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The plant proteolytic machinery and its role in defence

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The diverse roles of plant proteases in defence responses that are triggered by pathogens or pests are becoming clearer. Some proteases, such as papain in latex, execute the attack on the invading organism. Other proteases seem to be part of a signalling cascade, as indicated by protease inhibitor studies. Such a role has also been suggested for the recently discovered metacaspases and CDR1. Some proteases, such as RCR3, even act in perceiving the invader. These exciting recent reports are probably just the first examples of what lies beneath. More roles for plant proteases in defence, as well as the regulation and substrates of these enzymes, are waiting to be discovered.

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Abbreviations

AEBSF	p-aminoethylbenzenesulphonyl fluoride
Avr	avirulence gene
CDR1	CONSTITUTIVE DISEASE RESISTANCE-1
E-64d	trans-epoxysuccinyl-leucylamido-3-methylbutane ester
ER	endoplasmic reticulum
HR	hypersensitive response
LapA	acidic leucine aminopeptidase
MCP	metacaspase
Mir1	maize inbred resistant-1
NO	nitric oxide
P69	69-kDa PR protein
PCD	programmed cell death
PR	pathogenesis-related
RCR3	REQUIRED FOR *Cladosporium* RESISTANCE-3
RD21	RESPONSIVE TO DESICCATION-21
TMV	tobacco mosaic virus
VPE	vacuolar processing enzyme
YCA1	yeast caspase-1

Introduction

With hundreds of genes encoding proteases, plants are equipped with a large proteolytic machinery that irreversibly regulates the fate of proteins. This machinery has generally been viewed in a housekeeping role, serving to remove non-functional proteins and to release of amino acids for recycling. However, proteases also appear to play key roles in the regulation of biological processes in plants, such as the recognition of pathogens and pests and the induction of effective defence responses.

Indications of roles of plant proteases in defence came from observations that subtilisin-like proteases (P69 [for 69-kDa PR protein]) accumulate in viroid-infected tomato plants [1], and that acidic leucine aminopeptidase (LapA) activity increases during insect feeding [2]. Associations between the induction of protease genes and defence have also been found for genes that encode metallo, aspartic and cysteine proteases [3–5].

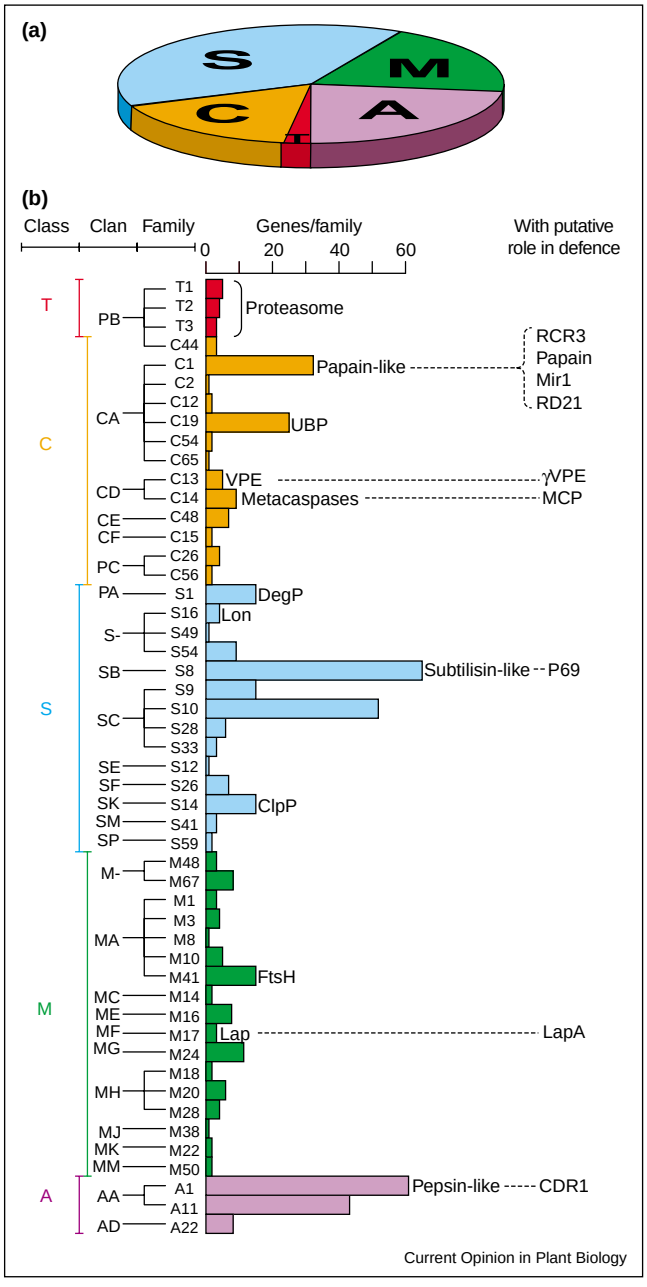
Apart from these correlations, there are other reasons to expect that proteases could be involved in plant defence. Certain cysteine proteases called the caspases play a key role in animal apoptosis, a form of programmed cell death (PCD). Apoptosis has many features in common with the plant hypersensitive response (HR), a defence mechanism that involves PCD [6]. The involvement of plant proteases in signalling during HR has been predicted on the basis of a seemingly conserved 'death pathway'. Although plant caspase genes have not yet been identified, their existence is indicated by many studies in which caspase inhibitors blocked the HR and other defence responses.

Further evidence that plant proteases are involved in defence emerged recently with the identification of RCR3, a secreted cysteine protease that is required for the function of the resistance gene *Cf-2* (for *Cladosporium fulvum resistance-2*) [7••], and CDR1, a secreted aspartic protease that regulates defence responses [8••]. More recent studies have revealed additional roles for proteases in plant defence. In this review, we summarise and comment on the current data on the roles of proteases in plant defence, drawing upon background information when appropriate.

The non-proteasome plant proteolytic machinery

At least 488 protease genes, divided into five catalytic classes (Figure 1a), can been identified within the *Arabidopsis* genome sequence. An extremely helpful updated classification of all known proteases is contained within the MEROPS database (http://merops.sanger.ac.uk; [9]). In this database, proteases are placed within the same family if they share sufficient sequence homology, and families that are believed to have a common ancestor are placed within the same clan. The *Arabidopsis* genome encodes proteases of 50 different families, divided over

Figure 1



Classification of the *Arabidopsis* proteases. **(a)** Pie diagram of the catalytic types of *Arabidopsis* proteases. A total of 488 proteases can be distinguished within the encoded *Arabidopsis* genome, most of which are also represented in the MEROPS database. Proteases can be subdivided into catalytic types on the basis of the residues used to cleave a peptide bond. The *Arabidopsis* genome encodes 198 serine (S), 112 aspartic (A), 95 cysteine (C), 80 metallo (M) and 12 threonine (T) proteases. **(b)** The number of *Arabidopsis* proteases belonging to the different clans and families. Each protease class consists of several clans of proteases, which are identified by a letter following the catalytic class (e.g. clan CA within class C). Members of a single clan are believed, on the basis of their conserved tertiary structure and order and spacing of catalytic residues, to have a common evolutionary origin. Proteases of clans starting with the letter 'P' can have different catalytic residues. Each clan of proteases consists of several families, which are identified by a number following the catalytic type (e.g. family C1 within clan CA). Not all clans and families of plant proteases are represented in *Arabidopsis*. The number of proteases belonging to each family is indicated by bars, and the classification of well-studied proteases is indicated. DegP, Lon, ClpP and FtsH proteases are localised in chloroplasts and mitochondria [58]. UBPs are de-ubiquitinating enzymes. Detailed phylogenies of papain-, subtilisin-, and pepsin-like proteases have recently been published elsewhere [10••].

27 clans (Figure 1b). Amongst the largest families are the S8 (subtilisin-like), C1 (papain-like), and A1 (pepsin-like) groups, which have been well described recently [10••]. As this classification is based on evolutionary relationships, it is likely that the functions of proteases are similarly clustered between clans and families. This classification also helps to predict the effect of protease inhibitors on the members of each group of proteases.

Protease inhibitors: leading or misleading?

Pharmacological research has provided a wealth of inhibitors of animal proteases. The same inhibitors have been used to investigate the role of plant proteases in defence, as summarised in Table 1. The location at which the protease inhibitors are supposed to act is summarised in Figure 2. Conclusions drawn from these studies involving protease inhibitors should be taken with caution, however, as many of these inhibitors are not as specific as was originally believed. In addition, many reports on protease inhibitors seem to be contradictory, perhaps because of differences between the plant species and/or inducing agents that were used. For example, caspase inhibitors but not leupeptin effectively suppressed HR in one study [11], whereas in another study, PCD was inhibited effectively by leupeptin but not by caspase inhibitors [12]. Despite the discrepancies, however, these inhibitor studies indicate the involvement of various proteases in the regulation of defence-associated cell death.

Serine protease inhibitors

The involvement of serine proteases in plant defence signalling was indicated by studies using the serine protease inhibitor aprotinin, which blocked elicitin-induced cell death in tobacco cell cultures [13], and AEBSF, which blocked both H_2O_2-induced cell death in soybean cells [14] and xylanase-induced cell death in tobacco cell cultures [12]. However, it is not possible to conclude with any certainty that serine proteases are involved in defence signalling on the basis of the latter experiments because AEBSF also inhibits cysteine proteases such as papain [15].

Papain inhibitors

Papain-like cysteine proteases (family C1) are inhibited by cystatin and by E-64d. Co-infiltration of E-64d with avirulent cowpea rust spores in resistant cowpea leaves delayed the death of invaded cells [16]. In addition, the overexpression of cystatin in soybean cell suspensions

Table 1

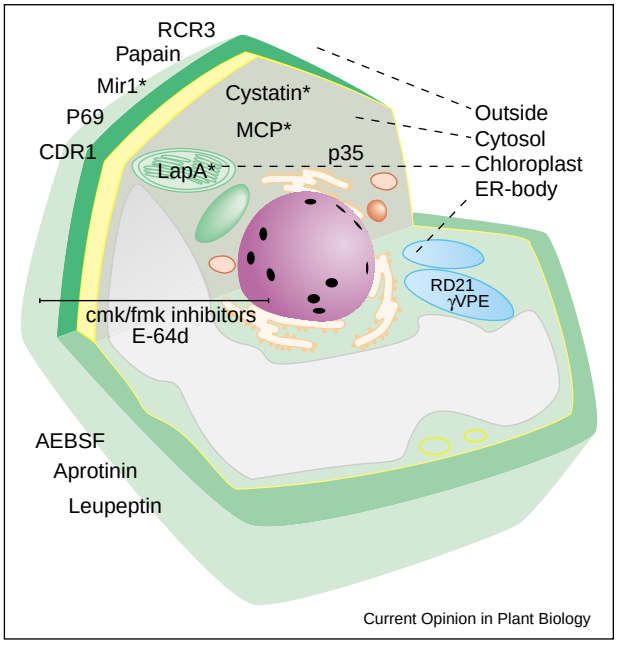
Effects of protease inhibitors on defence-related responses.

Plant tissue	Inhibitor	Action[b]	Inhibits cell death induced by:[c]	Reference
Soybean cell culture	AEBSF	Serine	H_2O_2	[14]
Tobacco cell culture	Leupeptin, AEBSF	Cysteine/serine	Xylanase	[12]
Tobacco cell culture	Aprotinin	Serine	Elicitin	[13]
Cowpea leaf	E-64d	Cysteine	Avirulent cowpea rust	[16]
Soybean cell culture	Cystatin	Cysteine	H_2O_2/avirulent *Psg*	[15]
Tobacco leaf	Cystatin	Cysteine	Avirulent *Psp*	[17••]
Arabidopsis cell culture	Cystatin	Cysteine	H_2O_2/avirulent *Psm*	[17••]
Tobacco leaf	DEVD[a], YVAD[a]	Caspase	Avirulent *Psp*	[11]
Arabidopsis cell culture	YVAD[a]	Caspase	NO and H_2O_2	[54]
Tobacco/bean leaf	DEVD[a], YVAD[a]	Caspase	Avirulent *Psp*, *Pst* and *Psa*	[55]
Tobacco cell culture	zVAD[a], BocD[a]	Caspase	Xylanase	[56•]
Soybean cell culture	DEVD[a]	Caspase	Chitosan	[57]
Tobacco leaf	TATD[a]	Caspase	Avirulent TMV	[21••]
Tobacco leaf	p35	Caspase	Avirulent TMV	[23•]

[a]Caspase inhibitors such as fluoro-/chloromethylketone or aldehyde derivatives were used. [b]Protease class on which the protease inhibitor is supposed to act. [c]Cell-death-inducing treatments are abbreviated as *Psa*, *Psg*, *Psm*, *Psp* and *Pst* for *Pseudomonas syringae* pv. *angulata, glycinea, maculicola*, *phaseolicola* and *tabacci*, respectively.

blocked PCD and induced protease activity in response to H_2O_2 or avirulent bacteria [15]. Both studies also revealed a stress-induced activity of cysteine proteases in the cytosol. The latter study was followed up recently with the ectopic overexpression of an *Arabidopsis* cystatin that is induced in leaves by wounding, avirulent bacteria and nitric oxide (NO) [17••]. This cystatin inhibits papain activity and is one of six identified *Arabidopsis* cystatin genes. Overexpression of this cystatin in *Arabidopsis* cell cultures blocked cell death in response to avirulent bacteria and NO. Furthermore, overexpression of this cystatin in tobacco plants blocked the HR induced by avirulent bacteria. These cystatin studies indicate a key role in plant defence not only for cysteine proteases but also for endogenous protease inhibitors that counterbalance the action of proteases.

Figure 2



Presumed localisation of inhibitors and proteases. Protease inhibitors (Table 1) have been expressed in the cytosol (cystatin and p35) or administered extracellularly. Some of these inhibitors are probably membrane-permeable (E-64 and peptide-based caspase chloro-/fluoromethylketone [cmk/fmk] inhibitors), whereas others are probably not (AEBSF, aprotinin and leupeptin). The localisations of inhibitors and proteases were determined either experimentally or by using the PSORT prediction program (the latter being indicated by *).

Caspase inhibitors

Mammalian caspases are cysteine proteases (family C14) that cleave substrates after aspartate residues (hence their name) and play a role in apoptosis [18]. Frequently used caspase inhibitors are small, membrane-permeable peptides that have a carboxy-terminal aspartate and a chloro-/fluoromethylketone group. Indications of an ancient signalling pathway that leads to PCD have provoked many investigators to use these caspase inhibitors to test whether caspase-like proteases also play a role in signalling leading to PCD in plants [19]. Indeed, defence-associated cell death that has been induced in various biological systems can be blocked by caspase inhibitors (Table 1). Careful interpretation of these results is needed, however, as these inhibitors are based on human caspase substrates, and because methylketone-based inhibitors are also able to inhibit papain-like cysteine proteases [20].

In the most recent hunt for caspase-like proteases in plants, a new caspase inhibitor was developed that is based on a caspase-like cleavage site in VirD2 [21••]. VirD2 is a virulence protein of *Agrobacterium* that is injected into the plant cytoplasm and that mediates the

transport of *Agrobacterium* T-DNA into the nucleus. Cleavage of VirD2, resulting in the loss of its nuclear localisation signal, can be mediated by both human caspase-3 and an endogenous plant cysteine protease activity that is induced during HR. Using an elegant assay with green fluorescent protein (GFP)-tagged VirD2, it was found that the nuclear localisation of the fusion product was lost when HR was induced. The identified cleavage site in VirD2 (TAVD/S) was used to generate a specific caspase inhibitor (biotinyl-TATD-CHO) that was found to delay HR in leaves inoculated with tobacco mosaic virus (TMV). Although these data are solid and interesting, the caspase-like protein remains to be identified.

The involvement of a caspase-like protease in defence signalling was also indicated with p35 transgenic tobacco plants. p35 is a caspase inhibitor from baculovirus that is effective against all known caspases from animal model systems. The cleavage of p35 by caspase results in a p35–caspase complex that inhibits caspase activity [22]. Expression of p35 in tobacco resulted in a delayed HR and the loss of TMV resistance, which depended on the presence of the caspase cleavage site in p35 [23•].

Proteases implicated in defence

Several studies on particular proteases and their role in defence have recently been published. Some of these proteases were investigated because they were differentially regulated, some were identified genetically, and others were identified by genomic approaches. These proteases are also mentioned in Figure 1b and their presumed localisation is summarised in Figure 2.

Papain-like proteases (clan CA)

Papain (C1) is a small protease with broad substrate specificity and is one of many cysteine proteases that were identified long ago in the latex of various plant species. However, its role in defence against herbivorous insects became clear only recently. Artificial diets containing papain, or bromelain or ficin (which are also C1 cysteine proteases from latex), in concentrations that occur in latex are toxic for silkworm larvae [24••]. In addition, the larvae died when fed on fig leaves, but not when the latex was removed from these leaves by washing or when the cysteine proteases were inactivated by E-64d. The feeding experiments show that proteases are a vital part of an arsenal of defence proteins in the latex that pours out of wounded sites during insect feeding.

The role of papain-like proteases has become increasingly intriguing with the genetic identification of RCR3, a secreted papain-like protease (C1) of tomato. RCR3 is required for the function of *Cf-2*, a resistance gene that mediates recognition of the *Avr2* avirulence gene of the fungal pathogen *C. fulvum* [7••]. The apoplastic localisation of RCR3, and its requirement for the function of Cf-2 but not of the highly homologous Cf-5 protein, suggests that RCR3 may act upstream of Cf-2, perhaps even in mediating the perception of the Avr2 protein. Various possibilities for the role of RCR3 have been proposed. RCR3 may cleave Avr2, Cf-2 or other plant proteins to activate the defence response. Alternatively, RCR3 may be inhibited by Avr2; in which case resistant plants may carry the Cf-2 protein to 'guard' RCR3 and detect such inhibition.

Mir1 is another papain-like cysteine protease (C1) that plays a role in defence against herbivorous insects. The abundance of Mir1 is increased dramatically at the site of larval feeding and in response to wounding [25]. Significantly, the overexpression of Mir1 in maize callus used for feeding experiments decreased larval growth by 80%. Mir1, like papain, may be directly toxic to larvae, but its proteolytic activity may also result in the release of other toxic compounds or essential signalling intermediates.

RD21 from *Arabidopsis* is a papain-like protease (C1) that accumulates in vesicles that originate from the endoplasmic reticulum (ER; hence called ER bodies [26••]). An RD21-like protease is also induced in potato upon infection with *Phytophthora infestans* [5]. A role for ER bodies in defence against insects was suggested by the observation that ER bodies are induced by wounding and insect feeding [27•], and that they fuse with the vacuole upon stress [28]. ER bodies may therefore be involved in defence against insects, and this suggests that RD21 may also play a role in this defence [26••].

Caspase-like proteases (clan CD)

Vacuolar processing enzymes (VPEs in family C13) are also cysteine proteases, but they probably evolved independently of papain-like proteases. Four VPEs that have been identified in *Arabidopsis* mediate the processing of seed storage proteins [29•,30•]. Interestingly, one of these *VPE* genes (*γVPE*) is induced upon wounding [31], and (like RD21) the γVPE protein accumulates in wound-induced ER bodies [28]. In addition, the active site of VPEs, and probably also their protein folding, resembles that of the caspases [32,33], suggesting that VPEs are evolutionarily related to mammalian caspases. These observations are intriguing, and it is tempting to speculate that VPEs also have a role in plant defence.

Metacaspases (MCPs; family C14) are relatives of caspases and were discovered in yeast and plants by iterative PSI-BLAST searches [33]. Their homology to human caspases is not restricted to sequences surrounding the catalytic residues but also extends into the secondary structure. Caspase-like proteolytic activity was recently shown *in vivo* for the yeast metacaspase YCA1. Overexpression of YCA1 in yeast enhanced H_2O_2-induced cell death, and its disruption blocked H_2O_2-induced cell

death [34••]. In addition, heterologous expression of a metacaspase from *Trypanosoma brucei* in yeast caused growth inhibition, mitochondrial dysfunction and clonal death [35]. Nine MCPs have been identified in the *Arabidopsis* genome, two of which are upregulated by bacterial pathogens [36•]. Interestingly, both of these metacaspases carry an additional amino-terminal zinc-finger domain that is also found in LSD1 (LESIONS SIMULATING DISEASE RESISTANCE-1), a negative regulator of HR [37]. In addition, a tomato metacaspase was found to be upregulated upon infection with *Botrytis cinerea* [38]. Much evidence is still needed to assign a role for plant metacaspases in regulating HR, but studies with caspase inhibitors and indications of a functionally conserved 'death pathway' hint at an important role for metacaspases in regulating HR-associated cell death.

Other proteases

P69 proteases are subtilisin-like proteases (family S8), that accumulate during infection as pathogenesis-related (PR) proteins [1,39]. Significantly, LRP, a leucine-rich repeat protein that accumulates in diseased tomato plants, is processed during pathogenesis in a way that could be mimicked by the activity of purified P69 [40]. The S8 family is the largest protease family in *Arabidopsis* with 54 members [10••]. Some of these proteases may act as convertases, releasing signalling intermediates. This has been suggested to be the case for SDD1 (STOMATAL DENSITY AND DISTRIBUTION-1), which regulates stomatal density [41], and ALE1 (ABNORMAL LEAF SHAPE-1), which is required for proper formation of the embryo epidermis [42]. The vast number of subtilisin-like proteases, their observed pathogenesis-related accumulation and their proposed roles as convertases raise the possibility that they could be involved in defence.

Acidic leucine aminopeptidases (LapA in family M17) specifically accumulate in tomato upon wounding and insect feeding [2,43]. Silencing of *LapA* genes in tomato did not, however, impact the growth and development of tobacco hornworm larvae [44]. Therefore, the role of these genes in defence remains unknown. It is both puzzling and interesting to note, however, that the aminopeptidase inhibitor bestatin is sufficient to induce wound-responsive genes in tomato [45].

Another interesting protease was recently discovered by activation tagging. CDR1 is an aspartic pepsin-like protease (A1) that, when overexpressed, causes constitutive disease resistance to *Pseudomonas* bacteria [8••]. CDR1 overexpressing lines are also dwarfed and exhibit elevated levels of salicylic acid and PR gene expression, whereas mutations in the protease active sites abolished these overexpression phenotypes. Resistance to *Pseudomonas* was compromised in antisense lines, indicating that CDR1 is required for induced resistance. Intriguingly, CDR1 appears to release a small, mobile signalling molecule that remains to be identified.

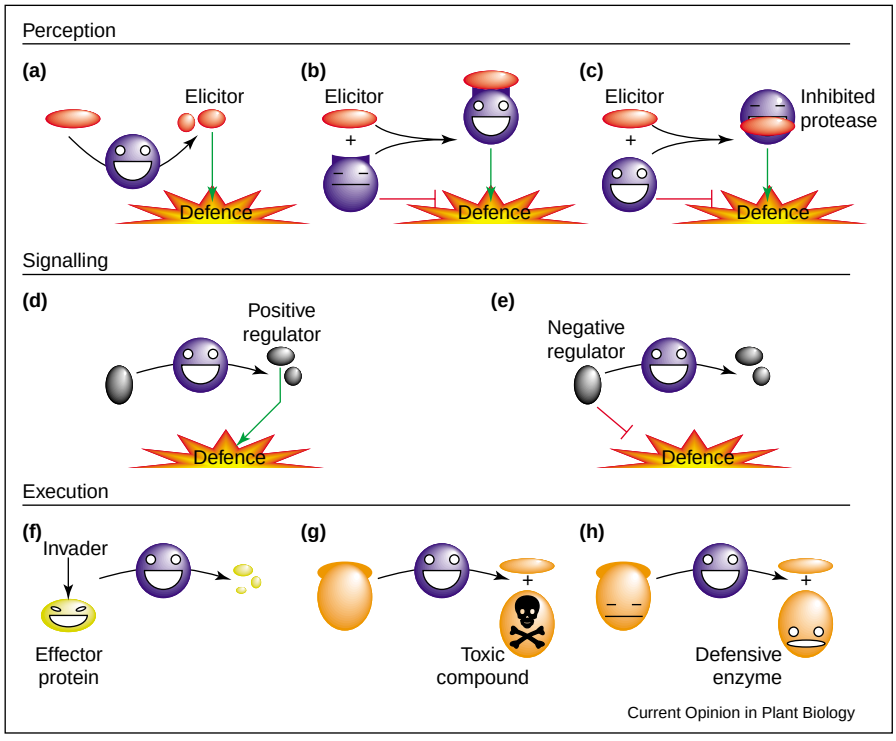
Possible roles for proteases in defence

The examples described above indicate that proteases have many different roles in defence. They can act at the level of perception, signalling and execution, each according to different models as summarised in Figure 3.

The role of proteases in perceiving the invader is perhaps unexpected, but numerous possibilities exist. First, proteases may release elicitors from the invader that are subsequently recognised elsewhere (Figure 3a). A antigen presentation on major histocompatibility complex-I (MHC-I) involves a similar process, mediated by aminopeptidase ERAAP [46]. Second, specific binding of elicitors may activate the protease, which may activate downstream signalling components by proteolytic cleavage (Figure 3b). This mechanism was recently found to regulate factor C, a serine protease of horseshoe crab that becomes activated upon binding of pathogen-derived lipopolysaccharide (LPS). This activation leads to a proteolytic cascade that results in a defence response that includes blood clotting [47•]. Third, binding of the elicitor to the protease may inhibit its activity, and the elicitor–protease complex or altered proteolytic activity might induce signalling (Figure 3c). This mechanism could easily evolve if the inhibition of the protease activity is advantageous to the invader; for example, when the protease is part of a defence response that needs to be repressed. This inhibited protease then serves as bait in plants that have evolved resistance genes that 'guard' such a virulence target, a process that may result in durable resistance [48]. This model may well apply to RCR3 (as explained above). Another striking example is indicated by P34, an inactive homologue of a cysteine protease, which binds the syringolide elicitor and probably confers recognition of this elicitor in resistant plants [49].

Signalling proteases may act by releasing positive regulators (Figure 3d) or by degrading negative regulators (Figure 3e). Mammalian caspases, for example, activate themselves and downstream caspases, and inactivate poly(ADP-ribose)polymerase (PARP) [19]. In addition, the *Drosophila* persephone (psh) is a secreted serine protease that is required for the activation of the Toll pathway after fungal infection [50•]. Metacaspases (C14) are strong candidates for fulfilling caspase-like signalling roles in plant defence responses [34••], but VPEs (C13) should not be excluded. In addition, CDR1 may play a role in releasing signalling peptides [8••]. The existence of signalling proteases is also indicated by the series of secreted peptide wound hormones (systemins) that probably need proteolytic activation of their precursor proteins [51•].

Figure 3



Models for the various roles of proteases in plant defence. **(a–c)** Proteases (blue balls) may act in perception, **(d,e)** signalling or **(f–h)** execution. Green arrows and red t-bars indicate positive and negative signalling to defence responses, respectively.

Proteases may also execute the defence response. They can directly degrade proteins from the invader (Figure 3f), release peptide-based toxins (Figure 3g), or activate enzymes from their precursor proteins (Figure 3h). Proteases that accumulate to high levels at the site of the invasion are strong candidates for this role. These include the papain and papain-like proteases Mir1 and RD21, as well as the subtilisin-like P69, metalloprotease LapA, and many other proteases that have been identified by transcriptomic approaches. Measuring their contribution, however, can be difficult as these proteases may be part of an array of cooperative defence responses.

Conclusions and perspectives

Exciting as recent data are, we only have just begun to appreciate the different roles of proteases in defence. Proteases are implicated in perception, signalling and execution leading to plant defence. Papain-like cysteine proteases (clan CA) may be involved in each of these steps, whereas caspase-like cysteine proteases (metacaspases and VPEs [clan CD]) might play a role in signalling. Studies with protease inhibitors, despite their seemingly contradictory results, strongly indicate crucial roles for (especially cysteine) proteases in signalling, even though the corresponding protease genes have not yet been identified. The expected sites of action of the protease inhibitors that have been used, as well as the localisation of well-studied proteases, indicate roles for proteases present at different subcellular locations, such as the cytosol, apoplast and organelles (Figure 2).

The huge number of proteases, their redundancy and their posttranscriptional regulation makes this field a challenge to explore. A new functional proteomic technology, called protease-activity profiling, which displays multiple protease activities rather than their transcript or protein abundance [52], provides an excellent tool with which to study the activities of proteases during defence responses, and has recently been introduced in plants [53••]. This technology, combined with detailed studies of candidate proteases and other approaches, will uncover proteases that are involved in defence. Another exiting question that remains to be solved is how these proteases are regulated and what their substrates are. It can be expected that research in the next decade will bring us astonishing discoveries on the roles of the proteolytic machinery in plant defence responses.

Note added in proof

An exiting new report shines a different light on the identity of the caspase-like activities that have often been detected [59••]. Purification of caspase-like proteases that are secreted during victorin-induced cell death in oat revealed sequences of subtilisin-like serine proteases

(family S8). Once again, this hints at important roles for this protease family and stresses caution in interpreting studies that use inhibitors and substrates.

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