

Review

Multifunctional role of plant cysteine proteinases

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Cysteine proteinases also referred to as thiol proteases play an essential role in plant growth and development but also in senescence and programmed cell death, in accumulation of storage proteins such as in seeds, but also in storage protein mobilization. Thus, they participate in both anabolic and catabolic processes. In addition, they are involved in signalling pathways and in the response to biotic and abiotic stresses. In this review an attempt was undertaken to illustrate these multiple roles of cysteine proteinases and the mechanisms underlying their action.

Proteolysis in plants is a complex process involving many enzymes and multifarious proteolytic pathways in various cellular compartments, with cysteine proteinases playing an essential role. Their share in total proteolysis depends on the kind of plant and its organ. It amounts up to 30% of total proteolytic activity in mature non-senescent organs. However, the activities of cysteine proteinases respond dramatically to different internal and external stimuli and in some cases

they rise to 90% of the total proteolytic activity (Wiśniewski & Zagdańska, 2001). They are involved in protein maturation, degradation, and protein rebuilt in response to different external stimuli and they also play a house-keeping function to remove abnormal, misfolded proteins. In each case, the proteolysis by cysteine proteinases is a highly regulated process. The aim of this review is to illustrate the progress in the determination of the enzyme structure, localisation and gene

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Abbreviations: ER, endoplasmic reticulum; PCD, programmed cell death; SH-EP, sulfhydryl-endo-peptidase; VPE, vacuolar processing enzymes.

expression and to highlight the great diversity of cellular processes in which the cysteine enzymes are involved.

CLASSIFICATION OF CYSTEINE PROTEINASES

Peptide hydrolases (peptidase or protease) 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 (Barrett, 1994). 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. Classification of endopeptidases (proteinases) is based on the kind of active site residue (cysteine-, serine-, aspartic-, and metallo-types), and not on the type of their substrate (Barrett, 1986). The reason for such a classification criterion is the often unknown nature of the substrate. Cysteine proteinases (EC 3.4.22), endopeptidyl hydrolases with a cysteine residue in their active center are usually identified basing on the effect of their active site inhibitors (iodoacetate, iodoacetamide and E64) and activation of the enzymes by thiol compounds. Most cysteine proteinases show acidic pH optima.

Rawlings & Barrett (1993), who based classification of proteinases on the structural and evolutionary relationship, have grouped the enzymes into families and clans. A clan comprises a group of families of an evolutionary relationship. The classification proposed by those authors is continuously brought up to date (MEROPS peptidases database, <http://merops.sanger.ac.uk>). Cysteine proteinases being labeled with the prefix C and comprise more than 40 families of peptidases grouped into at least six superfamilies or clans. Most plant cysteine proteinases belong to the papain (C1) and legumain (C13) families. The members of cysteine proteinases:

caspsases (family C14) and calpains, the calcium-dependent proteinases (family C2), have recently been found in plants. Two additional families of cysteine proteinases: ubiquitin C-terminal hydrolases (family C12) and ubiquitin-specific proteinases (C19), the components of the ubiquitin-proteasome-dependent pathway that catalyze deubiquitination of proteins, have also been detected in plants (Vierstra, 2003).

CHARACTERIZATION OF CYSTEINE PROTEINASES

The family of papain-type proteinases is the most thoroughly investigated among all the cysteine proteinases. Papain is characterized by a two-domain structure with the active site between the domains. These enzymes are synthesized as inactive or less inactive precursors which comprise 38–250 amino-acid prosequence, an N-terminal signal sequence and the 220–260 amino-acid mature enzyme. Activation takes place by limited intra- or intermolecular proteolysis (Wiederanders, 2003). To date, the amino-acid sequences of more than 50 papain-like and 15 legumain-like plant proteinases have been established (Fischer *et al.*, 2000).

The *Arabidopsis thaliana* genome encodes 32 papain-type (C1 family) cysteine proteinases which can be classified into eight main groups (senescence- and stress-induced, aleurain, cathepsin-b like, bromelain-like, KDEL, telo sequences, actinidain-like) based on the sequence similarity to other cysteine proteinases (Simpson, 2001). Plant papain-type enzymes (C1) are synthesized as small preproteins of 40–50 kDa prepropeptides that undergo proteolytic processing of the pre and pro peptides to yield mature, fully active enzymes (22–35 kDa). The structural relationships between eukaryotic papain proteinase proparts and their role in regulation of enzyme activity, correct intracellular targeting and folding of the mature enzyme

has been described recently by Wiederanders (2003). The prerequisite for catalysis by papain are not only the Cys 25 and His 159 residues but also the Asn 175 residue important for the proper orientation of the His side chain (Beers *et al.*, 2004). Besides, it has been found that members of the papain group of proteinases preferentially cleave peptide bonds with Arg in P₁ position (Fischer *et al.*, 2000) or Phe at the P₂ position (Menard & Storer, 1998).

Calpains (C2) are cytoplasmic, calcium-dependent cysteine proteinases requiring micro- or millimolar concentrations of Ca²⁺ for activity, with a highly conserved molecular structure in the catalytic site. To date, only three reports on the identification of calcium-dependent enzymes in plants have been published: i.e. in *Arabidopsis* roots (Safadi *et al.*, 1997), and in root tips (Subbaiah *et al.*, 2000) and the grain aleurone layer of *Zea mays* (Wang *et al.*, 2003).

Legumains are a newly discovered group of cysteine proteinases (C13) isolated from maturing *Ricinus communis* seeds (Hara-Nishimura *et al.*, 1991), *Glycine max* cotyledons (Scott *et al.*, 1992), germinating *Vicia sativa* seeds (Becker *et al.*, 1995) and in different organs of *A. thaliana* (Kinoshita *et al.*, 1995a; 1995b). These enzymes belong to the asparaginyl-specific subclass of the cysteine endopeptidase family which cleave peptide bonds with Asn or Asp (less efficiently) in the P₁' positions at the C-terminal flank (Becker *et al.*, 1995). They are active only at acidic pH (Müntz & Shutov, 2002). Plant legumains are usually called vacuolar processing enzymes (VPE) but they are also present in the cell wall and their function is not restricted to precursor protein processing but also includes protein breakdown in the vacuole or cell wall (Müntz *et al.*, 2002). Comparison of sequences and gene expression showed that *Arabidopsis* legumains can be divided in two subfamilies: those specific for seeds (β VPE) and others (γ VPE and α VPE) specific for vegetative organs. This division is consistent

with the classification of plant vacuoles into protein-storage and lytic vacuoles (Kinoshita *et al.*, 1995a; 1995b; 1999). An immunocytochemical analysis confirmed the specific localization of β VPE in the protein storage vacuoles and γ VPE in the lytic vacuoles. However, there are a few exceptions, e.g. barley grain legumain (nucellain), although found in the cell wall of the nucellus, is related to the seed-specific enzymes (Linnestad *et al.*, 1998).

Caspases, belonging to the C14 class of specific cysteine proteinases show a high specificity with an absolute requirement for an Asp residue adjacent to the cleavage site and a recognition sequence of at least four amino acids N-terminal to the cleavage site. The available data suggest that a true caspase-like proteolytic activity is present in plants (Woltering *et al.*, 2002). Caspase-like proteinases in plants are inhibited by specific caspase inhibitors and are resistant to typical cysteine proteinase inhibitors (del Pozo & Lam, 1998; Lam & del Pozo, 2000).

SYNTHESIS AND INTRACELLULAR TRANSFER OF CYSTEINE PROTEINASES

Cysteine proteinases are synthesized at membrane bound polysomes in the cytoplasm as large precursors with short N-terminal and much longer C-terminal propeptides. The inactive proenzymes enter the lumen of the endoplasmic reticulum (ER) and are transported to the vacuole or cell wall. Most soluble plant proteins have a C-terminal KDEL or HDEL tetrapeptide sequence recognized by the ERD2-KDEL receptor on the Golgi apparatus (Okamoto *et al.*, 2003) and are transported to the *trans*-Golgi network. However, certain proteinases, e.g. of the papain-type, containing an ER retention signal are transported into large vesicles (termed KDEL vesicle) that bud off from the ER and bypass the Golgi to fuse directly with protein storage vac-

uoles (Toyoka *et al.*, 2000; Okamoto *et al.*, 2003). It seems that the KDEL/HDEL sequences in plants function not only as a protein retention signal in the endoplasmic reticulum but may also regulate the delivery of proteins to other compartments. Sequential removal of the N- and C-terminal propeptides in vacuoles is required to produce the mature form (Yamada *et al.*, 2001).

Papain-like cysteine proteinases have the ER retention signal KDEL (Okamoto & Minamikawa, 1999) at the C-terminus of their cDNA-deduced amino-acid sequences removed post-translationally (Fischer *et al.*, 2000). *Arabidopsis* has more than one gene coding for potential receptors of the KDEL tetra peptide (Frigerio *et al.*, 2001). HDEL and KDEL possibly have different receptors which may be separately distributed in the Golgi complex (Frigerio *et al.*, 2001). KDEL has been detected in SH-EP (sulfhydryl-endopeptidase), a vacuolar proteinase involved in degradation of seed storage proteins accumulated in protein storage vacuoles (Okamoto & Minamikawa, 1998). SH-EP is synthesized as an inactive zymogen of 43 kDa with a carboxy-terminal KDEL motif (Okamoto & Minamikawa, 1998). Processing of the pro-proteinase results in an activation of the enzyme of 33 kDa which is sequestered into endoplasmic reticulum-derived electron-dense vesicles (KDEL-tailed Cys-proteinase accumulating vesicles or KV) that reach the vacuole *via* a pathway that bypasses the Golgi complex (Toyooka *et al.*, 2000; Okamoto *et al.*, 2003). The post-translational processing of SH-EP includes removal of the KDEL motif prior to translocation and activation of the mature protein (Okamoto *et al.*, 1999). The KDEL-containing proteinases appear to be a distinct plant-specific enzyme subset linked to the endoplasmic reticulum-derived precursor protease vesicles (Chrispeels & Herman, 2000) and have been identified exclusively in higher plants (Toyooka *et al.*, 2000; Okamoto *et al.*, 2003).

In 14 members of the papain family extension sequences at the C-terminus have been found (Gietl *et al.*, 2000). The C-terminal extension sequences are composed of two domains: a Pro-rich domain and a domain of high homology to animal proteins of the epithelin/granulin family (Bhandari *et al.*, 1992). Epithelins and granulins are small proteins of about 6 kDa that modulate the intensity of growth of animal cells, whereas plant granulins have a 4 kDa insertion with two cysteine residues. The function of these proteins remains to be established (Bhandari *et al.*, 1992; Yamada *et al.*, 2001).

At their N-terminal propeptides the papain family proteinases have the ERFNIN (EX₃RX₃FX₂NX₃I/VX₃N) motif (about 110 residue propeptide) which is strongly conserved and seems to function as an autoinhibitory domain (Beers *et al.*, 2000).

Legumains are also synthesized on the rough endoplasmic reticulum. The N-terminal signal peptide is removed during segregation the nascent polypeptide into the lumen of endoplasmic reticulum and from there prolegumains may be packed directly and transported in an inactive state in dense vesicles to the vacuole or to the cell wall (Fischer *et al.*, 2000; Schlereth *et al.*, 2001). Processing and activation of legumains occur when they reach the final destination. Recently it has been shown that maturation of these enzymes consists of two proteolytic cleavages of the precursor (56 kDa): the first cleavage consists of a self-catalytic removal of the C-terminal pro-peptide, which is able to auto-inhibit the enzyme activity. The second cleavage removes the N-terminal peptide that might contribute to appropriate folding and targeting of the enzyme (Kuroyanagi *et al.*, 2002).

The cysteine proteinase activation *via in vitro* and *in vivo* processing is discussed in details by Wiederanders (2003).

PROTEINASE SUBCELLULAR LOCALIZATION

Plant cysteine proteinases are localized in vacuoles and in the cell wall. It still remains unclear whether the cysteine proteinases present in chloroplasts are involved in chloroplast protein degradation. A proteinase degrading a 16 kDa protein associated with photosynthetic water oxidation has been purified from PSII enriched membranes of spinach but it is inhibited and not activated by sulfhydryl reducing agents and, besides, it is not inhibited by the conventional inhibitors of cysteine proteinases (Kuwabara & Hashimoto, 1990). Another proteinase which is associated with the thylakoid membrane of spinach and is responsible for LHC II (light-harvesting chlorophyll a/b-protein of PSII) degradation is probably of cysteine type (Lindhall *et al.*, 1995). Therefore, identification of chloroplast proteinases, their corresponding genes and their specific functions remain to be elucidated.

Legumains have been found both in the seed-storage vacuoles and in lytic vacuoles of cells of vegetative organs (Becker *et al.*, 1995; Kinoshita *et al.*, 1995a; Hara-Nishimura *et al.*, 1998a; Schlereth *et al.*, 2001). Recently, it has been shown that the "bodies" of endoplasmic reticulum may store proteinases (Yamada *et al.*, 2001; Royo *et al.*, 2003).

A plant caspase-like activity has been found in the cytosol but not in vacuoles (Korthout *et al.*, 2000).

NATURAL INHIBITORS

Proteinaceous inhibitors of cysteine proteinases, widely distributed among plants, animals and microorganisms are subdivided into three families (stefin, cystatin and kininogen) based on their sequence homology, the presence and position of intrachain disulfide bonds, and the molecular mass of the protein (Turk & Bode, 1991). The stefin family con-

sists of proteins of about 100 amino-acid residues and molecular mass about 11 kDa, which do not contain disulfide bonds or carbohydrate groups. The cystatin family groups proteins of about 120–126 amino-acids residues and molecular mass about 13.4–14.4 kDa. The cystatins contain four conserved cysteine residues forming two disulfide bonds and most of them are not glycosylated (Grzonka *et al.*, 2001). The third family, kininogens, is composed of larger glycoproteins from 60 to 120 kDa. Recently, a novel class of cysteine protease inhibitors, staphostatins, has been discovered in *Staphylococcus aureus* (Dubin, 2003; Dubin *et al.*, 2003; Rzychon *et al.*, 2003). The plant cystatins (phytocystatins), homologous to animal cysteine protease inhibitors, have been characterized in several monocots and dicots. Plant cysteine proteinase inhibitors have sequence similarities to stefins and cystatins but do not contain free Cys residues (Fernandes *et al.*, 1993; Zhao *et al.*, 1996). The unique feature of phytocystatin superfamily is a highly conserved region of G58 residue, the QVVAG motif (amino-acid positions 102–106) and the PW motif (positions 132–133). The last residue mediates binding of this inhibitor domain to the enzyme. Phylogenetic analysis of 63 cystatins, 32 of them being phytocystatins, and their comparison to the animal cystatins and stefins revealed the existence of a typical [LVII]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N consensus sequence, exclusive of plant cystatins (Margis *et al.*, 1998). The analysis of the phylogenetic tree indicated that phytocystatins constitute a single branch statistically distinct from that of cystatins and stefins (Margis *et al.*, 1998).

Plant cysteine proteinase inhibitors are encoded by gene families (Fernandes *et al.*, 1993; Waldron *et al.*, 1993) but little is known about regulation of these genes. Expression of the proteinase inhibitor genes is usually limited to specific organs or to particular phases during plant growth: germination (Botella *et al.*, 1996), early leaf senescence

(Huang *et al.*, 2001), drought (Waldron *et al.*, 1993) or cold and salt stresses (Pernas *et al.*, 2000; Van der Vyver *et al.*, 2003). A similar pattern of gene expression is evoked by wounding or methyl jasmonate (Botella *et al.*, 1996). The cytosolic localization of the inhibitors suggests that they are involved in plant defense against insects (Botella *et al.*, 1996; Zhao *et al.*, 1996). Transgenic potato expressing two cystatin genes (Cowgill *et al.*, 2002) and transgenic rape plants (Rahbe *et al.*, 2003) expressing rice cystatin 1 became resistant to a nematode (potato), coleopteran insects (potato) and aphids (rape). These facts confirm the above supposition.

EXPRESSION OF CYSTEINE PROTEINASE GENES AND THEIR REGULATION

In *A. thaliana*, the vacuolar processing enzymes (VPE) gene family is composed of four genes, i.e. α VPE, β VPE, γ VPE and δ VPE (Kinoshita *et al.*, 1995a; 1995b; Gruis *et al.*, 2002). Several members of this gene family have been identified in other plants including seeds of *Ricinus communis*, *Glycine max* and *Canavalia ensiformis* and besides, two cDNAs were cloned from germinating seeds of *Vicia sativa* L. and ripening *Citrus* fruit (Gruis *et al.*, 2004). On the basis of sequences and evolutionary analysis, three major gene clusters of plant legumains were distinguished: those of seeds (S), vegetative tissues (V) and early embryogenesis-specific (E) legumains (Müntz *et al.*, 2002). The S cluster legumains are expressed during maturation of seeds, e.g. β VPE in *Arabidopsis*, whereas the V cluster legumains are expressed only in vegetative organs, e.g. γ VPE and α VPE in *Arabidopsis* (Hara-Nishimura *et al.*, 1998a; 1998b) and in fruit tissues and cotyledons (Müntz *et al.*, 2002). Besides, cluster S may be supplemented by nucellain. Cluster E, a sister group of clusters S and V, includes two legumains from *Nicotiana tabacum* L. expressed in seeds

only during early embryogenesis and from *Lycopersicon esculentum* L. The recent findings that vegetative type VPE genes are expressed significantly in developing seeds of *Arabidopsis* (Royo *et al.*, 2003; Shimada *et al.*, 2003; Gruis *et al.*, 2004) did not confirm the concept of two separate VPE clusters: of the seed and vegetative types. It appears that seed-type VPEs are involved in seed protein maturation and vegetative-type VPEs constitute a subfamily of proteinases involved in senescence and mobilization of protein reserves in germinating seeds (Gruis *et al.*, 2004).

Expression of rice cysteine proteinase gene, *OsEP3A*, is regulated by multiple mechanisms: it is hormonally regulated in germinating seeds, spatially and temporarily regulated in vegetative tissues and nitrogen regulated in suspension-cultured cells (Ho *et al.*, 2000).

It has been shown that the cysteine proteinase (EP-B) secreted into the endosperm during germination of barley is encoded by at least four genes (Koehler & Ho, 1990a). In various senescing plants, a number of cDNA clones encoding cysteine proteinases are up-regulated: *Arabidopsis* *SAG2* and *SAG12*, *BnSAG12* of *Brassica napus*, *See1* of *Zea mays*, *SENU2* and *SENU3* of *Lycopersicon esculentum*, *SmCP* of *Solanum melongena*, *See1* of *Lolium multiflorum* and *NTCP-23* of *Nicotiana tabacum* (Chen *et al.*, 2002). The senescence-associated gene of *Ipomoea batatas*, *SPG31*, encodes a pre-pro-protein of 341 amino acids with a predicted molecular mass of 37 kDa with the catalytic triad at positions Cys 147, His 284 and Asn 305 and with conserved ERFNIN motif (Chen *et al.*, 2002). Thus, *SPG31* can be classified as a member of the papain family. The enzyme precursor is cleaved between Ser 121 and Ser 122 to generate the mature form of the enzyme. In the promoter region of the 5'-upstream sequence of 899 bp, a number of putative ethylene, wound-, heat-, and light-responsive elements were found (Chen *et al.*, 2002). Southern blot analysis revealed 3–8 bands suggesting that this gene belongs to a small multigene family

of closely related sequences in the hexaploid *I. batatas* genome.

Expression of genes that encode cysteine proteinases has been shown to respond to cold, heat and water deficiency. Cloning and nucleotide sequence analysis of cDNAs that accumulated following exposure of tomato fruit to low or high temperature showed that one cDNA displayed a significant similarity to plant and animal cysteine proteinases of the papain family (Schaffer & Fischer, 1990). The *15a* clone encoding a 363 amino-acid protein in pea plants (Guerrero *et al.*, 1990) and clones *rd19* and *rd21* encoding different cysteine proteinases in *Arabidopsis* were induced by water deficit and were also responsive to salt stress but not to heat and cold (Yamaguchi-Shinozaki *et al.*, 1992; Koizumi *et al.*, 1993). Further study revealed that RD21, the corresponding protein, contains a C-terminal extension sequence composed of a 2 kDa proline-rich domain and a 10 kDa domain homologous to the domain of granulin family proteins (Yamada *et al.*, 2001). The RD21 protein is synthesized as a pre-pro-protein of 58 kDa and processed *via* an intermediate of 38 kDa into a mature protein of 33 kDa. The inactive RD21 precursor of 38 kDa accumulates in the endoplasmic reticulum bodies that fuse with vacuoles during stress action and thus becomes active (Hayashi *et al.*, 2001). *Arabidopsis* RD21 is a cysteine proteinase classified as belonging to the papain family (Yamada *et al.*, 2001). Comparison of sequences revealed a high similarity between *rd21* and *C14* (cDNA for a tomato gene induced by low and high temperature) and rice oryzains α and β (Koizumi *et al.*, 1993). RD19 has the highest homology to pea *15a* cysteine proteinase which is induced by water deficit and does not contain the C-terminal sequence (Koizumi *et al.*, 1993). A phylogenetic tree indicated that RD19 and RD21 appear to be different types of cysteine proteinases belonging to different subfamilies. Four putative dehydration-responsive cysteine proteinase cDNAs (*BoCP1*, *BoCP2*, *BoCP3* and *BoCP4*) have

been isolated from *Brassica oleracea* L. florets (Coupe *et al.*, 2003). These cDNAs are most similar (73–89% at the amino-acid level) to the dehydration-responsive RD19 and RD21 cysteine proteinases from *Arabidopsis*.

Genes encoding caspase-like proteinases have been identified in the genomic sequences of *Arabidopsis* and other plants (Uren *et al.*, 2000) but their function in plants as cell death initiators remains to be established.

A single gene encoding a calcium-dependent cysteine proteinase (calpain) has been identified recently in maize (Lid *et al.*, 2002) and was found to determine the development of aleurone cells in the endosperm of maize kernels. Calpain homologous sequences were found in 11 different mono- and dicot plant species: *Zea mays*, *Hordeum vulgare*, *Triticum aestivum*, *Oryza sativa*, *Glycine max*, *Medicago sativa*, *Gossypium hirsutum*, *Solanum tuberosum*, *Lycopersicon esculentum*, *Sorghum* sp. and *Arabidopsis thaliana* (Margis & Margis-Pinheiro, 2003). Calpain full-length cDNA sequences were detected in *A. thaliana*, *O. sativa* and *Z. mays* (Margis & Margis-Pinheiro, 2003). The nature of the different cDNA libraries from which the clones were isolated showed that phytocalpains exhibit a ubiquitous tissue expression pattern. Despite a significant homology within the catalytic domain between animal and plant calpains, phytocalpains lack the conserved calcium-binding domain IV and phytocalpains of *A. thaliana*, *O. sativa* and *Z. mays* have an N-terminal transmembrane receptor-like domain (Lid *et al.*, 2002).

INVOLVEMENT OF CYSTEINE PROTEINASES IN DEPOSITION AND DEGRADATION OF STORAGE PROTEINS

Cysteine proteinases participate in limited proteolysis. This is especially significant since it concerns storage proteins deposited

in the cotyledon mesophyll of dicotyledonous plants, the endosperm of cereals, and embryonic axis. Cysteine proteinases determine storage of proteins in both kinds of vacuoles: storage in seeds and lytic ones in vegetative tissues. Vacuolar processing enzymes, β VPE and γ VPE, are the key proteinases responsible for maturation of various proteins in protein-storage vacuoles of maturing seeds (Hara-Nishimura *et al.*, 1995; Kinoshita *et al.*, 1999). The endoproteolytic cleavage of 11S globulins, the major seed storage proteins, into two chains linked by a disulfide bridge is evolutionarily conserved in conifers, monocots and dicots (Nielsen *et al.*, 1995). VPE-mediated cleavage at the conserved asparagine site is required to convert the oligomeric trimer formed in the endoplasmic reticulum to the mature vacuole-localized hexamer (11S) storage protein (Jung *et al.*, 1998). Thus, the additional processing of storage proteins may serve for preparation of proteins for dense packing within the vacuole. Other seed proteins mature similarly through processing by VPE (Herman & Larkins, 1999). 11S globulins (legumins), 2S albumins and 7S globulins (vicilins) frequently co-exist with VPE in protein bodies of mature seeds but the conformation of native proteins is resistant to legumain attack (Müntz *et al.*, 2002). The occurrence of γ VPE mRNA in vegetative organs of *Arabidopsis* supports the possibility that a similar system to that in seeds may function in lytic vacuoles (Kinoshita *et al.*, 1999). A homolog of the vacuolar processing enzyme, VmPE-1, is involved in post-translational processing of the cysteine proteinase SH-EP activating it in germinating cotyledons of *Vigna mungo* (Okamoto & Minamikawa, 1995). It is likely that vacuolar processing enzymes may be involved in maturation and activation of the inducible cysteine proteinases in the lytic vacuoles of senescing and degenerating tissues (Kinoshita *et al.*, 1999).

Besides the above role of cysteine proteinases in deposition of proteins in vacuole

cysteine proteinases are the most abundant group of proteinases responsible for degradation and mobilization of storage proteins. In germination of barley seeds 42 proteinases are involved and among them 27 are cysteine proteinases (Zhang & Jones, 1995). In germinating maize (de Barros & Larkins, 1994) and wheat (Bottari *et al.*, 1996) cysteine proteinases account for about 90% of the total degradation activity of prolamins, the major storage proteins of cereals. Two cysteine proteinases have been purified from germinated barley so far (Pouille & Jones, 1988) and two from barley aleurone layers (Koehler & Ho, 1988; 1990a; 1990b). They were designated EP-A (Koehler & Ho, 1990a; 1990b) and EP-B, respectively (Koehler & Ho, 1988) and classified as papain-like enzymes. EP-A occurs in three and EP-B in two isoforms (Koehler & Ho, 1990a; 1990b). It has been revealed that the barley cysteine proteinases catalyze cleavage at Leu, Val, Ileu and Met or Phe, Tyr, Trp side chains at P₂ (Davy *et al.*, 2000). Also it has been suggested that barley cysteine proteinases mediate the release of β -amylase from its bound form during germination (Koehler & Ho, 1990a; 1990b). Examination of the substrate specificity of EP-B with synthetic peptides revealed that only the C-terminal domain of the native 60 kDa β -amylase is accessible to proteolytic attack (Davy *et al.*, 2000). In the cotyledons of germinated *Vigna mungo* seeds a cysteine proteinase (SH-EP) is responsible for the breakdown of globulin (Okamoto & Minikawa, 1998). It is also possible that KDEL-tail proteinases, e.g. SH-EP is responsible for the massive protein mobilization from cotyledon to hypocotyl and from senescing organs to maturing seeds (Toyooka *et al.*, 2000).

For mobilization of storage proteins in cotyledons of dicotyledonous seeds so far investigated the following enzymes were shown to be involved: four papain-like (CPR1, CPR2, proteinase A and CPR4) and two legumain (VsPB2 and proteinase B) cysteine proteinases (Fischer *et al.*, 2000; Schlereth *et al.*,

2000; 2001). Moreover, Tiedemann *et al.* (2001) provided immunohistochemical evidence that in mobilization of bulk globulins in vetch (*Vicia sativa* L.) two papain (CPR1 and CPR2) and one legumain-like (proteinase B) proteinases were involved. Stored proteinases formed during late embryogenesis initiate mobilization of stored proteins at first in embryonic axes before *de novo* synthesis of proteases (Tiedemann *et al.*, 2001). Papain-like cysteine proteinases (CPR1 and CPR2) are responsible for the mobilization of vicilin, the 7S storage globulin of *Vicia faba* L. The complexity of germination process in dicotyledonous seeds and the function of cysteine proteinases in degradation of storage proteins have been discussed in details by Fischer *et al.* (2000), Schlereth *et al.* (2000; 2001), Müntz *et al.* (2002) and Tiedemann *et al.* (2001).

THE RESPONSE OF CYSTEINE PROTEINASES TO ABIOTIC AND BIOTIC STRESSES

In response to environmental abiotic and biotic factors cellular proteins should be rebuilt. Misfolded and damaged proteins are eliminated by protein degradation and replaced by newly formed proteins, i.e. cold and heat shock proteins, dehydration-induced proteins and pathogenesis-related proteins. The protein substrates of cysteine proteinases induced by cold and water deficiency have not been defined. Degradation of damaged or denatured proteins under stress is closely coupled with the synthesis of new proteins (Schaffer & Fischer, 1988; 1990) since the released amino acids are reused for the synthesis of new proteins and/or adjust the cell osmotically to water limitation (Guerrero *et al.*, 1990; Vincent & Brevin, 2000).

In response to water deficit, cysteine proteinases are induced in wheat leaves both non-acclimated and acclimated to drought and their contribution in the total proteolytic

activity increases significantly upon drought (Zagdańska & Wiśniewski, 1996). Extension of the experiments to cultivars of different drought resistance revealed that although a general induction of cysteine proteinases takes place the level of their induction is negatively related to the drought resistance and positively correlated with extravacuolar ATP-dependent proteolysis (Wiśniewski & Zagdańska, 2001). This latter activity in genotypes resistant to drought compensated the lower activity of cysteine proteinases. Both responses were highly statistically significant. The experiments carried out on seedlings of 55 cultivars and strains of winter wheat (Grudkowska *et al.*, 2002) proved that this genetic relation also holds for frost tolerance.

In the case of plant response to biotic stresses, a defense mechanism in plants against insects is resolved in maize genotypes by induction of a cysteine proteinase without involvement of a cysteine proteinase inhibitor (Pechan *et al.*, 2000). In response to larval feeding a unique 33 kDa cysteine proteinase, encoded by *mir1*, accumulates in the whorl of maize genotypes resistant to *Lepidoptera*. This enzyme inhibits larval growth although the mechanism involved remains unknown.

SENESCENCE AND PROGRAMMED CELL DEATH

Programmed cell death (PCD) in multicellular organisms occurs as a part of normal development and is one of the plant defense mechanisms in pathological processes. In plants, PCD is connected with developmental changes and differentiation of plant organs is associated with induction of cysteine proteinases such as during xylogenesis in *Zinnia* (Minami & Fukuda, 1995) or differentiation of tracheary elements in *Arabidopsis* (Funk *et al.*, 2002), during leaf and flower senescence (Eason *et al.*, 2002; Rabiza-Świder *et al.*, 2003), and senescence of unpollinated pea

ovaries (Cercós *et al.*, 1993). Generally, PCD in plants takes a different course than in other organisms (Korthout *et al.*, 2000; Kuriyama & Fukuda, 2002). Plant cysteine proteinases in senescing tissues are members of the papain-type distinguished by the C-terminal KDEL motif (Schmid *et al.*, 1999). A similar proteinase was found in the maturing pods of *Phaseolus vulgaris* in which senescence of hulls is associated with development of seeds (Tanaka *et al.*, 1993). Induction of a cysteine proteinase (EP-A) with a C-terminal TDEL motif in barley aleurone cells during germination (Kuo *et al.*, 1996) and Cys-EP in ricinosomes of *Ricinus communis* L. (Schmid *et al.*, 1999) are both related to PCD of aleurone and endosperm disintegrating during germination. Cys-EP is a papain-type cysteine proteinase with a molecular mass of 35 kDa for a mature enzyme and precursor protein of 45 kDa with a presequence for cotranslational targeting into the endoplasmic reticulum, an N-terminal propeptide, a C-terminal KDEL motif and a very high homology to the cysteine proteinases typical for senescing tissues (Schmid *et al.*, 1999). Cys-EP is approximately 50–100 times less active than its 45 kDa proform.

Little is known about the biochemistry and molecular biology of the infection process and nodule formation in the roots of actinorhizal plants. However, a nodule-specific cysteine proteinase similar to other enzymes of the papain family was found in the root nodules of *Alnus glutinosa* (Goetting-Minesky & Mullin, 1994). Suggested roles for this enzyme are as a defense response to root invasion by *Frankia*, a nitrogen-fixing actinomycete, as an integral component of tissue remodeling in root and nodule tissues, and/or as an agent of recycling of nitrogenous compounds invested in the symbiosis.

Caspase-like proteinases are involved in the control of plant cell death, as was discovered in tobacco tissues infected with tobacco mosaic virus (del Pozo & Lam, 1998). Later, induction of proteolytic activity in cowpea

plants in response to cowpea rust fungus was also shown (D'Silva & Poirier, 1998). Poly(ADP-ribose) polymerase (PARP) is involved in H₂O₂-induced programmed cell death in plants (Amor *et al.*, 1998) and degradation of plant PARP is dependent on cytochrome *c* release into the cytosol and could be inhibited by specific caspase 3 inhibitors (Sun *et al.*, 1999). The presence of caspase 3-like activity in plants has been shown for the first time in *Chara corallina* cells (Korthout *et al.*, 2000).

CONCLUSIONS

Recent results show the complexity of cellular regulation in plants by proteolysis. The rapidly growing amount of information indicates that cysteine proteinases participate in degradation of storage proteins, turnover of proteins in response to abiotic and biotic stresses and in programmed cell death accompanying the hypersensitive response to pathogen attack, tracheary element differentiation and organ senescence. Thus, cysteine endopeptidases are involved in such diverse processes as protein processing (limited proteolysis) before deposition of storage proteins in developing seeds and subsequent breakdown of storage proteins during mobilization of seed reserves during germination and seedling growth. Regulation of this dual function of these enzymes should be a subject of further studies.

To date, our attention has been focused on protein synthesis but probably the complex regulation of protein degradation should attract equal attention. However, taking into account that the *Arabidopsis* genome sequence revealed over 2000 genes involved in proteolysis it seems that it will take some time before we understand the complexity of cellular regulation by proteolysis.

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