Chapter

Programmed Cell Death in Seeds: An Adaptive Mechanism Required for Life

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Abstract

The regeneration of the mother plant through germinative process is the main reason that evolutionarily justifies the existence of a viable seed. Current knowledge indicates that the control of germination is a sophisticated process mainly controlled by hormones and reactive oxygen species (ROS), among other endogenous factors. One of the events that directly participate in the germination is the degradation of storage proteins (SPs). Thus, vacuolar processing enzymes (VPEs) contribute to SPs' degradation and mobilization due to direct proteolysis or through the activation of other peptidases. In parallel, the relationship between VPEs and programmed cell death (PCD) is beyond doubt. As an alternative to VPEs, the formation of vesicles called ricinosomes containing papain-like Cys-proteases (PLCPs) and located in the reserve tissues of some germinating seeds also collaborates to protein degradation. Finally, there are increasing evidences linking nucleases to PCD in different tissues of seed. However, its state of the art is still little developed. Together, this current overview illustrates a part of the complexity of PCD in seeds, a puzzle far from being solved.

Keywords: Cys-endoproteases, endosperm, lytic vacuoles, nucleases, papain-like Cys-proteases, ricinosomes, seeds, seed storage proteins, vacuolar processing enzymes

1. Introduction

The life cycle of organisms requires targeted cell types to be removed in a predictable and genetically organized way. This process of cellular suicide, named programmed cell death (PCD), occurs from embryogenesis to senescence and is an essential part of development and cell homeostasis of any multicellular organism [1–3]. Thus, PCD has been observed from the onset of zygotic embryogenesis until the germinative process ends [4–6]. The mechanism through which specific cells are targeted for PCD without affecting neighboring cells has not yet been resolved. Notable cellular compartments (i.e., mitochondria, chloroplasts, Golgi complex, endoplasmic reticulum (ER), and vacuoles) have been shown to be involved in PCD [7]. Plant PCD exhibits several hallmarks: (i) DNA laddering and strong chromatin condensation [8]; (ii) sometimes, release of cytochrome-c from the mitochondria to the cytosol, and its subsequent degradation, which is dependent on reactive oxygen species (ROS) and caspase-like activity [9]; (iii) generation of autophagic vacuoles due to the absence of an active phagocytosis system [10, 11];

(iv) degradation of organelles such as the plastidome, mitochondria, and peroxisomes [11]; (v) extensive vacuolation (i.e., appearance of a large vacuole) [12]; (vi) sometimes, development of ricinosomes concomitantly with the progression of nuclear DNA fragmentation [13, 14]; and (vii) contribution of nucleases and ROS [15, 16]. At the end of PCD, the cell is completely digested, and the remaining protoplast is surrounded by the cell wall (CW), which finally becomes disorganized and disintegrates in a coordinated and regulated way [17]. Because plants have CWs, they have developed their own PCD process, thus not requiring the apoptotic regulators and phagocytic processes present in animal cells. At the cellular level, plant PCD can be non-autolytic or autolytic (i.e., formation of large lytic vacuoles and rapid clearance of cytoplasm due to tonoplast rupture and the release of active hydrolases) [18]. Thus, developmental PCD (dPCD) is autolytic and is critical for many vegetative and reproductive processes [2, 19, 20]. However, environmental PCD (ePCD) is non-autolytic and is involved in responses to biotic and abiotic stresses. In this latter form of PCD is involved the hypersensitive response (HR), which prevents the growth and spread of pathogens into healthy tissues [21–23]. Recently, it has been suggested that dPCD and ePCD are characterized by separate regulatory pathways. In fact, a conserved core of transcriptionally controlled dPCD-associated genes has been defined [24]. Because plants and animals have different molecular mechanisms for PCD, an evolutionary parallelism of PCD pathways in plants and animals has been postulated [25].

The involvement of PCD has been described in various plant life processes, including the emptying of xylem tracheary elements [26], aerenchyma formation [25, 27], and dynamic turnover of the root cap [28]. In addition, PCD is an integral part of the seed development and germination (i.e., dPCD), during which cells of the integuments, nucellus, suspensor, and endosperm face death [5, 6]. The following text presents an update on the substantial progress that has been made to our understanding of PCD through the life of the seed, an entity that represents the dispersal unit of the spermatophytes securing their survival and perpetuation. The role of papain-type KDEL-Cys-endoproteases (PLCPs), vacuolar processing enzymes (VPEs) and nucleases, is carefully reviewed.

2. The role of plant-specific KDEL-Cys-endopeptidases in seed development and germination

2.1 Ricinosomes

Cys-endopeptidases (Cys-EPs) are the most abundant group of proteases responsible for degradation and the mobilization of storage proteins (SPs), being the SPs of seeds the most affected [29]. Cys-EP is a member of a unique group of papain-type Cys-EPs found specifically during senescence. The ER-derived vesicles (e.g., protein bodies, glyoxysomes, and ricinosomes) accumulate in seeds, among other compounds, specific SPs, (e.g., prolamin and zein) and KDEL-tailed and papain-type proteases [30, 31]. The SPs' accumulation process is mediated by ER chaperones such as the luminal binding protein (BiP) and protein disulfide isomerase (PDI). Interestingly, BiP can function either as a negative or a positive modulator of PCD events and also participate in innate immunity. Besides, in the seeds of castor bean, the immature 11S globulin was aggregated and then packaged in vesicles from ER [32]. That is, the ER-derived vesicles are thought to function as repositories of specific proteins until they are required for the cellular metabolism.

The ricinosomes (**Figure 1**) are spherical plant-specific organelles that have been firstly documented in senescing germinating endosperms of castor bean

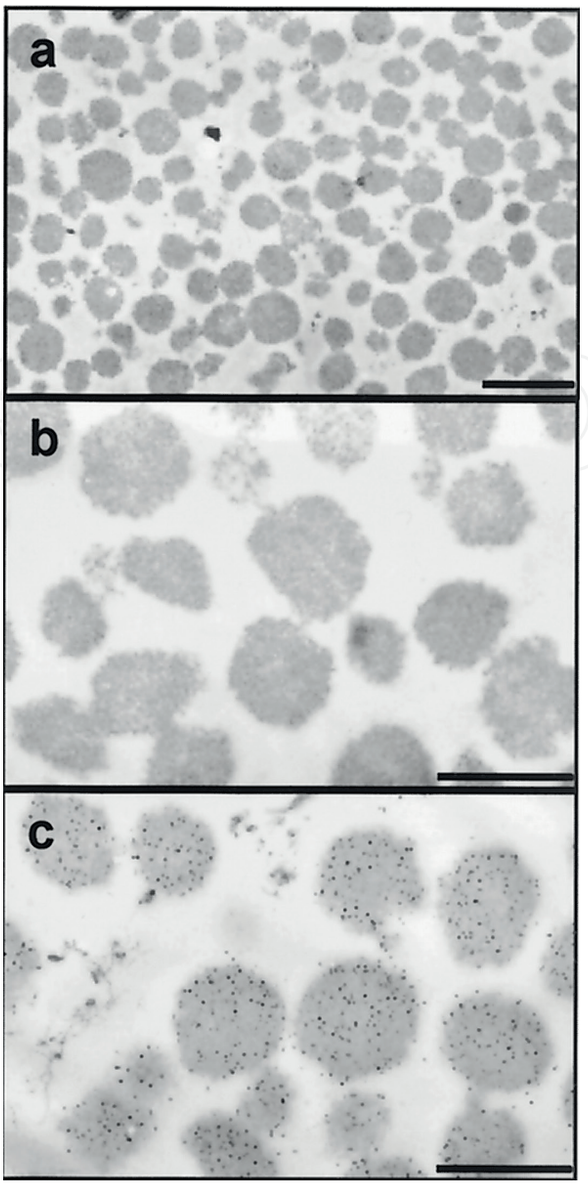

Figure 1.
Ultrastructure of ricinosomes purified from 5-day-old castor bean endosperm and immunolocalization of their marker enzyme Cys-EP. Electron micrographs (a, ×4400; b, ×12,000) and immunocytochemistry by using α-CysEP (c, ×12,000). Scale bar: a = 1.0 μm; b and c = 0.5 μm. From Schmid et al. [30] with permission of PNAS (USA).

[30, 33–35]. They are present prior to the appearance of other subcellular changes related to PCD and appear at the beginning of PCD and deliver large amounts of papain-type Cys-EPs in the final stages of cellular disintegration [13]. The ricinosomes contain large quantities of a 45-kDa pro-Cys-EP with a C-terminal KDEL (ER retention signal), and they are specifically for plant PCD [30, 36]. The ricinosomes are surrounded by a single ribosome-studded membrane and are directly sorted toward vacuoles through a Golgi-independent pathway to get involved in the PCD. These vesicles bud off from the ER in senescing tissues concomitantly with the progress of nuclear DNA fragmentation and have Cys-EPs as marker enzymes [37, 38]. KDEL-Cys-EPs are synthesized as inactive or weakly active pre-proenzyme which usually include a KDEL and an auto-inhibitory pro-domain that is cotranslationally transferred into the ER and then stored in ricinosomes because the pro-domain prevents premature activation of the protease [39]. Upon cytosolic acidification due to the LV collapse, the KDEL-Cys-EPs autocatalytic activation occurs [40]. This activation has been confirmed by in vitro acidification experiments of isolated ricinosomes and implies the cleavage of the N-terminal

pro-peptide and the C-terminal KDEL motif. The presence of mature Cys-EP is consistent with the loss of tonoplast integrity. The mature and enzymatically active KDEL-Cys-EPs exhibit unusual broad substrate specificity (**Figure 2A**). This characteristic is due to the fact that the active site accepts a wide variety of amino acids, including proline and glycosylated hydroxyproline (e.g., extensions) from the glycoproteins of the CW [41]. When ricinosomes disintegrate and release their content into the cytoplasm, the cells that contain them are going to die [13, 14, 42]. More specifically, these ER vesicles are present prior to the appearance of other subcellular changes related to vacuolar cell death, one of the two classes of PCD previously defined [13, 35, 42, 43]. Interestingly, the ricinosomes, but not the enzymes within them, have also been implicated in the PCD of *Solanum lycopersicum* [35]. Likewise, anther dehiscence in tomato has also been linked to dPCD, and accumulation of ricinosome-like vesicles and the dPCD-associated SlCys-EP has been observed in the dehiscence zones of tomato anthers along with nuclear condensation and cytoplasmic retraction [13]. In year 2014, the first evidence for the existence of ricinosomes in *Arabidopsis* has been documented [44].

2.2 Involvement of papain-like KDEL-Cys-EPs in seed life

Papain-like Cys-EPs (PLCPs; often called cathepsins in animals) are essential and central hubs of plant immunity, germination, development, and senescence [45, 46]. Thus, when activated, PLCPs induce a broad spectrum of defense responses, including PCD [46]. On the other hand, PLCPs constitute one of the most abundant groups of the proteases responsible for the degradation and mobilization of SPs in seeds [47]. Their role during germination has been reported in a wide range of both monocot and dicot plants [48]. PLCPs in plants are divided

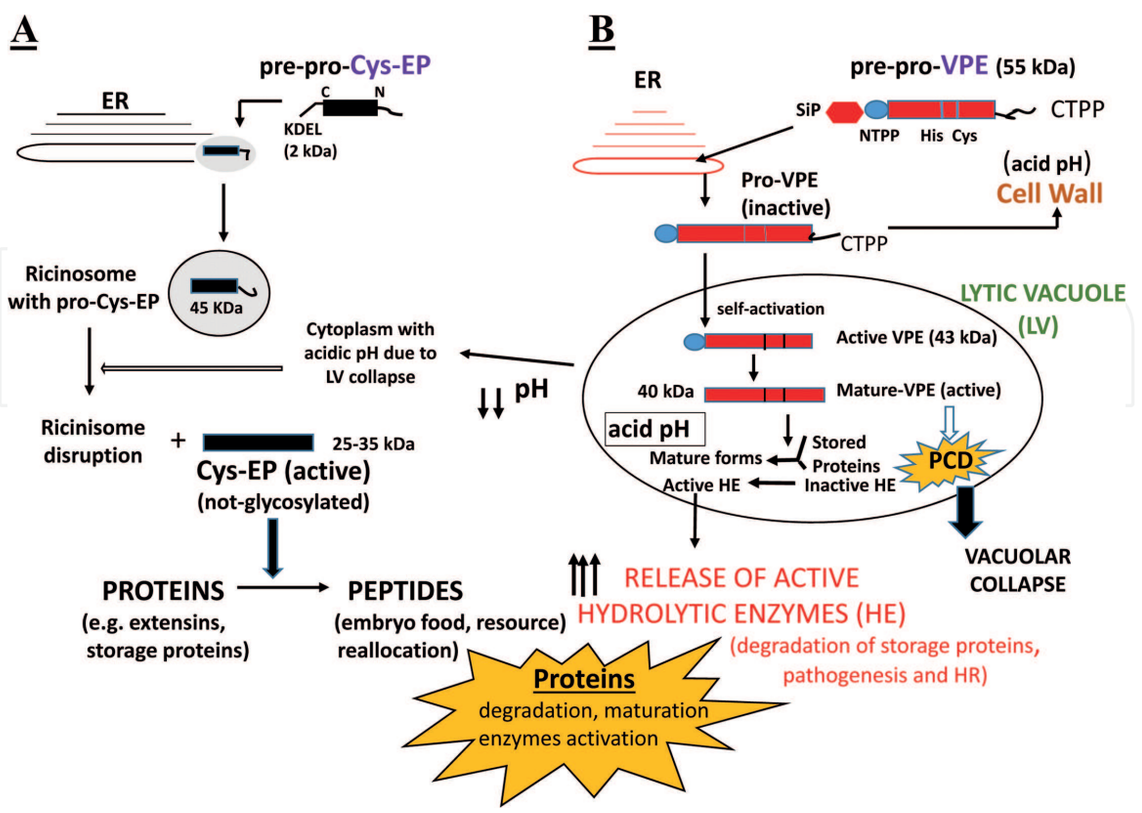

Figure 2.
Maturation, activation, and involvement of papain-type KDEL-Cys-EPs (A) and γVPE (B) in plant PCD. Endoplasmic reticulum (ER), N-terminal pro-peptide (NTPP), self-inhibitory C-terminal pro-peptide (CTPP), signal peptide (SiP), and storage protein (SP) (see text for more details).

into nine subfamilies. Thus, 32, 41, and 45 PLCPs' members have been identified in *Arabidopsis*, barley, and rice, respectively [49]. PLCPs have no structural relationship to the caspases, and its natural competitive and reversible inhibitors are the phytocystatins which are evolutionarily well conserved [50]. Recent results support the bifunctional ability of carboxy-extended phytocystatins in regulating legumain proteases via its carboxy-extended domain and PLCPs by its amino-terminal domain [51]. The activities of phytocystatins and PLCPs need to maintain a relatively balanced level to ensure the normal seed germination [29].

KDEL-tailed Cys-protease SH-EP is the first Cys-EP found to have a KDEL tail in spite of the fact that the protease localizes in the protein storage vacuoles [52]. KDEL-tailed protease-accumulating vesicles in germinating mung bean (*Vigna mungo*) cotyledons are similar to ricinosomes in that they accumulate the KDEL-tailed cysteine protease SH-EP [53, 54]. During the seeds' life, the ricinosomes accumulate PLCPs for the degradation of seed storage materials in both cotyledons and endosperm [30, 53]. Upon cell death, the content of ricinosomes (i.e., PLCPs) is released into the cell corpse where the proteinases are activated and proceed to degrade any remaining protein for the growing seedling in the case of nutritive seed tissues. Alternatively, PLCPs can also digest CW extensions in the final stage of PCD when the cell collapses and tissue breaks down [55]. Thus, the absence of ricinosomes during seed development (e.g., perisperm, integuments, chalaza, and pericarp) may be due to the fact that the CWs remain intact until germination, at which time these tissues are finally dismantled [56]. Interestingly, area micropylar of *Chenopodium quinoa* seeds does not have ricinosomes [6, 56]. KDEL-Cys-EPs are unique in digesting the extensions that form the basic scaffold for CW formation [55] (**Figure 2A**). So, KDEL-CPs like AtCEP1 are considered as late-acting proteases that digest CW proteins during the final stages of PCD and tissue remodeling after cellular disintegration [55, 57].

During seed germination, SPs are degraded to nourish the growing seedlings. This process is mainly triggered by PLCPs [29]. As a example, during both *Zea mays* and *Triticum aestivum* germination, the activity of Cys-EPs increases up to 90% of the total proteolytic activity. During barley seed germination, PLCPs were secreted from the scutellar and the aleurone layers to the endosperm to degrade the endosperm Sps [58]. Recently, the results of overexpression and silencing of *HvPap-1*, a gibberellin (GAs)-induced PLCP gen, indicated that PLCPs are important factors in mobilizing SPs to promote seed germination, and their expression and/or activity are regulated by GAs, ABA, and cystatins [49]. Ricinosomes and nuclear DNA are fragmented during PCD. In *Arabidopsis*, three KDEL-Cys-EPs called AtCEP1, AtCEP2, and AtCEP3 have been expressed in tissues undergoing PCD. Thus, the first gen is expressed in senescing ovules, the second in the vascular vessels, and the third in maturing siliques [55, 57–59]. Recently, AtCEP2 storing ricinosomes in *Arabidopsis* seedlings seems to be—like ER bodies—exclusively localized in epidermal cells [44]. The accumulation of KDEL-Cys-EPs and the appearance of ricinosomes may predict the occurrence of PCD during late seed development [37]. The ricinosomes containing pro-Cys-EP have been observed in anther tissues prior to PCD [13] and in the endosperm cells of imbibed tomato seeds (*Solanum lycopersicum*) where the reserve mobilization, Cys-EP accumulation and processing, is GA-induced [60]. Cereal aleurone PCD is controlled by phytohormones: the PCD promoting GAs and the antagonistically acting ABA [61]. The presence of ABA- and GA-responsive genes encoding proteases confirms their notable role in regulating the growth of cereal seeds [5, 62]. The endosperm in cereal seeds undergoes PCD during development, and, with the exception of the aleurone layer, is a dead tissue at maturity. In *Ricinus communis* the KDEL-Cys-EPs and ricinosomes were detected for the first time not only in the senescing endosperm of germinating seeds [30] but

also in the nucellus of seeds during maturation [36, 63]. Ricinosomes with the pro-form of KDEL-Cys-EPs are also present in imbibed tomato seeds [60]. The presence of KDEL-Cys-EPs has been also demonstrated in (i) the hypogeous cotyledons of *Vicia sativa* [64]; (ii) the seed coat of *Phalaenopsis* [65]; (iii) the megagametophyte cells after germination of *Picea glauca* seeds [66]; (iv) the epigeous cotyledons of *Vigna mungo* [52]; (v) the senescing endosperm of germinating castor bean seeds [30, 67]; (vi) the nucellus in maturing castor bean seeds, where the endosperm expands at the expense of the nucellus cells [36]; (vii) the endosperm cells of imbibed tomato seeds [60]; and (viii) the germinating mung bean (*Vigna mungo*) cotyledons in that they accumulate the KDEL-tailed cysteine protease SH-EP [53]. Recently, an attractive PLCP protein called NbCP14 was characterized in *Nicotiana benthamiana*. This autocatalytically activated enzyme seems to be a Cath-H-like protease with great importance for the execution of PCD during plant development [68]. Previous to the NbCP14 identification, NtCP14 was also described in *Nicotiana tabacum* as a key component in triggering of PCD during the early stages of embryogenesis [69]. In the tobacco suspensor, PCD is antagonistically regulated by NtCP14 and its cystatin inhibitor NtCYS. Both silencing of NtCP14 and overexpression of NtCYS delay PCD [69].

3. Entailment of vacuolar processing enzymes with plant PCD

The cellular vacuoles execute essential functions for plant growth, development, and adaptation to biotic and abiotic stresses. In the absence of macrophages, the unwanted material for plant PCD is only degraded through vacuole-released hydrolytic enzymes, located in LVs (acidic pH) [70]. The formation of LVs involves the coalescence of protein storage vacuoles (PSVs, with a pH near neutrality), vacuolar lumen acidification, and intracellular material mobilization (i.e., cytoplasm engulfing). Only cells with high vacuolation resulted in PCD [12]. In brief, PSVs contain large amounts of defensive and SPs to be used during seed germination, while LVs contain hydrolytic enzymes [50]. Therefore, the degree of vacuolation can reflect the intensity of the PCD process. A clear example of vacuolization takes place in the aleurone cells during cereal seed germination [5, 12]. Mature cereal seeds consist mainly of dead cells, and only the embryo and aleurone layer are still alive. The PCD of aleurone cells is an essential process for the successful completion of post-germination storage mobilization, which is associated with the vacuole destruction [70]. Vacuolation and PCD of aleurone cells are initiated near the embryo and then gradually reach the distal area of the embryo [5, 12]. Similarly to the micro- or macroautophagy processes, the disruption of LVs and the concurrent release of various hydrolytic enzymes indicate that the PCD has been triggered [12]. The bursting of the tonoplast leads to a rapid cytoplasmic acidification and hydrolysis of the remaining cellular contents [71]. So much so, this vacuolar collapse needs to be rigorously organized to achieve PCD at a suitable timing [72]. Because the vacuolar collapse releases hydrolytic enzymes, the vacuole rupture is used as an indicator of PCD initiation [73]. Therefore, the tonoplast breakage is considered a point of no return during plant PCD [2]. In brief, the tonoplast rupture and vacuolar collapse are two important features of plant PCD. Finally, the plasmalemma integrity is maintained until the vacuole collapses [12, 74–76].

Plant PCD is accompanied by the upregulation of a heterogeneous group of vacuolar hydrolytic enzymes, being the vacuolar processing enzymes (VPEs), also called legumains, closely involved in its activation. VPEs originate from prokaryote pro-legumains. The VPEs have properties similar to animal caspases and fulfill relevant vacuolar functions in seeds [77]. Nevertheless, VPEs are

directly involved in plant development and environmental stress responses [50]. Earlier studies in pumpkin seeds have demonstrated the identification of VPE as a vacuolar Cys-EP protein probably responsible for degradation of vacuolar SPs during germination [78]. Thus, the VPEs in monocots (i) are required for processing of glutelins that are the dominant seed SPs in rice [79], and (ii) they also process other seed SPs such as albumins, globulins, and ricins in storage vacuoles in seeds of pumpkin and castor bean [80]. However, VPE deficiency does not affect storage protein degradation in germinating seeds [81]. VPE was the first identified enzyme in plants with both caspase-like activity and activity against caspase-1-substrate [82]. Recent review contains the contributions of VPEs to plant PCD and its role in vacuole-mediated cell death [83]. Thus, the VPE4 expression pattern in the developing pericarp of *Nicotiana benthamiana* coincides with the profile of the caspase-1-like activity [84, 85]. Once vacuolar hydrolytic enzymes are activated, the proteolytic cascade leading PCD begins [70, 71]. However, although it is beyond question that VPE is an initiator of the vacuolar processing system, the mechanism by which VPE controls the vacuolar breakage and the execution of a variety of plant PCD is still unclear. In this regard, it was suggested that the disruption of the vacuole may be mediated by VPE in conjunction with protein kinases [86]. It has been also proposed that VPE and cathepsin-B (Cath-B), which have, respectively, caspase-1-like and caspase-3-like activity, may promote coalescence, accelerating the process of vacuolation and thus triggering vacuolar collapse during the PCD [87, 88]. However, no research yet has integrated the action of both VPE and Cath-B in the PCD pathway. VPE of *N. benthamiana* has been reported to mediate virus-induced HR by regulating tonoplast collapse [87, 89]. The PCD triggered by vacuolar collapse is unique to plants and has not been seen in animals (**Figure 2B**). As a result of this collapse and the liberation of active vacuolar hydrolases, the chromatin structure crumbled, the DNA is fragmented, and the plasma membrane disabled. Finally, the disintegration of the nuclear envelope starts [90], and the protoplast rapidly collapses and dies [5, 91].

Autophagy is a process known to mediate the degradation of residual proteins and aggregates of insoluble proteins and lipids and to remove damaged organelles. Likewise, autophagy is essential for vacuolation of cells undergoing developmental PCD and is activated by type-II metacaspases (McIIPa) [92]. Thus, during spruce embryogenesis McIIPa is transported from cytosol to the nucleus, where its presence is correlated to DNA fragmentation. These data reinforce that McIIPa is directly involved in a pathway which generates nuclear degradation, an event present in most programs of eukaryotic PCD. This McIIPa metacaspase can play a role on the cleavage of nuclear proteins [71, 93]. Besides metacaspases, VPE has been also described as another class of Cys-EPs involved besides in different types of PCD and also in development and immunity [70, 87, 94, 95]. The VPE contains a His-Cys catalytic dyad and cleaves a peptide bond at the C-terminal side involving an Asn residue, hence the name of asparaginyl endopeptidases [96]. VPEs are evolutionarily related to caspases and preferably localized in vacuoles (i.e., maximal activity at acidic pH) and are specific for plants [38, 70, 97]. Therefore, the plants might have evolved a VPE-mediated vacuolar system as a cellular suicide strategy. Plants encode at least four functional isoforms of VPEs, which are located in the vacuole ([77] and refs. therein). Plant VPEs are classified into vegetative, embryogenic, and seed-expressed types [98–100] (**Figure 3**). The genome of tomato has 14 VPE genes [95]. However, the genome of *Arabidopsis* has four VPE genes: α-VPE and β-VPE play a key role in the processing of SPs during seed maturation [80, 94], while γ-VPE and δ-VPE are expressed at early stage of seed development being involved in the formation of the inner integuments of the seed coat [101–103].

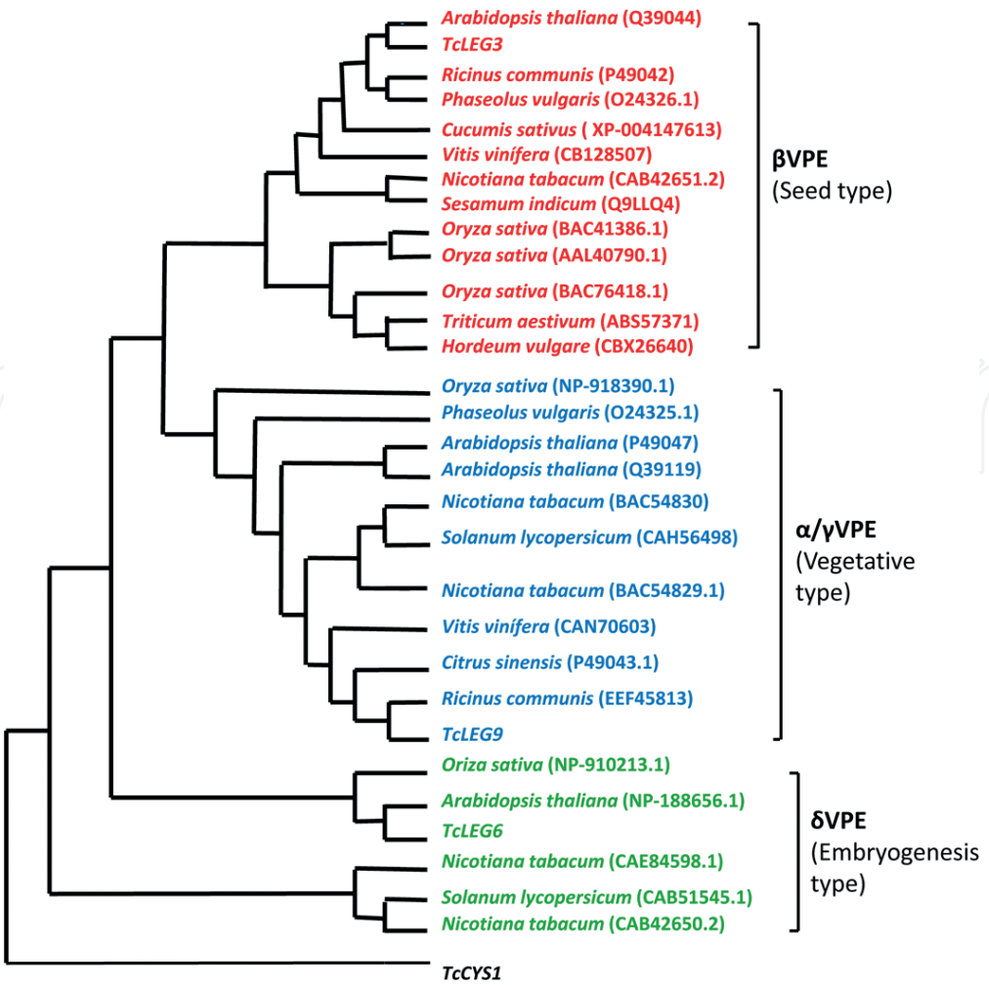

Figure 3.
Dendrogram of VPEs of several plant species. VPEs (access numbers are within parentheses) were separated into three groups: βVPE (seed type), α/γVPE (vegetative type), and δVPE (embryogenesis type). Signal peptides were excluded from the sequences. Adapted mainly from Nakaune et al. [103] and other recent publications.

Interestingly, in spite of delayed vacuolation, *Arabidopsis* γ-VPE mutants have a normal germination phenotype. This suggests that vacuolation does not trigger, but rather is a consequence of germination [104].

The δ-VPE was originated early during dicotyledonous diversification [103]. Regarding the maturation of γ-VPE in *A. thaliana* (**Figure 2B**), it is to know that the N-terminal signal peptide of VPE pre-protein precursor is cotranslationally removed in the ER to produce VPE pro-protein. The transfer of pro-protein precursor to the acidic vacuole causes the self-catalytic conversion into an intermediate isoform by removal of the C-terminal inhibitory pro-peptide. The subsequent removal of the N-terminal pro-peptide produces the mature γ-VPE [96]. In the case of the seed SPs, the VPEs' vacuolar maturation is of major importance, as it conditions the establishment of vigorous seedlings [105]. A quadruple-KO mutant with no detectable VPE activity strongly suggests that there are no other proteases with a similar activity in *Arabidopsis* [106]. When the VPE genes were knocked out, no characteristics belonging to cell death were observed [12]. VPE orthologs are widely distributed in land plants including mosses (e.g., *Physcomitrella patens*) and ferns (e.g., *Ceratopteris richardii*) [83]. In rice, five VPE (OsVPE) genes are found [83, 107]. Phylogenetic analyses and gene expression studies have demonstrated that OsVPE2 (OsaLeg2) and OsVPE3 (OsaLeg3) are involved in H_2O_2-induced PCD and in salt-stressed seeds, whereas OsaLeg1, OsaLeg4, and OsaLeg5 would act as vegetative-related legumains [83]. The barley (*Hordeum vulgare*) genome contains

eight VPE genes (HvVPEs) which are differentially expressed during vegetative and reproductive development [98].

The first increase in a cascade of caspase-1-, caspase-3-, caspase-4-, caspase-6-, and caspase-8-like activities in the endosperm of *Hordeum vulgare* seeds may be related to PCD in the nucellus [84, 85, 108]. The importance of pericarp PCD for proper development of the endosperm has been recently described [84]. The increase in caspase-1-like activity may be acquired by HvVPE2a (called nucellain), HvVPE2b, and HvVPE2d proteases which is exclusively expressed in nucellus and nucellar projection. The expression patterns of the HvPhS2 and HvPhS3, which are exclusively active in the nucellar projection, coincide with the caspase-6-like activity profile in the early endosperm fraction indicating that HvPhS1 and HvPhS2 may be responsible for the caspase-6-like activity [84, 85]. Caspase-1-, caspase-3-, and caspase-6-like activities are also localized in the degenerating nucellus of *Sechium edule* [109]. In the degenerating nucellar tissue of castor bean, proteomic analyses identified multiple proteases and protease inhibitors [108]. The MADS-box transcription factor called MADS29 has been suggested to promote nucellar degeneration through the regulation of Cys-EP expression in rice and maize [61]. The α, β, γ, and δ-VPEs of *Arabidopsis* appear to share no direct one-to-one relationships of orthology with VPEs from gymnosperms. The VPE protein and its transcripts increase at the beginning of the HR reaction in the tobacco leaf, in which the cells showed typical PCD characteristics, and both the VPE inhibitor ESEN-CHO and the caspase-1-like activity inhibitor Ac-YVAD-CHO inhibit the appearance of PCD [98, 99]. These and other recent results [100] reaffirm that VPE is a protease with caspase-1-like activity in plants. VPE activation was started once the leaves of tobacco were infected with the TMV, leading to vacuole disruption and activation of PCD to prevent the proliferation of virus [70, 87]. Likewise, PCD during HR is critical for the removal of biotrophic pathogens, whose growth depends on the living host tissues [87, 99]. Together, VPE deficiency suppresses vacuolar collapse, leading to mycotoxin-induced cell death [83]. Interestingly, the results of Zhan's group using the NbVPE silencing suggest that VPE plays an important role in elicitor signaling in plants of *Nicotiana benthamiana* [89]. Finally, and based on the results to date, (i) transcriptome sequence information has permitted the identification of new VPE genes than having a cyclization function rather a protease function ([77] and refs. therein), and (ii) VPEs and other vacuolar enzymes once released from LV to cytosol through a barely known route promote a VPE-mediated vacuolar disruption and constitute a fundamental piece in the plant PCD puzzle whose organization is far from unravelling.

4. Nucleases: the next frontier for knowledge of PCD in plants

As shown throughout this review, the PCD process involves the selective removal of unwanted cells and the mobilization of cellular debris, including the products of DNA fragmentation, which is a known hallmark of PCD [6, 17, 93, 110, 111]. In plants, genomic DNA is actively degraded during dPCD (e.g., during seed coat formation [103] and barley pericarp development [112]). Although the enzymes directly involved in nuclear dismantling are unknown, there is increasing evidence linking proteases and nucleases to plant PCD [20]. What is known is that during PCD, different nucleases are induced, including a set of S1-type (Zn^{+2} dependent) endonucleases that are synthesized regardless of tissue type [113, 114]. While these nucleases are cytoplasmic and lack a canonical nuclear localization signal, upon induction of cell death, they become nuclear [115]. Thus, nucleases are tightly associated with different plant PCD processes, including HR [116],

endosperm development aleurone cell death [4, 117], and xylogenesis [118]. It has been hypothesized that PCD-associated nucleases help to recycle DNA from dead cells by degrading it into smaller fragments so that it can be taken up to be reused by neighboring cells. In cereal seeds, the progression of endosperm PCD is accompanied by an increase in nuclease activity and the degradation of nuclear DNA at internucleosomal sites [4, 115]. PCD in the endosperm precedes PCD in the suspensor, suggesting that the endosperm and suspensor either receive different chemical signals or interpret them differently [119]. A nuclear-localized GA-induced nuclease was found to be active just prior to the appearance of DNA laddering in wheat aleurone cells undergoing PCD. Interestingly, this GA-induced nuclease is not detected in GA-insensitive mutants or when GA synthesis is inhibited [120]. Furthermore, aleurone layers that have not been treated with GAs do not complete PCD.

Foundational biochemical experiments revealed that plant nucleases are localized to a variety of different cellular spaces [5, 116, 120, 121]; for example, in barley aleurone, nuclease activity was found in the ER, Golgi, protein body, and vacuole [122]. A nuclease that is a promising candidate for involvement in PCD is the bifunctional nuclease-1 (BFN1). In *Arabidopsis*, the *BFN1* gene is induced during senescence, abscission, and dPCD [123]. Recent studies revealed that the BFN1 protein, which possesses RNase and DNase activity, is responsible for rapid cell-autonomous corpse clearance and DNA fragmentation during root cap cell death [118, 124]. TUNEL assays showed a delay in nucleic acid degradation in both the nuclei and the cytoplasms of *BFN1* mutants [123]. ORE1, a NAC (ANAC092) transcription factor that positively regulates leaf senescence, has been demonstrated to control the *BFN1* expression [125]. ORE1 is located downstream of the ethylene signaling cascade. Considering this data, it is not surprising that BFN1 is an accepted marker of both plant senescence and PCD. Another nuclease, known as *Zinnia* endonuclease-1 (ZEN1), which shares a number of similarities with BFN1, has been directly implicated to function in PCD. ZEN1 is localized to vacuoles, which collapse before DNA is degraded. ZEN1 was demonstrated to be responsible for nuclear DNA fragmentation during PCD associated with xylem development [126]. Furthermore, silencing *ZEN1* prevented the degradation of nuclear DNA, but did not affect vacuole collapse in a *Zinnia elegans* cell suspension culture. While these findings support the notion that ZEN1 may play a central role in plant DNA fragmentation [126], evidence exists that suggests that multiple nucleases are involved in plant PCD [15].

Given the limited number of nucleases known to be involved in PCD, it has proven to be a challenge to identify these PCD-associated endonucleases. Once identified, other hurdles remain. Based on a study of the role of the nucleases in the process of leaf senescence [127], future studies must explore whether the nuclease is involved in cell death in different tissues in a same plant or in different processes (e.g., fertilization, zygotic embryogenesis, and seed dormancy and germination), is subcellularly localized to the nucleus, or possesses a PCD phenotype—an activity that involves creation of a mutant for the nuclease followed by experiments to observe the impact on genomic DNA during PCD. Additionally, these studies must consider how different nucleases work together to degrade nuclear and organelle DNA.

5. Concluding remarks and future perspectives

Although the biochemical and molecular understanding of plant PCD has increased over the last decade, the mechanisms of action are still very limited and restricted to determine species and some organs and cell compartments. Given its importance, the origin and evolution of genes involved in PCD still need to be resolved. For example, all through seed evolution, PCD has played a fundamental

role. In my opinion, eight important goals are key for the best knowledge of plant PCD: (i) the molecular components used in its execution, (ii) the components that have been conserved during evolution, (iii) specific components of PCD, (iv) temporal and spatial expression of Cys-EPs involved in PCD, (v) subcellular Cys-EPs localization and interaction with other proteins, (vi) how the different proteases orchestrate PCD and if there is functional redundancy between the different gene families, (vii) activation of relevant Cys-EPs, and (viii) knowledge of the in vivo protein substrates. An example that justifies what has been said previously can be that VPEs are proposed to control indirectly tonoplast rupture during PCD. However, the detailed mechanism by which VPEs control tonoplast rupture is still diffuse. Together, the intense research carried out in the last decade on PCD in seeds is a strong scientific support to understand the coexistence between death and life.

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Conflict of interest

The authors declare no conflict of interest.

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