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RESEARCH

Identification of Genes Related to Endosperm Balance Number Insensitivity in *Paspalum notatum*

M. B. Depetris, C. A. Acuña, F. I. Pozzi, C. L. Quarin, and S. A. Felitti*

ABSTRACT

The endosperm balance number (EBN) theory states that genomic contribution ratio should be kept at 2:1 maternal/paternal (m:p) ratio for normal endosperm development. However, the endosperm formation in apomictic *Paspalum notatum* Flüggé does not depend on the EBN. The aim of this work was to characterize gene expression during seed formation from apomictic and sexual *P. notatum* using complementary DNA-amplified fragment length polymorphism methodology. To induce the formation of seeds with different m:p genomic ratios, crosses were made between genotypes with different ploidy levels and reproductive modes. RNA was isolated from ovaries 24 h after pollination, when maximum endosperm growth rate was expected. Some of the 49 differentially expressed transcript-derived fragments (DETDFs) provided relevant information. Three of those were found in ovaries of apomictic plants with an EBN different from 2:1. A DETDF was predicted to be involved in sucrose metabolism during the accumulation of hexose and starch in the endosperm and might be related to EBN insensitivity. Twenty-two DETDFs were found in crosses where sexual plants were used as the female parent and the predicted m:p ratio was not 2:1. One of those was related to the failure of fusion of the polar nuclei in *Arabidopsis thaliana* (L.) Heynh. Finally, three transcripts presented similarity with a casein kinase II that regulates the accumulation of storage products in seeds of *A. thaliana*. Both processes might be involved in endosperm development in *P. notatum.*

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Abbreviations: ABA, abscisic acid; AFLP, amplified fragment length polymorphism; cDNA, complementary DNA; CK2, casein kinase II; DETDF, differential expression transcript-derived fragment; *e*, similarity of sequences; EBN, endosperm balance number; EDTA, ethylenediaminetetraacetic acid; m:p, maternal/paternal; PCR, polymerase chain reaction; SUS1, Sucrose Synthase 1; TDF, transcript-derived fragment.

Apomixis is a natural route of plant reproduction that circumvents meiosis and fertilization to generate an embryo that is solely maternal in genotype (Koltunow et al., 2013). Various research groups have made important contributions to the understanding of the biology and inheritance of apomixis in different species (reviewed by Ozias-Akins and van Dijk, 2007; Tucker and Koltunow, 2009; Pupilli and Barcaccia, 2012). Most have been focused on the identification of genes involved in the formation of clonal embryos in different apomictic species. However, few studies have attempted to understand the endosperm development in these systems (Quarin, 1999; Albertini et al., 2004; Polegri et al., 2010; Sharbel et al., 2010; Felitti et al., 2015). The possibility of transferring apomixis to economically important species requires understanding of the development of apomictic endosperm.

Apomixis offers a unique opportunity; any apomictic superior plant would produce seeds through open pollination by an unlimited number of generations without changes in the genotype (Hanna

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and Bashaw, 1987). Apomixis is present only in some species of agronomic interest (i.e., many tropical forage grasses, Kentucky bluegrass [*Poa pratensis* L.], mango [*Magnifera indica* L.], strawberry [*Fragaria* × *anasassa* Duchesne], apple [*Malus domestica* Borkh.], citrus, and cassava [*Manihot esculenta* Crantz]), although is widespread in flowering plants (Dwivedi et al., 2010). The transfer of this trait to the major crops would have an impact on agriculture because it will allow the maintenance and reproduction by seeds of superior varieties and hybrid combinations (Vielle Calzada et al., 1996; Dwivedi et al., 2010). Due to the increasing commercialization of hybrid seeds and the high cost of production that they represent, cloning genotypes through apomixis offers a great potential, valid for autogamous species (rice [*Oryza sativa* L.], cotton [*Gossypium hirsutum* L.]) and cross-pollinated species (maize [*Zea mays* L.], sunflower [*Helianthus annuus* L.]).

Paspalum is a large grass genus widely distributed in tropical and subtropical areas. Polyploidy and apomixis have affected the speciation and evolution of this genus. Most polyploid species are apomictic and chiefly tetraploids (Quarin, 1992). In the tetraploid cytotype ($2n = 4x = 40$) of *Paspalum notatum* Flüggé (bahiagrass), gametophytic apomixis includes apospory (nucellar cells develop unreduced embryo sacs), parthenogenesis (embryo development without fertilization of the egg cell), and pseudogamy (endosperm formation after fertilization of the polar nuclei) (Martínez et al., 2003). In this species, apospory is inherited as a single dominant Mendelian factor with distorted segregation (Martínez et al., 2001). It is located in a genomic region with preferential chromosome pairing and suppression of recombination (Martínez et al., 2003; Stein et al., 2004, 2007). In sexual plants, the endosperm has a maternal genomic contribution in concordance with its ploidy level. Seed is produced solely when the maternal/paternal genomic ratio (m:p) is 2:1 (endosperm balance number [EBN] is effective). Nevertheless, in apomictic plants, there is an evident EBN insensitivity. In pseudogamous apomictic 4*x* of *P. notatum*, the endosperm develops independently of the ploidy level of the male parent, allowing seed production even if the pollinator belongs to a different species (Quarin, 1999). The triple fusion involving two unreduced polar nuclei with 40 chromosomes and a reduced male gamete with 20 chromosomes forms the endosperm with 100 chromosomes by pseudogamy.

To characterize the gene expression during early stages of seed development and comprehend more about the EBN sensitivity and insensitivity in *P. notatum*, RNA isolation was performed from ovaries at different times after pollination. Previously, complementary DNA (cDNA)-amplified fragment length polymorphism (AFLP) methodology was used to characterize gene expression of bahiagrass ovaries 3 h after pollination (allowing fertilization to occur) (Felitti et al., 2015). Forty-six of the 100 DETDFs (differentially expressed transcript-derived fragments [TDFs]) identified in that study were found in apomictic ovaries in which EBN was different from 2:1. Also, 12 of the DETDFs presented high similarity with proteins in *Arabidopsis* cell suspension cultures expressed in response to changes in the levels of extracellular ATP. This molecule controls organellar energy metabolism and activates gene expression related to developmental programs and specific growth, acting in plants as a molecular switch. The results suggest that the regulation of endosperm development could be related to extracellular ATP-mediated signaling (Felitti et al., 2015). Presently, we use the same methodology to characterize gene expression of bahiagrass ovaries 24 h after pollination (allowing mitotic division of embryo and endosperm).

Classical methods of crossing in *P. almum* Chase, *P. brunneum* Mez, *P. rufum* Nees, *P. intermedium* Munro ex Morong, and *P. quadrifarium* Lam. have allowed investigating the intraspecific crossability using 2*x* female and 4*x* male cytotypes. In 2*x* sexual plants after self-pollination, the pollen tube development stopped at the stigma or style levels, supporting that 2*x* plants were self-incompatible. The pollen from 4*x* apomictic cytotypes germinated and penetrated the pistils of diploid cospecific cytotypes. Most ovules have manifested development of endosperm and embryo 24 h after fertilization. Notwithstanding, in 2*x* × 4*x* crosses at 91 h postfertilization, the endosperms collapsed and embryos detained their development. Therefore, postzygotic abortive system causes low crossability in diploid-tetraploid crosses (Norrmann et al., 1994). The first zygotic division in *Triticum aestivum* L. is seen at ~22 h after pollination, whereas the endosperm consists of ~16 nuclei 24 h after pollination (Bennett et al., 1973). In *Hordeum vulgare* L., the first zygotic division occurs 22 to 24 h after pollination, similarly to wheat (Marshall and Grace, 1992). The embryo develops slowly compared to the endosperm. The latter may be partially or totally reabsorbed, resulting in exalbuminous seeds, or may persist until germination resulting in albuminous seeds like in *Poaceae* (Brown and Lemmon, 2007).

The aim of this work was to characterize gene expression in apomitic and sexual plants of *P. notatum* during early stages of seed development, with special emphasis in the identification of genes associated with endosperm formation. We expected to observe DETDFs in ovaries of apomictic and sexual plants at these stages. As low fertility is given by a postzygotic abortion, the analysis was performed 24 h after pollination, when endosperm growth rate was expected to be at its maximum level and before the endosperm collapse. A great diversity of endosperm genome contribution ratios (EBN) was achieved by designing a set of experimental crosses including sexual and apomictic scenarios in *P. notatum*. The gene expression patterns of the

endosperm were compared in sexual and apomictic ovaries to perform the analysis. The availability of *P. notatum* races with different ploidy levels and reproductive modes was an advantage in accomplishing this study.

MATERIALS AND METHODS

Plant Material, Chromosome Numbers, and Crosses

To induce the development of seeds with different EBN, experimental crosses were made using several races of *P. notatum*. Parental genotypes differed in reproductive systems and ploidy levels: two were apomictic tetraploid (4*x* A1 and 4*x* A2), two were sexual tetraploid (4*x* S1 and 4*x* S2), four were sexual diploid (2*x* S1, 2*x* S2, 2*x* S3, and 2*x* S4), and one was an apomictic hexaploid (6*x* A). Description of the different accessions used in this study (ploidy levels, mode of reproduction, and origin) and crosses are described in Tables 1 and 2, respectively. The parental combinations were selected based on the information generated by Quarin (1999). Prior observations indicated that pollen grains take ~15 min (after pollination) to germinate on stigma papillae, whereas the pollen tube takes 90 to 150 min to penetrate through the micropyle and reach the ovule (Fernández, 1990). According to microscopic observations of ovaries, most ovules showed embryo and endosperm development 24 h after fertilization (data not shown). Thus, ovaries were isolated from the flowers, under magnifying glass and with dissecting forceps, 24 h after pollination to detect differences during formation of *P. notatum* seeds. A minimum of 20 ovaries for each time point were harvested and froze in liquid nitrogen (Felitti et al., 2015).

Chromosome numbers of accessions H398 and Q4308 were determined by transmitted light microscopy in root tips stained with Feulgen reaction, squashed on a slide, and mounted with the aid of a drop of Acetocarmine under a glass cover. The chromosome numbers of other used materials were previously known and reported as follow: 'Tifton 9' is a forage grass cultivar (Burton, 1989) developed from Pensacola bahiagrass, which is the popular name for the diploid ($2n = 20$) cytotype of *P. notatum*, officially recognized as the botanical variety *P. notatum* var. *saurae* Parodi; the accessions Q4084-8 ($2n = 20$), C4-2x ($2n = 20$), and C4-4x ($2n = 40$) were reported by Quarin et al. (2001), whereas Martínez et al. (2001) reported accessions Q3775, Q4117, and Q4188, all with $2n = 40$ chromosomes.

RNA Isolation and cDNA Synthesis

The protocol reported by Felitti et al. (2015) was used with minor modifications. Briefly, using SV Total RNA Isolation System (Promega), the total RNA was extracted from frozen ovaries according to the manufacturer's protocol. The quality and yield of total RNA was determined by an agarose gel and

Table 1. Plants of *Paspalum notatum* identified according to accessions, ploidy levels, mode of reproduction, and origin.

Plant ID†	Accession	Chromosome no.	Mode of reproduction	Origin
2*x* S1	H398	20	Sexual	Empedrado, Corrientes, Argentina
2*x* S2	Tifton 9	20	Sexual	An individual plant of the commercial cultivar 'Tifton 9'
2*x* S3	Q4084-8	20	Sexual	An individual plant collected in a natural population at Cayastá, Santa Fe, Argentina
2*x* S4	C4-2x	20	Sexual	Experimental origin from a nonduplicated callus sector obtained by tissue culture and colchicine treatment (see 4*x* plant C4-4x)
4*x* A1	Q4117	40	Apomictic	Río Grande do Sul, Brazil
4*x* A2	Q3775	40	Apomictic	Municipality of Gómez, Tamaulipas, Mexico
4*x* S1	Q4188	40	Sexual	Sexual hybrid, parents: Q3664 (4*x* predominantly sexual, Tifton, USA) × Q3853 (Capivarí, Rio Grande do Sul, Brazil)
4*x* S2	C4-4x	40	Sexual	Induced tetraploid derived from a chromosome-duplicated callus sector obtained by tissue culture and colchicine treatment of a diploid
6*x* A	Q4308	60	Apomictic	BIII hybrid obtained by $2n + n$ fertilization of apomictic 4*x* accession Q4023

† Plants are ordered by ploidy levels (2*x* = diploid, 4*x* = tetraploid, 6*x* = hexaploid) and reproductive systems (S = sexual, A = apomictic).

Table 2. Crosses of *Paspalum notatum* between genotypes with different ploidy levels and reproductive systems

Female parent†	Pollinator†	Expected ploidy of the embryo	Expected ploidy of the endosperm	Expected maternal/paternal genome ratio in the endosperm
4*x* A1	2*x* S1‡	4*x* ($2n + 0$)	9*x* ($2n + 2n + n$)	8:1
	4*x* A2‡	4*x* ($2n + 0$)	10*x* ($2n + 2n + n$)	8:2
	4*x* S2‡	4*x* ($2n + 0$)	10*x* ($2n + 2n + n$)	8:2
	6*x* A‡	4*x* ($2n + 0$)	11*x* ($2n + 2n + n$)	8:3
4*x* S1	2*x* S1§	3*x* ($n + n$)	5*x* ($n + n + n$)	4:1
	4*x* A2‡	4*x* ($n + n$)	6*x* ($n + n + n$)	2:1
4*x* S2	2*x* S3§	3*x* ($n + n$)	5*x* ($n + n + n$)	4:1
2*x* S4	2*x* S2‡	2*x* ($n + n$)	3*x* ($n + n + n$)	2:1
4*x* A2	4*x* A1‡	4*x* ($2n + 0$)	10*x* ($2n + 2n + n$)	8:2

† Female parents and pollinators are ordered by ploidy levels (2*x* = diploid, 4*x* = tetraploid, 6*x* = hexaploid) and also by reproductive system (S = sexual, A = apomictic).

‡ Crosses that are expected to set seed.

§ Crosses that are not expected to set seed.

spectrophotometer as described by Sambrook and Russell (2001). Biotinylated oligo-dT primer and SuperScript Reverse Transcriptase (Invitrogen Life Technologies) allowed the synthesis of first-strand cDNA using 10 μL of total RNA for each sample (~0.08 μg) as template, according to the manufacturer's instructions. Then, 140 μL of the second-strand cDNA synthesis mix (16 μL 10× second strand buffer, 3 μL 10 mM deoxynucleotide mix, 0.16 μL ribonuclease H [10 U μL^{-1}] [Invitrogen Life Technologies], 2.5 μL DNA polymerase I [10 U μL^{-1}] [Invitrogen Life Technologies], and 118 μL H_2O) were added and incubated for 1 h at 12°C, followed by 1 h at 22°C.

cDNA-AFLP Analysis

The cDNA-AFLP was performed as described by Felitti et al. (2015). Double-stranded cDNA was purified using the Nucleospin Extract II kit (Macherey-Nagel) and eluted in 20 μL. The first restriction enzyme, *Cvi*AII (New England Biolabs), was used to digest the cDNA in a final volume of 40 μL for 2 h at 25°C. Then, to the solution of digested cDNA fragments, 40 μL (100 μg) of resuspended streptavidin-coated Dynabeads (Promega) was added, giving a final volume of 80 μL, and the mixture was then incubated for 30 min at room temperature. Magnetic separator allowed immobilizing and collecting the biotinylated 3′-terminal cDNA fragments. The supernatants were removed with a pipette. The tubes were released from the magnetic separator, and the beads were washed four times with 100 μL 1× STEX buffer (1 M NaCl, 10 mM tris-HCl [pH 8.0], 1 M ethylenediaminetetraacetic acid [EDTA, pH 8.0], and 1 mL 100 mL^{-1} Triton X-100). Finally, the STEX buffer was removed and the beads were resuspended in 30 μL tris-EDTA (TE) buffer (10:0.1). Ten microliters of the second digestion mix (10× buffer, 10 U *Taq*I and deionized H_2O [New England Biolabs]) were added to the 30-μL bead suspension and incubated with gentle agitation (to ensure that the beads are resuspended) for 2 h at 65°C. The beads were collected again using the magnet, and the supernatant was transferred to a new tube containing the template fragments for the adaptor ligation. The sequences of primers and adapters used for cDNA-AFLP analyses were described by Felitti et al. (2015).

Each cDNA sample was preamplified and then used as template in a second selective amplification using 16 primer combinations. The selective primers used during the selective amplifications were identical to the preamplification primers but were extended at the 3′ end by one selective nucleotide. Preamplified samples were diluted by one-third and used as templates in reactions of selective amplification. The reaction conditions were as follows: 95°C for 5 min; 12 cycles of 94°C for 30 s, 65°C (decrease of 0.7°C each cycle) for 30 s, and 72°C for 1 min; and 24 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by a final extension step (72°C for 10 min) as indicated by Felitti et al. (2015). Loading buffer (98% [w/v] formamide, 10 mM EDTA [pH 8.0], bromophenol blue, and xylene cyanol) was added and the samples were denatured at 94°C for 5 min and loaded in 6% (w/v) denaturing polyacrylamide gels. To estimate the molecular weights of the bands of DETDFs, a molecular weight marker (100–1500 bp, New England Biolabs) was loaded into the polyacrylamide gels. Electrophoresis was conducted for ~3 h at 60 W using 0.5× TBE (100 mM tris-HCl, 90 mM boric acid, and 1 mM EDTA [pH 8.0]) in the upper tank and 1× TBE and lower tank, both buffers with pH 8.0. The DNA Silver Staining System (Promega) was used to stain gels. To assess the reproducibility of the electrophoresis results, technical replicates were run in different gels (two aliquots for each polymerase chain reaction [PCR]).

In the analysis at 3 h after pollination, the validation was performed by real-time PCR (Felitti et al., 2015), and *β-tubulin* was selected as a suitable reference gene to analyze gene expression levels in *P. notatum* ovaries (Pfaffl et al., 2004). Validation showed that the DETDFs identified by cDNA-AFLP analysis were indeed differentially expressed in the crosses under study. Since for 24 h after pollination the same technique and molecular experiments were performed in the same laboratory, we considered the validation experiments conducted for the samples 3 h after pollination conclusive.

Isolation and Sequencing of Differential Expression Transcript-Derived Fragments

To isolate DETDFs, the amplification reactions obtained using the most informative primer combinations were run in acrylamide gels. In addition, technical replicates from separate PCR reactions employing the same selective primer pair were run to check reproducibility. The TDFs with an interesting polymorphism classified as DETDFs were identified. They differed on the m:p contribution ratio in the endosperm and expected seed formation. The bands present and showing higher intensity in at least one cross compared with the other crosses in the same gel were hydrated with a drop of distilled water, excised from the polyacrylamide gel by crushing with a micropipette tip, and incubated in 30 μL of elution buffer with occasional vortexing (0.5 M $NH_4CH_3CO_2$, 1 mM EDTA [pH 8.0]) for 4 h at 37°C. A reamplification reaction was conducted using 1 μL of the eluted sample as a template and the same conditions described for the selective amplification reactions. Agarose gels 2% (w/v) were conducted to check the resulting PCR products, as described by Sambrook and Russell (2001). Those bands presenting the correct size and quality were sent for sequencing analysis to Macrogen (Korea).

Sequence Bioinformatics Analysis

The sequences and chromatograms provided by Macrogen (Korea) were analyzed with the program Sequencher version 4.1.4 (Gene Codes Corporation, 2004). The corresponding specific primer sequences were detected and eliminated. The sequences shorter than 50 bp were not included. Unique sequences with high quality were analyzed using parameters of best similarity to infer protein functions. The similarity analysis was performed using the BLAST 2.2.25 NCBI site program (NCBI, 2017). Searches were confined to plant species (plants taxid: 3193) and examination of the Expressed Sequence Tags BLASTn (blasted on the nonhuman, nonmouse expressed sequence tag), and then the best expressed sequence tags were blasted on nonredundant protein sequences BLASTx. The similarity search with the genome of *Arabidopsis thaliana* (L.) Heynh. was conducted using the tool BLASTp 2.2.8 of the TAIR *Arabidopsis* Information Resource site (TAIR, 2017).

RESULTS

The races of *P. notatum* used differed in ploidy levels and reproductive systems (Table 1). Crosses were performed

to characterize the gene expression profiles at the start of endosperm development and seed formation (Table 2). This allowed us to obtain a diversity of endosperm genomic ratios (EBN) and seed set, including both sexual and apomictic reproduction systems. RNA was isolated from ovaries 24 h after pollination, and gene expression characterization was conducted using cDNA-AFLP. For this study, a total of 16 primer combinations were used (Felitti et al., 2015). On average, patterns of ~30 fragments had been produced by each combination of primers, with molecular weights ranging from 100 to 800 bp. Figure 1 shows a section of a typical cDNA-AFLP polyacrylamide gel.

A total of 3780 scorable TDFs were detected. The TDFs were considered differentially expressed (DETDFs) when differed in either the presence or absence or intensity along the different crosses. Of these, 107 were isolated because they were highly polymorphic between samples differing on the m:p contribution ratio in the endosperm and the expected seed formation. The expression profiles were highly reproducible. Fragment sizes were confirmed in agarose gels, leaving 91 DETDFs for sequencing (data not shown). Forty-nine of the ninety-one DETDFs were successfully sequenced, with an average length of 219 bp and presenting a size range of 57 to 380 bp. The rest of the DETDFs (42 of 91) were sequenced but have been discarded for bioinformatic analysis because they have <50 bp with poor quality of the chromatograms.

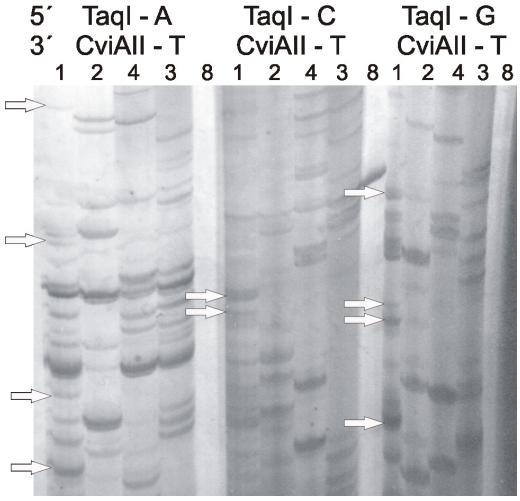

Fig. 1. Gel results using the complementary DNA-amplified fragment length polymorphism methodology. Transcript-derived fragments resulting from selective amplification used the next three combinations of primers: forward primers *Taq*IselA, *Taq*IselC, and *Taq*IselG and reverse primers CviAIIselT (indicated in the top of the figure). Rows correspond to five crosses: 4*x* S1 × 2*x* S1 (1), 4*x* S1 × 4*x* A2 (2), 4*x* A1 × 2*x* S1 (4), 4*x* A1 × 4*x* A2 (3), and 4*x* A1 × 6*x* A (8). Arrows indicate examples of some of the differential expression transcript-derived fragments that were only present in cross 4*x* S1 × 2*x* S1 (1) where seed set is not predicted (endosperm balance number different from 2:1 in sexual ovaries).

Classification of Differential Expression Transcript-Derived Fragments

The 49 DETDFs were classified in 11 functional categories. Most of the genes were predicted to be involved in signal transduction (15%), metabolism (8%). and energy (6%). Fewer DETDFs belonged to the transcription and growth/division (4% each), intracellular traffic, disease/defense, cell structure, transport, and protein synthesis (2% each). The rest of the sequences analyzed (53%) were included in the unknown function category (Fig. 2).

Twenty-nine of the forty-nine DETDFs were expressed in crosses where a sexual plant was used as female parent (Table 3, Classes A–C). Six of these DETDFs were expressed only when the predicted m:p ratio in the endosperm was 2:1 (Table 3, Class A). This was considered as the sexual situation, and seeds could be expected to be formed. These sequences were classified as being involved in energy, disease/defense, and unknown categories (Supplemental Table S1, Class A). The other 22 DETDFs were expressed only when the predicted m:p ratio in the endosperm was 4:1 and the female parent was a tetraploid sexual (Table 3, Class B). In this case, no seeds were expected to be formed because the EBN was different from 2:1 ratio necessary to fulfill the EBN theory. These sequences belonged to the transcription, protein synthesis, energy, growth/division, signal transduction, metabolism, transporters, and unknown functional categories (Supplemental Table S1, Class B). Finally, one DETDF was expressed when the predicted m:p ratio in the endosperm was 2:1 and 4:1 (Table 3, Class C). It was present in the two situations (m:p ratios 2:1 and 4:1) and was considered as control. In this case, the sequence belonged to unknown function (Supplemental Table S1, Class C).

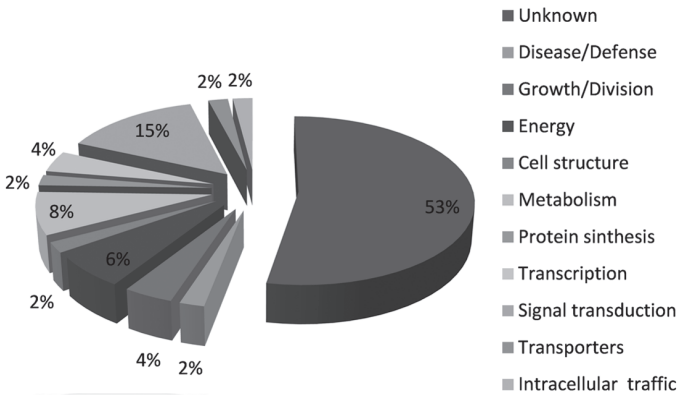

Fig. 2. Functional categories assigned to 49 differential expression transcript-derived fragments identified by complementary DNA-amplified fragment length polymorphism analysis. The transcripts were isolated and sequenced from ovaries obtained at 24 h after pollination from the different crosses already described. The sequences of the best matching proteins were blasted using BLASTp from the *Arabidopsis* informative resource (TAIR) protein database. To search putative molecular functions and related biological processes, the best-hit proteins were submitted to the GO annotation tool of TAIR.

Table 3. Classes of differential expression transcript-derived fragments (DETDFs) based on the comparative gene expression patterns.

Expression pattern	Expected seed set	Class	Reproductive mode					Total of DETDFs
			Apomictic			Sexual		
			4:1†	8:1†	8:3†	2:1†	4:1†	
Sexual only (EBN‡ sensitive)	Yes	A				X		6
	No	B					X	22
	Yes/no	C				X	X	1
Apomictic only (EBN insensitive)	Yes	D	X					3
Apomictic and sexual	Yes	E	X	X	X	X		4
	Yes	F	X			X		9
	Yes/no	G	X				X	2
	Yes/yes/yes/yes/no	H	X	X	X	X	X	2
Total								49

† Predicted maternal/paternal ratio in the endosperm.

‡ EBN, endosperm balance number.

On the other hand, 3 of the 49 DETDFs were expressed when an apomictic plant was used as a female parent and the predicted m:p ratio in the endosperm was different from the ratio 2:1 present in most angiosperms and sexual plants of the species (Table 3, Class D). This situation was interesting because it could be related with the EBN-insensitive seed set. The sequences belonged to metabolism (Supplemental Table S1, D2) and unknown categories.

Also, 17 of the 49 DETDFs were found in crosses derived from apomictic and sexual females (Table 3, Classes E–H). Four were found using an apomictic tetraploid female (Table 3, Class E), where endosperm relationships were 4:1, 8:1, and 8:3, and also when the female was a sexual tetraploid and genomic ratio was 2:1. The transcripts were classified in signal transduction, energy, metabolism, and unknown processes (Supplemental Table S1). Additionally, 9 of the 17 were expressed in ovaries from apomictic tetraploid (EBN 4:1) and sexual tetraploid females (EBN 2:1) in which seeds were expected to develop (Table 3, Class F). In this case, the transcripts corresponded to the categories signal transduction, growth/division, cell structure, transcription, and unknown (Supplemental Table S1). Two DETDFs were present in crosses where the female was a sexual tetraploid and was not expected to set seed (EBN 4:1), and in a fertile apomictic female that still produces seed despite its insensitivity to EBN (Table 3, Class G). These transcripts were present in two situations (EBN 4:1 and insensitivity to EBN), so they were considered as controls with unknown and signal transduction functions. Finally, two DETDFs were present when the female parent was an apomictic tetraploid and the EBN was 4:1, 8:1, or 8:3, and when the female was a sexual tetraploid with genomic ratios of 2:1 and 4:1 (Table 3, Class H). The functional categories corresponded to metabolism and intracellular traffic (Supplemental Table S1, Class H).

DISCUSSION

The understanding of endosperm development in apomictic species is a crucial aspect to transfer apomixis to cereals, as nonapomictic crops do not produce grain if the EBN relationship varies from 2:1. Apomixis would enable the setting, maintenance, and multiplication of genotypes of interest by seed, with great impact on the economy. As there is no information about differential expression of genes during the endosperm development in apomictic species like *P. notatum*, our research was conducted.

Norrmann et al. (1994) performed crosses in *P. rufum* and *P. intermedium* between 2*x* and 4*x* ploidy levels. The endosperm chromosome number of the progeny was $2n = 30$, explaining the contribution of the polar nuclei $n = x = 10$ and sperm nuclei with $n = 2x = 20$. Another piece of evidence is that we have worked with the same species as in Quarin (1999), who previously counted the endosperm chromosome numbers in 20 nuclei of the self-pollinated apomictic 4*x* accession. They showed 100 chromosomes at mitotic metaphase. The results indicated that *P. notatum* contributed two unreduced polar nuclei ($2n + 2n = 4x + 4x = 80$) and a reduced sperm that is the same as in sexual plants ($n = 2x = 20$). Additionally, in a cross between two sexual 4*x* plants, the progeny had 40 chromosomes, implying that both the sperm and egg nuclei provide 20 chromosomes ($n = 2x = 20$). The endosperm derived from fertilization of two reduced polar nuclei ($n + n = 2x + 2x = 40$) with a reduced sperm nuclei ($n = 2x = 20$) gave an endosperm $2n = 6x = 60$ with EBN = 2:1. Also, Martínez et al. (2007) made several crosses in *P. notatum* and *P. simplex* Morong involving different ploidy levels and counted the chromosome number for each hybrid. They concluded that from chromosome counts of each progeny it is possible to infer the chromosome number of the male gamete. It is important to mention that two accessions (Q4084 and Q4188) are exactly the same as those we used in this study. We consider that it is enough evidence to expect the ploidy levels and m:p genome ratios in the endosperm from ploidy levels

of the offspring. Given that our sample was random, we would not expect major changes.

The classification of transcripts in this study was similar to the functional categories proposed in the analysis corresponding to 3 h after pollination (Felitti et al., 2015). In that case, the 100 DETDFs identified were classified in 12 functional categories. The most numerous categories corresponded to transcription (10%), signal transduction, and metabolism and cell structure (8% each). Lesser categories were involved in protein destination and storage (7%), intracellular traffic (6%), and regulation of gene expression (4%). It was predicted that the remaining 12% of the sequences participated in disease/defense, transporters, secondary metabolism, and protein synthesis (Felitti et al., 2015). In this study, the 49 DETDFs were sorted into 11 functional categories, and the most important sets of genes corresponded to the functional groups involved in signal transduction (15%), metabolism (8%), and energy (6%) (Fig. 2).

It is worth mentioning that there is similitude at the biological level between both analyses. Considerable proportions of the identified transcripts in both studies belong to functional categories involved in metabolism, energy, and cell structure. Within those functional categories, their possible function was related to amino acid metabolism and transport, and cell wall and cytoskeleton organization. These processes play key roles during the active cell division that occurs in the early seed development. Above all, the cytoskeleton is essential in the development pathway in the endosperm. Changes in microtubule leading to a set of specialized tissues such as transfer cells, the starchy endosperm and the aleurone layer have been observed (Brown and Lemmon, 2007). It is possible that the transcripts detected 3 (Felitti et al., 2015) and 24 h after pollination in *P. notatum* are related to the accumulation of reserves to complete the endosperm formation.

In this study, DETDFs B3, B7, B8, and B10 (Supplemental Table S1) were isolated from sexual ovaries where the EBN was not 2:1, and endosperm formation was not expected to occur. Particularly, B7 was classified in the category of metabolism and showed high similarity (e^{-123} BLASTp, were *e* is a measure of the similarity of sequences; the lower the *e* value, the higher the congruity of your query sequence and the retrieved sequence; *e* values of zero mean that there is an exact match for sequences) with an *A. thaliana* sequence (AT1G19520) involved in ribonucleotide biosynthetic processes, more precisely, pyrimidines. This locus is known as *Nuclear Fusion Defective 5* and is involved in processes of karyogamy or nuclear fusion, essential for sexual reproduction (Portereiko et al., 2006). In most angiosperms, karyogamy occurs twice during double fertilization and once when the two polar nuclei fuse to form the central cell (Portereiko et al., 2006). In *Arabidopsis thaliana*, nine female gametophyte mutants have been identified, known as *nuclear fusion defective* (nfd1 to nfd9), which have defective fusion of the polar nuclei. In the nfd1 to nfd6 mutants, the only defect detected during megagametogenesis was the failure of fusion of the polar nuclei (Portereiko et al., 2006). However, in *Paspalum,* there is no fusion of the polar nuclei during female gametophyte development. Rather, the two polar nuclei remain unfused several hours after pollination, when they join the sperm nucleus in a simultaneous triple fusion (Ortiz et al., 1997; Fig. 1A and 1B). In this study, B7 was expressed in *P. notatum* after pollination according to when the polar nuclei would fuse with the sperm nuclei. This result could indicate that the failure in the fusion of the polar nuclei after pollination would be the cause of nonseed production when the female is sexual and EBN differs from 2:1.

The other three transcripts (B3, B8, and B10) were classified into the signal transduction functional category (Supplemental Table S1) and showed high similarity (e^{-148} BLASTp) with a *Casein Kinase* II (AT2G23080) involved in signaling and response to physiological and environmental conditions (protein phosphorylation), cell division, and DNA replication in *A. thaliana*. In plants, casein kinase II (CK2) is involved in relevant processes such as growth and development. In fact, CK2 is implicated in abscisic acid (ABA) signaling and related processes, such as seed maturation, dormancy, and germination. During seed maturation, ABA regulates the synthesis of seed storage proteins and lipids (Finkelstein et al., 2002; Wang et al., 2014; Vilela et al., 2015). In maize, a strong activity of the CK2 in the endosperm was demonstrated (Grasser et al., 1989). Zein genes encoded the primary protein products, and CK2 regulated zein gene expression because *Opaque2* is phosphorylated by CK2 during seed development. Opaque2 is a transcriptional activator that regulates the expression of zein seed storage protein gene family through the sequence-specific binding to the zein gene promoter. The participation of the Opaque2 in regulating zein gene is controlled by CK2 in response to different physiological and environmental conditions (Ciceri et al., 1999; Kemper et al., 1999; Łebska et al., 2010).

The next interesting DETDF, D2 (Supplemental Table S1) was found specifically in crosses where apomictic plants were used as female parents, which produce seed regardless of presenting an endosperm ratio of 2:1. The D2 DETDF was classified as metabolism and showed high similarity (e^{-111} BLASTp) to a sucrose synthase (AT5G20830) from *A. thaliana*. The *Sucrose Synthase 1* (*SUS1*) gene (AT5G20830) is expressed during the morphogenesis of the seed. It corresponds to the phase of the massive accumulation of hexoses and starch (Angeles-Núñez et al., 2014). Mutation of *SUS1* affects mainly hexoses/sucrose ratio, resulting in a decrease of starch levels during early stages of seed development and significant accumulation of fatty acids (Angeles-Núñez et al., 2014). The failure in endosperm

cellularization makes the central vacuole the main sucrose destination of developing seed instead of the embryo. This is consistent with a high hexose to sucrose ratio in mutant seeds where the embryo is arrested. When cellularization is restored and hexose levels decrease, progress is allowed in the development of the embryo through the channeling of sucrose to that destination (Hehenberger et al., 2012). According to our results, the increase in the content of hexoses is related to the failure of endosperm development in *A. thaliana*. In conclusion, there is a correlation between increased hexose level, failure of endosperm cellularization, and embryo arrest in *Arabidopsis* (Hehenberger et al., 2012) that might be related to EBN insensitivity in ovaries derived from apomictic female with an endosperm ratio different from 2:1.

Upon hybridization of species that differ in ploidy, the failure in endosperm development and seed lethality are observed. When EBN is different from 2:1 m:p, genomic imprinting generates parental conflict. In *Arabidopsis*, the failure of the function FERTILIZATION INDEPENDENT SEED-PRC2 causes defects in endosperm cellularization, the paternally expressed imprinted genes are deregulated, and the embryo aborts (Lafon-Placette and Kohler, 2016). We suggest that the differences in genomic imprinting could be a main reason for insensitivity or sensitivity to EBN in apomictic or sexual ovaries.

Siena et al. (2016) characterized an apomixis-linked sequence of *P. simplex,* homologous to Subunit 3 of a multiprotein complex that controls cell differentiation and DNA replication in eukaryotes. The sequence was called ORIGIN RECOGNITION COMPLEX (ORC3). The specific copy for the apomictic genotype, *PsORC3a*, was a pseudogene that was constitutively expressed at low levels in all developmental stages of apomictic flowers. Mutants in rice and *Arabidopsis* had shown an interruption of embryo and endosperm development in early stages of growing, and the authors suggested it was related to the downregulation of its functional homolog and with the development of apomictic endosperm that deviates from EBN 2:1. In our study, we found transcripts assigned to the division/growth category that could be related to the aforementioned downregulation of the functional homolog of *PsORC3a*, giving failure or successful endosperm development in early stages of seed production in *Paspalum*.

E2 and F3 DETDFs were present in crosses where seed production is expected. Both had high similarity (e^{-165}) with the *casein kinase II* (AT2G23080). They could be involved in the beginning in ABA signaling and activated transcriptional factors in response to physiological and environmental condition signaling pathways, as were reported in B3, B8 and B10. This *casein kinase II* accumulation may trigger both the success (E2 and F3) and failure (B3, B8, and B10) of seed production.

The isolated sequence B7 was present in sexual ovaries with an EBN different from 2:1. This sequence has high similarity to a sequence involved in the *nfd* that causes a failure in the polar nuclei fusion in *A. thaliana*. The polar nuclei fusion is necessary for the endosperm development in sexual and pseudogamous apomictic plants. Given the existence of facultative apomictic plants that could take both sexual and apomictic pathways, it is believed that an alteration in sexual regulation could trigger the omission of key steps resulting in apomictic seed production (Nogler, 1984; Koltunow et al., 2013). However, it is important to mention that the apomictic genotypes used in these experiments were previously classified as highly apomictic (Ortiz et al., 1997). Genotypes Q4117 and Q3775 (4*x* A1 and 4*x* A2, respectively) had >92% of their ovules with only aposporous embryo sacs, and the rest of their ovules had a single meiotically derived embryo sac, or they had both aposporous and meiotically derived embryo sacs in the same ovule. The probability of obtaining a sexually derived offspring from one of these apomictic genotypes should be considered very low, since it has been shown that the fraction of the progeny resulting from sexuality in a facultative plant is lower than what is potentially observed on mature embryo sacs (Hojsgaard et al., 2013). If the genes that control sexual or apomictic pathways are the same, maybe the mechanisms of differential regulation may trigger either failure or successful sexual endosperm development and pseudogamy in *Paspalum* apomictic plants. However, there is an important difference between *A. thaliana* and *P. notatum* in the timing of the polar nuclei fusion. In *A. thaliana*, the fusion occurs during the embryo sac development much earlier than when they are joined by the sperm nucleus after pollination. Thus, the beginning of endosperm development requires two previous steps: the fusion of polar nuclei during embryo sac development, and the fusion with one sperm nucleus after pollination. In *P. notatum*, the polar nuclei remain uncoupled in mature embryo sacs, and after anthesis and pollination, they join the sperm nucleus in a triple fusion event. Therefore, it would be necessary to understand the real significance of B7 sequence in *Paspalum*.

Finally, it is worth mentioning that specific transcripts existing in some crosses and absent in others do not necessarily mean that these transcripts are associated or involved in endosperm development and/or seed production. Certainty about the biological function of the DETDFs will be achieved by obtaining gene knockout or knockdown *P. notatum* plants by transgenesis and/or phenotypic analysis of specific mutants of related species. Nevertheless, the results allowed identifying transcripts potentially related to the success or failure of endosperm development in *P. notatum*. This will help us to understand the mechanism by which this system produces seeds, regardless of the strict maternal and paternal genomic ratio (2:1 m:p) present in most grass species.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material for this article is available online.

Acknowledgments

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