Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Specific expression of apomixis-linked alleles revealed by comparative transcriptomic analysis of sexual and apomictic *Paspalum simplex* Morong flowers

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Abstract

Apomixis is defined as clonal reproduction by seed. A comparative transcriptomic analysis was undertaken between apomictic and sexual genotypes of *Paspalum simplex* Morong to identify apomixis-related polymorphisms at the level of mRNA. cDNA-AFLP (amplified fragment length polymorphism) profiling of apomictic and sexual flowers at several stages of development yielded 202 amplicons that showed several kinds of expression specificities. Among these, the large majority consisted of amplicons that were present only in specific stages of development of the apomictic flowers. Ten percent of polymorphic amplicons were present with almost identical intensity in all stages of the apomictic flowers and never in the sexual flowers. Reverse transcription-PCR (RT-PCR) and Southern analyses of these amplicons showed that they belong to constitutively expressed alleles that are specifically present on the apomixis-controlling locus of *P. simplex*. The most frequent biological functions inferred from the sequence homology of the apomixis-linked alleles were related to signal transduction and nucleic acid/protein-binding activities. Most of these apomixis-linked alleles showed nonsense and frameshift mutations, revealing their probable pseudogene nature. None of the amplicons that were present only in specific stages of development of the apomictic flowers co-segregated with apomixis, indicating they did not originate from additional apomictic alleles but more probably from differential regulation of the same allele in apomictic and sexual flowers. The molecular functions inferred from sequence analysis of these latter amplicons were related to seed storage protein and regulatory genes of various types. The results are discussed regarding the possible role in apomictic reproduction of the differentially expressed genes in relation to their specificity of expression and inferred molecular functions.

Key words: Apomixis, cDNA-AFLP, RNA profiling, gene silencing.

Introduction

Genetic recombination through meiosis coupled with egg cell fertilization is the most formidable system that life forms have adopted to enable new genetic assortments, allowing for the evolution and adaptation of species. Nevertheless, some plants have evolved alternative reproductive systems that circumvent sexual reproduction, allowing for clonal propagation of the intact mother plant's genotype; among these, gametophytic apomixis involves the formation of an unreduced embryo sac (skipping anaphase I or II of meiosis) and parthenogenetic development (avoiding fertilization) of the embryo from the unreduced

egg cell. For these reasons apomixis is defined as 'clonal reproduction by seed without contribution of the paternal gamete' (Nogler, 1984), because it allows the production of individuals that are genetically identical to the mother plant.

Harnessing apomixis in crop species could revolutionize agriculture and seed production practices because, among other reasons, it would allow each farmer to self-produce hybrid seed without performing hybridization between elite lines (Spillane *et al.*, 2004). Despite the wide distribution of apomixis in the plant kingdom, it is absent among crop

species with the exception of some fruit trees and a few forage species. Efforts aimed at transferring apomixis to crop species through hybridization with their apomictic wild relatives have resulted in limited success (Spillane *et al.*, 2004). Therefore, there is a need to understand the molecular bases of apomictic reproduction to enable the development of an apomixis system in crop species.

Paspalum simplex, the model system used in the present study, is an apomictic grass that is considered an important species in South America for domestication and plant improvement due to its forage potential (Urbani *et al.*, 2002). In this context the isolation of genes linked to apomictic reproduction in this species could serve to develop a molecular marker-based system for the assisted selection of apomixis in this biological system. *Paspalum simplex* has a number of characteristics that makes it amenable for apomixis gene cloning via a map-based strategy. Chief among them are its relatively small genome size and the existence in nature of sexual and apomictic cytotypes that are sexually compatible (Pupilli *et al.*, 2001). Nevertheless, as the apomixis-controlling locus (ACL) of *P. simplex* is characterized by a strong repression of recombination (Pupilli *et al.*, 2001; Labombarda *et al.*, 2002), its physical dissection followed by sequencing of large DNA fragments appeared to be the obligatory route to identifying candidate apomixis genes in this species. However, as recombination repression has probably caused an accumulation of migrating DNA, the rare genes present at the ACL are likely to be embedded in a large 'genomic sea' of repetitive and transposable elements and non-coding DNA.

Comparative mapping studies have revealed strong synteny of the ACL of *P. simplex* with the telomeric region of the long arm of chromosome 12 of *Oryza sativa* (Pupilli *et al.*, 2001, 2004). This fact led to apomixis gene isolation in *Paspalum* to be approached through comparative mapping with rice; however, as gene colinearity between the two species was frequently interrupted by rearrangements of various types, apomixis gene cloning via comparative mapping is not a suitable strategy in *P. simplex* (Calderini *et al.*, 2006).

These results prompted the combination of the structural information available with a transcriptional analysis of the ACL to enhance the probability of identifying the genetic determinants of apomixis in this species. Based on the assumption that the effects of the ACL-linked genes on the reproductive system are likely to be related to their transcription, the idea was to identify apomixis-related polymorphisms at the mRNA level by a comparative transcriptomic analysis with the aim of isolating apomixis-linked genes that are transcriptionally active. Transcriptional profiling of reproductive organs in apomictic and sexual relatives has been used since the latter half of the 1990s to detect differentially expressed genes. Since the pioneering work of Vielle Calzada *et al.* (1996) in *Pennisetum squamulatum*, other analogous studies have been reported in *Brachiaria* (Leblanc *et al.*, 1997; Rodriguez *et al.*, 2003), *Panicum* (Chen *et al.*, 1999; Yamada-Akiyama *et al.*, 2009), *Poa* (Albertini *et al.*, 2004), *Eragrostis*

(Cervigni *et al.*, 2008), *Paspalum* (Laspina *et al.*, 2008), and *Pennisetum ciliare* (Singh *et al.*, 2007), and among non-grass systems in *Hieracium* (Guerin *et al.*, 2000) and *Boechera* (Sharbel *et al.*, 2009). Major drawbacks frequently encountered while performing comparative transcriptional analyses of apomictic and sexual lines were: (i) the lack of genuine near isogenic lines; (ii) the lack of a microarray reference system that allows for high throughput analysis of gene expression; and (iii) the difficulty of functionally analysing the selected candidate genes because of the scarcity of transformation systems in wild apomictic species. In fact, although several candidate genes have been identified via transcriptional profiling and studied further (Albertini *et al.*, 2005; Chen *et al.*, 2005), to date there are no reports of genuine apomictic genes being isolated from natural apomictics and proven to be involved in apomictic processes by functional analysis. Furthermore, none of the differentially expressed genes identified so far in the above-mentioned studies co-segregated with apomictic reproduction, indicating that these genes probably act downstream of the apomixis-linked genes.

According to Carman's hypothesis (Carman, 1997), apomixis is the result of deregulation of the place and time of expression of sex-related genes as a consequence of heterochronic expression of these genes due to hybridization. This hypothesis has been confirmed by cytoembryological observations in *Tripsacum* (Grimanelli *et al.*, 2003) and, more recently, by comparative transcriptomic analysis in *Boechera* (Sharbel *et al.*, 2009). To identify these asynchronously regulated genes, it would be necessary to compare the transcriptional profiles of apomictic and sexual reproductive organs over several developmental stages encompassing the whole process of apomictic reproduction, starting from the formation of the apomeiotic initial to early stages of seed formation. In this way, a set of genes that show a strikingly deregulated pattern compared with their sexual counterparts, evaluated not only in a single stage as the presence or absence of bands but across the whole process of seed formation, are expected to be identified. To overcome the problem of genotype heterogeneity, mRNAs representative of each of the developmental stages considered were pooled from several genotypes belonging to the same segregating population. The cDNA-amplified fragment length polymorphism (cDNA-AFLP) technology (Bachem *et al.*, 1996) is expected to enhance the probability of detecting apomixis-specific alleles because it combines the site-specific cleavage of restriction enzymes together with the highly specific 3' matching of selective primers. The aim of the present work was to identify transcriptionally active apomixis-related alleles by comparing the cDNA-AFLP profiles of apomictic and sexual flowers of *P. simplex* at several stages of development. This technology allowed the identification of a set of genes showing stage- and/or phenotype-specific expression. Among these, a class of alleles that show a characteristic specificity of expression were mapped (for the first time in an apomixis system) as 100% linked to apomixis.

Materials and methods

Plant material and staging

Plants used for nucleic acid extractions belonged to a BC1 population segregating for apomixis described previously (Pupilli *et al.*, 2001). Plants were maintained in a growth chamber with constant temperature (28 °C day and 22 °C night), a 16 h photoperiod, and 60% humidity. Inflorescences of *P. simplex* are composed of several racemes that differ in their developmental stages according to their position. Each raceme is formed by several rows (2–4) of florets. Flower development varies according to the position of the florets in the inflorescence. Apical florets and racemes develop earlier compared with those located in basal positions. To standardize the developmental differences between reproductive phenotypes and among developmental stages, the florets on the side rows located in the distal quarter of the central racemes were collected from 9:00 h to 10:00 h. Staging was estimated by taking into account previous cytoembryological analyses (Caceres *et al.*, 1999), where A denotes apomictic and S sexual ovules: stage I, inflorescence completely enveloped in the flag leaf sheath/no differentiated meiotic cells (A and S); stage II, inflorescence emerged for one-third of its length/beginning of meiosis (A and S) and appearance of aposporic initials (A); stage III, 2–4 d before anthesis as predicted by anthesis initiation in apical racemes/degeneration of the functional megaspore (A), development of two- or four-nucleated megaspores (A and S); stage IV, 1–2 d before anthesis predicted as in stage III/development of eight-nucleated (S) and aposporic *Hieracium*-type (A) embryo sacs; stage V, anthesis/same as in stage IV; stage VI, 1–2 d after anthesis/early stages of embryo and endosperm development (A and S); and stage VII, 3–6 d after anthesis/early stages of seed formation (A and S). Florets used for RNA extraction were left to open pollinate in the growth chamber so that they shared a common pool of pollen. Florets were stored at –80 °C until used for RNA extraction.

cDNA-AFLP

Total RNA was extracted from ~35–50 mg of florets at the same developmental stage using the NucleoSpin® RNA kit (Macherey-Nagel) following the manufacturer's protocol. Equal amounts of RNA were pooled from florets at the same developmental stage belonging to four or five genotypes of the same reproductive phenotype. Pooled RNAs were purified of residual DNA by DNase treatment using the DNA-*free*™ kit (Ambion). The presence of residual DNA was checked by PCR using universal primers designed on the internal transcribed spacer (ITS) genomic sequence of the rDNA gene as described by White *et al.* (1990). mRNA was purified from total RNA with the GeneElute mRNA miniprep kit (Sigma-Aldrich) following the instruction manual. The first strand of cDNA was synthesized using the SuperScript III kit (Invitrogen). Newly formed first-strand cDNA served as the template for the second-strand synthesis reaction consisting of 20 μl of the first-strand mixture, 15 μl of 10× cDNA II buffer (Invitrogen), 3 μl of DNA polymerase I (10 U μl^{-1}, Invitrogen), 1.5 μl of RNase H (10 U μl^{-1}, Invitrogen), and 1 μl of dNTPs (25 mM each) in 150 μl total volume. The reaction was incubated at 16 °C for 2 h.

After purification with one phenol and one chroroform/isoamyl alcohol extraction, cDNA was precipitated with 0.6 vol. of isopropanol, incubated at –20 °C overnight, centrifuged, and resuspended in 35 μl of water after washing in 70% ethanol. cDNA was quantified by both spectrophotometry and PCR amplification in non-saturating conditions using primers (fw 5′-AGGCCGCC-CACGCATCTCTT-3′; rev 5′-ACAACAACTGCACCAAATGG-3′) designed from the open reading frame (ORF) of a gene coding for a cytidine deaminase (*PsCDA* GenBank Accession no. AM400871) that was isolated from developing flowers of *P. simplex* and proven to be constitutively expressed in various tissues (Calderini *et al.*, unpublished data) and therefore taken as an internal standard. The PCR conditions were: 94 °C 2 min; 28 cycles of 94 °C 20 s, 50 °C 30 s, 72 °C 1 min; and 72 °C for 7 min.

A 500 ng aliquot of cDNA was used to reveal AFLPs according to the classical protocol of Vos *et al.* (1995) as modified by Labombarda *et al.* (2002). After selective PCR amplification, the AFLP amplicons were separated on a 6% denaturing polyacrylamide gel and visualized in the Genomyx SC Scanner (Beckman). The picture was collected as a TIFF image and the grey levels were adjusted manually with the software Adobe Photoshop CS2 version 9.0 to enhance the sharpness of the image. The positions of the AFLP bands showing differences in intensity or presence/absence between the two reproductive phenotypes were recorded with the aid of the coordinate system of the Genomyx SC scanner. The gel sectors containing the selected bands were excised from the gel with a scalpel, soaked in 100 μl of distilled water, incubated at –80 °C for 30 min, then vigorously vortexed for 30 s, spun down, and stored overnight at +4 °C as described in Brugmans *et al.* (2003). A 5 μl aliquot of the supernatant was used as template for PCR amplification using the same primer combinations and thermocycling conditions as in the pre-selective amplification. After gel analysis to check the molecular weight, PCR products were purified according to the polyethylene glycol (PEG) precipitation protocol reported below.

A 15 μl aliquot of PEG solution (20% PEG, 2.5 M NaCl) was added to an equal volume of PCR product and incubated at 37 °C for 15 min. After centrifugation at maximum speed for 15 min, the supernatant was discarded and the pellet was rinsed twice with chilled 80% ethanol and centrifuged for 2 min. After discarding the supernatant, the pellet was dried in a SpeedVac for 3 min and resuspended in 8 μl of TE buffer. After gel quantification, 10–20 ng of purified fragments were sequenced bidirectionally using the same primer combinations as in pre-selective amplification with the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The reactions were run on an ABI Prism 3130 sequencing apparatus (Applied Biosystems).

Reverse northern, RT-PCR, and Southern analyses

A total of 78 differentially expressed transcript-derived fragments constituted by single sequences (ssDETDFs) were re-amplified from the excised bands using the appropriate selective primer pairs and related AFLP amplification conditions in 100 μl total volume. *PsCDA* was amplified from flower cDNA as described above and used as an internal control. A 5 μl aliquot of each amplification mixture was run on a 1% agarose gel to check the molecular weight and to normalize the amount of DNA visually. Depending on the amount of DNA, 10–20 μl were blotted in triplicate onto Amersham Hybond™-XL membranes using the Bio-Dot® SF (Bio-Ras) apparatus following the supplier's instruction. Equal amounts of mRNAs from the four stages before anthesis and the three stages after anthesis were bulked and used as template, together with the mRNA corresponding to the anthesis stage to synthesize three labelled probes for each phenotype. For each probe, 500 ng of mRNA was annealed for 10 min with 20 pmol of oligo(dT)$_{18}$ at 65 °C in a total volume of 15 μl. This mixture was diluted to a final volume of 50 μl containing 400 U of SuperScript III reverse transcriptase (Invitrogen), 1× reaction buffer (supplied), 5 mM dithiothreitol (DTT), 1 mM each of dATP, dGTP, and dTTP, 10 μM dCTP, and 50 μCi of [α-^{32}P]dCTP. The mixture was incubated for 1 h at 42 °C. The labelled probes were purified from unincorporated nucleotides by gel filtration chromatography on laboratory-built G-50 (Sephadex) gel columns. The volume of the eluate was adjusted to 300 μl; 75 μl of 1 N NaOH was added and the mixture was incubated at 42 °C for 30 min.

For neutralization, 75 μl of 1 N HCl together with an equal volume of 20× SSC was added to the mixtures and the cDNA probes were denatured for 10 min at 100 °C. Blots containing the amplicons were hybridized for 42 h at 65 °C in 10 ml of

hybridizing buffer consisting of 5× SSC, 5× Denhardt's solution, 0.5% SDS, 1 mg of denatured salmon sperm DNA, and 1 mg of denatured tRNA. Hybridized blots were washed as follows: I (2× SSC, 0.1% SDS), room temperature, two washes of 15 min each; II (1× SSC, 0.1% SDS), 65 °C, one wash, 15 min; and III (0.5× SSC, 0.1% SDS), 65 °C, one wash, 15 min. Blots were exposed to X-ray films for 1 week at –80 °C.

The developed films were scanned with an HP SCANJET G4050 (resolution=600 dpi) and the intensity of each dot in the TIFF images was converted into numerical values (integrated pixel density) with the aid of the software ImageJ (Rasband WS, ImageJ, US-NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/ 1997–2009) using the Dot-Blot Analyser option. The relative abundance of transcripts was expressed as arbitrary RNA units (Fig. 1) corresponding to the ratio of the integrated density of each dot divided by the same parameter corresponding to the house-keeping gene. Averages and SD were computed and converted into histograms with Microsoft Excel software.

Reverse transcription-PCR (RT-PCR) was performed in 20 μl total volume using 0.5 U of TaKaRa ex Taq and 5 μl of the pre-selective amplification mixtures (diluted 1:20) as the template. One of the primers was designed to anneal internally to the amplified sequence while the other was either the *Eco*RI- or *Mse*I-selective AFLP primer depending on the orientation of the internal primer. PCR conditions varied according to the T_m of the selected primers. Genomic DNA (5–7 μg) from the parental lines was digested overnight with 20 U of *Eco*RI, *Hin*dIII, *Bam*HI, *Eco*RV, and *Bgl*II (New England Biolabs) to detect informative polymorphisms and with the appropriate enzyme for linkage analysis in all the BC individuals. Electrophoresis and blotting procedures were as described by Pupilli *et al.* (2001). Restriction fragment length polymorphism (RFLP) probes were developed from PCR amplification of the isolated AFLP bands using internal primers designed for their sequences. Amplicons were checked for molecular weight on agarose gels, purified with the QIAquick PCR purification kit, and labelled with ^{32}P using the Ready-To-Go labelling kit (GE

Healthcare). Hybridization, washing, and exposure conditions were as described by Pupilli *et al.* (2001).

Bioinformatics

High quality, unique sequences were analysed for best homology to infer protein functions. Sequences were submitted to a non-redundant protein sequence database (NCBI) first using the BLASTX function and then BLASTN on the non-human, non-mouse expressed sequence tag (EST) database. The best EST hits were blasted on a non-redundant protein sequence database using BLASTX. To infer the most likely biological function, the sequences of the best matching protein found in all searches were blasted against the *Arabidopsis* informative resource (TAIR) protein database using BLASTP. Best-hit proteins were submitted to the GO annotation tool at TAIR to search for putative molecular functions and related biological processes. *In silico* mapping onto rice chromosomes was performed with the best hits on The Institute for Genomic Research (TIGR) database using the BLASTN option.

Results

cDNA-AFLP

On the basis of information gained by previous AFLP analyses carried out at the genomic level in *P. simplex* (Labombarda *et al.*, 2002; Calderini *et al.*, 2006), the primer combinations *Eco*+G/*Mse*+T and *Eco*+A/*Mse*+C were chosen for the pre-selective amplifications, whereas the selective amplifications were performed with *Eco*+3/*Mse*+2 or +3, where 2 and 3 are the number of selective bases used in each of the possible combinations. A total of 76 selective primer combinations, of which 30 were *Eco*+G/*Mse*+T and
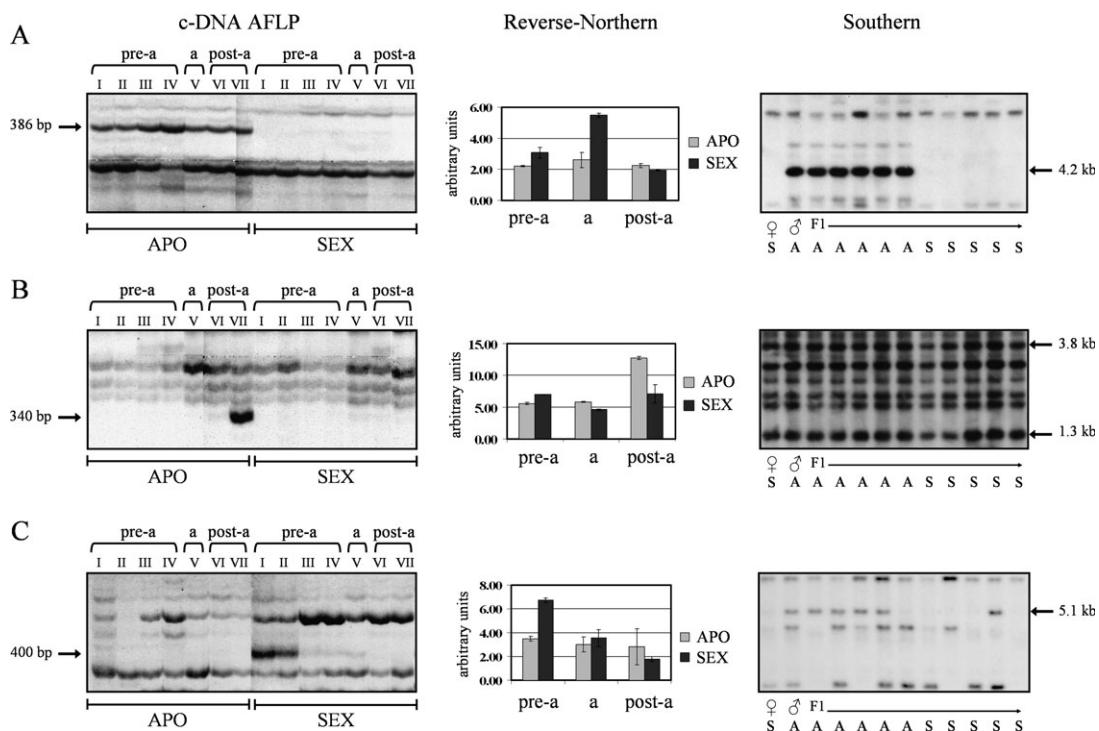

Fig. 1. Reverse northern and Southern blot characterization of ssDETDFs identified by cDNA-AFLP analysis. (A–C) Three representative examples of ssDETDFs belonging to classes A, B, and C, respectively (Table 1). Genomic DNA was digested with *Hin*dIII in A and B and with *Eco*RI in C.

46 were *Eco*+A/*Mse*+C, were used on the cDNA synthesized from mRNA extracted from flowers at seven stages of development in both apomictic and sexual phenotypes. A total of 4480 scorable transcript-derived fragments (TDFs) were detected whose molecular weights ranged between 100 bp and 700 bp. Of these, 202 differed in either presence/absence or intensity along the same stages of flower development in both phenotypes and therefore were considered to be differentially expressed (DETDFs). These DETDFs were classified into five classes according to their specificity of expression (Table 1). Classes B–E were divided into subclasses (1–4) according to the homogeneity of expression so that subclass 1 included stages I and II, subclass 2 included stages III and IV, subclass 3 stage V (anthesis) and subclass 4 included stages VI and VII. DETDFs that were present only in specific stages of apomictic flowers (class B, *n*=102) were the most represented while only 32 DETDFs were present in specific stages of the sexual flowers only. Thirty-four DETDFs were expressed more in the apomictic than in the corresponding sexual stages (class D) and 12 showed the opposite trend (class E). The most interesting class (A) included 22 DETDFs (~10% of the total detected DETDFs) that were constitutively expressed in apomictic flowers only. Among the stages considered, the last stages of the apomictic and the early stages of the sexual flowers showed the most abundant DETDFs. Among the 202 DETDFs, 74 showed a clear unique sequence, of which 11 were duplicates, 52 did not amplify successfully or show a clear sequence, and 76 showed a multiple sequence profile. To select the sequences showing specificity of expression in the cDNA-AFLP pattern among the multisequence amplicons, the eluted bands were re-amplified with the same *Eco*+3 primer in combination with each of four *Mse*I-selective primers each carrying an additional selective base at the 3' end after two or three degenerate (N) bases instead of the selective ones. The use of degenerate bases allowed the use of the same set of *Mse*I primers for all the primer combinations exploited. All the amplicons re-selected from multiple-sequence AFLPs were subjected to two levels of validation: (i) molecular weight comparison; and (ii) confirmation of expression specificity. Fifteen amplicons satisfied these conditions and were therefore selected for further analysis.

Reverse northern, RT-PCR, and Southern analyses

To investigate whether the specificity of expression detected at the level of cDNA-AFLP depended on the existence of apomixis-specific alleles or on the alternative expression of the same gene in the two reproductive phenotypes, reverse northern and Southern analyses were carried out. The templates for the reverse northern were the 78 (63+15) ssDETDFs and the probes were synthesized by reverse transcription of mRNAs extracted from the flowers at all developmental stages considered and pooled into three bulk lots including all the pre-anthesis (probe 1), anthesis (probe 2), and post-anthesis (probe 3) stages for both reproductive phenotypes. The ssDETDFs that were constitutively present

only in all stages of apomictic flower development (class A) did not show specificity of expression when subjected to reverse northern analysis. As an example, the class A DETDF reported in Fig. 1 showed a comparable level of hybridization signal for all three RNAs in apomictic flowers (pre-anthesis, anthesis, and post-anthesis), while a significant increase of expression was detected in pre-anthesis and anthesis RNAs in the sexual compared with the apomictic flowers (Fig. 1A, reverse northern). This suggested that: (i) the specificity of expression detected at the level of cDNA-AFLP was likely to be due to allelism rather than to differential gene expression; and (ii) although the expression of sex-related alleles was time regulated that of the apomixis-linked alleles appeared to be stage independent.

If apomixis-specific alleles do exist at the mRNA level, then a corresponding allele should exist at the genomic level. To verify this hypothesis, all the ssDETDFs (Table 1) belonging to class A and 10 each of classes B and C were subjected to Southern analysis. All the ssDETDFs of class A except one (for which a smearing-type unreadable hybridization pattern was obtained) evidenced at least one restriction fragment linked 100% *in cis* to apomixis that should belong to the apomixis-linked allele of the corresponding gene. Most of the class A ssDETDFs (11 of 12) showed a single- or low-copy hybridization pattern and a low level of overall expression.

To verify whether the homogeneity of expression of apomixis-linked alleles was specific to flower tissues, an RT-PCR analysis of all ssDETDFs of class A was carried out on RNA extracted from flowers, leaves, and roots of both apomictic and sexual genotypes using pre-selective amplifications as the template. All the class A ssDETDFs were present in all the organs analysed of the apomictic plants, indicating that apomixis-linked alleles were expressed constitutively (Fig. 2 displays a representative example). Conversely, when ssDETDFs of class B were analysed by reverse northern, their specificity of expression was confirmed in almost 100% of the cases. Figure 1B shows a representative example of these ssDETDFs, in which a significant increase of expression was detected in the last stages of apomictic flower development consistent with the cDNA-AFLP profile (Fig. 1B, reverse northern). When the corresponding amplicon was used as an RFLP probe, no apomixis-linked fragments were detected, as documented by Fig. 1B (Southern). This confirmed that the significant differences in the relative abundance of transcripts detected between developmental stages and between phenotypes were in these cases probably due to differential gene expression rather than to the existence and expression of apomixis-linked alleles.

When the sex-specific cDNA-AFLP (class C) results were considered, no constitutively expressed amplicons were detected. Sex- and stage-specific ssDETDFs appeared mainly in the early stages of flower development. Reverse northern analysis confirmed in most cases the stage specificity of expression in sexual flowers (Fig. 2C, reverse Northern), whereas a rather constant expression of the same ssDETDFs was detected in apomictic flowers. This suggested that either

Table 1. Classes of differentially expressed fragments detected and corresponding AFLP patterns

Expression pattern	class	N[b]	ssDETDFs	APO I[a] (1[c])	APO II (1)	APO III (2)	APO IV (2)	APO V (3)	APO VI (4)	APO VII (4)	SEX I (1)	SEX II (1)	SEX III (2)	SEX IV (2)	SEX V (3)	SEX VI (4)	SEX VII (4)
Constitutively expressed in apomicts only	A	22	12	■	■	■	■	■	■	■							
	tot	22	12														
Apomixis stage-specific	B1	10	5	■	■												
	B1-2	2	1		■	■	■										
	B2	5	2			■	■										
	B2-3	3	1			■	■	■									
	B3	12	4					■									
	B3-4	3	2					■	■	■							
	B4	67	22						■	■							
	tot	102	37														
Sex stage-specific	C1	23	8								■	■					
	C2	1	1										■	■			
	C3	4	1												■		
	C3-4	3	1												■	■	■
	C4	1	-													■	■
	tot	32	11														
Stronger expression in apomicts	D	1	1	■	■	■	■	■	■	■	□	□	□	□	□	□	□
	D1	5	2	■	■						□	□					
	D1-2	2	1		■	■	■					□	□	□			
	D2	2	1			■	■						□	□			
	D2-3	2	2			■	■	■					□	□	□		
	D3	8	3					■							□		
	D4	14	3						■	■							□	□
	tot	34	13														
Stronger expression in sexuals	E	2	1	□	□	□	□	□	□	□	■	■	■	■	■	■	■
	E1	6	4	□	□						■	■					
	E2	1	-			□	□						■	■			
	E3-4	1	-					□	□	□					■	■	■
	E4	2	-						□	□						■	■
	tot	12	5														
	TOT	202	78														

[a] Stage I, inflorescence completely enveloped in the flag leaf sheath/no differentiated meiotic cells (A and S); stage II, inflorescence emerged for one-third of its length/start of meiosis (A and S) and appearance of aposporic initials (A); stage III, 2–4 d before anthesis as estimated by anthesis initiation in apical racemes/degeneration of the functional megaspore (A), development of two- or four-nucleated megaspores (A and S); stage IV, 1–2 d before anthesis, estimated as in stage III/development of eight-nucleated embryo sac (S) and *Hieracium*-type embryo sac (A); stage V, anthesis/same as in stage IV; stage VI, 1–2 d after anthesis/early stages of embryo and endosperm development (A and S); stage VII, 3–6 d after anthesis/early stages of seed formation (A and S).
[b] Number of bands corresponding to each class.
[c] The numbers 1–4 represent the subclass of DETDFs expressed in specific stages (subclass 1 corresponds to stages I and/or II, subclass 2 to stages III and IV, subclass 3 to stage V, and subclass 4 to stages VI and/or VII).
■ indicates medium-high intensity of the AFLP fragment on the acrylamide gel.
□ indicates low intensity of the AFLP fragment on the acrylamide gel.

an apomixis-specific allele might be present and thus not revealed at the level of cDNA-AFLP or the stage-specific expression might be related to differential gene expression as for class B amplicons. In no cases were apomixis-linked alleles detected by Southern analysis, with the exception reported in Fig. 1C, where a 5.1 kb restriction fragment appeared to segregate non-independently from apomixis. When this probe was hybridized against the digested DNA of a larger population (*n*=42), non-independent segregation (χ^2=13.83, significant at P ≤0.05) and a map distance of

0.1 cM (Liu, 1998) were recorded for this fragment and apomixis. This indicates that, at least in this case, an apomixis-linked allele is present that can affect the expression of the sexual TDF in apomictic flowers.

Sequence analysis

Out of the 78 non-redundant ssDETDFs analysed, 25 did not show any significant hits (defined as an e-value <10^{-2}) in any of the searched databases and another three showed highly
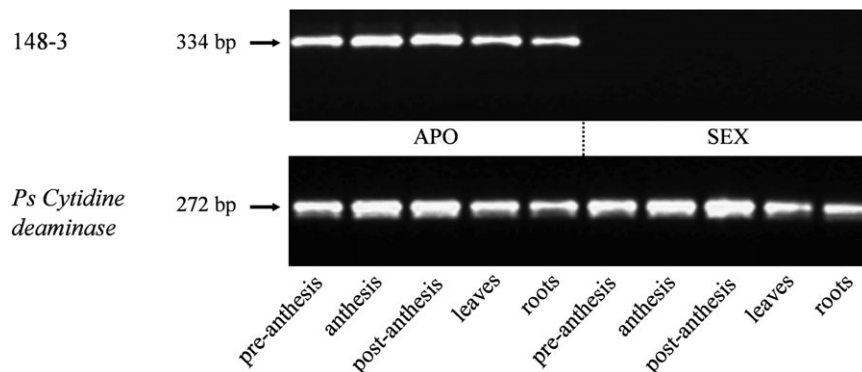
Fig. 2. RT-PCR of an ssDETDF belonging to class A. *PsCDA* was used as the internal housekeeping gene and pre-selective amplification was used as the template.

significant matches with the EST database only. Out of these 25 sequences, four belonged to class A, 17 to class B, none to class C, three to class D, and one to class E. In total, out of 25 non-matching sequences, 21 were specifically expressed only in apomictic flowers together with an additional three sequences that were quantitatively expressed more in apomictic flowers compared with their sexual counterparts. Almost all the sex-specific ssDETDFs showed high levels of similarity with known genes, indicating that 'sexual' genes showed a higher tendency to conserve homology and probably function compared with the 'apomictic' genes. Table 2 lists all the ssDETDFs that showed significant homology with at least one of the queried databases.

Among the apomixis-specific ssDETDFs (classes A+B), the most frequent putative function detected was represented by nucleic acid and protein binding, followed by kinase activities. Of the latter, two were constitutively expressed in apomictic flowers only (class A), two were stage specific (class B), and two were expressed more in some stages of apomictic flowers compared with their sexual counterparts (class D). This may suggest that genes related to signal transduction play a role in apomictic reproduction. Only one apomixis-linked ssDETDF was related to a transposable element (TE). Most of the apomixis- and stage-specific ssDETDFs (22 of 37) appeared in post-anthesis stages, four were expressed in anthesis, and eight in pre-anthesis stages (see Table 1). Out of the class B ssDETDFs that were expressed in post-anthesis phases, three are related to genes involved in endosperm-specific biological functions. Of these, two are homologous to the maize 19 kDa α-zein gene family that codes for the most abundant class of seed storage proteins in this species (Sabelli and Larkins, 2009), and the other is homologous to a gene coding for a glucose-6-phosphate translocator involved in starch accumulation in *Vicia* (Rolletschek *et al.*, 2007). When blots containing genomic DNA of apomictic and sexual plants were probed with one of the α-zein-related amplicons, the resulting hybridization pattern was highly conserved between apomictic and sexual plants, typical of multigene families but without any apomixis-linked fragments (Fig. 1B, Southern). Other ssDETDFs appearing in

the later developmental stages of the apomictic flowers showed homology only with genes related to putative hydrolase (two) and transcription regulation (one) functions. Of the six amplicons that were specifically expressed in the early stages of the apomictic flowers, two showed homology to nucleic acid-binding genes belonging to the class of auxin response factors (ARFs). Among those ssDETDFs that showed a more intense signal in the apomictic than in the sexual flowers, the most abundant functions detected were again those related to kinases (two of 10) and transport-associated proteins (two of 10), followed by nucleic acid-binding proteins (one) and hydrolases (one). In summary, the vast majority of the ssDETDFs that were specifically present in apomictic flowers belong to regulatory genes related to processes involved in cell differentiation and development. Curiously, despite the high density of TEs detected at the ACL (Calderini *et al.*, 2006), only one amplicon related to TEs was found within class A, suggesting that many of the apomixis-linked TEs are transcriptionally silent.

Out of the 11 ssDETDFs detected in only specific stages of sexual flower development, eight were present in the first two stages. Among these, three were homologous to nucleic acid/protein-binding genes, one to a hydrolase, three to various enzyme activities, and one to a gene coding for an unknown protein. The ssDETDFs specific to the later stages of sexual flower development are related to transport (anthesis and latest post-anthesis) and to a GTP-binding protein (anthesis). Once again, most of the differentially expressed amplicons were related to regulatory genes. However, striking differences were noticed between sexual and apomictic modes of reproduction for the interstage distribution of DETDFs (class B versus class C; Table 1); class B ssDETDFs (apomixis specific) were more abundant in the latest stages of flower development, whereas those of class C (sex specific) were more abundant in the earliest stages. Rice homologues of *Paspalum* ssDETDFs found in the TIGR database did not show any biased distribution toward chromosome 12, for which clear synteny with the ACL of *P. simplex* was previously reported (Pupilli *et al.*, 2001; Calderini *et al.*, 2006).

Table 2. Sequence search results of clones with significant homologies

Class	DETDF	Direct protein by BLASTX	TIGR CDS with BLASTN	EST with BLASTN	Protein by BLASTX of the EST best match	TAIR BLASTP[a]	Processes
		ID; name (e-value)	ID; name (e-value)	ID; name (e-value)	ID; name (e-value)	ID (GO function)	
A	121-2	AAM51833; unknown protein [*Oryza sativa*] ($7e^{-29}$)	LOC_Os05g11820; retrotransposon protein ($1.2e^{-28}$)	FL938061; *Panicum virgatum* root ($3e^{-58}$)	EEE65370; hypothetical protein [*Oryza sativa*] ($3e^{-44}$)	n	Unknown
A	121-12	n	n	FL440822 ; *Zea mays* mixed male/female infl., root ($7e^{-06}$)	NP_001105460; gamma-tubulin [*Zea mays*] ($1e^{-64}$)	AT5G05620 (nucleotide binding)	Developmental processes
A	123-11	n	n	AW000408; *Zea mays* root 3–4 days old ($4e^{-12}$)	LOC100193645; hypothetical protein [*Zea mays*] ($8e^{-06}$)	AT3G08720 (kinase activity)	Response to abiotic or biotic stimulus
A	124-3	AAL07816; NBS-LRR-like protein [*Hordeum vulgare*] ($5e^{-07}$)	n	n	–	AT3G14460 (nucleotide binding)	Response to stress
A	128-12	n	n	FL996398; *Panicum virgatum* callus ($1e^{-28}$)	n	n	Unknown
A	148-3	EEE57187; hypothetical protein [*Oryza sativa*] ($5e^{-20}$)	LOC_Os04g35700; mitogen-activated kinase kinase kinase ($2.6e^{-23}$)	DV478092; *Brachypodium distachyon* stem plus sheath ($1e^{-46}$)	BAD15457; MEK kinase [*Oryza sativa*] ($9e^{-78}$)	AT1G53570 (kinase activity)	Protein metabolism
A	148-4	EAZ18827; hypothetical protein [*Oryza sativa*] ($4e^{-08}$)	LOC_Os01g27740; dnaJ domain containing protein ($2.9e^{-10}$)	CF572339; *Saccharum officinarum* stem ($4e^{-35}$)	EAY81410; hypothetical protein [*Oryza sativa*] ($5e^{-42}$)	AT5G18750 (protein binding)	Protein metabolism
A	150-5	n	n	FL804898; *Panicum virgatum* late flowering buds+developing seeds ($5e^{-08}$)	LOC100281492; tubby-like protein [*Zea mays*] ($1e^{-30}$)	AT1G76900 (transcription factor)	Transcription
B1	124-1	EEC77345; hypothetical protein [*Oryza sativa*] ($2e^{-28}$)	LOC_Os04g36054; auxin response factor 1 ($1.0e^{-39}$)	CA091552; *Saccharum officinarum* apex ($2e^{-132}$)	Q0JCZ4; auxin response factor 9 [*Oryza sativa*] ($1e^{-65}$)	AT1G59750 (DNA or RNA binding)	Transcription
B1	127-1	EAZ10449; hypothetical protein [*Oryza sativa*] ($2e^{-08}$)	LOC_Os01g04409; OsWAK receptor-like cytoplasmic kinase ($1.7e^{-16}$)	FL982291; *Panicum virgatum* callus ($3e^{-48}$)	NP_001148074; OsWAK receptor-like cytoplasmic kinase [*Zea mays*] ($4e^{-90}$)	AT1G25390 (kinase activity)	Protein metabolism
B1	153-1	Q6YVY0; auxin response factor 7 [*Oryza sativa*] ($3e^{-48}$)	LOC_Os02g35140; auxin response factor 1 ($1.7e^{-64}$)	CA074938; *Saccharum officinarum* apical meristem ($2e^{-166}$)	LOC100285977; auxin response factor 1 [*Zea mays*] ($1e^{-89}$)	AT1G59750 (DNA or RNA binding)	Transcription
B1	153-4	NP_00104826; conserved hypothetical protein [*Oryza sativa*] ($2e^{-08}$)	LOC_Os02g53210; expressed protein ($4.2e^{-15}$)	CA191538; *Saccharum officinarum* root tips ($1e^{-41}$)	EEC74085; hypothetical protein [*Oryza sativa*] ($2e^{-62}$)	AT2G32970 (unknown molecular functions)	Unknown
B1–2	150-8	LOC100285062; 6-phosphogluconolactonase [*Zea mays*] ($8e^{-46}$)	LOC_Os07g41280; 6-phosphogluconolactonase ($8.2e^{-47}$)	CA202197; *Saccharum officinarum* developing inflorescence ($3e^{-143}$)	LOC100285062; 6-phosphogluconolactonase [*Zea mays*] ($1e^{-68}$)	AT1G13700 (hydrolase activity)	Other metabolic processes

Table 2. *Continued*

Class	DETDF	Direct protein by BLASTX	TIGR CDS with BLASTN	EST with BLASTN	Protein by BLASTX of the EST best match	TAIR BLASTP[a]	Processes
		ID; name (e-value)	ID; name (e-value)	ID; name (e-value)	ID; name (e-value)	ID (GO function)	
B2	144-6	ACG36854; ocs element-b inding factor 1 [*Zea mays*] (3e^{-13})	LOC_Os12g37410; ocs element-binding factor 1 (6.7e^{-10})	CA254822; *Saccharum officinarum* developed inflorescence and rachis (2e^{-45})	542394 obf; octopine synthase-binding factor1 [*Zea mays*] (2e^{-25})	AT3G62420 (transcription factor activity)	Transcription
B2–3	144-7	BAD16525; putative CRK1 protein [*Oryza sativa*] (3e^{-32})	LOC_Os02g35300; transposon protein, putative, unclassified (8.4e^{-28})	FL812314; *Panicum virgatum* late flowering buds+seed development (1e^{-95})	dbjlBAD16525; CRK1 protein [*Oryza sativa*] (1e^{-51})	AT1G09600 (kinase activity)	Protein metabolism
B3	145-14	n	n	FL458747; *Zea mays* mixed male/ female infl., root (4e^{-09})	n	n	Unknown
B3–4	122-2	NP_001148885; epoxide hydrolase 2 [*Zea mays*] (4e^{-14})	LOC_Os01g15130; epoxide hydrolase 2 (1.9e^{-29})	BG466125; *Sorghum propinquum* rhizomes (2e^{-84})	NP_001148885; epoxide hydrolase 2 [*Zea mays*] (2e^{-82})	AT4G02340 (hydrolase activity)	Unknown
B3–14	144-5	n	n	CA166109; *Saccharum officinarum* shoot–root transition zone (2e^{-13})	n	n	Unknown
B4	120-3	n	n	MZCCL10144C05.g; *Zea mays* endosperm 10–25 days after pollination (1e^{-08})	LOC100281048; glucose-6-phosphate/phosphate translocator 2 [*Zea mays*] (3e^{-25})	AT1G61800 (seed storage activity)	Endosperm development
B4	122-1	EAY93154; hypothetical protein [*Oryza sativa*] (2e^{-23})	LOC_Os10g25210; F-box protein interaction domain-containing protein (8.7e^{-09})	ES304806; *Cynodon dactylon* normalized cDNA library (0.002)	EAY93154; hypothetical protein [*Oryza sativa*] (3e^{-23})	AT3G57580 (unknown functions)	Unknown
B4	126-3	XP_661903; hypothetical protein [*Aspergillus nidulans*] (1e^{-37})	n	FD739333; *Picea sitchensis* tissues attached by *Pissodes strobi* (3e^{-67})	XP_001934443; hypothetical protein [*Pyrenophora tritici-repentis*] (8e^{-60})	n	Unknown
B4	128-8	n	n	FE669305; *Cicer arietinum* leaf 10 days old (6e^{-14})	dbjlBAB33421; putative senescence-associated protein [*Pisum sativum*] (3e^{-48})	n	Unknown
B4	137-2	ACP43629; chitinase [*Musa* AB Group] (4e^{-18})	LOC_Os06g51060; basic endochitinase 1 precursor (6.3e^{-20})	CI103398; *Oryza sativa* panicles 1 day after flowering (3e^{-42})	CAA39535; chitinase [*Oryza sativa*] (4e^{-60})	AT3G12500 (hydrolase activity)	Signal transduction
B4	148-5	CAA47640; 19 kDa zein [*Zea mays*] (2e^{-27})	n	CO461411; *Zea mays* endosperm multiple stages (6e^{-27})	AAL16985; 19kD alpha zein [*Zea mays*] (1e^{-58})	n (seed storage activity[b])	Endosperm development
B4	150-2	EEE51510; hypothetical protein [*Oryza sativa*] (2e^{-24})	LOC_Os11g01872; regulatory associated protein of mTOR (1.3 e^{-22})	DN150261; *Panicum virgatum* callus (2e^{-57})	AAR82959; transducin/WD-40 repeat protein [*Oryza sativa*] (6e^{-47})	AT3G08850 (protein binding)	Developmental processes
B4	154-3	EEC79532; hypothetical protein [*Oryza sativa*] (2e^{-26})	LOC_Os05g44080; expressed protein (5.1e^{-15})	EC902099; *Zea mays* multiple stressed tissues (1e^{-79})	XP_002467871; hypothetical protein SORBIDRAF [*Sorghum bicolor*] (7e^{-56})	AT1G77220 (unknown functions)	Unknown

Table 2. *Continued*

Class	DETDF	Direct protein by BLASTX	TIGR CDS with BLASTN	EST with BLASTN	Protein by BLASTX of the EST best match	TAIR BLASTP[a]	Processes
		ID; name (e-value)	ID; name (e-value)	ID; name (e-value)	ID; name (e-value)	ID (GO function)	
B4	156-2	n	LOC_Os05g34600; NAC transcription factor ($6.1e^{-07}$)	n	–	AT2G02450 (transcription activity)	Developmental processes
B4	15-5	n	LOC_Os11g33000; prolamin (0.0073)	CD433479; *Zea mays* endosperm ($5e^{-15}$)	100037759 azs4-18; zein-alpha precursor [*Zea mays*] ($5e^{-76}$)	n (seed storage activity[b])	Endosperm development
C1	151-1	NP_001140844; hypothetical protein [*Zea mays*] ($2e^{-57}$)	LOC_Os03g46410; transmembrane protein 56, ($1.9e^{-58}$)	EC899012; *Zea mays* multiple stressed tissues ($1e^{-155}$)	LOC100283418; transmembrane protein 56 [*Zea mays*] ($1e^{-117}$)	AT1G31300 (unknown functions)	Unknown
C1	121-7	ACN29144; unknown [*Zea mays*] ($2e^{-10}$)	LOC_Os01g26000; acyltransferase/catalytic ($3.9e^{-10}$)	FL816570; *Panicum virgatum* late flowering buds+seed development ($2e^{-28}$)	ACN29144; unknown protein [*Zea mays*] ($1e^{-125}$)	AT1G54570 (transferase activity)	Other biological processes
C1	126-1	CAD54633; NADP-dependent malate dehydrogenase [*Paspalum paniculatum*] ($4e^{-46}$)	LOC_Os08g44810; malate dehydrogenase 1, chloroplast precursor ($2.8e^{-45}$)	GD048623; *Panicum virgatum* abiotic and biotic stress treatments ($7e^{-114}$)	NP_001105420; malate dehydrogenase6 [*Zea mays*] ($2e^{-110}$)	AT5G58330 (other enzyme activity)	Other cellular/metabolic processes
C1	128-1	NP_001055069; [*Oryza sativa*] ($2e^{-38}$)	LOC_Os05g19150; epoxide hydrolase 2 ($2.0e^{-32}$)	CK213145; *Triticum aestivum* multiple stressed tissues ($3e^{-67}$)	NP_001055069; hypothetical protein [*Oryza sativa*] ($3e^{-90}$)	AT4G02340 (hydrolase activity)	Other cellular processes
C1	131-9	EEE59807; hypothetical protein [*Oryza sativa*] ($9e^{-08}$)	LOC_Os03g50510; threonine dehydratase biosynthetic ($2.4e^{-10}$)	CA262523; *Saccharum officinarum* lateral buds ($4e^{-54}$)	ABF98530; threonine dehydratase biosynthetic [*Oryza sativa*] ($3e^{-29}$)	AT3G10050 (other enzyme activity)	Other cellular/metabolic processes
C1	135-1	n	LOC_Os03g52594; CCR4–NOT transcription complex subunit 2 ($1.1e^{-07}$)	CI114344; *Oryza sativa* panicles 2 weeks after flowering ($3e^{-10}$)	ABF98743; NOT2/NOT3/NOT5 family protein, [*Oryza sativa*] ($2e^{-62}$)	AT5G59710 (protein binding)	DNA or RNA metabolism
C1	150-7	EEE54332; hypothetical protein [*Oryza sativa*] ($7e^{-67}$)	LOC_Os01g17170; magnesium-protoporphyrin IX monomethyl ester cyclase ($9.4e^{-68}$)	FE649788; *Panicum virgatum* 4 day old seedlings ($2e^{-171}$)	BAA87823; putative ZIP [*Oryza sativa*] ($2e^{-140}$)	AT3G56940 (DNA or RNA binding)	Other cellular/metabolic processes
C1	151-3	EEC76327; hypothetical protein [*Oryza sativa*] ($1e^{-04}$)	LOC_Os03g58160; heat shock factor protein HSF30 ($2.3e^{-06}$)	FL757933; *Panicum virgatum* early floral buds+reproductive tissue ($6e^{-20}$)	LOC100281501; heat shock factor protein HSF30 [*Zea mays*] ($1e^{-64}$)	AT3G22830 (transcription factor activity)	Transcription
C2	128-4	AAA97556; urf209; orf25 [*Sorghum bicolor*] ($2e^{-29}$)	LOC_Os12g14930; mitochondrial 22 kDa protein ($4.9e^{-37}$)	GO852730; *Festuca pratensis* inflorescence with fungal stroma ($9e^{-94}$)	AAA97556; urf209; orf25 mitochondrion [*Sorghum bicolor*] ($1e^{-63}$)	ATMG00640 (transporter activity)	Electron transport
C3	135-7	n	LOC_Os03g50520; dynamin-related protein 1C ($2.4e^{-08}$)	CA252112; *Saccharum officinarum* developing inflorescence ($1e^{-22}$)	LOC100280709; dynamin-related protein 1C [*Zea mays*] ($6e^{-146}$)	AT1G14830 (GTP binding)	Cell organization/biogenesis
C3–4	151-8	NP_001148930; potassium transporter 17 [*Zea mays*] ($2e^{-06}$)	LOC_Os08g39950; potassium transporter 17 ($6.5e^{-15}$)	FL154498; *Zea mays* mixed male/female infl., root ($1e^{-28}$)	LOC100282550; potassium transporter 17 [*Zea mays*] ($1e^{-56}$)	AT4G13420 (transport activity)	Transport

Table 2. *Continued*

Class	DETDF	Direct protein by BLASTX ID; name (e-value)	TIGR CDS with BLASTN ID; name (e-value)	EST with BLASTN ID; name (e-value)	Protein by BLASTX of the EST best match ID; name (e-value)	TAIR BLASTP[a] ID (GO function)	Processes
D	144–8	EEE68177; hypothetical protein OsJ_26310 [*Oryza sativa Japonica Group*] (1e^{-43})	LOC_Os08g09200; aconitate hydratase (1.1e^{-53})	CA175217; *Saccharum officinarum* apical stalk internodes (1e^{-141})	BAD05751; aconitate hydratase [*Oryza sativa*] (1e^{-91})	AT2G05710 (nucleotide binding)	Response to stress
D1	124-8	NP_001149246; acid phosphatase [*Zea mays*] (3e^{-48})	LOC_Os03g60370; acid phosphatase (1.1e^{-50})	CA264201; *Saccharum officinarum* lateral buds (3e^{-150})	LOC100282868; acid phosphatase [*Zea mays*] (2e^{-71})	AT1G09870 (hydrolase activity)	Unknown
D1	142-9	002439970; hypothetical protein SORBIDRAFT [*Sorghum bicolor*] (7e^{-11})	LOC_Os05g40270 expressed protein (3.1e^{-11})	CA202537; *Saccharum officinarum* developing inflorescence (3e^{-36})	XP_002439970; hypothetical protein SORBIDRAFT [*Sorghum bicolor*] (5e^{-90})	AT1G06840 (protein tyrosine kinase activity)	Signal transduction
D2	137-3	NP_001141937; hypothetical protein [*Zea mays*] (5e^{-39})	LOC_Os02g24430; phosphatidylinositol transporter (7.0e^{-48})	FL767660; *Panicum virgatum* early floral buds (3e^{-112})	BAD16989; SEC14 cytosolic factor [*Oryza sativa*] (9e^{-91})	AT2G21540 (transporter activity)	Developmental processes
D2–3	131-11	n	n	FL823945; *Panicum virgatum* late flowering buds (3e^{-24})	NP_001042704; PI3K-like domain [*Oryza sativa*] (2e^{-19})	AT2G40850 (kinase activity)	Unknown
D2–3	137-7	NP_001143127; hypothetical protein [*Zea mays*] (4e^{-15})	LOC_Os06g44810; expressed protein (5.5e^{-10})	CO530544; *Zea mays* multiple tissues (2e^{-50})	LOC100275605; hypothetical protein [*Zea mays*] (2e^{-132})	n	Unknown
D3	142-16	n		GD034445; *Panicum virgatum* abiotic and biotic stress treatments (4e^{-10})	LOC100282521; NFU3 [*Zea mays*] (1e^{-27})	AT5G49940 (structural molecule activity)	Cell organization/ biogenesis
D4	142-7	MGG_14430; hypothetical protein [*Magnaporthe grisea* 70-15] (7e^{-46})	LOC_Os04g04060; interferon-induced GTP-binding protein (7.1e^{-06})	CF883326; *T. reesei* mycelial culture (2e^{-96})	FG07453; hypothetical protein [*Gibberella zeae*] (5e^{-98})	AT1G60500 (GTPase activity)	Unknown
D4	153-8	NP_001054191; [*Oryza sativa*] ubiquitin-conjugating enzyme (6e^{-33})	LOC_Os02g16040; ubiquitin-conjugating enzyme (1.1e^{-32})	FL979051; *Panicum virgatum* callus (2e^{-97})	BAB89355; ubiquitin-conjugating enzyme [*Oryza sativa*] (7e^{-66})	AT4G27960 (ubiquitin-protein ligase activity)	Protein metabolism
D4	146-2	XP_710281; hypothetical protein [*Candida albicans*] (2e^{-06})	n	DR179219; *Pinus taeda* roots minus micronutrients (5e^{-53})	XP_001230225; hypothetical protein [*Chaetomium globosum*] (2e^{-26})	n	Unknown
E	120-1	NP_001043162; hypothetical protein [*Oryza sativa*] (4e^{-27})	LOC_Os01g49300; retrotransposon protein (2.1e^{-28})	n	–	AT4G23160 (kinase activity)	Protein metabolism
E1	124-6	NP_001142144; hypothetical protein [*Zea mays*] (5e^{-32})	LOC_Os10g33970; double-stranded RNA-binding motif family protein (4.1e^{-47})	DN560822; *Zea mays* developing embryo (7e^{-133})	AAK21352; putative extensin [*Oryza sativa*] (4e^{-84})	AT2G28380 (double-stranded RNA binding)	Unknown biological processes
E1	126-6	n	n	CA256898; *Saccharum officinarum* (3e^{-43})	ACG26907; tubulin alpha-3 chain [*Zea mays*] (4e^{-57})	AT4G14960 (protein binding)	Cell organization/ biogenesis
E1	153-9	NP_001149132; GMP synthase [*Zea mays*] (3e^{-41})	LOC_Os08g38170; methyladenine glycosylase (1.4e^{-45})	FL797578; *Panicum virgatum* late flowering buds (2e^{-134})	LOC100282754; GMP synthase [*Zea mays*] (3e^{-48})	AT1G13635 (methyladenine glycosylase I activity)	DNA repair

[a] BLASTP on TAIR database of the best hit.
[b] As inferred by the best hit of non-TAIR databases.
n, no significant hit found.

Discussion

Transcriptomic analysis of the developing flowers of apomictic and sexual genotypes of *P. simplex* allowed identification of three main categories of DETDFs that differed in their specificity of expression (A, B, and C, see Table 1). Reverse northern analysis confirmed the specificity of expression detected by cDNA-AFLPs for most of the ssDETDFs belonging to classes B and C, to lesser extents for classes D and E, but never for those that were specifically amplified from cDNA from all stages of apomictic flowers (class A). In the latter case, three main features were noticed: (i) the overall expression level of the corresponding genes was in general low in both apomictic and sexual flowers; (ii) the expression of these genes was almost always lower in apomictic flowers compared with the corresponding developmental stages of sexual flowers; and (iii) these differences were more marked in those stages for which the expression level in the sexual flowers was higher. The latter finding could be explained assuming the existence of a silencing mechanism of the 'sexual' allele operated by the silencer 'apomictic' allele acting in a dosage-dependent manner, that is when the abundance of the sexual mRNA target is sufficiently high to trigger the silencing machinery, as has previously been observed in transgenic systems (Schubert *et al.*, 2004; Bleys *et al.*, 2006) and in the endogenous silencing mechanism of the chalcone synthase gene family mediated by small interfering RNA (siRNA) in soybean (Tuteja *et al.*, 2004, 2009). Southern analysis demonstrated the presence of a putatively silenced 'sexual' allele for all the apomixis-linked alleles. Conversely, those TDFs that were apomixis and stage specific (class B) showed, in general, a higher level of expression in the apomictic flowers compared with the corresponding sexual stages, suggesting in this case that the related genes have functional rather than silencing roles.

The expression of some of these genes appeared to be time shifted between the sexual and apomictic genotypes. An example of this type of heterochrony could be represented by the apomixis-specific expression of genes homologous to maize seed storage proteins. α-Zeins are typical seed storage proteins of the maize endosperm whose synthesis is developmentally regulated at the transcriptional level (Sabelli and Larkins, 2009, and references therein). Maize genes for α-zeins are organized as a large gene family (Feng *et al.*, 2009) and are transcribed between 10 d and 45 d after pollination (Kodrzycki *et al.*, 1989). Apomictic flowers of *P. simplex* began to accumulate zein homologue transcripts at ~5 d after pollination, while these transcripts were absent in the sexual flowers. This suggested that the transcription of zein homologues in *P. simplex* may begin earlier in apomictic developing endosperm compared with sexual developing endosperm.

As a parthenogenetic embryo does not need fertilization, embryo development in apomictic plants could be accelerated compared with their sexual counterparts, and consequently this could send positive signals to the central cell of the embryo sac, which is 'primed' for faster cell cycle activity. Among these signals, those committing to earlier storage protein accumulation might be included. Recent findings in *Arabidopsis* have indicated that such embryo–endosperm communication mechanisms do exist (Nowack *et al.*, 2006). Although it is not known if a similar mechanism operates in the grass family, the presence of supernumerary polar nuclei prior to fertilization in the embryo sac of some grasses and of the maize mutant *indeterminate gametophyte* suggests some potential for premature endosperm proliferation that is repressed in sexual seed development (Sabelli and Larkins, 2009).

Among genes showing apomixis- and stage-specific expression, those showing putative regulatory functions (transcription factors, nucleic acid binding, and kinase activities) were the most represented. Among these, two ssDETDFs showing highly significant homology to ARF1 of *Arabidopsis* were both expressed in the early stages of apomictic flower development. ARFs are transcription factors that regulate the expression of auxin-responsive genes in both activation and repression modes (Guilfoyle and Hagen, 2007). ARF1 has been recognized as a transcriptional repressor *in vitro* (Tiwari *et al.*, 2003) and *in vivo* (Ellis *et al.*, 2005), and together with its sister gene ARF2 it has various effects on plant development (Ellis *et al.*, 2005). Attia *et al.* (2009) reported the higher transcript abundance of *OsARF1* in embryogenic compared with non-embryogenic rice tissues and the low growth and fertility of *OsARF1* antisense transgenic lines, indicating its key role in cell differentiation and growth. The auxin response mechanism has been suggested to have a role in apomictic ovule development in *Hieracium* (Koltunow *et al.*, 2001). Yamada-Akiyama *et al.* (2009) also found an auxin-responsive protein expressed in pistils of apomictic *Panicum maximum*. In the present findings, a homologue of *Arabidopsis* ARF1 was expressed in the early stages of apomictic ovule formation, suggesting that auxin response may affect the differentiation of the aposporic initial from nucellus cells, perhaps by repressing a class of auxin-responsive genes that are committed to maintaining the undifferentiated state of nucellar cells once the megaspore mother cell is formed. The developmental fate of non-reproductive cells has been switched in female gametophytes of *Arabidopsis* by manipulating auxin response genes (Pagnussat *et al.*, 2009).

The most striking difference observed in the expression of TDFs between apomictic and sexual flowers of *P. simplex* was that the alleles located on the ACL were expressed in all the stages of apomictic flower development analysed, whereas no cases of such constitutive expression were noticed in sexual flowers. This feature could be related to the highly rearranged nature of the ACL of *P. simplex*, which, as a consequence of the reduced recombination, is characterized by large and small INDELs, translocations, and the insertion of a TE (Calderini *et al.*, 2006). All of these types of rearrangements can deregulate transcriptional activity. Genetic rearrangements following gene migration or the inversion/translocation of large chromosomal areas can relocate genes to the proximity of *cis*-regulatory elements that can deregulate their transcription. However,

as all the apomixis-linked alleles detected in this study showed deregulation, a mechanism that affects the ACL as a whole should be invoked. The ACL of *P. simplex* presents a number of similarities with the better studied Y chromosome of dioecious plants; chief among them, repression of recombination, accumulation of TEs, and gene degeneration (Jamilena *et al.*, 2008). Most of the Y-linked genes that have been characterized for their expression in *Mercanthia polymorpha* are housekeeping genes (Yamato *et al.*, 2007), as are almost all the genes isolated from the Y chromosome of *Silene latifolia* (Jamilena *et al.*, 2008). TE insertions have been reported to affect the transcription of neighbouring genes in mice (Peaston *et al.*, 2004), yeast (Servant *et al.*, 2008), humans (Medstrand *et al.*, 2005), and plants (Kashkush *et al.*, 2003). TE insertions into regulatory regions have been implicated in the reactivation of ancient disease resistance genes in rice (Hayashi and Yoshida, 2009), the ectopic expression of tandemly repeated Myb genes in maize (Robbins *et al.*, 2008), and modulation of the transcriptional regulation of the Cg1 allele involved in maize development (Chuck *et al.*, 2007). In addition, TE insertion into resistance-like genes has been shown to generate pseudogenes that can enter into silencing regulatory pathways (Hernàndez-Pinsòn *et al.*, 2009). Several mechanisms have been suggested to explain how TEs can interfere with gene regulation; for example, TEs may contain sequences able to bind a regulator protein or, once they are inserted, they may create new binding sites that act as transcriptional regulators (Lerat and Sémon, 2007, and references therein).

Although no definitive conclusion can be reached on the basis of the data available to date regarding the coding or non-coding nature of apomixis-linked ssDETDFs, few of the apomixis-linked alleles showed the potential for coding a functional protein, whereas most of them showed frequent premature stop codon insertions and frameshift mutations, revealing their pseudogene nature. In any case, using cDNA-AFLP analysis, a set of genes that showed differences in their expression between apomictic and sexual flowers and that showed at least an apomixis linked-allele were selected for the first time in an apomixis system. Both mutated and non-mutated apomixis-linked alleles appear to be transcribed in developing flowers in a stage-independent manner. Among all the genes located on the ACL, the genetic determinants of apomictic reproduction in *P. simplex* should be present; mutated, non-functional alleles may have a role in silencing their corresponding sexual alleles, while the genetic factors triggering apomictic reproduction could be included among functional alleles. In this regard, genes that are differentially expressed but unlinked to apomixis could also be involved in apomictic reproduction as downstream actors that are pleiotropically regulated by the apomixis-linked factors. As Leblanc *et al.* (2009) pointed out, the introduction of apomictic reproduction in a crop species by the single-step transfer of the chromosomal area that controls the whole locus would probably be unsuccessful because of the high (epi)genetic load of this region. Moreover, on the basis of the present results, even if the key genes necessary to trigger apomictic reproduction might be few, the sexual recipient genome should be prepared to adapt the regulation of a plethora of genes acting downstream of the apomixis-linked factors to be converted into an apomictic crop. This aspect should be taken into account when attempting to develop an apomixis system for those crops for which closely related apomictic relatives do not exist. Wild apomictic systems composed of sexual and asexual cytotypes could serve as models to understand the complex functional network based on gene-to-gene communications that converts a sexual plant to an asexual apomictic plant after it has received an apomictic controlling locus. In the model system used in this study it is now possible to distinguish, among all the genes that are differentially expressed between apomictic and sexual flowers, those that are located at the apomixis locus from those that act downstream. The present work constitutes a basal study to develop a high-throughput strategy to identify all these genes and their interactions. The expected results should indicate which genes are worthy of being cloned and modified in the effort to develop apomixis systems for crop species.

Finally, transcriptionally active apomixis-linked alleles could also be used to develop a platform of functional markers for the assisted selection of apomixis within the *Paspalum* genus. The ACL can be introgressed into a sexual overperforming genotype of *P. simplex* to fix its superior characteristics, and the functioning of the locus could be followed during the selection cycles with the aid of functional markers.

Acknowledgements

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