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Xenia in bahiagrass: gene expression at initial seed formation

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Abstract

Xenia is the direct effect of pollen genotype on the development and characteristics of the seed and fruit in the period that spans from fertilization to seed germination. Xenia effects cause phenotypic variations in the seed and fruit, which have importance for seed and fruit production but are not heritable to the progeny. Two hypotheses have been proposed as a mechanism for xenia: the hormonal hypothesis and the mobile mRNAs hypothesis. Although xenia effects have been studied in seeds and fruits in many crops, its effects and mechanism have been poorly studied at the molecular level. The aim of this work was to perform an initial screening of the xenia effect in the hybrid endosperm at the molecular level by differential gene expression among different pollen genotype sources from *Paspalum notatum* Flüggé. In order to characterize xenia effects and mechanisms, crosses were made between an emasculated mother plant with donors from two different pollen genotypes. RNA was isolated from ovaries 3 h after pollination. Some of the 24 differentially expressed transcript-derived fragments (DETDFs) provided relevant information. Four of those DETDFs were related to germination, pollen tube growth and pollen–pistil interaction. Seven DETDFs were associated with seed development and production. Finally, four DETDFs were predicted to encode for mobile mRNAs. These DETDFs might be involved in xenia effects and mechanisms in *P. notatum*.

Introduction

Xenia is defined as the direct effect of pollen genotype on the development and characteristics of the seed and fruit (including embryo, endosperm and maternal tissues) in the period that spans from fertilization to seed germination (Rieger *et al.*, 1968; Denney, 1992). As a consequence of xenia effects, some variations in the phenotypic characteristics of the seeds or fruit from the same maternal parent are the result of fertilization by different pollen sources, but they are not inherited to the progeny. This effect is of great agronomic importance for the production of fruits and seeds, having applications in plant breeding, and in the research to increase grain yield and/or the size and quality of the fruit (Denney, 1992; Bullant and Gallais, 1998; Dogterom *et al.*, 2000; Kaczmarek *et al.*, 2001; Weingartner *et al.*, 2002; Liu, 2008; Liu, 2018).

The xenia effect has been demonstrated in crops such as alfalfa (*Medicago sativa* L.), where pods of larger size and greater number of seeds were obtained after cross-fertilization (Bullant and Gallais, 1998). The results were similar in rye (*Secale cereale* L.). In this species the comparison of the size and weight of 1000 grains of an inbred line and the same line crossed with a tester, rendered the highest levels of the variable for the cross-pollinated plants. In addition, it was shown that the smaller the seed resulting from an inbred line, the higher the effect of the pollen exerted in cross-pollination (Kaczmarek *et al.*, 2001). In blueberry (*Vaccinium corymbosum* L.), xenia increased the number of seeds and also the mass and quality of fruits (Dogterom *et al.*, 2000). Xenia was demonstrated in tomato (*Solanum galapagense* S. and *Solanum lycopersicum* L.), where a wild male parent increased the density of trichomes of the fruit and decreased the size of the seeds when it was crossed with cultivated genotypes (Piotto *et al.*, 2013). Most studies about xenia have been made in maize (*Zea mays* L.), due to its pollination mode. It was demonstrated that the xenia effect in this crop contributed an increase of 35% in grain yield due to increments of the levels of zeins proteins. This increase in protein accumulation was attributed to the elongation of the effective filling period of grain (Bullant and Gallais, 1998). It was also shown that the highest grain yield for this crop was obtained by combining male sterility with xenia. In addition, it has been proposed that the effects of pollen are greater when more genetic distance exists between the female and male parent (Weingartner *et al.*, 2002).

Although xenia remains a poorly understood biological process at the molecular level, many authors have proposed a mechanism for this effect, and elaborated different hypotheses

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to explain its occurrence. Many of them point to molecules that diffuse through the maternal tissues whose synthesis depends on the pollen genotype that fertilizes the embryo sac. These molecules were called gemmules by Darwin (1868), or hormones by Swingle (1928) and Dag and Mizrahi (2005), but Denney (1992) proposed that the cause of xenia may be the mRNAs as mediators hypothesis. Evidence suggested that these molecules would be represented by mRNAs and microRNAs (miRNAs) (Kim *et al.*, 2001; Kudo and Harada, 2007). The RNA molecule can act within the cell where it was generated or as a signal molecule, moving between cells and regulating gene expression of other tissues. Hence they are able to cause phenotypic effects in the development of the target tissue (Kim *et al.*, 2001; Kudo and Harada, 2007; Piotto *et al.*, 2013). During fertilization, pollen would release mRNA molecules, which would diffuse into the tissues of the mother plant causing changes in colour, size and chemical composition of seeds and fruits. These changes depend on the particular male parental (Liu, 2008; Liu, 2018).

Endosperm results from fertilization of the central cell by one sperm cell, and this tissue is an important source of energy and proteins for humans and animals (Brown and Lemmon, 2007). The value of cereals, such as maize and wheat, are largely determined by the endosperm. Parental imprinting affects endosperm development and thereby seed size and chemical composition (Sabelli and Larkins, 2009). In addition, this tissue has been central throughout the evolution of the concept of xenia. In consequence, studies on endosperm development with different pollen donors are fundamental for understanding the xenia effect (Denney, 1992). Therefore, *Paspalum notatum* Flüggé (bahiagrass) is the species used in this study to analyse the xenia effect at the molecular level in the hybrid endosperm. This species is used as a model in studies of plant reproductive genetics. *Paspalum notatum* is multiploid, including a diploid cytotype and several polyploids, of which 98% are tetraploid (Tischler and Burson, 1995; Pozzobon and Valls, 1997). The apomixis in the polyploid cytotypes is gametophytic and includes apospory (nucellar cells develop unreduced embryo sacs), parthenogenesis (embryo development without fertilization of the egg cell), and pseudogamy (endosperm formation following fertilization of the polar nuclei) (Martínez *et al.*, 2003; Depetris *et al.*, 2018). Therefore, in apomictic individuals, fertilization of the egg cell is not required, although fertilization of polar nuclei (pseudogamy) is necessary for endosperm development (Burton, 1948, Quarin, 1999). Thus, the contribution of the direct effect of pollen genotype (xenia effect) on the hybrid endosperm could be evaluated in this species by analysing crosses in which apomictic plants are used as female parents. In our approach, the use of such pseudogamous aposporic species to study xenia effects is necessary because of technical difficulties to separate the embryo from other seed tissues to obtain RNA samples. As the embryo originates directly from maternal cells, heritable to progeny pollen effects are avoided and only pollen genotype effects on endosperm and other seed tissues (i.e. xenia effects) can be assessed at the molecular level by means of the plant materials employed in this experiment.

Due to the importance of the xenia effect in plant breeding of species of agronomic interest, the absence of information on its mechanism of action at the molecular level, and the importance of the endosperm in seed production, the aims of this work were: (1) to perform an initial screening of a xenia effect in hybrid endosperm development at the molecular level by studying the differential gene expression in crosses among the same pseudogamous aposporic maternal plant with two different paternal genotypes; (2) to evaluate if the differentially expressed genes detected exert functions during the development of the seed; and (3) to explore the metabolic processes and the signalling molecule (s) that mediate the xenia effect in the endosperm in this species.

Table 1. Crosses and unpollinated control mother plant genotypes

Sample[a]	Parental genotypes[b]	
	Female	Male
C1	Q4117 (4*x* A)	H398 (2*x* S)
C2	Q4117 (4*x* A)	Q3775 (4*x* A)
T	Q4117 (4*x* A)	–

[a]C1, cross1; C2, cross2; T, unpollinated mother plant (control). [b]Parental genotypes were classified according to ploidy level (2*x* = diploid, 4*x* = tetraploid) and reproduction mode (S = sexual, A = apomictic).

Materials and methods

Plant material and crosses

Experimental crosses from *P. notatum* were made to obtain fertilized embryo sacs with two different pollen genotypes: Q4117xH398 (4*x* pseudogamous aposporic × 2*x* sexual) and Q4117xQ3775 (4*x* pseudogamous aposporic × 4*x* apomictic). In addition, an emasculated and unpollinated mother plant (genotype Q4117, 4*x* apomictic) was used as the control (Table 1). The crosses were carried out at the Instituto de Botánica de Noreste (IBONE – CONICET), Corrientes.

Female plants were emasculated by eliminating the anthers immediately after anthesis in a humid chamber (Burton, 1948). Pollen from the plants used as the male parent was collected at anthesis by introducing the inflorescences into an envelope. Finally, this pollen was used to pollinate the previously emasculated inflorescence.

In order to detect the xenia effect at endosperm level during the formation of *P. notatum* seeds, ovaries were isolated from the flowers with dissecting forceps under a stereo microscope, 3 h after pollination. At this stage, the fertilization of the polar nuclei has already occurred (Burson, 1987). A minimum of 20 ovaries from each cross were harvested and frozen in liquid nitrogen (Felitti *et al.*, 2015).

RNA isolation, cDNA synthesis and cDNA-AFLP analysis

The cDNA-AFLP (DNA copy-Amplified Fragment Length Polymorphism) profiles were obtained from the mRNA retrotranscription of the Q4117 (T), Q4117xH398 (C1) and Q4117xQ3775 (C2) samples, according to the protocol described by Vuylsteke *et al.* (2007) and Xiao *et al.* (2009). The combination of restriction enzymes used was *Cvi*AII/*Taq*I, selected through the AFLPinSilico program (Stölting *et al.*, 2009). The combinations of the primer pairs were used for pre-selective and selective PCR programs (see supplemental table S1) (Vuylsteke *et al.*, 2007). Each cDNA sample was pre-amplified and then used as template in a second selective amplification using 16 primer combinations. Pre-amplified samples were diluted 1/3 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; 72°C for 1 min, and

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24 cycles of 94°C for 30 s; 55°C for 30 s; 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% m v^{-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% m v^{-1} denaturing polyacrylamide gels. In order to estimate the molecular weights of the bands of DETDFs (differentially expressed TDFs [transcript-derived fragments]), a molecular weight marker (100 to 1500 b.p.; New England Biolabs, Ipswich, MA, USA) was loaded into the 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, both buffers with pH 8.0. The DNA Silver Staining System (Promega, Madison, WI, USA) was used to stain gels. Technical replicates were performed to determine the reproducibility of the technique (data not shown). Biological replicates of samples and validation of the technique by real-time PCR were reported by Felitti *et al.* (2015): total RNA was obtained using SV Total RNA isolation system (Promega). Two biological replicates (different RNA extractions from two experimental crosses) were used in real-time PCR experiments. One of the RNA samples was the same as that used in the cDNA-AFLP analysis, and the second RNA sample was obtained from ovaries of a replicated cross of *P. notatum* genotypes. cDNA was synthesized, DETDF-specific primer sequences were designed with Primer3 v 0.4.0 software (http://primer3.sourceforge.net). Real-time PCR analysis was performed using the Rotor-Gene Q (Qiagen, Hilden, Germany) thermal cycler. Reactions were performed on two biological replicates (different RNA extractions from two experimental crosses), using six technical replicates. 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 suitable reference gene to analyse gene expression levels in *P. notatum* ovaries (Pfaffl *et al.*, 2004). Normalized expression value for each gene was calculated based on amplification efficiency (E) and Cq in comparison with the reference gene according to Simon's formula (Simon, 2003). Data were tested for statistical significance using the Kruskal–Wallis test. Statistical analyses were performed using agricolae package of R software (http://www.r-project.org/) (Felitti *et al.*, 2015).

In apomictic *P. notatum*, embryo develops by parthenogenesis (clonal embryo) but endosperm develops following fertilization of the polar nuclei (hybrid endosperm) (Martínez *et al.*, 2003). Therefore, for the analysis of the differential expression of genes in respect to xenia, the TDFs simultaneously present in T, C1 and C2 were considered as maternal origin. TDFs present in both C1 and C2 were then analysed as an effect of the interaction between the clonal embryo and the hybrid endosperm and just those TDFs differentially present in C1 or C2 were assigned to pollen genotype effect (xenia) at endosperm or other seed tissues level. TDFs were considered differentially expressed (DETDFs) when they differed in either presence/absence or intensity along the different crosses (C1 and C2) and the control mother plant (T).

Isolation, sequencing of DETDFs and bioinformatic analysis

DETDFs considered as a paternal (or xenia) effect were isolated, 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 and excised from the polyacrylamide gel, crushed with a micropipette tip and incubated in 30 µl of elution buffer (0.5 M NH_4Ac, 1 mM EDTA, pH 8.0) with occasional vortexing for 4 h at 37°C. A re-amplification 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% (m v^{-1}) 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 Inc. (Korea). The sequences and chromatograms provided by Macrogen Inc. were analysed. The corresponding specific primer sequences were detected and eliminated using CleanBSequences package of R software (http://www.r-project.org/). The sequences shorter than 41 b.p. were not included. Unique sequences of high quality were analysed using parameters of best similarity to infer protein functions. The similarity analysis was performed using the BLAST 2.2.25 NCBI site program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Searches were confined to plant species (plants taxid: 3193) and examination of the following databases: Expressed Sequence Tags (ESTs), BLASTn (blasted on the non-human, non-mouse expressed sequence tag) and then, the best ESTs were blasted on non-redundant protein sequences (nr) BLASTx. The similarity search with the genome of *Arabidopsis thaliana* was conducted using the tool BLASTp 2.2.8 of the TAIR Arabidopsis Information Resource Site (http://www.arabidopsis.org/Blast/).

Xenia effect observations

In order to characterize xenia effect in seeds of *P. notatum* at phenotypic level, seeds of genotypes Q4064 and Q4294 (4*x* pseudogamous aposporic) were collected after self- and open-pollination to compare seed set, and to measure the length and width of the seed. The measurements were made using ImageJ2 software (Rueden *et al.*, 2017). Data were tested for statistical significance using Student's *t*-test. Three replications were made for each treatment.

Results

In order to characterize xenia effect at endosperm level by studying the differential gene expression between crosses involving different paternal genotypes using cDNA-AFLP analysis, two genotypes of *P. notatum* were used as pollen donors. On average, patterns of approximately 60 fragments had been produced by each primer combination, with molecular weights ranging from 100 to 800 b.p. Figure 1 shows a section of a typical cDNA-AFLP polyacrylamide gel.

Differential gene expression for study of xenia effect

Based on the analysis of 16 primer combinations, 11 of them showed amplification products. Combinations *Cvi*AII/*Taq*I: A/C, A/G and G/T were removed from the analysis because they did not show amplification products in sample C2 (Fig. 1). Of the total TDFs detected, 39 were considered as maternal origin, 10 were considered as interaction between the clonal embryo and the hybrid endosperm effect, and 33 (28 corresponding to C1 and five corresponding to C2) were considered as xenia effect at endosperm level and were isolated from the gel. Twenty-four of the 33 DETDFs (23 to C1 and one to C2) were successfully re-amplified, sequenced and classified by function (Table 2).

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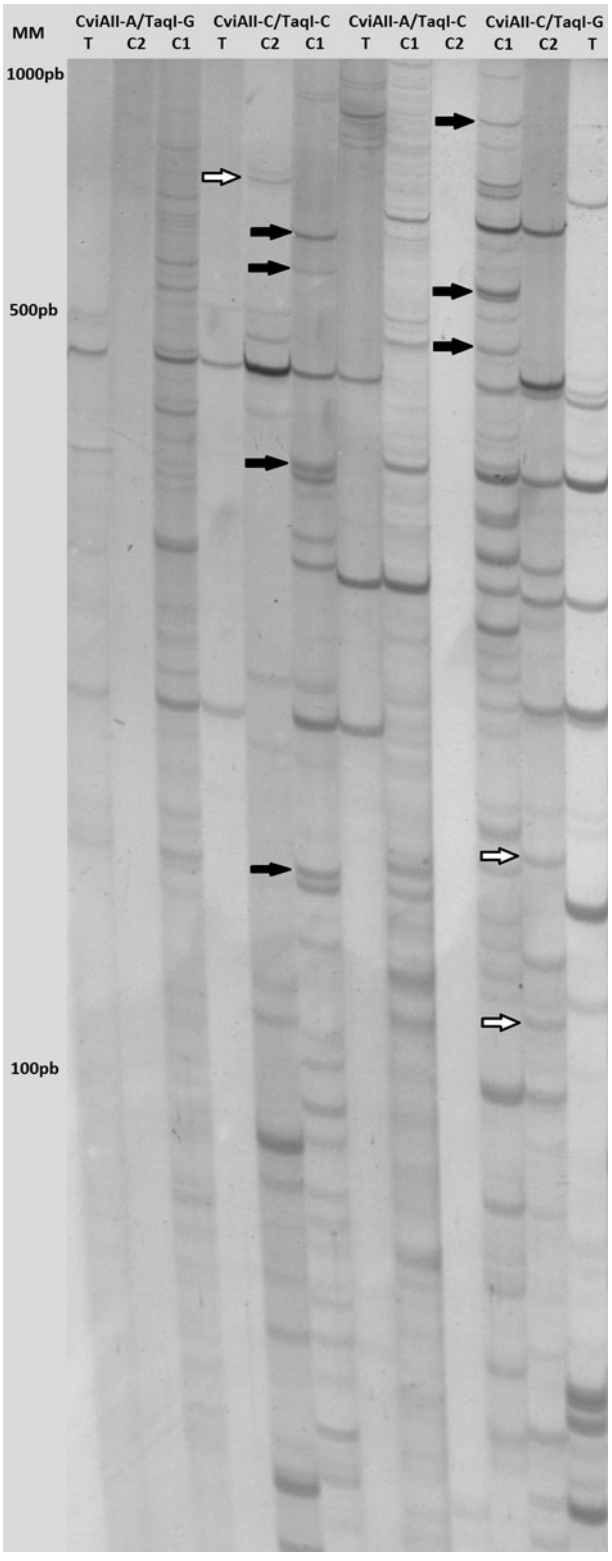


Fig. 1. Polyacrylamide gel with typical band pattern using the cDNA-AFLP methodology. Transcript-derived fragments resulting from selective amplification used the next four combinations of primers (CviAII/TaqI): A/G, C/C, A/C and C/G (indicated at the top of the figure). Rows correspond to unpollinated mother plant (T): Q4117 (4*x* A) and two crosses: Cross 1 (C1): Q4117 (4*x* A) × H398 (2*x* S) and Cross2 (C2): Q4117 (4*x* A) × Q3775 (4*x* A). A/G and A/C primers combinations did not show amplification products in sample C2 (removed from the analysis). In C/C and C/G primer combinations, black arrows indicate examples of some of DETDFs that were only present in C1 cross, and white arrows indicate examples of some of DETDFs that were only present in C2 cross.

Sequence analysis

The 24 DETDFs obtained were expressed only in one or another analysed cross and not in the control, therefore they were considered as related to xenia effect in *P. notatum* (Table 2). The sequences CG7, GG5, GA1 and TG2 were characterized in *Arabidopsis thaliana* and it was determined that they were related to pollen effect in the metabolic processes during germination and development of the pollen tube and pollen–pistil interaction. The sequences CG5, GA4, GA6, GG5, CG3, TG2 and CC1 were related to the number and size of seeds (Wang *et al.*, 2008a; Hajduch *et al.*, 2010; Bourdenx *et al.*, 2011; Missbach *et al.*, 2013; Voiniciuc *et al.*, 2015; Salas-Muñoz *et al.*, 2016). The sequences CC3, GA4, GA6 and GA2 were associated with *A. thaliana* loci encoding mobile mRNAs that can be moved cell to cell in the same tissue, or to nearby or distant tissues via phloem (Thieme *et al.*, 2015). The expression patterns of some DETDFs were analysed by real-time PCR and published in Felitti *et al.* (2015). The expression profiles were confirmed for four out of the five DETDFs selected.

Xenia effect at phenotypic level in P. notatum

The comparisons of self- and open-pollinated seeds within genotypes Q4064 and Q4294 in *P. notatum* are presented in Table 3. The results showed that the higher values for percentage seed set, length and width of seeds were obtained from open-pollinated plants, although the differences in percentage of full seeds between self- and open-pollinated seeds were not significant. In the Q4064 genotype, the mean value of the number of full seeds was 41 and 123 in self- and open-pollinated types, respectively. In the Q4294 genotype, the mean value of the number of full seeds was 50 and 151 in self- and open-pollinated types, respectively (Fig. 2).

Discussion

Numerous studies have reported xenia effects on seed and fruit characteristics in species of agronomic interest. These studies showed the importance of the effect of pollen on grain yield, size, colour and chemical characteristics, and their implications for breeding and seed production (Bullant and Gallais, 1998; Dogterom *et al.*, 2000; Kaczmarek *et al.*, 2001; Weingartner *et al.*, 2002; Piotto *et al.*, 2013; Wang *et al.*, 2013).

However, only one study of xenia at the molecular level in an apomictic species has been reported (Wang *et al.*, 2013). There appeared to be great phenotypic variability (length, diameter and weight) in seeds and fruits of hickory depending on the pollen used. The authors found that the progeny showed substantial differences when compared with the male progenitor. However, when they analysed the offspring with random amplified polymorphic DNA (RAPD) and AFLP markers they did not find so many differences compared with the female progenitor. It is essential to provide more molecular information on xenia in order to generate new tools to assist plant breeding in the search for higher grain yield and better fruit quality. Thus, in the present work, biological function was assigned to DETDFs obtained for each cross used when compared with each other and with an unpollinated control. Different pollen pools generated different patterns of gene expression. It was determined that some DETDFs were associated with reproductive mechanisms, specifically identifying genes associated with double fertilization and development of the

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Table 2. Transcript classes and sequence search results of clones with significant homologies

Cross	DETDFs	EST with BLASTn ID; name; (e-value)	Protein by BLASTx of the EST best match ID; name; (e-value)	TAIR BLASTp ID; GO function; (e-value)	Processes
C1	CC1	FL254388.1; 16661026 CERES-504 *Zea mays* cDNA clone; $4e^{-04}$	NP_001148104.1; uncharacterized protein LOC100281712 [*Zea mays*]; $5e^{-09}$	AT5G67230; Transferase activity; $2e^{-60}$	Metabolism
	CC3	CT857629; *Oryza sativa* Indica Group cDNA clone OSIGCFA222E11; $1e^{-1}$	PREDICTED: D-3-phosphoglycerate dehydrogenase 1, chloroplastic [*Oryza sativa* Japonica group]; $4e^{-30}$	AT1G17745; Protein binding; 0.0	Metabolism
	CG1	GW883551.1; Ti_Con_121 Timothy drought stress Library *Phleum pratense* cDNA; $8e^{-02}$	N	n	Unknown
	CG3	GR510905.1; ELPLPC2010F06-b1FSP_20010821 Perennial ryegrass 3 wk seedlings, cold-acclim. for 7 days at 4 deg. library *Lolium perenne* cDNA; $8e^{-1}$	XP_003558043.1; PREDICTED: dnaJ protein homolog [*Brachypodium distachyon*]; $6e^{-92}$	AT3G44110; Protein folding; e^{-160}	Protein synthesis
	CG4	n	_	_	Unknown
	CG5	BE358268.1; DG1_27_A06.b2_A002 Dark Grown 1 *Sorghum bicolor* cDNA; $3e^{-73}$	KRH17766.1; hypothetical protein GLYMA_13G013900 [*Glycine max*]; $3e^{-7}$	AT1G15440; snoRNA binding; $1e^{-02}$	Protein synthesis
	CG7	FL185963.1; 15193031 CERES-502 Zea mays cDNA clone; $5e^{-1}$	XP_008653125.1; PREDICTED: phosphatidylinositol N-acetylglucosaminyltransferase gpi3 subunit-like isoform X5 [*Zea mays*]; $9e^{-23}$	AT3G45100; Transferase activity; e^{-122}	Metabolism
	CG8	n	_	_	Unknown
	CG9	BI956709.1; HVcDNA0015 *Hordeum vulgare* subsp. *vulgare* cDNA clone; $1e^{-1}$	EMS61840.1;Xyloglucan endotransglucosylase/hydrolase protein 24 [*Triticum urartu*]; $2e^{-26}$	AT4G30270; Hydrolase activity; $4e^{-67}$	Metabolism
	GA1	BG365200.1; HVcDNA0010 (20 DAP) *Hordeum vulgare* subsp. *vulgare* cDNA clone; $1e^{-2}$	ADW78607.1; heat shock protein 17 [*Hordeum vulgare* subsp. *spontaneum*]; $6e^{-33}$	AT5G59720; Response to heat shock; $5e^{-39}$	Disease/ defence
	GA2	GW883549.1; Ti_Con_119 Timothy drought stress Library *Phleum pratense* cDNA; $7e^{-1}$	CAD30024.2; ferredoxin-NADP(H) oxidoreductase [*Triticum aestivum*]; $3e^{-25}$	AT1G20020; poli U-RNA binding; e^{-148}	Disease/ defence
	GA4	JZ515962.1; GG13 ovaries 3 h after pollination *Paspalum notatum* cDNA; $2e^{-3}$	ACF84459.1; unknown [*Zea mays*]; $8e^{-12}$	AT2G47470; Protein disulfide isomerase activity; $5e^{-52}$	Protein synthesis
	GA6	JZ515962.1; GG13 ovaries 3 h after pollination *Paspalum notatum* cDNA; $2e^{-1}$	ACF84459.1; unknown [*Zea mays*]; $8e^{-12}$	AT2G47470; Protein disulfide isomerase activity; $5e^{-52}$	Protein synthesis
	GG1	CI653564.1; CI653564 *Oryza sativa* Japonica group cDNA clone; $6e^{-1}$	XP_015613138.1; PREDICTED: chlorophyllase-2, chloroplastic [Oryza sativa Japonica Group]; $1e^{-37}$	AT5G43860.1; Chlorophyllase activity; $1e^{-63}$	Metabolism
	GG2	HS557334.1; CCPN1718.g1 developing embryos (L) *Wrightia tinctoria* cDNA clone; $3e^{-2}$	KZV22603.1; COBW domain-containing protein 1 [*Dorcoceras hygrometricum*]; $3e^{-58}$	AT1G80480; Molecular function; $5e^{-57}$	Unknown
	GG3	GR391418.1 ICC1882 dehydration stressed root cDNA library *Cicer arietinum* cDNA clone; $1e^{-22}$	ACL54967.1; MADS FLC-like protein 3 [*Cichorium intybus*]; $1e^{-05}$	AT5G10140; DNA binding; $2e^{-56}$	Transcription
	GG5	EV007516.1; BNAEN3GH *Brassica napus* cDNA; $4e^{-33}$	CDY10992.1; BnaA03g46510D [*Brassica napus*]; $6e^{-29}$	AT4G24190; ATP binding; 0.0	Protein synthesis
	TG1	n	–	–	Unknown
	TG2	GE562482.1; CCHT20496 Niger seed *Guizotia abyssinica* cDNA clone; $8e^{-1}$		AT1G02205; aldehyde	Metabolism

(*Continued*)

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Table 2. (*Continued.*)

Cross	DETDFs	EST with BLASTn	Protein by BLASTx of the EST best match	TAIR BLASTp	Processes
		ID; name; (e-value)	ID; name; (e-value)	ID; GO function; (e-value)	
			XP_015866072.1; PREDICTED: protein ECERIFERUM 1-like [*Ziziphus jujuba*]; $8e^{-92}$	catabolic process 0.0	
	TG3	n	_	_	Unknown
	TG4	EY748269.1; sweet orange flower, greenhouse plant *Citrus sinensis* cDNA; $1e^{-1}$	KDO43347.1; hypothetical protein CISIN_1g0336531 mg [*Citrus sinensis*]; $9e^{-24}$	AT5G43500; molecular function; $7e^{-1}$	Regulation of gene expression
	TG5	n	_	_	Unknown
	TG6	n	_	_	Unknown
C2	CG2	JZ515963.1; GG15 ovaries 3 h after pollination *Paspalum notatum* cDNA; $7e^{-03}$	XP_015639675.1; PREDICTED: probable leucine-rich repeat receptor-like protein kinase At5g49770 [*Oryza sativa* Japonica group]; $1e^{-06}$	AT1G79620; ATP binding; 0.0	Signal transduction

_, no hit; n, similarity below the threshold used (e-value less than 1); Cross, grouping of DETDFs based on cross analysed (C1 and C2). DETDFs: CC, CG, GA and TG were indicated selective primer combinations.

Table 3. Analysis of xenia effect in self- and open-pollinated seeds

	Q4064		*P*-value	Q4294		*P*-value
	SP	OP		SP	OP	
Seed set (%)	12.29	24.24	0.0292*	20.27	32.25	0.1912
Seed length	0.30	0.34	0.0067*	0.32	0.38	0.0069*
Seed width	0.24	0.26	0.0193*	0.23	0.25	0.0174*

SP, self-pollinated; OP, open-pollinated. *Significant at $\alpha = 0.05$.

seed *per se*. In addition, xenia effects at endosperm level began at very early stages of development of the *P. notatum* seed, as this effect was already detected after 3 h of pollination.

Pollen effects in the metabolic processes during germination and development of the pollen tube and pollen–pistil interaction

The sequences CG7, GG5, GA1 and TG2 were related to *A. thaliana* loci associated with germination, pollen tube growth and pollen–pistil interaction. CG7 showed high similarity (e^{-122} BLASTp) to the AT3G45100 locus (alternative name: SETH2) that encoded glycosylphosphatidylinositol anchor (GPI anchor), a protein involved in pollen grain germination and pollen tube growth (Lalanne *et al.*, 2004). SETH2 mutation blocked male transmission and pollen function, associated with abnormal callose deposition, suggesting the important role of GIP in the formation and metabolism of the pollen tube (Lalanne *et al.*, 2004). GG5 showed high similarity (0.0 BLASTp) to AT4G24190 locus. Constant gene expression levels of these loci were shown during the period between the desiccated mature pollen grains to developed pollen tubes (Wang *et al.*, 2008b). GA1 sequence showed high similarity ($5e^{-39}$ BLASTp) to AT5G59720 locus, which encoded a low molecular weight heat shock protein 18.2 (HSP18.2). Gene expression of this locus was demonstrated during pollen grain germination and pollen tube growth (Wang *et al.*, 2008b). In addition, it was shown that gene expression levels increased from the mature pollen condition desiccated to the hydrated mature pollen condition, where the transcript levels were maintained at a constant maximum level until pollen development (Wang *et al.*, 2008b). Finally, TG2 presented high similarity (0.0 BLASTp) to AT1G02205 locus (alternative name: CER1), which was important in the pollen–pistil interaction, and was associated with the structure and composition of the pollen wax, which in turn, was involved in the development and fertility of the pollen grain (Preuss *et al.*, 1993). In mutants where the pollen wax layer was removed, it was found that the stigma placed in contact with this pollen did not expose callose and did not carry out the fertilization (the pollen did not germinate), and thus interrupted the pollen–pistil interaction. These results demonstrated that wax was necessary for fertilization (Aarts *et al.*, 1995).

Pollen effects in metabolic processes during seed development and production (xenia effect)

The sequences CG5, GA4, GA6, GG5, CG3, TG2 and CC1 were related to loci of *A. thaliana* associated with seed development and production. CG5 showed similarity ($1e^{-02}$ BLASTp) to AT1G15440 a locus that encoded a protein acting as cofactor in the biogenesis of the 40S subunit of the ribosome (alternative name: PWP2) (Missbach *et al.*, 2013). In studies with heterozygous mutants in *A. thaliana* (PWP2 +/–) it was observed that

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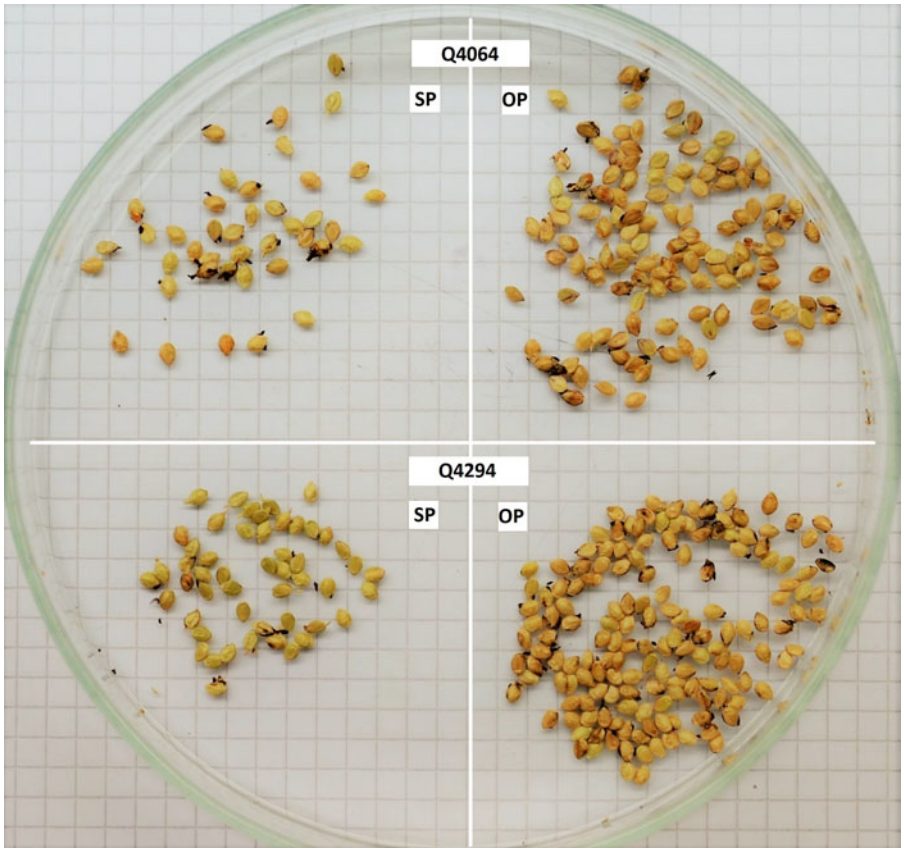


Fig. 2. Seed set obtained from self- and open-pollinated Q4064 and Q4294 plants. SP, self-pollinated; OP, open-pollinated.

the siliques reduced its size by 25% and the seeds were very small or had aborted early, so the number of seeds was reduced by 50% (Missbach *et al*., 2013). In a transcriptome analysis that included different organs and developmental moments, the highest expression of PWP2 was found in reproductive and strongly dividing tissues (such as flowers, seeds and siliques) (Missbach *et al*., 2013). The CG3 sequence showed high similarity (e^{-160} BLASTp) to AT3G44110 locus (alternative name: ATJ3). Loss of ATJ3 function was associated with small seed production. Studies in mutant seeds showed a reduction of 24% in size and weight of 500 seeds, compared with the wild-type (Salas-Muñoz *et al*., 2016). In addition, it was observed that the lack of transcription of the gene altered the columella form of the seeds. The ATJ3 gene could be an important factor in seed development (Salas-Muñoz *et al*., 2016). On the other hand, the CC1 sequence showed high similarity ($2e^{-60}$ BLASTp) to AT5G67230 (alternative name: IR14-L) locus. IR14-L encoded a member of the family glycosyltransferases43 (GT43) protein family and was essential for the synthesis of highly branched xylan in seed coat epidermal cells and was more abundant in the mucilage of the *A. thaliana* seed and required to maintain its architecture (Voiniciuc *et al*., 2015). These unique branches appeared to be necessary for the attachment of pectin to the seed surface, while the xylan backbone maintains the cellulose distribution. The expression for this locus was demonstrated at different moments and tissues of the seed development (Voiniciuc *et al*., 2015).

The GA4 and GA6 sequences were highly similar ($5e^{-52}$ BLASTp) to AT2G47470 locus, which encoded a protein disulfide isomerase 2-1 (PDIL2-1) that is involved in the maturation of plasma membrane and secreted proteins (Wang *et al*., 2008a). Mutant phenotypes of this locus of *A. thaliana* showed reduced seed production, small siliques and reduced seed numbers. In addition, it was shown that in these mutants there is an interruption in the funicular and micropylar pollen tube guidance, altering the orientation of the pollen tube (Wang *et al*., 2008a). Although previous studies had attributed the role of correct pollen tube growth to PDIL2-1 (Wang *et al*., 2008a; Boavida *et al*., 2009), gene expression studies during *A. thaliana* seed development had shown that the maximum expression level of this gene was at 5 days after flowering (DAF) and then the expression levels decreased (Hajduch *et al*., 2010). The results suggested that this gene played an additional role besides the guidance of the pollen tube to the micropyle of the ovule. Taking into account that fertilization of *A. thaliana* occurs 3 h after flowering (Mansfield and Briarty, 1991) and that the period of greater storage of oils and protein, synthesis and deposition in *A. thaliana* seeds is between 5 and 13 DAF (Hajduch *et al*., 2010), it is probable that this gene plays an important role during the development of *A. thaliana* seed.

Similar to GA4 and GA6 sequences, whose functions are exerted at moments prior to fertilization (development of the pollen tube) and during seed development, the GG5 sequence seemed to behave in the same way in *A. thaliana* (Hajduch *et al*., 2010). A similar behaviour to GA4, GA6 and GG5 was shown by the TG2 sequence which, in addition to fulfilling an important function in the pollen–pistil interaction (Preuss *et al*., 1993; Aarts *et al*., 1995), also performed functions during the development of seed and fruit (Bourdenx *et al*., 2011). Previous studies had shown that waxes are not only implicated in dehiscence processes but also that the relative abundance of transcripts for CER1 showed the highest levels of expression in flowers and siliques, in addition to CER1 being involved in the biosynthesis of flower waxes and fruits (Bourdenx *et al*., 2011).

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Molecules related to the xenia effect

Liu (2008) suggested two hypotheses for the mechanism of xenia, the first of which was that the effect would be mediated by hormones that are secreted by the embryo and/or endosperm and that these hormones would depend on the male parent. The second hypothesis was that the molecules mediating xenia would be mobile mRNAs, which would be released during fertilization from the pollen tube and diffuse out into maternal tissues of the seed and fruit, producing phenotypic variations. It has been shown that mRNAs can move long distances via phloem or plasmodesmata and are also involved in signaling between the male and female parent, which would affect the characteristics of the seed and/or fruit, causing phenotypic changes of the tissues in development (Kim *et al.*, 2001; Kudo and Harada, 2007; Liu, 2008; Wang *et al.*, 2008a; Piotto *et al.*, 2013; Thieme *et al.*, 2015). Piotto *et al.* (2013) supported the hypothesis of mRNA as a signalling molecule, as its diffusion could explain the transmission of the signal within the tomato fruit and, therefore, could be involved in the observed expression of xenia. Wang *et al.* (2013) found that genetic differences (by analyses using AFLP and RAPD markers) exist between the female progenitor and F1progeny, but not between F1 individuals and the male parent. Therefore, they concluded that the effect of pollen is not genetic (supporting either hormone-mediated or another type of mechanism).

In the present work, four DETDFs (CC3, GA4, GA6 and GA2) were detected for the pollen grain genotype used in *A. thaliana* to be encoded for mobile mRNAs. Of these, CC3 sequence was highly similar (0.0 BLASTp) to AT1G17745 locus that encoded D-3-phosphoglycerate dehydrogenase (PGDH2), a member of the PGDH isoenzyme family. PGDHs are components of the glycolytic phosphoserine (PS) pathway and phosphorylation pathway of serine biosynthesis (PPSB), playing important roles in plant development and metabolism. PPSB play an essential role in actively diving cells such as embryos and anthers (Toujani *et al.*, 2013). In addition, an alteration of the PGDH family gene expression affected the primary metabolism of *A. thaliana*, so that mobile mRNA might be involved in seed development and primary metabolism of the seed (Benstein *et al.*, 2013). GA4 and GA6 were associated with an *A. thaliana* locus (AT2G47470) involved in protein synthesis. Mutants for this locus showed interference in signalling between the pollen tube and the micropyle of the ovule during fertilization (Wang *et al.*, 2008a). These results were consistent with the second hypothesis proposed by Liu (2008) and could be added to the results obtained by Piotto *et al.* (2013) supporting the hypothesis that mobile mRNAs would be involved in the mechanism of xenia functioning as a signal between the male and female parents.

Xenia effects in seeds obtained from self- and open-pollinated plants

The values for seed set, length and width of seeds obtained from self- and open-pollinated plants of Q4064 and Q4294 genotypes demonstrated the effects of the pollen genotype (xenia) at phenotypic level. The results of xenia in seeds obtained from self- and open-pollinated *P. notatum* plants were consistent with the observations reported in crops such as alfalfa, rye (Kaczmarek *et al.*, 2001) and maize (Bullant and Gallais, 1998; Weingartner *et al.*, 2002). The different genotypes of the pollen source resulted in measurable changes during the development of the seed, such as in the filling and grain size (length and width). Also, the results obtained in the present work show a connection between the genes (CG5, GA4, GA6, GG5, CG3, TG2 and CC1) predicted to be involved in xenia effect and the phenotypic differences in seed set and seed size in *P. notatum*.

Conclusions

In conclusion, the results of the initial screening of xenia effect showed evidence of the pollen genotype effect in the hybrid endosperm at molecular level in *P. notatum*. Additionally, the effect of pollen genotype in *P. notatum* could be one of the factors that determine differential expression of genes related to number, size and weight of seed. Therefore, this effect of pollen genotype may be associated with grain yield. In addition, four out of 24 DETDFs were particularly interesting, as they are mobile mRNAs that mediate signalling pathways between maternal and paternal tissues. The results present evidence that these mobile mRNAs could be the signals involved in the xenia effect at endosperm level in the species. Given the implication of the genotype of the pollen grain on plant genetic improvement in species of agronomic interest such as maize, alfalfa, rye, tomato and blueberry, we consider that the results obtained in this work are among the first contributions to the description of xenia in the endosperm at the molecular level. In addition, xenia effects were demonstrated at the phenotypic level in *P. notatum* seeds.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0960258518000375.

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