The roles of proteases during developmental programmed cell death in plants

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Abstract

Proteases are among the key regulators of most forms of programmed cell death (PCD) in animals. Also in plants, many PCD processes have been associated with protease expression or activation. However, the functional evidence of the roles and actual modes of action of plant proteases in PCD remains surprisingly limited. In this review, we give an update on protease involvement in the context of developmentally regulated plant PCD. To illustrate the diversity of protease functions, we focus on several prominent developmental PCD processes, including xylem and tapetum maturation, suspensor elimination, endosperm degradation and seed coat formation, as well as plant senescence processes. Despite the substantial advance in the field, protease functions are still often only correlatively linked to developmental PCD, and the specific molecular roles of proteases in many developmental PCD processes remain to be elucidated.

Keywords:

plant, programmed cell death (PCD), developmental PCD (dPCD), protease, development, protein degradation

One sentence abstract

We review recent developments on the role of proteases in key developmental programmed cell death contexts, and discuss their modes of action.

Abbreviations

PCD	Programmed cell death
dPCD	Developmentally controlled PCD
ePCD	Environmentally triggered PCD
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
PLCPs	Papain-like cysteine proteases
ROS	Reactive oxygen species
SI	Self-incompatibility or Self-incompatible
SI-PCD	Self-incompatibility induced programmed cell death
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TE	Tracheary element cell in the xylem
VPEs	Vacuolar processing enzymes

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1. Introduction

Programmed cell death (PCD) designates different forms of genetically encoded, tightly controlled processes to eliminate no longer needed, damaged or harmful cells in a targeted fashion. There is a plethora of different PCD pathways, and many of them are vital for the development and fitness of animals and plants. In plants, PCD occurs in the course of regular development (dPCD), as well as a part of various stress responses to the biotic and abiotic environment (ePCD) (Daneva *et al.*, 2016; Huysmans *et al.*, 2017).

During plant development, a surprising number of – partly still putative – dPCD processes have been recognized in different stages of vegetative and reproductive development (Van Hautegem *et al.*, 2015). A paradigm for dPCD in plants occurs during the development of the water-conducting xylem network, whose efficiency relies on the formation of hollow xylem cells that die and become functional in water transport (Escamez and Tuominen, 2014). A precise control of xylem cell death in early vascular plants was a key evolutionary prerequisite for the successful colonialization of terrestrial habitats. Another well-established example of dPCD is the degeneration of the tapetum layer in the floral anther. Several mutants have been characterized in which tapetum differentiation and degeneration is altered, which almost invariably leads to abortion of pollen development and thus male sterility (Wilson and Zhang, 2009). A form of dPCD also disposes of embryonic suspensor cells, which has been shown to be important for embryo development in some plant species (Smertenko and Bozhkov, 2014). After seedling germination, the organ size of the root cap in Arabidopsis (*Arabidopsis thaliana*) is controlled by a precisely timed dPCD event, which is important for optimal root growth and the patterning of the root system (Fendrych *et al.*, 2014; Xuan *et al.*, 2016). Finally, reproductive self-incompatibility (SI) to avoid inbreeding relies on the specific execution of a cell death program in incompatible self-pollen in some plant taxa (Eaves *et al.*, 2014) (see also article in this Special Issue by Wang et al.). These selected examples of dPCD processes in plants highlight the importance of PCD for successful plant growth and reproduction. Notably, PCD has to be tightly controlled in any context, as ectopic or untimely cellular death can be as detrimental to plant development as the delay or lack of PCD.

Despite substantial advances achieved over the last two decades in the discovery and characterization of plant PCD processes, we still lack a coherent mechanistic understanding of dPCD pathways. In many cases there seems to be a strong link between tissue differentiation and execution of PCD as the ultimate differentiation step (Figure 1). A number of transcription factors have been identified in the coordination of differentiation and dPCD preparation(Cubria-Radio and

Nowack, 2019; Van Durme and Nowack, 2016), emphasizing the importance of transcriptionally regulated dPCD-associated genes as a starting point for the generation of research hypotheses. In a large-scale meta-analysis of commonly regulated genes in diverse PCD contexts, we identified a core set of dPCD-associated genes (Olvera-Carrillo *et al.*, 2015). Among these, there is a substantial number encoding hydrolases (Table 1). Though transcriptional reporters based on some of these genes provide a convenient tool for dPCD research (Olvera-Carrillo *et al.*, 2015), there is still little insight into the mechanisms of action of most of the corresponding proteins. Despite substantial efforts, unveiling targets and precise functions of proteases remains a challenging task (Sueldo and van der Hoorn, 2017).

As described in more detail in other articles of this Special Issue, proteases (also called peptidases or proteinases) are enzymes that cleave target proteins, generally via hydrolysis-driven proteolysis. There are hundreds of protease-encoding genes in the genomes of multicellular organisms, for instance, there are more than 800 in Arabidopsis (van der Hoorn, 2008). This vast number of proteases can be grouped according to their catalytic residues as aspartic-, cysteine-, serine-, threonine-, glutamic-, asparagine-, or metalloproteases (Oda, 2012). In the MEROPS database (http://merops.sanger.ac.uk/, (Rawlings *et al.*, 2010)), proteases are grouped in families (based on amino-acid sequence similarity) and in clans (based on tertiary structure), with some groups comprising over 100 individual members.

Proteases likely originated in the earliest protein-producing organisms as a result of the need to recycle amino acids (Poręba *et al.*, 2013). In addition to this ancestral housekeeping function, proteases have adopted novel functions as posttranslational modifiers, thus becoming key components in the regulation of biological processes. Hydrolytic cleavage irreversibly alters target proteins; they can be activated or inactivated, stabilized or destabilized, their structure can be modified, or targets can be re-localized to different subcellular compartments (Schaller, 2004). Owing to this versatile regulatory capacity, proteases have been identified as regulators of a plethora of biological processes, including, but not restricted to, many steps of plant vegetative and reproductive development, as well as the orchestration of local and systemic defense responses (van der Hoorn, 2008).

Proteases have also been implicated in the regulation of many plant PCD processes. In fact, proteases are key regulators and executors of several cell death processes in animal systems, including apoptosis (caspase-mediated PCD) and pyroptosis (caspase-1 dependent proinflammatory PCD) (Fink and Cookson, 2005). In these contexts, the most prominent proteases are cysteine-

dependent, aspartate-specific proteases (caspases). The activation of initiator caspases occurs both in the extrinsic and the intrinsic apoptosis pathway and leads to the activation of executioner caspases. Thus, caspases form a proteolytic cascade that eventually leads to the direct or indirect cleavage of hundreds of target proteins (Poręba *et al.*, 2013). Although a few key caspase targets have been identified, it seems that the concerted cleavage of many proteins is decisive for the execution of caspase-mediated cell death (Julien and Wells, 2017). More recently, alternative caspase-independent pathways have been described. Necroptosis, for instance, does not require caspase activity to be initiated; on the contrary, caspase-8 is an important inhibitor of necroptosis (Fuchslocher Chico *et al.*, 2017). On the other hand, caspases have also been implicated in other, non-PCD-related cellular remodeling events (Julien and Wells, 2017).

Plant scientists started to investigate the role of proteases in cell death regulation over two decades ago. In several plant PCD systems, specific activity probes reported the presence of caspase-like activities, and, in some of these systems, chemical caspase inhibitors were shown to alter PCD progression (Bonneau *et al.*, 2008). The quest for caspase homologs in plants took a turn with the publication of whole genome sequences, which revealed that caspases are not conserved outside animals (Uren *et al.*, 2000), leading to the study of the structurally related metacaspases and paracaspases, grouped in the same family (C14) and clan (CD) of cysteine proteases. Metacaspases are subdivided into types I, II, and III, depending on their overall structure. Only type I and II metacaspases are found in plants. Type I metacaspases may contain an N-terminal extension, whereas type II metacaspases lack extra N-terminal motifs and generally have a longer linker region between the p10 and p20 motifs. The diversity of caspase homologs has been recently reviewed (Klemenčič and Funk, 2018). Although metacaspases became prime candidates in the search of functional caspase analogs in plants, it was finally shown that plant metacaspases lack aspartate specificity, and rather cleave after basic arginine or lysine residues (Vercammen *et al.*, 2004). Hence, although some metacaspases have been implicated in types of plant PCD (Minina *et al.*, 2017), they cannot be directly responsible for the observed caspase-like activities. Instead, several non-C14 family proteins have been shown to generate caspase-like activities in the plant PCD context, among them vacuolar processing enzymes (VPEs, (Hatsugai *et al.*, 2015), phytaspases (Galiullina *et al.*, 2015; Reichardt *et al.*, 2018), cathepsin B (Cai *et al.*, 2018), and subunits of the 26S-proteasome (Hatsugai *et al.*, 2009).

In this review, we will explore the roles of proteases that have been implicated in the regulation or progression of developmentally regulated PCD processes in plants. We will focus on dPCD occurring in the xylem, the tapetum, the embryonic suspensor, the endosperm and the seed

coat, and finally in senescing plant organs (Figure 2). Other PCD processes, including the Arabidopsis root cap turnover (Huysmans *et al.*, 2018; Kumpf and Nowack, 2015; Xuan *et al.*, 2016), lace plant (*Aponogeton madagascariensis*) leaf perforation (Dauphinee *et al.*, 2014; Lord *et al.*, 2013), or root aerenchyma formation (Takahashi *et al.*, 2015; Yamauchi *et al.*, 2016) will not be included, as there is still very little functional data available on the proteases involved in these processes. Although in many dPCD processes, proteases and protease activities have been found to be associated with different stages of cell death progression, evidence of the precise function and the specific mode of action of these proteases is rare. We attempted not only to summarize up-to-date information on dPCD proteases, but also to point out areas in which we still have to make further progress to understand dPCD regulation and execution. Of note, the roles of proteases in the context of plant immunity- and stress-related PCD processes will not be covered, and we refer to a number of excellent reviews focusing on these topics that have been recently published (Balakireva and Zamyatnin, 2018; Hou *et al.*, 2018; Thomas and van der Hoorn, 2018).

2. Proteases in xylem PCD

Long distance water transport in vascular plants is facilitated by the xylem. In angiosperms, the xylem is composed of an interconnected network of tracheary elements (TEs) and tracheids, as well as parenchyma and fiber cells. The differentiation of TEs and tracheids involves extensive secondary cell wall deposition and lignification. PCD and the ensuing *post-mortem* degradation of the protoplast, while maintaining the reinforced cell walls, is essential for the final function of the xylem as an efficient water transport system (Daneva *et al.*, 2016). The progression of PCD in TEs is marked by the release of vacuolar contents caused by tonoplast rupture, triggering the deterioration of the cytoplasm, the dismantling of the endomembrane system, and mitochondrial degradation (Groover *et al.*, 1997; Kuriyama, 1999; Yu *et al.*, 2002).

Xylem-expressed proteases have been identified and implicated in xylem differentiation and PCD in different plant systems (Böllhoner *et al.*, 2018; Hao *et al.*, 2008; Liu *et al.*, 2015; Obudulu *et al.*, 2016; Petzold *et al.*, 2012; Sueldo and van der Hoorn, 2017; Zhang *et al.*, 2015). In fact, xylem differentiation and PCD are tightly coordinated. VND6 (VASCULAR-RELATED NAC-DOMAIN 6) and VND7 are two central NAC transcription factors implicated in xylem differentiation in Arabidopsis and poplar (*Populus* spec.) (Nakaba *et al.*, 2015; Ohashi-Ito *et al.*, 2010; Yamaguchi *et al.*, 2010). Closely related NAC transcription factors have been implicated in xylem development of other species, including gymnosperms and non-vascular plants (Duval *et al.*, 2014; Laubscher *et al.*, 2018; Xu *et al.*, 2014), suggesting a high degree of evolutionary conservation of xylem differentiation. Although the ectopic overexpression of *VND6* or *VND7* leads to the expression of several genes believed to control differentiation and PCD, among them several proteases (Ohashi-Ito *et al.*, 2010; Valdivia *et al.*, 2013; Yamaguchi *et al.*, 2010; Yamaguchi *et al.*, 2011; Zhong *et al.*, 2010), only few xylem proteases have been functionally investigated in detail to date.

XCP1 (XYLEM CYSTEINE PROTEASE 1) and XCP2 have been identified as xylem-expressed papain-like cysteine proteases (PLCPs) that are produced in preparation for PCD, accumulate in the vacuolar lumen and are to a smaller extent associated with the endoplasmic reticulum (ER) and Golgi of Arabidopsis TEs (Avci *et al.*, 2008; Zhao *et al.*, 2000). Although simultaneous deletion of both gene functions does not lead to developmental disturbances or PCD impairment, transmission electron microscopy showed a delay in the PCD-associated cell clearance (Avci *et al.*, 2008). Consistent with this role in Arabidopsis, Tr-cp14, a cysteine protease with homology to XCP1 and XCP2, has been identified in white clover (*Trifolium repens*) and shown to accumulate in the ER prior to vacuolar collapse (Mulisch *et al.*, 2013). Although accumulation of XCPs seems to be part of xylem

differentiation, their activation seems to be confined to precise developmental stages. Despite the transcriptional accumulation of *PsgXCP1*, *PsgXCP2A*, and *PsgXCP2B* in poplar, ray parenchyma cells (radially arranged cells in woody tissues) remained alive for extended periods of time, even after secondary cell wall deposition (Nakaba *et al.*, 2015). Despite the fact that ray parenchyma cells might follow differentiation programs distinct from TEs, this finding suggests that XCP proteases have to be post-transcriptionally activated in order to affect *post-mortem* clearance.

In poplar, the activity of the proteasome has been linked to the caspase 3-like activity observed during TE differentiation and PCD (Han *et al.*, 2012). Although chemical inhibition of proteasome activity by clasto-lactacystin β-lactone in Zinnia (*Zinnia elegans*) and Arabidopsis xylogenic cell cultures suppressed PCD, it was difficult to separate this effect from a *post-mortem* clearance effect or a potential disruption of early xylem differentiation (Han *et al.*, 2012; Woffenden *et al.*, 1998). Reinforcing the complexity of separating the potential pro-PCD effect of proteases from a role in early differentiation, chemical caspase inhibitors have been shown to affect the kinetics of TE differentiation in Zinnia cell cultures, but not to block PCD itself (Twumasi *et al.*, 2010).

Albeit not exerting caspase-like activity, metacaspases have been implicated in xylem PCD. In Arabidopsis, an *atmc9* mutant displays delayed xylem *post-mortem* corpse clearance, but PCD onset is not affected. Although *post-mortem* clearance is eventually achieved in triple *atmc9 xcp1 xcp2* mutants, activity profiling of PLCPs in whole-plant extracts showed overall reduction in PLCP activity in the triple mutants in a manner that cannot be explained by *atmc9* or *xcp1 xcp2* mutations alone (Bollhöner *et al.*, 2013), pointing to an interplay between the different proteolytic activities. AtMC9 has been localized to nucleocytoplasm, the apoplast, and the vacuolar lumen (Böllhoner *et al.*, 2018; Escamez *et al.*, 2016; Tsiatsiani *et al.*, 2013; Vercammen *et al.*, 2006) (Figure 3). Further supporting a *post-mortem* role, the acidic pH optimum for the cytoplasmic-localized AtMC9 might only be achieved after vacuolar collapse as a committing step toward PCD (Bollhöner *et al.*, 2013). . Whereas the lowering of cytoplasmic pH might enhance the activity of the cytoplasmic pool of AtMC9 without necessarily changing its abundance, the apoplastic pool might be independently regulated by the abundance of apoplastic Serpin1 (Vercammen *et al.*, 2006). Serpin1 is a suicide protease inhibitor that has also been localized to the cytoplasm and shown to interact with other proteases such as the PLCP RESPONSIVE TO DISSECATION 21 (RD21), likely after vacuolar collapse as RD21 accumulates in the vacuole (Lampl *et al.*, 2013; Lema Asqui *et al.*, 2018). In agreement with a non-essential role of MC9 in xylem PCD, the downregulation of putative *MC9* poplar homologs, *PttMC13* and *PttMC14*, is not sufficient to alter xylem differentiation (Böllhoner *et al.*, 2018). However, the activity of *PttMC13* and *PttMC14* coincides with the processing of RD21, again suggesting a participation of metacaspase

activity in a proteolytic cascade after vacuolar collapse potentially aiding in corpse clearance (Böllhoner *et al.*, 2018). Similarly, downregulation of *AtMC9* in Arabidopsis xylogenic cell cultures fails to alter the timing or extent of TE-PCD, though in accordance with the *atmc9* mutant, *post-mortem* autolysis is affected. Interestingly, *AtMC9* downregulation causes an accumulation of autophagic bodies in TE-cell vacuoles, and is associated with an increased death rate of parenchymatic non-TE cells (Escamez *et al.*, 2016). Non-TE cells do not express *AtMC9* in these cell cultures and, in contrast to TE-cells, survive xylogenic induction. Concomitant downregulation of *AtMC9* and the autophagy-related gene *ATG2* in TE cells alleviates the ectopic cell death phenotype, suggesting that the level of autophagy in TE cells influences the survival rate of neighboring non-TE cells (Escamez *et al.*, 2016).

Although xylem PCD is one of the most intensively studied dPCD processes, and many proteases and protease activities have been linked to xylem PCD, our knowledge of roles and mechanistic details of plant protease functions in this process remains limited. For instance, although a general program of xylem differentiation including upregulation of NAC transcription factors with subsequent upregulation of protease genes has been observed for the different species studied, the subcellular localization and target identification of these proteases need better elucidation. Moreover, with most of the phenotypes of analyzed mutants being related with *post-mortem* degradation, the role of proteases in earlier TE PCD steps remains to be unraveled.

3. Proteases in tapetum PCD

Pollen development is essential for successful sexual reproduction in plants. Pollen formation takes place within the anthers and is dependent on the proper development of the tapetum, a layer of cells lining the anther locule (Daneva *et al.*, 2016). Pollen development and pollen exine (tough outer walls of pollen grains) formation depend on correct tapetum differentiation, which induces dPCD of tapetal cells at a specific stage of pollen development (Ariizumi and Toriyama, 2011; Plackett *et al.*, 2011). Prior to dPCD, sporopollenin components are exported from intact tapetum cells, whereas the pollen coat is deposited later when tapetum dPCD occurs and their cellular content is released in the locule cavity in which the pollen grains develop. The precise timing of differentiation and PCD is crucial, as precocious or delayed tapetum differentiation and PCD almost invariably result in male sterility (Bedinger, 1992; Kawanabe *et al.*, 2006; Lam *et al.*, 2000). Nevertheless, as differentiation and degeneration of the tapetum is tightly linked, it is difficult to distinguish if tapetum differentiation and early secretion or later dPCD-related defects affect pollen development in mutant plants. In this context, several protease activities have been associated with tapetum

development (Hierl *et al.*, 2012; Omidvar *et al.*, 2017; Phan *et al.*, 2012; Sheoran *et al.*, 2009; Shukla *et al.*, 2016; Solís *et al.*, 2014), but few of them have been functionally analyzed in detail.

Arabidopsis CYSTEINE ENDOPEPTIDASE 1 (CEP1), a KDEL-tailed cysteine protease from the PLCP family, has been identified as a key element of tapetum PCD (Zhang *et al.*, 2014). *CEP1* is expressed in the tapetum from stage 5 to 11 of anther development. CEP1 is transported in vesicles as an inactive proenzyme to the vacuole, where maturation occurs, and the active form is released to the cytosol after vacuolar rupture. Knock-out of *CEP1* leads to abnormal and delayed tapetal PCD, and reduction in pollen fertility. Conversely, the overexpression of *CEP1* under the 35S promoter causes premature tapetal PCD and, as in the *cep1* mutant, a reduction in the number of viable pollen grains (Zhang *et al.*, 2014). Interestingly, transcriptome analyses of *cep1* mutant flower buds revealed the upregulation of hundreds of genes in comparison to the wild type already in early stages of anther development before tapetum PCD (Zhang *et al.*, 2014), suggesting an additional role for CEP1 in early tapetum differentiation.

Another cysteine protease, *AtCP51*, has been identified as being expressed during anther development (Yang *et al.*, 2014). Its expression was observed in different parts of the anther with strong promoter activity detected in tapetal cells and microspores. Contrarily to CEP1, AtCP51 seems to have a pro-survival function, because silencing of *AtCP51* by RNAi results in male sterility as a result of early tapetum degradation. Homologs of *AtCP51* have been found in various plant species such as *OsCP1* in rice (*Oryza sativa*) and *NtCP56* in tobacco (*Nicotiana tabacum*) (Yang *et al.*, 2014). Analysis of promoter activity suggested *OsCP1* expression in late anther development, including pollen and tapetum. The *oscp1* mutant shows overall delay in plant growth, and abnormal pollen development with reduced male fertility (Lee *et al.*, 2004). However, whether OsCP1 controls pollen viability directly or via tapetum cell death has not been established. *OsCP1* together with *Osc6*, encoding a putative protease inhibitor, have been shown to be directly regulated by the tapetum-specific transcription factor *TDR* (*Tapetum Degeneration Retardation*). Knock-out of *TDR* results in a delayed tapetum degeneration in rice, characterized by lower *OsCP1* and *Osc6* levels (Li *et al.*, 2006), confirming the link between *OsCP1* expression and tapetal PCD.

Next to cysteine proteases, aspartic proteases have been implicated in PCD processes throughout plant development, including tapetum PCD (Gao *et al.*, 2017a; Gao *et al.*, 2017b; Ge *et al.*, 2005). Two aspartic proteases in rice, *OsAP25* and *OsAP37*, have been found to be under direct transcriptional control of ETERNAL TAPETUM1 (EAT1) (Niu *et al.*, 2013). EAT1 is a tapetum-expressed basic helix-loop-helix transcription factor and *EAT1* knock-out leads to a delay in PCD, causing male

sterility. Both *OsAP25* and *OsAP37* are strongly downregulated in *eat1* mutants, and transient expression of both proteases causes ectopic cell death in *Nicotiana benthamiana* leaves, which was blocked in presence of the aspartic protease inhibitor pepstatin A. These results suggest that the protease activities of OsAP25 and OsAP37 are necessary and sufficient to drive cells into PCD. (Niu *et al.*, 2013)

In contrast to OsAP25 and OsAP37, the Arabidopsis aspartic protease UNDEAD is a negative regulator of tapetum PCD. *UNDEAD* expression is controlled by the tapetum transcription factor AtMYB80, a key regulator of tapetum differentiation and PCD (Phan *et al.*, 2011). Promoter activity analysis showed expression in the tapetum throughout the development of anthers, but mainly in young floral stages. The presence of a putative mitochondrial targeting signal suggests that UNDEAD might be localized to, and act in, mitochondria (Figure 3). RNAi-mediated knock-down of *UNDEAD* mimics the *myb80* phenotype of premature tapetal degeneration, characterized by an early onset of chromatin degradation in tapetum cells (Phan *et al.*, 2011). These results establish UNDEAD as a pro-survival protease that fine-tunes the timing of tapetum PCD downstream of MYB80. It is tempting to speculate that UNDEAD targets and inactivates mitochondria-localized pro-PCD factors, whose premature accumulation leads to early cell death in *myb80* and *undead* mutants. Future studies with *UNDEAD* overexpression might help to elucidate its pro-survival role downstream of MYB80.

4. Proteases in seed development-related PCD

The plant seed is a complex organ consisting of both zygotic fertilization products (embryo and endosperm) as well as maternally derived tissues (seed integuments, nucellus). While the embryo develops to carry on life to a new sporophytic generation, all other seed tissues die at specific stages during seed development (Daneva *et al.*, 2016; López-Fernández and Maldonado, 2015). In the following section, we will review the role of proteases in cell death processes occurring in the different seed compartments during seed development and germination.

4.1 Proteases in embryonic suspensor PCD

The embryonic suspensor is formed after the asymmetric division of the zygote. The terminal cell gives rise to the embryo proper, the basal cell to the suspensor cells. The suspensor promotes early embryo growth and undergoes a form of dPCD when the embryo enters the maturation phase (Kawashima and Goldberg, 2010; López-Fernández and Maldonado, 2015; Smertenko and Bozhkov, 2014). Although suspensor dPCD has only been investigated and described in few species, it is clear

that correct PCD progression in suspensor cells can be critical for embryo development (Daneva *et al.*, 2016).

One of the major model systems to study suspensor dPCD is the suspensor of somatically generated embryos of Norway spruce (*Picea abies*). In this *in-vitro* system, the large spruce suspensor is easily accessible for biochemical, pharmacological, and genetic approaches (Filonova *et al.*, 2008). Caspase-like VEIDase activity has been detected specifically in embryos at the onset of suspensor PCD, and its inhibition has been found to disturb embryo development by interfering with suspensor differentiation (Bozhkov *et al.*, 2004). In this context, a type II metacaspase, mcII-Pa, has been identified as being expressed in suspensor cells that are committed to PCD (Suarez *et al.*, 2004). While silencing of *mcII-Pa* leads to a reduced caspase-like activity on a VEID-based substrate (Suarez *et al.*, 2004), it has been shown that *mcII-Pa*, like other metacaspases, does not possess an intrinsic caspase activity (Bozhkov *et al.*, 2005). Nonetheless, mcII-Pa has been described to localize to the nucleus in dying cells (Figure 3), recombinant mcII-Pa is able to promote the degradation of nuclei in suspensor cell extracts, and its silencing reduces the frequency of DNA degradation as seen by TUNEL staining (Bozhkov *et al.*, 2005; Suarez *et al.*, 2004). A recent RNA-sequencing-based transcriptomics analysis revealed that several dPCD-associated genes are expressed in suspensor cells of Norway spruce. Those include the transcription factors XND1 and ANAC075, as well as putative spruce homologs of MC9, CEP1, and cathepsin B-like genes (Reza *et al.*, 2018).

An interplay between mcII-pa and autophagy during the development of suspensor cells has been observed in Norway spruce, pointing to a regulatory role for protease activities. The downregulation of autophagy has been shown to phenocopy embryo and suspensor development defects and leads to aberrant suspensor cell differentiation with divergent cell death (Minina *et al.*, 2013). Moreover, the downregulation of *mcII-Pa* leads to reduced autophagy, potentially reducing the cytoprotective activity of the latter in the rapidly differentiating suspensor cells. Although the mechanisms for the action of mcII-Pa in autophagy are not yet clear, a role for this metacaspase in the inactivation of an autophagy repressor has been suggested by Minina and colleagues (2014).

In tobacco, the differentiation and final PCD of suspensor cells have been linked to the opposing activity of NtCP14, a cathepsin H-like PLCP, and the interacting cystatin protease inhibitor NtCYS (Zhao *et al.*, 2013). While upregulation of *NtCP14* is sufficient to induce embryonic cell death and abortion, both overexpression of *NtCYS* or silencing of *NtCP14* leads to a delay in the onset of suspensor PCD. However, downregulation of *NtCYS* also leads to increased caspase 1-, 3-, and 6-like activities that do not seem to be caused by NtCP14, suggesting other targets or indirect effects (Zhao

et al., 2013). NtCYS has also been shown to localize, together with NtCP14, to the ER (Zhao *et al.*, 2013), suggesting either a subcellular confinement of its action to the ER or the existence of roles that are only fulfilled after the release of ER content (Figure 3). Nine other cystatins have been identified in tobacco and their differential localizations among the cytoplasm, ER, and vacuolar lumen can be a hint at different targets and processes (Zhao *et al.*, 2014). Interestingly, the control of suspensor PCD by *NtCYS* in tobacco interspecific hybrids is controlled in a parent-of-origin dependent fashion. The levels of the maternally, but not the paternally, inherited allele are controlled to ensure the correct timing of suspensor PCD (Luo *et al.*, 2016).

In contrast to the spruce tobacco suspensors, little is known about the regulation of dPCD in the Arabidopsis 7- to 9-cell suspensor (Peng and Sun, 2018), and even less about the role of proteases in this process. *CEP1* is expressed in suspensor cells prior to PCD execution where it localizes to subdomains of the ER (Zhou *et al.*, 2016). This localization prompts the hypothesis that CEP1 is stored in these structures until triggering of PCD leads to its release, unleashing its proteolytic activity (Figure 3). However, its actual role in the dying suspensor remains uncertain, as no suspensor phenotype has been identified in the respective mutants (Zhou *et al.*, 2016). KISS OF DEATH (KOD), a PCD-inducing peptide expressed in Arabidopsis suspensor cells, has been shown to be involved in suspensor PCD. Loss-of-function *kod* mutants have a reduced proportion of suspensor cells initiating PCD. In agreement with a pro-PCD role, ectopic overexpression of *KOD* in both tobacco leaves and Arabidopsis seedlings is sufficient to induce cell death. Interestingly, the expression of *KOD* leads to increased caspase 3-like activity, and co-expression of the pan-caspase inhibitor *p35* with *KOD* causes a reduction of cell death (Blanvillain *et al.*, 2011). These data suggest that the enhancement of caspase-like activity is crucial for KOD-induced death, though the mechanism of peptide signaling in the regulation of proteolytic activity is yet poorly understood.

Further suggesting a role for proteases in suspensor development and its final PCD, caspase-like activities have also been observed in the large suspensor of runner bean (*Phaseolus coccineus*) (Lombardi *et al.*, 2007). Altogether, an enhancement of proteolytic activity during suspensor development culminating in PCD seems to be a general phenomenon affecting embryo development.

4.2 Proteases in endosperm PCD

Next to the embryo, the endosperm is the second fertilization product generated by the double fertilization process in seed plants. In contrast to the embryo, however, the endosperm does not survive past the seed stage. Nevertheless, the endosperm has important functions as hybridization barrier and as nutritive tissue for the embryo during seed development and

germination (Becraft and Gutierrez-Marcos, 2012; Lafon-Placette and Köhler, 2016). Because of its role as nutrient storage in mature cereal seeds, several cereal species have been domesticated over the past millennia and now form the most important source of carbohydrate nutrition for humankind.

Several instances of dPCD terminate the life of different endosperm tissues at specific stages of seed development (Costa *et al.*, 2004; Daneva *et al.*, 2016; López-Fernández and Maldonado, 2015). In many plant species, the endosperm adjacent to the developing embryo will disappear, providing space for the growing embryo (Sabelli and Larkins, 2009). In cereals, most of the nutrients for seedling germination are stored in the starchy endosperm, which undergoes PCD after seed filling is completed (Sabelli and Larkins, 2009). In contrast to lytic endosperm death, starchy endosperm death preserves the intact starch-filled cell corpses until seed germination. Unlike the central endosperm, the peripheral aleurone layer of the endosperm shows similarities to the embryo in that both survive desiccation during seed maturation (Becraft and Gutierrez-Marcos, 2012). In cereals, the aleurone organizes nutrient remobilization during germination by secreting lytic enzymes into the dead mass of the starchy endosperm (Domínguez and Cejudo, 2014). When this function has been fulfilled, the aleurone as last surviving endosperm tissue will in turn undergo cell death (Bethke *et al.*, 2007; Sabelli and Larkins, 2009). Though many protease genes and specific protease activities have been found to be expressed in association with endosperm degeneration (Domínguez and Cejudo, 1999; Domínguez *et al.*, 2002; Rocha *et al.*, 2013; Sreenivasulu *et al.*, 2006; Szewińska *et al.*, 2016; Tran *et al.*, 2014), our knowledge about functional aspects of these proteases is still very restricted.

During lytic endosperm breakdown in the embryo-surrounding region of Arabidopsis, the bHLH transcription factor ZHOUPI (ZOU) appears to have a central role, as *zou* mutant endosperm persists, severely impeding embryo growth (Waters *et al.*, 2013). The action of ZOU appears to result in a weakening of endosperm cell walls, allowing the growing embryo to exert mechanical pressure on the endosperm cells (Fourquin *et al.*, 2016), causing either a passive disruption, or a mechanically triggered active PCD process in the embryo-surrounding endosperm. The expression of several proteases depends on ZOU, including the PCD-associated PUTATIVE ASPARTIC PROTEINASE A3 (PASPA3) and the subtilisin-like protease ABNORMAL LEAF SHAPE1 (ALE1) (Fourquin *et al.*, 2016; Yang *et al.*, 2008). Although the functional role of PASPA3 is still unknown, ALE1 is not involved in endosperm breakdown, but in the epidermal surface formation of the developing embryo (Yang *et al.*, 2008). Recently, putative *ZOU* orthologs have been described in other species in association with endosperm development (Dou *et al.*, 2018; Feng *et al.*, 2018; Zhang *et al.*, 2017b), but whether the role of ZOU in endosperm breakdown is conserved in all plant clades is unclear.

During starchy endosperm PCD in cereal seeds, the pattern of cell death progression varies (Becraft and Gutierrez-Marcos, 2012). In wheat, a rather stochastic cell death pattern has been observed (Young and Gallie, 1999), whereas maize (*Zea mays*) and rice endosperm cell death is initiated in the central part and progresses outward (Kobayashi *et al.*, 2013; Young *et al.*, 1997). In several cases, caspase-like activities have been detected in association with different instances of starchy endosperm cell death. For example, an increase in several caspase-like activities has been detected in the maturing endosperm of barley (*Hordeum vulgare*) and found to coincide with the expression of protease-encoding genes such as *HvVPE1* and *HvPhS1*, and with nuclear DNA degradation in the starchy endosperm (Tran *et al.*, 2014). Previously, VEIDase (caspase 6-like) activity has been detected, though preferentially in young, rapidly developing tissues of the barley caryopsis, not corresponding to the onset of DNA degradation (Borén *et al.*, 2006). Also in the starchy endosperm of rice, VEIDase activity has been detected prior to the onset of endosperm degradation (Kobayashi *et al.*, 2013). In the later stage of starchy endosperm development, several protease-encoding genes are upregulated. Endosperm-expressed proteases include metalloproteases and subunits of the 20S/26S proteasome complex. Interestingly, serine and aspartic protease transcripts that are present in pericarp tissues undergoing cell death are not expressed in the starchy endosperm (Sreenivasulu *et al.*, 2006), suggesting that the preservation of starchy endosperm corpses might depend on the lack of protease activity. Specific protease activity might be further regulated by protease inhibitors that are expressed in the endosperm, but not in the degenerating pericarp (Sreenivasulu *et al.*, 2006). Despite these expression data, to date there is still little knowledge about the role of proteases in the direct regulation of cell death in the starchy endosperm in cereals.

In cereals and other taxa, the aleurone endosperm layer is the only endosperm tissue that survives seed development. In developing seeds of wheat (*Triticum aestivum*), both the embryo and the aleurone express a cystatin protease inhibitor named WC5 (Wheat Cystatin 5) (Corre-Menguy *et al.*, 2002), possibly to protect these tissues from death-associated protease activities. Nevertheless, the aleurone finally dies during germination. Aleurone PCD is a well-established process, characterized by an accumulation of reactive oxygen species and the occurrence of internucleosomal DNA fragmentation (Domínguez and Cejudo, 2014). Before dying, the aleurone secretes large quantities of enzymes, including proteases, to mobilize the storage starch and proteins in the dead bulk of the starchy endosperm (Domínguez and Cejudo, 1999; Martinez *et al.*, 2009). Recently, the N-end rule pathway controlling targeted proteolysis has been implicated in the storage reserve mobilization process in Arabidopsis (Zhang *et al.*, 2018). However, proteases involved in aleurone

PCD remain little investigated. In dying barley aleurone cells, a spectrum of nuclease and protease activities has been described (Fath *et al.*, 2000). The aleurone-expressed wheat carboxypeptidase CPIII has also been found to be associated with xylem TE differentiation and PCD in wheat seedlings, suggesting a common PCD-relevant function in both tissues (Domínguez *et al.*, 2002). However, whether CPIII is functionally involved in xylem cell death and might have other roles than the mobilization of endosperm proteins during germination, remains to be investigated.

In other plant taxa, for instance, castor bean (*Ricinus communis*), the bulk of the endosperm undergoes cell death only during germination (Schmid *et al.*, 1999). In castor bean, endopeptidases with a C-terminal KDEL retention motif have been identified in specific ER-derived compartments, dubbed ricinosomes (Schmid *et al.*, 1998; Schmid *et al.*, 2001). Ricinosomes accumulate the KDEL-proteases and release them during PCD into the cytoplasm, where they become activated by cleavage of a pro-peptide (Schmid *et al.*, 1999). Similar KDEL-proteases, CEP1, CEP2, and CEP3, have also been identified in Arabidopsis as being expressed in association with dPCD (Hierl *et al.*, 2014; Zhou *et al.*, 2016) and involved in pathogen defense (Höwing *et al.*, 2017; Höwing *et al.*, 2014), though their precise mode of action remains to be elucidated. CEP2 was found to accumulate in ricinosome-like vesicles and to be activated by a pH-dependent proteolytic step (Hierl *et al.*, 2014). Similarly, in the endosperm of germinating tomato (*Solanum lycopersicum*) seeds, ricinosome-like structures and KDEL-tailed cysteine protease activities have been detected (Trobacher *et al.*, 2013).

Although there is ample evidence of protease expression and activity during the different cell death and degradation processes that occur in the endosperm, it remains challenging to define the precise mechanism of protease action in control of the cell death process.

4.3 Proteases in seed integument and nucellus PCD

The seed integuments are a multi-layered structure of maternal tissue that surrounds the developing embryo and endosperm in seed plants. During seed development and maturation, the different layers follow individual differentiation programs (Beeckman *et al.*, 2000). All cell layers eventually undergo dPCD, leading to the formation of the seed coat or testa, a tough protective layer surrounding the mature embryo (Daneva *et al.*, 2016). Further to its protective role, the seed coat stores an abundance of different proteins, including proteases, that might fulfil important functions during seed dormancy, protection, and germination (Raviv *et al.*, 2017).

Accumulation and upregulation of proteases concomitant with the development of the seed coat and PCD have been reported for different species. Several proteases are upregulated during

seed coat formation in the oil-producing tree *Jatropha curcas*, including cysteine and serine proteases, and subtilases (Rocha *et al.*, 2013; Soares *et al.*, 2017). In tomato, five *VPE* genes, named *SlVPE1* through *SlVPE5*, have been identified. Based on phylogenetic and expression analysis, *SlVPE1* and *SlVPE2* are expressed in the seed coat, similarly to *δVPE* in Arabidopsis. However, even with the downregulation of all five *SlVPEs* by RNAi, the seed coat is able to differentiate normally (Ariizumi *et al.*, 2011). In rapeseed (*Brassica napus*), BnCysP1, a putative cysteine protease that lacks a KDEL motif and is structurally related to the leaf senescence-associated genes BnSAG-12-1 and BnSAG-12-1, has been identified to be expressed only in the inner integument prior to cell death (Obermeier *et al.*, 2009; Wan *et al.*, 2002).

In Arabidopsis, the seed coat develops from five integument layers that differentiate and finally undergo cell death in a specific succession (Haughn and Chaudhury, 2005). The Arabidopsis genome codes for four different *VPE* genes, with *δVPE* being specifically expressed in the inner integument (Nakaune *et al.*, 2005). While the other three Arabidopsis VPEs (αVPE, βVPE, and γVPE) have been shown to localize to vacuoles, δVPE was found in aggregates between the cell wall and plasma membrane of shrinking integument cells (Figure 3). In the *δvpe-1* mutant, PCD characterized by nuclear degradation and cellular breakdown is delayed. However, mature *δvpe-1* seeds show a similar appearance, dormancy, and germination rate as wild-type seeds. Using a YVAD-based substrate, δVPE has been shown to have caspase 1-like activity (Nakaune *et al.*, 2005). In the Arabidopsis seed coat, loss of function of the also inner integument-accumulated PDI5 (Protein Disulfide Isomerase 5), a Cys protease inhibitor, causes premature PCD of the inner seed coat layer and loss of embryo viability (Andème Ondzighi *et al.*, 2008). The *pdi5* mutant phenotype suggests that the regulation of proteolytic activity during seed coat development is crucial for proper seed set. PDI5 has been shown to interact with the Cys proteases RD21, CP43, and CP19 in integument cells. Both RD21 and PDI5 traffic together through the endomembrane system towards the vacuole, where these accumulate. Accumulation of PDI5 has been observed in flowers, stems, leaves, siliques, and immature seeds (Andème Ondzighi *et al.*, 2008). The reduction of PDI5 levels prior to the onset of PCD might indicate that PDI5 has a pro-survival role by maintaining the suppression of proteolytic activity to avoid premature seed coat PCD.

The nucellus is a tissue of maternal origin that gives rise to and supports the early female gametophyte and developing seed, and in many species degenerates through PCD (Lu and Magnani, 2018). In aubergine (*Solanum melongena*), a putative cysteine protease similar to tobacco's CYP-8 has been identified and is expressed in nucellar cells as well as in several other tissue types that undergo PCD (Xu and Chye, 1999). In castor bean, the expression of CysEP, a cysteine protease found

in ricinosomes in senescing tissues, coincides with nucellar PCD (Greenwood *et al.*, 2005). In cardoon (*Cynara cardunculus*), aspartic proteases named cardosins accumulate in flowers. These are traditionally used for cheese manufacture in the Iberian Peninsula. The nucellus of cardoon has been shown by TUNEL staining to undergo PCD and also to accumulate cardosin B (Figueiredo *et al.*, 2006). This protease localizes to the extracellular space in the stigma and the transmitting tissue in the style during floral development (Vieira *et al.*, 2001). In quinoa (*Chenopodium quinoa*), the nucellus gives rise to a layer of nutritive tissue called perisperm. It consists of dead, starch-filled cells and takes over the role of the nutritive endosperm in some angiosperms. In the forming perisperm, caspase 6-like activity has been found when starch accumulation starts and few cells already show signs of nuclear degradation (López-Fernández and Maldonado, 2013). In the degenerating nucellus of chayote (*Sechium edule*), caspase 1-, 3-, and 6-like activities have been found to be upregulated during cell death (Lombardi *et al.*, 2007). The nucellus has also been shown to produce nitric oxide and hydrogen peroxide during its development, and exogenous applications of these compounds enhances the observed caspase-like activity and cell death (Lombardi *et al.*, 2010).

In barley, seven *VPE* genes, named *HvVPE1* through *HvVPE7*, have been found to be expressed in developing grains. *HvVPE2a* and *HvVPE2b,* as well as two phytaspases *HvPhS1* and *HvPhS2, and* the aspartic protease nucellin are expressed during preparation and initiation of PCD in the nucellus (Chen and Foolad, 1997; Radchuk *et al.*, 2011; Tran *et al.*, 2014). Interestingly, HvVPE2a/nucellain has been found to localize to the cell wall (Linnestad *et al.*, 1998), complicating the understanding of its role in *pre-mortem* intracellular events (Figure 3). In rice, suppression of the transcription factor MADS29 leads to the downregulation of PCD-related genes in the nucellar projection, among them one encoding a Cys protease, causing defective nucellar PCD and abnormal seed development with shrunken seeds containing abnormal starch granules (Yin and Xue, 2012). These results suggest a role for transcriptionally regulated proteolytic activity in the developmentally controlled degradation of the nucellus. The investigation of PCD progression in many plant tissues, including the nucellus, is hampered by the low accessibility of the tissue. To circumvent this issue, a system using maize nucellus protoplasts has been developed to allow the rapid manipulation of the cellular environment. In this system, overexpression of *MADS29* leads to the upregulation of Cys proteases, as was observed in rice (Chen *et al.*, 2015), indicating that nucellar protoplasts might be promising to aid gene discovery and characterization in nucellar dPCD.

While the current studies showcase a significant and developmentally controlled proteolytic activity during seed coat and nucellus development, functional links between these activities and the occurring PCD are still limited. Further studies will be necessary to untangle the roles of the identified

proteases to understand if their activity is responsible for triggering or executing cell death, or whether these are responsible primarily for *post-mortem* corpse degradation activities.

5. Proteases in senescence-induced PCD

Senescence is an integrative final stage of plant development that can also be induced or accelerated by a variety of environmental stresses. Senescence can occur on the level of individual organs, but can also encompass the entire plant at the end of its life cycle. Of interest, leaf and flower senescence are tightly controlled and are crucial for the degradation of high-weight macromolecules and their remobilization to actively growing parts of the plant (Diaz-Mendoza *et al.*, 2016).

5.1 Leaf senescence

Leaf senescence is an intensely studied phenomenon in which a large number of genes have been implicated (Li *et al.*, 2017). Proteolysis is among the most important catabolic processes occurring during leaf senescence and is central in the recycling of nitrogen compounds. Accordingly, genes encoding proteases are among the most upregulated genes during leaf senescence (Guo *et al.*, 2004). Although some proteases, like SENESCENCE-ASSOCIATED GENE 12 (SAG12), represent important genetic markers for senescence, their actual functions often remain unknown, likely due to extensive genetic or functional redundancy (Pružinská *et al.*, 2017). Generally, it is assumed that protein breakdown in senescing leaves involves protease activities in different cellular compartments, maximizing the catabolism of proteins into transportable units (Diaz-Mendoza *et al.*, 2016; Schippers *et al.*, 2015).

Although often used interchangeably, leaf senescence and PCD do not refer to the same process. Whereas nutrient mobilization is an active cellular program depending on cellular viability, PCD marks the endpoint of the senescence process (Thomas, 2013). As such, senescence-induced cell death needs to be tightly coordinated with the catabolic senescence processes to optimize recuperation of nutrients (Kim *et al.*, 2016), with nuclei and mitochondria remaining active until the very late stages of senescence (Lim *et al.*, 2007). Levels of the phytohormone salicylic acid (SA) increase during senescence. *SAG12* expression and senescence-associated cell death are delayed in the SA signaling mutant *pad4*, indicating that SA has a role in the control of gene expression during developmental senescence, and that protease activity downstream of SA can have an effect on late senescence and cell death (Morris *et al.*, 2000).

Several protease-encoding genes have been shown to be differentially regulated during leaf senescence (Bhalerao *et al.*, 2003; Gregersen and Holm, 2007; Guo *et al.*, 2004; Kinoshita *et al.*, 1999; Parrott *et al.*, 2010; Roberts *et al.*, 2012). However, untangling proteases that act primarily on nutrient mobilization from those acting on the initiation and execution of senescence-induced PCD remains problematic. While the role of proteolysis in nutrient mobilization has been extensively reviewed (Diaz-Mendoza *et al.*, 2016; Roberts *et al.*, 2012; Schippers *et al.*, 2015), here we focus on the few instances in which proteases have been implicated in senescence-associated cell death.

One approach to identify PCD-regulating genes expressed during plant organ senescence is to identify genes that are also expressed in other, senescence-unrelated PCD processes. A meta-analysis of different PCD-associated transcriptional signatures revealed the upregulation of senescence-associated genes also during osmotic stresses, the hypersensitive response, and cell-differentiation induced PCD (Olvera-Carrillo *et al.*, 2015). Thus, especially proteases that are co-regulated during leaf senescence and in cell types that undergo differentiation-induced death might represent interesting candidates for death-promoting proteases during senescence. For instance, SERINE CARBOXYPEPTIDASE-LIKE48 (SCPL48), PASPA3, and some VPEs are upregulated in organ senescence as well as diverse cell types that undergo cell death as final differentiation step (Olvera-Carrillo *et al.*, 2015).

Additionally, several proteases linked with senescence-induced PCD have been reported in different protease groups. The cucumber (*Cucumis* sativus) metalloprotease Cs1-MMP is expressed in the final stage of cotyledon senescence just prior to DNA fragmentation (Delorme *et al.*, 2000). This late upregulation suggests a role in PCD regulation rather than in nutrient recycling. Additionally, the expression of the *At2-MMP* metalloprotease in Arabidopsis increases with organ age and senescence, and senescence-induced PCD is accelerated in *at2-mmp-1* mutants (Golldack *et al.*, 2002). Similarly, mutants of the mitochondrial metalloprotease *FtSH4* display early leaf senescence, higher autophagic activity and cell death, suggesting a pro-survival function for FtSH4 and At2-MMP (Zhang *et al.*, 2017a). Naturally, the effects of these loss-of-function mutants might be pleiotropic and the influence on leaf viability might be only indirect.

Next to protease activity, also autophagy has been involved in nutrient remobilization during leaf senescence (Havé *et al.*, 2017). Interestingly, the autophagy-deficient *atg5* mutant has been found to display higher levels of protease activity, which was attributed to the proteasome, and cytosolic and vacuolar cysteine proteases (Havé *et al.*, 2018). Although these might act directly in

promoting cell death, it is possible that these activities are back-up proteolytic systems to compensate for the absence of canonical autophagy.

5.2 Floral organ senescence

Leaf and floral senescence share the upregulation of PCD- and senescence-associated genes (Trivellini *et al.*, 2016). In comparison with leaf senescence, floral senescence might be more dependent on developmental than on environmental stimuli. For example, the lifespan of the stigma is tightly controlled by NAC transcription factors, which induce PCD to terminate flower receptivity for pollen (Gao *et al.*, 2018). Also, pollination itself is an important trigger for floral organ senescence, often mediated by the phytohormone ethylene (Rogers, 2006, 2013).

Ageing floral organs display certain PCD hallmarks, including degradation of total protein content, DNA fragmentation, vacuolar rupture and protoplast shrinkage (Battelli *et al.*, 2011). The activity of serine-, cysteine-, and metalloproteases, and of KDEL-like and caspase-like proteases has been shown throughout floral senescence (Battelli *et al.*, 2014; Pak and Van Doorn, 2005; Tripathi *et al.*, 2009; Zhou *et al.*, 2016), but functional evidence for involvement in PCD is still limited. For instance, senescing lily (*Lilium longiflorum*) flowers develop caspase-like activities during late senescence, and YVADase, VEIDase, and DEVDase inhibitors strongly decrease this activity (Battelli *et al.*, 2011). However, none of the three inhibitors affect flower longevity or the progression of senescence(Battelli *et al.*, 2011). In another study on lily flowers, signs of PCD including decreasing protein content and DNA degradation have been described in mesophyll and epidermal cells (Mochizuki-Kawai *et al.*, 2015). During early senescence, mesophyll cells show an upregulation of the KDEL-tailed cysteine protease LoCYP and display signs of PCD. Only during later flower senescence, epidermal cells start to undergo PCD, with the simultaneous upregulation of the lily *SAG12* homolog, *LoSAG12* (Mochizuki-Kawai *et al.*, 2015).

6. Conclusion

Based on the enormous regulatory potential of targeted protein modification and degradation by proteases, it comes as no surprise that proteases are involved in the regulation of most biological processes, including PCD. A major question is, however, if there are plant proteases that are similarly central to plant PCD as caspases are to apoptosis and other animal cell death types, or whether proteolytic events merely accompany plant PCD without being central for its actual regulation.

In this review, we aim to provide an overview of existing data on proteases in the context of dPCD and cover a selection of relatively well-described PCD processes in which the functional involvement of proteases has been demonstrated (Figure 2). (Dauphinee *et al.*, 2014; Huysmans *et al.*, 2018; Kumpf and Nowack, 2015; Lord *et al.*, 2013; Takahashi *et al.*, 2015; Xuan *et al.*, 2016; Yamauchi *et al.*, 2016)But even in the intensively studied PCD model systems, most available data are still associative in that they describe the expression of protease-encoding genes or activity of proteases in connection with PCD processes. Only in a limited number of cases functional data exist that allow to draw causal relationships between proteases and/or protease activity and dPCD (Table 2).

The determination of the actual mode of action of PCD-associated proteases remains a key challenge. VPEs for instance have been implicated in tonoplast breakdown as a committing step in PCD (Hatsugai *et al.*, 2015), but how tonoplast disintegration is achieved remains unresolved. A substantial limitation might be presented by the genetic and functional redundancy of proteases – many plant proteases exist in large families and even higher-order mutants might fail to produce phenotypes that would allow an inference of the proteases' function. The development and application of synthetic protease inhibitors have the potential to overcome such redundancy. However, the limited and often overestimated selectivity can restrict the utility of chemical approaches (Poręba *et al.*, 2013). A way forward would consist in comprehensive approaches combining pharmacology and reverse genetics. Protein localization is key to protease function, especially regarding the sequestration of inactive zymogens and the re-localization of activated proteases (Figure 3). So far, only a few publications describe the dynamic subcellular localization of proteases, for instance by fusion proteins that take activation cleavage sites into account (Hierl *et al.*, 2014). Additionally, strategies to identify the protease targets in quantitative degradomics studies (Demir *et al.*, 2018; Huesgen and Overall, 2012) will be an important path to a functional understanding of the role proteases play in the control and execution of dPCD in plants.

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Table 1: Proteases commonly upregulated during Arabidopsis dPCD (Olvera-Carrillo *et al.*, 2015).

AGI	Name	Annotation	Co-expressed with*	Upregulated in dPCD**
AT5G04200	AtMC9	Metacaspase 9	7 genes	b.c.
AT4G04460	PASPA3	Saposin-like aspartyl protease family protein	6 genes	12 conditions
AT3G45010	SCPL48	Serine carboxypeptidase-like 48	5 genes	12 conditions
AT4G35350	XCP1	Xylem cysteine peptidase 1	4 genes	b.c.
AT2G25940	αVPE	Alpha-vacuolar processing enzyme	3 genes	8 conditions
AT1G20850	XCP2	Xylem cysteine peptidase 2	3 genes	b.c.
AT1G23460	n.n.	Pectin lyase-like superfamily protein	3 genes	b.c.
AT1G63120	ATRBL2	RHOMBOID-like 2	3 genes	b.c.
AT5G50260	CEP1	Cysteine proteinases superfamily protein	2 genes	b.c.
AT1G01900	ATSBT1.1	Subtilase family protein	n.a.	8 conditions

* based on a Genevestigator co-expression analysis with other dPCD-associated genes
** dPCD conditions covered in meta-analysis
n.n. no name
n.a. not applicable
b.c. below cutoff value of 8 conditions

Table 2: A selection of proteases implicated in dPCD processes.

Tissue/Process	Name	Species	MEROPS clan/family/ID	Catalytic type	Localization	Mode of action	Reference
Xylem	XCP1	*At*	CA/C1/C01.065	Cysteine	Vacuole and to a lesser extend in cytoplasm	Post-mortem clearance	(Avci *et al.*, 2008)
	XCP2	*At*	CA/C1/C01.065	Cysteine	Vacuole and to a lesser extend in cytoplasm	Post-mortem clearance	(Avci *et al.*, 2008)
	Tr-cp14	*Tr*	CA/C1/C01.065	Cysteine	ER	n.d.	(Mulisch *et al.*, 2013)
	AtMC9	*At*	CD/C14/C14.034	Cysteine	Nucleocytoplasm, apoplast, and vacuole	Post-mortem clearance	(Vercammen *et al.*, 2006); (Escamez *et al.*, 2016); (Bollhöner *et al.*, 2013)
Tapetum	CEP1	*At*	CA/C1/A03	Cysteine	Vacuole	Pro-PCD	(Zhang *et al.*, 2014)
	AtCP51	*At*	CA/C1/A26	Cysteine	n.d.	Pro-survival	(Yang *et al.*, 2014)
	OsAP25	*Os*	AA/A1/UPW	Aspartic	n.d.	Pro-PCD	(Niu *et al.*, 2013)
	OsAP37	*Os*	AA/A1/UPW	Aspartic	n.d.	Pro-PCD	(Niu *et al.*, 2013)
	UNDEAD	*At*	AA/A1/A01.A49	Aspartic	Predicted mitochondrial	Pro-survival	(Phan *et al.*, 2011)
Suspensor	CEP1	*At*	CA/C1/C01.A03	Cysteine	Subdomains of ER	n.d.	(Zhou *et al.*, 2016)
	NtCP14	*Nt*	CA/C1/C01.A13	Cysteine	ER	Pro-PCD	(Zhao *et al.*, 2013)
	NtCYS	*Nt*	n.p.	inhibitor	ER	Pro-survival	(Zhao *et al.*, 2013)
	mcII-Pa	*Pa*	CD / C14 / C14.036	Cysteine	Nucleus	Promotes nuclei degradation	(Bozhkov *et al.*, 2005)
Endosperm	CEP2	*At*	CA/C1/C01.A01	Cysteine	Ricinosome-like vesicles	n.d.	(Hierl *et al.*, 2014)
Inner integument	RD21A	*At*	CA/C1/C01.064	Cysteine	Vacuole	n.d.	(Andème Ondzighi *et al.*, 2008)
	PDI5	*At*	n.p.	inhibitor	Vacuole	Pro-survival	(Andème Ondzighi *et al.*, 2008)
	δVPE	*At*	CD/C13/C13.A01	Cysteine	Cytosol, Apoplast	Pro-PCD	(Nakaune *et al.*, 2005)
Nucellus	CysEP	*Rc*	CA/C1/C01.010	Cysteine	Ricinosomes	n.d.	(Greenwood *et al.*, 2005)
	HvVPE2a	*Hv*	CD/C13/C13.001	Cysteine	Apoplast	n.d.	(Linnestad *et al.*, 1998)
Leaf senescence	At2-MMP	*At*	MA/M10/M10.012	Metallo	Predicted plasma membrane	Pro-survival	(Golldack *et al.*, 2002)
Floral senescence	FtSH4	*At*	MA/M41/M41.004	Metallo	Mitochondria	Pro-survival	(Zhang *et al.*, 2017a)

n.d. not determined; n.p. not present; *At - Arabidopsis thaliana; Tr - Trifolium repens; Os - Oryza sativa; Nt - Nicotiana tabacum; Pa - Picea abies; Rc - Ricinus communis; Hv - Hordeum vulgare*

Figure Legends

Figure 1. Hypothetical regulatory network of the plant dPCD process. Coordinated with cellular differentiation, dPCD competency is achieved at least partly on the transcriptional level. A range of PCD-associated genes are upregulated, and their proteins accumulate in cells that are preparing to undergo PCD. Once competent, a hypothetical trigger starts the PCD execution process. Finally, partial or complete corpse clearance is achieved by activity of degrading enzymes that are activated during PCD and complete corpse clearance post mortem. Conceivably, proteases might play roles in all of these steps, but so far only few protease functions in dPCD have been characterized in detail.

Figure 2. Proteases implicated in selected PCD processes occurring during plant development. PCD processes are an integral part of vegetative and reproductive plant development. They occur during xylem and tapetum differentiation, in various tissues during seed development and germination, and during plant organ senescence. We indicate proteases that have been implicated with aspects of these cell death processes in the individual tissues. For some of the PCD processes listed, no functional data for protease involvement exists to date. Abbreviations are: A, aleurone layer; E, endosperm; ESR, embryo-surrounding region of the endosperm; I, seed integuments; S, suspensor.

Figure 3. Selection of dPCD-associated proteases with known or predicted subcellular localizations. Many dPCD-associated proteases accumulate as inactive proenzymes or in specific subcellular compartments before they are activated. Especially the vacuole, but also the ER and ER-derived vesicles can serve as storage space for inactive proteases. Once PCD execution is triggered, the breakup of cellular compartments is thought to release these proteases into the same compartments as their targets. The specific chemical environment of subcellular compartments before and after compartment breakup can be an additional cue to activate PCD-associated proteases. Abbreviations are: A, apoplast; C, cell wall; Ch, chloroplast; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; PM, plasma membrane; R, ricinosome; V, vacuole.

Fig. 1

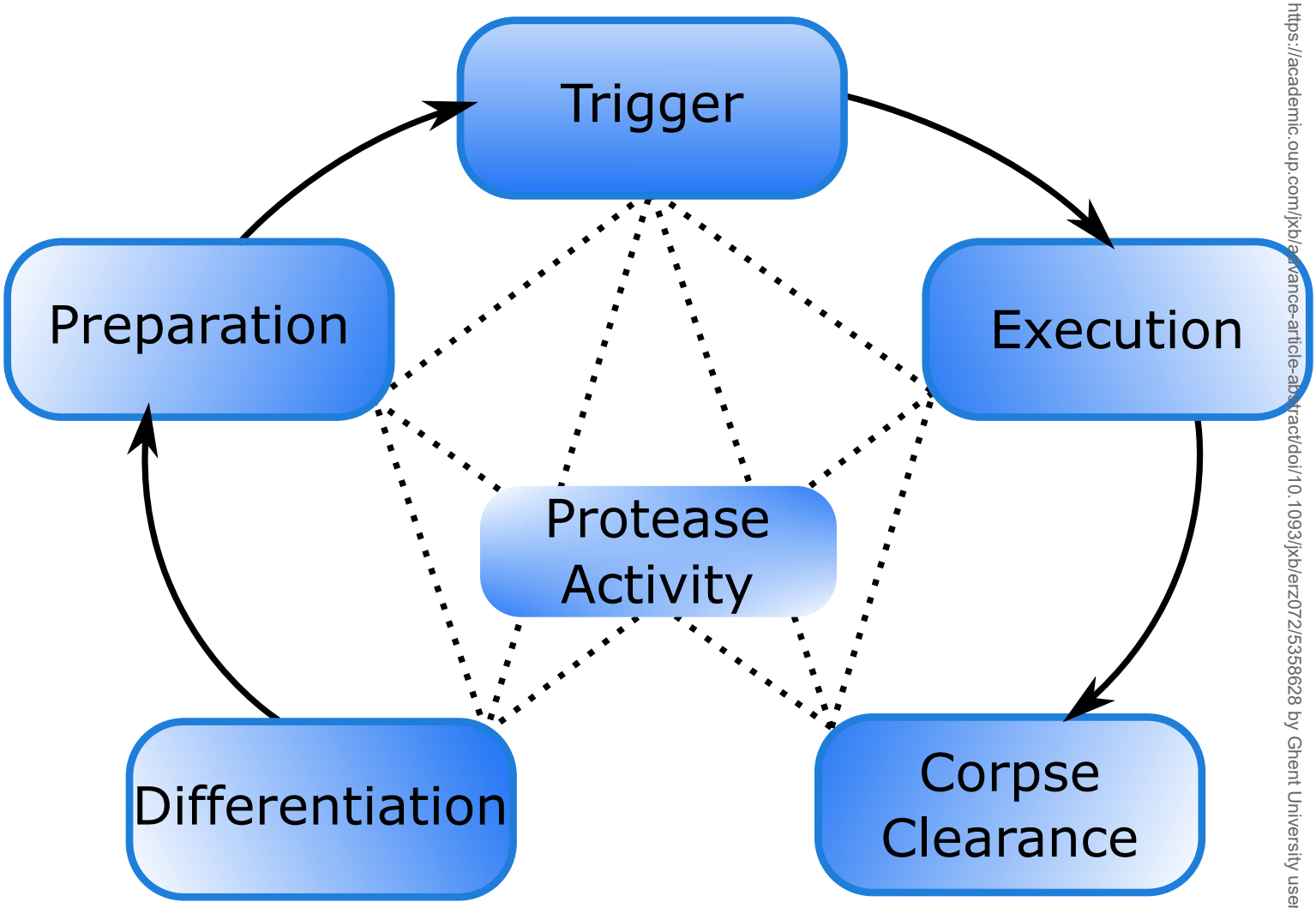

Fig. 2

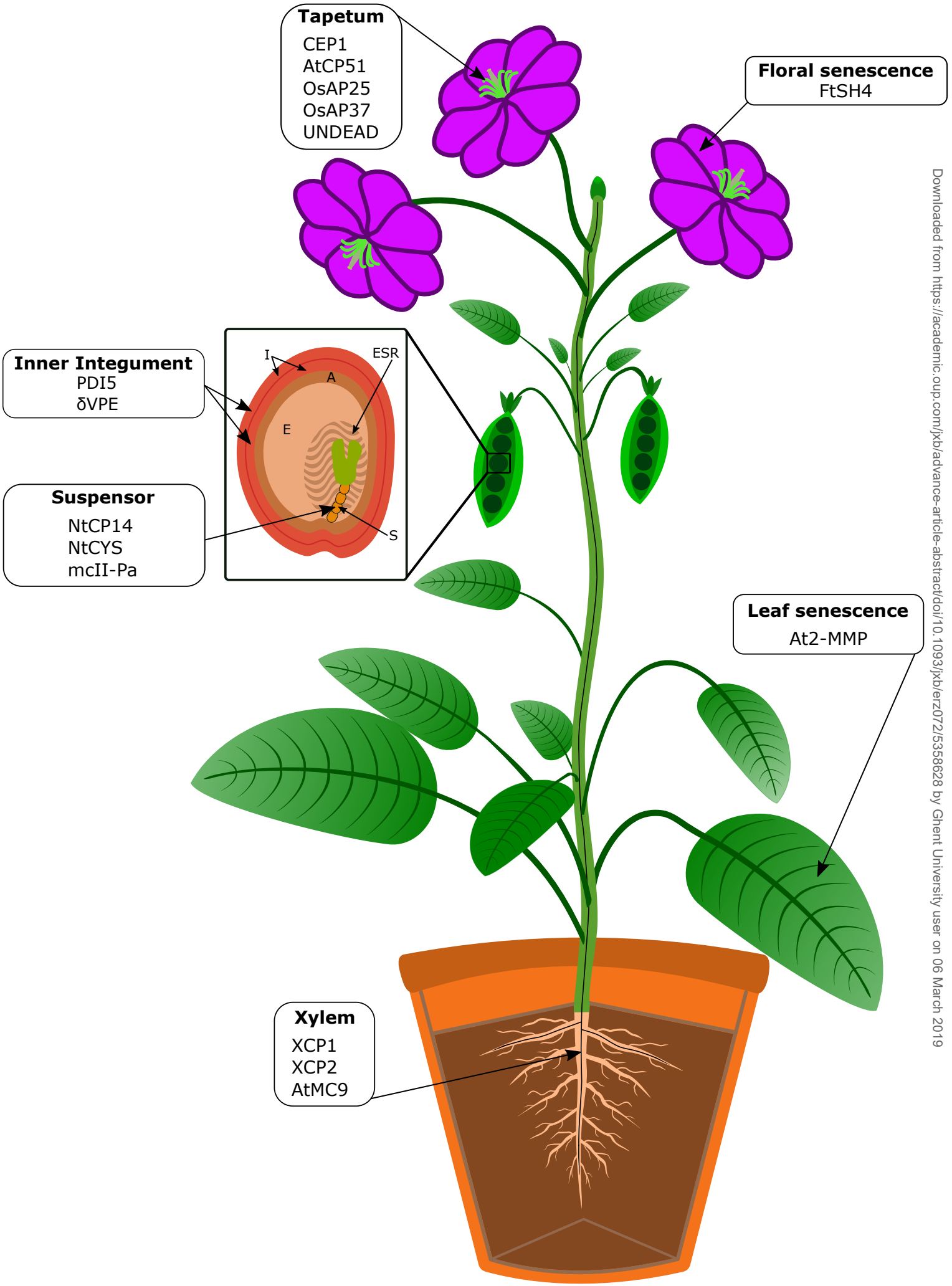

Fig. 3