Türk Bilimsel Derlemeler Dergisi 1 (1): 65-78, 2008
ISSN:1308-0040, www.nobel.gen.tr

Proteolytic Enzymes in Plant Programmed Cell Death

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Abstract

Programmed cell death (PCD) is required for the development and morphogenesis of almost all multicellular eukaryotic organisms. In cell death mechanisms proteolytic enzymes have very diverse roles. The recent findings point to the existence of different plant caspase-like proteolytic activities involved in cell death. Cysteine proteases, specifically caspases, have emerged as key enzymes in the regulation of animal PCD. Although plants do not have true caspase homologues, several instances of caspase-like proteolytic activity with aspartate-specific cleavage have been demonstrated in connection with PCD in plants. Because of the caspase-like activity in plants, the researcher's main goal is to determine which molecular components may be used in the execution of PCD in plants that have been conserved during evolution. In the present review, examples of serine, cysteine, aspartic, metallo- and threonine proteinases are explained which provide background information about their roles as regulators of animal PCD, and linked to plant PCD in developing flowers, senescing organs, differentiating tracheary elements and in response to stress.

Key Words: Apoptosis, proteases, caspase, caspase-like activity.

INTRODUCTION

Programmed cell death (PCD) is a functional process which plays an important role in development and morphogenesis in the multicellular organisms to control cell number, and as a defensive strategy to remove mutated, infected or damaged cells. This process is essential to ensure that it is only activated in the required cells at the proper moment and involved in many aspects of development as well as in responses to external stimuli. Because of the ubiquitous occurrence of PCD throughout nature and the coincident morphological and functional features in eukaryotes, it is conceivable that PCD evolved from a common ancestral cell death process and thus plants, fungi, and animals may share common regulatory mechanisms [1]. Building on the ancestral form of PCD, plants are expected to have evolved their own pathways to cope with plant specific features such as the presence of cell walls that prevent dead cells from being phagocytosed by neighboring cells. However, at the molecular level, very few regulatory proteins or protein domains have been identified as conserved across all eukaryotic PCD forms [2].

In animal systems PCD, more commonly apoptosis, is reported to result in the disassembly of cells involving condensation and fragmentation of the nucleus, internucleosomal cleavage of DNA, cell shrinkage, blebbing of the plasma membrane and fragmentation of the cell into cellular debris-containing vesicles called "apoptotic bodies" that are being phagocytosed by the neighboring cells or the macrophages [3]. Thus, there are no remnants of cell corpses left. Apoptosis is mediated by a class of cysteine proteases specific for the target sites containing aspartate residues called cysteinyl aspartate-specific proteinases (caspases) [1] and they function as molecular switches to activate the cell death program [4]. Caspases are synthesized in the cell as inactive precursors or procaspases. Once activated caspases, cleave and activate other procaspases. Some of the activated caspases start to cleave other key proteins in the cell. This irreversible activation triggers an amplifying proteolytic cascade that turns on enzymes involved in cell death [1].

There are numerous examples of cell death during plant development that conform to the general definition of PCD such as cell death during xylogenesis, aerenchyma formation, plant reproductive processes, leaf and petal senescence and endosperm development. Furthermore, cell death in response to pathogen attack and a variety of abiotic factors such as ozone and UV radiation also fall within the definition of PCD [5]. The most convincing evidence for the origin of PCD is that some basic morphological and biochemical features are conserved in animals and plants such as compaction and shrinkage of the cytoplasm and nucleus, DNA and nuclear fragmentation and formation of apoptotic-like bodies which are small membrane sealed packets

containing DNA [6]. Unlike animal cells, dying plant cells indicate the occurrence of cytoplasmic condensation and shrinkage but not its breakage into small pieces; there are evidences of vacuolar autophagy in most cases which accounts for the elimination of the cytoplasm [5]. Another difference between animal and plant cell death, is the presence of cell walls acting as physical barriers preventing the recycling of cellular material from dead cells via apoptotic bodies [8].

Although no functional homologs of animal caspases have been identified in plants, a vast amount of indirect evidences, suggesting the existence in plants of true caspase-like activity and its functional involvement in plant cell death, have accumulated [1]. Caspase-like activities have been detected in plants during the hypersensitive response (HR) [9] or after a heat shock of suspension cells [10]. In support of these caspase-like activities in plant PCD, experiments in tobacco protoplasts showed that during menadione-induced PCD, caspase inhibitors could block the induction of DNA fragmentation and of Poly (ADP-ribose) polymerase (PARP) cleavage [11]. Caspase inhibitors (Ac-DEVD-CHO or Ac-YVAD-CHO) have also been shown to block PCD after pathogen induction [9]. Expression of p35, a caspase inhibitor, has been reported to reduce the initiation of apoptosis in embryonic maize callus [12]. However, despite the completion of the *Arabidopsis* genome sequence, only a few plant genes have been identified as orthologues of mammalian genes involved in apoptosis. Although the absence of evident homologs of caspases in plants, increased proteolytic caspase-like activity in dying plant cells has proposed that there are special plant proteases which are homologous and functionally equivalent to animal caspases [13].

The aim of this review is to explain the roles of proteolytic enzymes in plant PCD providing background information about their roles as regulators of animal PCD.

PROTEASES AND PLANT CELL DEATH

Proteolysis provides a controlled gene expression and plays a fundamental role in development, homeostasis, physiology and survival at the organismal level. There are several terms commonly used to describe specific proteolytic enzymes functioning in multiple regulatory pathways found in all organisms [14]. Peptidases or proteases (peptide hydrolases) comprise two groups of enzymes: the endopeptidases which act on the interior of peptide chains and the exopeptidases which cleave peptide bonds on termini of peptide chains. Exopeptidases have been differentiated according to their substrate specificity as aminopeptidases, acting at a free N-terminus, and carboxypeptidases, which degrade peptides at the C-terminus [15].

The most thoroughly characterized cell death proteases are endopeptidases. Although the exact nature of the involvement of cell death proteases are still not definite, it has been reported that cysteine proteases with specificity for aspartate residues (the caspases) and other proteolytic systems (the 26S proteasome, granzyme B, calpain, cathepsin D and matrix metalloproteinases) are important to PCD processes [16].

Proteolysis in plants is a complex process involving many enzymes and multifarious proteolytic pathways in various cellular compartments and assumed to function in the random autolysis of intracellular proteins rather than being regulatory in an ordered breakdown process [17]. Different types of proteolytic enzymes are known to be associated with developmental and pathogen- and stress-induced PCD in plants. Many supporting reports have shown that extracts from plants undergoing cell death contain activities that are capable of cleaving a variety of synthetic caspase substrates, such as the human caspase-1 substrate YVAD-AMC and human caspase-3 substrate DEVD-AMC. In addition, natural caspase substrates such as bovine and plant PARP are cleaved by plant proteases at caspase cleavage sites. Similarly, it has been shown that

caspase inhibitors such as YVAD-CHO and DEVD-CHO markedly suppress plant cell death and associate morphological and biochemical features of apoptosis in several systems [2]. Plant cell death can also be blocked by heterologous expression of the baculovirus macromolecular caspase inhibitors IAP, Op-IAP and p35 [18]. These observations strongly suggest that cell death associated, caspase-like proteases exist in plants. According to Rawlings and Barret [19] endopeptidases are divided into the following sub-subclasses based on the kind of active site residue (cysteine-, serine-, aspartic-, threonine- and metallotypes), and not on the type of their substrate: serine-endopeptidases (EC 3.4.21), cysteine-endopeptidases (EC 3.4.22), aspartic-endopeptidases (EC 3.4.23), metallo-endopeptidases (EC 3.4.24) and threonine-endopeptidases (EC 3.4.25).

Serine endopeptidases (EC 3.4.21)

Serine endopeptidases (Ser proteases) have a widespread occurrence. They are distinguished by the characteristic arrangement of the catalytic histidine, aspartate, and serine residues that conform the catalytic triad. These enzymes are divided into two major groups; subtilisin-like serine proteases (subtilases) and (chymo)trypsin-like serine proteases. Subtilisin-like serine proteases represent an ancient protein family with homologs in such diverse organisms as Archae, bacteria, fungi, yeast, and higher eukaryotes including plants and are associated with developmental process, defence response and PCD. Based on the difference in the amino acid sequences, subtilisin-like serine proteases are further classified into six families: subtilisin, thermitase, kexin, pyrolysin, proteinase K, and lantibiotic peptidases [20].

Evidences from animal models supporting a role for Ser proteases during PCD are limited to granzyme B, an S1 family, trypsin-type enzymes [21]. Groover and Jones [22] detected the existence of a secreted 40 kDa peptidase which has a trypsin-like acvtivity in *Zinnia* mesophyll cell culture. While the peptidase inhibited by soybean trypsin inhibitor, PCD of tracheary elements was prevented also.

Plant subtilisin-like serine endopeptidases have been the focus of several investigations recently. The lily subtilisin, LIM9, accumulates in anther tapetum which undergoes degeneration leading to dehiscence [23]. The expression of subtilisin LeSBT1 in roots and flowers of tomato may indicate a role for this plant subtilisin during PCD in these organs [24].

Isolates of the necrotrophic fungus *Cochliobolus victoriae* that produce the toxin victorin activate a PCD response in susceptible *Avena sativa* cultivars, leading to disease progression [25]. Coffen and Wolpert [26] reported that, victorin-induced PCD is associated with caspase-like activities that can be differentiated by their sensitivity to caspase inhibitors and general protease inhibitors (e.g. leupeptin and E64). Using the synthetic caspase substrates DEVD-AFC and zVAD-AFC, protein fractions specifically cleaving these substrates in extracts from victorin-treated oat leaves were isolated. The fractions with DEVDase activity did not show activity towards zVAD-AFC; the fractions with zVADase activity showed no activity towards DEVD-AFC. Both caspase-like activities were not affected by general protease inhibitors (leupeptin and E64). This caspase-like protease was purified and partial sequence of the coeluting protein showed that there are two proteases which have nearly identical sequences homologous to subtilisin-like Ser proteases. Characterization of the purified proteases showed that their substrate specificities are strict for aspartate at the P_1 position and thus they are distinct from all other known Ser proteases. Because of their aspartate specificity (aspase) and active-site Ser residue, they have been termed saspases [1]. The saspases function in a PCD-induced signaling cascade involving other proteases that leads to the proteolytic processing of photosynthetic enzyme Rubisco. They are also constitutively present in the cell, not being transcriptionally or translationally activated during the response, but released into the extracellular fluid upon induction of PCD. Furthermore heat shock–induced PCD displays similar biochemical features as victorin-induced PCD, including

DNA laddering, Rubisco proteolysis, and release of the saspases into the extracellular fluid [26].

Cysteine endopeptidases (EC 3.4.22)

Cysteine endopeptidases, also referred to as thiol proteases, are involved in protein maturation, degradation, and protein rebuilt in response to different external stimuli. They also play a house-keeping role to remove abnormal, misfolded proteins. Furthermore, cysteine proteases take a major role in PCD, more commonly apoptosis [17]. Cysteine proteases, being labeled with the prefix C, comprise more than 40 families of peptidases grouped into at least six superfamilies or clans. Recently, it has been suggested that legumains (C13), caspases (metacaspases - C14) and papains (C1) have a direct relation with plant PCD [27].

Legumains (Vacuolar processing enzymes-VPEs).

Legumains are newly discovered group of cysteine proteinases (C13) isolated from different plant organs [17]. They have been extensively studied for their role in the maturation of proteins in seed storage vacuoles, where they account for the mass of processing activity [28]. Although plant legumains are usually called vacuolar processing enzymes (VPEs), they are also present in the cell wall. Their function is not restricted to precursor protein; they also include protein breakdown in the vacuole or cell wall [29]. Recently, it was reported that members of this family are expressed in vegetative tissues where they are localized in protease precursor vesicles (PPVs), vacuoles [30] and organelles, closely associated with PCD in plants.

Comparison of sequences and gene expression showed that *Arabidopsis* legumains can be divided in two subfamilies: those specific for seeds ($_{\beta}$VPE) and others (γVPE and αVPE) specific for vegetative organs. This division is consistent with the classification of plant vacuoles into protein-storage and lytic vacuoles. An immunocytochemical analysis confirmed the specific localization of $_{\beta}$VPE in the protein storage vacuoles and γVPE in the lytic vacuoles [31].

These enzymes belong to the asparaginyl-specific subclass of the cysteine endopeptidase family which cleave peptide bonds with asparagine or aspartate (less efficiently) in the P_1 positions at the C-terminal side [32] and show significant structural homology to animal caspases. Modeling of the three-dimensional structure of *Arabidopsis thaliana* γVPE predicts a close alignment of its catalytic residues with caspase-8 which is the key initiator caspase in the death-receptor pathway. Furthermore, Rojo et al., [33] has showed γVPE regulate the protein degradation during senescence, a type of PCD. These data suggest that VPEs may encode caspase-like activities associated with PCD in plants. Two recent reports have provided direct evidences that VPEs have caspase-like activity and regulate cell death in *Nicotiana* and *Arabidopsis* [34]. Tobacco (*Nicotiana tabacum*) plants carrying the N-resistance gene activate an acute PCD response when infected by tobacco mosaic virus (TMV), which is blocked by treatment with VPE-inhibitors or caspase1-inhibitors. When VPEs are silenced in *Nicotiana benthamiana*, the induction of caspase activity in response to TMV is suppressed, vacuolar collapse and PCD are blocked, and TMV proliferation increase [35]. These results suggest that the VPEs from *Nicotiana* may display caspase-like activity and initiate PCD during TMV infection by promoting vacuolar rupture a common process to most cases of PCD in plants and possibly constitutes an irreversible step in cell death. Although the localization of *Nicotiana* VPEs has not been reported, they are likely localized in vacuoles, and activate PCD from the vacuolar lumen. In addition, VPEs may act after disruption of the tonoplast by processing cytosolic enzymes involved in PCD execution. This function would be similar to the role of animal cathepsins, which activate caspase cascades in the cytosol, triggering apoptosis [36]. The temperature-sensitive N-TMV tobacco plant–pathogen system allows synchronized cell death. At 30^{o}C, TMV can systemically infect N tobacco plants because

induction of cell death and defence gene expression is completely suppressed. When the temperature decreased to 23°C, cell death appears throughout the infected plant. A study of the *Arabidopsis* γVPE gene has provided direct evidence of its caspase-like activity and genetic evidence for the involvement of γVPE in disease resistance and cell death [34]. An increase in caspase activity, simultaneous with a rapid PCD initiation response, is observed in *Arabidopsis* plants infected with an incompatible strain of *Pseudomonas syringae* pv tomato DC3000 (Pst). The early induction of this caspase activity is compromised in γVPE mutants and they are more susceptible to infection with the incompatible strain of Pst. The γVPE mutants are also susceptible to infection with turnip mosaic virus (TuMV), a pathogen that does not induce a classical PCD but rather affects the viability of infected cells. Thus, VPEs negatively regulate the growth of several biotrophic pathogens (TMV, Pst, TuMV), most likely by promoting cell death. These researches suggest that VPEs are caspase orthologs that activate PCD in plants and influence the outcome of a wide range of interactions with pathogens [37].

Caspases (Metacaspases)

Caspases, belonging to the C14 class of specific cysteine proteinases show a high specificity with an absolute requirement for an aspartate residue adjacent to the cleavage site and a recognition sequence of at least four amino acids N-terminal to the cleavage site. They are essential in cells for apoptosis, a main type of PCD, in development and most other stages of adult life and have been termed "executioner" proteins for their roles in the cell. Eleven human caspases have been identified so far. They can be classified as the initiator caspases (caspases 2, 8, 9 and 10), effector caspases (caspases 3, 6 and 7) and the other caspases (caspases 1, 4, 5 and 11). Recently, it has been reported that there are two identified genes encoding two ancestral families of caspase-like proteins: paracaspases which found in metazoans (e.g. human, *Caenorhabditis elegans*) and *Dictyostelium*, metacaspases which found in plants, fungi and protozoa [38].

Caspase-like proteolytic activity was recently shown *in vivo* for the yeast metacaspase YCA1. Overexpression of YCA1 enhanced H_2O_2-induced cell death, and its disruption blocked H_2O_2-induced cell death. These results suggest that YCA1 encodes a caspase-like proteinase that activates PCD in yeast [39]. In addition, heterologous expression of a metacaspase from *Trypanosoma brucei* in yeast causes growth inhibition, mitochondrial dysfunction and clonal death [40].

Plant genomes contain an extensive complement of metacaspases, which have been classified as type I and type II based on their sequence and structural features. Type I metacaspases are predicted to be localized in mitochondria and chloroplasts. Type II metacaspases do not contain signal peptides or transmembrane domains, so they are predicted to be cytosolic and thus would function in the same location as animal caspases [1]. *Arabidopsis thaliana* genome contains 9 metacaspases: 3 type I (AtMCP1a–1c) and 6 type II (AtMCP2a–2f) metacaspases [41]. *Arabidopsis* metacaspases show that AtMCP2d is the most abundantly expressed metacaspase, and AtMCP1b expression is induced during compatible and incompatible interactions with *Pseudomonas syringae* pv tomato DC3000 (pst). AtMCP1c is also induced in response to infection with compatible and incompatible strains of Pst at a higher-fold level than AtMCP1b. Interestingly, both AtMCP1b and AtMCP1c are induced when infected by a mutant of Pst during a nonhost interaction with *P. syringae* pv phaseolicola, indicating that their expression is responsive to pathogen-associated molecular patterns (PAMPs) present in the bacterial surface rather than to virulent factors injected into the plant cell. Consistent with this, treatment with the flagellin peptide Flg22, a well-characterized bacterial PAMP [42], induces the expression of AtMCP1b and AtMCP1c. AtMCP2e, for which there was no previous evidence of expression [43], is also induced by *Pseudomonas* infection or

by treatment with Flg22. Moreover, infection with *Phytophthora infestans* or treatment with NPP1, a peptide PAMP from *Phytophthora* [44], induces the expression of AtMCP1b, AtMCP1c, and AtMCP2e. Thus, these three genes are coordinately induced by PAMPs from different pathogens and may be part of the innate immune response of plants, which in some cases includes the activation of cell death processes. AtMCP2f is induced in senescing organs of the flower and in senescing cell cultures, indicating that it may play a role in this type of PCD. Interestingly, AtMCP2a, AtMCP2c, and AtMCP2d, are expressed at higher levels in roots than in aerial organs, suggesting that they may play a specialized role in these organs. Moreover, AtMCP2a is induced in roots under salt, drought, and genotoxic stress conditions that activate cell death [37]. Similarly, it has been reported that a tomato type II metacaspase (LeMCA1), which is most similar to the *Arabidopsis* AtMCP2a to 2d gene cluster, is induced during infection with *Botrytis cinerea* [45], suggesting that these genes may play a role in the PCD induced by this pathogen.

Bozhkov et al. [46] reported that activation of proteases with the preferential cleavage of VEID sequence-containing caspase substrate (VEIDase activity) is essential for PCD and embryogenesis in the gymnosperm Norway spruce (*Picea abies*). VEID amino-acid sequence corresponds to the site of lamin A cleaved by mammalian caspase-6 during apoptosis [47]. Apart from similar substrate specificity, both spruce VEIDase and caspase-6 can be inhibited by Ac-VEID-CHO, a caspase inhibitor, and exibit high sensitivity to pH changes, ionic strength and Zn^{2+} concentration [48]. Significantly, *in vitro* activation of spruce VEIDase is crucial in autophagic cell death during plant embryo pattern formation, at a pH substantially lower than the vacuolar pH of plant cells may be indicative of the involvement of this proteolytic activity in the degradation of cytoplasm inside Golgi- and plastid-derived acidic vesicles, the earliest event of the execution phase of autophagic cell death in embryo-suspensor [49]. The substrate specificity of the Norway spruce VEIDase appears to be similar to that of the yeast metacaspase YCA1 [39] suggesting that the plant VEIDase involved in cell death is a metacaspase.

Bozhkov et al. [46] reported that there is another caspase-like activity (*mcII-Pa*) in *Picea abies.* Silencing of *P. abies* metacaspase gene *mcII-Pa* inhibited VEIDase activity, suppressed PCD in the embryos, and blocked suspensor differentiation [13]. *mcII-Pa* is not VEIDase, because active *mcII-Pa* does not retain aspartate-specific proteolytic activity typical for animal caspases but prefers substrates containing arginine as the C-terminal amino acid. Moreover, recombinant *mcII-Pa* does not cleave caspase substrates, including the VEID sequence-containing substrate, indicating that VEIDase activity is caused by different protease(s). The proteolytic activity of *mcII-Pa* is important for the terminal differentiation and PCD of the suspensor. Immunolocalization analyses and functional assays show that *mcII-Pa* accumulates in the nuclei of the suspensor cells and is directly involved in the execution of nuclear degradation, which is a key event of most of the eukaryotic cell-death programs [50].

Despite the differences in primary structure and substrate specificity of plant metacaspases and human caspases, they serve common cellular functions as executioners of PCD, demonstrating evolutionary parallelism of the cell-death pathways in plants and animals.

Papain-type endopeptidases

Plant papain-type endopeptidases (C1 family) are the largest and most widely represented plant endopeptidase family. For instance; *Arabidopsis thaliana* genome encodes 32 papain-type cysteine proteases which can be classified into eight main groups (senescence- and stress-induced, aleurain, cathepsinB-like, bromelain-like, KDEL, telo sequences, actinidain-like) based on the sequence similarity to other cysteine proteases [51]. Papain-type enzymes can be divided into two subfamilies: the first similar to animal cathepsin H and L,

and the second similar to cathepsin B. The prodomains of these two subfamilies show no sequence homology, and yet, crystal structures of enzymes in these subfamilies are similar [52].

Plant papain-type proteases are zymogens characterized by small-size, acidic pH optimum, wide *in vitro* substrate specificity, sensitivity to cysteine protease inhibitors (e.g. leupeptin, E-64, TPCK). It has been found that members of the papain group of proteases preferentially cleave peptide bonds with arginin in P_1 position [53] or phenylalanine at the P_2 position [54]. Numerous examples of increases in papain-type protease activity in developing and germinating seeds and growing seedlings have been reported [55]. Both cathepsin L/H-type [56] and cathepsin B-type [57] papain homologues have been cloned from embryos and germinating seeds. The degradation of seed storage proteins by papain-type proteases has been recognized for several plant species [58]. In recent experiments, papain homologues which are similar to mammalian cathepsin H and L rather than to cathepsin B, are expressed in a diverse set of senescing organs, tissues and cell types undergoing PCD [16].

Some papain-type proteases are targeted to the large central vacuole or other intracellular lytic compartments. Release of hydrolases into the cytosol, after tonoplast rupture, may serve a signaling function or provide cell death/autolysis effectors. Plasma membrane or tonoplast rupture is a recognized marker for some cell death programs, such as synergid death, suspensor death, pith autolysis, tracheary element differentiation, leaf and petal senescence [59] and programmed death of barley aleurone cells [60]. The role played by extracellular papain-type enzymes in the events surrounding PCD is even less certain.

The partially purified PCD-associated cysteine protease is sensitive to cystatin, a protein inhibitor of papain-type proteases. Ectopic expression of cystatin effectively blocks H_2O_2-induced PCD. Synthetic inhibitors of papain-type proteases, however, do not effectively block H_2O_2-induced PCD [61]. Overexpression of cystatin, a papain-type proteases inhibitor, in *Arabidopsis* cell cultures blocks cell death in response to avirulent bacteria and NO. Furthermore, overexpression of this cystatin in tobacco plants blocks the HR induced by avirulent bacteria [62]. A peptide-aldehyde inhibitor of papain-type proteases prevents the complete removal of intracellular contents of differentiated tracheary elements [63], suggesting that papain-type proteases are necessary for autolysis during plant PCD [16].

Aspartic endopeptidases (EC 3.4.23)

Phytepsins (plant pepsin-type enzymes) are the only plant aspartic endopeptidases implicated in plant PCD. Cleavages of model substrates (insulin B chain, glucagons and melittin) by barley phytepsin occur between two residues retaining large hydrophobic side-chains or next to one hydrophobic residue [64]. Phytepsins also cleave aspartate-threonine [64] and aspartate-aspartate [65] bonds. They are encoded by small gene families and synthesized as inactive propeptides that exhibit a high degree of similarity to animal cathepsin D which has been linked with PCD [66]. High levels of antisense cathepsin D or pepstatin, a phytepsin inhibitor, protected cells from interferon-γ- and Fas/APO-1-induced cell death [67]. Substrate analogues that inhibit cathepsin D are also effective inhibitors of phytepsin [68].

Most of the available information about phytepsins comes from characterizations of enzymes from barley, rice, *Cynara cardunculus*, *Arabidopsis* and *Brassica*. It has been reported that aspartic peptidase mRNA levels increase during senescence of leaves [69] and petals [70]. Similarly, tomato phytepsin expression is increased in response to wounding and methyl jasmonate and systemin application [71]. In barley, phytepsin is detected in seeds, seedlings, flowers, stems, leaves and roots [72]. A novel gene encoding phytepsin is expressed in nucellar cells after pollination, concomitant with nucellar degeneration in barley [73]. Barley phytepsin has been characterized which based on its primary sequence [74], three-dimensional model [75], substrate specificity [65], sensitivity to inhibitors [69] as well as

vacuolar localisation [76]. Thereafter, it has been observed that phytepsin is highly homologous to mammalian cathepsin D and yeast vacuolar proteinase A. Runeberg-Roos and Saarma [77] have been reported that in serial transverse sections of the vascular cylinder, starting from the root tip, phytepsin is expressed in root cap cells in the tracheary elements of early and late metaxylem, and in the sieve cells of the protophloem and metaphloem during partial autolysis of sieve cells.

Phytepsin has been localized to vacuoles in leaves, roots and stigmatic papillae cells and to protein bodies in aleurone cells [76] where it may process seed storage proteins [65] or lectins [76]. A pepstatin-sensitive peptidase has been localized to cell walls where they degrade pathogenesis-related proteins [78].

Metalloendopeptidases (EC 3.4.24)

Compared to the other endopeptidases little is known about plant metalloendopeptidases. Graham et al. [79] has reported that SMEP1, a soybean metalloendopeptidase, originally identified as an azocoll-degrading, extracellular, EDTA-sensitive enzyme, was purified from leaves of soybean and shown to be a 19kDa protein localized almost exclusively to the apoplast of leaves and most abundant during the late stages of leaf expansion. The purified protein was sequenced and found to share approximately 40% identity with animal matrix metallopeptidases (MMPs) [80]. Consequently SMEP1 and recently cloned MMP from *Arabidopsis* [81] have been listed with the M10A family of MMPs. Family M10A includes a number of animal endopeptidases such as collagenases, gelatinases and stromalysins, involved in the degradation of extracellular matrix (ECM) proteins. Although the roles of M10 peptidases in plants are not yet known, work with animal MMPs suggests that plant enzymes such as SMEP1 and AtMMP may be important regulators of growth and development and may even participate in plant PCD [16].

Threonine endopeptidases (EC 3.4.25)

Proteasome endopeptidase complex (EC 3.4.25.1) belongs to the threonine endopeptidases and called proteasome briefly. Under this title we will provide a brief introduction to the proteasome, particularly 26S proteasome, as a regulator of growth, development and PCD. The inhibitor sensitivities of the assembled proteasome and the amino acid sequences for its subunits were unlike those of known peptidases; therefore, the proteasome's catalytic mechanism is not completely clear. The 26S proteasome locating in nucleus and cytoplasm is responsible for degrading proteins covalently bound to ubiquitin (Ub) molecules. Polyubiquitination of proteins is sufficient to target them for degradation by the 26S proteasome. The 26S complex is thus seemed to be the main nonlysosomal proteolytic pathway of eukaryotic cells [16].

One of the best studied examples of animal PCD occurs at the intersegmental muscle (ISM) degeneration during metamorphosis of the hawkmoth (*Manduca sexta*). Polyubiquitin gene expression increases [82], the levels of ubiquitinated proteins raise and enzymes involved in Ub attachment to cellular proteins induce [83] corresponding with commitment of ISMs to death [16]. Expression of genes encoding Ub-proteasome pathway components is upregulated in some plant PCD models. Increases in mRNA for Ub-conjugating enzymes (E2s) and Ub [84] and in GUS expression driven by an Ub promoter [85] were observed in senescing leaves. Other reports, however, do not support a role for the proteasome during senescence. Levels of Ub and/or ubiquitinated proteins also increased during tracheary element differentiation in wounded *Coleus* stems [86] and members of one *Arabidopsis* E2 family display vascular tissue-specific expression [87], while members of other *Arabidopsis* E2 families have more generalized expression patterns [14]. As a result of these experiments with senescing organs, degenerating anthers and differentiating tracheary elements, no clear model about the role of the proteasome in plant PCD existed [16].

In the *Zinnia* tracheary element experiment, application of the proteasome inhibitor lactacystin at culture initiation completely

prevents tracheary element differentiation [63]. Proteasome activity inhibition results in a delay in development. The tracheary element cell death program was neither blocked nor induced prematurely in these experiments. That specific proteasome inhibition during differentiation does not prevent autolytic clearing of tracheary elements. This is consistent with the conclusion that the proteasome does not participate in bulk autolysis of differentiating tracheary elements [16]. These studies support a role for the proteasome in some aspects of plant growth and development possibly including PCD.

This review provides basic information about five mechanistically distinct endopeptidases taking an important role on plant proteolytic activity and PCD. It is possible that undiscovered proteolytic systems, perhaps caspase homologues or analogues will be identified in near future.

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