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REVIEW

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# A cut above the rest: the regulatory function of plant proteases

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**Abstract** Proteolytic enzymes are intricately involved in many aspects of plant physiology and development. On the one hand, they are necessary for protein turnover. Degradation of damaged, misfolded and potentially harmful proteins provides free amino acids required for the synthesis of new proteins. Furthermore, the selective breakdown of regulatory proteins by the ubiquitin/proteasome pathway controls key aspects of plant growth, development, and defense. Proteases are, on the other hand, also responsible for the post-translational modification of proteins by limited proteolysis at highly specific sites. Limited proteolysis results in the maturation of enzymes, is necessary for protein assembly and subcellular targeting, and controls the activity of enzymes, regulatory proteins and peptides. Proteases are thus involved in all aspects of the plant life cycle ranging from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs. This article reviews recent findings for the major catalytic classes, i.e. the serine, cysteine, aspartic, and metalloproteases, emphasizing the regulatory function of representative enzymes.

**Keywords** Aspartic protease · Cysteine · Serine · Metalloprotease · Proteolysis · Regulation of plant development

**Abbreviations** ale1: *Abnormal leaf shape 1* · AP: Aspartic protease · cdr1: *Constitutive disease resistance 1* · CYS-EP: Cysteine endopeptidase · LAP: Leucine aminopeptidase · PCD: Programmed cell death · PSV: Protein storage vacuole · SCP: Serine carboxypeptidase · sdd1: *Stomatal density and distribution 1* · SH-EP: Sulfhydryl endopeptidase · SLD: Saposin-like domain · SPP: Stromal processing peptidase · VPE: Vacuolar processing enzyme · Zn-MP: Zinc metalloprotease

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## Introduction

Plant scientists have long recognized protein turnover as a fundamental component in plant development. Research interests, however, have traditionally focused only on physiological processes relevant for agriculture, including the breakdown of storage proteins during seed germination, and protein remobilization upon the onset of leaf senescence, concomitant with the reallocation of N resources to reproductive organs (Ryan and Walker-Simmons 1981; Huffaker 1990). While these are still highly active areas of research, we now begin to appreciate the importance of selective protein degradation for the regulation of all aspects of plant development. This appreciation results from recent studies linking the main proteolytic pathway in eukaryotes, the ubiquitin/proteasome pathway, to numerous developmental processes including embryogenesis, photomorphogenesis, circadian rhythms, flower and trichome development, as well as hormone signaling, including the responses to auxins, cytokinins, gibberellins, abscisic acid and jasmonates (Vierstra 2003). In hindsight, Judy Callis's statement in her 1995 review article that "regulated proteolysis is probably directly or indirectly involved in most cellular processes" (Callis 1995) proves to be truly prophetic, since indeed, it is difficult to find a biological process in plants that does not have some connection to ubiquitinylation (Bachmair et al. 2001). The ubiquitin/proteasome pathway, however, is by no means the only proteolytic system potentially involved in the regulation of plant growth and development. In addition to the 1,300 genes devoted to the ubiquitin/proteasome pathway, more than 600 protease genes have been annotated in the *Arabidopsis* genome that may contribute not only to protein turnover and the control of protein half-life

by complete degradation, but also to protein trafficking, proprotein processing, and the regulation of protein activity by limited, site-specific proteolysis. The tantalizing developments in the ubiquitin/proteasome field have been summarized recently in several excellent articles (Frugis and Chua 2002; Serino and Deng 2003; Sullivan et al. 2003; Vierstra 2003; Dharmasiri and Estelle 2004; Smalle and Vierstra 2004) and will not be covered in the present review. I will rather focus on the remaining proteases and discuss recent findings for selected examples from each of the main classes of proteolytic enzymes.

The hydrolytic cleavage of peptide bonds catalyzed by peptidases (peptide hydrolases, proteases) is achieved by nucleophilic attack at the carbonyl carbon supported by the donation of a proton to the NH group of the scissile peptide bond. While exoproteases, i.e. those acting on the termini of (poly)peptide chains, can be classified according to their substrate specificity as amino or carboxypeptidases, the classification of proteases acting on the interior of (poly)peptides (endoproteases or proteinases) is based on the respective catalytic mechanism. In serine, threonine, and cysteine proteases, the hydroxyl or sulfhydryl groups of the active-site amino acids act as the nucleophile during catalysis. Aspartic and metalloproteases, on the other hand, rely on an activated water molecule as nucleophile (Table 1). This is also true for glutamic proteinases, recently identified as a novel class of acid proteases with glutamate rather than aspartate as the catalytic residue (Fujinaga et al. 2004). According to the classification used in the MEROPS database (http://merops.sanger.ac.uk/; Rawlings et al. 2004) the peptidases of each of these catalytic types are assigned to families on the basis of sequence similarity. Families may be diverged so far that relationship cannot be firmly established by comparison of their primary structures. If common ancestry can be demonstrated by similarity of the three-dimensional structure the respective families are grouped into clans. Therefore, the number of different clans is an indication of structural diversity within the major groups of proteases (Table 1). As of 30 June 2004, the MEROPS database (release 6.70) lists 628 proteases for *Arabidopsis* and 138 presumably inactive homologs (in the following, only those genes predicted to encode functional proteases will be considered), which fall into 30 clans of 72 families representing all of the main peptidase groups except the glutamic proteases.

**Table 1** The main classes of peptidases. For the five catalytic classes of peptidases (proteases) the nucleophile involved in catalysis is indicated. For cysteine, serine, and threonine proteases, the nucleophile is part of the protein and, consequently, a covalent acyl-enzyme intermediate is formed in the reaction cycle. The number of clans (MEROPS classification) identified in *Arabidopsis* is an indication of the structural diversity within a class of proteases. The clans discussed in this article are indicated

| Catalytic class | Nucleophile | Covalent intermediate | Number of clans | Clans discussed |
|---|---|---|---|---|
| Cysteine | Thiol | Yes | 4 | CA, CD |
| Serine | Hydroxyl | Yes | 8 | SB, SC |
| Threonine | Hydroxyl | Yes | 1 | – |
| Aspartic | Water | No | 2 | AA |
| Metallo | Water | No | 12 | ME, MF |

## Cysteine proteases

Cysteine (sulfhydryl) proteases shall be the first ones to be discussed, since much of the initial work on plant proteases focused on this class of enzymes, and cysteine proteases were actually believed to be the predominant class of proteases in plants. In terms of numbers, however, they fall far behind serine proteases: 106 and 248 *Arabidopsis* genes are listed in the MEROPS database for cysteine and serine proteases, respectively.

The recent characterization of the d*efective* k*ernel 1* (*dek1*) mutant in maize led to the intriguing discovery of a cysteine protease that is required for the perception/response to positional cues in the specification of cereal aleurone identity (Becraft and Asuncion-Crabb 2000; Lid et al. 2002). DEK1 is related to animal calpains, calcium-dependent cysteine proteases with multiple roles in cell proliferation, apoptosis, and differentiation. Plant calpain homologs, i.e. phytocalpains, have been identified in 11 species. Functional characterization in maize and in *Nicotiana benthamiana* indicated a regulatory role in numerous developmental processes, including embryonic pattern formation and shoot apical meristem formation, cell fate specification in the endosperm and leaf epidermis, and regulation of the balance between cell differentiation and proliferation (Becraft et al. 2002; Margis and Margis-Pinheiro 2003; Ahn et al. 2004). As opposed to their animal homologs, phytocalpain appears to be a singular enzyme, as it is represented by just one gene in all species investigated (Margis and Margis-Pinheiro 2003). Its protease domain, however, resembles that of papain and has the most common fold in plant cysteine proteases (clan CA, 76 genes in *Arabidopsis*)

Papain was the first cysteine protease to be discovered in the latex and fruit of *Carica papaya* and served for a long time as a model for mechanistic and structural studies on cysteine proteases (Drenth et al. 1968; Asboth et al. 1985). Papain-like enzymes (reviewed by Beers et al. 2000, 2004) are involved in protein degradation and N-mobilization during seed germination and leaf senescence (Kato and Minamikawa 1996; Noh and Amasino 1999; Gietl and Schmid 2001; Chen et al. 2002a; Wagstaff et al. 2002; Okamoto et al. 2003) and have been implicated in a number of different cell death events (Solomon et al. 1999; Beers et al. 2000; Funk et al. 2002; Wan et al. 2002; Belenghi et al. 2003). They also contribute to plant resistance against pathogens (Krüger et al. 2002) and insects (Pechan et al. 2000; Konno et al. 2004). Second to papain-like enzymes, the legumains (family C13 in clan CD) are the most intensively studied among the plant cysteine proteases. Legumains are involved in the processing of seed storage proteins

and—similar to papain-like proteases—have been implicated in storage protein breakdown and tissue senescence (Kinoshita et al. 1999; Schlereth et al. 2001; Müntz et al. 2002; Müntz and Shutov 2002; Kato et al. 2003; Shimada et al. 2003b). Recently, by the use of multiple gene knock-outs in *Arabidopsis*, it has been possible to clearly define the contribution of different cysteine proteases to the synthesis of storage proteins during seed maturation and their degradation upon germination.

## Cysteine proteases in the accumulation and degradation of storage proteins

In maturing seeds, storage proteins are accumulated in dedicated compartments (protein storage vacuoles, PSVs; Shimada et al. 2003a) to serve as a source of reduced nitrogen for the germinating seedling. In *Arabidopsis*, there are two classes of seed storage proteins, the legumin-type globulins (12S globulins) and the napin-type albumins (2S albumins). For both classes, processing at conserved asparagine (Asn) residues is required for the assembly into multimeric structures and accumulation of the mature storage proteins. The search for an Asn-specific activity involved in storage protein processing resulted in the identification of the legumains as a novel class of cysteine proteases, also called asparaginyl-endopeptidases or, most commonly, vacuolar processing enzymes (VPEs; Hara-Nishimura et al. 1993; Ishii 1994). Processing of storage proteins by VPEs in vitro has been demonstrated in several species (Hara-Nishimura et al. 1993; Yamada et al. 1999). Until recently, however, it was unclear which VPE, if any, is responsible for processing and accumulation of seed storage proteins in vivo.

In *Arabidopsis*, the VPE gene family comprises four members, $\alpha$, $\beta$, $\gamma$, and $\delta$VPE. Based on gene expression and phylogenetic analyses, these genes had been assigned to two subfamilies, the seed-type VPEs ($\beta$VPE and $\delta$VPE) thought to be responsible for storage protein processing and assembly, and the vegetative VPEs ($\alpha$VPE and $\gamma$VPE) for which roles in storage protein mobilization and tissue senescence were proposed (Kinoshita et al. 1995, 1999; Gruis et al. 2002). This classification has to be reconsidered, however, as evident from the functional analysis of the two seed-type VPEs in *Arabidopsis* mutants. Using transposon-insertion lines, $\beta$VPE was in fact confirmed to be involved in seed storage protein processing (Gruis et al. 2002). Screening 28,000 T-DNA-tagged *Arabidopsis* lines for defects in seed storage protein maturation, Shimada et al. (2003b) isolated six $\beta$VPE mutants, thus supporting the specific involvement of this particular VPE. Surprisingly, however, normal processing was observed for $>80\%$ of the seed storage protein in the $\beta$VPE single and $\beta/\delta$VPE double knockouts, indicating the presence of redundant processing activities (Gruis et al. 2002). Triple and quadruple knockouts lacking seed-type and vegetative VPEs $\alpha$, $\beta$, and $\gamma$, on the other hand, lacked correctly processed storage proteins. In the absence of VPEs, processing of storage protein precursors was observed at alternative sites, resulting in the accumulation of abnormal polypeptides (Shimada et al. 2003b; Gruis et al. 2004). These findings demonstrate unequivocally (i) the exclusive responsibility of VPEs for Asn-specific processing of 12S globulins and 2S albumins and (ii) the contribution of both seed-type and vegetative VPEs to seed storage protein processing and assembly during seed maturation. Interestingly, however, the rate of storage protein mobilization in germinating seedlings lacking VPEs was unaffected (Shimada et al. 2003b; Gruis et al. 2004).

Seed storage proteins stably accumulate in PSVs where they coexist with active VPEs. They seem to be protected from unlimited proteolysis by VPEs through conformational stabilization: Asn-residues potentially susceptible to proteolytic cleavage by VPEs are located in structurally ordered regions where they are protected from uncontrolled proteolysis by legumains (Müntz et al. 2002). Upon germination, this conformational stabilization appears to be lost, resulting in the complete degradation of seed storage proteins. The role of legumains in this process may be direct or indirect: processing inactive zymogens, legumains may activate proteinases which are then responsible for storage protein degradation, or else, after initial cleavage and destabilization by other proteases (most likely papains), they may contribute directly to the complete degradation of the storage proteins (Müntz et al. 2002). There is experimental evidence for both scenarios: (i) A legumain from mung bean was shown to activate pro-sulfhydryl endopeptidase (proSH-EP), the major papain-like cysteine proteinase responsible for the breakdown of storage globulins, by Asn-specific cleavage in vitro (Okamoto et al. 1999). Also, *Arabidopsis* vegetative $\gamma$VPE was shown to be required for the processing of vacuolar carboxypeptidase Y (AtCPY) upon leaf senescence (Rojo et al. 2003). (ii) The 11S globulin from vetch seeds becomes susceptible to degradation by proteinase B (a legumain) only after destabilization by limited proteolysis (Müntz et al. 2002). Notwithstanding the evidence for a direct and/or indirect contribution of legumains to seed storage protein mobilization, redundant mechanisms must exist, as indicated by the apparently unaffected degradation of storage proteins in multiple VPE knockouts in *Arabidopsis* (Shimada et al. 2003b; Gruis et al. 2004).

## Cysteine proteases in senescence and programmed cell death (PCD)

Cysteine proteinases also serve important functions during senescence. Not surprisingly, there are many parallels to the mobilization of protein reserves in germinating seedlings, which eventually also leads to senescence and ultimately the death of the storage

organs. In 1970, specialized organelles were identified in the senescing endosperm of germinating castor bean and termed "dilated cisternae" to emphasize their development from the ER or "ricinosomes" to indicate their apparently unique occurrence in castor bean (*Ricinus communis*; Mollenhauer and Totten 1970; Vigil 1970). Ricinosomes are now known to be jam-packed with the precursor of cysteine endopeptidase (Cys-EP), a member of a subgroup of papain-like cysteine proteases characterized by the presence of a signal for ER retention (KDEL) at the carboxy terminus (Schmid et al. 1998, 2001). ProCys-EP is targeted to and retained in the ER by virtue of an amino-terminal signal peptide and the carboxy-terminal KDEL-motif, respectively. The proenzyme is then packaged into ricinosomes as they bud off from the ER (Schmid et al. 2001). ProCys-EP is processed and activated upon release from the ricinosomes to degrade cytosolic constituents during the final stages of cell death (Schmid et al. 1999). The recent elucidation of the Cys-EP crystal structure suggests that this enzyme may be particularly well suited to degrade a wide variety of proteins during cell death. The active-site cleft of Cys-EP, as compared to that of other papain-like enzymes, was found to be more open and can thus accommodate diverse substrates with many different amino acids, including proline, on both sides of the scissile bond (Than et al. 2004).

It has become increasingly clear in recent years that ricinosomes are not restricted to castor bean but may be a more general feature of senescing tissues where they appear to act as a "suicide bomb". Furthermore, KDEL-tailed cysteine proteases, marker enzymes of ricinosomes, were frequently shown to be associated with senescence (Schmid et al. 1999; Gietl and Schmid 2001, and references therein). Among the KDEL-tailed proteases, SH-EP from mung bean has been characterized in greatest detail. This papain-type enzyme has already been mentioned in the previous paragraph as a major player in the degradation of storage globulins (Okamoto and Minamikawa 1998). As for other KDEL-tailed cysteine proteases, proSH-EP is targeted to the secretory pathway co-translationally. It accumulates in the ER, from where it is packaged into 200- to 500-nm vesicles, named KDEL-vesicles (Toyooka et al. 2000). Unlike ricinosomes, which discharge their content into the cytosol during the final stages of cell death, KDEL-vesicles are transport vesicles in a Golgi-independent pathway for vacuolar protein targeting: KDEL-vesicles were shown to fuse with the PSV membrane to release proSH-EP into the PSV where it is converted into the active enzyme for storage protein degradation (Toyooka et al. 2000; Frigerio et al. 2001; Okamoto et al. 2003). Despite this apparent difference in function, the two types of vesicle are strikingly similar in terms of biogenesis and protein content, and they also serve similar purposes, i.e. the degradation of proteins for the mobilization and recycling of N-resources. Clearly, ricinosomes and KDEL-vesicles are highly related and may even be identical: the apparent discrepancy in function will possibly be resolved when more data become available on the function of ER-derived vesicles in senescence and other cell death events.

Developmental cell death resulting, for example, in the elimination of unpollinated ovules or the disintegration of the tapetum, the suspensor, integuments, the nucellus, and the aleurone layer, i.e. tissues that are needed for only a limited period of time during pollen, embryo, and seedling development, respectively, is a genetically programmed integral part of a plant's life cycle. Other examples include the formation of aerenchyma, the differentiation of xylem vessels, the shaping of lobed leaves, and the localized cell death at sites of pathogen infection, i.e. the hypersensitive response. Hydrolytic enzymes, including the KDEL-tailed cysteine proteases, are the executioners of cell death, catalyzing the hydrolysis of cellular constituents for resource reallocation. However, there is a growing body of evidence for an involvement of proteases, and cysteine proteases in particular, not only in the execution of the cell death program but also in its initiation (Beers et al. 2000).

The initiation of the cell death program in animals (apoptosis) involves a proteolytic cascade of caspases, i.e. a family of cysteine proteases specifically cleaving after aspartic acid residues. Triggered by either one of two canonical pathways involving (i) death receptors or (ii) the release of cytochrome *c* from mitochondria, dormant initiator caspases [caspase-8 (i) and -9 (ii)] are activated to directly process and activate downstream executioner caspases (caspases 3, 6, 7, and 14). The latter enzymes process specific target molecules such as the inhibitor (ICAD) of caspase-activated DNase (CAD), which ultimately leads to the hallmarks of apoptosis, including the shrinkage of the cytoplasm, nuclear condensation, DNA fragmentation, blebbing of the plasma membrane, and the formation of apoptotic bodies (Enari et al. 1998; Lam and del Pozo 2000; Hoeberichts and Woltering 2002; Woltering et al. 2002). Despite considerable efforts, no enzyme structurally and functionally related to animal caspases has yet been characterized from any plant source. There is ample evidence, however, for the involvement of Asp-specific proteases in the regulation of PCD also in plants.

In many instances of plant PCD, the induced proteases were able to cleave the specific peptide substrates of animal caspases (Beers et al. 2000; Lam and del Pozo 2000; Woltering et al. 2002; Belenghi et al. 2004; Danon et al. 2004). The plant enzyme(s), like animal caspases, are strongly inhibited by synthetic caspase inhibitors and have low sensitivity towards general inhibitors of cysteine proteinases (like E64), which has often been taken as support for a structural similarity beyond the substrate binding site. This conclusion, however, is not justified since synthetic caspase inhibitors are chloromethylketones (CMKs; e.g. Ac-YVAD-CMK for caspase-1) or peptide aldehydes (CHOs; e.g. Ac-DEVD-CHO for caspase-3). The peptide moiety of these inhibitors resembles the substrate and provides for the specificity of the inhibitor. The CMK and CHO reactive groups, on

the other hand, target cysteine, serine, as well as threonine proteases. Hence, the inhibitor studies yield information about the substrate specificity but not the catalytic type or structure of the protease. More meaningful results in this respect were obtained by the use of the baculovirus p35 inhibitor of caspases. The p35 gene product suppresses cell death in baculovirus-infected insect host cells to allow completion of viral replication (Clem et al. 1991). As for synthetic caspase inhibitors, specificity is provided by a tetrapeptide motif (DQMD) located in an exposed surface loop of the p35 protein that binds to the active site of the target protease. Cleavage of p35 results in suicide inhibition: a stable inactive complex is formed between the protease and the inhibitor fragments. P35 residues outside the DQMD pseudosubstrate motif were shown to contribute to complex formation, providing exclusive specificity and excluding non-caspase proteases (Fisher et al. 1999). Interestingly, expression of the p35 protein in transgenic tomato plants blocked PCD induced by host-selective mycotoxins or necrotrophic pathogens, and in transgenic tobacco, a partial inhibition of hypersensitive cell death was observed, indicating a role for caspases structurally related to the animal enzymes in the regulation of plant PCD (Lincoln et al. 2002; del Pozo and Lam 2003). In a similar approach, however, papain-like cysteine proteases were also implicated in PCD because inhibitors of papain-like enzymes (i.e. cystatins) were found to suppress PCD in soybean cells and in transgenic tobacco and *Arabidopsis* plants (Solomon et al. 1999 Belenghi et al. 2003).

While the available data support an involvement of plant proteases with caspase-like properties and cleavage specificity in the initiation of PCD in plants, it is still unclear whether or not these enzymes are also structurally related to their animal counterparts. Indeed, close plant homologs of animal caspases apparently do not exist. Recently, however, a family of distantly related putative cysteine proteases has been identified, i.e. the metacaspases (Uren et al. 2000). Metacaspases are represented by nine genes in *Arabidopsis* and a tomato metacaspase was found to be upregulated during pathogen-induced cell death (Hoeberichts et al. 2003). While metacaspases are candidate enzymes for a caspase-like function in plants, a direct involvement in PCD by reverse-genetic approaches and caspase-like activity remains to be demonstrated.

As compared to the candidate gene approach, the question of whether or not plant cysteine proteases with Asp-specificity (i.e. caspases) exist is more directly addressed by the purification of such activities from plant tissues and the identification of the respective enzymes. This approach has recently been taken by Chichkova et al. (2004). In this study, the *Agrobacterium* VirD2 protein was identified as a potential caspase substrate in a computer-assisted search, and shown to be processed by human caspase-3. Using a green fluorescent protein (GFP)–VirD2 fusion protein transiently expressed in tobacco leaves as a reporter, the induction of a caspase-like activity during tobacco mosaic virus (TMV)-induced hypersensitive cell death was monitored in vivo. A synthetic peptide inhibitor was designed based on the cleavage site in the VirD2 protein and was found to counteract TMV-induced cell death. The activity has been partially purified from tobacco leaf tissue and was tentatively confirmed as a cysteine protease (Chichkova et al. 2004). A more detailed characterization of this interesting enzyme seems to be only a matter of time. The last word, so far, in this controversial issue was had by Coffeen and Wolpert (2004) who purified two highly similar Asp-specific proteases from oat leaves that had been treated with the mycotoxin victorin to induce cell death. These enzymes, apparently involved in a proteolytic cascade leading to PCD, turned out not to be cysteine proteases but serine proteases and, in analogy to animal caspases, were named saspase-1 and -2 (Asp-specific serine protease). Saspases belong to the S8 family of subtilisin-like serine proteases, which will be discussed in more detail in the following section.

## Serine proteases

The class of serine proteases appears to be the largest class of proteases in plants. The MEROPS database lists 248 enzymes for *Arabidopsis* representing 8 of the known 12 clans. Three of them, the chymotrypsin PA(S), subtilisin (SB), and carboxypeptidase D (SC) clans, share a common reaction mechanism based on a well-characterized ''catalytic triad'' comprising a serine, an aspartic acid, and a histidine residue (Fig. 1). While functionally similar, the protein folds of the three clans and the arrangement of the three catalytically important residues in the primary structure are different, providing a striking example of convergent evolution. The enzymes selected for discussion in this chapter belong to family S8A in clan SB (subtilases) and family S10 in clan SC (carboxypeptidases), which together comprise more than half of all the serine proteases in *Arabidopsis*. The size of both of these families, being much larger in plants as compared to animals, may point to the acquisition of new, plant-specific functions in the course of evolution.

### Serine carboxypeptidases

A well-established function of serine carboxypeptidases (SCPs) is protein turnover for the mobilization of N-resources as it occurs during seed germination and wound stress, and part of their diversity has been attributed to differences in substrate specificity (Walker-Simmons and Ryan 1980; Mehta et al. 1996; Moura et al. 2001; Dal Degan et al. 1994; Granat et al. 2003, and references therein). SCPs have also been implicated in the autolysis of cellular constituents during PCD (Domínguez and Cejudo 1998; Domínguez et al. 2002), in brassinosteroid signaling (Li et al. 2001), and seed development (Cercos et al. 2003). The

surprising diversity of SCPs, however, may rather be explained by the acquisition of novel, non-proteolytic functions: apparently, in the course of evolution, SCPs were recruited by plant secondary metabolism to catalyze the transfer of acyl groups (transacylations; Steffens 2000; Milkowski and Strack 2004). SCP-like enzymes were shown to catalyze the regiospecific formation of diacylglucose (1,2-di-*O*-acylglucose), sinapoylmalate, and sinapoylcholine using the respective glucose esters (1-*O*-acylglucose, 1-*O*-sinapoylglucose) as acyl donors (Lehfeldt et al. 2000; Li and Steffens 2000; Shirley et al. 2001; Shirley and Chapple 2003; Milkowski et al. 2004). Considering the chemical similarity between ester and peptide bonds, this finding is easily reconciled with the reaction mechanism of serine proteases: during catalysis, the active-site serine is acylated as a consequence of its nucleophilic attack at the carbonyl carbon of either peptide or ester bonds. A covalent acyl-enzyme intermediate is formed which is then hydrolyzed, resulting in proteolytic or esterolytic activity of the enzyme (Fig. 1). In the case of transacylation, on the other hand, the acyl-enzyme intermediate is cleaved not by water (step 3 in Fig. 1) but by the second substrate (acylglucose, choline, or malate; Steffens 2000). To favor cleavage by the acyl acceptor and to prevent hydrolysis, SCP-like acyltransferases appear either to shield the active site and eliminate water or else to activate the hydroxyl group of the acceptor rendering it a stronger nucleophile as compared to water. Experiments designed to differentiate between the two possible mechanisms give a still controversial picture, supporting either one of the two scenarios (Milkowski and Strack 2004). Possibly, both mechanisms have been implemented by nature during the evolution of different SCP-like acyltransferases.

The limited data available do not allow an identification of SCPs as peptidases or acyltransferases merely on the basis of their amino acid sequence. Therefore, some conclusions relating to the function of carboxypeptidases may have to be re-evaluated. For example, the *BRS1* gene product in *Arabidopsis*, coding for an SCP-like enzyme, was shown to suppress brassinosteroid insensitivity in a brassinosteroid perception mutant (*bri1*). Active-site mutants of BRS1 (S181F and H438A of the catalytic triad) lacked this activity and it was concluded that a carboxypeptidase processes a protein involved in an early step of brassinosteroid signaling (Li et al. 2001). Since the proteolytic and transacylase activities of SCP-like enzymes rely on the same mechanism for catalysis, these mutations would have affected both activities equally, and BRS1 may as well be a transacylase rather than a carboxypeptidase.

Considering the versatility of the catalytic triad of serine proteases (Fig. 1), the size of the S10 family of carboxypeptidase-like enzymes, and the demands of a complex secondary metabolism, a broader role of SCP-like enzymes, and possibly also subtilases, in the formation of secondary plant compounds may be anticipated (Milkowski and Strack 2004).

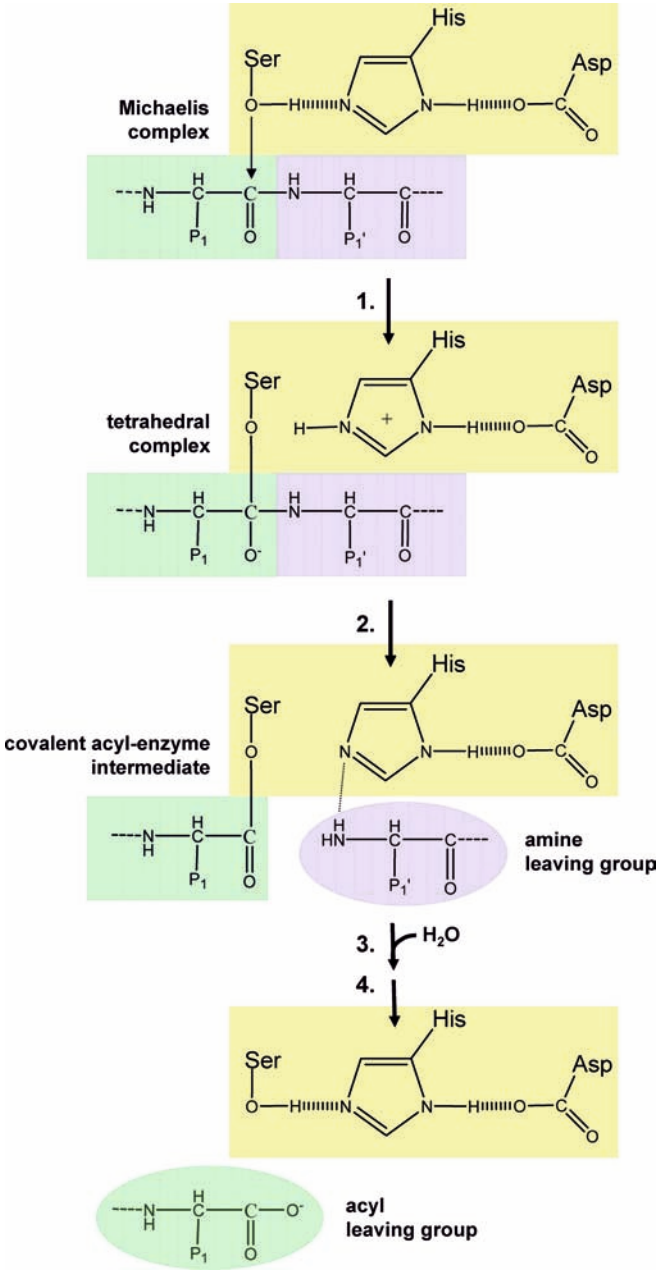


**Fig. 1** reaction mechanism of a serine protease. The catalytic triad of the protease is highlighted in *yellow*, the parts of the substrate amino- and carboxy-terminal to the scissile bond in *green* and *purple*, respectively. *Broken lines* indicate hydrogen bonds and the continuation of the polypeptide chain of the substrate. The reaction is initiated by a nucleophilic attack of the active-site serine at the carbonyl C of the scissile bond and transfer of a proton from the serine hydroxyl to the active site histidine (*1.*). The proton transfer is facilitated by the catalytic triad with aspartate stabilizing the developing positive charge. The proton is then donated to the nitrogen atom and the peptide bond is thus cleaved (*2.*). The amino-terminal part of the substrate is esterified to the enzyme serine in a covalent acyl intermediate and the carboxy-terminal part is released. This completes the acylation stage of the reaction (*1.* and *2.*). The deacylation (*3.* and *4.*) is a reversal of the acylation, with $H_2O$ substituting for the amine component of the substrate

## Subtilases

Subtilases are characterized by a unique arrangement of the Asp, His, and Ser residues in the catalytic triad of the active site and constitute the S8 family within the clan SB of serine proteases. The MEROPS database groups subtilases into two subfamilies, those of true subtilisins (S8A subfamily), including the prototypical subtilisin Carlsberg from *Bacillus licheniformis*, and kexins (S8B subfamily). Kexin was identified in 1988 as the first eukaryotic subtilase, required in yeast for the processing of the precursors of the α-mating factor pheromone and of killer toxin (Fulleret al. 1988). The interest in subtilases was greatly stimulated when it was discovered in the 1990s that the mammalian homologs of kexin are the long-sought proprotein convertases, responsible in mammals for the formation of peptide hormones, growth factors, neuropeptides, and receptor proteins from inactive proproteins by limited proteolysis at highly specific sites (Barr 1991; Seidah et al. 1994). Nine subtilases have been identified in mammals, seven in the kexin subfamily and two in the subtilisin subfamily (Sakai et al. 1998; Seidah et al. 1999, 2003). While kexins appear to be absent from plants, 56 genes predicted to encode functional subtilisins have been annotated in the *Arabidopsis* genome (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcawp/psdb.html; for detailed information on the organization of the gene family the reader is referred to the recent article of Beers et al. (2004) and genes and/or cDNAs of 15 subtilisin-like proteases have been cloned from tomato (Meichtry et al. 1999). Despite their prevalence, our current understanding of subtilase function in plants is still very limited.

A number of proteases have been purified from plant tissues and classified as subtilases based on their catalytic properties and/or primary structure (see e.g. Bogacheva 1999; Fontanini and Jones 2002; Popovic et al. 2002, and references therein). Most of these enzymes are highly abundant and exhibit broad substrate specificity. They are thus believed to function as non-selective enzymes in general protein turnover. Examples of degradative subtilases are cucumisin, which constitutes up to 10% of the soluble protein in melon fruit and cleaves a broad variety of peptide and protein substrates at the carboxy side of charged amino acids (Kaneda and Tominaga 1975; Yamagata et al. 1994; Uchikoba et al. 1995), and macluralisin from the fruits of *Maclura pomifera* for which similar characteristics have been reported (Rudenskaya et al. 1995). Considering the complexity of the subtilase gene family, which seems to exceed the needs for merely degradative functions, it is tempting to speculate that plant subtilases, like their mammalian homologs, may perform more specific roles in the processing of precursor proteins to regulate growth and development by limited proteolysis. Such processing proteases would be expected to exhibit much more stringent substrate requirements as compared to degradative enzymes in non-selective protein turnover. Biochemical characterization has in fact revealed high selectivity for specific cleavage sites for the subtilases Ara12 from *Arabidopsis* (Hamilton et al. 2003), LeSBT1 from tomato (Janzik et al. 2000) and C1 from soybean (Boyd et al. 2002), and for two highly related saspases from oat (Coffeen and Wolpert 2004). The saspases require Asp in the $P_1$ position of their substrates and have been implicated in a proteolytic cascade for programmed cell death (Coffeen and Wolpert 2004). The C1 subtilase, showing strong preference for Glu–Glu and Glu–Gln bonds, was shown to initiate the breakdown of soybean storage proteins (α-conglycinin) by limited proteolysis in the N-terminal region (Boyd et al. 2002). The LeSBT1 and Ara12 subtilases show a preference for Gln and hydrophobic residues in the $P_1$ positions of their respective substrates (Janzik et al. 2000; Hamilton et al. 2003). Their physiological substrates, however, have not been identified and the suggested role as proprotein convertase-like processing enzymes remains to be demonstrated.

This is true for the majority of plant subtilases for which certain physiological roles are supported only by circumstantial evidence mostly derived from stress-induced-, tissue-, or development-specific patterns of expression. Tomato P69, for example, has been implicated in pathogen defense as it was identified initially as a pathogenesis-related protein and was later shown to be one of several closely related subtilases that are specifically induced following pathogen infection (Vera and Conejero 1988; Tornero et al. 1996; Jordá et al. 1999). The P69s form a distinct subgroup among the 15 cloned tomato subtilases, most of which are expressed in a tissue-specific manner (Meichtry et al. 1999). Tomato tmp, like its ortholog in lily (Lim9) has been implicated in microsporogenesis based on its highly tissue- and development-specific expression in anthers during late stages of pollen development (Taylor et al. 1997; Riggs and Horsch 1995). The *Arabidopsis* subtilases XSP1 and AIR1 appear to be involved in xylem differentiation and lateral root formation, as indicated by the specific expression in the respective tissues (Neuteboom et al. 1999; Zhao et al. 2000). Tissue-specific and developmentally regulated expression was also shown for the *Arabidopsis* subtilases SLP2 (subtilisin-like protease 2), SLP3, and ARA12 (Golldack et al. 2003; Hamilton et al. 2003). Other examples include ag12, which appears to be involved in *Alnus* in the development of root nodules for nitrogen fixation (Ribeiro et al. 1995), and a seed coat-specific subtilase in soybean (Batchelor et al. 2000). Indirect evidence was obtained also for the involvement of a subtilase in the processing of systemin (Schaller and Ryan 1994) and the presence of subtilases in the plant secretory pathway (Kinal et al. 1995, Jiang and Rogers 1999). In all of the above examples however, a clear cause-and-effect relationship between the observed pattern of expression and the suspected role in defense or development remains to be established.

Strongest support for a role as processing proteases is derived from the characterization of *Arabidopsis* loss-of-function mutants, which identified subtilases as

highly specific regulators of plant development. In the *Arabidopsis sdd1* mutant (*stomatal density and distribution 1*) the pattern of stomata formation is disrupted, resulting in the clustering of stomata and a higher than normal stomatal density. The *SDD1* gene was identified by positional cloning and was found to encode a subtilase (Berger and Altmann 2000). The SDD1 protease is transiently expressed in the precursors of stomates, i.e. meristemoids and guard mother cells. It is secreted into the apoplast where it is thought to act as a processing protease in the generation of a signal responsible for regulation of stomatal density (von Groll et al. 2002). Likewise, the gene disrupted in the *ale1* mutant (*abnormal leaf shape 1*) was cloned and found to encode a subtilase. *ALE1* is required for cuticle formation and epidermal differentiation during embryo development in *Arabidopsis*. A role for ALE1 in the generation of a peptide signal required for the proper differentiation of the epidermis was suggested (Tanaka et al. 2001).

The mutant phenotypes of *sdd1* and *ale1* demonstrate that (at least some) subtilases serve highly specific functions in plant development. Their mode of action in the regulation of the respective developmental processes is still unknown but has been interpreted within the framework set by mammalian subtilases: SDD1 and ALE1 may be required for the generation of (poly)peptide signals which act non-cell-autonomously to control plant development. This notion is supported by the interesting observation that SDD1 depends on TMM (Too Many Mouths), a member of the large leucine-rich repeat receptor-like kinase (LRR-RLK) family in *Arabidopsis* and required for regulation of stomatal density (Nadeau and Sack 2002; von Groll et al. 2002). In LRR-RLKs, the extracellular LRR domain is a typical protein–protein-interaction module thought to be responsible for the binding of (poly)peptide ligands leading to kinase activation (Yin et al. 2002). SDD1 and TMM appear to be involved in the same cell-to-cell communication system and it is an intriguing possibility that the subtilase SDD1 is involved in the processing of the ligand for the TMM receptor kinase.

## Aspartic proteases

The MEROPS database lists 158 aspartic proteases (APs) for *Arabidopsis* in 3 families, i.e. the family of presenilins (A22), uncharacterized in plants but crucial to the development of Alzheimer's disease in humans, the large family of retrotransposon proteases (A11), and the pepsin family (A1). The 59 A1 proteases from *Arabidopsis* have recently been annotated and grouped into five distinct subfamilies (Beers et al. 2004) one of which, the phytepsins, has been investigated in considerable detail (Chen et al. 2002b). Phytepsins, originally characterized from barley seeds (Runeberg-Roos et al. 1991; Sarkkinen et al. 1992), are most closely related to mammalian cathepsin D and yeast vacuolar proteinase A. They are distinguished from their non-plant homologs by an insertion of approximately 100 amino acids, the so-called plant-specific insert, C-terminal of the two D-T/S-G active-site motifs. The name is not quite appropriate, however, since the plant-specific insert is absent not only from mammalian and microbial APs but also from other members of the plant A1 family. The plant-specific insert is homologous to saposins (i.e. lysosomal sphingolipid-activating enzymes in mammalian cells) and, therefore, more adequately described as the saposin-like domain (SLD). The SLD may contribute to the substrate specificity of APs (Payie et al. 2003) and, because of its membrane-permeabilizing activity, a role in plant defense has been proposed (Egas et al. 2000). Most importantly, the SLD was shown to be required for the exit of the proenzyme from the ER and targeting to the vacuole (Ramalho-Santos et al. 1998; Kervinen et al. 1999; Törmäkangas et al. 2001). In the vacuole, prophytepsins are proteolytically processed to yield the active enzyme. The SLD may be retained resulting in a monomeric AP or processed to yield a heterodimeric enzyme of two disulfide-linked polypeptide chains (Mutlu and Gal 1999).

Biochemical and structural analyses have shed light on the mechanisms involved in zymogen processing, the function of the prodomain in the inactivation of prophytepsins, and the role of the SLD in protein sorting (reviewed by Simões and Faro 2004). Our current knowledge about the physiological roles of phytepsins (and other APs), however, is still very limited and based mostly on indirect evidence derived from specific expression patterns, co-localization studies with putative protein substrates, and degradation or processing of the suspected substrates in vitro. Phytepsins have thus been implicated in the processing of seed storage proteins in numerous plant species, in protein degradation for the mobilization of nitrogen resources during seed germination, organ senescence, and cell death, in defense against microbial pathogens and insect herbivores, and in pollen/pistil interaction. These data have been reviewed in several recent reviews and will not be covered here in any more detail (Mutlu and Gal 1999; Beers et al. 2004; Simões and Faro 2004). I will rather discuss the few novel APs described recently outside the phytepsin family.

Carnivorous pitcher plants (*Nepenthes*) accumulate a digestive fluid containing APs in their insect-trapping organs. Two APs, nepenthesins I and II have recently been purified and characterized from the pitcher fluid of *N. distillattoria* and the corresponding cDNAs have been cloned from *N. gracilis* (Athauda et al. 2004). The two nepenthesins lacked the SLD of phytepsins and were found to belong to a distinct subfamily of APs. Nepenthesins appear to be well adapted to their digestive function in an extreme environment: they were found to be extraordinarily stable up to 50°C over a wide pH range and exhibited a somewhat broader substrate specificity as compared to other APs. Nepenthesin I cleaved the insulin B chain at five major sites as compared to three cleavage sites for pepsin A and

cathepsin D (Athauda et al. 2004). Nepenthesins may be just two of a battery of APs in the digestive fluid of pitcher plants, since two phytepsin-type APs were previously found to be expressed specifically in the digestive glands in the lower part of the pitchers, which is consistent with the proposed role in the digestion of the insect prey (An et al. 2002).

Despite their prevalence in the *Arabidopsis* genome, very few APs without SLD have hitherto been characterized from plants. Other than nepenthesins, they include nucellin and CND41 implicated in the PCD of the nucellus and the negative regulation of chloroplast gene expression, respectively (Chen and Foolad 1997; Murakami et al 2000). The latest addition to this group of APs is CDR1, which functions in *Arabidopsis* disease resistance signaling (Xia et al. 2004). The *CDR1* gene (*CONSTITUTIVE DISEASE RESISTANCE 1*) was identified by T-DNA activation tagging in a screen for gain-of-function mutants that show enhanced resistance to bacterial infection. Loss of *CDR1* function, on the other hand, compromised the resistance to avirulent bacteria and increased susceptibility to virulent strains. CDR1 was found to encode an extracellular AP the activity of which is required for the mounting of salicylic-acid-dependent disease resistance. Intriguingly, the local activation of CDR1 was shown to mediate a mobile signal for the induction of systemic defense responses, suggesting that the long-sought signal molecule for long-distance signaling of systemic acquired resistance may be a peptide released by the proteolytic activity of CDR1 (Xia et al. 2004).

## Metalloproteases

Metalloproteases rely on a divalent cation for activity, most commonly zinc, but also cobalt or manganese, which functions in catalysis by activating a water molecule for nucleophilic attack of the scissile peptide bond. Of the six catalytic types of protease, metalloproteases are the most diverse in terms of both structure and function. More than 50 families have been identified in 24 clans. The *Arabidopsis* genome comprises 81 genes for metalloproteases in 12 different clans. Metalloproteases in plants include exo- and endopeptidases in many different subcellular locations, with merely degradative or highly specific processing function. Only very few of these enzymes have been characterized to date. I will concentrate here on two of them, leucine aminopeptidase and stromal processing peptidase.

### Leucine aminopeptidase

Leucine aminopeptidases (LAPs; M17 family, clan MF) are ubiquitous enzymes, conserved between prokaryotes, animals, and plants (Bartling and Weiler 1992). In all organisms investigated, active LAPs are oligomers of six identical subunits. The primary structure of the subunits is conserved as well, in particular the C-termini containing the invariant zinc-binding residues. The involvement of these residues in catalysis and the requirement of homo-hexamer formation for activity were confirmed by site-directed mutagenesis for tomato LAP-A (Gu and Walling 2002). The similarity of LAPs from different kingdoms extends to the reaction mechanism and the substrate specificity: a comparison of substrate specificity between porcine, *Escherichia coli*, and tomato LAPs showed that all three enzymes efficiently cleave N-terminal Leu, Arg and Met but not Asp or Gly residues. The three enzymes were found to be similar also in their preference for amino acids in the penultimate position but differed in their tolerance of aromatic amino acids at the N-termini of their substrates (Gu and Walling 2000, 2002).

The function of eukaryotic LAPs is not entirely understood. LAP in animals is an abundant protein of the eye lens and may be involved in the degradation of crystalline protein deposits (Taylor et al. 1982). The remarkable stability of LAPs is a prerequisite for their role as eye lens proteins in animals and is also observed for LAPs of plant origin (Bartling and Weiler 1992; Herbers et al. 1994; Gu et al. 1999). Both animal and plant LAPs may be important for the regulation of protein half-life, which is largely influenced by the N-terminal amino acid residue (the ''N-end-rule''; Varshavsky 1996). Cleavage of the N-terminal amino acid may either remove or expose a destabilizing residue and result in the stabilization of the respective protein or in targeting for degradation by the ubiquitin/proteasome system. Antisense suppression of LAPs in tomato and comparison of protein extracts between wild-type and antisense plants, however, did not reveal reproducible differences in protein composition during two-dimensional gel electrophoresis (Pautot et al. 2001). Likewise, in potato, no phenotypic alterations were observed between wild-type and LAP antisense plants (Herbers et al. 1994). In antisense-inhibited potato plants, LAP transcripts were reduced below the detection limit while 80% of the activity persisted in leaf extracts. Therefore, the lack of a discernable phenotype may be explained by additional peptidases with redundant activities (Herbers et al. 1994). Indeed, the analysis of the *Arabidopsis* genome indicates the existence of further aminopeptidases in families M1 (alanyl aminopeptidases), M18 (aspartyl aminopeptidases), and M24 (methionyl aminopeptidases and aminopeptidase P) of metalloproteases. The only plant enzyme characterized from any of these families, however, aminopeptidase P from tomato (Hauser et al. 2001), is specific for amino-terminal Xaa-Pro bonds and is thus not likely to contribute to the residual LAP activity in antisense-inhibited potato leaves.

In addition to the common LAP (LAP-N) which is constitutively present in all plant species (Bartling and Nosek 1994; Chao et al. 2000), some plants of the nightshade family have additional LAPs (LAP-A1 and A2 in tomato) expressed in the reproductive organs

during normal development and upregulated via the octadecanoid signaling pathway under several stress conditions, including osmotic stress, wounding, and pathogen infection (Hildmann et al. 1992; Pautot et al. 1993, 2001; Schaller et al. 1995; Chao et al. 1999). LAP is thought to facilitate the mobilization of C and N resources from cells committed to die and to enhance protein turnover to allow for metabolic reprogramming in response to herbivore or pathogen attack (Gu et al. 1999). A similar role during jasmonic acid-induced senescence has also been suggested (Herbers et al. 1994).

### Stromal processing peptidase and other M16 peptidases

The vast majority of chloroplast proteins are nuclear-encoded, translated in the cytosol as larger precursor proteins, and posttranslationally imported into the plastids. Import of the precursor proteins relies on an amino-terminal targeting signal, the transit peptide, which is proteolytically removed in the plastid stroma. Considering, on the one hand, the abundance of chloroplast proteins and, on the other hand, the importance of plastids for photosynthesis, plant development, and the biosynthetic capacity of plants, cleavage of the transit peptide may well be the most important post-translational protein modification in the plant cell (Richter and Lamppa 2002). Removal of the transit peptide is catalyzed by stromal processing peptidase (SPP), a soluble metalloprotease similar to pitrilysin, insulin-degrading enzyme, and the $\beta$-subunit of mitochondrial processing peptidase (family M16 in clan ME of metalloproteases; VanderVere et al. 1995; Richter and Lamppa 1998). Precursor-protein processing involves at least three steps. The initial recognition of the precursor protein is independent of SPP activity but depends on an interaction of the N-terminal domain of the protease and the C-terminal 10–15 residues of the transit peptide. Subsequently, in a first endoproteolytic reaction, the mature protein is released but the transit peptide remains bound to SPP. A second cleavage reaction disrupts the binding site in the C-terminal region of the transit peptide and the subfragments are released. Both proteolytic events depend on the HXXEH Zn-binding motif close to the amino-terminus of SPP (Richter and Lamppa 1999, 2003). Antisense suppression of the single SPP gene in *Arabidopsis* showed that SSP is an essential component of the chloroplast protein import machinery and necessary for chloroplast biogenesis and plant survival (Zhong et al. 2003). Interestingly, chloroplast import of GFP equipped with an amino-terminal transit peptide was impaired in SPP-antisense lines, indicating that SPP-mediated maturation of the protein in the chloroplast stroma is not an isolated event but affects earlier steps in the import pathway (Zhong et al. 2003).

Despite the enormous amount of protein imported into plastids—an uptake rate of $2.5\times10^4$ proteins per minute per chloroplast has been estimated for the ferredoxin precursor in vitro (Pilon et al. 1992)—cleaved transit peptides have not been observed to accumulate in vitro or in vivo. Hence, a proteolytic system for selective elimination of the transit peptide from the chloroplast stroma has been postulated and tentatively identified as a soluble ATP-dependent metalloprotease (Richter and Lamppa 1999, 2002). Similarly in mitochondria, a protease(s) must exist for the removal of targeting peptides, which are potentially harmful due to their membrane-permeabilizing activity. In fact, a presequence-degrading protease (PreP) has recently been isolated chromatographically from potato tuber mitochondria and identified as a zinc-dependent metalloprotease (Zn-MP; Ståhl et al. 2002). Intriguingly, the *Arabidopsis* homolog (AtZn-MP) was found to cleave both mitochondrial and chloroplast targeting peptides in vitro and, furthermore, was targeted to both mitochondria and chloroplasts in vivo (Bhushan et al. 2003; Moberg et al. 2003). The data strongly indicate that the degradation of targeting sequences is ATP-independent and catalyzed by Zn-MP, a metalloprotease with dual function and ambiguous targeting to plastids and mitochondria.

Incidentally, the two proteases catalyzing the removal and the degradation of the targeting sequence are highly related. SPP and Zn-MP belong to subfamilies M16B and M16C in clan ME, respectively. The remaining subfamily, M16A, comprises homologs of the mammalian insulin-degrading enzyme with two representatives in the *Arabidopsis* genome. The tomato homolog has been cloned in a screen for proteases able to cleave the polypeptide wound hormone systemin (Pearce et al. 1991), suggesting a role in the regulation of the wound response (Strassner et al. 2002). While the recombinant enzyme expressed in *E. coli* was shown to cleave and inactivate systemin in vitro, such a function remains to be demonstrated in vivo (Strassner et al. 2002). A distinguishing feature of all the proteases in the M16 family seems to be the lack of preference for certain amino acids as determinants of substrate specificity. Potential substrates appear to be recognized by higher-order structural features (or the lack thereof) rather than specific motifs in the primary amino acid sequence (Kurochkin 2001; Richter and Lamppa 2002; Moberg et al. 2003).

## Concluding remarks

Facing more than 600 different proteases in the *Arabidopsis* genome, this review does little more than touch the tip of the iceberg. This is partly the fault of the author who selected proteases from the main catalytic groups according to personal preference and interest, albeit trying to highlight areas that have seen substantial progress in recent years, or discuss subjects that are still a matter of controversy. Nevertheless, the choice is a subjective one and I am guilty of neglecting other important enzymes. Omission of the catalytic group of threonine peptidases may be justifiable since they are typical of the 26S proteasome, which has been the focus

of many recent review articles (see Introduction for references). Also neglected were large groups of proteases in chloroplasts and mitochondria, like the ATP-dependent Clp, Lon, and FtsH proteases, as well as the DegP-like enzymes, which may be equally important in the regulation of physiological processes and development (Kuroda and Maliga 2003). For more information on these interesting proteolytic systems the reader is referred to recent review articles (Adam et al. 2001; Adam and Clarke 2002). Furthermore, there are emerging fields like that of plant matrix metalloproteases (matrixins), which have not been covered here but are likely to attract considerable attention in the future (Maidment et al. 1999; Golldack et al. 2002). Notwithstanding the omissions, I hope that the selected topics give an impression of the mechanisms, the proteases involved, and also the potential for proteases in the control of plant development. Stimulated by the recognition of proteolysis as an important regulator in all stages of the plant life cycle, the whole field is rapidly expanding and we are likely to witness in the near future the discovery of many more proteases intricately involved in plant development.

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