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Programmed cell death in seeds of angiosperms

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Abstract During the diversification of angiosperms, seeds have evolved structural, chemical, molecular and physiologically developing changes that specially affect the nucellus and endosperm. All through seed evolution, programmed cell death (PCD) has played a fundamental role. However, examples of PCD during seed development are limited. The present review examines PCD in integuments, nucellus, suspensor and endosperm in those representative examples of seeds studied to date.

Keywords: Caspases; endosperm; KDEL-CysEP; metacaspases; nucellus; nucleases; programmed cell death; perisperm; ricinosomes
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Minireview

INTRODUCTION

In the angiosperms, fertilization results in the formation of the seed from the ovule. This involves the activation and coordination of the distinct developmental pathways leading to form an embryo, an endosperm and a seed coat. Programmed cell death (PCD) is an integral part of the seed development, during which cells of the integuments, nucellus, suspensor and cells of the endosperm, face death. Different cell death types are often defined by morphological characteristics because precise molecular mechanisms behind the regulation and execution of cell death processes are still limited.

Seed morphological diversity mainly comprises endospermic, exendospermic and perispermic seeds. The existence of different types of seeds suggests the occurrence of very different development programs. In some of them, cells should die to form specialized tissues. In other cases, cells die because they have fulfilled their role and/or are no longer necessary.

To date, PCD during seed development has been well-studied in the nucellus of ricinus (Greenwood et al. 2005), *Sechium edule* Sw (Lombardi et al. 2007a), wheat (Domínguez et al. 2001) and barley (Radchuk et al. 2011) and also in the endosperm of quinoa (López-Fernández and Maldonado 2013b), rice (Lan et al. 2004; Kobayashi et al. 2013), wheat (Young and Gallie 2000), maize (Young and Gallie 2000) and barley (Borén et al. 2006). Figure 1 summarizes the main events studied in the seeds of such species, most of which are involved in the activation of proteases and the induction of specific nucleases. Van Doorn et al. (2011) classify cell death in plant tissues according to their biochemical and morphological characteristics and kinetics, recognizing two main types: (i) vacuolar type, and (ii) necrosis.

A number of recent studies have described the importance of vacuoles in plant cell death (Hara-Nishimura and Hatsugai 2011). Vacuoles have emerged as key sources for factors that mediate cell lysis and as reservoirs for a variety of metabolites. Similar to the function of lysosomes in animals, plant vacuoles can be used to recycle part of their cell content. During cell death, lytic vacuoles increase their volume by engulfing the cytoplasm with the consequent degradation of its content, which is a crucial mechanism of cell dismantling similar to the micro- or macro-autophagy processes. Tonoplast disassembly represents the last step of the vacuolar cell death, because it involves the release of vacuolar hydrolases affecting chromatin structure and initiating DNA fragmentation and disintegration of the nuclear envelope (van Doorn et al. 2011; Domínguez and Cejudo 2014); finally, hydrolases destroy the residual cell content and, in some cases, the entire cell including the cell wall. Mitochondria and other organelles as well as the plasma membrane are maintained until the tonoplast breaks (van Doorn et al. 2011 and references therein). In cereals, cells of the starchy endosperm accumulate storage reserves like protein and starch and die, but their "corpse" remains unprocessed until germination (Young and Gallie 2000). This also occurs during perisperm maturation in quinoa, where cells accumulate starch and die (López-Fernández and Maldonado 2013a). In both cases, there is a delay between cell death and cell "corpse" processing, which is the time (sometimes years) between seed ripening and the beginning of germination.

On the other hand, the necrotic mode of cell death differs in several cytological features, i.e., changes in mitochondria, early loss of plasma membrane integrity (leading to protoplast contraction) and lacking lytic vacuoles (leaving the necrotic corpse unprocessed) (van Doorn et al. 2011; Galluzzi et al. 2012). This type of cell death occurs from hours, during

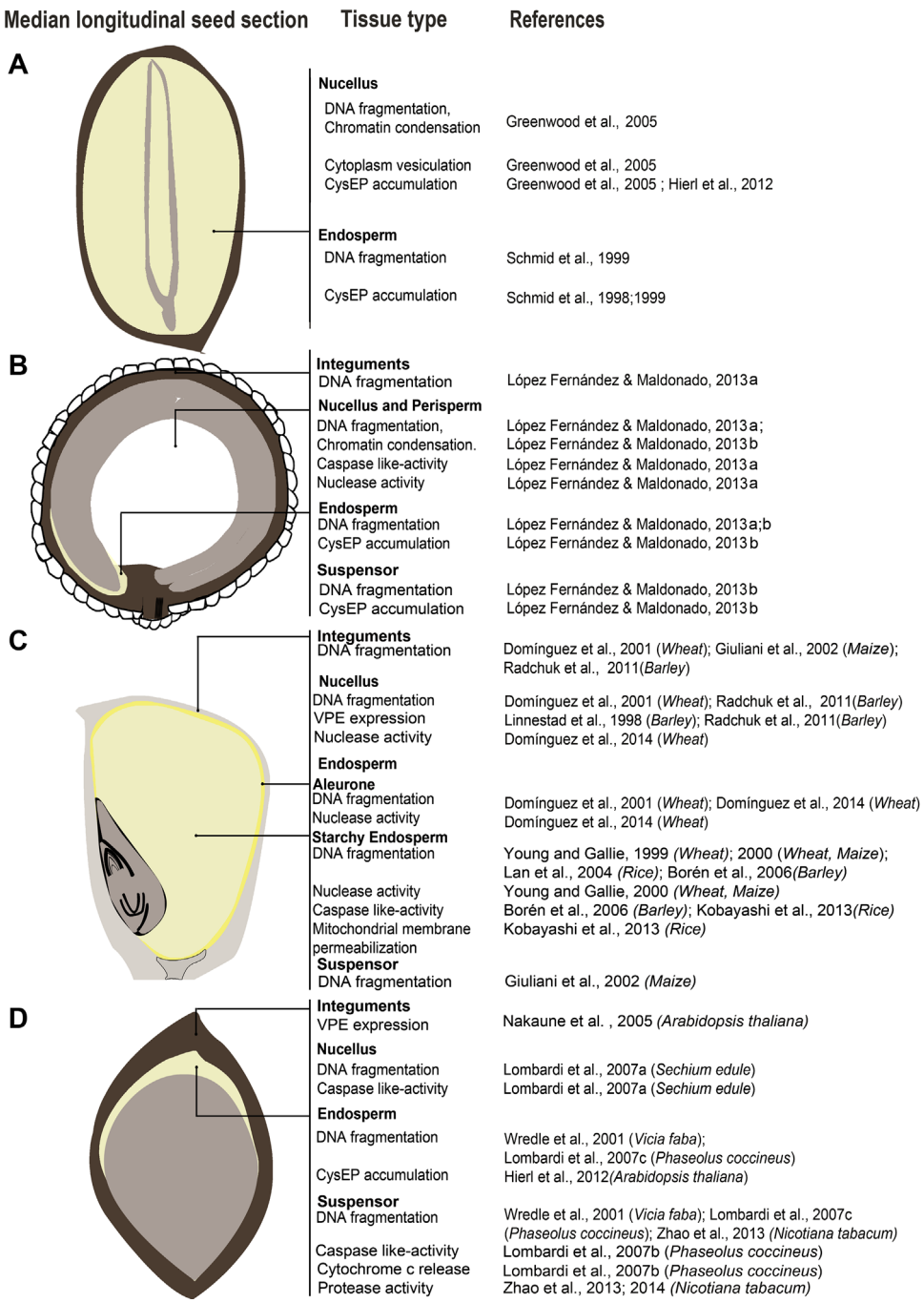

Figure 1. Key events that are being reported during programmed cell death (PCD) in representative examples studied to date (**A**) Castor bean seed. (**B**) Quinoa seed. (**C**) Cereal (maize, wheat, barley, rice) seed. (**D**) *Sechium edule* seed.

treatment response, to a day, as seen all through hypersensitive response (HR). Therefore, the necrosis is a rapid process in contrast to vacuolar cell death.

PROTEASES

Seed developmental PCD involves diverse classes of proteases, including cysteine proteases, serine proteases, and aspartic proteases, and special functions have been described for vacuolar proteases (Radchuk et al. 2011; Hara-Nishimura and Hatsugai 2011), metacaspases (Tsiatsiani et al. 2011) and a group of papain-type cysteine endopeptidases (KDEL CysEPs) (Schmid et al. 1998; Greenwood et al. 2005).

Caspases are a group of proteins from the family of cysteine aspartate proteases, which mediate and control the apoptotic response in animal cells. Caspases play a key role in PCD initiation and execution, introducing specific breaks after

aspartate residues in target proteins. To date, no structural animal caspase homologs with specific cleavage and similar function have been found in plant genomes. However, caspase-like activities can be detected in plants, suggesting that enzymes structurally distinct to classical caspases can operate as proteases-like caspases in plants (Borén et al. 2006; López-Fernández and Maldonado 2013a).

Plants encode only metacaspases that are distant caspase homologs. Different to caspases, metacaspases display arginine or lysine protease substrate specificity (Watanabe and Lam 2005). In plants, two metacaspase groups have been identified in sequence and structure bases: *type I*, which contain an N-terminal prodomain with a proline-rich and a Zn-finger motif typical of plant proteins that function in the hypersensitive response pathway, and *type-II*, which lack the N-terminal prodomain, but have a linker region between the p20 and p10 catalytic subunits (Uren et al. 2000). Metacaspases have no specific caspase activity, but increasing evidence points to a role in PCD regulation (Bozhkov et al. 2005b; Watanabe and Lam 2005; Minina et al. 2013; Coll et al. 2014).

Other proteases that have been related to developmental PCD (Radchuk et al. 2011) in different tissues are Vacuolar Processing Enzymes (VPEs), also called legumains; these proteases specifically cut after an asparagine or aspartic amino acid residue to eventually be transported to the vacuole or cell wall, where they become active (Martínez et al. 2007). As mentioned above, PCD in plants is also carried out by a single group of papain-type cysteine endopeptidases (Cys-EP) with a C-terminal KDEL endoplasmic reticulum (ER) retention signal (Schmid et al. 1999; 2001). The pro-form of this Cys-EP is located in an ER-derived vesicle, the "ricinosome" (Schmid et al. 2001). It is speculated that in plant cells undergoing PCD where ricinosomes are involved, the acidification of the cytosol results in autocatalytic processing of the mature enzyme to its active form (Schmid et al. 1999). At the same time, ricinosomes swell and burst, releasing these highly active enzymes and acting in the final dismantling of the cell body (Schmid et al. 2001). These enzymes are not structurally related to caspases and no homologous genes are present in mammals or yeast (Helm et al. 2008). From 25 species of monocots, dicots and one species of gymnosperm, Hierl et al. (2012) recognize a highly conserved amino acid sequence, which is decisive for the proteolytic activity of KDEL-CysEP. Both ricinosome and its enzyme have been associated with different PCD processes such as germination, in castor bean endosperm (Schmid et al. 1999) and development, in quinoa endosperm (López-Fernández and Maldonado 2013a) and castor bean nucellus (Greenwood et al. 2005). According to Helm et al. (2008) KDEL-CysEP are expressed in tissues that eventually collapse, either disappearing or remaining as crushed layers. This could explain the absence of ricinosomes in the perisperm of quinoa and probably in the starchy endosperm of cereals.

NUCLEASES

There is a strong association between nucleases and DNA fragmentation (Domínguez and Cejudo 2014). These enzymes have a crucial role during nucleus dismantling because they generate chromatin condensation, internucleosomal DNA fragmentation, and nuclear membrane disruption. Several seed tissues undergo PCD as part of their normal development, which is accompanied by an increase in nuclease activity; some examples can be found in the endosperm of tomato (Farage-Barhom et al. 2008), perisperm of quinoa (López-Fernández and Maldonado 2013a), nucellus of wheat (Domínguez and Cejudo 2014) and, endosperm of maize (Young and Gallie 2000). The nucleases, detected in the nuclei of the aleurone cells of wheat during germination, are regulated by gibberellins and requires a functional signal transduction pathway in response to this hormone (Domínguez et al. 2004; Domínguez and Cejudo 2014).

Two classes of endonucleases have been identified: those dependent on Zn^{2+} and those dependent on Ca^{2+}. Zn^{2+}-dependent endonucleases exhibit maximum activity in a pH range from 5.0 to 6.5, which is consistent with their location in acidic cell compartments such as the vacuole or the extracellular space, and both RNA and single-stranded DNA (ssDNA) are their preferred substrate; however, under suitable conditions, any Zn^{2+}-dependent endonuclease can generate a nick and linearize supercoiled double stranded DNA (dsDNA) (Sugiyama et al. 2000). Zn^{2+}-dependent endonucleases are believed to act after tonoplast or membrane collapse.

Ca^{2+}-dependent endonucleases are active mainly in the nucleus. Most Ca^{2+}-dependent endonucleases have optimum pH in the neutral region and prefer ssDNA as substrate (Sugiyama et al. 2000).

However, recent evidence shows an exception to this rule, as a Zn^{2+}-dependent endonuclease in the nucleus is responsible for DNA laddering of wheat scutellum cells undergoing PCD (Domínguez et al. 2012). These examples show the participation of different nucleases in a wide diversity of plant PCD systems.

PCD AND INTEGUMENTS

Differentiation of the seed coat from the ovule integuments, although ultimately ending in the death of the seed coat cells, includes some of the significant cellular changes observed during seed development. By the time the seed coat is mature, cells of all layers are dead. The death of the various layers occurs at different times of development and in a specific sequence (which is characteristic of a species, genus or family) suggesting that the cell death is programmed as part of the differentiation process, and involves cell-cell signaling.

At some developmental points, which may vary depending on the species, the cells of each layer of the integument layer initiate a program of cell death that can lead to a total disappearance of the cell layer (death without a "corpse") or the persistence of dead cells (death with "corpse"); each integument layer has its own cell death program, which is a proper taxonomic character of the species, genus or family. In *Arabidopsis* seeds, after fertilization, the seed coat consists of an outer integument composed of two cell layers and an inner integument made up of three layers. These integuments are reduced to an outer and inner integument layers when matured. During early seed development, DNA fragmentation is detected. The expression of dVPE in the integument layers that are eliminated is a key step during seed coat formation

(Nakaune et al. 2005). In quinoa, the seed coat consists of an outer and inner integument, each one made up of two layers thick. During seed development, one layer of the outer integument and both layers of the inner integument are consumed. At early seed developmental stages, the inner integument is the first layer to show TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) signal (López-Fernández and Maldonado 2013a).

In some dry fruits with a single seed, such as caryopsis (in Poaceae), achenes (in pseudocereals) (Amaranthaceae, Polygonaceae), or cypsele (in Asteraceae), the seed enclosed in the pericarp is the dispersal unit. The seed coat, attached to the pericarp plays a strategic role in coordinating endosperm and embryo growth during early seed development. In cereals, the pericarp is adhered to the remains derived from the ovule integuments, and usually is reduced to a thin layer (Morrison 1976). The first signs of pericarp PCD start very early in development. Biochemical and morphological analyses show the presence of proteolytic activities and nuclei fragmentation (Domínguez et al. 2001; Giuliani et al. 2002; Radchuk et al. 2011). The quinoa pericarp is also reduced; at maturity it is constituted by two cell layers, the outer layer cells are large and papillose and those of the inner layer are tangentially stretched (Prego et al. 1998).

PCD AND NUCELLAR DEVELOPMENT

In angiosperms, a succession of cell death programs occurs throughout the development of the embryo sac, before anthesis, and the endosperm, after fertilization, affecting the nucellar tissue, which is consumed partial or totally. Lombardi et al. (2007a) studied the nucellar development in *Sechium edule* and showed that, after fertilization, the nucellus degenerates through a cell death program in which a set of proteases such as caspases-like proteases emerge and play a relevant role. Recently, Lombardi et al. (2012) demonstrated that ethylene, released in high concentration from the young endosperm, plays a role in this nucellar PCD leading to DNA fragmentation.

In *Ricinus communis*, nucellar PCD has been described by Greenwood et al. (2005) from both the morphological and ultrastructural points of view. Some features observed include DNA fragmentation, chromatin condensation, cytoplasm vesiculation, generation of ricinosomes and vacuolar collapse. Progressively, the expanding endosperm compresses the nucellus; finally, the nucellus disappears and only residues of the walls of their cells are found.

The perisperm, which originates from the nucellar tissue, is the main storage tissue in perispermic seeds. In quinoa, the mature perisperm (also named basal body) consists of dead cells. As a storage tissue, the cells of the perisperm accumulate starch and degenerate (simultaneously), and both processes are mediated by a controlled cell death program. PCD in quinoa seeds was evaluated by morphological and biochemical cell changes in the nucleus and cytoplasm, i.e., nuclear DNA degradation, endopolyploidy, caspase-like proteases and nuclease activities (López Fernández and Maldonado 2013a). The PCD occurring during seed development in perispermic tissue has been studied only in quinoa, but a basal body with similar features is present in other members of the Caryophyllales (Werker 1997). It can thus be hypothesized that the perisperm or basal body of other Caryophyllales, as well as the perisperm of members of Piperaceae and Nymphaeaceae, and genera of Liliaceae or Zingiberaceae (Werker 1997), would share the same development pattern as that described in quinoa. This is a topic worth investigating in the future.

During wheat grain development, nucellar cells undergoing PCD show nucleus fragmentation as assessed by TUNEL assays, and also ultrastructural changes such as disruption of plasma and nuclear membranes and increase of chromatin levels (Domínguez et al. 2001). The barley nucellus initiates starch accumulation at the anthesis stage and undergoes degradation as early as 2 d after fertilization (Radchuk et al. 2009). Degradation of nucellar tissue provides space for the growing coenocytic endosperm, which expands and becomes cellular. The nucellain (HvVPE2a), a nucellar VPE (Linnestad et al. 1998), together with the other two VPEs (HvVPE2b and HvVPE2d), are involved in these cell death programs. According to Radchuk et al. (2011) HvVPE2b and HvVPE2d are present in the nucellus and participate in its disintegration. The coordinated developmental regulation of the nucellar breakdown and probable relationship to the coenocytic endosperm nourishment might regulate the final cell number of the endosperm, which is an important determinant of storage capacity (Sreenivasulu et al. 2010).

PCD AND ENDOSPERM DEVELOPMENT

During the embryogenesis, the growing embryo consumes the endosperm partially (in endospermic seeds) or totally (in exendospermic seeds). Cells of this ephemeral endosperm die by a program of vacuolar cell death from the innermost layers surrounding the embryo towards the nucellar tissue.

In *Ricinus communis* mature endosperm constitutes a live tissue that stores proteins and lipids to be used during germination. During development, the endosperm consumes the nucellus and the embryo consumes a small portion of the surrounding endosperm (Schmid et al. 1999). In germinating seeds, endosperm PCD starts in the cell layers closest to the cotyledon and goes through the opposite side of the tissue. In these cells the development of ricinosomes is concomitant with the progression of nuclear DNA fragmentation (Schmid et al. 1999).

In the exendospermic seeds of *Phaseolus coccineus* (Lombardi et al. 2007c) and *Vicia faba* (Wredle et al. 2001), the endosperm is a short-lived tissue that is almost totally consumed by the developing embryo. In both species DNA fragmentation in the endosperm precedes DNA fragmentation in the suspensor. Wredle et al. (2001) proposed that this could be due to different signals affecting the endosperm and suspensor or that a single signal triggers different death programs. In the endospermic seeds of *Trigonella foenumgraecum* (other species of Fabaceae), the endosperm is constituted by a living aleurone layer and an underlying dead tissue that accumulates galactomanannans as a source of carbohydrate reserve, but nothing is known about the simultaneous occurrence of cellular death associated with deposition of hemicelluloses in the cell walls of this tissue (Reid 1985).

In the perispermic seeds of quinoa, the endosperm is partially consumed by the embryo during development, but a lasting endosperm, which forms a micropylar cone, covers the radicle (López-Fernández and Maldonado 2013a; Burrieza et al. 2014). The lasting endosperm is a live tissue that stores lipids and proteins and dies during germination. On the other hand, chromatin condensation, DNA fragmentation, cell vacuolization and formation of ricinosomes containing Cys-EP are the main PCD diagnostic markers in the ephemeral endosperm during development (López-Fernández and Maldonado 2013b).

IIn cereals, the mature endosperm is constituted by the aleurone layer, a living tissue that stores proteins and lipids, and the starchy endosperm, a dead tissue that stores starch and proteins. Cells of the starchy endosperm die during seed development, and the PCD involves both cell death and starch synthesis. Young and Gallie (1999, 2000) study how hormones such as abscisic acid (ABA), ethylene, and gibberellic acid (GA) regulate PCD in the developing maize and wheat starchy endosperm. Progression of PCD starts in a specific location within rice and maize, as opposed to wheat, where it appears to be a random process (Young and Gallie 1999, 2000). In maize, along with DNA fragmentation, three nucleases are identified (Young et al. 1997). DNA fragmentation has been also reported in the starchy endosperm of barley (Borén et al. 2006), rice (Lan et al. 2004), and wheat (Young and Gallie 1999); in all cases studied it was the first sign of PCD. Borén et al. (2006a) identified a caspase- like protease (VEIDase) throughout the development of the barley starchy endosperm and associated VEIDase activity with several PCD events. It should be noted that, in contrast to what occurs during perisperm PCD where DNA fragmentation is coupled to VEIDase activity, in barley endosperm, DNA fragmentation and VEIDase activity are uncoupled. VEIDase activity has also been detected in rice by Kobayashi et al. (2013) together with other PCD hallmarks such as mitochondrial membrane permeabilization. According to Lan et al. (2004) the earliest sign of PCD in rice starchy endosperm is the nuclear fragmentation, which starts as soon as cellularization is completed.

During germination, the aleurone layer produces enzymes to hydrolyze the starchy endosperm and, after completing hydrolase secretion, cells of the aleurone layer die (Young et al. 1997; Young and Gallie 1999). PCD in aleurone layer is tightly regulated by GA and ABA (Bethke et al. 1999). The reactive oxygen species produced in response to GA indirectly stimulate PCD in barley aleurone cells (Aoki et al. 2014). In barley, during the first days of germination, DNA fragmentation is confirmed by TUNEL and DNA laddering assays; by contrast, during wheat germination, nuclei degrade towards the end of the process (Domínguez and Cejudo 2014). Likewise, PCD in aleurone cells drives the accumulation of a wide range of nucleases and proteases (Domínguez and Cejudo 2014).

PCD AND SUSPENSOR

The suspensor anchors the embryo to the endosperm and serves as a nutrient conduit for the developing embryo. During embryogenesis, the suspensor accomplishes its function and is subsequently removed by genetically controlled cell death (Bozhkov et al. 2005a). The suspensor cells are the first cells of the embryo determined to PCD not contributing to the next plant generation (Bozhkov et al. 2005a; Zhao et al. 2013).

Lombardi et al. (2007) use a combination of morphological and biochemical approaches to analyze PCD in the suspensor of *Phaseolus coccineus*. The DNA fragmentation starts in the neck cells, when the embryo reached the early cotyledonary stage, and subsequently spreads to the basal cells i.e., the knob region. During suspensor death, DNA fragmentation, activation of caspase-like proteases and cytochrome c release from the mitochondria has been reported (Lombardi et al. 2007b, 2007c).

DNA fragmentation is also detected during maize (Giuliani et al. 2002), *Vicia faba* (Wredle et al. 2001), and *Chenopodium quinoa* (López-Fernández and Maldonado 2013b) suspensor cell death becoming the first PCD hallmark to be observed. Suspensor PCD in *Nicotiana tabacum* is controlled by two antagonistically acting proteins, a pro-death cathepsin-like protease and its cystatin inhibitor, which co-localize to the basal-most cell of the suspensor. In addition, *Nicotiana tabacum* suspensor PCD exhibits DNA fragmentation, nuclear envelope disassembly, and is accompanied by the activation of proteases with caspase-like specificity (Zhao et al. 2013, 2014).

CONCLUSION

Although the biochemical and molecular understanding of plant PCD has increased over recent years, the mechanisms of PCD are still very limited and are restricted to only a few species.

Future research should aim to identify the mechanisms that control the time of execution of PCD in nucellus and endosperm during seed development. This may help to explain the coexistence of endospermic and exendospermic species within the same family as in Leguminosae (Reid 1985) or even within a same genus as in *Bulnesia* (Maldonado et al. 1997) and/or of species with and without perisperm within the same family, as in Chenopodiaceae (Shepherd et al. 2005) and also to understand the evolution of similar patterns in phylogenetically distant families. It is not a short-term goal, but when achieved, it will not only improve our understanding of seed development but also of angiosperm phylogeny and evolution.

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