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Gene expression prior to post-zygotic endosperm collapse in tetraploid bahiagrass

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Abstract

The endosperm is the storage tissue of seeds and is an important source of nutrients for humans and animals. In the previous work, the gene expression was characterized at 3 and 24 h after pollination (AP). The results suggested that eATP would act as a signalling molecule at the beginning of endosperm development and that sucrose metabolism could be related to EBN insensitivity. In addition, differentially expressed transcripts derived fragments (DETDFs) were related to the failure of fusion of the polar nuclei and the accumulation of storage products in seeds of *Arabidopsis thaliana*. The objective of the present study was to identify genes related to endosperm development in apomictic and sexual ovaries of *Paspalum notatum* 48 h AP, a stage at which development is prior to post-zygotic collapse. The cDNA-AFLP analysis was carried out to analyse different crosses and DETDFs categorized according to their function. The main cellular functions at 48 h AP were *metabolism* and *signal transduction*. Fourteen out of 39 DETDFs with relevant functional information were found in crosses for which normal endosperm development was expected. Three DETDFs were found in crosses where viable and unviable seeds were predicted and presented similarity with a casein kinase II (CK2), an enzyme that governs the accumulation of storage proteins in seeds of *A. thaliana* and *Zea mays*. The results obtained at 3, 24 and 48 h AP suggest that CK2 is involved in early endosperm development in *P. notatum*.

Introduction

The endosperm is a storage tissue of seeds and determines the qualitative and quantitative value of crops of agronomic interest. It is an important source of nutrients for humans and animals and, therefore, is of considerable economic importance (Sabelli and Larkins, 2009). Endosperm results from fertilization of polar nuclei in the central cell by one sperm cell (Brown and Lemmon, 2007; Sabelli and Larkins, 2009). In addition to nourishing the embryo during its development and at the time of seed germination, it has the function of detecting hybrids and inadequate polyploidies, thus avoiding energy expenditure in the production of non-viable seeds. Around 70% of flowering plants have a 3*n* endosperm, suggesting a high ploidy level is evolutionarily advantageous (Brown and Lemmon, 2007; Bewley et al., 2013).

Endosperm development is essential for seed formation. The most important role of this tissue is to transfer nutrients to the embryo, and additionally, it is involved in the imprinting of genes. The endosperm develops at a speed considerably greater than the embryo since during the early stages of development of the nuclear type (observed in cereals, legumes and *Arabidopsis thaliana*, among other species), there is no cytokinesis or cell wall formation (Sabelli and Larkins, 2009).

Four phases for the development of the endosperm have been described in grasses: (1) Syncytial phase: characterized by a high mitotic rate. (2) Cellularization phase: begins when the mitotic rate of the syncytial phase tends to zero. (3) Growth and differentiation phase: different cell types that will be part of the endosperm are differentiated: aleurone cells, transfer cells, starchy endosperm cells and the cells that surround the embryo. In this way, the different parts that will be present in the mature endosperm are defined during this phase. The cellular types observed are: (A) Aleurone: formed by one or more layers of cells surrounding the endosperm. It has several functions, such as the protection against desiccation, the synthesis of hormones and the induction of gene expression for proteolytic and hydrolytic proteins that digest the walls of the endosperm by mobilizing the stored starch and proteins during the imbibition of the seed. (B) Transfer Cells: they are involved in the transport of metabolites, such as amino acids and carbohydrates, between the maternal vascular system and the rest of the endosperm, facilitating the absorption of these metabolites by the endosperm tissue. (C) Starchy Endosperm: it stores mainly starch in amyloplasts. Therefore, it requires a high energy

expenditure in the form of ATP molecules in order to accumulate starch. Its development is closely related to a high metabolic rate of carbohydrates, high levels of cell signalling and the regulation of energy states. (D) Embryo-surrounding region (ESR): it is constituted of several layers of cells that surround the embryo. Only vestiges of this region were observed 12 d after pollination (AP) in maize. The cells of this region are metabolically very active since they supply sugars to the embryo, in addition to participating in the defence against pathogens and in the signalling between the endosperm and the embryo. (4) Maturation Phase: programmed cell death occurs in this phase, dormancy (in some cases) and drying of the seed (Brown and Lemmon, 2007; Nguyen et al., 2007; Sabelli and Larkins, 2009; Bewley et al., 2013).

Apomixis refers to a form of asexual reproduction through seeds, which produces progenies genetically identical to the mother plant (Nogler, 1984). This type of reproduction has been described in more than 300 species belonging to 35 different families of angiosperms (Hanna and Bashaw, 1987). Regardless of its wide distribution in flowering plants, apomixis occurs only in some species of agronomic interest. Although many studies have made important contributions to the understanding of apomixis, their studies have aimed to generate knowledge about genes implicated in the formation and development of the clonal embryo (Tucker and Koltunow, 2009; Ortiz et al., 2013), while few studies have aimed to study genes implicated in the development of the apomictic endosperm. The understanding of endosperm development is crucial to be able to transfer apomixis to the most important crops, allowing to maintain hybrid vigour through reproductive cycles (Felitti et al., 2015; Depetris et al., 2018).

Tetraploid *Paspalum notatum* Flüggé (bahiagrass) reproduces by gametophytic apomixis and pseudogamy is required for endosperm development (Burton, 1948; Quarín, 1999). In most angiosperms, the development of the endosperm depends on the maternal:paternal genomic contributions (m:p), which, according to the theory of the Endospermic Balance Number (EBN), must maintain a 2:1 ratio. However, in apomictic *P. notatum*, the endosperm develops independently of these contributions (EBN insensitive) (Quarín, 1999; Felitti et al., 2015). Therefore, understanding the development of the endosperm in *P. notatum* is fundamental to the perspective of incorporating apomixis in major crops that require a 2m:1p genomic contribution (EBN sensitive) for normal development of the endosperm (Quarín, 1999; Felitti et al., 2015; Depetris et al., 2018). On the other hand, a post-zygotic abortive system has been described in *Paspalum*, where it was observed that after conspecific interploid ($2x \times 4x$) pollinations, the endosperm and the embryo develop, but 96 h later, the endosperm collapses and the embryo arrests its development (Norrmann et al., 1994).

Previously, the gene expression in sexual and apomictic plants of *P. notatum* was characterized at 3 and 24 h AP (immediately after fertilization and syncytial endosperm, respectively) (Felitti et al., 2015; Depetris et al., 2018). Felitti et al. (2015) found DETDFs [differentially expressed TDFs (transcript-derived fragments)] related to proteins that responded to changes in the levels of extracellular ATP (eATP) in *A. thaliana*. This result suggests that eATP would act as a signalling molecule at the beginning of endosperm development (Felitti et al., 2015). Depetris et al. (2018) found DETDFs expected to be involved in sucrose metabolism during the accumulation of carbohydrates in the endosperm and could be related to EBN insensitivity, DETDFs related to the failure of fusion of the polar nuclei in *A. thaliana* and DETDFs associated with a casein kinase II that regulates the accumulation of storage products in seeds of *A. thaliana*. In the present work, we study genes expressed in sexual and apomictic plants ovaries of *P. notatum* 48 h AP, a final stage prior to post-zygotic seed abortion (Norrmann et al., 1994). For this stage, a cellular endosperm was expected (Felitti et al., 2015).

Based on the interest in incorporating apomixis in crops of agronomic importance and due to the need for a deeper knowledge of the development of the endosperm in sensitive and insensitive EBN individuals, the objective of this study was to characterize gene expression in apomictic and sexual plants of *P. notatum* previous to post-zygotic collapse (48 h AP) and the identification of genes related to endosperm development. The results will complement the two prior studies (Felitti et al., 2015; Depetris et al., 2018) and would allow for the description of the complete process of endosperm development in this species.

Materials and methods

Plant material and crosses

Ploidy levels, modes of reproduction, chromosome number and geographic origin of the plant material are described in Table 1 (Martínez et al., 2001; Quarín et al., 2001, 2003; Felitti et al., 2015; Depetris et al., 2018). In order to generate seeds with different ploidy levels in the endosperm, crosses are carried out as detailed in Table 2 and according to Depetris et al. (2018). Ploidy levels of the embryo and the m:p genome ratio in the expected endosperm were obtained from Norrmann et al. (1994), Quarín (1999) and Martínez et al. (2007). Unlike Depetris et al. (2018) in the present work, the ovaries were isolated from the flowers 48 h AP because this moment is preceding the post-zygotic endosperm collapse (96 h AP) (Norrmann et al., 1994). In addition, according to microscopic observations of ovaries 48 h AP, most ovules showed large embryo and endosperm that occupied much of the seed space (Fig. 1).

cDNA-AFLP and functional analysis

The cDNA-AFLP (complementary DNA-amplified fragment length polymorphism) analysis was obtained from the mRNA retrotranscription of the C1, C2, C4, C5 and C6 samples (Table 2), according to Vuylsteke et al. (2007) and Xiao et al. (2009). The combination of restriction enzymes used was *Cvi*AII/*Taq*I, selected through the AFLPinSilico programme (Stölting et al., 2009). Each cDNA sample was pre-amplified and then used as a template in a second selective amplification using 16 primer combinations. The reaction conditions were as follows: 95°C, 5 min; 12 cycles of 94°C, 30 s; 65°C (decrease of 0.7°C each cycle), 30 s; 72°C, 1 min, and 24 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 1 min; followed by a final extension step (72°C, 10 min) as indicated by Felitti et al. (2015). Loading buffer (98% mv^{-1} 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% mv^{-1} denaturing polyacrylamide gels. Electrophoresis was conducted for approximately 3 h at 60 W using 0.5× TBE (100 mM Tris-HCl, 90 mM boric acid, 1 mM EDTA pH 8.0) in the upper tank and 1× TBE and lower tank. The DNA Silver Staining System (Promega, Madison, WI, USA) was used to stain gels.

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

Plant identification[a]	Accession	Chromosome number	Mode of reproduction	Origin
2x S1	H398	20	Sexual	Empedrado, Corrientes, Argentina
2x S2	Q4084-8	20	Sexual	An individual plant collected in a natural population at Cayastá, Santa Fe, Argentina
4x A1	Q4117	40	Apomictic	Río Grande do Sul, Brazil
4x A2	Q3775	40	Apomictic	Municipality of Gómez, Tamaulipas, Mexico
4x S1	Q4188	40	Sexual	Sexual hybrid: Parents: Q3664 (4x predominantly sexual, Tifton, USA) × Q3853 (Capivarí, Rio Grande do Sul, Brazil)
4x S2	C4-4x	40	Sexual	Induced tetraploid derived from a chromosome duplicated callus sector obtained by tissue culture and colchicine treatment of a diploid

[a]Plants are ordered by ploidy levels (2x = diploid, 4x = tetraploid) and reproductive systems (S, sexual; A, apomictic).

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

Female parent[a]	Pollinator[a]	Sample[b]	Expected ploidy of the embryo	Expected ploidy of the endosperm	Expected m:p genome ratio in the endosperm
4x A1	2x S1[c]	C1	4x ($2n + 0$)	9x ($2n + 2n + n$)	8:1
	4x A2[c]	C2	4x ($2n + 0$)	10x ($2n + 2n + n$)	8:2
4x S1	2x S1[d]	C4	3x ($n + n$)	5x ($n + n + n$)	4:1
	4x A2[c]	C5	4x ($n + n$)	6x ($n + n + n$)	2:1
4x S2	2x S3[d]	C6	3x ($n + n$)	5x ($n + n + n$)	4:1

[a]Parents are ordered by ploidy levels (2x = diploid, 4x = tetraploid) and also by reproductive systems (S, sexual; A, apomictic).
[b]C1: Cross 1, C2: Cross 2, C4: Cross 4, C5: Cross 5 and C6: Cross 6.
[c]Crosses that are expected to set seed.
[d]Crosses that are not expected to set seed.

C1, C2 and C4 crosses were used as biological replicates for cDNA-AFLP analysis. Real-time PCR analysis was performed using the Rotor-Gene Q (Qiagen®, Hilden, Germany) thermal cycler as reported by Felitti et al. (2015). Reactions were performed on two biological replicates (different RNA extractions from two experimental conditions), using three technical replicates. Each cycle consisted of denaturation for 15 s at 94°C, annealing for 60 s at 53°C (depending on the primer pair, Supplementary Table S1), and extension for 60 s at 72°C. Quantification cycle (Cq) and efficiency (E) for each amplicon were obtained from the Comparative Quantitation software supplied by Corbett Research for Rotor Gene. β-tubulin was selected as a reference gene to analyse gene expression levels in *P. notatum* ovaries (Pfaffl et al., 2004; Felitti et al., 2015). Normalized expression values were calculated based on amplification efficiency (E) and Cq in comparison to the reference gene according to Simon's formula (Simon, 2003). Data were tested for statistical significance using the Wilcoxon test (Mann–Whithey U), using the Infostat student version software (Di Rienzo et al., 2016).

Obtained TDFs were analysed by the presence or absence of bands in electrophoretic patterns (DETDFs), when the different crosses were compared (C1, C2, C4, C5 and C6), considering the m:p contribution ratio in the endosperm and expected seed formation. TDFs considered differentially expressed were isolated. A re-amplification reaction was conducted using 1 µl of the eluted sample and the same conditions described for the selective amplification reactions. Those bands were sent for sequencing to Macrogen Inc. (Korea). The primer sequences were detected and eliminated using CleanBSequences package of R software (http://www.r-project.org/). The similarity analysis was performed using the BLAST 2.2.25 NCBI site programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The similarity search with the genome of *A. thaliana* was conducted using the tool BLASTp 2.2.8 of the TAIR Arabidopsis Information Resource Site (http://www.arabidopsis.org/Blast/) (Pozzi et al., 2018).

Results

To characterize endosperm development 48 h AP in seeds with different ploidy levels originated by the sexual or apomictic modes, a study of differential gene expression was carried out among crosses that involve different maternal and paternal genotypes, using the cDNA-AFLP technique. An average of 60 fragments was obtained for each primer combination, displaying molecular weights from 100 to 800 bp (Fig. 2; Supplementary Table S2).

Gene expression in endosperm development 48 h AP

Based on the analysis of 16 primer combinations, 66 DETDFs were detected (Table 3) and were considered as differentially expressed because they were polymorphic with respect to the m:p contribution ratio in the endosperm and predicted seed formation. Twelve of the 66 DETDFs were found when the predicted m:p ratio in the endosperm was 4:1 and the female parent was a sexual tetraploid (Table 3, Class A). In this situation, seed formation is not supposed to happen because the m:p genomic contributions are different from 2:1, necessary for endosperm development in a sexual plant of *P. notatum*. Finally, four DETDFs

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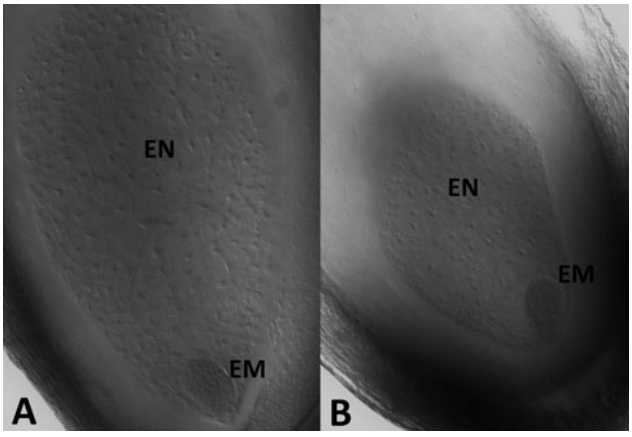

Fig. 1. Embryo (EM) and endosperm (EN) observed in bahiagrass 48 h AP. (a) Seed from a 4x A1 plant (Q4117) pollinated by a 2x S plant (Tifton 9). (b) Seed from a 4x S1 plant (4118) that has been pollinated by a 2x S plant (Tifton 9). Magnification: ×200.

were obtained when the assumed m:p genomic contributions in the endosperm were 2:1 (Table 3, Class B). In this case, seeds could be expected to be formed. Conversely, 10/66 DETDFs were detected when the mother plant was apomictic and the m:p ratio was different from 2:1 (EBN insensitive) (Table 3, Class C). Also, 4/66 DETDFs were obtained when the female plant was apomictic (Table 3, Class D), with 4:1 and 8:1 m:p genomic contributions and also when the mother plant was sexual and tetraploid with the m:p ratio 2:1. Two DETDFs were expressed in crosses where the mother plant was sexual and tetraploid; hence, seed production was not expected (due to the 4:1 m:p ratio and its EBN sensitivity) (Table 3, Class E). Sixteen DETDFs were observed in crosses where the m:p ratio was 4:1, with a sexual tetraploid female (seed production was not assumed), and in an apomictic female that still produce seed despite its insensitivity to EBN (Table 3, Classes F and I). Two DETDFs were obtained in crosses where the mother plant was sexual tetraploid (m:p ratio 2:1), and in a fertile apomictic female with the 8:1 m:p ratio, set seed was predicted (Table 3, Class G). Additionally, 12/66 were detected in crosses from apomictic tetraploid (m:p ratio 4:1) and sexual tetraploid mother plants (m:p ratio 2:1). In both cases, seed development was expected (Table 3, Class H). In closing, two DETDFs were observed in crosses involving apomictic tetraploid females with the predicted m:p ratio of 4:1 and 8:1 (EBN insensitive) and two more DETDFs when the mother plant was sexual and tetraploid, resulting in genomic ratios 2:1 and 4:1 (Table 3, Class J).

Thirty-nine of the 66 isolated DETDFs were precisely sequenced and functionally categorized (Supplementary Table S3). The rest of the DETDFs (27/66) were discarded because they presented short sequences (less than 50 pb) and poor quality. 14/39 DETDFs were observed in crosses for which seed production is predicted. Four of them belonged to Class D (AC1, AC2, AC3 and GG1), two to Class G (GG2 and GG3) and eight to Class H (AC8, AA2, AA3, AA4, AA6, AA7, AA8 and AA11), respectively (Table 3; Supplementary Table S3). Two of the DETDFs obtained from crosses for which seed production is not expected belonged to Class E (AC4 and AC5) (Table 3; Supplementary Table S3). In addition, it was determined that the majority (40/66) of the DETDFs obtained correspond to crosses for which seed production is expected (Table 3; Supplementary Table S3). The technique was

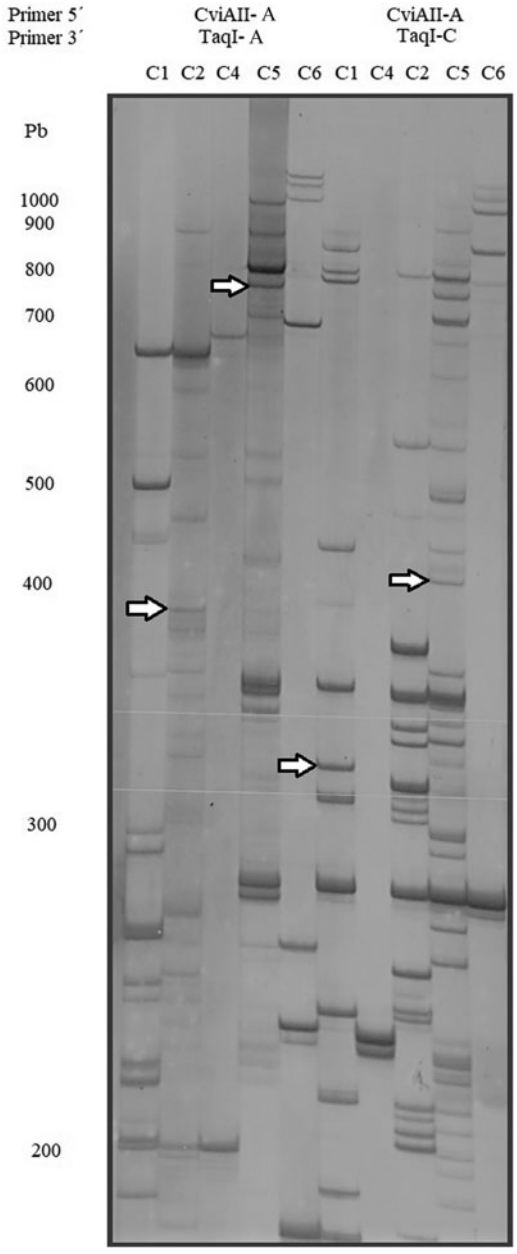

Fig. 2. Polyacrylamide gel with a typical band pattern using the cDNA-AFLP methodology. Transcript-derived fragments resulting from selective amplification used the next two combinations of primers (*Cvi*AII/*Taq*I): A/A and A/C (indicated in the top of the figure). Rows correspond to six crosses: Cross 1 (C1): Q4117 (4x A1) × H398 (2x S1), Cross2 (C2): Q4117 (4x A1) × Q3775 (4x A2), Cross 4 (C4): Q4118 (4x S1) × H398 (2x S1), Cross 5 (C5): Q4118 (4x S1) × Q3775 (4x A2) and Cross 6 (C6): C4-4x (4x S2) × Q4084-8 (2x S2). Arrows indicate examples of some of DETDFs obtained. Combination primers A/A: AA2, AA3, AA4, AA6, AA7, AA8, AA11 and AA17 DETDFs were obtained. Combination primers A/C: AC1, AC2, AC3, AC4, AC5 and AC8 DETDFs were obtained.

validated for the real-time experiment: expression profiles were confirmed for AC3 ($P = 0.0040$; $\alpha = 0.05$; Class D), AC5 ($P = 0.0079$; $\alpha = 0.05$; Class E) and GG2 DETDFs ($P = 0.0381$; $\alpha = 0.05$; Class G) (Fig. 3).

Functional categories

Thirty-nine DETDFs were arranged in nine functional categories. The majority of the genes were involved in metabolism (23%), signalling (13%), transcription (10%) and protein synthesis (8%).

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Table 3. Classes of DETDFs based on the comparative gene expression patterns

Expression pattern	Expected seed set	Class	APO[a]		SEX[a]		Total of DETDF's
			4:1[b]	8:1[b]	2:1[b]	4:1[b]	
Sexuals only (EBN sensitive)	Yes	A				X	12
	No	B			X		4
Apomictics only (EBN insensitive)	Yes	C	X	X			10
Apomictics and Sexuals	Yes/Yes/Yes	D	X	X	X		4
	No/No	E				X	2
	Yes/No/No	F	X			X	13
	Yes/Yes	G		X	X		2
	Yes/Yes	H	X		X		12
	Yes/No	I	X			X	3
	Yes/Yes/Yes/Yes/No	J	X	X	X	X	4
Total							**66**

[a]Reproductive mode of the female parent.
[b]Predicted maternal:paternal ratio in the endosperm.

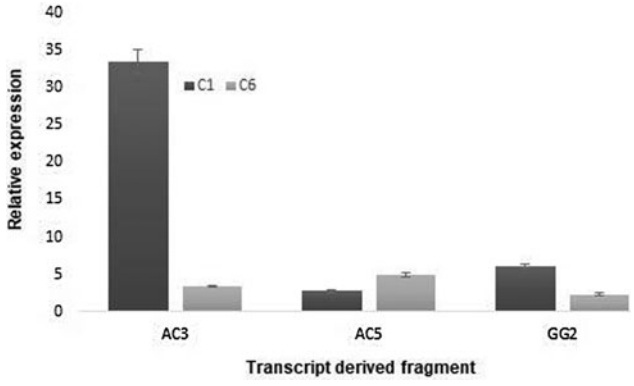

Fig. 3. Real-time PCR showing the expression levels for selected TDFs that are indicated below the axis. Each TDF was analysed separately. Columns show relative expression values (normalized expression relative to the lowest normalized value for each gene) and bars indicate errors. Crosses are indicated in the figure.

Fewer DETDFs were associated with the categories disease/defence, proteins of transport and storage, intracellular transport (5% of each category) and finally to the category cell structure (3%). The rest of the analysed sequences (28%) were included in the category unknown function (Supplementary Table S3; Fig. 4).

The DETDFs AC1, AC2, AC3, AC4, AC5, GG1 and GG3 (Supplementary Table S3) were studied in *A. thaliana* and was diagnosed that they were involved in signal transduction, response to abscisic acid (ABA), transcription and protein synthesis functions during endosperm development, especially in late stages of seed formation (maturation and dormancy).

Discussion

Appropriate endosperm development is crucial for the growth of the embryo and assembly of a viable seed. Therefore, knowing the mechanisms that regulate endosperm development is of significant agronomic importance for seed production because it would allow an increase in the yield and quality of the grains. Also, the transfer of apomixis into sexual crops is a prime target because it would maintain hybrid combinations of interest across reproduction cycles, with great impact on the economy. Thus, in order to transfer apomixis to crops where the viable seed must satisfy the EBN 2:1 condition, it is fundamental to understand in depth the development of the endosperm. Due to the above, in the present work, the differential gene expression of endosperm development (48 h AP) in apomictic and sexual *P. notatum* plants was evaluated. The last time point analysed (48 h AP) corresponded to cellular endosperm and is prior to the post-zygotic collapse (Norrmann et al., 1994). In addition, this analysis differed regarding the state of endosperm development with respect to 3 h AP (immediately after fertilization) (Felitti et al., 2015) and 24 h AP (syncytial endosperm) analysed stages (Depetris et al., 2018).

In the present work, DETDFs were functionally categorized similar to the ones proposed in the analysis corresponding to 3 h AP (Felitti et al., 2015) and 24 h AP (Depetris et al., 2018). In Felitti et al. (2015), the majority of DETDFs were grouped in the categories: *transcription* (10%), *signal transduction*, *metabolism* and *cell structure* (8% each). In Depetris et al. (2018), the higher number of DETDFs were grouped in *signal transduction* (15%), *metabolism* (8%) and *energy* (6%). In this study, 39 DETDFs were identified and classified into nine functional categories. The majority of DETDFs were classified into two functional categories: *metabolism* (23%) and *signal transduction* (13%) (Fig. 4). These results are similar to those found at 3 and 24 h after occurrence of pollination in *P. notatum* (Felitti et al., 2015; Depetris et al., 2018). The fact that the highest percentage of DETDFs identified belong to the functional categories *metabolism* and *signal transduction* is consistent with what was expected for development of the seed in grasses and cereals, since the energy requirements increase, besides the extracellular signals that would be triggering responses that lead to the formation (or not) of seeds (Brown and Lemmon, 2007; Nguyen et al., 2007; Sabelli and Larkins, 2009; Depetris et al., 2018).

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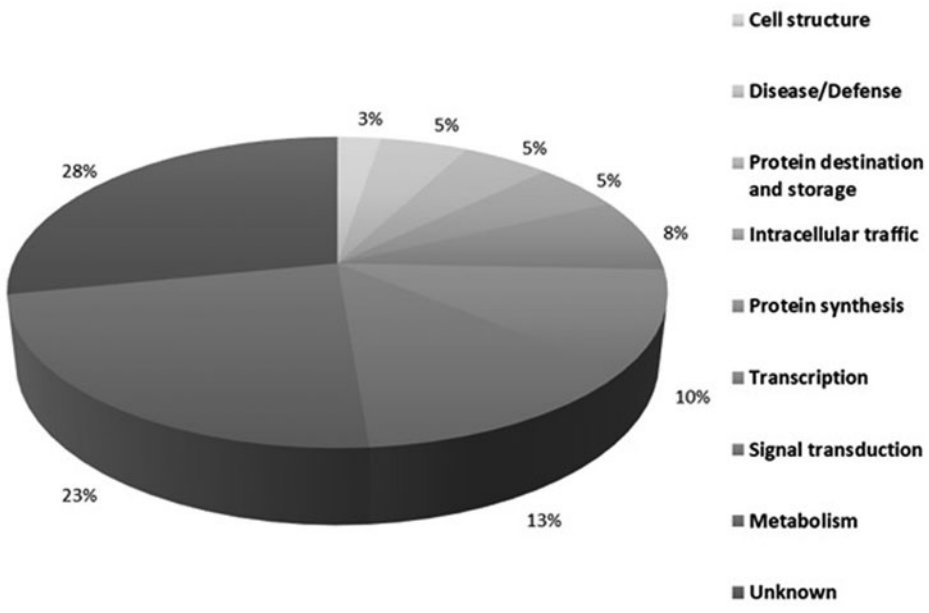

Fig. 4. Functional categories assigned to 39 differential expression transcript-derived fragments identified by complementary DNA-amplified fragment length polymorphism analysis. The transcripts were isolated and sequenced from ovaries obtained at 48 h AP from the different crosses already described. 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.

In this study, DETDFs AC1, AC2, AC3, AC4, AC5, GG1 and GG3 (Supplementary Table S3) offered relevant information about endosperm development in seeds of *P. notatum*. AC1, AC4 and AC5 showed a high similarity with a *locus* of *A. thaliana* (AT5G67380; e^{-110} Blastp) that codes for a casein kinase alpha1 (CKA1), which is part of the casein kinase 2 complex (CK2). In *A. thaliana*, CK2 is implicated in signalling and in the response to environmental and physiological conditions, in cell division and in DNA replication. CK2 is related to plant growth and development, in addition to being involved in signalling mediated by ABA (Finkelstein et al., 2002; Wang et al., 2014; Vilela et al., 2015; Depetris et al., 2018). In maize, CK2 phosphorylates the transcription factor Opaque2 (O2), which activates zein genes. This activation leads to an increase in the amount of storage proteins (in the form of zeins) in the endosperm of the seed. Under different environments and physiological conditions, CK2 controls the participation of Opaque2 in the regulation of zein genes (Grasser et al., 1989; Ciceri et al., 1999; Kemper et al., 1999; Łebska et al., 2010; Depetris et al., 2018). DETDFs AC4 and AC5 correspond to Class E and AC1 to Class D. Thus, their functions are related to crosses in which no seeds were expected to be formed for Class E, and crosses were expected to set seed for Class D (Supplementary Table S3). In crosses where seed production was not expected, two DETDFs were found. However, in crosses expected to set seeds, a single DETDF was found. This could indicate the presence of a mutation in the *locus* that codes for CK2. This mutation could have generated a new cut-off site for the restriction enzymes used in the cDNA-AFLP technique, or, alternatively, it could come from a differential splicing of genes. This mutation or differential splicing may be generating, in crosses for which seed production is not expected, an aberrant CK2 protein which would lead to the seed not developing due to problems in the accumulation of endosperm proteins (Depetris et al., 2018). That is, the accumulation of casein protein kinase II (AC1, Class D) would trigger success in the production of seeds, or the accumulation of its mutated form (AC4 and AC5, Class E), would trigger the failure in the formation of the seeds. In the analyses carried out at 3 and 24 h AP in *P. notatum* (Felitti et al., 2015; Depetris et al., 2018), sequences associated with CK2 were found in crosses in which seed production was expected and in crosses in which seed production was not expected. These results were consistent with the results obtained in this study at 48 h AP and demonstrated the importance of casein kinase II during the different stages of seed development.

The AC2 DETDF (Class D, Supplementary Table S3) showed high similarity to AT1G74520 *locus* ($8e^{-35}$ Blastp) that encodes a HVA22 protein that was expressed in the presence of ABA. HVA22 is required for maturation of the seed and to maintain seed dormancy. ABA is essential to maintain seed dormancy, and gibberellin (GA) is necessary for seed germination. The expression of the *HVA22* barley gene coincides with the dormant state of the seed: the HVA22 mRNA progressively accumulates in the aleurone layer (endosperm) during the late stage of seed maturation, when the levels of biosynthesis of endogenous ABA are elevated in order to maintain seed dormancy. HVA22 accumulation is induced by ABA, which negatively regulates the formation of GA-mediated digestive vacuoles, an important aspect in cell death programmed in aleurone cells. In this way, the mobilization of nutrients towards the embryo is prevented, slowing the germination of the seeds and the growth of the seedlings (Guo and Ho, 2008).

The AC3 DETDF (Class D, Supplementary Table S3) showed a high similarity to a *locus* of *A. thaliana* (AT5G22650; $2e^{-28}$ Blastp) that encodes a histone deacetylase 2B (HD2B), which modifies the structure of chromatin. Such changes determine the accessibility of transcriptional factors to target DNA and contribute to the regulation of gene expression. This epigenetic regulation is closely connected with cell differentiation (Lee et al., 2016). The *HD2B* gene has been identified as a genetic factor associated with seed dormancy and germination in *A. thaliana* (Yano et al., 2013). It has been shown that HD2B of wheat acts as a substrate and is phosphorylated by CK2 (Dennis and Browning, 2009). Moreover, the study of the expression of the HD2 genes showed that they are expressed in all the tissues of the barley seed and at all stages of its development. These results suggest a close interconnection between the expression of these genes and the enlargement of the seed (Demetriou et al., 2009).

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In addition to the above, it was shown that these genes react to exogenous hormones, such as jasmonic acid (JA), ABA and salicylic acid (SA), all of them related to stress situations for the plant. These observations would indicate that these genes are associated with resistance to abiotic stress (Demetriou et al., 2009). In addition, it has been suggested that *HD2* genes would have a possible role in epigenetic regulation during seed development (Demetriou et al., 2009).

The GG1 DETDF (Class D, Supplementary Table S3) showed high similarity to AT2G24260 *locus* ($2e^{-57}$ Blastp) that codes for the Defective Pollen Region 1 protein (DROP1). Zhang et al. (2017) demonstrated through RNA-seq experiments that DROP1 is expressed mainly in seeds. On the other hand, the mutants for DROP1 in *A. thaliana* produce pollen without sperm cells but which behaved like the wild-type. These results showed that the sperm cells are dispensable for the normal development of the pollen tube (Zhang et al., 2017).

The GG3 DETDF (Class G, Supplementary Table S3) was highly similar ($2e^{-36}$ Blastp) to AT5G12020 *locus*, which encodes a 17.6 kDa Class II heat-shock protein (HSP17.6II). *Hsp* genes increase their expression from stage 7 to stage 10 of seed development in *A. thaliana* (Kotak et al., 2007). The abundance of the *Hsp* genes during the late maturation of the seed has demonstrated the important role of these genes in the development of the seed (Kotak et al., 2007).

Finally, the results of gene expression characterization at 48 h AP in plants of *P. notatum* showed evidence that some of the most important cellular functions at that moment of seed development were *metabolism* and *signal transduction*. During this stage of endosperm development, metabolic processes predominate, especially those associated with the synthesis of carbohydrates and the signalling between the endosperm and embryo and between seed tegument and endosperm (Brown and Lemmon, 2007; Sabelli and Larkins, 2009). Also, the DETDFs AC1, AC2, AC3, AC4, AC5, GG1 and GG3 were expressed at different stages of seed development and would probably be related to endosperm development and to the presence or absence of viability of the seed in *P. notatum*. Some DETDFs were associated with late stages of seed development (maturation and dormancy) and demonstrated to be ABA sensitive, suggesting that *P. notatum* seeds begin expressing genes related to these processes 48 h AP. In addition, the results obtained at 3, 24 and 48 h AP indicate that CK2 protein might play an important role in seed viability in *P. notatum* (Felitti et al., 2015; Depetris et al., 2018). Therefore, the present work included DETDFs related to the success or failure of the development of the endosperm and, consequently, to the occurrence or absence of seed formation. These results allow a deeper comprehension of endosperm development and normal seed formation in systems independent of the 2:1 EBN, enabling an approach to introducing apomixis into sexual crops.

Supplementary material. To view supplementary material for this article, please visit: https://doi.org/10.1017/S096025852000015X.

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Conflicts of interest. The authors declare that they have no conflict of interest.

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