

Megasporogenesis and programmed cell death in *Tillandsia* (Bromeliaceae)

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Abstract The degeneration of three of four meiotic products is a very common process in the female gender of oogamous eukaryotes. In *Tillandsia* (and many other angiosperms), the surviving megaspore has a callose-free wall in chalazal position while the other three megaspores are completely embedded in callose. Therefore, nutrients and signals can reach more easily the functional megaspore from the nucellus through the chalazal pole with respect to the other megaspores. The abortion of three of four megaspores was already recognized as the result of a programmed cell death (PCD) process. We investigated the process to understand the modality of this specific type of PCD and its relationship to the asymmetric callose deposition around the tetrad. The decision on which of the four megaspores will be the supernumerary megaspores in angiosperms, and hence destined to undergo programmed cell death, appears to be linked to the callose layer deposition around the tetrad. During supernumerary megaspores degeneration, events leading to the deletion of the cells do not appear to belong to a single type of cell death. The first morphological signs are typical of autophagy, including the formation of autophagosomes. The

TUNEL positivity and a change in morphology of mitochondria and chloroplasts indicate the passage to an apoptotic-like PCD phase, while the cellular remnants undergo a final process resembling at least partially (ER swelling) necrotic morphological syndromes, eventually leading to a mainly lipidic cell corpse still separated from the functional megaspore by a callose layer.

Keywords Bromeliaceae · Gametophyte · Megasporogenesis · Plant reproduction · Programmed cell death · *Tillandsia*

Introduction

The degeneration of three of four meiotic products is a very common process in the female gender of oogamous eukaryotes, both in animals (for instance Cupisti et al. 2003) and plants (Bell 1996; Yang et al. 2010). This phenomenon occurs both during female gametophyte development in heterosporous plants (haplo-diplontic life cycle), and during female gametes development in animals (diplontic life cycle). The degeneration of the three supernumerary meiotic products is hence a case of developmental programmed cell death (PCD) and it is of interest to investigate its features with the aim of checking resemblance to apoptotic morphological syndrome as described in general and as described for plants.

In plants, the retention of one functional megaspore of four is considered by some authors as the most important requisite for the seed habit (Herr 1995). This process occurs through PCD, which is a type of cell death caused by a series of genetically controlled events leading to a controlled and organized destruction of the cell (Lockshin and Zakeri 2004). In some cases, the disruption regards only the

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cytoplasm as for PCD of the xylem elements (Fukuda 2000; Neill 2005), or part of it (as for sieve elements maturation).

In animals, apoptosis is driven by the activation of a family of cysteine proteases known as caspases. Activation of caspases can occur through extrinsic death receptors signaling, or alternatively, intrinsic pathways, controlled in part by the mitochondria and the release of cytochrome *c* (Kroemer et al. 2007). The release of cytochrome *c* drives the assembly of the apoptosome, a caspase activating complex located in the cytoplasm (Reape and McCabe 2008; Reape et al. 2008). Another effect of cytochrome *c* release is the production of reactive oxygen species (ROS), which are considered as regulators of PCD in both animal and plant cells (Jabs 1999; Jones 2001; Doyle et al. 2010). The loss of mitochondrial membrane integrity can be caused or inhibited by apoptosis regulators (Green and Reed 1998; Adrain and Martin 2001).

Genes homologous to caspases are not present in plants (Sanmartin et al. 2005; Reape and McCabe 2010). Nevertheless, analogous proteins with a role similar to that of caspases and inhibited by some of the same molecules inhibiting caspases, can be found, as the senescence-induced cysteine protease vacuolar processing enzymes, in which homology to the tertiary structure of animal caspases can be observed, and the metacaspases (Sanmartin et al. 2005).

Kerr et al. (1972) initially described by ultrastructural investigations two forms of cell death: apoptosis and necrosis. Apoptosis was described by specific morphological features, as cell and nucleus shrinkage, nuclear condensation, DNA fragmentation, and finally, in animals, the breakup of the cell into ‘apoptotic bodies’, eventually engulfed by phagocytes.

Main currently recognized morphological cell death types are three, on the basis of ultrastructure: apoptosis, autophagy, and necrosis (Lockshin and Zakeri 2004; Reape et al. 2008). PCD is largely used to indicate the processes of apoptosis and autophagy (Van Doorn and Woltering 2005). Autophagy, originally named type II lysosomal cell death (Schweichel and Merker 1973), differs from apoptosis since it works engulfing cytoplasmic constituents into double-membraned vesicles called autophagosomes, which are then targeted to the vacuole or to lysosomes for degradation (Patel et al. 2006; Zakeri et al. 2008). Necrosis is considered a chaotic type of cell death, without a genetic control by the cell, possibly caused by prompt external cellular stress. The execution time is so short that the cell cannot activate the PCD pathways. The ultrastructural morphology of necrosis is different from the PCD syndromes, since swelling rather than shrinkage is the main feature of the morphological changes involving endoplasmic reticulum, mitochondria, and the cytoplasm (Krysko et al. 2008). The swelling is probably caused by the loss of the

plasma membrane osmoregulation capability, resulting in water and ions freely entering into the cell (Lennon et al. 1991; Reape et al. 2008).

PCD in plants shows partially overlapped morphological features with that in animals, often resembling apoptosis in cases of deletional PCD, in which the cell undergoing the PCD genetic program is destined to disappear, as in tapetum degeneration (Papini et al. 1999), in supernumerary megaspores deletion in gymnosperms (Cecchi Fiordi et al. 2002), in cells elimination in leaf perforations of *Aponogon madascariensis* (Gunawardena et al. 2007), and synergids elimination (Rogers 2006). The situation is largely diverging and with a very heterogeneous range of morphological features, in cases of developmental PCD as in tracheary elements differentiation and *Tillandsia* trichome development (Papini et al. 2010). Moreover, the formation of apoptotic bodies in plants is rare (Reape et al. 2008) and, when present, they are not comparable to those observed in animals, since they cannot be removed by macrophages. The absence of this last phenomenon does not allow recognition of real apoptosis in plants (Van Doorn and Woltering 2005). This fact induced Reape et al. (2008) to coin the term apoptotic-like PCD (AL-PCD) for PCD in plants.

The term senescence in plants, at the cell level, as defined by Noodén (1988): ‘an endogenously controlled degenerative process’, in terms of morphological syndrome by transmission electron microscope, largely overlapped with the concept of PCD in plants, including both autophagy and AL-PCD. Rogers (2006) decided to use the two terms, senescence and PCD, interchangeably for PCD of flowers organs. A resume on morphological markers of the different types of cell death in plants is reported in Table 1.

Bell (1996) investigated the degeneration of the supernumerary megaspores in a few plants starting from *Marsilea*, a heterosporous fern, proposing a genetic model of the control of the degeneration where a bipartite locus would be involved in PCD control at the megaspores tetrad level. He recognized the abortion of three of four megaspores as the result of an apoptotic process, based on preliminary morphological data on *Marsilea*.

Tillandsia is an important genus belonging to family Bromeliaceae characterized by the lack of an absorbing root system, substituted by the presence of absorbing trichomes specialized for water and other nutrients uptake. The reproductive apparatus and the PCD mechanisms have been extensively investigated in this genus (Papini et al. 1999; Milocani et al. 2006; Brighigna et al. 2006), and hence, the present results can clarify the peculiarities of the PCD in meiotic products. Hence, we studied

The aim of our investigation was to study the degeneration of three of four megaspores after meiosis by transmission electron microscope (TEM), light/fluorescence microscope, and TUNEL assay, to analyse structural

Table 1 Morphological markers of cell death in plants

	Protoplast	Plasma membrane	Nucleus	Chromatin/DNA	Mitochondria	Plastids	ER	Vacuole(s)	Specific structure
PCD, including senescence (Nooden 1988)	Shrinkage (Papini et al. 1999)	Integrity retained	Permanence of the nucleolus (Krysko et al. 2008); fragmentation	Condensation at the margins; DNA laddering	Some survive until the final stage		Early disappearance (Van Doorn and Woltering 2005)	Integrity retained until the very last stage	
Apoptosis or AL-PCD (Reape and McCabe 2008)		Integrity retained							Autophagosome (Van Doorn and Woltering 2005)
PCD, including senescence (Nooden 1988)		Loss of integrity		Condensation in irregular patches (Krysko et al. 2008)	Early damage	Early damage	Swelling	Loss of integrity, release of lytic enzymes	
Necrosis	Swelling (Potten and Wilson 2004)								

characters as the PCD events and the cytoplasmic changes in *Tillandsia aeranthos* and *Tillandsia meridionalis*. We should consider that apoptosis has been demonstrated in the absence of DNA fragmentation, questioning the universality of this character (Sanders and Wride 1995; Schulze-Osthoff et al. 1994), hence ultrastructural investigation remains of fundamental importance for studying apoptotic events (Wyllie et al. 1980; Tinari et al. 2008) and can be considered a ‘golden standard’ in cell death research (Krysko et al. 2008), particularly for autophagic death (Zakeri et al. 2008).

Material and methods

Anatomy and ultrastructure

Plants of *T. aeranthos* (Loiseleur) L. B. Smith and *T. meridionalis* Baker were collected in the experimental station of Las Gamas (Santa Fe, Argentina). Flowers were dissected at various developmental stages before and after anthesis. Pieces of ovary were prefixed overnight in 1.25% glutaraldehyde, at 4°C in 0.1 M phosphate buffer (pH 6.8). The samples were fixed in 1% OsO4 in the same buffer for 1 h. After dehydration in an ethanol series and a propylene oxide step, the samples were embedded in Spurr’s epoxy resin (Spurr 1969). Transverse sections approximately 80 nm thick were cut with a diamond knife, stained with uranyl acetate (Gibbons and Grimstone 1960), and lead citrate (Reynolds 1963), then examined with a Philips EM300 TEM at 80 kV. Semithin sections (1–5 µm), obtained using glass knives, were stained with Toluidine blue, 0.1%, observed and photographed with a light microscope. Some sections at the tetrad stage were stained with PAS+Aniline Blue (Currier and Shih 1968) for callose localization and observed with a fluorescence microscope.

In situ detection of DNA fragmentation

A subset of the material after the glutaraldehyde fixation step was embedded in Technovit 7,100 resin (Kulzer, Germany). Semithin sections (5–8 µm) were obtained with glass knives with a Reichert Om U3 ultramicrotome. In situ Cell-Death-Detection Kit (Roche Diagnostics, Mannheim, Germany) was applied to the sections (TUNEL assay). The sections were digested for 15 min at RT with proteinase K (10 mg ml⁻¹), briefly rinsed twice with a phosphate buffer 0.1 M at pH 6.8, then incubated in a humidified atmosphere with fluorescein labeling mix (10 U TdT, 0.5 nM dig-11-dUTP, in 50 µl of TdT buffer per slide) for 1 h at 37°C in the dark. We prepared also negative controls using only label solution of TUNEL Roche reaction kit but without terminal transferase (data not shown). The reaction was

terminated by rinsing the slides three times with the phosphate buffer. Samples were observed in a drop of buffer under fluorescent microscope Leika DM RB Fluo in the range of 515–565 nm (green).

Results

Stage 1

A *T. aeranthos* ovule at the stage of megaspores mother cell (MMC) is shown in Fig. 1 (1). The MMC was surrounded by two to three nucellus layers. The teguments did not yet completely cover the nucellus.

The MMC could be easily distinguished from the nucellar tissue because of its larger dimension and its central position within the ovule (Fig. 1 (1)). The wall was thin when compared to the surrounding nucellar cells. The nucleus was huge, euchromatic, and with a scarcely electron dense matrix. The nucleolus was clearly visible and apparently active (Fig. 1 (2)). The nuclear membrane was frequently interrupted by pores. Some electron dense bodies appeared to enter in contact with the nuclear membrane (Fig. 1 (2a)). Short tracts of ER and small vacuoles, sometimes merging together, were observable in the cytoplasm (Fig. 1 (2)); mitochondria were numerous and small, with a rarified matrix and rare cristae; plastids were subtle, elongated, and amoeboid; both mitochondria and plastids were often in division, and lipid droplets were frequent (Fig. 1 (3)). A certain prevalence of chloroplasts and lipid droplets appeared to be at the chalazal pole (Fig. 1 (2 and 5)). When the chromosomes began to coil in the nucleus, the nuclear membrane began to lose regularity (Fig. 1 (4)), the cytoplasm resulted enriched in lipid droplets and we could observe a remarkable increase of the ER network. Some ER cisternae were running in parallel to the nuclear envelope (Fig. 1 (5)).

Stage 2

At the dyad stage the nucellus layers were three to four, while the inner tegument was almost completed (Fig. 1 (6)). A callose layer surrounded the two cells derived from the first MMC division. The two cells derived from the first meiotic division did not show difference, but for a higher fragmentation of the vacuolar compartment in the chalazal cell (Fig. 1 (6)). In this last cell, the vacuoles appeared to be located mainly at the micropylar pole.

Stage 3

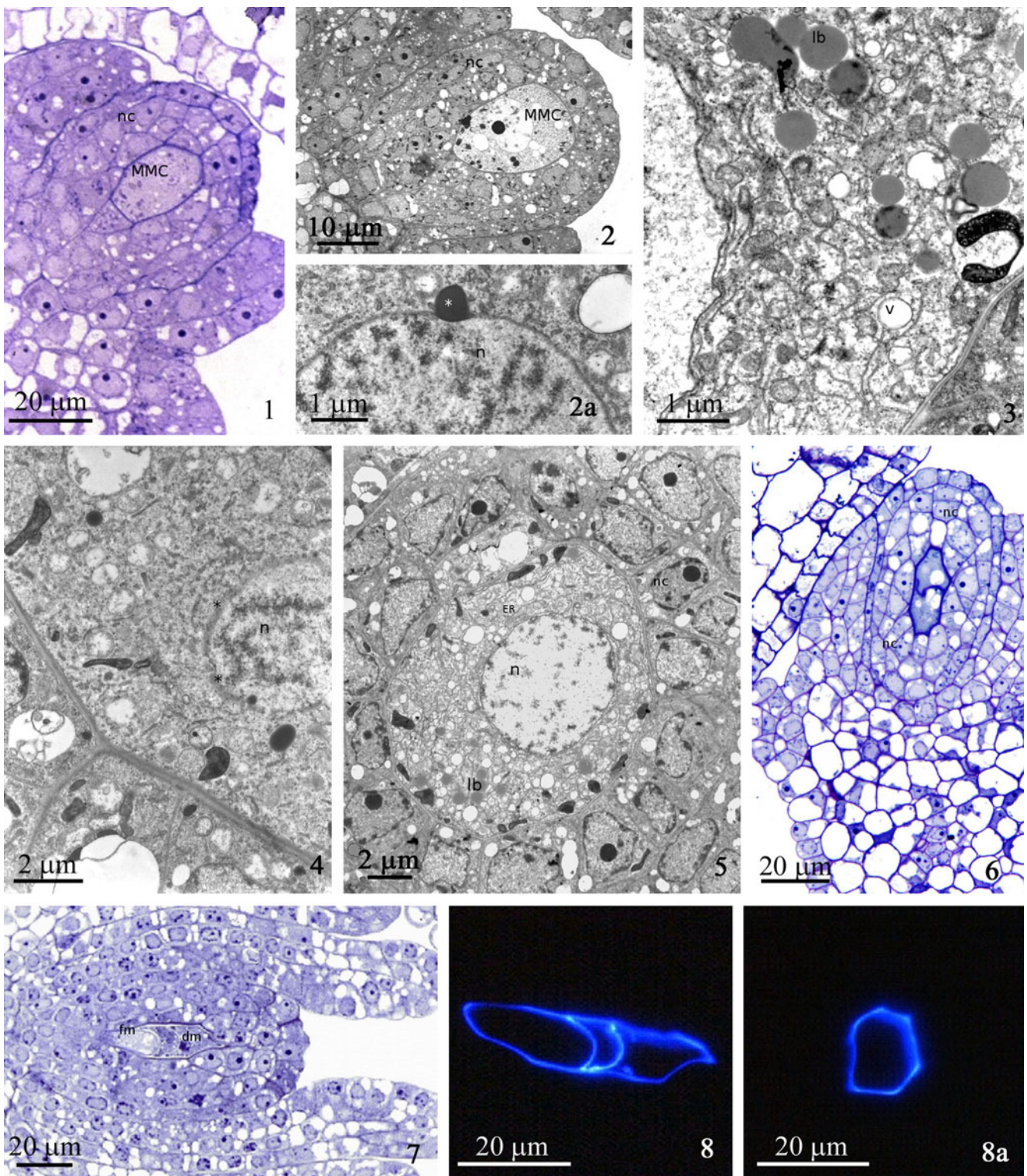
At the tetrad stage, the surviving megaspore was in chalazal position with respect to the other three degenerating spores.

Fig. 1 1 *T. aeranthos* ovule at the stage of megaspores mother cell (MMC) in prophase. The MMC is surrounded by two nucellus layers. The teguments did not yet completely cover the nucellus. *Bar* = 2 μm . 2 Same sample as Fig. 1, TEM image. The nucleus is huge, heterochromatic and with a low electron density matrix and a big, active nucleolus. *Bar* = 10 μm . 2a Same stage as 2. An electron dense body (*white asterisk*) is entering in contact with the nuclear membrane. *Bar* = 1 μm . 3 Same stage as 2. Mitochondria and plastids are often in division. Lipid bodies are frequent. *Bar* = 1 μm . 4 Late MMC stage. When the chromosomes start coiling, the nuclear membrane (*asterisks*) begins to lose regularity. *Bar* = 2 μm . 5 Late MMC stage. The cytoplasm is enriched in lipid droplets, and we could observe a remarkable increase of the ER network. Some ER cisternae are running in parallel to the nuclear envelope. *Bar* = 2 μm . 6 Dyad stage. The nucellus layers are three to four while the inner tegument is almost completed. A callose layer is built along the dyad walls. *Bar* = 20 μm . 7 Tetrad stage. The functional megaspore is in chalazal position with respect to the other three degenerating spores. The three supernumerary spores appear to have a denser cytoplasm with respect to the functional one (Toluidine blue). *Bar* = 20 μm . 8 Tetrad stage in *T. meridionalis* (PAS+Aniline blue staining). Three spores appeared surrounded by a callose layer. The tetrad is in longitudinal section. *Bar* = 20 μm . 8a The same as in 8, but in transverse section. *Bar* = 20 μm . MMC megaspores mother cell, *n* nucleus, *nc* nucellus cell, TEM transmission electron microscope, *lb* lipid body, *v* vacuole, *fm* functional megaspore, *dm* degenerating megaspore

The three supernumerary spores appeared to have a denser cytoplasm with respect to the surviving one (Fig. 1 (7)). Three spores appeared to be surrounded by a callose layer (Fig. 1 (8 and 8a)). A callose layer separated also the surviving megaspore from the other degenerating ones (Fig. 2 (9)).

The surviving megaspore was characterized by a huge heterochromatic nucleus with an active nucleolus (Fig. 2 (10)). Bell-shaped plastids were numerous in the cytoplasm, which was of the same electron density of that of the nucellar cells. Mitochondria were rare (Fig. 2 (10)). Apparently, the nucleus was first localized close to the degenerating megaspores (Fig. 2 (9)), while later it moved to chalazal position.

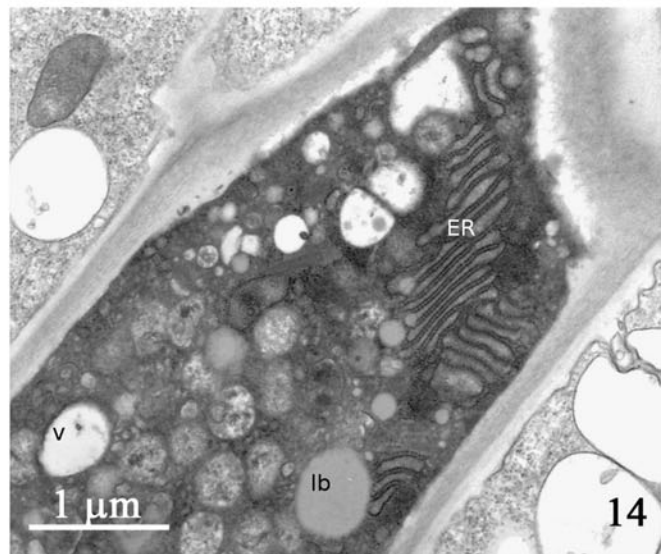
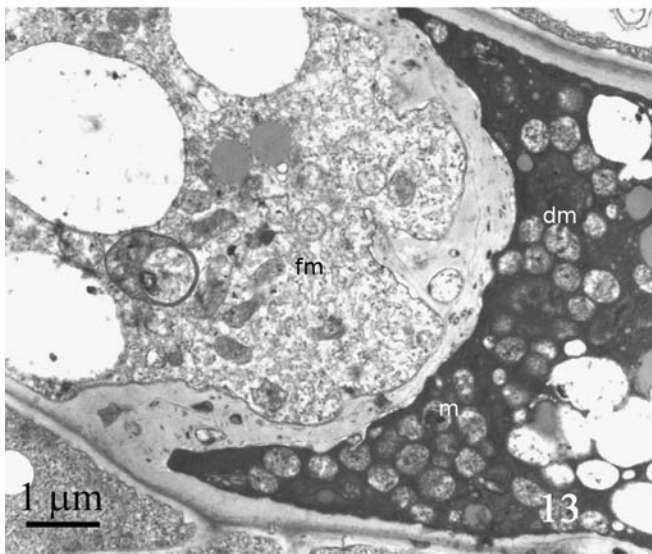
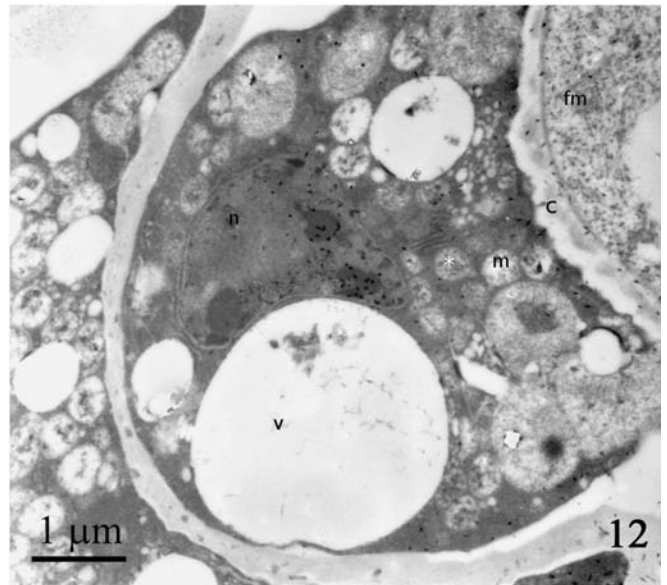
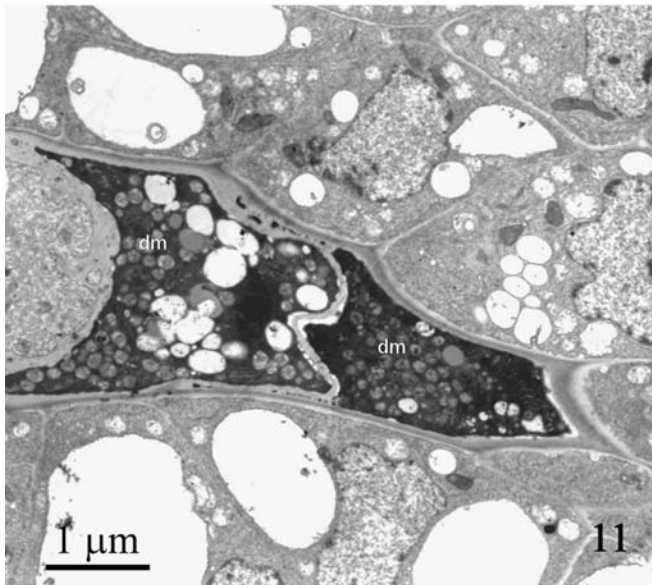
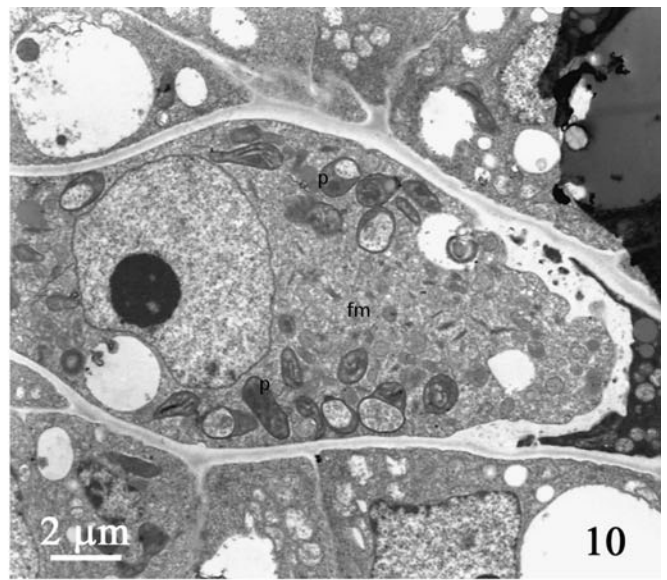
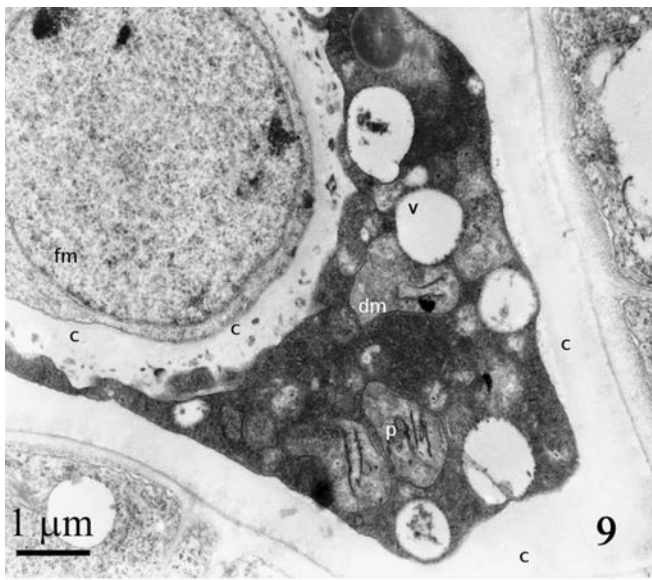
The general aspect of the supernumerary degenerating megaspores is shown in Fig. 2 (11). The nucleus appeared very electron dense and with condensed chromatin in blocks. About 15–25% of the cytoplasm observed in TEM images was occupied by vacuoles of variable dimensions. The vacuoles appeared to engulf organelles and cytoplasm regions. Some of the vacuoles appeared to join together. Many lipid bodies are visible in the cytoplasm. The plastids appeared different from those of the functional megaspore: the dimension here was larger, with few thylakoids and very rare starch granules (Fig. 2 (9)). Two types of organelles were tentatively related to mitochondria in the degenerating megaspores: one with a more rarefied matrix, similar to those observed in the functional megaspore, and another type with a more electron dense matrix (Fig. 2 (12)). Mitochondria were much more numerous than in the functional megaspore (Fig. 2 (13)). The ER appeared swollen and producing lipid droplets (Fig. 2 (14)). In a



later moment of the stage, the degenerating megaspores appeared to be mainly converted to lipid material of variable electron density. Remnants of the nucleus and of swollen ER were still visible in the very electron dense cytoplasm (Fig. 3 (15)).

Stage 4

At the gametophyte stage, the nucellar cells surrounding the gametophyte showed shrunk and condensed cytoplasm with respect to the gametophyte cells (Fig. 3 (16)).



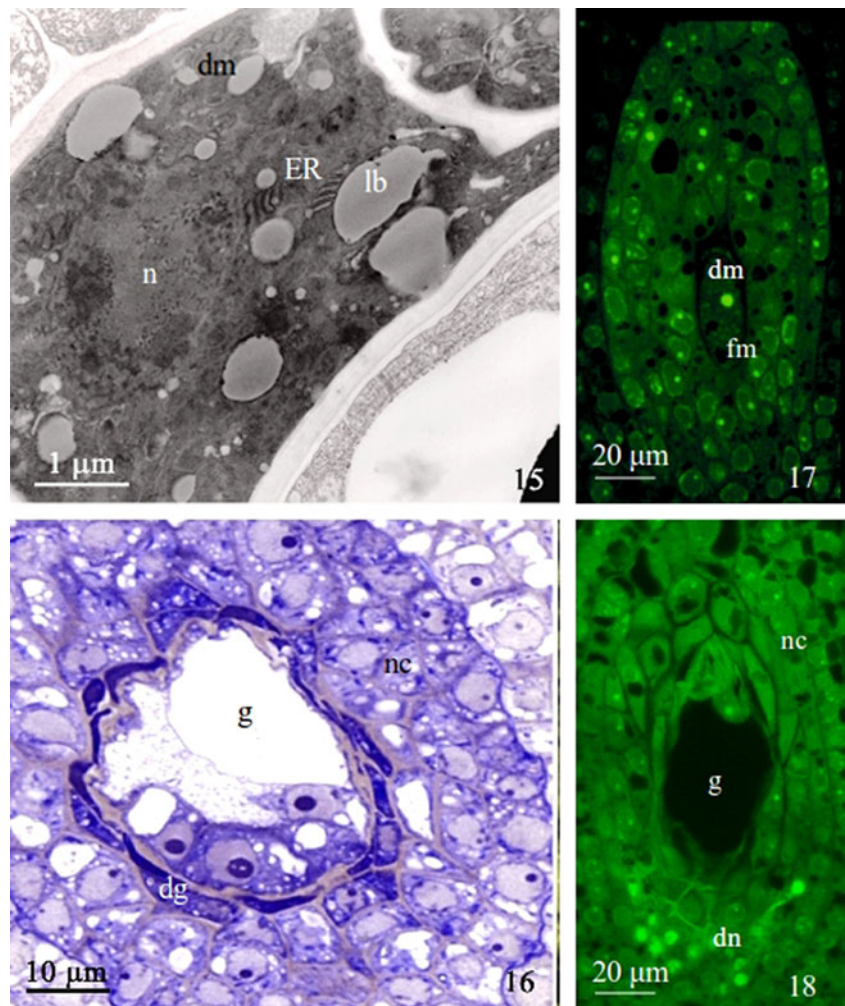
◀ **Fig. 2** 9 Tetrad stage, TEM image. A callose layer separates also the surviving megaspore from the other degenerating ones. *Bar* = 1 μm . 10 Tetrad stage, functional megaspore. The heterochromatic nucleus is huge and with an active nucleolus; bell-shaped plastids are numerous in the cytoplasm, while mitochondria are rare. *Bar* = 2 μm . 11 Tetrad stage, degenerating megaspore. The nucleus appears very electron dense and with condensed chromatin in blocks. About 15–25% of the cytoplasm is occupied by vacuoles of variable dimensions. Vacuoles appear to engulf organelles and cytoplasm regions. Some of the vacuoles appear to join together. Many lipid bodies are visible. *Bar* = 1 μm . 12 Tetrad stage, degenerating megaspore. Two types of organules are related to mitochondria in the degenerating megaspores: one with a very rarefied matrix and another type with a more electron dense matrix (*asterisk*). *Bar* = 1 μm . 13 Tetrad stage, degenerating megaspore. Mitochondria were much more numerous than in the functional megaspore. *Bar* = 1 μm . 14 Tetrad stage, degenerating megaspore. The ER (resembling a Golgi apparatus, but with more cisternae) appears swollen and producing lipid droplets. *Bar* = 1 μm . ER endoplasmic reticulum, *v* vacuole, *lb* lipid body, *fm* functional megaspore, *c* callose layer, *dm* degenerating megaspore, *p* plastid, *m* mitochondrion, *n* nucleus

The TUNEL reaction at the tetrad stage reacted positively in the supernumerary megaspores nuclei (only one visible in the cut slice of Fig. 3 (17)), but not in the surviving one; also some nucellus cells resulted TUNEL positive (Fig. 3 (17)). The position of the degenerating megaspores with respect to the surviving one could be assessed because they were at the micropylar pole: the general shape and the consequent spatial relationship between the megaspores was the same observed with TEM in the same stage (Fig. 2 (9, 11 and 13)). At the gametophyte stage, the nucellus cells at the chalazal pole of the gametophyte resulted TUNEL positive (Fig. 3 (18)).

Discussion

The ovule in *T. meridionalis* and *T. aeranthis* is bitegmic and crassinucellate as in other Bromeliaceae (Rao and Wee 1979). The gametophyte development in *Tillandsia* corresponds to the Polygonum type (Willemse and Van Went 1984), that is with a final stage of eight nuclei and seven cells.

Fig. 3 15 Tetrad stage, degenerating megaspore in an advanced phase. The degenerating megaspore content appears to be mainly converted to lipidic material of variable electron density. Remnants of the nucleus and of swollen ER are still visible in the very electron dense cytoplasm. *Bar* = 1 μm . 16 Gametophyte stage, light microscope. The nucellar cells surrounding the gametophyte show shrunk and condensed cytoplasm with respect to the gametophyte cells. *Bar* = 10 μm . 17 Tetrad stage, fluorescence microscope. The degenerating megaspores nuclei (only one visible in the figure) are TUNEL positive. Also some nucellus cells nuclei are TUNEL positive. *Bar* = 20 μm . 18 Gametophyte stage, fluorescence microscope. The degenerating nucellus cells nuclei around the female gametophyte are TUNEL positive. *Bar* = 20 μm . *nc* nucellus cell, *g* gametophyte, *dn* degenerating nucellus, *dm* degenerating megaspore, *ER* endoplasmic reticulum, *lb* lipid body, *n* nucleus, *fm* functional megaspore



The aspect of the MMC in the initial stage is typical of a high activity in protein synthesis preceding the meiosis. A prevalence of mitochondria was observed in other angiosperms at the chalazal pole (Ingram 2010). In *Tillandsia*, mitochondria were numerous in the whole MMC, and hence, it was difficult to establish a polarity of these organelles. At the chalazal pole, we observed more prevalence of plastids and lipid droplets.

The MMC undergoes an autonomous genetic program divided in two stages. A first stage begins with the occlusion of plasmodesmata, with the consequent symplastic separation of the MMC from the surrounding nucellar cells. This separation is related to the blockage of signaling through plasmodesmata and leads eventually to meiosis (which is limited to the MMC). In *Arundo donax*, a persistence of plasmodesmata in the chalazal area is reported (Jane and Chiang 1996).

A second sign of further autonomous genetic program is the presence of callose surrounding the MMC, starting before the dyad stage. After Schwab (1971) and Bouman (1984), the callose deposition begins at meiosis prophase I.

This second mechanism of signaling blockage is more probably related to the PCD events that will occur from this stage on. The callose would act as a molecular filter after Southworth (1971), and we can suppose that PCD signaling molecules are not allowed to flow through the callose layer. Callose is observed also around pollen tetrads (for instance, Brighigna and Papini (1993)). The situation is different from pollen development (where no spore undergoes PCD), since callose here separates not only the megaspores from the nucellar cells, but also the surviving megaspore from the degenerating ones (see Fig. 1 (8)).

Callose has been detected in megasporogenesis in all investigated species with mono- and bisporic embryo sac development (Rodkiewicz 1970). In tetrasporic embryo sac development, no callose is observed and all the four spores survive (Maugini 1953; Schwab 1971; Willemse and Van Went 1984; Madrid and Friedman 2010). No cytokinesis occurs, and all the four female nuclei will contribute to the final gametophyte that will have therefore a chimeric nature: four starting nuclei, each with a different genome. Callose is known to occur first in the meiotic prophase in the chalazal part of the MMC wall and, by the first meiotic metaphase, the whole cell is enveloped in a callose-containing wall. Later, callose decreases at the chalazal pole (Rodkiewicz 1970). In plants where the micropylar megaspore is the surviving one, as in *Oenothera*, the decrease in callose fluorescence begins just at the micropylar pole (Rodkiewicz 1970).

The callose distribution observed in *Tillandsia* is comparable to that observed by Russell (1979) in *Zea mays*, where the aniline blue fluorescence is intense in nonfunctional megaspores walls; whereas in the functional

megaspore, intensity decreases at the chalazal end. In *A. donax*, callose deposition starts instead at the micropylar end (Jane and Chiang 1996). Nevertheless, no aniline blue fluorescence was seen later in the chalazal half of the functional megaspore, where plasmodesmata were also present (Jane and Chiang 1996). The final situation in this species would not hence be different from the general pattern.

The general shrinkage of the cell protoplast and the condensation of the cytoplasm and particularly of the nucleus observed in the degenerating supernumerary megaspores are signs of PCD (Pennell and Lamb 1997). The PCD of the supernumerary megaspores in angiosperms is a deletional PCD, since the developmental program leading to the female gametophyte formation and maturation implies their disappearance. The PCD program runs quite fast, since, even observing a lot of samples, it was very difficult to record the very first stage of the degeneration process.

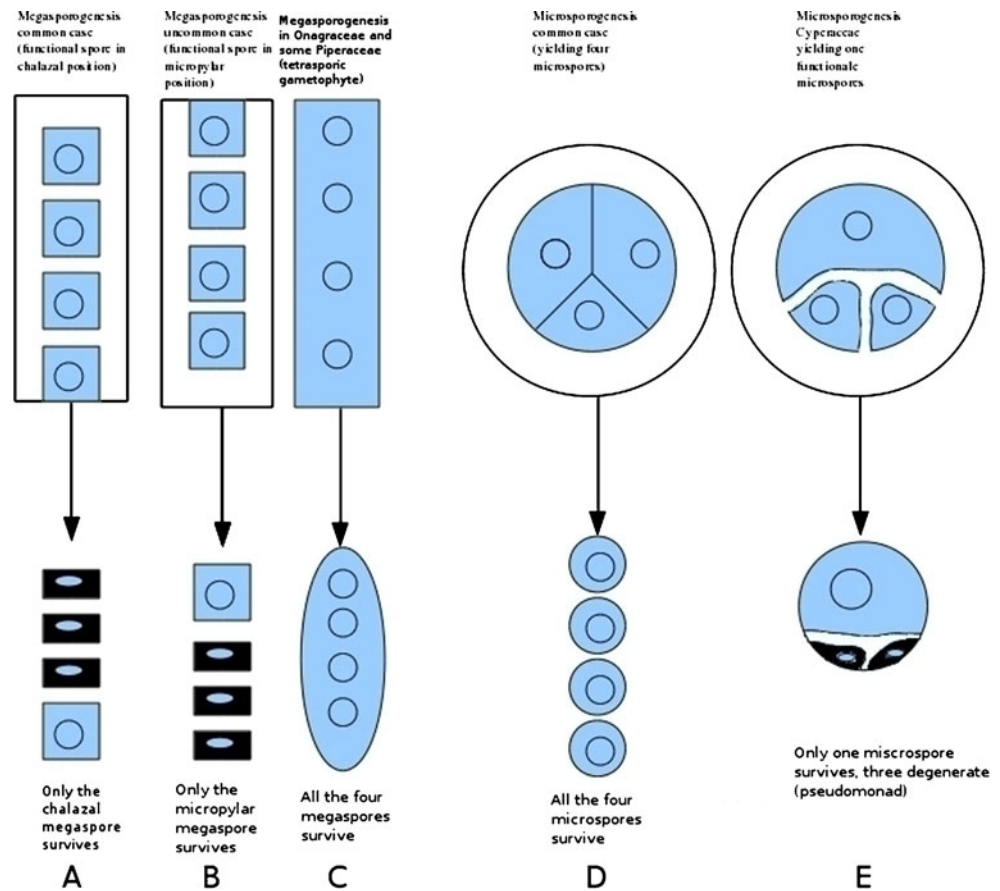
The early disappearance of callose around the functional megaspore and the degeneration of the megaspores undergoing PCD are common features in angiosperms (Rodkiewicz 1970). Even if the callose layer is not impermeable to all substance (Rodrigues-Garcia and Majewska-Sawka 1992), we can suppose that the presence of callose is related to the necessity of blocking PCD inhibiting signaling towards the meiospores.

A role for the callose in directing the megaspores destiny after meiosis was also proposed by Bouman (1984), who considered the uneven distribution of callose in the tetrad clearly connected with the polarization of the cells since the beginning of callose deposition. Its disappearance corresponded to the localization of the functional megaspore in the tetrad.

An indirect support to this speculation may be the fact that in tetrasporic embryo sac development, no megaspore nucleus degenerates nor callose can be found around them (Schwab 1971). Another indirect confirmation of the role of callose in PCD control in megaspores comes from the situation of microsporogenesis in Cyperaceae. In Cyperaceae, three microspores of four degenerate after meiosis, and this pattern is linked to an unusual distribution of callose thickenings around the microspores, lasting until late tetrad stage (Ranganath and Nagashree 2000). Callose in this family was found also around the three degenerated megaspores enclosed within the pollen grain (Dunbar 1973; Strandhede 1973), even if this presence was not considered linked to the degeneration itself. A resume on the occurrence of callose around spores in plants is reported in Scheme 1.

The proposal of a genetic model for triggering PCD in megaspores with two cistrons activating the degeneration program after meiosis (Bell 1996) would suggest an

Scheme 1 Callose deposition effect on tetrads. **a** More common megasporogenesis ending with a monosporic gametophyte, functional megaspore in chalazal position. **b** Megasporogenesis with functional megaspore in micropylar position, ending with a monosporic gametophyte (*Oenothera*). **c** Megasporogenesis ending with a tetrasporic gametophyte. No cytokinesis occurs after meiosis. **d** More common microsporogenesis ending with four living spores. **e** Microsporogenesis in Cyperaceae with three degenerating microspores, ending with a pseudomonad. Callose deposition in *white*; degenerated cells in *black*; living cells in *blue*



internal activation of the PCD. Nevertheless, the heterogeneous callose thickenings may allow unidentified PCD inhibitors to reach the functional megaspore (with no callose thickenings) but not the supernumerary megaspores. The arrival of a 'survival' signal to the chalazal pole of the tetrad from the nucellus was proposed also by Bell (1996) who did not link this theoretical opinion to the callose thickening patterns. Also the model by Calderon-Urrea and Dellaporta (1999) for the selective elimination by PCD of floral organs during the unisexual flower formation in maize, suggested that a mechanism with two genes, *tasselseed1* and *tasselseed2*, is required for the death of pistil cells. Functional pistils are protected from cell death by the action of the *silkless1* gene inhibiting *tasselseed*-induced cell death. On the contrary, Ingram (2010) proposed that the three supernumerary megaspores could simply be starved of nutrients and energy, triggering cell death. Further investigations would be useful to clarify if the mechanism acting by surviving megaspores selection during megasporogenesis in angiosperms works with the same genes and inhibitors.

The development and activity of vacuoles in the degenerating megaspores was structurally related to autophagosomes, markers of autophagic cell death, even if, due to the high electron density of the cytoplasm, it was very

difficult to assess the presence of a vacuolar double membrane as indicated by Zakeri et al. (2008).

The last stage of degeneration of the supernumerary megaspore ends in a lipidic corpse still partially embedded with callose. This last developmental stage shows some necrotic features, as the swelling of endoplasmic reticulum (Krysko et al. 2008), even if the plasma membrane does not break, or at least fuses with the cell lipidic content. The lipidic content of the corpse and the presence of callose could be necessary to avoid contact between this corpse and the surviving megaspore.

The method of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) for the identification of DNA fragmentation is one of the most used to confirm apoptotic syndromes in cells, since it is linked to the internucleosomal DNA cleavage caused by caspase-activated DNase (Nagata 2000) and the analogous enzymes in plants. The observation of TUNEL-positive nuclei in the degenerating supernumerary megaspores after meiosis confirmed that this type of PCD in plants shows resemblance to animal apoptosis for the mechanism of DNA fragmentation and for some of the ultrastructural features.

Also, some nucellar cells and later all those surrounding the developing embryo sac at the fourth stage (from mature

megaspore on) showed TUNEL positivity, possibly indicating a PCD genetic program activated by the end of the meiosis and later by the growing gametophyte. The loss of the callose layer would permit a faster entrance of nutrients to the gametophyte and probably a PCD-inducing signal from the gametophyte to the nucellus. The developmental aim is probably related to the necessity of increasing the free space for the growing gametophyte, and later for the embryo, within the ovule. The PCD of the nucellus in *Tillandsia* showed some morphological similarity with the hypersensitive response (*sensu* Krishnamurthy et al. 2000), probably due to the necessity of a very fast process following the increase in dimension of the gametophyte (Brighigna et al. 2006).

Of the two types of organelles tentatively related to mitochondria observed in the degenerating megaspores, those with a more electron dense aspect might be linked to the mitochondria-linked PCD pathway (Vianello et al. 2007), while those with a more rarefied matrix would be still (at least partially) functional, and rather related to the energy needs of a cell undergoing a genetic program leading to an organized disruption. After Tinari et al. (2008), a rarefaction of the mitochondrion matrix is common during necrosis. Anyway, in *Tillandsia* megaspore (and other plant tissues), mitochondria with matrix rarefaction are present also in the functional megaspore, that is not destined to undergo neither necrosis nor PCD. Hence, in the degenerating megaspore, these mitochondria would rather be those still providing some energy to the cell undergoing its genetic death program. Indeed, the ATP level has been indicated as a possible switch between PCD (high ATP) and necrosis (low ATP) at least in animals (Lemasters 1999). The permanence of mitochondria until late stage of PCD in plants has been shown in many tissues, such as the angiosperm tapetum (Papini et al. 1999), and could be necessary to maintain high ATP levels until late stages of the PCD process. The plastids of the degenerating spores in a first stage (afterwards they degenerate and are hardly recognizable) appear different from those of the functional megaspore. The change in shape/dimension, the loss of starch storage, and the reduction in thylakoid apparatus may be linked with the generation of ROS. The production of ROS by chloroplasts in cells undergoing AL-PCD has been suggested by Doyle et al. (2010), even if no particular ultrastructural change was recorded. After Mullineaux and Karpinski (2002), the chloroplasts are likely candidates for a sensor of stress and initiator of cellular signalling that leads to stress responses. Even after Danon et al. (2006), chloroplastic generation of stress-related ROS may regulate PCD pathways in plant cells.

The degeneration begins with the increase of the vacuolar apparatus; but in the final stages, the vacuoles do not occupy the main cell volume as indicated for autophagy

(van Doorn and Woltering 2005), and eventually, they reduce their size until the final stage in which the cellular remnant appears to be of mainly lipidic nature. At the gametophyte stage, the cellular remnants of the nonfunctional megaspores are hardly visible, and we can suppose that, even if no periplastic bodies were released outside the plasma membrane as in *Larix* megasporogenesis (Cecchi Fiordi et al. 2002), a secondary necrosis process may occur leading to in situ lysis.

Conclusions

In *Tillandsia*, the decision on which will be the three of four megaspores destined to undergo PCD is linked to the pattern of callose deposition around the tetrad. This conclusion can be extended to many other angiosperms.

During supernumerary megaspores degeneration, events leading to the deletion of the cells do not appear to belong to a single type of cell death. The first morphological signs are typical of autophagy, with the formation of autophagosomes. Mitochondria morphology and the TUNEL positivity indicate the passage to an AL-PCD phase, while the cellular remnants undergo a final process resembling, at least partially (ER swelling), a necrotic morphological syndrome, eventually leading to a mainly lipidic cell corpse still separated from the functional megaspore by a callose layer.

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

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