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Review

Role of metacaspases and autophagy in developmental programmed cell death in plants

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ABSTRACT

During the life cycle of plants, the demise of certain cells occurs when they have fulfilled their task. This selective and tightly regulated adaptive mechanism, known as programmed cell death (PCD), contributes to life and is fundamental for the maintenance of cellular homeostasis. Proteases are one of the key performers of PCD-mediated processes and, although no caspases have been identified in plants, caspase-like activities are associated with PCD in different tissues, including developing seeds. The role of caspase-like proteases was first demonstrated with the identification of their phylogenetically closest proteins in plants, the metacaspases. Autophagy, which regulates different forms of PCD and plays key roles in cellular homeostasis, has been recently conceptualized as a mediator of life and death. Thus, autophagy genes are key determinants during this cellular suicide response. Although great advances have been made in the study of PCD, the molecular signals that trigger it remain unknown. Taken together, this review, referred to as the role of metacaspases and autophagy in PCD, illustrates the complexity and diversity of mechanisms that control PCD in plants.

KEYWORDS: autophagy, autophagy-related genes, cargo, cathepsins, cell wall, Cys-proteases, programmed cell death, storage proteins.

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INTRODUCTION

The life cycle of organisms requires specific cell types to be eliminated in a predictable and genetically regulated way. This process, referred to as programmed cell death (PCD), is essential for life [1, 2]. The mechanism through which specific cells are targeted for PCD without affecting neighboring cells has not yet been defined. Notable cellular compartments, such as mitochondria, chloroplast, Golgi complex, endoplasmic reticulum (ER) and vacuole, have been shown to be involved in the control of PCD [2]. This process of cellular suicide occurs at every stage of life: from embryo till death [3]. For example, PCD is required during the onset of zygotic embryogenesis for proper seed development [3, 4]. Plant PCD exhibits several hallmarks, which include: 1) DNA laddering and strong chromatin condensation [5]; 2) 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 [6]; generation of autophagic vacuoles due to the absence of an active phagocytosis system [7]; degradation of organelles such as the plastidome, mitochondria and peroxisomes [8]; extensive vacuolation (i.e. appearance of a large vacuole) [9]; and sometimes the development of ricinosomes concomitantly with the progression of nuclear DNA fragmentation [10, 11]. At the end of PCD, the cell is completely digested and the remaining cytosol is surrounded by the cell wall (CW), which finally becomes disorganized and disintegrates [2]. That is, since

the plants have CWs, they have developed their own PCD process. At the cellular level, plant PCD can be autolytic (i.e. formation of large lytic vacuoles and the rapid clearance of cytoplasm due to tonoplast rupture and the release of hydrolases) or non-autolytic [12]. Developmental PCD (dPCD) is autolytic and critical for many vegetative and reproductive processes [2, 13]. However, environmental PCD (ePCD) is non-autolytic and is involved in responses to biotic and abiotic stresses and in the hypersensitive response (HR), which prevents the growth and spread of pathogens into healthy tissues [14-16]. While dPCD has been the focus of various studies [17], to date, a comprehensive understanding of the molecular pathways governing PCD during development is lacking. 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 [18]. The involvement of PCD has been described in various plant life processes, including the emptying of xylem tracheary elements [19], aerenchyma formation [20, 21], and dynamic turnover of the root cap [22]. Because plants and animals have different molecular mechanisms for PCD, an evolutionary parallelism of PCD pathways in plants and animals has been postulated [20]. Here, an update on the recent and substantial progress that has been made to our understanding on PCD and its importance to plant development is provided. The role of metacaspases and autophagy is carefully reviewed.

The task of proteases in plant PCD

In plants, like in animals and several branches of unicellular eukaryotes, proteases (http://merops.sanger.ac.uk/) play a regulatory role in a variety of processes that are essential for growth, development, reproduction, immune response, embryogenesis, photosynthesis and PCD [23, 24]. Plant genomes encode about 500 to 800 proteases from all four classes (i.e. metalloproteases, and serine (Ser-EPs) (e.g. subtilases), cysteine (Cys-EPs) and aspartic proteases (Asp-EPs) (e.g. nucellin-like ASP-EPs), which are named according to their active site residues) [25-29]. The degradome of *Arabidopsis thaliana* contains more than 800 proteases from 60 families while that of *Oryza sativa* contains more than 600 proteases. Ser-EPs are the most abundant proteases in plants: they comprise 14 families and nine clans. However, Cys-EPs are divided into 15 families of five clans [30]. Thus, plant genomes encode more than 100 Cys-EPs that act in the cytoplasm, endomembrane system and apoplast. In plants, special functions have been described for the following types of Cys-EPs:

(i) Papain-like Cys-EPs (PLCPs) are the most abundant family of Cys-EPs (e.g. plant cathepsins; derived from the Greek kathepsein -to digest). They are divided into family C1B (cytosolic) and C1A (apoplastic). These globular enzymes can be found in cellular vesicles, such as ricinosomes. PLCPs are involved in protein storage and mobilization (e.g. during seed life) and stress responses [31, 32]. Specifically, plant cathepsins are involved in processes such as senescence, abscission, fruit ripening, PCD, and in the mobilization of proteins accumulated in seeds and tubers [33]. Thus, the cathepsin-B (Cath-B) is a Cys-lysosomal-EP possessing caspase-3-like activity in Arabidopsis that is inhibited by caspase-3-specific inhibitors and with a central role in regulating ER stress-induced PCD and development-induced PCD [33, 34]. Although in *A. thaliana* Cath-B-like proteases are encoded by a gene family comprising three members (*AtCath-B1*, *AtCath-B2* and *AtCath-B3*), only the *AtCath-B3* gene is highly induced upon seed germination and at the early post-germination stage [35]. In addition, recent findings in Arabidopsis revealed that: (i) AtCath-B downregulation reduces reactive oxygen species (ROS) accumulation; (ii) a mutant in AtCath-B displayed unchanged tonoplast breaking; (iii) AtCath-B and the proteasome subunit PBA1 contribute to caspase-3-like activity during endoplasmic reticulum (ER) stress; and (iv) AtCath-B activation is independent of vacuolar processing enzymes (VPEs; see below). The authors conclude that AtCath-B may execute its function after tonoplast dismantling and that this Cys-protease, which does not control the rupture of the tonoplast, acts in parallel with VPEs [36]. On the other hand, it is possible that Cath-B is involved in plant disease resistance; a hypothesis that is supported by previous findings that have implicated Cath-B in pathogen-induced PCD [37]. The fact that Cath-B regulates PCD in both plants

and animals suggests that this Cys-EP may be part of an ancestral PCD pathway that evolved before the plant/animal divergence [34]. Moreover, C1A apoplastic proteases actively participate in proteolysis induced by biotic and abiotic stresses [38-40]. Cystatins and serpins are well known endogenous inhibitors of PLCPs [41]. PLCPs carry a signal peptide important for their transport to the apoplast as well as an auto inhibitory prodomain prior to the active C1-protease domain [27]. Some PLCPs possess a signal for retention in the ER at the C-terminal (i.e. KDEL) [42, 43], whereas other PLCPs possess a signal at the N-terminus of the pro-protein for vacuolar targeting (i.e. NPIR) [44]. Recently, a root-specific PLCP has been characterized and differences between the salicylic acid-dependent activation of PLCPs in roots and leaves have been identified [45].

(ii) Vacuolar processing enzymes (VPEs), also called legumains, are the most likely candidates for serving as caspase-like executioners during hypersensitive response (HR) [46]. HR is a mechanism, used by plants, to prevent the spread of infection by microbial pathogens and it is characterized by the rapid death of cells in the local region surrounding a pathogenic infection. Recent review contains the contributions of VPEs to plant PCD and its role in vacuole-mediated cell death [47]. VPEs are required for vacuole disruption [42] and vacuolar disruption-triggered cell-death, a process that while not present in animal cells [48], has been observed in the slime mold *Distyostelium discoideum*. Vacuolar disruption is also indispensable for dPCD, which has been shown to be independent of all caspase, paracaspase and metacaspase activity [49]. Although it is beyond question that VPEs are initiators of the vacuolar-processing system, the mechanism by which VPEs controls the vacuolar breakage and the execution of a variety of plant PCD is still unclear.

(iii) Metacaspases, a phylogenetically distant family of caspases, are found within several plant subcellular locations [50-52]. Some are Ca^{+2}-activated and unlike caspases do not require dimerization or the presence of adaptor proteins [53, 54]. VPEs and metacaspases display identical fold topologies to their animal counterparts [55]. Since the metacaspases play a prominent role in plant PCD, a detailed update on them is found below.

(iv) Subtilisin-like serine proteases (SLSPs), also called subtilases (SBT), are the largest group of proteases in plants [56, 57]. Eukaryotic SBT constitute the S8 family within the SB clan of Ser-EPs and plant SBT correspond to S8A subtilisin subfamily forming an extensive group of enzymes (i.e. 63 genes known in the *Oryza sativa*, 56 genes in *Arabidopsis thaliana* and at least 15 in *Lycopersicon esculentum* genomes [58, 59]. Although SLSPs orthologue sequences from *A. thaliana* were recently published [59], the function of the majority of these subtilases remains unknown. SLSPs are involved in a number of plant-specific functions as seed development and germination, cuticle formation, xylem development, organ abscission and senescence [57]. Another outstanding feature of SLSPs is their involvement in plant PCD and triggering of HR response. That is, several SLSPs are associated to plant defence responses against the most diverse pathogens (e.g. pathogenic recognition and resistance and plant immune priming) [57, 60].

(v) Ubiquitin is a highly conserved protein found in all eukaryotes and is involved in almost all aspects of plant physiology, including immunity [61]. Attachment of ubiquitin to cellular proteins (i.e. ubiquitination) is important for regulating distinct cellular processes (e.g. DNA repair, cell-cycle progression, stress responses, signal transduction, PCD) [62]. Deubiquitinating enzymes (DUBs), which reverse the process of ubiquitination, are proteases that make up part of the ubiquitin-26S proteasome complex and that mediate caspase-3-like activity in the cytoplasm. Given the characteristics of deubiquitinating enzyme Cys-EPs, in plants it appears that caspase-3-like activity is, at least in part, due to proteasome activity [63, 64]. Unexpectedly, although proteasome associated proteases and Cath-B contribute to the increase in total caspase-3-like activity [64], they play antagonistic roles on PCD. Thus, Cath-B promotes PCD whereas PBA1 negatively regulates it. This highlights the fact that increased caspase-3-like activity does not always translate into increased plant PCD and suggests that more complex mechanisms are at play [36]. It was recently shown that inhibition of AtCath-B3 abolished

caspase-3-like activity and prevented PCD from occurring in Arabidopsis [34]. Regarding this fact, deubiquitinating enzymes AtUBP12 and AtUBP13 and their tobacco homologue NtUBP12 are negative regulators of plant immunity. The target protein of UBP12/13 is yet unknown. Loss of AtUBP12 and AtUBP13 are lethal. AtUBP12 and NtUBP12 are functionally interchangeable and their deubiquitinating activity is required to suppress PCD [65].

Finally, an intriguing trait of PCD-associated proteases is that, despite their highly destructive potential, they are able to travel through the cell's secretory pathway without causing damage. This trait highlights how tightly regulated their activity is in the cell. In any case, whether Cys-EPs universally control plant PCD remains an open question [66]. This is because the link between Cys-EP activity and PCD was not observed in all species or in all organs studied. Taking into account that this correlation is not conserved across different species, we must be cautious when it comes to judging the role of the Cys-proteases in PCD mechanisms.

Involvement of metacaspases in plant PCD

a) Structural, biochemical and evolutionary characteristics

The cytosolic Asp-specific proteases, also known as caspases, are involved in the initiation and execution of metazoan PCD and are only present in animals. All members of caspases family contain the p20 catalytic domain, which harbours the catalytic dyad formed by two amino acid residues (i.e. His and Cys) (Figure 1). As compared to animals, not much was known about the regulators of PCD until caspase-like proteins, also known as metacaspases, were discovered in bacteria, algae and plants where they regulate the autophagic activity that controls aging, immune responses, terminal differentiation of cells and post-mortem cell clearance [67]. Cys-endopeptidases are further divided into two groups, vacuolar processing enzymes (VPEs) and metacaspases [51]. Although no caspase orthologous genes have been detected in plants [68], numerous studies have identified structural similarities between caspases and metacaspases. However, functionally they differ on the basis of their different substrate specificity (i.e. Arg- or Lys-specific). At the structural level, metacaspases are divided into several types [50, 51]. Thus, type-I metacaspases contains a Pro/Gln-rich prodomain (80-120 residues) in the N-terminal region. This prodomain, resembling the nuclear Arabidopsis LSD1 (Lesion Simulating Disease Resistance 1) [69, 70], comprises two Zn-finger motifs at N-terminal site which are found in plant proteins within the HR responses [70, 71]; type-II are conserved Cys-proteases lacking the above prodomain at its N-terminal site, are found in plants and green algae (e.g. Chlamydomonas

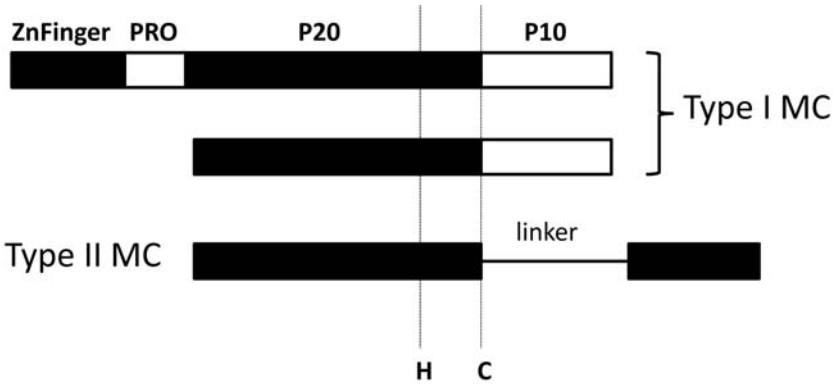

Figure 1. Scheme of metacaspases (MC) type I and II. The type I could present or not present a prodomain rich in proline, include a zinc finger motif in the N-terminus region, and necessarily have a metacaspase domain at the C-terminus region. Type II does not have the prodomain and the zinc finger motif, but feature an insert (linker) between the p20 and p10 subunits. The catalytic amino acid regions containing histidine (H) and cysteine (C) residues are shown as dotted lines.

and Volvox) and have autoprocessing sites, whose cleavage seems to be necessary for their full activation [71], and a large loop region (90-150 residues) located between p10 (10 kDa) and p20 (20 kDa) catalytic subunits [31, 67, 71]; biochemical and genetic studies have demonstrated that: (i) only type-II metacaspases undergo autocatalytic activation, similar to the phenomenon observed for caspases; (ii) some members in this family could be involved in oxidative stress-induced cell death in higher plants; and (iii) type-III metacaspase recently found in the cryptophyte *Guillardia theta* (GtMC2), which have undergone secondary endosymbiosis. GtMC2, is an active endopeptidase that contains two Ca^{+2}-binding sites and is functionally related to plant type-I metacaspases [72, 73]. Regarding Zn-finger motive, it was conjectured that its acquisition seems to have happened later during the metacaspase evolution. The absence of this motive seems to be a characteristic of the ancient condition based on the fact that no Zn-finger domains were found in typical algae [51]. The studied organisms also showed the presence of metacaspase-like proteases which possess only the p10 domain. These proteases are found in bacteria and may represent an evolutionary connection.

Higher plants contain a large family of metacaspase genes [70]. From the results so far, it has been found that the number of these genes vary in different plant species. For instance, in Arabidopsis there are nine members [50], whereas in grapes, rubber, and tomato and potato there are six, nine and eight metacaspase genes found, respectively [70 and ref. therein]. At the biochemical level, metacaspases have these remarkable properties: (i) lack the Asp specificity that characterizes caspases [50, 68]; (ii) possess the same catalytic pair (i.e. Cys/His) as caspases [73]; (iii) are unable to cleave synthetic substrates of caspases [51]; (iv) are insensitive to caspase inhibitors [51]; (v) some type-II metacaspases are Ca^{2+}-dependent under neutral pH (e.g. McII-Pa of *Picea abies* and AtMC1, AtMC4 and AtMC5 of Arabidopsis) [71]. This Ca^{2+} dependence is associated with essential amino acids located in its p20 domain. However, AtMC9 is active only under mildly acidic pH, regardless of the availability of Ca^{2+}; and (vi) although are proteases phylogenetically closest to caspases, do not have caspase activity [71]. At the evolutionary level, it has been hypothesized that type-I represents the ancient form of the metacaspase family and that the evolution of type-II had occurred before the emergence of multicellular plants from their photosynthetic, unicellular ancestors [73, 74]. More specifically, metacaspases must have been acquired by eukaryotes through primary mitochondrial and plastidic endosymbiosis [50]. The discovery of two ancestors of caspases (i.e. paracaspases and metacaspases) in different organisms [67] suggests that the paracaspase and metacaspase genes may represent an initial and ancestral set of core genes that led to the emergence of the PCD machinery. Likewise, the similarities observed between the PCD systems of bacteria, animals and plants suggests a common origin, one that is presumably the consequence of the endosymbiotic acquisition of bacteria by eukaryotes [74]. That is, metacaspase-like proteins are present not exclusively in α-proteobacteria but also in all bacteria groups, such as cyanobacteria, the known ancestors of plant chloroplasts [73]. In the context of Cys-protein evolution, canonical caspases may have originated from ancient metacaspase-like proteins, conceivably through horizontal gene transfer from α-proteobacteria to early multicellular organisms [63, 67, 69]. Recently, results in potato (*Solanum tuberosum*) indicated that most of metacaspase genes (*SotubMCs*) and its orthologs might have evolved after the speciation events of the Solanaceae plants [70]. Taken together, molecular evidences have indicated that the PCD machinery has evolved since very early steps of evolution, and that this evolution has been processed through expansion and innovation of protein recruitment domains, as well as through the derivation of effector domains and horizontal gene transference events.

Likewise, caspase activity has been measured in plant extracts using caspase substrates from animal cells [75], and caspase-specific inhibitors (e.g. Ac-DEVD-CHO) have been shown to prevent some forms of plant PCD [76]. On the other hand, caspase-like activities have been detected during plant development [76-78] and also induced during the HR, a process that can be blocked by caspase inhibitors [75, 76, 79].

Although metacaspases have no specific caspase activity, increasing evidence points to their role in the regulation of PCD [51]. In others words, although metacaspases may not be directly involved in PCD, they might be indirectly involved in the signaling cascades that lead to PCD [14]. Other proteases with caspase-like activities may also be involved in PCD [73]. A number of studies have indicated that the expression of the metacaspase genes can be regulated by developmental cues and induced by a wide range of abiotic and biotic stresses [50, 51].

The identification and characterization of substrates is a key feature to understanding function of any protease. What is the current situation with respect to metacaspases targets? Although metacaspases seem to be essential for PCD in plants, data on its natural substrates remain even scarce. The first metacaspase biological target was identified by the Bozhkov's group. Thus, metacaspase of Norway spruce (*Picea abies*) McII-Pa was found to cleave, *in vivo* and *in vitro*, the phylogenetically conserved protein TSN (Tudor Staphylococcal Nuclease). This study was carried out during both developmental and stress-induced PCD and also revealed functional conservation between metacaspases and caspases as TSN was shown to be processed also by the human caspase-3 [80]. Other functional conservated targets include the Poly (ADP-ribose) polymerase and glyceraldehyde 3-P-dehydrogenase (GAPDH), a specific substrate of yeast metacaspase [79, 80]. In addition, the nucleocytoplasmic Arabidopsis type-II metacaspase-9 (AtMC9) was found to cleave the gluconeogenesis enzyme phosphoenolpyruvate carboxykinase-1 (PEPCK1). In this case, the *in vivo* cleavage of PEPCK1 enhances its activity, leading to stimulation of the glucose *de novo* synthesis pathway during PCD [81]. Interestingly, proteins involved in seed development, such as several LEA proteins, are frequently found among the MC9-cleaved proteins (i.e. constituting part of the MC9 degradome) [81]. Recently, other natural substrates for MC9 have also been identified [82, 83 and ref. therein]. On the other hand, regarding the control of metacaspases at the posttranslational level, the proteolytic activity of AtMC9 is regulated by S-nitrosylation and autocatalytically activated by proteolytic separation of its p20 and p10 subunits [82, 84]. Likewise, it has been proposed that S-nitrosylation at the level of the catalytic Cys (Cys-147) can be used as a regulatory strategy under basal NO levels. The S-nitrosylation on AtMC9 on its mature processed form does not affect its activity. This happens because other cysteine (Cys-29) residue can act as alternative nucleophile. Despite this, the enzyme can be kept inactive through S-nitrosylation, and otherwise, become active only under conditions of disturbance in cellular redox balance. Interestingly, type-II metacaspase AtMC9 is involved in xylem differentiation [85].

b) Mechanism of action

Recent studies have demonstrated that metacaspases are involved in regulating the dPCD and eDCP in plants. However, the mechanism by which metacaspases affect plant PCD still has many gaps. The involvement of metacaspases in PCD is supported, among others approaches, by works with plants under pathogen attack. That is, pathogen attack can induce PCD through HR [15], and metacaspases seem to be involved as a consequence of cellular ROS increase and through proteolytic activation of other metacaspases and degradative enzymes, among other causes [86-89]. Several genes involved in HR have been identified in different species [51 and ref. therein]. During avirulent pathogen infection, plant resistance (R) proteins, which are characterized by a conserved leucine-rich repeat (LRR) domain, recognize pathogen avirulence (Avr) factors and initiate the HR and a localized PCD at the site of infection [15, 16, 90, 91]. In pepper (*Capsicum annuum*), CaMC9 was suggested to act as a positive regulator of cell death upon infection by *Xanthomonas campestris* [89, 92, 93]. The overexpression of *CaHIR1* in Arabidopsis leads to elevated defense responses during infection with virulent bacterial pathogens (i.e. *Pseudomonas syringae* pv. tomato and the oomycete *Hyalopenospora arabidopsidis*); while the *CaHIR1* silencing disrupts hypersensitive and susceptible PCD in pepper plants [93, 94]. Likewise, studies suggest that host-controlled PCD is also closely associated with the onset of susceptible cellular death and disease development in plants [92 and ref. therein]. The enhanced expression of type II metacaspase suggest a crucial role of these

Cys-proteases in maize leaf response to O_3 and age-mediated senescence [15 and ref. therein]. Likewise, the increasing expression level of a type II metacaspase on tomato leaves under *Botrytis cynerea* infection, and the detection of cell death phenotype, confirms the involvement of metacaspases on PCD [86].

First evidence for the involvement of metacaspase in plant development comes from Norway spruce study. Thus, downregulation of Ca^{2+}-dependent McII-Pa suppressesed vacuolar PCD in suspensor cells from an embryonic culture from *Picea abies*. This type of PCD also requires autophagy, which occurs downstream to McII-Pa [68, 95]. That is, autophagy is essential for vacuolation of cells undergoing dPCD and is activated by McII-Pa [96]. More information on McIIPa indicates that the McII-Pa protein is transported during the above dPCD from the cytosol to the nucleus, where its presence is correlated to DNA fragmentation. These data reinforce the notion that this metacaspase is directly involved in a pathway leading to nuclear protein degradation, a process that takes place during most types of eukaryotic PCD. Accordingly, McII-Pa has been shown to play a role in the cleavage of nuclear proteins [96, 97]. Likewise, evidences about the Arabidopsis metacaspases indicate that: (i) AtMC1 and AtMC2 regulate PCD antagonistically. That is, AtMC1 acts as a positive regulator requiring conserved caspase-like putative catalytic residues for its function; whereas AtMC2, a negative regulator of PCD, acts independent of the putative catalytic residues [69]; Ser-protease inhibitor AtSerpin1 constitutes a negative regulator of AtMC1 self-processing [79, 84 and ref. therein]; (ii) AtMC4 acts as a positive mediator of PCD under biotic and abiotic stress [98]; (iii) AtMC8 positively regulates PCD induced by oxidative stress, UV and H_2O_2 [99]; and (iv) AtMC9 facilitates the post-mortem clearance of cell contents after vacuole rupture in xylem vessel elements [100]. On the other hand, it has been demonstrated in *Nicotiana tabacum* that reactive carbonyl species, which are downstream products of ROS, can directly activate caspase-1-like and caspase-3-like proteases to initiate PCD in plant cells [101]. Finally, knowledge on the cellular localization of plant metacaspases is critical for understanding their modes of action. For example, AtMC9 is known to be localized to the apoplast, nucleus and cytoplasm; and this localization changes during late autolysis [102]. The Bozhkov's group points out that the execution of plant PCD is controlled by two groups of Cys-proteases with separated cellular localization. One of them is accumulated on lytic compartments and vacuoles, and the other has cytoplasmic-nuclear localization, as in the case of MCII-Pa [97]. Future work is required to unravel subcellular localization for other metacaspases in order to define their specific roles during PCD, their substrate specificities and the modes through which they operate and interact with one another.

Autophagy and its relationship with plant PCD

The autophagy (from Greek: 'self-eating'), is an evolutionarily conserved, highly selective and multi-step process that is crucial to various aspects of plant life, such as germination, seedling establishment, reproduction, senescence, the stress response and disease resistance [7, 8 and ref. therein]. This process is considered to be the major cell recycling system in eukaryotes and contributes significantly to plant metabolic homeostasis. The hallmark of autophagy is the engulfment of cargo by a double-layered membrane called phagophore to form the autophagosome (Figure 2). The cargo comprises the cytoplasmically targeted constituents, including protein aggregates and damaged or superfluous organelles (e.g. mitochondria, chloroplasts and peroxisomes) as well as infectious organisms [7, 8]. Once formed, the autophagosome is mobilized toward an endolithic compartment (i.e. plant vacuole or mammalian lysosome) and becomes enriched in hydrolases before cargo degradation and recycling [8, 103 and ref. therein]. Although this complex set of membrane trafficking and fusion events is an integral aspect of the autophagy process [104, 105], very limited data exist about degradation process within lytic compartment and how the cargo is recycled and mobilized. Likewise, how autophagy recognizes its cargos in the plant cell is also unknown. Much research remains to be done in this area. Autophagy and the ubiquitin-proteasome system (UPS) are two major protein degradation pathways implicated in the response

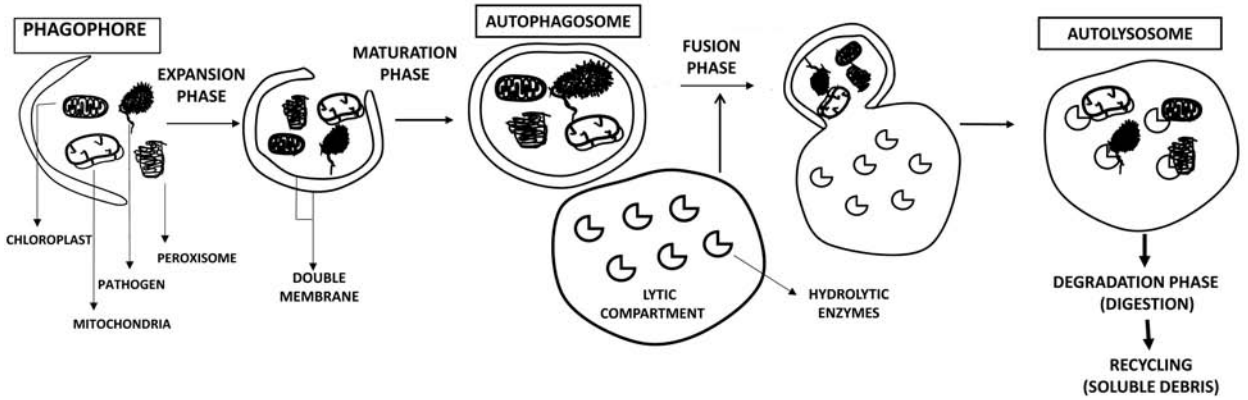

Figure 2. Autophagy is a membrane-trafficking pathway involved in the degradation and transport of cellular material. There are several essential stages in the selective autophagy process. During the nucleation phase, the phagophore assembly site (PAS) is involved and an isolation membrane known as the phagophore engulfs proteins or damaged cellular organelles. The isolation membrane then extends, and the phagocytic membrane elongates to form a bilayer vesicle, named the autophagosome. Next, the autophagosomes fuse with lytic vacuoles to form autolysosomes, which degrade vesicular contents. After degradation, heterogeneously degraded components are released into the cytoplasm and recycled.

to microbial infections in eukaryotes. Nevertheless, it is now considered that while the UPS is responsible for the turnover of short-lived damage proteins, the autophagy process is more specifically dedicated to degradation of long-lived or aggregated proteins [7, 106 and ref. therein]. Regarding the proteasome, it is worth pointing out that the UPS protease complex may be degraded *via* an autophagy process known as proteaphagy [107]. Thus, proteaphagy in Arabidopsis can develop *via* non-selective and selective routes. Unlike non-selective autophagy, which is induced by starvation [108], selective proteaphagy is induced by inhibition of the proteasome and requires the ubiquitination of inactive proteasome [109].

The genes participating in the autophagy process, the *ATG* genes, were originally discovered in *Saccharomyces cerevisiae* (yeast) [110 and ref. therein]. More than 30 *ATG* genes acting in concert are required for autophagy [111 and ref. therein]. However, how transcription of these genes is regulated is not known in detail. Together, autophagosome initiation and completion are mediated by autophagy-related and conserved ATG proteins and require two ubiquitin-like conjugation systems to produce ATG12-ATG5 and ATG8-phosphatidylethanolamine (ATG8-PE) conjugates (Figure 3). ATG8-PE conjugation involves the Cys-proteinase ATG4 and E1-like protein ATG7, through which ATG8 is lipidated and translocated *via* autophagosomes to the vacuole [7, 104]. Thus, membrane-anchored ATG8 acts as an important docking site for selective autophagy receptors that deliver a multitude of substrates to the growing autophagosome, including single or aggregated proteins, entire organelles and invading microbes. However, the exact origin of the membranes of the autophagosome is not known. Recent work has begun to shed light on this knowledge gap: ATG9 is known to play a critical role in the origin of autophagosomes from the ER [112]. In contrast to most other ATG proteins, ATG9 is the only transmembrane protein and moves rapidly as numerous distinct compartments throughout the cytoplasm. Identifying and characterizing ATG9-interacting proteins would certainly facilitate the understanding of how ATG9 is involved in this process. Recently, it was observed that: (i) ATG5 exerts its effect on ATG8 lipidation by directly controlling the rate of ATG12–ATG5 conjugation; and (ii) overexpression of *ATG5* or *ATG7* facilitates ATG8 lipidation, leading to autophagosome formation and autophagy flux [113]. Many insights have been obtained through functional analyses of autophagy-deficient Arabidopsis mutants impaired in the expression of different *ATG* genes. It is noteworthy that *ATG6* represents the only *ATG* gene in plants whose loss of function results in

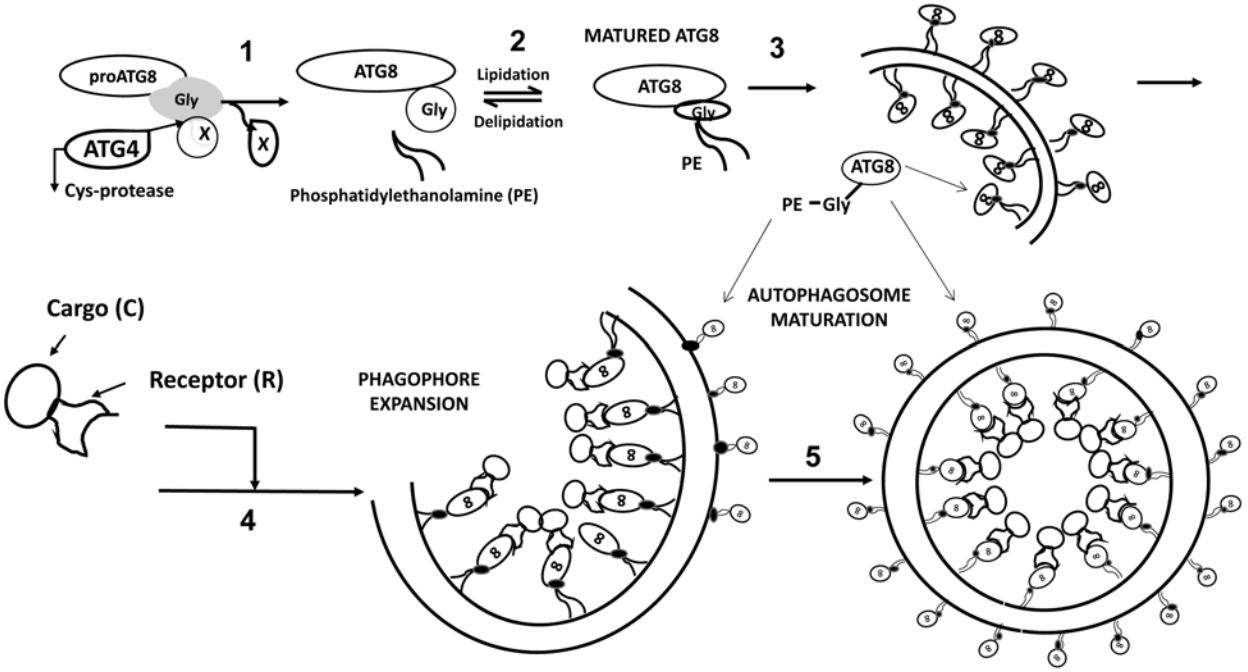

Figure 3. Selective autophagy requires autophagy-related protein 8 (ATG8) and cargo (C) receptors: (1 and 2) ATG8 conjugated to PE (ATG8 lipidation) during autophagy plays an important role not only in autophagosome biogenesis but also in cargo (C) recruitment. ATG8 lipidation requires processing of the C-terminal conserved glycine (Gly) residue in ATG8 by the ATG4 (Cys-protease-mediated processing of ubiquitin-like ATG8 proteins); (3) ATG8-GlyPE complex (mature ATG8) is ready to bind to the phagophore membrane; (4) once bound, the mature ATG8-R-C complex is formed, followed by phagophore expansion and (5) phagosome maturation.

embryogenic lethality. On the other hand, unlike the above proteins, the lipids of autophagic membranes remain far less well defined [114]. Recently, a number of questions have been raised concerning lipids: where do autophagosomal lipids originate? How are lipids transported to the autophagy pathway and how this supply is regulated [115 and ref. therein]? Future work should clarify how cytoskeleton activities are coordinated for autophagosome formation.

The relationship between autophagy and PCD also remains unclear, although a number of links have been made. Some evidence indicates the involvement of selective autophagy in PCD [96, 116, 117 and ref. therein]. Thus, a crucial role for autophagy has been identified in cell death of suspensor cells during normal embryogenesis in Norway spruce. Also, a strong link between autophagy and metacaspase activity has been identified during tracheary differentiation in Arabidopsis [85, 118]. Likewise, it has been shown that autophagy contributes to the modulation of immunity and HR [119, 120]. Other examples include a recent study in barley, which indicated that autophagy plays a role in cell death at early stages of stress-induced microspore embryogenesis [33], during which glyceraldeyde-3-phosphate dehydrogenase (GAPDH) seems to be involved as a direct regulator of autophagy. Also, GAPDH in *Nicotiana benthamiana* has been shown to directly interact with the ATG3 protein to downregulate autophagy. Interestingly, when NbGAPDH1 and NbGAPDH2 are silenced, autophagy is induced, whereas their overexpression inhibits autophagy [7, 121]. Overall, these findings reveal that autophagy promotes or limits different forms of PCD [122 and refs. therein]. However, the puzzle of how PCD and autophagy are linked (i.e. the underlying mechanisms) in plants is far from solved. The generation of mutants that specifically suppress PCD or autophagy will contribute significantly to advancing our understanding of this cell degradation and recycling system. Future research is needed to

identify how autophagy is regulated by proteasome ubiquitinization. Likewise, the role and underlying mechanisms of *ATG* genes and proteins, and the targeting of the various cell organelles in autophagy degradation constitute some of the most important goals in this field.

FUTURE PERSPECTIVES

Although our understanding of the biochemical and molecular aspects of plant dPCD has increased over the last decade, there is still no experimentally validated mechanism of action that adequately integrates the various players and processes involved in PCD, such as proteases and nucleases, autophagy, cellular redox—let alone one that accounts for differences between specific tissues and phases of the life cycle. Given their importance, knowledge of the origin and evolution of key genes involved in the dPCD still needs to be defined. Related to this task is study of the underlying genetic features that have been evolutionarily conserved. Added to this are other key questions such as: How do different proteases and nucleases work together to orchestrate dPCD and does functional redundancy exist? How are the relevant Cys-EPs activated? Also, how do the PCD mechanisms vary between model organisms?

Other questions remain regarding autophagy. Thus, future work should clarify how cytoskeleton activities are coordinated for autophagosome formation. On the other hand, although a number of essential genes that function in autophagy (i.e. *ATG* genes) have been identified in plants, the upstream cellular pathways that regulate the process still remain little known. Interestingly, Arabidopsis ATG1 and ATG13 form a complex that regulates autophagy, suggesting a conserved function for ATG1/13 in plants [121 and refs. therein]. The recent isolation of the target of rapamycin (i.e. TOR; an evolutionarily conserved Ser/Thr kinase) as a negative regulator of autophagy in Arabidopsis opens new avenues for the study of autophagy [122, 123 and refs. therein]. Identifying conserved components in the TOR cascade will help to shed light on how TOR regulates the plant cell growth. Improved experimental design and tools have enabled comprehensive analyses that have demonstrated pivotal roles of TOR in plant development. Thus, detailed comparative analysis of Arabidopsis ATG mutants that are sensitive to rapamycin or with reduced levels of TOR represents powerful new tools to aid in this endeavor [121 and refs. therein]. Recently, it was demonstrated that TOR regulates autophagy induced by nutrient starvation, salt or osmotic stress, but not oxidative or ER stress, indicating that TOR-dependent and -independent pathways for regulation of autophagy exist in plants [124]. Likewise, it is worth noting that auxin regulates plant stress responses through the TOR signaling pathway [124]. Future work is required to identify the upstream stress sensors that repress TOR activity to allow activation of autophagy and the components of the TOR-independent autophagy activation pathway. For an overview of the future prospects of research on autophagy it is interesting to see the recent opinion of Dr. Bozhkov (2018) [125].

In summary, the extensive research that has been carried out over the last decade in plant PCD/ dPCD lays a strong scientific foundation — one that will, along with emerging techniques, enable a better understanding of this essential process. A challenge over the next decade will be to define the molecular modes of function of putative dPCD regulators and the posttranslational modifications that lead to the rapid execution of cell death, which have been observed in various plant model systems. Thus, emerging morphological, molecular genetics and cell biological analyses, will lead the way to a more comprehensive understanding of PCD as a fundamental cellular process in plants.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest.

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