Rosa-Angélica Sánchez-Díaz

Ana-María Castillo

María-Pilar Vallés *

Microspore embryogenesis in wheat: New marker genes for early, middle and late stages of embryo development

Sánchez-Díaz RA

Castillo AM

Vallés MP *

Departamento de Genética y Producción Vegetal, Estación Experimental Aula Dei, Consejo Superior de Investigaciones Científicas (EEAD-CSIC), Av Montañana 1005, 50080 Zaragoza, Spain.

* Corresponding author:

e-mail: valles@eead.csic.es

Phone 34 976716084

Fax 34 976716145

ABSTRACT

Microspore embryogenesis involves reprogramming of the pollen immature cell towards embryogenesis. We have identified and characterized a collection of 14 genes induced along different morphological phases of microspore-derived embryo development in wheat (*Triticum aestivum* L.) anther culture. *SERK* and *FLA* genes previously associated to somatic embryogenesis and reproductive tissues, respectively, were also included in this analysis. Genes involved in signalling mechanisms such as *TaTPD1-like* and *TAA1b*, and two glutathione *S*-transferase (*GSTF2* and *GSTA2*) were induced when microspores had acquired a “star-like” morphology or had undergone the first divisions. Genes associated with control of plant development and stress response (*TaNf-YA*, *TaAGL14*, *TaFLA26*, *CHI3*, *XIP-R*; *Tad1* and *WAL16*) were activated before exine rupture. When the multicellular structures have been released from the exine, *TaEXPB4*, *TaAGP31-like* and an unknown embryo specific gene *TaME1* were induced. Comparison of gene expression, between two wheat cultivars with different response to anther culture, showed that the profile of genes activated before exine rupture were shifted to earlier stages in the low responding cultivar. This collection of genes constitutes a value resource for study mechanism of intra-embryo communication, early pattern formation, cell wall modification and embryo differentiation.

Keywords: bread wheat, microspore embryogenesis, embryo development, gene expression, marker genes, double haploid

INTRODUCTION

Microspore embryogenesis involves reprogramming of the pollen immature cell towards embryogenesis. In this process, the microspores at mid-late uninucleate stage, after exposure to a stress treatment, dedifferentiate, acquire cell totipotency and under appropriate culture conditions, divide, develop into an embryo and finally into a plant.

Microspore embryogenesis has proven to be an important tool for plant breeding and genetic studies due to its ability to generate homozygous lines (doubled haploids, DH) in a single generation (Forster et al. 2007). Due to the economic importance of wheat (*Triticum aestivum* L.), many studies have been focused on the development of DH production techniques (for review see Zheng 2003; Jauhar et al. 2009). Despite significant efforts to improve the efficiency of wheat microspore embryogenesis (Soriano et al. 2007, 2008; Broughton 2008; Redha and Suleman 2011), genotype dependency, albinism and chromosome doubling are still the main bottlenecks (for review see Jauhar et al. 2009; Lantos et al. 2013). In fact, there are many agronomically important bread wheat genotypes that show very low (less than 1 plant /100 anthers) or no green plant regeneration (Lantos et al. 2013).

During the last years, interest has been focused on the search for marker genes whose induction could be associated with key steps of microspore embryogenesis in model species as rapeseed, tobacco and barley. In early studies, only few genes associated with the reprogramming phase and the early stages of embryogenesis were described such as heat shock proteins (HSPs) and napin gene (for review see Boutilier et al. 2005; Seguí-Simarro and Nuez 2008). In barley genes coding for a non-specific lipid transfer protein (ECLTP), a glutathione-S-transferase (ECGST) and an arabinogalactan-like protein (AGPs) (EARLY CULTURE ABUNDANT 1, ECA1) were reported in early embryogenesis (Vrinten et al. 1999). The *BABY BOOM* (*BBM*) was the first gene involved in the induction of rapeseed microspore embryogenesis (Boutilier et al. 2002).

More recently, the use of functional genomic tools has allowed the identification of higher numbers of genes associated with different steps of microspore embryogenesis. During the stress-treatment stage genes associated with metabolism, chromosome remodelling, transcription, translation, and signalling were identified in tobacco (Hosp

et al. 2007); large changes in the expression of genes related to central metabolism, stress response, proteolysis and the suppression of the gametophytic developmental pathway have been revealed in barley (Maraschin et al. 2006; Muñoz-Amatriáin et al. 2006, 2009). Genes related to metabolism, cell wall and membranes, control of cellular organization, cellular communication and signal transduction were identified in early stages of rapeseed embryogenesis (Joosen et al. 2007; Malik et al. 2007; Tsuwamoto et al. 2007; Stasolla et al. 2008).

Limited information is available for wheat microspore embryogenesis, probably due to the difficulties in obtaining high efficiency in microspore and anther cultures. In the only study conducted in wheat, five genes were associated with differentiation of embryoids (Reynolds and Kitto 1992). One of them was further characterized as an early cysteine-labeled metallothionein (EcMt) that was proposed as a molecular marker for microspore embryogenesis (Reynolds and Crawford 1996).

Despite the number of studies describing major changes in gene expression in this process, the underlying control mechanisms are largely unknown (for review see Soriano et al. 2013, in this issue). Therefore more studies are needed to clarify the molecular control of microspore embryogenesis, especially in an economically important crop such as wheat.

In this study, genes induced along wheat anther culture have been identified. The expression patterns of these genes have been analysed at different stages of microspore-derived embryo development in two wheat cultivars that present a different response to anther culture, and has been compared to zygotic embryogenesis. For the first time, genes activated from the first mitotic division until embryogenic globular structures have been identified and characterised in wheat.

MATERIALS AND METHODS

Vegetal material and growing conditions

The spring bread wheat cultivars Pavon and Caramba were used as the source of donor plants for anther and isolated microspore culture. Pavon is a high-responding cultivar to microspore embryogenesis whereas Caramba is an agronomical important cultivar in Spain, with low microspore embryogenesis response, mainly due to its low rate of dividing microspores (Soriano et al. 2008). Anther donor plants were grown as described by Soriano et al. (2007).

Anther and microspore culture

Anthers containing the majority of microspores at mid- to late-uninucleate stage were excised from the flowers for stress treatment in 0.7 M mannitol. For anther culture, anthers were inoculated in 1.5 ml modified MS3M medium and supplemented with 200 gl^{-1} Ficoll type 400 (MS3MF200). Medium was conditioned for 5 days with 10 ovaries at 25 °C. For isolated microspore culture, microspores were isolated as described by Castillo et al. (2000) and cultured in an ovary preconditioned MS3MF200 medium at a final density of $8\text{-}10 \times 10^4$ microspores/ml. Anthers and isolated microspore cultures were maintained in the dark at 25 °C. After 10 to 12 days, plates were replenished with 1.5 ml of modified MS3M supplemented with 400 gl^{-1} Ficoll type 400 (for details see Soriano et al. 2007).

Cytological studies

Morphological changes associated with microspore embryogenesis in the cultivar ‘Pavon’ were characterized after 5 days of mannitol treatment (5dp), and 5, 10 and 15 days after culture (5dC, 10dC and 15dC). Anthers were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in cacodylate buffer 0.05 M (pH 7.2) at RT for 3 hours and at 4 °C overnight. Samples were embedded in Unicryl. Semithin sections 1 μm thick were cut using a RM2164 rotary microtome (Leica) and stained with 0.05% toluidine blue. The *in vivo* characterization was performed in isolated microspore cultures since microspores are not accessible for observation in anther cultures. The characterization was performed with the microscope Nikon Eclipse T300.

Selection of candidate genes, primer design and sequence analysis

Candidate genes were selected based on a previous gene expression study performed in barley (unpublished results). In that study, microarray analysis using the Affymetrix Barley1 GeneChip was carried out on anthers before and after 4 days of mannitol stress treatment and after 4 and 8 days of culture following the procedures described in Muñoz-Amatriaín et al. (2006). Differentially expressed genes between anthers after 4 days of culture and anthers before and after stress treatment were selected. For annotation purpose, blastx (e-value cutoff = e^{-10}) data was exported from HarvEST:Barley version 1.83 (<http://www.harvest.ucr.edu>). Wheat homologous to the barley genes were identified based on sequence homology by FASTA-EMBL-EBI (Nucleotide similarity search) (<http://www.ebi.ac.uk>), HarvEST wheat 1.59 version and PLEXdb (Plant Expression Database) (<http://www.plexdb.org>) (Supplemental Table S1). Out of selected genes, 14 with a high level of expression were used for subsequent analysis: TaAffx.3154.1 (*TAPETUM DETERMINANT 1-like*, *TaTPD1-like*), Ta.9528.1 (a fatty acyl CoA-reductase, *Ta.ANTH-ER-SPECIFIC1*, *TAA1b*), Ta.1775.1 (*GLUTATHIONE-S-TRANSFERASE-F2*, *GSTF2*), Ta.21342.1 (*CHITINASE-3*, *CHI3*), Ta.303.2 (*GLUTATHIONE-S-TRANSFERASE-A2*, *GSTA2*), Ta.28319.1 (*Ta.DEFENSIN-1*, *Tad1*), Ta.5024.1 (*WHEAT ALUMINUM INDUCED-6*, *WAL16*), Ta.10047.1 (*NUCLEAR FACTOR Y-A7*, *TaNF-YA7*), Ta.1839.1 (*FASCICLIN-26*, *FLA26*), Ta.18801.1 (*ARABINOGALACTAN PROTEIN 31-LIKE*, *TaAGP31-like*), Ta.7773.1 (*MICROSPORE EMBRYOGENESIS-1*, *TaME1*), Ta.13785.1 (*XYLANASE INHIBITOR PROTEIN-R2*, *XIP-R1*), Ta.6411.1 (*AGAMOUS LIKE-14*, *TaAGL14*) and Ta.3749.1 (*TaEXPANSIN-B4*, *TaEXPB4*). Other genes previously described associated to somatic embryogenesis or reproductive tissues were also included in this analysis: wheat homologous to *SERK1* (Ta.12817.1) and *SERK2* (Ta.6832.1) (*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1* and *2*) and the wheat arabinogalactan fasciclin genes (*FASCICLIN-14*, *FLA14*, Ta.1852.1 and *FLASCICLIN 25*, *FLA25*, Ta.28834.1).

The consensus sequence of wheat genes based on HarvEST:Wheat version 1.59 was used for designing specific primer pairs by PRIMEREXPRESS software (Applied Biosystems) (Supplemental Table S2).

RNA Isolation and Semi-quantitative RT-PCR

Fresh anthers before stress treatment (0dp), anthers at 5dp, 5dC, 10dC, 15dC and 20dC from Pavon and Caramba, and mature ovaries (Ov), caryopsides (without palea and lemma) at 2, 4, 6 and 8 days after pollination (DAP) (C2, C4, C6 and C8), excised embryos and endosperm at 10, 12 and 14 DAP (E10, E12, E14, and En10d, En12 and En14d), and leaves (L) and roots (R) from a four leaves plantlet from cultivar Pavon were frozen in liquid nitrogen. Total RNA was isolated using TRIzol Reagent (Gibco BRL) and double-stranded cDNA was synthesized following protocols described in Muñoz-Amatriaín et al. (2006). PCR reactions were optimized to 94°C for 3 min, 33 amplification cycles at 94 °C for 1 min, the appropriate annealing temperature (Supplemental Table S2) for 1 min, 72 °C for 1 min, and a final extension of 10 min at 72°C. Amplified products were resolved on 1.5% agarose gels and visualized by SYBR staining (Invitrogen). 18S rDNA from wheat was used as control.

RESULTS AND DISCUSSION

In a previous study in barley anther culture, differentially expressed genes were selected based on comparison in the expression level between anthers at 4 days of culture and earlier stages (unpublished results). As anthers at 4 days of culture were characterized by tapetum degradation (Muñoz-Amatriaín et al. 2009) and a minimal expression of pollen genes (Maraschin et al. 2005a; Pulido et al. 2009), gene expression was associated with microspore-derived embryo development. Wheat homologous to the barley genes were identified based on sequence homology and their expression profile were characterised along anther culture of the high responding cultivar Pavon. SERK1 and SERK2 receptor-like kinases and two fasciclin-Like Arabinogalactan proteins genes *FLA14* and *FLA25* were also included in this analysis.

Morphological characterization of Pavon anther cultures showed that, after 5 days of mannitol treatment (5dp), the tapetum was degraded and microspores were randomly distributed inside the anther locus (Figure 1a). Microspores with a ‘star-like’ morphology were observed (Figure 1e). After 5 days of culture (5dC) the anther wall was degraded, most of the microspores presented a ‘star-like’ morphology or a dense cytoplasm, and some of them had undergone the second mitotic division (Figure 1b and

f), and less than 2% of microspores derived structures developed to pollen grain (data not shown). The 'star-like' morphology has been associated with the embryogenic potential in cereals, although, these structures were also described in microspores cultures that do not produce embryos (Indrianto et al. 2001, Maraschin et al. 2005a).

This stage has been poorly characterized at the molecular level. Out of the three genes identified at 3 days of barley isolated microspores cultures (Vrinten et al. 1999), *ECA1* and *ECLTP* have been postulated as signalling molecules in two-celled zygotic proembryos in wheat (Sprunck et al. 2005). Two genes induced at 5dC in our study, *TaTPD1-like* and *TAA1b*, have been associated to signalling mechanisms. *TaTPD1-like* has homology with *TAPETUM DETERMINANT1 (TPD1)* that encodes small secreted protein acting in signal tapetum cell fate in *Arabidopsis* (Yang et al. 2003). Recently, Leljak-Levanic et al. (2013, in this issue) described a *TPD1-like* gene as a marker for early zygotic reprogramming in wheat, with a proposed role in cell-to cell communication. The *TAA1b* gene encoded a Fatty acil-coA reductase that mediated the biosynthesis of long chains of fatty alcohols (VLCFA), forming sporopollenin and wax esters (Wang et al. 2002). It has been proposed that VLCFA derivatives function as signalling molecules to control cell division by communication from epidermal layer to internal tissue (Nobusawa et al. 2013). In microspore embryogenesis, due to the absence of sporophytic surrounding tissues, *TaTPD1-like* and *TAA1b* are probably implicated in intra-embryogenic structure communication.

Other genes activated at 5dC were two glutathione *S*-transferase, *GSTF2* and *GSTA2*, that encode for a phi (F) class (Cummins et al. 2003) and a pathogen-inducible one (Mauch and Dudler 1993), respectively. Up-regulation of GST genes have been previously described at different stages of microspore embryogenesis (Muñoz-Amatriain et al. 2006; Joosen et al. 2007, Malik et al., 2007; Tsuwamoto et al. 2007). This induction has been related to protection of the developing embryos against stress conditions in phases of active growth (Stasolla et al. 2008), but not with the acquisition of androgenic potential (Vrinten et al. 1999, Maraschin et al. 2005b).

At 10 days of culture (10dC) multicellular structures, most of them still confined inside the exine wall, have acquired an organized inner structure (Figure 1c and g). Although, exine rupture has been associated to the establishment of embryo polarity (for review

see Soriano et al. 2013, in this issue), in wheat polarity was established before rupture (Indrianto et al. (2001). Among genes induced at 10dC, genes associated with developmental control were identified, indicating probably the activation of the embryogenic program. One of these genes was *TaNF-YA7*, a member of the Nuclear Factor Y (NF-Y) complex (Stephenson et al. 2007). Recently Mu et al. (2013) showed that *NF-YA5* and 9, orthologous to *TaNF-YA7* (Cao et al. 2011), were sufficient to induce somatic embryogenesis and suggested that *NF-YA5* was in the same complex than *LEAFY COTYLEDON1 (LEC1)*, a key regulator for embryo identity (West et al. 1994). It is noteworthy that *LEC1* has been associated to microspore-derived embryo development in rapeseed (Malik et al. 2007; Stasolla et al. 2008). The *TaAGL14* gene encodes the transcription factor MADS-box type II (MIKC-type) (Zhao et al. 2006). *TaAGL14* and *TaAGL15* along with rice *OsMADS32*, implicated in floral organ identity (Sang et al. 2012,) form a new subfamily of type II genes that only exists in monocot (Zhao et al. 2006).

Arabinogalactan proteins (AGPs) have also been described as regulators in plant developmental processes (reviewed in Seifert and Roberts 2007). Specific AGPs genes have been identified in early microspore-derived embryos in barley (Vrinten et al., 1999), and in rapeseed (Joosen et al. 2007; Malik et al. 2007; Tsuwamoto et al. 2007). Also AGPs are known to be components of microspore conditioned medium from barley and maize (Paire et al. 2003; Borderies et al. 2004) and promoters of microspore embryogenesis in wheat (Letarte et al. 2006). Two AGPs have been identified in this study: *TaFLA26* induced at 10dC and *TaAGP31-like* at 15dC. *TaFLA26* encodes for a fasciclin-Like Arabinogalactan protein (FLAs) (Faik et al. 2006). Contrarily, the other two FLA included in the analysis, *TaFLA25* and *TaFLA14* were expressed specifically after stress treatment. This is the first time that FLAs proteins have been associated to microspore embryogenesis.

It is well known that SERKs are involved in multiple signalling programs, including switching on somatic embryogenesis (for review see Li 2010). SERK genes were also expressed in maize (Baudino et al. 2001), and rapeseed microspore embryogenesis (Malik et al. 2007). However in our study, neither *SERK1* nor *SERK2* expression was detected at any stage in Pavon.

At the stage of exine rupture initiation a group of genes associated to stress responses and/or cell wall loosening or modification was induced; *Tad1* belongs to a family of small cysteine-rich peptides known as plant defensins or δ -thionins (Koike et al. 2002); the XIP-type xylanase inhibitor is associated to the release of signalling elicitor-active arabinoxyl-oligosaccharides (Dornez et al. 2010). The Chitinase *CHI3* acts mainly over cell wall components (for review see Grover 2012). Finally, *WALI6*, a cysteine-rich serine protease inhibitor induced by wounding or metal stress, has homology to BBI proteases inhibitors (Richards et al. 1994). It was suggested that the expression of stress-related factors in microspore-derived embryos could be a consequence of taking over functions of the zygotic embryo surrounding tissues (Borderies et al. 2004, Boutilier et al. 2005).

At 15 days of culture (15dC), multicellular structures have been released from the exine, divided actively and consisted in a globular mass of small and rounded cells with rich cytoplasm showing the first signs of differentiation by the formation of protoderm (Figure 1d and h). At this stage new genes were induced: *TaEXPB4* encodes a member of the expansins family (Lin et al. 2005) whose expression probably responded to the demand of cell wall extensibility during the active growth and development; *TaAGP31-like* that presents sequence homology with Pollen Ole e I (Ole) domain-containing proteins and with a nonclassical arabinogalactan protein AGP31, that could be related with an initial vascular differentiation (Liu and Mehdy 2007); and the unknown embryo specific gene *TaME1*. At 20 days of culture when embryos were clearly visible under stereoscope (data not shown), most of the genes highly expressed in earlier stages were repressed, only *CHI3* and *TaAGP31-like* were highly expressed.

Similarity in gene expression profiles between microspore and zygotic embryogenesis was claimed (Boutilier et al. 2005). However, also important differences had been highlighted such as the level and the time of differentiation and maturation (for review see Maraschin et al. 2005b; Seguí-Simarro and Nuez 2008). Gene expression analysis in zygotic embryogenesis of Pavon was included in this study. (Figure 2, right side). As the whole caryopsis was analysed and the embryo is a minimal part of the structure, early stages of embryo development were not really covered with these samples. Excised embryo corresponded to stages of development equal or later to the later stages studied in microspore embryogenesis. Even so, 15 out of the 18 characterized genes

were expressed in excised zygotic embryo indicating that are involved in both embryogenic programs. Four of the genes were embryo specific (*Ta.TPD1-like*, *TaNFYA7*, *TaAGP31-like* and *TaME1*). The rest of the genes, although expressed in other organs, have the highest expression level at excised zygotic embryos at 12 DAP, except *WALI6* in caryopsis at 2 DAP and roots, *GSTF2* in roots and *CHI3* in near all organs. *SERK2* and *SERK1* genes, which were not expressed in microspore embryogenesis, showed a very low level and specific expression in excised zygotic embryos at 12 DAP. *Tad1* was the only gene that was specific of microspore-derived embryogenesis program.

In early stages of anther culture, morphological differences were observed between the high responding cultivar Pavon and the low responding cultivar Caramba. At 5dp, many microspores presented a 'star-like' morphology in Pavon (Figure 1e), whereas in Caramba most of them had still a large central vacuole and a clear cytoplasm (Figure 1i). At 5dC differences were mainly due to the lower number of surviving microspores in Caramba (Figure 1 f and j). A DAPI staining analysis determined differences also in the rate of divisions, being faster in Caramba (data not shown). At 10dC and 15dC structures with similar number of nuclei and morphology were observed in both cultivars (Figure 1 g ,k, h and l, and data not shown). However, the number of multicellular structures was significantly lower in Caramba (Figure 1 g ,k ,h and l).

When the expression profiles of genes induced at 5 and 10dC were compared between Pavon and Caramba, the profiles were shifted to earlier stages in Caramba (Figure 3). However, none of these genes were significantly induced before 5dC in this cultivar, except *WALI6*. Interestingly, as microspores from Caramba divided faster than those from Pavon at these stages, the shift in the expression profile in Caramba was in accordance with its advance in development. When expression profiles of genes induced at 15dC were compared between the two cultivars, not a general pattern of expression was observed. In Caramba, *TaME1* presented a significant shift to earlier stages; *TaAGP31-like* expression was not maintained at 20dC, and *TaEXPB4* showed significantly lower level of expression at 15dC. Also differences were observed for *XIP-R1* that was almost constitutively expressed in Caramba, and *SERK2* that was expressed at low level at 10dC. A detailed morphological characterization by live imaging of these

stages could contribute to associate gene expression profiles and developmental clues in the two cultivars.

In this study, we have successfully identified and characterized a collection of genes that are induced in microspore-derived embryos of wheat. Although some of the genes represented proteins previously associated with microspore embryogenesis as GSTs, chitinases and arabinogalactan, their expression was not reported before in wheat. Other genes as *Ta.TPD1-like*, *TAA1b*, *XIP-R1* and *WALI6* have not been previously described to be involved in microspore embryogenesis. A new gene *TaEM1* was identified in this study and *Tad1* gene was proved to be a specific for microspore embryogenesis. This collection of genes constitute a value resource to study mechanisms such as intra-embryo communication, early pattern formation, cell wall modification signalling and embryo differentiation.

To determine the functions of these genes during development of the microspore-derived embryo more studies are needed. However, the validity of these candidate genes was demonstrated in isolated microspore culture after cold stress treatment in four triticale cultivars by Zur et al. (2013, in this issue), suggesting that similar molecular mechanisms are involved in microspore embryogenesis in cereals.

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LEGENDS FOR FIGURES

Fig. 1 Morphological characterization of microspore embryogenesis in wheat. (a-d) Semithin sections of anther stained with toluidine blue. a) Anthers after 5 days of mannitol stress treatment (5dp) showing tapetum degradation (arrow), vacuolated microspores (*) and uninucleate microspores and first symmetrical division. b) Anthers at 5 days of culture (5dC) showing degradation of the anther wall (arrow) and the third mitotic division. c) Multicellular structure in anthers at 10 days of culture (10dC). d) Embryogenic multicellular structures showing exine rupture (arrow) at 15 days of culture (15dC). (e-n) Isolated microspore cultures of Pavon (e-i) and Caramba (j-n). e and j) Microspore cultures right after isolation (5dp). Many ‘star-like’ microspores (arrow) can be observed in Pavon(e) and mostly vacuolated microspores (*) in Caramba. f and k) Microspores cultures at 5dC with a high proportion of ‘star-like’ structures. g and l) Some embryogenic structures already breaking the exine (arrow) at 10dC. h and m) Proembryos (arrow) at 15dC. Bars=100 μ m

Fig. 2 Expression analyses by semiquantitative RT-PCR in cultivar Pavon of the 18 genes studied. Analysis of anther culture was performed in: excised anthers before mannitol treatment (0dp), 5 days after mannitol treatment (5dp) and 5, 10, 15 and 20 days of culture (5dC, 10dC, 15dC and 20dC) (left side). Analysis of zygotic embryogenesis and vegetative tissues was performed in: mature ovaries (Ov), caryopsides at 2, 4, 6 and 8 DAP (C2, C4, C6 and C8), excised embryos and endosperm at 10, 12 and 14 DAP (E10, E12, E14, and En10, En12, En14, respectively), leaves (L) and roots (R) from a four leaves plantlets (right side). 18S rDNA was used as control.

Fig. 3 Expression analyses by semiquantitative RT-PCR in cultivar Pavon (P) and cultivar Caramba (C) of the 18 genes studied. Analysis of anther culture was performed in: excised anthers before mannitol treatment (0dp), 5 days after mannitol treatment (5dp) and 5, 10, 15 and 20 after culture (5dC, 10dC, 15dC and 20dC). 18S rDNA was used as control.