen candidate sequences ($\sim 17\%$ of the sequences) and 2 equally-expressed endogenous reference sequences (endogenous genes histone H1-like and anthranilate synthase, isolated from *P. notatum* in previous work) (Martelotto et al. 2005). Three primer pairs failed to amplify from both samples and standards. The remaining 8

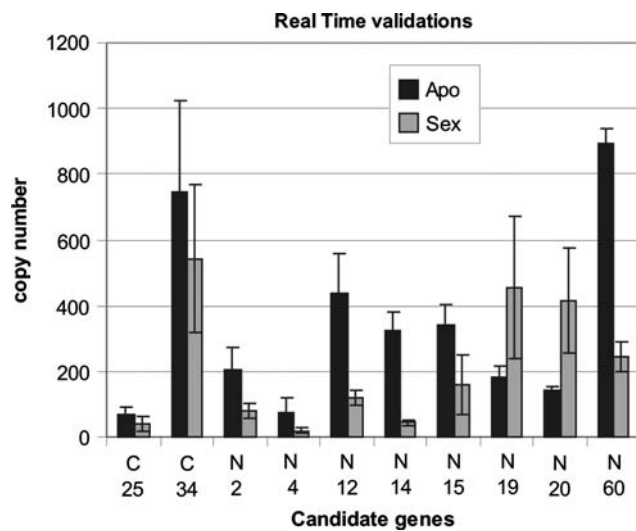


Fig. 4 Real-Time PCR validation of differentially expressed genes. C25 and C34 amplicons which originated from endogenous reference genes histone H1 and anthranilate synthase, which were used as controls of equal expression. Black bars represent amplification from the apomictic sample. Grey bars represent amplification from the sexual sample. Gene copy number was calculated based on two standard samples containing a known quantity of cloned insert. Copy numbers for clones C25 and C34 (n) were expressed in the graphic as n.10. Copy numbers for clones N19 and N60 (n) were expressed in the graphic as n.10⁻³

primer pairs amplified successfully, leading to the validation of their respective differentially expressed candidate gene, suggesting that the level of false positive detection was no longer significant. Dissociation curves in the real time PCR reactions indicated amplification of fragments presenting identical T_m for both types of plants (apomictic and sexual), which suggests that primers are detecting a single allele that is differentially expressed in both plants. Transcript copy numbers for the candidate and reference genes were schematized in Fig. 4.

Functional annotation

BLASTn and BLASTx analysis using the NCBI and Gramene tools allowed functional annotation for 45 sequences (69.2%). A list of sequences obtained, plant of origin and functional annotation (including E values and identification numbers for the best match) is provided in Table 1.

Several of the sequences listed in Table 1 seem to be involved in a signal transduction cascade of the ERK (extracellular receptor kinase) type, probably presenting sub-cascades controlled by a Ras ortholog and phospholipase C (Mishra et al. 2006). Other sequences show homology with genes related to cell cycle control, protein turnover, intercellular signalling, transposon activity, transcriptional regulation and endoplasmic reticulum (ER)

function. A tentative classification of the sequences into general functional groups is provided in Table 2.

In silico mapping of orthologous sequences onto the rice genome

Sequences identified as differentially expressed were mapped in silico onto the rice genome by using the BLAST sequence tool at the Gramene website (www.gramene.org). The position for each sequence was included in Table 1 (far right column). A distribution analysis (schematized in Fig. 5) indicated that the fragments over-expressed in the apomictic genotype appeared evenly scattered over the rice genome, with a representation slightly biased toward chromosomes 3 and 11. Contrastingly, sequences over-expressed in the sexual genotype (silenced in the apomictic one) clustered preferentially onto chromosome 2. A subset of genes mapping onto rice chr 2 (Subgroup 2: clones N5, N46, N19, N20 and N119) were particularly interesting since they were located in a region syntenic with the apospory locus in *Brachiaria brizantha* (Pessino et al. 1998) and *Paspalum notatum* (Pupilli et al. 2004). None of the clones over-expressed in the apo plant mapped to this region. The exact position of the clones mapping onto rice chr 2 (with a special reference to those located in the region syntenic to the apo locus) is provided in Table 3 and schematized in Fig. 6a. Clones integrating into this group were selected to perform mapping experiments in *Paspalum notatum*, in order to determine if they were effectively genetically linked to the apospory locus in the species.

Mapping in a *Paspalum notatum* segregating population

Gene specific PCR primers were designed for clones belonging to Subgroup 2, in order to detect informative SSCP markers between the parental plants (Q4117 and Q4188) and localize the corresponding genes in the *P. notatum* genetic map. Products were electrophoresed in MDE matrixes to detect polymorphisms due to conformational variation or minor molecular weight differences. Only primers corresponding to clone 119 amplified two polymorphic fragments, one specific to genotype Q4188 and the other to genotype Q4117. The rest of the primers did not reveal polymorphic bands in MDE gels.

SSCP markers corresponding to clone 119 were then assayed on 88 F₁ individuals of the *P. notatum* mapping population. Both SSCP markers were successfully mapped, one (sscp119-1) was located on linkage group F3a (corresponding to the sexual genotype (Q4188) and the other (sscp119-2) mapped to linkage group M17b (corresponding to the apomictic genotype, Q4117). M17b is one of the

Table 1 Sequences differentially expressed in apomictic and sexual genotypes

Unigen	Origin	ID ^a	E value	Annotation	Rice Chr
N2	A	YP_677180.1 (x)	3 e−4	Outer membrane receptor	12
N4	A	ZP_00049311.1 (x)	1 e−38	Methyltransferase	8
N5	S	AK068667 (g)	2.9 e−15	Lunapark B protein	2, 4
N7	S	n	–	n	7
N11	A	ZP_01062280.1 (x)	9 e−45	Hypothetical protein	6
N12	A	ABF99435.1 (x)	3 e−40	Cell-death suppressor protein	3
N13	A	n	–	n	10
N14	A	NP_817269.1 (x)	7 e−19	Ribosomal protein S12	10, 3, 8, 4
N15	A	NP_001044371 (x)	3 e−56	Meiotic serine proteinase	1
N16	A	Os02g30630 (g)	0.024	Acetolactate synthase III	2
N17	A	n	–	n	3
N18	A	n	–	n	–
N19	S	CAJ83813.1 (x)	3 e−8	CHK1 checkpoint homolog	2
N20	S	NP_001047962.1 (x)	1 e−53	GPI-anchored protein homolog	2
N22	A	n	–	n	–
N23	A	BAE73184.1 (x)	1 e−15	Phosphatidylinositol 4 kinase	11
N26	S	Os04g46100 (g)	5 e−7	Unknown protein	4
N31	S	CAB40774.1	0.25	Extensin-like protein	2
N35	S	BAD09364.1 (x)	0.021	Hypothetical protein	8
N38	S	n	–	n	–
N40	A	EAY75042.1 (x)	0.64	Cytochrome P450 family protein	1
N43	A	n	–	n	–
N44	S	AA062228.1 (x)	0.034	RNA polymerase beta subunit	3
N46	S	NP_001067464.1 (x)	5 e−54	MAP3KA	11, 4, 2
N49	A	n	–	n	–
N51	S	AY107249.1 (n)	2 e−5	VAP33-like protein	1
N54	S	NP_001054720.1 (x)	9 e−38	Polyubiquitin	1–10
N55	A	EAY75879 (x)	0.87	DNA pol Y family	1
N56	A	n	–	n	–
N58	A	n	–	n	11, 9
N60	A	Os03g52150 (g)	5.7 e−39	Major surface like glycoprotein	3
N66	A	AP008982.1 (n)	7 e−75	NADH dehydrogenase subunit	mit
N68	S	DQ667520.1 (n)	0.11	tRNA-Leu gene	–
N69	A	AAP12939.1 (x)	5 e−87	PRIP-interacting protein	3
N70	S	n	–	n	–
N73	S	ZP_00120240.1 (x)	2 e−26	ABC transporter component	–
N74	S	DQ984515.1 (n)	2 e−55	NADH dehydrogenase subunit	9
N75	A	DQ245701 (n)	0.70	Ribosomal protein L35-like	4
N77	S	n	–	n	–
N78	S	AAV33691.1 (x)	0.86	LRR family protein-like	1–12
N79	A	Os09g20970 (g)	0.93	LRR family protein	9
N82	A	n	–	n	–
N86	S	n	–	n	–
N87	S	n	–	n	–
N89	A	BAE90960 (x)	0.003	Unknown protein	7
N90	S	EAZ18807.1 (x)	4 e−9	PolyA polymerase family protein	3
N91	S	Os08g07720 (g)	0.12	Transferase family protein	8
N92	A	ABA98874.2 (x)	0.021	Retrotransposon protein	1–12

Table 1 continued

Unigen	Origin	ID ^a	E value	Annotation	Rice Chr
N95	A	Os02g38210 (g)	1.2 e-60	Elongation factor TU	2
N97	S	Os02g07320 (g)	0.39	Hypothetical protein	2
N98	S	PSU43403 (n)	5 e-22	Unknown protein	-
N99	S	n	-	n	-
N100	S	NP_773340.1 (x)	2 e-8	UDP glucosyl transferase	5
N102	A	Os08g35440 (g)	1.2	Ser/Thr protein phosphatase PP1	8
N104	A	n	-	n	-
N106	S	n	-	n	-
N108	S	AAX94831.1 (x)	1 e-36	Transposon protein	2
N109	S	AAT95867.1 (x)	6 e-20	Putative transposase	7
N110	A	L46847_1 (x)	2 e-6	Dehydroquinase synthase	5
N111	A	CAB89846.1 (x)	3 e-13	CMP-KDO synthetase	5
N114	A	Os11g44880 (g)	3.9 e-9	Kinesin motor protein	11
N115	A	AY107595.1 (n)	1 e-6	Unknown protein	11
N116	S	n	-	n	-
N119	S	n	-	n	2, 12
N120	A	ZP_00134229.2 (x)	2 e-28	Aldehyde dehydrogenase	-

^a ID corresponding to the best hit in the Blast Analysis, which were made with NCBI BLASTn (n), NCBI BLASTx (x) or Gramene Sequence BLASTn (g)

homologues to the linkage group carrying the locus controlling apospory in the species (M17a) (Stein et al. 2004, 2007)[k2]. Sscp119-2 was linked to the three markers located in this group that showed preferential chromosome pairing to the apo locus (Fig. 6b).

Since SSCP analysis did not produce polymorphisms for the rest of the clones, they were assayed by RFLP experiments using *EcoRI*, *HindIII* and *PstI* restriction enzymes. Clone N20 rendered one RFLP band (p20), generated from enzyme *HindIII*, that resulted in a polymorphic single-dose marker. Linkage analysis revealed that p20 mapped to linkage group M17a (the linkage group carrying the locus controlling apospory in the species), at a distance of 22 cM ($r = 0.20$, LOD 7.3) from the apo locus (Fig. 6b). The rest of the clones showed monomorphic bands between the parental genotypes and could not be mapped.

Ploidy vs. reproductive mode of gene expression control

A set of 48 sequences regulated by changes at the ploidy level in immature inflorescences of sexual *P. notatum* had been identified by our group in earlier work (Martelotto et al. 2005). The group of transcripts differentially expressed in inflorescences of diploid vs. tetraploid sexual plants (Martelotto et al. 2005) and apomictic vs. sexual tetraploid plants (reported here) were clustered and assembled to detect common sequences. Assorted contigs allowed the identification of four sequences that had been

isolated from both surveys. Sequences of clones N7 (unknown gene), N14 (ribosomal protein S12), N16 (acetolactate synthase) and N108 (transposon protein) corresponded to the sequences DDT32852x1, DDT32834x2, DDT32774x2 and DDT32884x, respectively (Martelotto et al. 2005). These preliminary observations suggest that at least some genes controlled by ploidy could be involved in aposporous development, and provide initial molecular evidence for the association between apomixis and polyploidy.

Tissue in situ hybridization

Two specific clones (N2 and N19) that had been validated using real time PCR were selected to perform tissue in situ hybridization experiments, in order to analyze more conclusively if differential expression between the appropriate tissues of sexual and apomictic progeny can be directly associated with the phenotype. N2 presents sequence similarity to an outer membrane receptor and maps in silico onto rice chr 12, while N19 is similar to a checkpoint kinase (chk1) and maps in silico onto rice chromosome 2. Both clones are positionally associated to rice genomic regions syntenic to the *Brachiaria brizantha* and *Paspalum notatum* apospory locus (see Table 1). N2 was shown to be overexpressed in aposporous plants, while N19 was activated in sexual ones (see Fig. 4). Sense controls originated from N2 and N19 and an equal expression control similarly expressed in sexual and aposporous plants (N47) were also

Table 2 Classification of sequences into functional groups

Category	Gene
Signal transduction cascade (ERK type)	LRR family protein-like (N79, Apo; N78: Sex)
	Outer membrane receptor (N2, Apo)
	GPI anchored protein (N20, Sex)
	Phosphatidylinositol 4K (N23, Apo)
	MAP3K (N46, Sex)
	Ser/Thr phosphatase (N102, Apo)
	PRIP-interacting protein (N69, Apo)
	Kinesin (N114, Apo)
Cell cycle control	CHK1 checkpoint homolog (N19, Sex)
	Cell-death suppressor protein (N12, Apo)
	Polyubiquitin (N54, Sex)
Protein turnover	Ribosomal protein S12 (N14, Apo)
	Ribosomal protein L35-like (N75, Apo)
	Meiotic serine proteinase (N15, Apo)
	Polyubiquitin (N54, Sex)
	tRNA-Leu gene (N68, Sex)
Intercellular signalling/adhesion/cell wall	Elongation factor TU (N95, Apo)
	Acetolactate synthase III (N16, Apo)
	Major surface like glycoprotein (N60, Apo)
	Extensin-like protein (N31, Sex)
Transposon activity	CMP-KDO synthetase (N111, Apo)
	RNA directed DNA polymerase (N66, Apo)
	Transposon protein (N108, Sex)
	Putative transposase (N109, Sex)
	Retrotransposon protein (N92, Apo)
Transcriptional regulation	RNA polymerase beta subunit (N44, Sex)
	PRIP-interacting protein (N69, Apo)
	PolyA pol family protein (N90, Sex)
ER function	VAP33-like protein (N51, Sex)
	UDP-glucosyl transferase (N100, Sex)
Others	Cytochrome P450 (N40, Apo)
	NADH subunit (N66, Apo; N74, Sex)
	ABC transporter component (N73, Sex)
	Transferase family protein (N91, Sex)
	Dehydroquinase synthase (N110, Apo)
	Aldehyde dehydrogenase (N120, Apo)

The setting up of the functional groups is only exploratory and might not correspond to an actual related biological role. Some unigenes could have been included in more than one group

assayed. Hybridization experiments were performed on spikelets which originated from inflorescences at stage I.

Both clones showed clear differential signals in the hybridization experiments. In aposporous plants N2 showed detectable signal in all ovary tissues (including the ovule), the anther tapetum and the epidermal cells, while

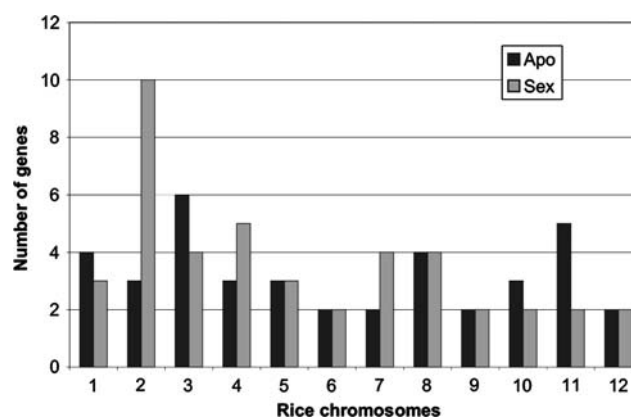


Fig. 5 Distribution of orthologous sequences onto the rice genome. Black bars represent genes isolated from the apomictic genotypes. Grey bars represent genes isolated from the sexual genotypes. A large group of genes silenced in the apo plants (over-expressed in the sexual ones) clustered onto rice chromosome 2

other tissues presented a more subtle level of expression. Meanwhile, sexual plants presented an even signal of low intensity in all tissues (data not shown). N19 showed a much stronger level of expression in comparison with N2, which correlated very well with the high expression levels observed in the real-time PCR experiments (see Fig. 4). In aposporous plants N19 hybridized evenly at a moderate level in all analyzed tissues. However, in sexual plants the signal was considerably stronger, and some tissues presented a particularly high expression level: the ovule (nucellus and integuments), the anther tapetum and pollen mother cells (see Fig. 7). The sense controls presented no hybridization signal and the equal expression control displayed a uniform signal in both aposporous and sexual plants (see Fig. 7).

Discussion

The identification of genes differentially expressed in inflorescences of sexual and apomictic plants might allow the isolation of candidates to be used in experiments aimed at the manipulation of apomixis and its introduction into sexual crops. The first attempts to isolate candidate genes specifically expressed or silenced during apomictic development targeted a few sequences with no clear functional role, and therefore the molecular nature of the pathways involved remained unclear for a while (Vielle-Calzada et al. 1996b; Chen et al. 1999; Pessino et al. 2001; Rodrigues et al. 2003). Later on, Albertini et al. (2004) carried out a comprehensive analysis of the inflorescence transcriptome in sexual and aposporous genotypes of *Poa*, which allowed the recognition of more than 170 sequences differentially expressed in association with aposporous development.

Table 3 Position of orthologous sequences onto rice chr 2

Unigen	Start	End	E value	Functional annotation
<u>5</u>	25229275	25229637	2.9 e-15	LunaPark B protein
	25229132	25229196	2.9 e-15	
<u>19</u>	28713827	28714146	3.9 e-84	CHK1 checkpoint homolog
<u>20</u>	29941545	29941964	1.7 e-65	GPI-anchored protein
	29943171	29943239	1.7 e-65	
31	2580469	2580645	0.00022	Extensin-like protein
<u>46</u>	27048263	27048344	6.3 e-5	MAP3KA
	27052698	27052789	6.3 e-5	
54	3341560	3341787	3.7 e-37	Polyubiquitin
	3341332	3341559	3.7 e-37	
	3341788	3342017	3.7 e-37	
	3340877	3341103	7.2 e-37	
	3340649	3340875	1.1 e-35	
78*	3324927	3326720	3.2 e-76	LRR family protein-like
	3333812	3335797	7.4 e-75	
97	3761184	3761308	0.39	Hypothetical protein
108	15977106	15977335	7.2 3-19x	Transposon protein
	15717289	15717850	1.2 e-13	
	17622965	17623518	5.1 e-7	
<u>119*</u>	30731059	30731502	2.2 e-74	n

* Position of unigenes 78 and 119 was inferred indirectly by locating the homologous sequences AAV33691 and EAZ02894, respectively. Underlined unigenes map onto a region syntenic to the apo locus of *Brachiaria* and *Paspalum*.

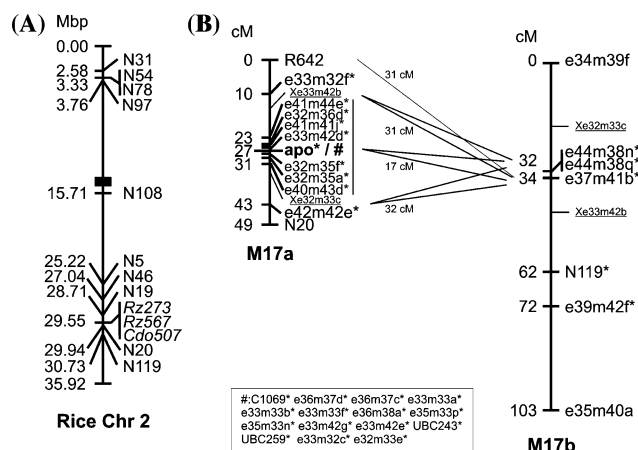
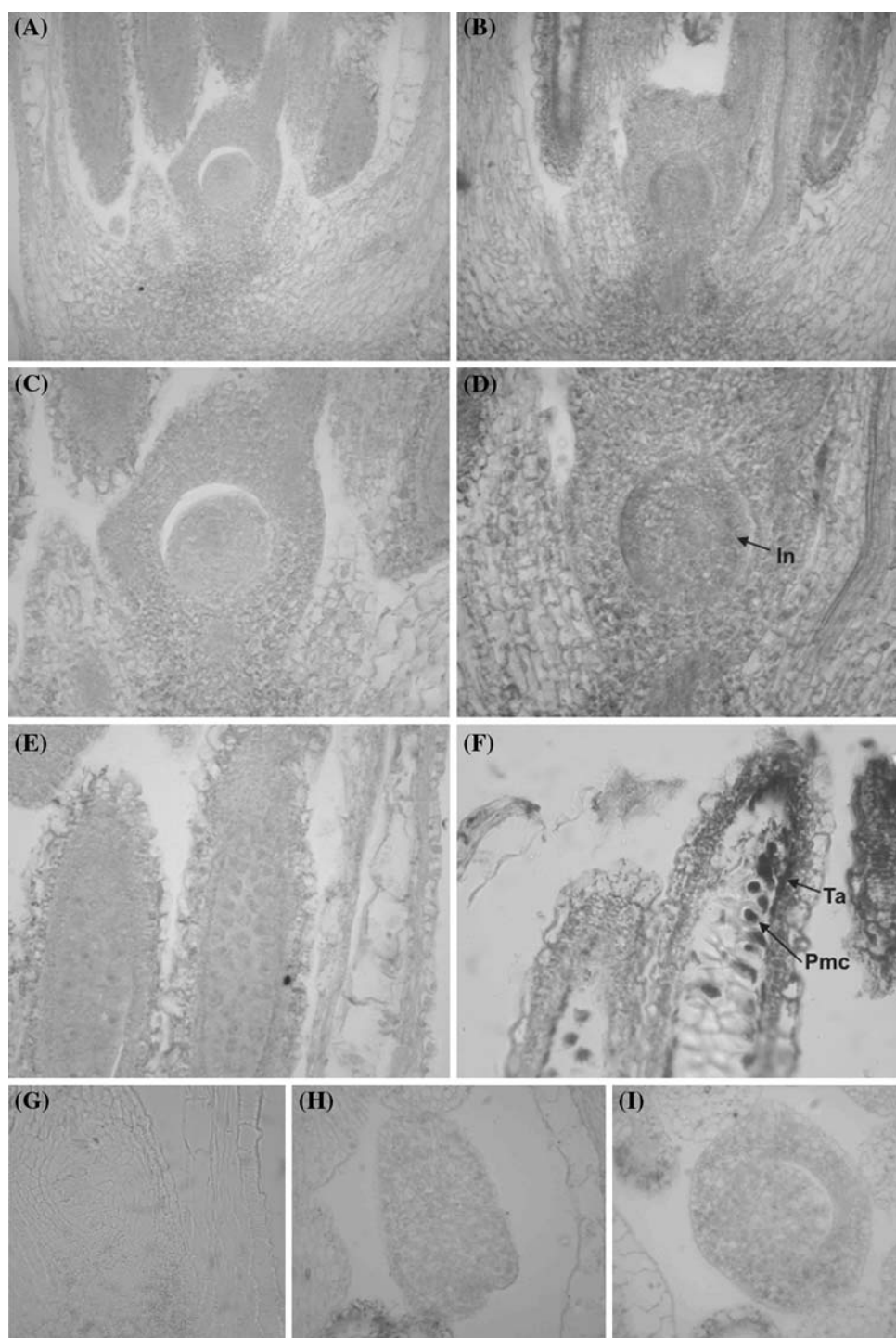


Fig. 6 Mapping of clones silenced in the apomictic genotype that clustered onto rice chromosome 2. Panel (a) In silico mapping of clones silenced in apo plants onto rice chromosome 2. A subgroup of clones (Subgroup 1) clustered at the rice chr 2 short arm. A second subgroup (Subgroup 2) clustered around the markers that were reported to be linked to the apospory locus in *Brachiaria brizantha* (rz273, rz567, cdo 507) (Pessino et al. 1998). Panel (b) Two of the clones from subgroup 2 (N20 and N119) could be mapped onto a *Paspalum notatum* family segregating for apospory. While clone N20 resulted linked in coupling to the apo locus at 22 cM, clone N119 mapped to the apo homologue presenting preferential pairing

Annotation comparisons among sequences reported by Albertini et al. (2004, 2005) and those reported here identified 5 genes with identical predicted function: outer membrane LRR receptor kinases (clones #6 and N79), kinesin motor protein (clones #172 and N114), GPI anchored protein (clones #84 and N20), UDP glucose glucosyl transferase (clones #5 and N100) and ribosomal protein S12 (clones #55 and N14).

Moreover, several of the genes differentially expressed in both *Paspalum* and *Poa* seem to be involved in a signal transduction cascade of the ERK type, with deviations controlled by a Ras ortholog and phospholipase C (Mishra et al. 2006). Here we report the isolation of an LRR (Leucine-rich repeat) family protein (N79), an outer membrane receptor-like protein (N2), a GPI anchored protein (N20), a phosphatidylinositol 4 kinase (N23), a MAP3K (N46), a Ser/Thr phosphatase (N102), a PRIP-interacting protein (N69) and a typical target of ERK cascades: a kinesin cytoskeleton protein (N114). Albertini et al. (2004, 2005) isolated an LRR family extracellular receptor (A #6), a GTP binding protein homologous to Ras (A#50), a Ras-GTPase activator protein binding protein (A#95), a GPI anchored protein (A#84), a protein kinase (#51) and a

Fig. 7 Tissue in situ hybridization experiments. Panel (a) panoramic view of floral organs corresponding to the apomictic genotype hybridized with antisense N19 probe. Panel (b) panoramic view of floral organs corresponding to the sexual genotype hybridized with antisense N19 probe. Note a general stronger hybridization signal in the sexual plant in comparison with the apomictic one (Panels a and b). Panel (c) detail of apomictic plant ovary and ovule tissues hybridized with antisense probe N19. Panel (d) detail of sexual plant ovary and ovule tissues hybridized with antisense probe N19. Note differential hybridization in the ovule integuments (In) and nucellus of the sexual plant (panel c and d). Panel (e) detail of apomictic plant anther tissues hybridized with antisense probe N19. Panel (f) detail of sexual plant anther tissues hybridized with antisense probe N19. Note differential hybridization in the tapetum (Ta) and pollen mother cells (Pmc) (panel e and f). Panel (g) ovary from the sexual plant hybridized with sense probe N19 (negative control). Panel (h) detail of apomictic plant ovary tissues hybridized with antisense probe N47 (equal expression control). Panel (i) detail of sexual plant ovary tissues hybridized with antisense probe N47 (equal expression control). Note a similar hybridization signal revealed with the equal expression control (panel h and i)



kinesin (A#72). Lack of complete coincidence between the reports might reflect particular developmental characteristics, an incomplete coverage of the whole transcriptome for both species, or perhaps the fact that the time of collection was not the same for *Paspalum* and *Poa* hence differences could be attributable to minor moves in the developmental timing at which flowers were sampled.

Recently, Stein et al. (2007) performed a detailed characterization of the locus that controls apospory in *P. notatum*. Mapping onto a progeny segregating for apomixis indicated that a single linkage group was associated with the trait. The genomic region defined by this group was characterized by a strong restriction of recombination and preferential chromosome pairing (Martínez et al. 2003;

Stein et al. 2004, 2007). Moreover, Pupilli et al. (2004) established that this region in the *Paspalum notatum* genome was syntenic to rice chromosome 2 long arm and to rice chromosome 12 short arm. Clones mapping onto rice chr 2 were also found linked to apospory in *Brachiaria* hybrids (Pessino et al. 1998). Orthologous sequences corresponding to transcripts differentially expressed in inflorescences of sexual and apomictic *P. notatum*, when mapped, were scattered across the rice genome. However, a small group of clones clustered around the rice genomic region syntenic to the apo region of *Brachiaria brizantha* and *Paspalum notatum*. Curiously, all the genes belonging to this small group (clones N5, N46, N19, N20 and N119) had been isolated from sexual plants (they were down-regulated in apomictic ones). Two of the clones (N20 and N119) presented polymorphisms between the parents of a *P. notatum* mapping population segregating for apomixis, and could therefore be mapped onto the genome of the species, showing that N20 was effectively linked to the trait and N119 belonged to the apo homologous group. These results suggest that the wide low-recombinant genomic region surrounding the apo locus might be silenced in *Paspalum*. The possibility of an association between this silencing phenomenon and the triggering of apospory should be further examined in the future.

An analysis of synteny focused on the apo locus among different species of the *Paspalum* genus (Pupilli et al. 2004) revealed only partial conservation of synteny. Pupilli et al. (2004) reported that in *Paspalum simplex* and *Paspalum malacophyllum* only probes which originated from rice chromosome 12 were found to be linked to apospory, while in *Paspalum notatum* probes from rice chromosome 2 and 12 cosegregated with apospory. This finding led the authors to conclude that candidate genes for apomixis are most probably located onto rice chromosome 12, and not onto rice chromosome 2. It was proposed that a translocation could be the cause of the linkage of chr 2 probes in *Paspalum notatum* (Pupilli et al. 2004). A similar rearrangement appears to have affected the *Brachiaria brizantha* genome, since probes located in the same region of rice chr 2 had been reported to be linked to apospory in this species by Pessino et al. (1998). Evidence presented in this work does not constitute direct evidence that the trigger/s of apospory are located in regions syntenic to rice chromosome 2. However, they clearly show that a region syntenic to rice chromosome 2 is placed close to the apo locus (in agreement with results reported by Pupilli et al. 2004), and it is probably silenced. Currently we are making efforts to reveal polymorphisms in our mapping population that would allow mapping of differentially expressed clones associated with rice chromosome 12, in order to determine if they are also linked to the trait.

In a former work we reported the isolation of several candidate genes controlled by ploidy in immature inflorescences of sexual *P. notatum*. At least 4 of the genes controlled by a change of the ploidy level (in plants with the same reproductive mode: sexual) also isolated here were differentially expressed between sexual and aposporous plants (in plants with the same ploidy: tetraploid). This observation indicates that several genes involved in aposporous development are also (and independently) ploidy-regulated. Hence, these genes might constitute a possible molecular association between apomixis and polyploidy. Similarly, in *Eragrostis curvula* a large group of genes activated or repressed in diplosporous plants with respect to sexual counterparts (all plants presented the same ploidy) were found to be regulated during ploidy level changes (in plants presenting the same reproductive mode: sexual) (Cervigni et al. 2008).

In situ hybridization of two selected clones (N2 and N19) (that map in silico to rice genomic regions syntenic to the *P. notatum* apo locus) confirmed that both of them are expressed in reproductive tissues. Differential expression in the ovule nucellus and integuments hints at a possible role in megagametophyte development that should be confirmed in the future by performing functional experiments (i.e. transgenesis aimed at silencing or activation). Interestingly, N2 and N19 are also expressed differentially in the anther tapetum and the pollen mother cells, suggesting a possible disparity in the developmental pathways aimed at the generation of microspores between sexual and apomictic plants. In fact, the highest level of expression was exhibited in this particular tissue. However, further experiments would be necessary to address the question of whether differential expression involving anther tissues effectively implies a consequential biological significance.

Besides providing a list of candidate genes related to apospory, our results suggest that apomixis in *Paspalum* (1) involves the altered expression of a signal transduction cascade; (2) might be triggered by the silencing of the large genomic region including the apo locus and (3) involves the modified expression of genes controlled by ploidy. Future research pointing towards the functional characterization of the genes isolated in this survey will allow us to determine if they may represent a toolbox for the genetic manipulation of the trait.

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