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Plant Proteases: From Phenotypes to Molecular Mechanisms

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Key Words

phytocalpain, papain-like cysteine proteases, deconjugating proteases, subtilases, serine carboxypeptidase-like proteases, pepsin-like aspartic proteases

Abstract

Plant genomes encode hundreds of proteases, which represent dozens of unrelated families. The biological role of these proteases is mostly unknown, but mutant alleles, gene silencing, and overexpression studies have provided phenotypes for a growing number of proteases. The aim of this review is to show the diversity of the processes that are regulated by proteases, and to summarize the current knowledge of the underlying molecular mechanisms. The emerging picture is that plant proteases are key regulators of a striking variety of biological processes, including meiosis, gametophyte survival, embryogenesis, seed coat formation, cuticle deposition, epidermal cell fate, stomata development, chloroplast biogenesis, and local and systemic defense responses. The functional diversity correlates with the molecular data: Proteases are specifically expressed in time and space and accumulate in different subcellular compartments. Their substrates and activation mechanisms are elusive, however, and represent a challenging topic for further research.

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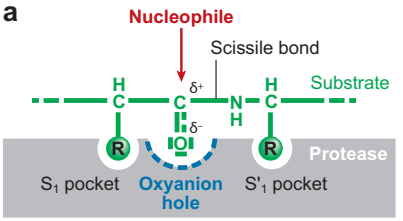
Contents

INTRODUCTION

As in the case of all other organisms, plants use proteases to degrade nonfunctional proteins into amino acids. This is common textbook knowledge, but there is more to proteases than this housekeeping function. Proteases are also key regulators. By irreversibly determining the fate of other proteins, they regulate different processes in response to developmental and environmental cues. This implies that proteases are substrate specific, and that their activity is tightly regulated, both in time and space. Testimony for the existence of regulatory proteases in plants is relatively recent and is summarized in this review.

Proteases cleave peptide bonds that can be internal (for endopeptidases), N-terminal (for aminopeptidases), or C-terminal (for carboxypeptidases). All proteases polarize the carbonyl group of the substrate peptide bond by stabilizing the oxygen in an oxyanion hole, which makes the carbon atom more vulnerable for attack by an activated nucleophile (**Figure 1*a***). Proteases can do this in four major ways, which gives the names to four catalytic classes: cysteine proteases, serine proteases, metalloproteases, and aspartic proteases (30) (**Figure 1*b***).

Proteases in the MEROPS protease database have been subdivided into families and clans on the basis of evolutionary relationships (**http://merops.sanger.ac.uk**) (69). The *Arabidopsis* genome encodes over 800 proteases, which are distributed over almost 60 families, which belong to 30 different clans (**Figure 2*b***). The distribution and the family size are well conserved within the plant



b

Catalytic class	Nucleophile	Oxyanion stabilizer
Cysteine proteases	**Cys**-His	-NH-(2x)
Serine proteases	**Ser**-His	-NH-(2x)
Metalloproteases	**H_2O**-Me^{2+}	Me^{2+}
Aspartic proteases	**H_2O**-Asp	H^+-Asp

Figure 1

Cleavage mechanisms of the four major catalytic classes of proteases. (*a*) The substrate protein (*green*) binds via amino acid residues (R) to the substrate binding site of the protease (*gray*) by interacting with substrate (S) pockets of the enzyme. The scissile peptide bond is adjacent to a carbonyl group, which is polarized by the enzyme by stabilizing the oxyanion hole (*blue*); this makes the carbonyl carbon vulnerable for nucleophilic attack. (*b*) The major differences between the catalytic classes are the nature of the nucleophile and oxyanion stabilizer. Cysteine and serine proteases use a Cys or Ser residue as nucleophile, activated by histidine (His) in the active site. The oxyanion hole is usually stabilized by two residues in the backbone of the protease. Metalloproteases and aspartic proteases use water as nucleophile, activated by electrostatic interactions with the metal ion (Me^{2+}) or aspartate (Asp), respectively. The oxyanion of these proteases is stabilized by Me^{2+} and Asp, respectively.

MEROPS: protease database, named after a tropical bird living in families and clans

Clan: group of protease families that share the same ancestor

Family: group of proteases that share a certain level of sequence homology

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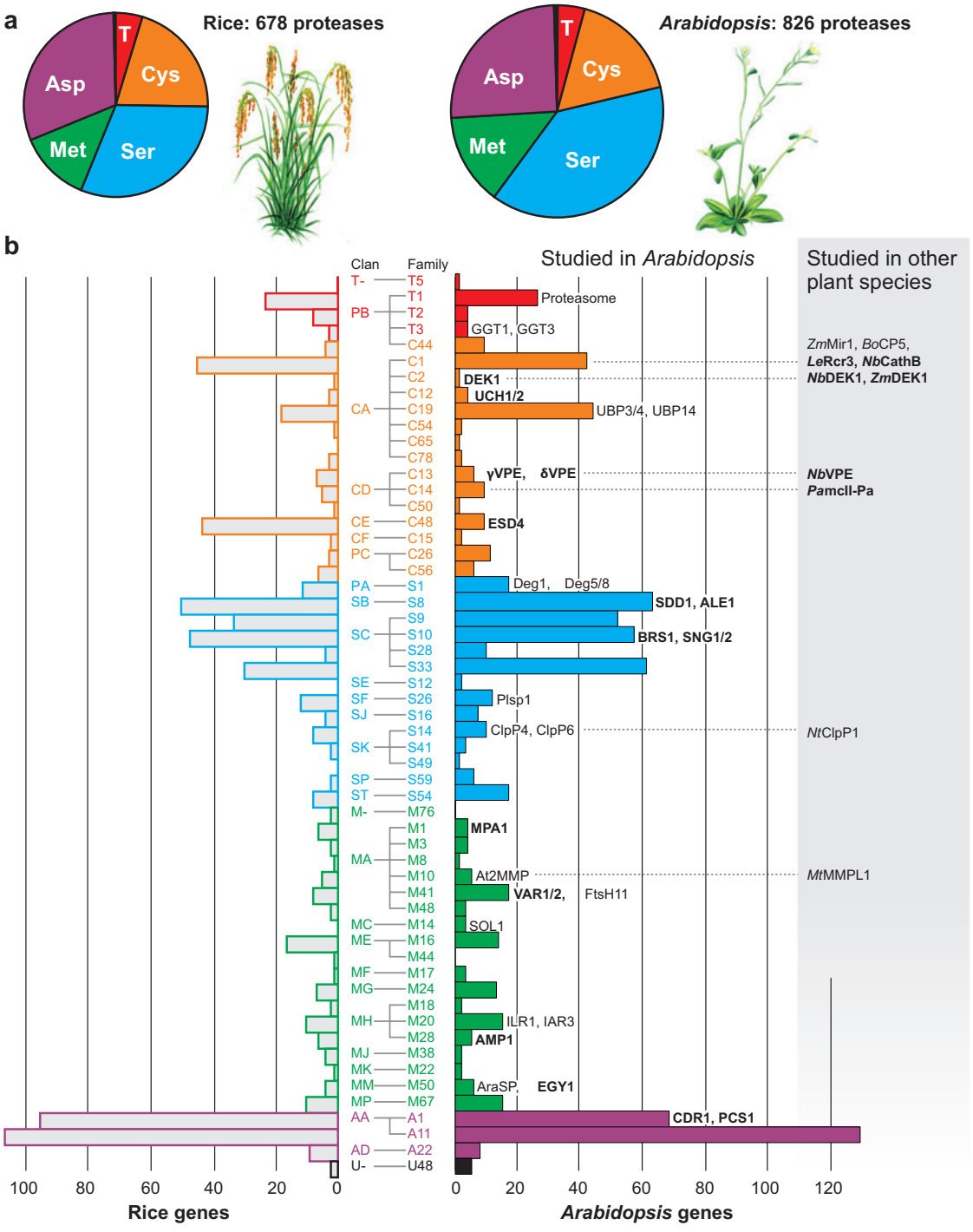

Figure 2

Distribution of rice (*left*) and *Arabidopsis* (*right*) protease genes over (*a*) the different catalytic classes, and (*b*) the different families (*right*) and clans (*left*). Proteases for which biological roles are known from genetic experiments are indicated on the right (see also **Table 1**). Proteases discussed in this article are indicated in bold.

Table 1 Proteases with known phenotypes

Name of protease[a]	Accession	Species[b]	Family	Phenotype observed[c]	Reference
GGT1	At4g39640	*At*	T3	KO: premature senescence after flowering	59
GGT3	At1g69820	*At*	T3	KO: reduced number of siliques and seeds	59
Rcr3	AF493234	*Le*	C1A	KO: loss of recognition of fungal pathogen	49
***Nb*CathB**	DQ492297	*Nb*	C1A	KD: suppressed hypersensitive cell death	35
Mir1	AAB70820	*Zm*	C1A	OE: inhibits caterpillar growth	67
*Bo*CP5	AF454960	*Bo*	C1A	KD: suppresses broccoli postharvest senescence	31
DEK1	AY061804	*Zm*	C2	KO: no/reduced aleurone on kernels	7
***Nb*DEK1**	AY450851	*Nb*	C2	KD: callus-like surface on all organs	1
***At*DEK1**	At1g55350	*At*	C2	KD: altered epidermal cell fate	43
UCH1/2	At5g16310	*At*	C12	KO: more branches; OE: less branches	101
UBP1/2	At1g177110	*At*	C19	KO: enhanced susceptibility to canavanine	100
UBP3/4	At4g39910	*At*	C19	KO: impaired pollen development	26
UBP14	At3g20630	*At*	C19	KO: embryos arrest at the globular stage	27
***Nb*VPE**	AB181187	*Nb*	C13	KD: blocked hypersensitive cell death	38
γVPE	At4g32940	*At*	C13	KO: reduced toxin-induced cell death	51
δVPE	At3g20210	*At*	C13	KO: delayed cell death in seed coat	65
mcII-Pa	AJ534970	*Pa*	C14	KD: reduced cell death during embryogenesis	12
ESD4	At4g15880	*At*	C48	KO: early flowering, pleiotropic effects	64
Deg1	At3g27925	*At*	S1	KD: reduced growth, early flowering	46
Deg5(/8)	At4g18370	*At*	S1	KO: reduced growth under high light	85
SDD1	At1g04110	*At*	S8	KO: altered stomata density and distribution	9
ALE1	At1g62340	*At*	S8	KO: lacks embryo cuticle	88
BRS1	At4g30610	*At*	S10	OE: enhanced BR sensitivity	54
SNG1	At2g22990	*At*	S10	KO: no sinapoylmalate biosynthesis in leaves	52
SNG2	At5g09640	*At*	S10	KO: no sinapoylcholine biosynthesis in seeds	82
Plsp1	At3g24590	*At*	S26	KO: reduced plastid internal membranes, lethal	42
ClpP4	At5g45390	*At*	S14	KD: bleached leaves; OE: chlorotic rosette leaves	80, 105
ClpP1	Z00044	*Nt*	S14	KO: ablation of shoot system	50
ClpP6	At1g11750	*At*	S14	KD: chlorotic young rosette leaves	83
MPA1	At1g63770	*At*	M1	KO: disturbed meiotic chromosome segregation	78
At2MMP	At1g70170	*At*	M10	KO: slow growth, late flowering, early senescence	36
MMPL1	Y18249	*Mt*	M10	KD: larger infection threads; OE: aborted infection	21
VAR1	At5g42270	*At*	M41	KO: variegated leaves, stems, and siliques	76
VAR2	At2g30950	*At*	M41	KO: variegated leaves, stems, and siliques	18
FtsH11	At5g53170	*At*	M41	KO: loss of thermotolerance	16
SOL1	At1g71696	*At*	M14	KO: suppressor of restricted root meristem	13
ILR1	At3g02875	*At*	M20	KO: insensitive to exogenous IAA-Leu	4
IAR3	At1g51760	*At*	M20	KO: reduced sensitivity to exogenous IAA-Ala	23
AMP1	At3g54720	*At*	M28	KO: oversized meristems, polycotyly, etc.	39
AraSP	At2g32480	*At*	M50	KD/KO: impaired chloroplast and seedling development	10
EGY1	At5g35220	*At*	M50	KO: reduced chlorophyl and gravitropism	15

(*Continued*)

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Table 1 (*continued*)

Name of protease[a]	Accession	Species[b]	Family	Phenotype observed[c]	Reference
CDR1	At5g33340	*At*	A1	OE: constitutive disease resistance; dwarfing	98
PCS1	At5g02190	*At*	A1	KO: lethality in gametophytes and embryos	34

[a]Proteases discussed in text are indicated in bold.
[b]*At*, *Arabidopsis thaliana*; *Bo*, *Brassica oleracea*; *Le*, *Lycopersicon esculentum*; *Mt*, *Medicago trunculata*; *Nb*, *Nicotiana benthamiana*; *Nt*, *Nicotiana tabacum*; *Os*, *Oryza sativa*; *Pa*, *Picea abies*; *Zm*, *Zea mays*.
[c]KO, knockout; KD, knockdown/silencing/RNAi; OE, overexpression; IAA, indole acetic acid.

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kingdom because poplar and rice have similar distributions (33) (**Figure 2*b***).

The biological functions of at least 40 proteases have been revealed through genetic studies (**Table 1**). The diversity of the biological functions is tremendous and stretches out over the entire spectrum of proteases. The proteases functionally described so far belong to ~20 different families of 14 clans (**Figure 2*b***). Although the phenotypes associated with altered expression of these proteases have been well described, research addressing their molecular mechanisms has only just begun. Interestingly, not all annotated proteases cleave peptide bonds in proteins. MEROPS peptidase T3 family members γ-glutamyltransferase 1 and 2 (GGT1 and GGT2), for example, hydrolyze the tripeptide glutathione and glutathione S-conjugates (59), whereas the MEROPS peptidase M20 family members indole acetic acid (IAA)-amino acid hydrolase (ILR1) and IAA-alanine resistant 3 (IAR3) hydrolyze auxin–amino acid conjugates (4, 23). One subclass of the S10 carboxypeptidases catalyzes acyltransferase reactions, rather than proteolysis (SNG1/2, discussed below).

This review summarizes the phenotypic data for a broad spectrum of plant proteases and discusses their molecular mechanisms. I focus on seventeen proteases that are relatively well described at a phenotypic level. The biological function of each of these proteases is so distinct that I choose to treat them separately and group them on the basis of the MEROPS classification. The summarized data illustrate that proteases play strikingly diverse regulatory roles in a broad spectrum of processes essential for a plant's life.

CYSTEINE PROTEASES

Cysteine proteases use a catalytic Cys as a nucleophile during proteolysis. Plant genomes encode for approximately 140 cysteine proteases that belong to 15 families of 5 clans (69). The structures of proteases from different clans are distinct, which implicates convergent evolution. Clans CA and CE contain proteases with a papain-like fold, whereas CD proteases have a caspase-like fold (explained below). Many cysteine proteases play a role in programmed cell death (PCD), in response to both developmental cues and pathogens. Other cysteine proteases regulate epidermal cell fate, flowering time, inflorescence architecture, and pollen or embryo development (**Table 1**). Seven of these proteases have been studied in detail and are discussed here.

Phytocalpain DEK1

Calpains (family C2, clan CA) are well studied calcium-dependent proteases in animals that usually act in the cytoplasm (74). Calpains are evolutionarily related to papain because they share the same fold and order of catalytic residues (Cys, His, Asn) (40). Calpains are folded as two lobes, one carries the catalytic Cys and the other carries His and Asn residues, and the catalytic triad is assembled between the lobes. In calpains, the distance between the lobes, and thereby the functionality of the catalytic triad, is regulated by calcium binding (40). Plant genomes

Convergent evolution: independent evolution toward a similar functional endpoint

Programmed cell death (PCD): cell death in which cell signaling is required for cells to die

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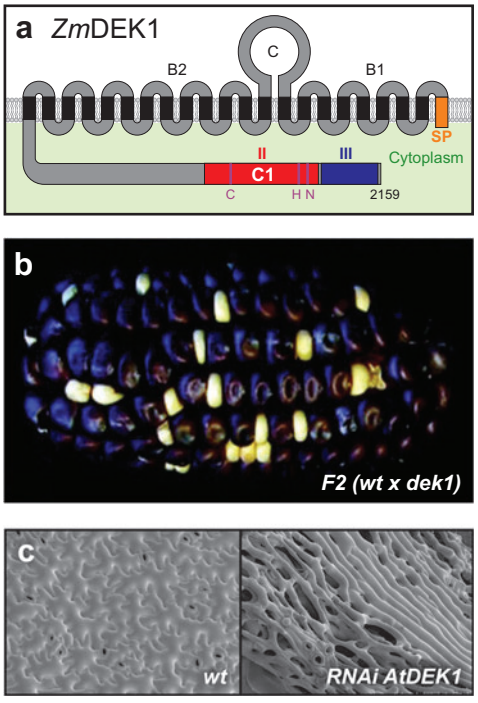

Figure 3

DEK1 (*a*) Predicted topology of the *Zea mays* defective kernel 1 (DEK1) protein. SP, signal peptide; B1, B2, transmembrane domains (*black*); C, extracytoplasmic loop; II, calpain protease domain (*red*); III, calpain domain III (*dark blue*); C, H, N, catalytic residues Cys, His, and Asn (*purple*). (*b*) Phenotype of a maize ear of a heterozygous *dek1* mutant in a genetic background that stains the aleurone layer black. The *dek1* mutation is recessive and causes loss of the aleurone layer in the homozygous state. Reprinted with permission from Reference 55, Copyright 2002, National Academy of Sciences, U.S.A. (*c*) Silencing of AtDEK1 in *Arabidopsis* results in loss of epidermal cell identity: Epidermal cells are gradually replaced by mesophyll-like cells. Pictures kindly provided by Dr. G. Ingham (Institute for Molecular Plant Sciences, Edinburgh, UK).

contain only one calpain, also called phytocalpain, which is unique in its structure (**Figure 3*a***) and essential for plant epidermis development.

DEK1. Defective kernel 1 (DEK1) (**Table 2**) is required for epidermal cell identity. The *dek1* mutation was originally identified in maize where it causes defective kernels that lack an aleurone cell layer (6) (**Figure 3*b***). Although the aleurone is initiated in young endosperm, it is not maintained in *dek1* mutants (55). A series of twelve maize *dek1* alleles was described with phenotypes that range from aborted embryos to viable plants that have crinkled leaves, shortened internodes, and bent nodes (7). Revertant sector analysis revealed that DEK1 functions cell-autonomously because wild-type cells cannot rescue the phenotype of adjacent *dek1* mutant cells, and *dek1* mutant cells cannot impose their phenotype onto adjacent wild-type cells (7). The maize *dek1* phenotypes are similar to those described in other plant species. *Arabidopsis dek1* mutants develop only a partial aleurone and the embryos abort during development (43, 56). Mutant embryos that develop to the globular stage show uncontrolled planes of cell division in the suspensor and embryo proper (43, 56). Suppression of *AtDek1* transcript levels via the use of RNAi permits the growth of viable plants. The phenotypes vary in severity from fused cotyledons to leaf epidermal cells that are gradually replaced by mesophyl-like cells that contain chloroplasts (43) (**Figure 3*c***). Suppression of *NbDek1* transcript levels via the use of virus-induced gene silencing in *Nicotiana benthamiana* results in hyperproliferation of epidermal cells and the formation of callus-like surfaces on leaves, stems, and flowers (1). Interestingly, despite the severe epidermal phenotypes caused by the loss of Dek1, the basic organization of inner leaf tissues is maintained, with normal palisade and mesophyll cells (1, 7, 56). *AtDEK1*-overexpressing *Arabidopsis* plants lack trichomes and show altered surface structures of leaves, ovules, and seeds (56). Taken together, these phenotypes indicate that *DEK1* is essential for epidermal cell identity, and epidermal cell identity is essential for the development of the embryo, the suspensor, and the shoot apical meristem, but not the endosperm and mesophyll (43).

The DEK1 protein contains an exceptionally high number of transmembrane domains (21), interrupted by a putative extracytoplasmic domain (55). The C-terminal domain is presumably cytoplasmic and shares homology with calpain (55). Apart from its unusual structure, DEK1 is also unique because it is highly

Table 2 Phytocalpain DEK1 (defective kernel 1)

Gene name	*DEK1*	*AtDEK1*	*NbDEK1*
Described alleles	*Dek1–1…12* (7)	*Dek1–1…4* (43, 56)	
Knockout phenotype	Aleurone deficient, embryo abortion (6, 7, 55)	Aleurone deficient, embryo abortion (43, 56)	Not reported
Knockdown phenotype	Not reported	Deformed plants lacking epidermis (43)	Callus formation on all surfaces (1)
Overexpression phenotype	Not reported	Loss of trichomes, different epidermal cell shape, but not in all ecotypes (56)	Not reported
Endogenous expression	Low levels, ubiquitous (55, 96)	Low levels, ubiquitous (56)	Low levels, ubiquitous (1)
Subcellular localization	Membrane (predicted)	Membrane (predicted)	Possibly in nuclear membrane (1)
Genetic interactors	Function of receptor-like kinase CR4 depends on Dek1 (7)	Receptor-like kinase ACR4 acts independent of Dek1 (56)	Not reported
Proteolytic activity	Domains II and III cleave casein in vitro, stimulated by Ca^{2+} (96)	Not reported	Not reported
Putative mechanism	May cleave transcription factors in response to signals from the surface of the organism (43)		

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conserved throughout the plant kingdom and is encoded by a single copy of the gene in each sequenced plant genome (55). The protease domain of DEK1 has proteolytic activity in vitro that can be enhanced by calcium (96). Different models exist for DEK1 function. An initial model proposed a role for maize DEK1 in the release of signals that are perceived by receptor-like kinase CR4, because maize *cr4* mutants share some of the *dek1* phenotypes, and *cr4*/*dek1* double mutants show *dek1* phenotypes (6). In *Arabidopsis*, however, *Arabidopsis thaliana homolog of crinkly 4* (*acr4*)/*dek1* double mutants show additive effects, which suggests that *dek1* and *acr4* act in different pathways of epidermis specification (43). In another model, DEK1 cleaves homeodomain–leucine zipper IV (HDZipIV) transcription factors, which regulate epidermal cell fate (43). This model is consistent with the cell-autonomous function of DEK1 and the fact that DEK1 carries nuclear targeting signals (1). However, the true subcellular localization of DEK1 remains to be investigated.

Papain-Like Proteases Rcr3 and *Nb*CathB

Papain-like proteases (family C1, clan CA) contain catalytic residues in the order Cys, His, Asn. As with calpain (family C2, clan CA), the fold consists of two domains (lobes) and the catalytic site lies between them (29). Family C1 has been subdivided into subfamily C1A, which comprises proteases that contain disulfide bridges and accumulate in vesicles, the vacuole, or the apoplast, and family C1B, which comprises proteases that lack disulfide bridges and are located in the cytoplasm (69). Plants only have C1A proteases. There are approximately 30 papain-like proteases in subfamily C1A encoded by *Arabidopsis*, subdivided into 8 subfamilies (8). These C1A proteases are produced as preproproteases (**Figure 4*a***). The autoinhibitory prodomain folds back onto the catalytic site cleft and is removed during the activation of the protease (90). Papain-like proteases are implicated in pathogen perception, disease resistance signaling, defense against insects, and senescence (**Table 1**).

Suspensor: connection between embryo and endosperm

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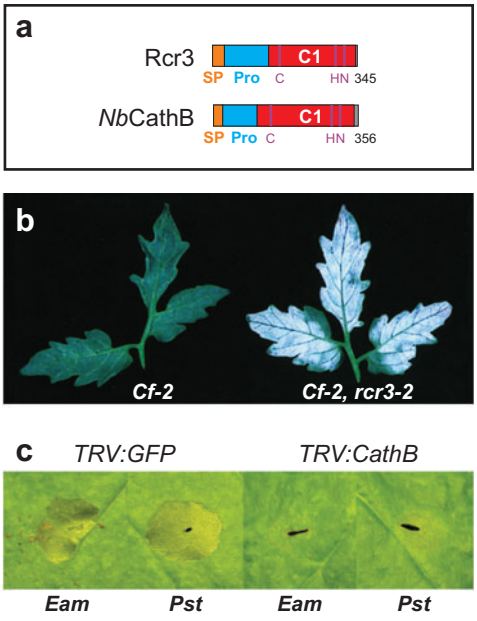

Figure 4

Rcr3 and NbCathB (*a*) Domains of Rcr3 (required for *Cladosporium* resistance 3) and *Nb*CathB (*Nicotiana benthamiana* cathepsin B) proteins. SP, signal peptide (*orange*); pro, autoinhibitory prodomain (*blue*); C1, protease domain (*red*); C, H, N, catalytic residues Cys, His, and Asn (*purple*). (*b*) Mutant *rcr3–2* plants have lost *Cladosporium fulvum* resistance gene-2 (*Cf-2*)-mediated resistance for the leaf mold fungus *Cladosporium fulvum*. Reprinted with permission from Reference 24, Copyright 2000, National Academy of Sciences, U. S. A. (*c*) Silencing of *NbCatB* suppresses the hypersensitive response induced by the nonhost bacterial pathogens *Erwinia amylovora* (*Eam*) and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). Figures kindly provided by Dr. E. Gilroy (Scottish Crop Research Institute, Dundee, Scotland).

Biotrophic: pathogen that feeds on living plant tissues

Hypersensitive response (HR): rapid programmed cell death that occurs at the site of pathogen infection

Nonhost resistance: when all genotypes of a host are resistant against all genotypes of a pathogen

Rcr3. Required for *Cladosporium* resistance 3 (Rcr3) (**Table 3**) is essential for the function of the resistance gene *Cf-2* in tomato. The *Cf-2* resistance gene was introgressed from wild tomato plants into cultivated tomato (*Lycopersicon esculentum*) by plant breeders to generate tomato plants that are resistant to the biotrophic leaf mold fungus *Cladosporium fulvum* carrying the avirulence gene *Avr2*. The resistance response involves a hypersensitive response (HR) of cell death at the site of infection, which prevents further pathogen growth. *Avr2* encodes a small, secreted, cysteine-rich protein without obvious homology to other proteins, and *Cf-2* encodes a receptor-like membrane protein (25, 58). *Rcr3* was identified in a forward genetic screen for *Cf-2* tomato plants that are susceptible to *C. fulvum* carrying *Avr2* (24) (**Figure 4*b***). *Rcr3* encodes a secreted papain-like cysteine protease with proven proteolytic activity (49) (**Figure 4*a***). Surprisingly, *Cf-2* plants contain the *Rcr3* allele from the wild tomato *Lycopersicon pimpinellifolium* ($Rcr3^{pim}$), which differs from the *Lycopersicon esculentum* allele ($Rcr3^{esc}$) in one amino acid deletion and six amino acid substitutions. $Rcr3^{esc}$ triggers necrotic responses in combination with *Cf-2*, but $Rcr3^{pim}$ does not (49). This explained a peculiar observation made by plant breeders in the early twentieth century. A necrosis-suppressing gene (*Ne*), whose identity was unkown, was introgressed from *L. pimpinellifolium* together with *Cf-2* to suppress autonecrotic responses induced by *Cf-2*. *Ne* proved to be $Rcr3^{pim}$ (49). Studies of the role of Rcr3 in Avr2 recognition revealed that Avr2 physically interacts with Rcr3 and inhibits its activity (72). Inhibition of Rcr3 by protease inhibitor E-64 or the absence of Rcr3 activity in *rcr3* mutants cannot trigger the resistance response mediated by Cf-2, suggesting that neither the product nor substrates of Rcr3, but the Avr2-Rcr3 complex or a specific conformational change in Rcr3, is required to trigger the resistance response (72). These data are consistent with the guard hypothesis, which predicts that resistance proteins (e.g., Cf-2) are guarding the virulence targets (e.g., Rcr3) of pathogen effector proteins (e.g., Avr2) (72, 91, 92). The upregulation of Rcr3 transcript levels during pathogen infection is consistent with a role in defense (49). However, whether Rcr3 contributes to pathogen resistance and how the Avr2-Rcr3 complex is recognized by Cf-2 remains to be investigated.

NbCathB. *Nicotiana benthamiana* Cathepsin B (*Nb*CathB) (**Table 3**) is required for the HR induced by nonhost pathogens. The potato *CathB* transcript level increases early during infection with the oomycete pathogen *Phytophthora infestans* (2). A similar quick transcriptional induction occurs with *NbCathB* in

Table 3 Papain-like proteases Rcr3 (required for *C. fulvum* resistance 3) and *Nb*CathB (*N. benthamiana* Cathepsin B)

Gene name	*Rcr3*	*NbCathB*
Described alleles	*rcr3—1. . .4* (24)	None
Knockout phenotype	Loss of resistance for fungus *Cladosporium fulvum* carrying Avr2 (24)	Not reported
Knockdown phenotype	Not reported	Suppresses hypersensitive cell death (35)
Overexpression phenotype	Not reported	Not reported
Endogenous expression	Higher expression in older plants, upregulated during pathogen infection (49)	Induced during hypersensitive cell death (2, 35)
Subcellular localization	Secreted into leaf apoplast (49)	Secreted (35)
Genetic interactors	Requires receptor-like protein Cf-2 (24)	Not reported
Proteolytic activity	Degrades casein and gelatin (49)	Not reported
Putative mechanism	May trigger activation of Cf-2-induced resistance response by complex formation with fungal inhibitor protein Avr2 (72)	May act in extracellular signaling to regulate hypersensitive cell death (35)

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N. benthamiana during the HR (35). Virus-induced gene silencing of *NbCathB* prevents the HR induced by two distinct nonhost bacterial pathogens (**Figure 4*c***). This loss of the HR is associated with further growth of the bacteria, which indicates that nonhost resistance is hampered. The HR induced by the combined expression of avirulence protein Avr3a with resistance protein R3a was also suppressed in silenced plants, but the HR induced by coexpression of avirulence protein Avr4 and resistance protein Cf-4 was unaltered (35). This indicates that *Nb*CathB is required for some, but not for all, resistance signaling pathways. *Nb*CathB is activated during secretion and is also active in noninfected plants. The data indicate that *Nb*CathB is an extracellular protease that acts in the transduction of signals during recognition of some, but not all, avirulent pathogens. How this protease mediates HR signaling is unknown, but it represents an exciting area for further research.

Deconjugating Enzymes UCH1/2 and ESD4

The conjugation of ubiquitin and small ubiquitin-like modifiers (SUMO) to lysine residues of target proteins is an important way to regulate the location, activity, and degradation of these proteins (28). Conjugation of ubiquitin and SUMO is mediated by specific E3 ligases, whereas deconjugation is catalyzed by different proteases that belong to MEROPS families C12, C19, and C48. Ubiquitin-specific proteases (UBPs; family C19, clan CA) and ubiquitin C-terminal hydrolases (UCHs; family C12, clan CA) interact with ubiquitin through electrostatic interactions and hydrolyze the bonds formed by the C-terminal glycine of ubiquitin in a highly selective manner, releasing ubiquitin from its precursors or from ubiquitinated proteins. SUMO-deconjugating enzymes (family C48, clan CE) are specific for the C-terminal glycine of SUMO and release SUMO from both precursors and conjugates. Most C12, C19, and C48 proteins are produced in the cytoplasm without a prodomain and move to the nucleus via nuclear localization signals (NLS) (**Figure 5*a***).

Although the C12/C19 and C48 families belong to different clans, the structure of the lobes and the position of the catalytic residues are similar between CA and CE clan proteases. However, the difference between CA and CE clan proteases is that the lobes are swapped in the primary sequence, possibly owing to an ancient gene rearrangement

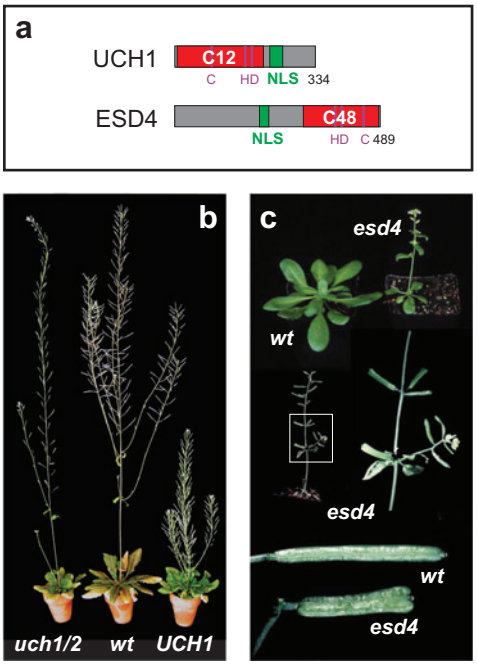

Figure 5

UCH1 and ESD4 (*a*) Domains of ubiquitin C-terminal hydrolase 1 (UCH1) and early in short days 4 (ESD4) proteins. NLS, nuclear localization signal (*green*); C12/C48, protease domain (*red*); C, H, D, catalytic residues Cys, His, and Asn (*purple*). (*b*) The *uch1–1/uch2–1* double mutant (*uch1/2*) shows less branching than wild-type, whereas the *UCH1*-overexpressing strain shows more branching than wild-type under short day conditions. The picture was kindly provided by Dr. R. Vierstra (University of Wisconsin, Madison). (*c*) *esd4–1* mutant plants flower earlier than wild-type when grown under short day conditions (*top*). *esd4–1* mutants develop siliques at unexpected positions (*middle*), and siliques are shorter and broader at the tip (*bottom*). Pictures kindly provided by Dr. N. Elrouby (Max-Planck-Institut für Züchtungsforschung, Cologne, Germany).

(63, 69). Members of the C19 family are required for pollen and embryo development (26, 27).

UCH1 and UCH2. Ubiquitin C-terminal hydrolase 1 and 2 (UCH1 and UCH2) (**Table 4**) regulate shoot architecture, probably by rescuing specific ubiquitinated proteins from degradation. Among the few UCHs encoded in the *Arabidopsis* genome, UCH1 and UCH2 share strong sequence similarity and were chosen for functional analysis. Although *uch1* and *uch2* single mutants have no phenotypes, the *uch1/uch2* double mutants show phenotypes that are often the opposite to those observed for *UCH1*-overexpressing (*35S:UCH1*) plants (101). Phenotypes in the rosette size, leaf shape, and flower organs were observed, but the strongest phenotype is displayed in the shoot architecture under short day conditions. *35S:UCH1* plants are short bushy plants covered with siliques, whereas *uch1/uch2* plants develop a less-branched primary inflorescence when compared with wild-type (101) (**Figure 5*b***). The specific phenotypes suggest that UCHs act on distinct ubiquitinated conjugates. Indeed, UCH2 can release ubiquitin from polyubiquitin precursors and from polyubiquitin conjugates, but the levels of ubiquitin conjugates are unaltered in the *35S:UCH1* and *uch1/uch2* plants (101). The phenotypes in shoot architecture suggest a possible link to auxin signaling. The phenotype of the auxin-insensitive mutants *axr1–3* and *axr2–1* is strongly enhanced by UHC1 overexpression, which indicates that auxin signaling may be affected by *UCH1/2*. Indeed, the stability of an AXR3/IAA17 reporter is stabilized in *35S:UHC1* lines (101). This result leads to the hypothesis that UCH1/2 proteins directly or indirectly rescue auxin/IAA proteins from degradation to dampen auxin signal strength or restore normal plant growth after auxin signaling. In addition, UCH1/2 proteins may also deubiquitinate proteins that are not related to auxin signaling.

ESD4. Early in short days 4 (ESD4) (**Table 4**) regulates many developmental processes, including flowering time, by modifying the sumoylation status of various proteins. The *esd4* mutant was identified in a screen for mutants that flower earlier in short day conditions. In addition to an earlier flowering time, the *esd4* mutant also has shorter internodes, smaller leaves, altered phylotaxy, fewer solitary flowers, and shorter siliques compared with wild-type (70) (**Figure 5*c***). The early flowering phenotype is partly explained by the fact that transcript levels of the floral repressor *FLOWERING LOCUS C* (*FLC*)

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Table 4 Deconjugating enzymes UCH1/2 (ubiquitin C-terminal hydrolase 1/2) and ESD4 (early in short days 4)

Gene name	*UCH1/2*	*ESD4*
Described alleles	*uch1–1*, *uch2–1* (101)	*esd4–1,2* (64, 70)
Knockout phenotype	Shorter petioles, smaller leaves, deformed petals, large stigmas, less fertile, less branched (101)	Early flowering in short days, smaller leaves, shorter internodes and siliques, altered phylotaxy, fewer leaves and flowers (64, 70)
Knockdown phenotype	Not reported	Not reported
Overexpression phenotype	Shorter petioles, smaller leaves, bushy plants covered with siliques (101)	No phenotype (64)
Endogenous expression	Ubiquitous (101)	Ubiquitous (64)
Subcellular localization	Nuclear and cytoplasmic (UCH1/2-GFP) (101)	Inner surface of nuclear envelope (ESD4-GFP) (64)
Genetic interactors	UCH1 overexpression enhances *axr1*/2 auxin mutant phenotypes (101)	In same pathway as nuclear pore anchor NUA (99)
Proteolytic activity	Cleaves ubiquitin from polyubiquitin and from ubiquitin conjugates (101)	Cleaves *At*SUMO1/2 from its precursor and from conjugates (20, 64)
Putative mechanism	May rescue specific ubiquitin-tagged proteins (e.g., AXR3) from degradation (101)	Regulates sumoylation status of different proteins involved in diverse developmental processes (64)

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are reduced in *esd4* mutants, which causes an upregulation of the flowering time genes *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*) (70). However, analysis of *flc/esd4* double mutants indicated that *ESD4* also regulates flowering time genes independently of *FLC* (70). *ESD4* encodes a desumoylating enzyme that can cleave certain *Arabidopsis* SUMO proteins from their precursor and from conjugates (20, 64). Consistent with the presumed role of ESD4 in desumoylating other proteins, SUMO conjugates accumulate in *esd4* mutants, and SUMO overexpression in *esd4* plants further enhances the accumulation of SUMO conjugates and the *esd4* mutant phenotype (64). ESD4 proteins localize to the inner surface of the nuclear envelope (64) and physically interact with a nuclear pore anchor (NUA) (99). *nua* mutants phenocopy *esd4* mutants and *nua/esd4* double mutants are indistinguishable from single mutants, which suggests that both genes act in the same pathway (99). Interestingly, besides an increased level of SUMO conjugates and a reduced *FLC* transcript level, *nua* mutants accumulate more mRNA in the nucleus, consistent with the role of NUA proteins as determined in yeast (99). Given the spectrum of different phenotypes of *esd4* mutants and the range of proteins that are regulated by sumoylation, ESD4 probably acts in multiple pathways to desumoylate different substrates, each involved in different processes, including flowering time.

Caspase-Like Proteases MCAs and VPEs

Caspases (family C14, clan CD) have been intensively investigated in animals because they regulate apoptotic cell death (73). Their fame is also a source of confusion because the hunt for caspase activities (cleavage after Asp) in plants resulted in the description of many "caspase-like proteases" that are probably not related to caspases (93). In this review, caspase-like proteases are defined as sharing sequence homology or at least structural homology to the animal caspases. Proteases that share sequence homology with animal caspases are absent from plant

Apoptotic cell death: a form of programmed cell death in animals

Caspase: cysteine protease that cleaves substrates after aspartic acid residues

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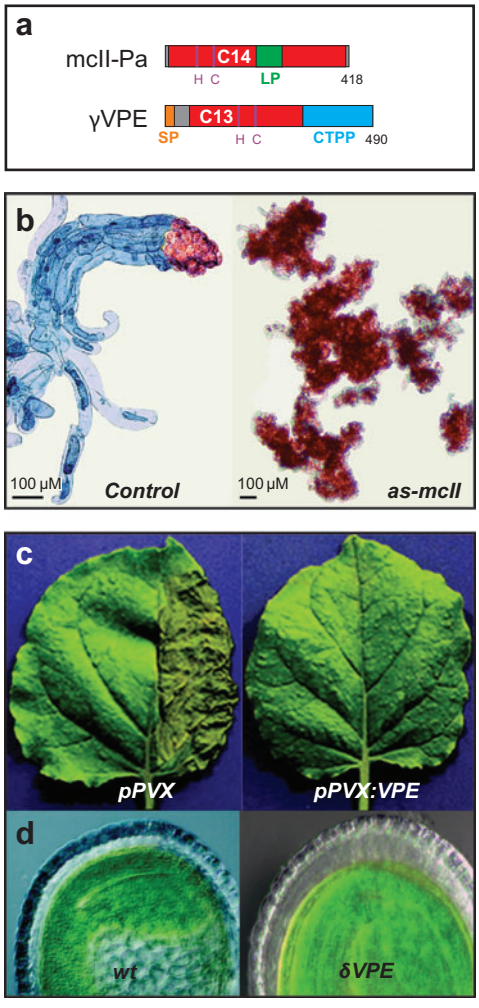

Figure 6

mcII-Pa and VPE (*a*) Domains of metacaspase type II of *Picea abies* (mcII-Pa) and γ vacuolar processing enzymes (γVPE) proteins. SP, signal peptide (*orange*); CTPP, autoinhibitory C-terminal propeptide (*blue*); C13/C14, protease domain (*red*); H, C, catalytic residues His and Cys (*purple*); LP, linker peptide (*green*). (*b*) Silencing of *mcII-Pa* prevents cell death induced during somatic embryogenesis, visualized by staining with acetocarmine (*red*, viable cells) and Evans blue (*blue*, dead cells). Picture kindly provided by Dr. P. Bozhkov (Sveriges Lantbruksuniversitet, Uppsala, Sweden). (*c*) *NbVPE* silencing blocks the hypersensitive response (HR), cell death induced by tobacco mosaic virus (TMV), in plants containing the *N* resistance gene. Collapsed tissue is visible 24 h after triggering HR. Reprinted from Reference 45 with permission from the American Association for the Advancement of Science. (*d*) Cell death in two cell layers during seed coat formation is delayed in Δ*vpe* mutants. Reprinted from Reference 65 with permission from the American Society of Plant Biologists.

genomes, but plants do contain metacaspases (MCAs; family C14) and vacuolar processing enzymes (VPEs; family C13). These caspase-like enzymes are unified in clan CD and use a catalytic Cys that is activated by the catalytic His for nucleophilic attack. Caspase-like enzymes are folded as an α/β/α sandwich (17). Clan CD proteases are highly selective for cleavage after specific residues: Asp for animal caspases, Arg for MCAs, and Asn for VPEs (69). Most CD clan proteases are produced with N- and C-terminal propeptides (**Figure 6*a***). Caspases and MCAs (family C14) are usually cytoplasmic or nuclear, whereas VPEs (family C13) are located in vesicles or in the vacuole. (Meta)caspases are produced with a linker protein that is proteolytically removed, which results in a heterocomplex of a p20 chain and a p10 chain (**Figure 6*a***). Given the evolutionary relationship with caspases, caspase-like enzymes in plants have long been suspected to regulate PCD. Published work from the past few years indicates that this is indeed the case (summarized below).

mcII-Pa. Metacaspase type II of *Picea abies* (mcII-Pa) (**Table 5**) mediates PCD during somatic embryogenesis in Norway spruce (*Picea abies*). Somatic embryogenesis of Norway spruce is an elegant system in which to study embryogenesis because embryo development can be synchronized by changing the hormone balance, and the embryos have large suspensor cells that undergo gradual, successive PCD (11). The fact that this PCD is accompanied by caspase activity and can be inhibited by a caspase inhibitor led to the identification of *mcII-Pa*, a metacaspase that is specifically expressed in suspensor cells that undergo PCD (84). Silencing *mcII-Pa* prevents PCD and suppresses caspase activity and the frequency of nuclear degradation (84) (**Figure 6*b***). However, biochemical characterization of the mcII-Pa protein revealed that it cleaves after Arg but not after Asp, which suggests that the caspase activity is not caused by mcII-Pa activity, but by enzymes activated by mcII-Pa. Interestingly, mcII-Pa

Table 5 Caspase-like proteases mcII-Pa (metacaspase type II of *Picea abies*) and VPE (vacuolar processing enzyme)

Gene name	*mcII-Pa*	*VPE*
Described alleles	None	$\alpha\beta\gamma\delta vpe$ (51); γvpe-*1* (71); δvpe-*1*, δvpe-*4* (65)
Knockout phenotype	Not reported	$\alpha\beta\gamma\delta vpe$: abolished toxin-induced cell death (51)
		γvpe: slightly decreased pathogen resistance (71)
		δvpe: delayed cell death during seed coat development (65)
Knockdown phenotype	No cell death in somatic embryos, no embryonic pattern formation (84)	*NbVPE:* blocks virus-induced hypersensitive cell death (38)
Overexpression phenotype	Not reported	Not reported
Endogenous expression	Only in somatic embryo cells that are committed to cell death and in procambial strands that lead to xylem differentiation (84)	*NbVPE*: upregulated during hypersensitive cell death (38)
		γ*VPE*: upregulated during programmed cell death (PCD) and pathogen infection (51, 71) δ*VPE*: only in maternal cell layers during seed development (65)
Subcellular localization	Cytoplasmic and nuclear (immunolocalization) (12)	In vacuole and vesicles (immunolocalization) (27)
Genetic interactors	Not reported	Not reported
Proteolytic activity	Cleaves after Arg, but not after Asp in vitro (12)	Cleaves after Asn (48)
Putative mechanism	May cleave nuclear structural proteins to disassemble the nuclear envelope during PCD (12)	May activate vacuolar enzymes and disintegrate the vacuolar membrane to release hydrolytic enzymes during PCD (37, 38, 51, 65)

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translocates from the cytoplasm into the nucleus during PCD and associates with chromatin and disassembling nuclear pore complexes (12). Nuclear disintegration can be induced by adding mcII-Pa protein to nuclei isolated from PCD-deficient cell lines (12). This nuclear disintegration can be inhibited by a mcII-Pa inhibitor and is absent if a catalytic mutant of mcII-Pa is added instead (12). The data lead to a hypothesis in which cytoplasmic metacaspases participate in PCD by degrading the nuclear envelope, which leads to nuclear degradation (12).

VPEs. Vacuolar processing enzymes (VPEs) (**Table 5**) are essential for PCD induced during disease resistance responses, by a fungal toxin, and during seed coat development. VPEs were initially studied for their role in the maturation of seed storage proteins, but the upregulation of these genes during different kinds of PCD prompted further phenotype investigation. Silencing of VPEs in *N. benthamiana* abolishes the hypersensitive cell death triggered by tobacco mosaic virus (TMV) in plants carrying the TMV-resistance gene *N* (38) (**Figure 6*c***). Cytological studies revealed that vacuolar collapse precedes PCD and both are prevented in VPE-silenced plants (38). *Arabidopsis* has four VPEs: α, β, γ, and δ. δ*VPE* is expressed specifically in two cell layers of the maternal inner integument of developing seed coats (65). These cell layers normally undergo PCD early during seed development, which results in the degradation of nuclei and shrinkage of the inner integument. However, this PCD is absent in δvpe mutants, although the final seed coat is normal (**Figure 6*d***). In contrast to δ*VPE*, the γ*VPE* gene is expressed throughout the plant, and γvpe mutant plants show a weak reduced resistance toward various pathogens

Metacaspases: proteases that share certain conserved sequence motifs with caspases

Integument: covering of an organ, in this case a seed

(71). *Arabidopsis* mutants lacking all four VPE genes ($\alpha\beta\gamma\delta vpe$) are insensitive to cell death induced by the fungal toxin Fumonisin B1 (FB1) (51). Of the single mutants, only γvpe shows a delayed FB1-induced cell death, which suggests that *VPE* genes act redundantly and that γVPE makes the largest contribution to FB1-induced PCD in leaves (51). VPE proteases act on caspase substrates and are inhibited by caspase inhibitors, which indicates that VPEs are the plant functional orthologs of animal caspases (38, 51, 65, 71). The mechanism of how VPEs act in PCD is unknown, but their location, and therefore probably their signaling role, is distinct from that of animal caspases. VPEs may activate vacuolar enzymes, which may trigger the collapse of the vacuolar membrane, resulting in the disintegration of cellular structures by released hydrolytic enzymes.

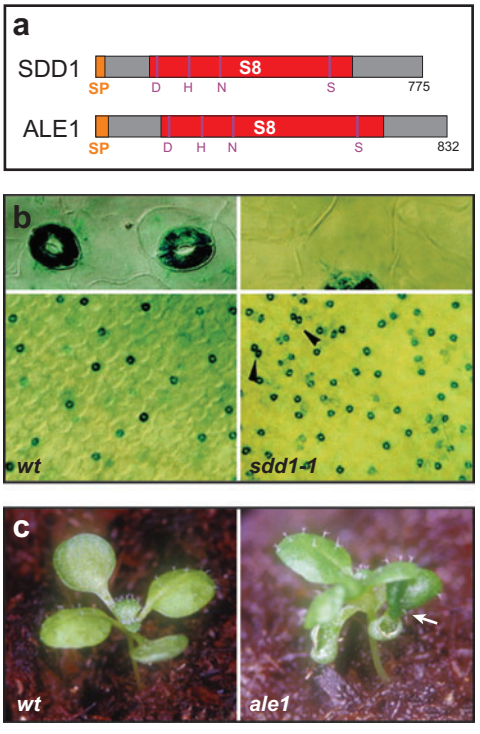

Figure 7

SDD1 and ALE1 (*a*) Domains of stomatal density and distribution 1 (SDD1) and abnormal leaf shape 1 (ALE1) proteins. SP, signal peptide (*orange*); S8, protease domain (*red*); D, H, N, S, catalytic residues Asp, His, and Ser (*purple*). (*b*) *sdd1–1* mutants have fourfold more stomata and stomata clusters than wild-type. Reprinted with permission from Reference 9, Copyright 2000, Cold Spring Harbor Laboratory Press. (*c*) 16-day-old seedlings of *ale1–1* have fused cotyledons that lack a cuticle (*arrow*). Reprinted from Reference 88 with permission from the Company of Biologists.

SERINE PROTEASES

Serine proteases use the active site Ser as a nucleophile. The catalytic mechanism is very similar to that of cysteine proteases, and some serine proteases are even evolutionarily related to cysteine proteases. With more than 200 members, serine proteases are the largest class of proteolytic enzymes in plants. Plant serine proteases are divided into 14 families. These families belong to 9 clans that are evolutionarily unrelated to each other. Families S8, S9, S10, and S33 are the largest serine protease families in plants, with each containing approximately 60 members. Biological functions for serine proteases have been described for some of the subtilases (SDD1 and ALE1; family S8, clan SB), carboxypeptidases (BRS1 and SNG1/2; family S10, clan SC), and plastid-localized members of the S1, S26, and S14 families (DegPs, Plsp1, and ClpPs; **Table 1**).

Subtilisin-Like Proteases SDD1 and ALE1

Subtilases (family S8, clan SB) contain a catalytic triad in the order Asp, His, Ser, and are folded as a seven-stranded β-sheet, sandwiched between two layers of helices. Subtilases are encoded as preproproteins and are usually secreted and processed at both the N and C terminus (68) (**Figure 7*a***). Most subtilases are endopeptidases. Some subtilases are expected to have a broad substrate range, others are considered to be specific prohormone convertases. The *Arabidopsis* genome encodes approximately 70 subtilases, which can be divided into three subfamilies (8). A biological role is known for only two *Arabidopsis* subtilases (see below). However, no macroscopic phenotypes were observed for

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Table 6 Subtilisin-like proteases SDD1 (stomatal density and distribution 1) and ALE1 (abnormal leaf shape 1)

Gene name	*SDD1*	*ALE1*
Described alleles	*sdd1–1* (9)	*ale1–1*, *ale1–2* (88)
Knockout phenotype	More stomata, also in clusters (9)	Embryo lacks cuticle (88)
Knockdown phenotype	Not reported	Not reported
Overexpression phenotype	2–3-fold decrease in stomata (95)	Not reported
Endogenous expression	Only in guard mother cells (95)	Only in endosperm, not in seedling (88)
Subcellular localization	Secreted (predicted) truncated SDD1-GFP in the plasma membrane (95)	Secreted (predicted)
Genetic interactors	Overexpression phenotype depends on receptor-like protein TMM (too many mouths) (95)	Acts independently of receptor-like kinases ACR4 (*Arabidopsis thaliana* homolog of crinkly 4) and ALE2 (Abnormal leaf shape 2) (88, 97)
Proteolytic activity	Not reported	Not reported
Putative mechanism	Could release signals from developing stomata to suppress the development of neighboring stomata (95)	Could activate signals or enzymes from the endosperm to stimulate cuticle formation on the embryo surface (88)

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knockout lines of 55 other subtilases, which indicates that these proteases act redundantly or have condition-specific roles (68).

SDD1. Stomatal density and distribution 1 (SDD1) (**Table 6**) specifically regulates the position of stomata development within the epidermis. The *sdd1–1* mutant was identified in a forward genetic screen for mutants with an altered stomatal density and distribution (9). The number of stomata in *sdd1–1* mutants is two- to fourfold higher than wild-type in all aerial organs except for the cotyledons (**Figure 7*b***). Many stomata are also clustered and almost every epidermal cell is in contact with at least one guard cell. No other morphological alterations are observed in *sdd1–1* mutant plants, consistent with the specific expression of the SDD1 gene: Transcripts are only detectable in guard mother cells during guard cell development (95). The *SDD1* gene encodes an S8 subtilisin-like serine protease (9) (**Figure 7*a***). The SDD1 protein is expected to be secreted, but localization studies with SDD1-GFP fusion proteins failed because subtilases are proteolytically processed at both the N and C terminus. A GFP fusion with a truncated SDD1 localizes to the plasma membrane, but it is unknown if this truncated fusion protein complements the *sdd1–1* phenotype (95). Overexpression of SDD1 results in a two- to threefold reduction in stomatal density in wild-type plants, and is accompanied by the formation of stomata that are arrested before the division into the two guard cells (95). *SDD1* overexpression does not change the increased number of stomata caused by a mutation in receptor-like protein TMM (too many mouths), which indicates that SDD1 acts upstream of TMM in the same signaling pathway (95). Although SDD1 remains to be investigated biochemically, the data are consistent with the model that SDD1 is localized at the plasma membrane of developing stomata mother cells and generates signals that move to neighboring cells to prevent the formation of nearby stomata, either by inhibiting the development of stomata or by promoting differentiation into epidermal cells (95). TMM may act as a receptor of this signal (95).

ALE1. Abnormal leaf shape 1 (ALE1) (**Table 6**) is responsible for cuticle development during embryogenesis. *ale1* mutants were identified during a forward genetic screen because they have an obvious phenotype: *ale1* seedlings die within three days after

germination in open air but they survive at high relative humidity, which suggests that the lethality is caused by water loss (88). Mutant *ale1* plants produce small, crinkled cotyledons and leaves that are often fused to each other (**Figure 7*c***). Once beyond the seedling stage, *ale1* mutants develop normally. Electron microscopy reveals that no cuticle is formed on *ale1* embryos, and that the endosperm remains attached to the embryo tissue (88). The lack of a cuticle in *ale1* mutant embryos explains the crinkled and fused cotyledons and excessive water loss, which causes seedling lethality. Interestingly, the *ALE1* gene is expressed only in the endosperm and not in the embryo or the seedling, which suggests that the endosperm plays a role in the formation of the cuticle of the embryo (88). The *ALE1* gene encodes a S8 subtilisin-like serine protease (88) (**Figure 7*a***). The biochemical properties and subcellular location of the ALE1 protein remain to be characterized, but ALE1 is predicted to be secreted and proteolytically active (88). *ale1* phenotypes are similar to phenotypes caused by mutations in the receptor-like kinases ACR4 and ALE2. However, *acr4* and *ale2* mutant alleles act synergistically with *ale1*, which indicates that ALE1 has a different mode of action (89, 97). The data suggest that the ALE1 protein is secreted by the endosperm and promotes cuticle formation on the embryo, e.g., by proteolytically activating enzymes involved in cuticle deposition (88).

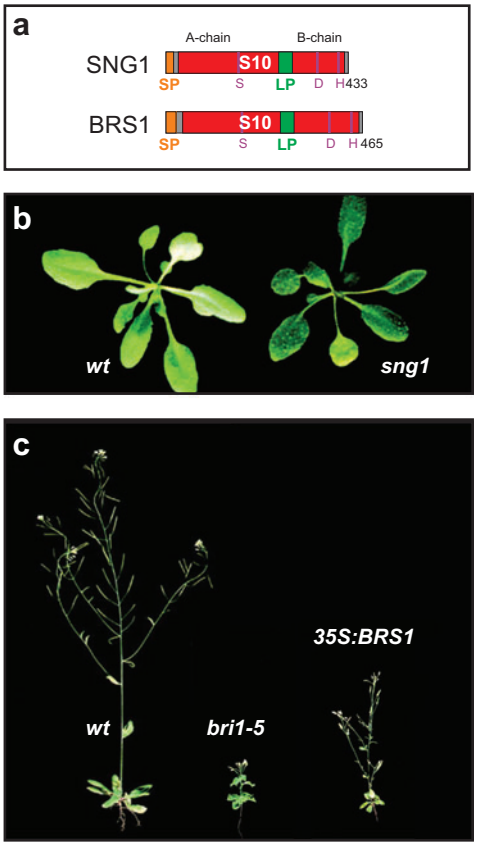

Figure 8

SNG1 and BRS1 (*a*) Domains of sinapoylglucose accumulator 1 (SNG1) and brassinosteroid insensitive 1 suppressor 1 (BRS1) proteins. SP, signal peptide (*orange*); LP, linker peptide (*green*); S10, protease domain (*red*); S, D, H catalytic residues Ser, Asp, His (*purple*). (*b*) *sng1* mutants are less fluorescent than wild-type under UV light because they do not accumulate the UV protectant compound sinapoylmalate. Reprinted from Reference 52 with permission of copyright holder, American Society of Plant Biologists. (*c*) *BRS1* overexpression suppresses the dwarfing caused by the *bri1–5* allele. Reprinted with permission from Reference 54, Copyright 2001, National Academy of Sciences, U. S. A.

Carboxypeptidase-Like Proteases SNG1/2 and BRS1

Serine carboxypeptidase protease-like proteins (SCPLs; family S10, clan SC) contain a catalytic triad in the primary sequence order Ser, Asp, His and fold as an α/β hydrolase, which is common to many other hydrolytic enzymes. SCPLs are distinct from other serine proteases in that they are active only at acidic pH. SCPLs are produced as preproproteases and often accumulate in the vacuole (**Figure 8*a***). Posttranslational removal of an internal linker peptide results in a disulfide-linked heterocomplex of A- and B-chains (**Figure 8*a***). There are nearly 60 SCPLs in the *Arabidopsis* genome, divided into different major subfamilies (32). Biological functions have been described for three SCPLs that belong to two different subfamilies. These proteins display a striking variety not only in phenotypes, but also especially in the reactions they catalyze.

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Table 7 Carboxypeptidase-like proteins SNG1/2 (sinapoylglucose accumulator 1/2) and BRS1 [brassinosteroid insensitive 1 (BRI1) suppressor 1]

Gene name	*SNG1; SNG2*	*BRS1*
Described alleles	*sng1–1…6* (52, 57) *sng2* (82)	*brs1–1* (54)
Knockout phenotype	*sng1*: less fluorescence of leaves under UV (52)	No phenotype (54)
Knockdown phenotype	Not reported	Not reported
Overexpression phenotype	Not reported	Suppression of *bri1–5* phenotype (54)
Endogenous expression	*SNG1*: in all organs (32) *SNG2*: in siliques only (32)	Ubiquitous, overlaps with *BRI1* expression (106)
Subcellular localization	Vacuolar (predicted)	Secreted (predicted) BRS1-GFP is in the cell wall (106)
Genetic interactors	Not reported	Suppresses phenotypes only of weak *bri1* alleles, not of strong *bri1* alleles or *er* (*erecta*) and *clv1* (*clavata 1*) mutants (54)
Proteolytic activity	No carboxypeptidase activity observed for either SNG1 (52) or SNG2 (81, 82)	Cleaves basic and hydrophobic synthetic dipeptides (106)
Putative mechanism	Catalyzes the transacylation of sinypolyesters via the use of sinapoylglucose as donor and malate (SNG1) or choline (SNG2) as acceptor (52, 82)	Could remove proteins that block brassinosteroid (BR) perception or activate proteins required for BR perception (106)

SNG1 and SNG2. Sinapoylglucose accumulator 1 and 2 (SNG1 and SNG2) (**Table 7**) are SCPLs that act as acyltransferases in the biosynthesis of sinapoyl esters, which provide UV-B protection (52, 57, 82). Leaves of *Arabidopsis sng1* mutants are less fluorescent under UV light, lack sinapoylmalate, and accumulate the donor molecule, sinapoylglucose (52, 57) (**Figure 8*b***). Similarly, seeds of *sng2* mutants lack sinypoylcholine and accumulate choline (81). The identification of the proteins encoded by *SNG1* and *SNG2* was surprising. Heterologous expression of the SNG1 and SNG2 proteins demonstrated that they catalyze the acyltransferase reaction but lack carboxypeptidase activity (52, 81, 82). SNG1 and SNG2 belong to a plant-specific clade of SCPL proteins that also includes 19 other *Arabidopsis* SCPLs and a tomato glucose acyltransferase (53), but not BRS1 (see below) (32). This suggests that this clade of SCPLs contains more acyltransferases that may contribute to the diversity of secondary metabolites in plants (32).

BRS1. Bri1 suppressor 1 (BRS1) (**Table 7**) contributes to the perception of brassinosteroid (BR) growth hormone, although phenotypes are observed only by overexpression analysis in a *bri1* mutant background. Receptor-like kinase BRI1 (BR insensitive 1) is essential for the perception of BR, and reduced BR perception in *bri1* mutants leads to a dwarfed phenotype. BRS1 was identified from an activation tagging screen for suppressors of *bri1–5*, a weak mutant allele of *BRI1* (54) (**Figure 8*c***). Overexpression of *BRS1* leads to suppression of the dwarf phenotype of *bri1–5* mutant plants, but not of kinase-dead mutant *bri1* alleles (54). The phenotype is specific because BRS1 overexpression does not cause phenotypes in wild-type plants and can not suppress phenotypes caused by mutations in the receptor-like kinases *clavata-1* (*CLV1*) and *erecta* (*ER*) (54). No phenotypes are observed in *brs1* knockout lines, which indicates that BRS1 homologs act redundantly (54). Overexpression of a catalytic mutant of BRS1 could not suppress the *bri1–5* phenotype, which

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indicates that catalytic activity is required for BRS1 function (54). BRS1 was characterized biochemically after purification from *Arabidopsis* plants overexpressing BRS1 (106). As with other S10 enzymes, BRS1 activation involves the posttranslational removal of a linker peptide, which results in A- and B-chains that remain linked through disulfide bridges. This activation step requires BRS1 activity because active site BRS1 mutants accumulate as proproteins. Active BRS1 can cleave synthetic dipeptides in vitro, which is significant because other S10 family members, such as SNG1 (see above), act as acyltransferases, rather than proteases. A role for endogenous *BRS1* in wild-type plants remains to be shown, but its expression pattern is ubiquitous and overlaps with that of *BRI1*, and BRS1-GFP fusion proteins are detected in the cell wall, which is different from most SCPLs (106). These details and the genetic data are consistent with the model that BRS1 acts upstream of BRI1 in BR signaling, either by activating proteins that assist in BR perception, or by removing proteins that block the BR binding site.

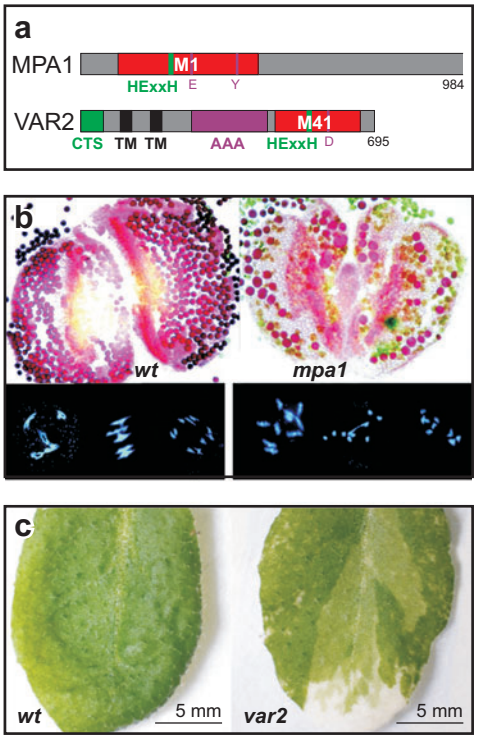

Figure 9

MPA1 and VAR1/2 (*a*) Domains of meiotic prophase aminopeptidase 1 (MPA1) and yellow variegated 1 (VAR1) proteins. CTS, chloroplast targeting sequence (*green*); TM, transmembrane domain (*black*); AAA, ATP-binding cassette (*purple*); M1/M41, protease domain (*red*); E, Y, D, catalytic residues Glu, Tyr, and Asp (*purple*); HExxH, zinc-binding motif (*green*). (*b*) The top panel shows that *mpa1* mutants have nonviable pollen (*green*). The bottom panel shows that during meiosis (diakinesis, metaphase I, anaphase I), chromosomes do not pair during metaphase I in the *mpa1* mutants, which leads to unequal division of the chromosomes. Reprinted from Reference 78 with permission from the American Society of Plant Biologists. (*c*) *var2* mutant lacks developed chloroplasts in the white sectors. Reprinted from Reference 47 with permission from the American Society of Plant Biologists.

METALLOPROTEASES

Metalloproteases contain catalytic metal ions that activate water for nucleophilic attack while stabilizing the oxyanion hole. Plant genomes encode approximately 100 metalloproteases that belong to 19 families. These families are diverse and are divided over 11 clans that are evolutionarily unrelated. Plant metalloprotease families usually contain fewer than 20 members. Metalloproteases are involved in nodulation, plastid differentiation, thermotolerance, regulation of root and shoot meristem size, sensitivity to auxin conjugates, and meiosis (**Table 1**). Four of these proteases are discussed below.

Clan MA Metalloproteases MPA1 and VAR1/2

Clan MA metalloproteases are united by the presence of a HExxH motif in which the two His (H) residues are ligands of a single zinc ion, and the Glu (E) provides a catalytic function (**Figure 9*a***). These proteases are folded as a bundle of helices and a β-sheet, and the active site is between two helices. In plants, clan MA contains six protease families. Members of the M1 family are mostly aminopeptidases, whereas family M41 members act progressively from both the N and C terminus. M41 proteases share homology with the

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Table 8 Clan MA metalloproteases MPA1 (meiotic prophase aminopeptidase 1) and VAR1/2 (yellow variegated 1/2)

Gene name	*MPA1*	*VAR2*
Described alleles	*mpa1* (78)	*var2–1. . .8* (18, 19, 60, 87)
Knockout phenotype	Impaired meiosis, reduced fertility, suppressed recombination (78)	Variegation: white sectors in all tissues, except cotyledons (19, 60)
Knockdown phenotype	Not reported	Not reported
Overexpression phenotype	Not reported	No phenotype (103)
Endogenous expression	Reproductive and vegetative tissues (78)	All green tissues (102)
Subcellular localization	Cytoplasm and nucleus (78)	Thylakoid membrane (18)
Genetic interactors	Not reported	*var2* phenotype is suppressed by *fug1*; *sco1*; *cplc2* and overexpression of *FtsH8* (61, 66, 102)
Proteolytic activity	Not reported	Not reported
Putative mechanism	May regulate complex assembly and disassembly required for chromosome pairing during prophase I of meiosis (78)	Dual: removes photodamaged D1 protein from photosystem II (3) and regulates thylakoid formation during chloroplast biogenesis (18)

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well-studied FtsH protease of *Escherichia coli*. FtsH proteases are membrane-bound, contain two transmembrane domains, and show ATP-dependent proteolytic activity.

MPA1. Meiotic prophase aminopeptidase 1 (MPA1) (**Table 8**) is essential for chromosome pairing and recombination during meiosis. *mpa1* mutants were identified from a forward genetic screen for mutants with reduced fertility (78). Siliques of *mpa1* mutants are smaller and contain only two or three seeds. A large proportion of *mpa1* pollen is not viable, smaller than wild-type, and deformed. Cytological analysis reveals that meiosis is impaired in both male and female gametogenesis (78). Homologous chromosomes fail to pair at the end of prophase I and the chromosomes separate unequally between the daughter cells (**Figure 9*b***). Homologous recombination is significantly repressed and only a few nuclei contain the proper five chromosomes by the end of anaphase II. *MPA1* encodes a metalloprotease of a M1 subfamily that is sensitive to the antibiotic purimycin and the noncompetitive fluorescent inhibitor DAMPAQ-22. Adding these inhibitors to wild-type plants can phenocopy the *mpa1* phenotype (78). Detailed immunolocalization studies with meiotic marker proteins indicate that the window of MPA1 activity occurs at an early stage in the recombination pathway, soon after the RecA homolog RAD51 is loaded onto the chromatin, but before loading of the mismatch repair protein MSH4 (78). These data indicate that MPA1 may be required for the assembly or disassembly of protein complexes that contain RAD51 or MSH4. MPA1 is also expressed in vegetative tissue, but its function there is unknown because *mpa1* plants grow normally.

VAR2. Yellow variegated 2 (VAR2) (**Table 8**) is crucial for chloroplast biogenesis and repair of photosystem II (PSII). Variegation is an obvious phenotype because parts of the green tissues appear white (**Figure 9*c***). Instead of chloroplasts, white tissues of *var2* mutants contain undifferentiated plastids that lack the typical thylakoids (19, 87). Plastids in dark-grown *var2* seedlings (etioplasts) resemble those in wild-type, but fail to differentiate in the light (18). The white sectors are initially yellow and are found in all green organs of the plant (leaves, stems, and siliques), except the cotyledons (19, 60). Eight *var2* alleles have been described, each displays different degrees of variegation (18, 19, 77, 87). *VAR2* is a nuclear gene that encodes an ATP-dependent FtsH metalloprotease (18, 87) (**Figure 9*a***).

Variegation: patchiness of pigmentation of leaves and other organs

Thylakoids: disc-shaped membrane vesicles in chloroplast stroma that carry the photosynthetic apparatus

VAR2 proteins accumulate in the thylakoid membrane with the domains for ATP binding and proteolysis facing the stroma (18).

Variegation in *var2* mutants also depends on VAR2 homologs. VAR2 (FtsH2) is one of 12 FtsH proteases encoded by the *Arabidopsis* genome (77, 102). The closest homolog of VAR2 is FtsH8. Although *ftsh8* mutants do not have a phenotype (77), *FtsH8* overexpression can suppress the *var2* phenotype (102) and the *var2/ftsh8* double mutant is completely white and can only survive on sugar-containing medium (104). These results suggest that VAR2 and FtsH8 act redundantly and support the hypothesis that FtsH8 compensates for the lack of VAR2 in the green sectors of *var2* mutant plants (102). Sector formation could arise from clonal propagation of cells that contain malfunctioning proplastids early during leaf development (102). VAR1 (FtsH5) is one of the other chloroplastic FtsH proteases, and *var1* mutant plants display a less severe variegation compared with *var2* (60). Similar to *var2*, the *var1* phenotype can be suppressed by overexpression of its closest homolog, *FtsH1* (103), and *var1/ftsh1* double mutants are completely white (104). VAR1 and VAR2 proteins form heterocomplexes that become unstable if one of the complex partners is missing (77, 102).

Apart from its role in chloroplast biogenesis, VAR2 is also essential for the repair of photosystem II by removal of the photodamaged D1 protein (3). However, hampered repair of photosystem II is probably not the cause of nonfunctional plastids in white sectors because these plastids are underdeveloped and not a result of photobleaching (47). This result suggests that VAR2 plays a role in chloroplast biogenesis, perhaps in the regulation of the formation of thylakoids by the accumulation of VAR2-containing complexes during chloroplast development.

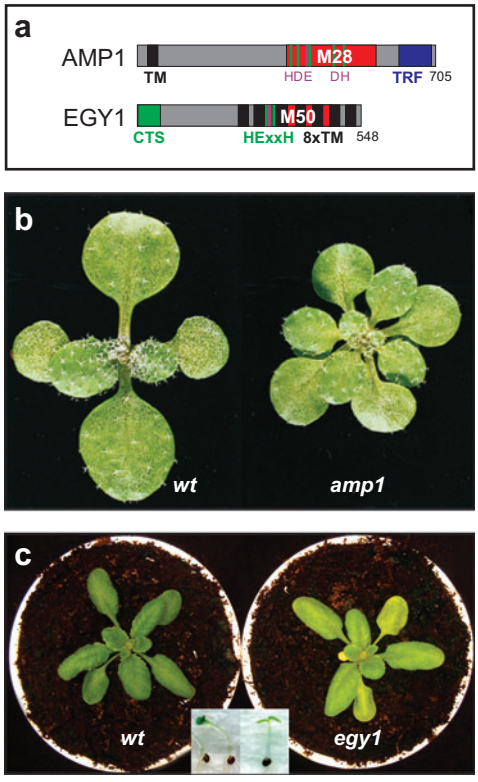

Figure 10

AMP1 and EGY1 (*a*) Domains of altered meristem program 1 (AMP1) and ethylene-dependent gravitropism-deficient yellow-green 1 (EGY1) proteins. CTS, chloroplast targeting sequence (*green*); TM, transmembrane domain (*black*); TRF, TATA binding protein–related factors (TRF) dimer motif (*dark blue*); M28/M50, protease domain (*red*); E, catalytic residue Glu (*purple*); HExxH, H, D, zinc-ion binding residues (*green*). (*b*) *amp1* mutants display an enlarged shoot apical meristem that generates more organs. Reprinted with permission from Reference 22, Copyright 1997, National Academy of Sciences, U. S. A. (*c*) *egy1* mutants display yellowish leaves with underdeveloped plastids and a hampered shoot gravitropism (*inset*). Pictures kindly provided by Dr. N. Li (Hong Kong University of Science and Technology, Hong Kong, China).

Metalloproteases AMP1 and EGY1

Two more metalloproteases are discussed here: AMP1 (family M28, clan MH) and EGY1 (family M50, clan MM). These clans are not related to each other evolutionarily or structurally. Family M28 of clan MH contains proteases that are folded as a six-stranded β-sheet surrounded by helices; the active site contains two cocatalytic zinc ions (**Figure 10*a***). Family M50 of clan MM contains proteases that are membrane-bound and contain a HExxH motif that probably binds a single zinc ion (**Figure 10*a***). The catalytic site may be in the membrane because it is part

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Table 9 Metalloproteases AMP1 (altered meristem program 1) and EGY1 (ethylene-dependent gravitropism-deficient yellow-green 1)

Gene name	*AMP1*	*EGY1*
Described alleles	*amp1–1. . .7; pt; hpt; cop2* (14, 22, 44, 75)	*egy1–1..3* (15)
Knockout phenotype	Polycotyly, more leaves, larger shoot apical meristem (14, 22, 44, 62)	Less chlorophyl, no gravitropism, fewer seeds (15)
Knockdown phenotype	Not reported	Not reported
Overexpression phenotype	Not reported	Not reported
Endogenous expression	Throughout the plant (39)	Throughout the plant, lower in roots (15)
Subcellular localization	Unknown	Chloroplast membrane (EGY1-GFP) (15)
Genetic interactors	Independent from *clv1* and *clv3* (*clavata 1* and *3*) (62); suppressor of monopterous (*mp*) (94)	Not reported
Proteolytic activity	Not reported	Degrades β-casein in vitro (15)
Putative mechanism	May release peptides that promote differentiation or inactivate peptides that suppress differentiation (39)	May regulate the assembly and maintenance of photosystem II complexes (15)

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of a predicted transmembrane domain, but so far no tertiary structure is available to confirm this topology.

AMP1. Altered meristem program 1 (AMP1) (**Table 9**) restricts the size of the shoot apical meristem by promoting differentiation. *AMP1* has many names because it was identified from many forward genetic screens and has a series of obvious phenotypes: *amp1* seedlings grow in the dark as though they are growing in the light [hence *cop2*, *constitutive photomorphogenesis* (14, 41)], frequently have more than two cotyledons [hence *hpt*, *hauptling* (44)], and generate leaves in whorls of three instead of one by one [hence *pt*, *primordia timing* (22)] (**Figure 10*b***). A reduced apical dominance also makes *amp1* mutant plants bushier, the rate of leaf formation is doubled, and the plants flower earlier (14, 22, 62). The *amp1* mutation causes male and female semisterility, which results in shorter siliques and fewer seeds (14, 62). The earliest *amp1* phenotype during embryogenesis appears at the second division of the zygote. The basal cell normally divides transversely to generate the suspensor, but in *amp1* mutant embryos the cells in the apical region of the suspensor undergo a series of vertical divisions to generate cells that become incorporated into an oversized shoot apical meristem (SAM) (22, 94). The SAM stays large throughout development and causes the initiation of mutiple organs. This phenotype suggests that in wild-type plants AMP1 promotes differentiation, which keeps the SAM small (94). Mutant *amp1* plants produce more cytokinin, probably as a result of the increased SAM size (14, 22, 39, 62). A larger SAM also occurs in *clavata* (*clv*) mutants. However, *amp1/clv1* and *amp1/clv3* double mutants show additive effects on SAM size, which indicates that AMP1 acts independently of the CLV1/3 pathway (62). AMP1 interacts genetically with MONOPTEROUS (MP), an auxin-response factor that acts with the Aux/IAA family in transcriptional regulation (94). Phenotypes of *mp* mutants are the opposite of *amp1* mutant phenotypes, and are suppressed in *amp1/mp* double mutants (94). This suggests that in wild-type plants, MP counteracts AMP1 by carving out meristematic niches by locally overcoming the differentiation-promoting activity of AMP1 (94). The data suggest that AMP1 releases signaling peptides that promote differentiation at the SAM border or inactivates signaling proteins that suppress differentiation (39, 94). Molecular details of these signaling pathways remain to be investigated.

Shoot apical meristem (SAM): population of dividing cells at the tip of the shoot axis

Gravitropic response: growth in relation to gravity

Grana: stack of thylakoids in chloroplasts

Amyloplast: colorless starch-forming plastid

EGY1. Ethylene-dependent gravitropism-deficient yellow-green 1 (EGY1) (**Table 9**) is a chloroplast intermembrane metalloprotease, essential for plastid development and shoot gravitropism. *egy1* mutants were identified from a genetic screen for mutants that were both pigmentation deficient and defective in ethylene-stimulated hypocotyl gravitropic responses (15). Instead of normal chloroplasts, *egy1* mutants contain plastids that have fewer stromal thylakoids, no grana and fewer starch grains. The *egy1* mutants also accumulate significantly fewer chlorophyll a/b binding (CAB) proteins, which are part of photosystem II antennae in the thylakoid membrane (15). Hampered photosynthesis explains the reduced growth rate, lower seed number, and the yellowish color (**Figure 10*c***). However, the absence of the shoot gravitropism response in *egy1* mutants is unexplained, although chloroplasts in the endodermis of *egy1* mutants may not differentiate into amyloplasts, which are the plastids required for the gravitropic response (15). EGY1 contains eight transmembrane helices and resides in the membrane of chloroplasts where it may regulate the maintenance and assembly of PSII complexes by intermembrane proteolysis. This is consistent with the accumulation of the EGY1 protein in chloroplasts in response to light (15). Interestingly, although EGY1 and VAR2 probably both act on the maintenance of photosystem II, their roles are likely different given the differences in phenotypes.

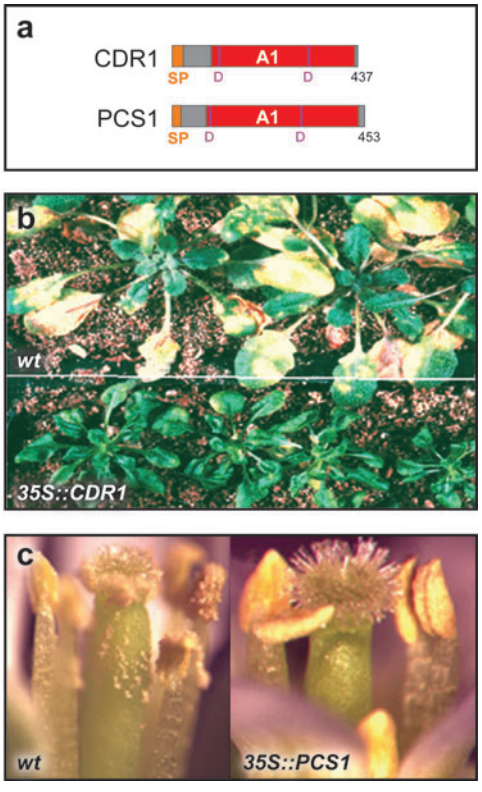

Figure 11

CDR1 and PCS1 (*a*) Domains of constitutive disease resistance 1 (CDR1) and promotion of cell survival 1 (PCS1) proteins. SP, signal peptide (*orange*); A1, protease domain (*red*); D, catalytic residue Asp (*purple*). (*b*) *CDR1*-overexpressing plants are semidwarfed and show enhanced disease resistance to infections with *Pseudomonas* bacteria. Reprinted from Reference 98 with permission from Macmillan Publishers Ltd. (*c*) *PCS1* overexpression prevents pollen release from anthers because programmed cell death is blocked in specific anther cell types. Reprinted from Reference 34 with permission from Macmillan Publishers Ltd.

ASPARTIC PROTEASES

Aspartic proteases contain two aspartic residues, which support a water molecule that acts as the nucleophile during proteolysis. Only three families of aspartic proteases exist in plants, but these families are so large that aspartic proteases make up the second-largest protease class in plants. Biological roles for aspartic proteases are only known for two pepsin-like proteases in family A1 of clan AA.

Pepsin-Like Proteases CDR1 and PCS1

Pepsin-like proteases (family A1, clan AA) are endopeptidases that are most active at acidic pH. The enzymes are produced as preproproteases and are often secreted from cells as inactive, glycosylated enzymes that activate autocatalytically at acidic pH (**Figure 11*a***). The three-dimensional structure reveals traces of ancient gene duplication: Pepsin-like proteases comprise

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two highly homologous lobes, each contains a homologous catalytic aspartate residue that forms the active site between the two lobes (69). The *Arabidopsis* genome encodes approximately 70 pepsin-like proteases, which can be divided into five subfamilies (8). CDR1 and PCS1 are typical pepsin-like proteases, with very different biological roles.

CDR1. Constitutive disease resistance 1 (CDR1) (**Table 10**) acts in disease resistance signaling. CDR1 was identified by activation tagging (98). *CDR1* overexpression suppresses disease caused by virulent strains of the pathogenic bacterium *Pseudomonas syringae* (**Figure 11*b***). This reduced susceptibility is explained by a constitutive upregulation of defense responses in *CDR1*-overexpressing plants, including microlesions, high levels of reactive oxygen intermediates (ROIs) and salicylic acid (SA), and constitutive expression of pathogenesis-related (PR) genes (86, 98). The constitutive defense response explains why *CDR1*-overexpressing plants are smaller and their leaves are darker and curled compared with wild-type (**Figure 11*b***). None of these phenotypes occurs when *CDR1* overexpressing plants also express the bacterial *NahG* gene, which encodes an enzyme that converts SA into cathechol. This result indicates that SA is required for *CDR1*-induced responses (98). CDR1-knockout lines are not available, but antisense *CDR1* lines are more susceptible to virulent *Pseudomonas* strains, which indicates that endogenous CDR1 also acts in defense responses (98). Active site mutants of CDR1 could not trigger resistance when overexpressed, which indicates that CDR1 protease activity is required for CDR1 function (98). The CDR1 protein displays proteolytic activity and accumulates in the extracellular space of plants during pathogen attack (86, 98). CDR1 activity may release small peptides in the apoplast that can systemically induce *PR* gene expression (98). Thus, CDR1 activity may lead to the generation of an endogenous extracellular elicitor that could act as a mobile signal for the induction of systemic acquired resistance (SAR).

PCS1. Promotion of cell survival (PCS1) (**Table 10**) is an endoplasmic reticulum (ER)-resident aspartic protease that prevents

Pathogenesis-related genes (PR genes): genes that are upregulated during pathogen infection

Systemic acquired resistance (SAR): activation of defense in uninfected parts of a plant

Table 10 Pepsin-like proteases CDR1 (constitutive disease resistance 1) and PCS1 (promotion of cell survival 1)

Gene name	*CDR1*	*PCS1*
Described alleles	Unknown	*pcs1* (34)
Knockout phenotype	Not reported	Degeneration of pollen and abortion of ovules and embryos (34)
Knockdown phenotype	Enhanced susceptibility to infection with *Pseudomonas* bacteria (98)	Not reported
Overexpression phenotype	Semidwarfing; constitutive disease resistance (86, 98)	Blocks programmed cell death in anther dehiscence (34)
Endogenous expression	Ubiquitous and slightly upregulated during pathogen infection (98)	Only in gametophytes and developing seeds (34)
Subcellular localization	Secreted (predicted) CDR1-GFP is in the cell wall and endoplasmic reticulum, and CDR1 is in the apoplast (86, 98)	Endoplasmatic reticulum (EndoH sensitive; PCS1-GFP) (34)
Genetic interactors	CDR1 overexpression phenotypes are absent in salicylic acid (SA)-deficient *NahG* lines (98)	Not reported
Proteolytic activity	Cleaves bovine serum albumin in vitro (86, 98)	Cleaves casein in vitro (34)
Putative mechanism	Could generate endogenous extracellular peptides that act as mobile signals for systemic acquired resistance (86, 98)	May release survival factors or inactivate death signals (34)

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cell death during gametogenesis and embryogenesis. When knockout lines for aspartic proteases were investigated, homozygous *pcs1* plants could not be obtained (34). Further investigation revealed that a third of the pollen and ovules from heterozygous *pcs1*/+ plants are degenerated, and the embryos die before the torpedo stage (34). Consistent with the observed phenotypes, *PCS1* gene expression is specific to developing gametophytes and developing seeds (34). However, when ectopically overexpressed, *PCS1* blocks PCD in the anther, which prevents the release of pollen because stobium and septum cells in the anther cell wall do not undergo PCD (34) (**Figure 11*c***). Biochemical studies show that PCS1 has proteolytic activity, but how this activity contributes to its function remains to be demonstrated. Intriguingly, deglycosylation experiments and experiments with GFP-fusion proteins demonstrated that PCS1 is localized to the ER (34). The phenotypes can be explained by a role for PCS1 in the prevention of cell death in certain cell types. PCS1 is likely involved in the proteolytic release of survival factors or the inactivation of death signals.

CONCLUSIONS

The biological roles of plant proteases are strikingly diverse (**Figure 12**). Protease functions have been identified for different stages in the life of a plant: meiosis (MPA1); gametophyte survival (PCS1); suspensor formation (mcII-Pa); embryo cuticle deposition (ALE1); seed coat formation (δVPE); meristem size (AMP1); epidermal cell fate (DEK1); stomata development (SDD1); chloroplast development (VAR2); plastid development (EGY1); growth (BRS1); UV

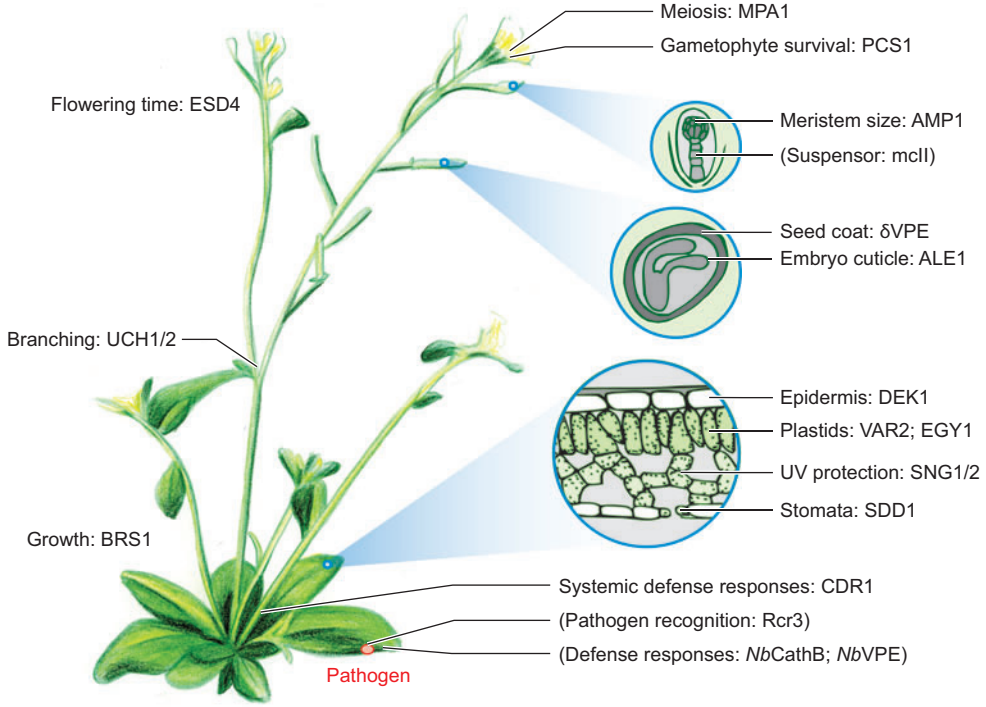

Figure 12

Summary of the biological roles of the discussed proteases. Proteases that were not studied in *Arabidopsis* are shown in parentheses.

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protection (SNG1); pathogen recognition (Rcr3); defense responses (*Nb*CathB, *Nb*VPE, γVPE); systemic defense responses (CDR1); flowering time (ESD4); and branching (UCH1/2).

Proteases are crucial for plants. Protease mutations are frequently lethal (e.g., *dek1*, *ale1*, and *pcs1*), and many result in severe fitness-reducing phenotypes (e.g., *esd4*, *var2*, *egy1*, and *mpa1*), whereas some proteases act redundantly (e.g., *VAR2*, *VPEs*, *BRS1*, and *UCH1/2*), and are lethal when both genes are mutated (e.g., VAR2/FtsH8). The redundancy and lethality associated with protease mutants limit the opportunities offered by forward and reverse genetics.

The biochemical roles and subcellular locations of proteases are often conserved within the clans. Clan CA/CE, for example, contains conjugating enzymes, and clans SB and AA contain secreted proteases. However, there are only weak correlations between biological function and evolutionary relatedness within protease clans. Clan CD, for example, contains proteases that regulate PCD, and CA clan proteases are often involved in pathogen-induced hypersensitive cell death. For most clans, however, biological functions are very different, e.g., BRS1 and SNG1/2 (clan SB), MPA1 and VAR2 (clan MA), and CDR1 and PCS1 (clan AA). These differences indicate that these protease families did not arise from the evolution of new biological processes, but were recruited from existing protease families during evolution.

Proteases are found at different subcellular locations (**Figure 13**). Of the 17 discussed proteases, six are secreted into the apoplast (Rcr3, *Nb*CathB, ALE1, SDD1, BRS1, CDR1), two are in the vacuole (VPEs, SNG1/2), two are in the chloroplast (VAR2, EGY1), one resides in the ER (PCS1), four are cytoplasmic/nuclear in localization (UCH1/2, ESD4, MPA1, mcII-Pa), and two have unknown subcellular localizations (AMP1, DEK1). Transmembrane domains are found in three of the proteases (DEK1,

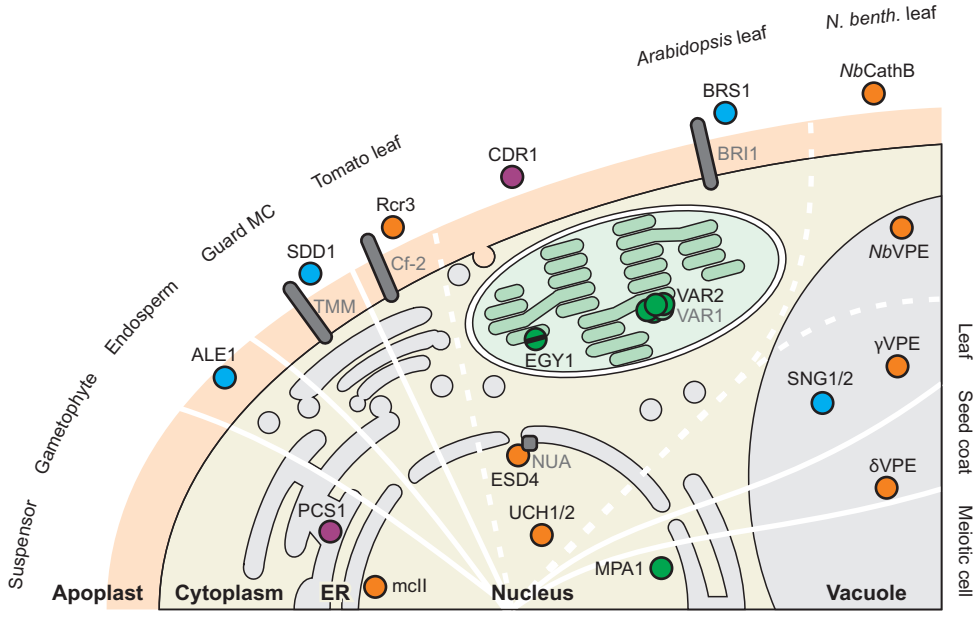

Figure 13

Summary of the subcellular locations of proteases and their interactors, as summarized in this review.

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VAR2, and EGYI), and the others are soluble or associated with membranes.

No biologically relevant substrate has been identified for any of the proteases described so far. However, some proteases do not act through their substrates (e.g., Rcr3), or act by catalyzing nonproteolytic reactions (e.g., SNG1/2). The absence of identified substrates for the other proteases is the major bottleneck for research because it hampers further progress in understanding the molecular basis of how these proteases function.

We know about other proteins that are required for the function of only a few proteases, although the molecular details of these interactions are not yet known. Functions of secreted proteases frequently depend on receptor-like proteins: BRS1 enhances signaling through BRI1, SDD1 signals through TMM, and Rcr3 inactivation by Avr2 is monitored by Cf-2. In contrast, VAR2 and ESD4 physically interact with VAR1 and NUA, respectively; these interactions are essential for their function.

FUTURE ISSUES

Proteases are crucial in plants, but this field has only just started to unfold. There are phenotypic data for many proteases, but there is still little understanding of their molecular mechanisms. More than 500 proteases remain to be functionally characterized. A daunting task lies ahead to understand the molecular mechanisms of these proteases. The challenge is summarized in the following issues:

1. Where are the proteases localized? This question has already been answered for most proteases, most often via the use of GFP fusion proteins, but for some proteases, like DEK1 and AMP1, this issue remains to be addressed. This task can be challenging because many proteases are processed, which makes it difficult to generate stable GFP-fusion proteins, as described for SDD1.
2. What are the substrates? This question is the hardest to resolve but also the most important. Proteases can cleave many proteins in vitro, but the biologically relevant target substrate is determined not only by substrate specificity, but also by its colocalization with the protease, in both time and space. If there is one major biologically relevant substrate to be cleaved, then this substrate might be identified through forward genetic approaches for suppressors of protease mutant phenotypes. Alternatively, characterization of the protease cleavage specificity and the subcellular location might be used to select and test candidate proteins on the basis of their predicted colocalization, expression, and putative cleavage sites. Other approaches for substrate identification are yeast two-hybrid screening, immobilized protein arrays, and differential proteomics (79), but each of these approaches has its limitations.
3. How are protease activities regulated? This is an intriguing question, but hardly resolved. Many proteases have autoinhibitory domains that are proteolytically removed during activation, but the molecular mechanism of this activation is often unclear. The activity of many proteases is probably also controlled by endogenous inhibitors, but their identity is also unknown. Another layer of regulation comes from environmental conditions such as pH, calcium ions, ATP, and redox status. These issues are poorly described, but are fundamental to understand when and where the protease is active. Fluorescent activity-based probes and substrates are useful tools to image the space and time of protease activities (5), but their potential remains to be exploited.

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4. How do proteases contribute to the phenotype? This bigger picture requires knowledge of not only the identity of the substrate, but also of other components that are part of the network in which the protease functions, such as receptors and transcription factors. Putting all this knowledge together should provide a systems biology model that explains how the protease is incorporated into the network that leads to the phenotype.
5. What is the biological function of the remaining >500 proteases? As described above, protease functions differ tremendously, even within families of related proteases. This makes it difficult to predict the biological function of a protease. Reverse genetics, via the use of RNAi approaches, T-DNA lines, or overexpression, may reveal phenotypes. This approach, however, may not be successful if the protease acts redundantly with family members. Pharmacological approaches can offer another approach to annotate functions to proteases. This approach also allows a choice of time point, dosage, and specificity of chemical interference.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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