Plant life needs cell death, but does plant death need Cys proteases?

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ABSTRACT

Caspases are key regulators of apoptosis in animals. This correlation has driven plant researchers for decades to look for caspases regulating PCD in plants. These studies revealed caspase-like activities, caspase-related proteases and other Cys proteases regulating PCD in plants, but no caspases, and no conserved death pathway as in apoptosis. Here, we critically review the evidence for Cys proteases implicated in cell death in plants. We discuss the role of papain-like Cys proteases, vacuolar processing enzymes and metacaspases in PCD during development of tracheary elements, seed coat, suspensor, tapetum and during the hypersensitive response. There are several convincing cases where these Cys proteases are required for PCD, but this requirement seems often not conserved across different plant species and there are also cases where Cys proteases contribute to PCD without being essential, or have other roles than regulating PCD. These data illustrate the need for caution generalizing the role of Cys proteases regulating PCD in plants, and calls for studies on other PCD regulators and other roles of plant Cys proteases.

INTRODUCTION

Life needs death. In virtually all forms of life, immunity and development depends on cell death. In metazoans, cell death occurs during innate immunity[1], cell differentiation, development of the nervous system[2] and gametogenesis. Cell death has been widely described in plants as well, where it contributes to responses to infection and to differentiation of tissues and organs[1,2].

As indicated by its name, programmed cell death (PCD) is regarded as an orchestrated combination of signalling events (a program) that culminates in cell death. In general terms, PCD involves DNA fragmentation, organelle 'degradation' and, in some cases, the clearance of cell remains. These events are not random and a wide variety of cell types are programmed to commit suicide. Based on the underlying genetic components and morphological features, several types of PCD have been described in animal systems, apoptosis being the most widely studied one[4,5].

Caspases are considered key regulators of apoptosis. Caspases rely on a cysteine (Cys) residue for their catalytic activity and cleave peptide bonds after aspartate (Asp) residues, hence their name. During

animal apoptosis, perception of a pro-apoptotic factor activates initiator caspases and these activate executioner caspases, which in turn initiate a suite of events that will culminate in cell death[6]. In addition to caspases, other Cys proteases, as well as aspartic and serine proteases (which rely on Asp and Ser residues for their catalytic activity, respectively), have also been implicated in PCD in animals[7,83].

The important role of caspases regulating apoptosis in animals has had a strong influence on plant research. Showing that caspases also regulate PCD in plants has for long been one of the 'Holy Grails' in plant science. The use of synthetic caspase substrates and inhibitors have driven the hypothesis that plants possess caspase-like proteases, because caspase substrates are cleaved during PCD in plants, and caspase inhibitors block PCD in plants [9]. However, plant genomes do not encode caspases. Meanwhile several other plant proteases have been identified that are either caspase-related proteases (metacaspases) or can convert caspase substrates and/or be inhibited by caspase inhibitors (e.g VPEs, saspases and proteasome). However, knockout lines of these proteases still undergo PCD, sometimes reduced or delayed, and these knockout lines have no obvious developmental phenotypes, suggesting that PCD in plants is not entirely dependent on these proteases. In this review we focus on some of these examples by discussing the roles of Cys proteases in five instances of PCD in plants (**Figure 1**). Based on our critical interpretation of the available data, we have classified the different Cys proteases in the following three categories (**Table I**):

1) **Required** for PCD: PCD is abolished or its onset is delayed (late PCD) in the protease null mutant;
2) **Contributes** to PCD: cell death still occurs in a protease null mutant, but PCD is slower (not later);
3) **Nonessential** for PCD: PCD is unaffected in protease null mutant.

Caspase-like proteases in plants

Caspases belong to family C14A in clan CD in the Merops peptidase database[10], but plant genomes do not code for caspases. However, the finding that PCD in plants can be suppressed by caspase inhibitors, and that caspase-like activities are detected during PCD in plants has sparked research lines aimed at the identification of caspase-like protease families that fulfil a role in plant PCD, similar to that of animal caspases. These families are briefly explained below and summarized in **Table II**.

Papain-like Cys proteases (PLCPs, family C1A of clan CA) are also Cys proteases, like caspases, but differ significantly in substrate specificity, as PLCPs are selective for sites two residues after a hydrophobic residue (P2=Ile/Leu/Phe/Val), whereas caspases target P1=Asp sites. In addition, PLCPs have a different fold and are normally secreted into the vacuole, vesicles or apoplast, whereas caspases are cytonuclear proteins.

Metacaspases (MCs, family C14B of clan CD) are evolutionarily related to caspases, but their substrate specificity is distinct from caspases as MCs prefer P1=Lys/Arg (basic residues) whereas caspases prefer P1=Asp, an acidic residue. MCs have, however, the same catalytic dyad (Cys and His) as caspases. Various subcellular locations have been described for plant MCs.

Vacuolar Processing Enzymes (VPEs, family C13 of clan CD) are structurally and evolutionarily related to caspases, but they are located in the vacuole. Their substrate specificity is distinctly after P1=Asn

residues and they are hence also called asparaginyl endopeptidases (AEPs). However, they can also cleave after P1=Asp at low pH, and therefore have caspase-like activity.

In conclusion, because the term 'caspase-like' has been used in different ways, the nomenclature has become very confusing. Caspase-like either means: 1) Cys proteases that belong to the same CD clan and are structurally and evolutionarily related to caspases (e.g. VPEs and MCs); 2) able to cleave caspase substrates or inhibited by caspase inhibitors (P1=Asp, e.g. VPEs); or 3) Cys proteases implicated in regulating PCD (e.g. PLCPs). Thus, it is important for the plant science community to use a more specific terminology in the future.

Table II: properties of caspases and 'caspase-like' proteases in plants

	Family	**Clan**	**Catalytic residue**	**Subcellular location**	**Substrate specificity**
Caspases	C14A	CD	Cys	Cytonuclear	P1=Asp
PLCPs	C1A	CA	Cys	Endomembrane	P2=Ley/Ile/Phe/Val
MCs	C14B	CD	Cys	Various	P1=Lys/Arg
VPEs	C13	CD	Cys	Vacuole	P1=Asn(/Asp)

Proteases associated with tracheary element PCD

The xylem is responsible for the movement of water and nutrients from the roots to the shoot of the plant. Tracheary elements (TEs), which constitute the xylem, differentiate from meristematic tissue and undergo deposition of a thick secondary cell wall and PCD, resulting in the formation of hollow tube-like structures that mediate water transport. Multiple components of TE differentiation and PCD have been identified using an *in vitro* system that induces differentiation of mesophyll cells of *Zinnia elegans* into TEs upon hormone treatment. The PCD process appears to be initiated through the activity of at least two NAC transcription factors: Vascular-related NAC Domain 6 (VND6) and VND7 in *Arabidopsis thaliana* (Arabidopsis) and poplar[11,12,13]. Loss of tonoplast integrity and the concomitant rupture of the vacuole are considered to be the actual moment of cell death during TE differentiation[14,15]. Tonoplast rupture would facilitate the release of vacuolar hydrolytic enzymes into the cytoplasm, causing autolysis and final clearance of the cell corpse.

Aimed at identifying Cys proteases that regulate TE-PCD, Beers and colleagues identified two Xylem-specific PLCPs (XCP1 and XCP2) that are specifically expressed in xylem tissues at the onset of PCD[16]. However, despite the expectation that these proteases would regulate PCD in TE-PCD, both the single *xcp1* and *xcp2* mutants and also the *xcp1/xcp2* double mutant showed normal TE-PCD and no developmental phenotype[17]. Further analysis using Transmission Electron Microscopy (TEM) revealed that

the Arabidopsis *xcp1* null mutant and the *xcp1/xcp2* double mutant accumulate non-degraded cell remnants, indicating these plants are impaired in autolytic processing that occurs *post mortem*[17]. Therefore, the role of XCP1 and XCP2 is dispensable during TE-PCD though this does not exclude the possibility that they act redundantly with other regulators.

Analysis of the expression pattern of metacaspase 9 from Arabidopsis (*At*MC9) suggested it might instead regulate TE-PCD[18]. However, electron microscopy analysis indicated that cell death started at the same time in *atmc9* mutant and wild-type plants, but cell clearance was reduced in *atmc9* mutant plants[18]. Furthermore, the *atmc9* mutant and also the *atmc9/xcp1/xcp2* triple mutant did not show a developmental phenotype, confirming that xylem differentiation is ultimately successful. In addition, microarray analysis of the *atmc9* mutant did not show altered expression of PCD-related genes. Thus, similar to XCP1, *At*MC9 participates in *post mortem* autolysis rather than in PCD. However, the story is more complicated as a recent report indicates that AtMC9 contributes to the confinement of PCD to the TE through regulation of autophagy[19].

In conclusion, the contribution of PLCPs and MCs in TE development appears to be more related to *post mortem* events rather than PCD. The fact that clearance of TE does occur in *atmc9/xcp1/xcp2* mutants, albeit incomplete, implies the involvement of other components like other proteases or hydrolases. For instance, proteasome inhibitors delay *in vitro* differentiation of mesophyll cells of *Z. elegans* and alter the expression and activity of Cys proteases[20]. Further studies in poplar and Arabidopsis indicate that the 20S proteasome causes the caspase-like activity detected during TE differentiation but genetic evidence is lacking to support this claim[21]. In addition, PCD during *in vitro* differentiation of mesophyll cells of *Z. elegans* coincides with the secretion of a 40 kDa serine protease which may be implicated in PCD[22]. However, this hypothesis has not been confirmed and the evidence supporting the role of this protease in PCD is only circumstantial[22]. Furthermore, also nucleases are released from the vacuole during TE development. ZEN1 (*Zinnia* Endonuclease-1) is an S1-type endonuclease mediating DNA fragmentation, though also this is likely a *post mortem* event[23–25]. Interestingly, BFN1 (bifunctional nuclease 1), the ortholog of ZEN1 in Arabidopsis, is also expressed during TE-PCD but so far it has only been implicated in cell clearance upon PCD in the lateral root cap[4].

Proteases implicated in seed coat PCD

The seed coat of Arabidopsis consists of inner and outer integuments, each composed of three and two cell layers, respectively. As embryogenesis progresses, the inner integument shrinks due to PCD of its two most internal cell layers (ii2 and ii3) in a process that involves VPEs[26,27]. Arabidopsis has four VPEs, but *δVPE* is the only VPE-encoding gene that is expressed in the seed coat at early developmental stages[27]. The integument of two independent *δvpe* null mutant lines of Arabidopsis remained thick throughout embryogenesis, supporting the role of this enzyme in seed coat development[27]. Likewise, nuclei were detected in these *δvpe* mutants at later stages of seed coat development, suggesting a delay in nuclear fragmentation and PCD. However, despite the delay in PCD, the inner integument of the fully developed

embryo of *δvpe* mutants did shrink and these plants produced seeds that were indistinguishable from wild type plants. Furthermore, neither germination nor dormancy was affected in *δvpe* mutant lines[27]. Though these data support a role for δVPE in PCD during seed coat development, PCD ultimately still occurs in the absence of δVPE, indicating that δVPE is not the key regulator of PCD during seed coat development.

There is an additional indication that proteases regulate PCD during seed development. Protein disulfide isomerase 5 (PDI5) is a redox-dependent chaperone, which is expressed in the integument in Arabidopsis seeds just before PCD. Yeast-two hybrid and co-immunoprecipitation assays showed that PDI5 interacts with three PLCPs while guiding them to the vacuole before PCD initiates. The *pdi5* mutant lines exhibited reduced seed set and viability[28]. Because PDI5 was able to suppress Cys protease activity in *in vitro* assays, it has been hypothesized that the PLCPs interacting with PDI5 mediate PCD once PDI5 is released. It is striking, however, that all three identified PLCPs interacting with PDI5 carry a Cys-rich granulin domain that is more likely to bind PDI5 instead of the protease domain of these PLCPs. Furthermore, genetic evidence to demonstrate the involvement of these PLCPs in PCD during seed coat development is still missing.

Proteases regulating suspensor PCD

Shortly after fertilization, the apical part of the plant embryo develops into the embryo proper, which will give rise to the future plant. The basal part will form the short-lived embryo suspensor, which connects the embryo proper to parental tissues during seed development. The suspensor facilitates transport of nutrients and growth factors to the embryo proper during the first cell divisions, after which it degenerates due to developmentally-regulated PCD. Embryogenesis can be induced in somatic cells by hormonal treatment, by mimicking the events that occur during zygotic embryogenesis (the consequence of egg fertilization). In fact, a large amount of our knowledge on suspensor PCD comes from the study of somatic embryogenesis in Norway spruce (*Picea abies*)[29–31].

McII-Pa is a MC that is exclusively expressed in the cells which differentiate into the suspensor in Norway spruce[29]. Cell lines with reduced expression of *McII-Pa* failed to undergo a normal somatic embryogenesis upon hormonal treatment[29]. These lines also had reduced MC activity and no DNA fragmentation associated with PCD[29,30]. However, *McII-Pa*-silenced lines also did not differentiate to produce suspensor cells that would undergo PCD. Interestingly, reduced expression of McII-Pa also resulted in reduced caspase-like activity. However, recombinant McII-Pa is unable to cleave caspase-like substrates *in vitro*, similar to other tested MCs[30,31]. These findings suggest that both MCs and a caspase-like activity are required for effective suspensor differentiation and/or PCD, possibly acting in the same signaling cascade.

Suspensor differentiation in tobacco (*Nicotiana tabacum*) provides a very strong example of a protease regulating PCD[32]. Timely PCD of the suspensor in tobacco requires a balance between *Nt*CP14 (a PLCP) and *Nt*CYS (a cystatin regulating *Nt*CP14). Knock-down of *Nt*CP14 results in delayed onset of suspensor PCD whereas reduced expression of *Nt*CYS causes premature PCD. Similar to what has been

described for Norway spruce, multiple caspase-like activities were detected. These caspase-like activities increased upon downregulation of *Nt*CYS or overexpression of *Nt*CP14, but cannot be caused by *Nt*CP14 itself, suggesting an interplay between *Nt*CP14 and caspase-like activities during embryo development in tobacco[32].

The striking finding that depletion of unrelated proteases (McII-Pa and *Nt*CP14) abolished or delayed suspensor PCD indicates that this process is differentially regulated in Norway spruce and tobacco, respectively[29,30,32]. Notably, none of these phenotypes have been reported for genetic studies in Arabidopsis, again suggesting that they might be specific to certain plant species. These studies also uncovered caspase-like activities during suspensor PCD but these are not caused by MCs or PLCPs. Identifying the protease(s) responsible for these caspase-like activities may further untangle the role of proteases during suspensor differentiation.

Proteases involved in tapetum PCD

Production of viable pollen requires the degeneration of the tapetum during anther development. Tapetal cells must undergo PCD to ensure release of nutrients and precursors for pollen wall synthesis[33]. Cysteine Endopeptidase 1 (CEP1) is an endoplasmic reticulum-localised PLCP that is expressed in the tapetum of Arabidopsis pollen grains at early stages of tapetum development, preceding PCD[34]. Importantly, *cep1* null mutant plants have a reduced pollen production, suggesting an involvement of CEP1 in pollen maturation[34]. Transmission electron microscopy analysis of pollen grains of the *cep1* mutant revealed that tapetal PCD is delayed in the absence of CEP1, as both the cell wall and the nucleus remain intact longer than in wild type plants. Importantly, *cep1* null mutant lines as well as transgenic CEP1 overexpressing lines displayed aberrant degeneration of the tapetum and reduced pollen germination[34]. However, despite the fact that CEP1 depletion delays tapetum PCD, tapetum cells of *cep1* mutant plants eventually undergo PCD, associated with TUNEL-positive nuclei. In addition, transcriptome analysis of *cep1* mutant plants revealed increased expression of genes related to cell wall biogenesis, whilst expression of PCD-related genes were unaffected. This raises the question whether CEP1 regulates other processes during tapetum degeneration, apart from PCD itself. Interestingly, recent work revealed the expression of *AtCEP1* and *AtCEP2* in Arabidopsis after ovule fertilization in tissues just before developmental PCD[5]. These findings pose the question as of whether CEP may be involved in other PCD-related processes, other than tapetum development.

Proteases implicated in Hypersensitive Cell Death

The hypersensitive response (HR) involves a form of PCD that occurs locally upon recognition of pathogen-derived effectors or toxins. HR-PCD depends on the activation of a signaling cascade that involves a Reactive Oxygen Species (ROS) burst, phosphorylation events, ion fluxes and transcriptional reprogramming[35,36]. HR-PCD occurs at the site of infection but is not always required for resistance.

Strong genetic and pharmacological evidence have implicated a role for proteases in HR-PCD. Infection of Tobacco Mosaic Virus (TMV) on tobacco carrying the *N* resistance gene triggers HR-PCD[37].

This TMV-induced HR-PCD is dependent on VPEs, as cell death can be blocked by either virus-induced gene silencing (VIGS) of VPEs, or using chemical inhibitors of VPE[38]. Likewise, fumonisin B1 (FB1), a toxin produced by the fungal pathogen *Fusarium moniliforme*, induces caspase-like activity and PCD and these responses are abolished in an Arabidopsis *vpe* null-mutant line lacking all four *VPE* genes[39]. Further examples support the involvement of VPEs in other cases of HR-PCD, though their participation in PCD clearly depends on the pathosystem. While HR-PCD elicited in tobacco by the bacterial elicitor harpin was dependent on VPEs, VPEs were not required for PCD induced by the fungal protein Nep1 (necrosis and ethylene-inducing protein) or the oomycete-derived elicitor boehmerin[40]. Likewise, the Arabidopsis *γvpe* mutant line showed reduced PCD and resistance to various pathogens but was still able to mount HR-PCD, indicating mechanisms regulating PCD are still in place[41]. Indeed, HR-PCD was also not affected in *vpe* null mutant Arabidopsis plant upon infection with avirulent *Pseudomonas syringae* pv. *tomato* carrying avrRpm1[42]. Hence, while involved in some cases, VPEs are not universally required for HR-PCD.

MCs have also been implicated as regulators of HR-PCD. The *atmc1* null mutant Arabidopsis shows reduced HR-PCD upon infection with avirulent *Pseudomonas syringae* pv. *tomato*, which triggers HR-PCD[43]. Interestingly, the role of *At*MC1 in HR-PCD is regulated by yet another MC, *At*MC2. The *atmc2* null mutant Arabidopsis shows increased PCD, though this is not dependent on the proteolytic activity of the enzyme[43]. Remarkably, the interplay between active and inactive proteases has also been described for animal caspases[43,44]. However, suppression of PCD is incomplete in *atmc1* null mutants, suggesting that HR-PCD also occurs independently of *At*MC1. The delay in HR-PCD in *atmc1* null mutants is enhanced in double mutants with reduced autophagy, suggesting these two pathways independently contribute to HR-PCD[45]. A dual participation of MCs and autophagy in PCD has also been described in xylem development and suspensor PCD[196].

Finally, Arabidopsis PBA1, the catalytic β1 subunit of the proteasome, also contributes to HR-PCD upon challenge with avirulent *Pseudomonas syringae* pv. *tomato*[42]. Arabidopsis interference RNA (iRNA) lines with reduced *PBA1* transcript levels displayed reduced caspase-like activity and HR-PCD, whereas other defence responses were not compromised, indicating that the contribution of the proteasome in immunity is through its participation in PCD. However, because HR-PCD was not abolished but only reduced upon *PBA1* suppression, it remains to be determined if this is due to residual *PBA1* expression in the iRNA lines or proteasome-independent PCD signaling cascades. In addition, because PBA1 is part of the proteasome complex were all subunits depend on each other, *PBA1* silencing is likely to reduce the overall proteasome activity and affect plant development, independent of caspase-like activity or PCD.

Concluding remarks

The examples discussed above show that some PLCPs, VPEs and MCs contribute to PCD, but that they are often not essential and their involvement depends on the instance of PCD and studied plant species. Therefore, whether Cys proteases are universally controlling PCD in plants remains an open question. Other proteases may also act in PCD. Serine proteases, mostly subtilases for instance, have also been associated

with plant PCD[46–50]. To convincingly demonstrate the role of proteases as regulators of PCD, or in any other biological process, it is paramount to perform genetic experiments and complementation assays with catalytically inactive variants of the candidate protease[45]. Application of Combined Fractional Diagonal Chromatography (COFRADIC) and other mass spectrometry-based proteomics techniques for substrate identification may address the role of proteases in PCD further[52,53].

However, given the evidence, we must consider the possibility that Cys proteases are not universal regulators of PCD in plants. It is possible that rather than relying on a signaling cascade based on the activity of caspase-like master regulators, plants have evolved multiple redundant proteolytic and non-proteolytic pathways that contribute independently to PCD – a strategy that would provide more phenotypic plasticity and a selective advantage[51]. Meanwhile, the overemphasis of the involvement of (Cys) proteases regulating PCD in plants may have hampered the identification of other factors regulating PCD in plants. Regulation of PCD in plants is still not well understood, and additional research activities are needed to elucidate the regulation and control of different instances of PCD in different plant species. Only then we will be able if plants have a conserved master regulator of life and death.

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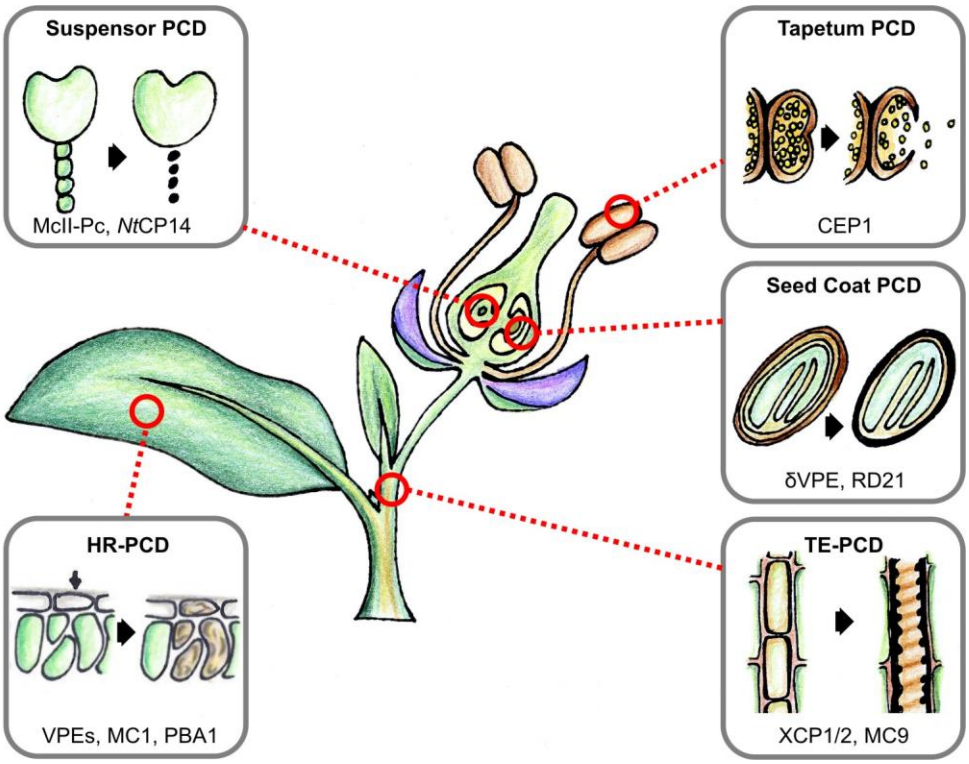

Figure 1. Cys proteases implicated in five instances of PCD in plants. This figure summarizes the processes and proteases that are discussed in this review.