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Micro-Review

Papain-like cysteine proteases: key players at molecular battlefields employed by both plants and their invaders

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ABSTRACT

Papain-like cysteine proteases (PLCPs) play crucial roles in plant–pathogen/pest interactions. During these parasitic interactions, PLCPs act on non-self substrates, provoking the selection of counteracting inhibitors and other means to evade proteolysis. We review examples of PLCPs acting on molecular battlefields in the extracellular space, plant cytoplasm and herbivore gut. Examples are maize Mir1 (Maize inbred resistance 1), tomato Rcr3 (Required for *Cladosporium* resistance-3), *Pseudomonas* AvrRpt2 and AurPphB, insect DvCAL1 (*Diabrotica virgifera* cathepsin L-like protease-1) and nematode MiCpl1 (*Meloidogyne incognita* cathepsin L-like protease 1). The data suggest that PLCPs cleave specific proteins and that their translocation, activation and inhibition of PLCPs are tightly regulated.

INTRODUCTION

Interactions of plants with their invaders are diverse. Plants are continuously challenged by bacteria, fungi, oomycetes, nematodes, insects and other pathogens and herbivores. It can be hypothesized that in these interactions the degradation of non-self proteins by both the plant and parasite plays an important role. Proteolysis during parasitic interactions, however, probably provokes the selection of counteracting inhibitors, non-cleavable substrates and other means to evade proteolysis. Therefore, the interactions of proteases with their substrates and inhibitors can be seen as a molecular battlefield. Intriguingly, both plants and their invaders use papain-like cysteine proteases (PLCPs) at these molecular battlefields.

PLCPs are usually 23–30 kDa in size, and use a catalytic cysteine residue to cleave peptide bonds in protein substrates. This catalytic cysteine is part of a catalytic triad situated in the middle of a cleft that binds the substrate through specific interactions (Drenth *et al*., 1968; Fig. 1a). PLCPs are produced with an auto-inhibitory prodomain that is folded back onto the substrate-binding cleft and needs to be proteolytically removed to activate the protease (Taylor *et al*., 1995). Many PLCPs of plants and their invaders are produced with a signal peptide (Fig. 1b), indicating that they are secreted or localized in the endomembrane system.

The MEROPS peptidase database (www.merops.sanger.ac.uk) groups PLCPs into 'clan CA' (Rawlings *et al*., 2006). Protease families that belong to this clan share the same papain-like three-dimensional structure, order of catalytic residues and other conserved features, indicating that they share a common ancestor. Clan CA proteases, however, can be very diverse at the protein sequence level. A subdivision of this clan is based on statistically significant sequence homology (Rawlings *et al*., 2006). PLCPs having sequence homology to papain itself have been grouped in family C1. The C1 family has been subdivided into extracytoplasmic PLCPs (subfamily C1A) and cytoplasmic PLCPs (family C1B). The C1A subfamily members include many of the plant, insect and nematode proteases discussed here (Fig. 1c). The PLCPs that are not homologous in sequence to papain have been assigned to other families. *Pseudomonas* PLCPs, for example, have been assigned to families C58, C70 and C72 (Fig. 1c).

The literature on the PLCPs produced by plants and their parasites is vast. In this paper, we focus on examples of PLCPs that act at the interaction interface between plants and their invaders (Table 1). We have identified three molecular battlefields in plant–pathogen/pest interactions in which PLCPs play a role. These battlefields are the plant apoplast, where PLCPs are part of a defence layer, the plant cytoplasm, where bacterial PLCPs act on host substrates, and the insect and nematode gut, where digestive PLCPs are inhibited by cystatins produced by plants. We review three PLCP examples for each of these molecular battlefields.

PLANT PLCPs ACT IN EXTRACELLULAR DEFENCE

Plants use PLCPs to protect themselves against pests and pathogen attack. Examples are papain from papaya and Mir1 (Maize inbred resistance 1) from maize, both acting against insect larvae.

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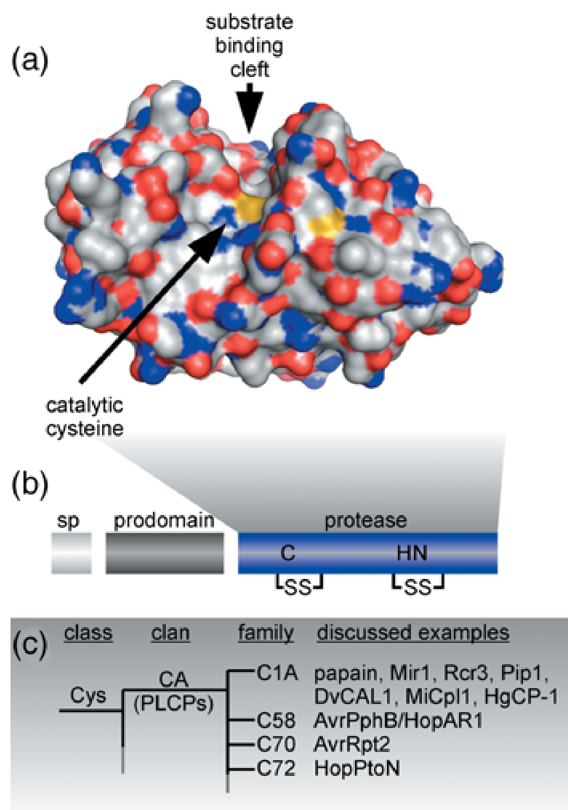

Fig. 1 Characteristics of papain-like cysteine proteases (PLCPs). (a) Surface representation of the crystal structure of papain, showing its wide substrate-binding cleft with the catalytic cysteine (yellow). (b) Domain structure of the open reading frame of PLCPs of subfamily C1A. The signal peptide (sp) targets the protein to the endomembrane system; the autoinhibitory prodomain needs to be removed to activate the protease. The protease domain contains three catalytic residues, cysteine (C), histidine (H) and asparagine (N), and often contains two disulphide bridges (SS). (c) Hierarchical subclassification of cysteine proteases according to the MEROPS database (Rawlings *et al.*, 2006). The cysteine protease class (Cys) consists of several clans of proteases. Clan CA contains PLCPs, and is subdivided into different (sub)families, such as C1A, of which papain is the type member. Only families containing the PLCPs discussed here are shown.

Tomato Rcr3 (Required for *Cladosporium* resistance-3) and Pip1 (*Phytophthora* inhibited protease-1) are produced upon pathogen attack and inhibited by pathogen-derived inhibitors.

Papain is a component of latex of papaya trees, which pours out of wounds, presumably as a defence response against herbivores (reviewed by El Moussaoui *et al.*, 2001). The structure of papain was one of the earliest resolved protein structures (Drenth *et al.*, 1968). Papain is produced as a preproprotein, and mechanical wounding of papaya fruit enhances papain accumulation and activation (Azarkan *et al.*, 2006; Moutim *et al.*, 1999). However, the mechanism of its accumulation and how it is activated are not yet clear. A role of papain in insect defence has been described only recently. Different lepidopteran caterpillars (*Samia ricini*, *Mamestra brassicae* and *Spodoptera litura*) showed reduced larval weight when fed with leaves containing papain (Konno *et al.*, 2004). This reduced growth was not observed when the latex was washed out or when the leaves were treated with the cysteine protease inhibitor E-64 (Konno *et al.*, 2004). This indicates that papain contributes to defence against herbivores.

Mir1 was identified because it was encoded by an abundant transcript in the callus of resistant, but not susceptible, maize when challenged with armyworms (*Spodoptera fugiperda*) (Jiang *et al.*, 1995; Pechan *et al.*, 1999). Like papain, Mir1 is translated as a preproprotein, suggesting that it is secreted or localized in vesicles. Mir1 protein accumulation occurs rapidly one hour after larval feeding, continues for 7 days and is most abundant at the feeding site (Pechan *et al.*, 2000). Tobacco budworm (*Heliothis virescens*) larvae fed with transgenic maize callus overexpressing the *Mir1* gene were significantly smaller than those fed with callus from control plants (Chang *et al.*, 2000; Pechan *et al.*, 2000). Feeding on Mir1-producing plants causes severe damage of the caterpillar peritrophic matrix, which is the chitin structure covering the insect gut surface, protecting it from chemical and physical damage (Pechan *et al.*, 2002). It has been suggested that Mir1 can bind to chitin, localizing the proteolytic activity to the insect gut (Pechan *et al.*, 2002).

Pip1 and Rcr3 are two secreted tomato PLCPs that accumulate in the apoplast (Kruger *et al.*, 2002; Tian *et al.*, 2007). The *Pip1* and *Rcr3* genes map at the same genetic locus of tomato and are transcriptionally up-regulated during pathogen challenge (Kruger *et al.*, 2002; Tian *et al.*, 2007). Both proteases are inhibited by pathogen-derived inhibitors. Pip1 is inhibited by Epic2B, a cystatin-like protease inhibitor secreted during infection by the oomycete *Phytophthora infestans* (Tian *et al.*, 2007). Rcr3 is inhibited by Avr2, a secreted, cysteine-rich protein produced by the leaf mould fungus *Cladosporium fulvum* (Luderer *et al.*, 2002; Rooney *et al.*, 2005). The Rcr3–Avr2 complex, and not Rcr3 inhibition itself, triggers the hypersensitive response mediated by the tomato resistance gene *Cf-2* (Rooney *et al.*, 2005). However, the specificity of inhibition by Avr2 and Epic2B for PLCPs and how *Cf-2* recognizes the Rcr3–Avr2 complex are not yet fully understood.

BACTERIAL PLCPs MANIPULATE DEFENCE RESPONSES IN THE PLANT CYTOPLASM

The well-studied Gram-negative pathogenic bacterium *Pseudomonas syringae* pv. *tomato* secretes effector proteins into plant cells via the type-III secretion system (TTSS). Many of the identified TTSS-secreted effector proteins, such as AvrRpt2, AvrPphB, HopPtoN and, most probably, HopX (Nimchuk *et al.*, 2007), are classified as clan CA proteases, and create a molecular battlefield in the host cytoplasm.

AvrRpt2 from *Pseudomonas syringae* pv. *tomato* DC3000 triggers Resistance to *P. syringae-2* (RPS2)-dependent defence

Table 1 Plant and pathogen papain-like cysteine proteases (PLCPs) discussed in this article.

Protease	(Sub)family	Origin	Remarks	Key references
Papain	C1A	Papaya	Present in wound-induced latex Activation during wounding	El Moussaoui *et al.* (2001) Azarkan *et al.* (2006)
Mir1	C1A	Maize	Accumulates on wounding Degrades insect gut surface	Pechan *et al.* (2000) Pechan *et al.* (2002)
Rcr3	C1A	Tomato	Required for fungal resistance Inhibited by fungal Avr2	Kruger *et al.* (2002) Rooney *et al.* (2005)
Pip1	C1A	Tomato	Closely related to Rcr3 Inhibited by oomycete Epic2B	Tian *et al.* (2007)
AvrRpt2	C70	*Pseudomonas syringae*	Cleaves RIN4(-like) proteins Activated by cyclophilin ROC1	Axtell and Staskawicz (2003); Coaker *et al.* (2005); Mackey *et al.* (2003)
HopPtoN	C72	*Pseudomonas syringae*	Suppresses cell death from both virulent and avirulent strains	Lopez-Solanilla *et al.* (2004)
AvrPphB (HopAR1)	C58	*Pseudomonas syringae*	Cleaves PBS1 and its orthologues X-Ray explains high specificity	Shao *et al.* (2003) Zhu *et al.* (2004)
DvCAL1	C1A	*Diabrotica virgifera*	Inhibited by soyacystatin One of many digestive PLCPs	Koiwa *et al.* (2000) Siegfried *et al.* (2005)
MiCpl1	C1A	*Meloidogyne incognita*	Inhibited by oryzacystatin RNAi: less feeding efficiency	Neveu *et al.* (2003) Shingles *et al.* (2007)
HgCP-1	C1A	*Heterodera glycines*	Inhibited by oryzacystatin RNAi: developmental role	Urwin *et al.* (1997a) Urwin *et al.* (2002)

responses, including hypersensitive cell death, by cleaving the *Arabidopsis* RIN4 protein (RPM1-interacting protein 4), which is monitored by the cognate resistance protein RPS2 (Axtell *et al.*, 2003; Mackey *et al.*, 2003). Mutation of the predicted catalytic residues in AvrRpt2 abolishes its ability to cleave RIN4, and therefore blocks the elicitation of RPS2-mediated resistance (Axtell and Staskawicz, 2003; Mackey *et al.*, 2003). Upon injection into the host cell, the activation of AvrRpt2 is mediated by plant cyclophilin ROC1, which probably acts by isomerization of the prolylisomeric bonds of AvrRpt2 (Coaker *et al.*, 2005, 2006). AvrRpt2 activation results in the processing of AvrRpt2 itself, followed by translocation of AvrRpt2 into the host plasma membrane, where RIN4 also resides (Coaker *et al.*, 2005, 2006; Takemoto and Jones, 2005). AvrRpt2 cleaves RIN4 at two sites, releasing RIN4 from the plasma membrane (Kim *et al.*, 2005). Interestingly, RIN4 homologues have conserved AvrRpt2 cleavage motifs and are also attached to the plasma membrane (Chisholm *et al.*, 2005; Takemoto and Jones, 2005). It is not yet clear whether these RIN4-like proteins are released from the membrane by AvrRpt2 or other proteases, or what is their function.

HopPtoN is another TTSS-secreted effector protein of *Pseudomonas syringae* pv. *tomato* DC3000 that has cysteine protease activity *in vitro* (Lopez-Solanilla *et al.*, 2004). Interestingly, HopPtoN acts inside the plant cell to reduce the necrosis associated with *Pseudomonas* infection of susceptible hosts. HopPtoN can be inhibited by the cysteine protease inhibitor E-64. Site-directed mutagenesis has revealed that all three catalytic residues are required for HopPtoN to suppress cell death induced by both virulent and avirulent *Pseudomonas*, implying that this effector protein is used to regulate the degree of death elicited in the host. The targets of HopPtoN are not yet known, and are especially interesting because they may be cell death regulators.

AvrPphB/HopAR1 has been identified from *Pseudomonas syringae* pv. *phaseolicola* and triggers the RPS5-mediated hypersensitive response in *Arabidopsis* (Simonich and Innes, 1995). AvrPphB is a *Yersinia* outer protein T (YopT)-like effector protein and has recently been renamed HopAR1 (Grant *et al.*, 2006). Catalytic residues are required for both autoproteolytic cleavage of AvrPphB and the elicitation of the hypersensitive response in plants carrying the resistance gene *RPS5* (Shao *et al.*, 2002). AvrPphB cleaves the *Arabidopsis* kinase PBS1 (AvrPphB susceptible-1), which is required for RPS5-mediated resistance (Shao *et al.*, 2003). Mutations in the kinase motif of PBS do not abolish the cleavage by AvrPphB, but block RPS5-mediated resistance (Shao *et al.*, 2003). No other factor is required for PBS1 proteolysis, as purified recombinant AvrPphB can cleave purified PBS1 *in vitro* (Shao *et al.*, 2003). The crystal structure of AvrPphB explains its high specificity and its potential binding mechanism to PBS1 (Zhu *et al.*, 2004). Indeed, AvrPphB can cleave PBS1 orthologues in monocots, but does not cleave PBS1 paralogues in *Arabidopsis* (Innes, 2003), suggesting a highly specific interaction of AvrPphB with PBS1. A recent report has shown that PBS1 forms a complex with the N-terminal coiled-coil domain of RPS5, even before exposure to AvrPphB (Ade *et al.*, 2007). The current model proposes that the cleavage of PBS1 activates RPS5 by a conformational change that removes the auto-inhibitory leucine-rich repeat domain of RPS5.

INSECT AND NEMATODE DIGESTIVE PLCPs ARE INHIBITED BY PLANT CYSTATINS

Although seemingly remote from interactions with living plant tissues, the herbivore digestive system is a real molecular battlefield in which PLCPs are inhibited by cystatins produced by plants on herbivory. Despite that cysteine protease activities in the guts of insects and nematodes have been reported many times (reviewed by Haq *et al.*, 2004), only a few PLCPs have been characterized at the molecular level.

DvCAL1 (*Diabrotica virgifera* cathepsin L-like protease-1) is one of the major digestive PLCPs in the larval midgut of western corn rootworm (WCR, *Diabrotica virgifera*) (Koiwa *et al.*, 2000). WCR larvae feed on the root tissue of maize and are a major pest of this crop. PLCPs of the WCR gut are effectively inhibited by a wound-induced protease inhibitor soyacystatin of soybean, but not by a constitutively expressed soyacystatin (Zhao *et al.*, 1996). Affinity purification of WCR gut proteases binding to immobilized soyacystatin revealed N-terminal protein sequences of five different PLCPs, one of which, DvCAL1, was cloned (Koiwa *et al.*, 2000). Several other DvCAL1-like proteases were cloned from midgut-derived cDNA and heterologously expressed for biochemical characterization (Bown *et al.*, 2004). Expressed sequence tag (EST) sequencing of WCR midgut-derived cDNA showed that more than 15 different PLCPs are expressed in larval midguts, where they may even comprise 10% of the expressed mRNA (Siegfried *et al.*, 2005). This illustrates the large repertoire of PLCPs in the digestive systems of insects.

MiCpl1 (*Meloidogyne incognita* cathepsin L-like protease 1) is a PLCP of the root-knot nematode *Meloidogyne incognita* (Neveu *et al.*, 2003). A role for digestive PLCPs in this nematode was indicated by the observation that the growth of these nematodes was severely reduced when feeding on *Arabidopsis* roots expressing the rice seed protease inhibitor oryzacystatin (Urwin *et al.*, 1997a). *MiCpl1* was cloned from *M. incognita* and encodes for a preproprotease, suggesting that it is a secreted protein (Neveu *et al.*, 2003). *In situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) showed that *MiCpl1* expression is limited to intestinal cells throughout the development of female nematodes (Neveu *et al.*, 2003; Shingles *et al.*, 2007). Silencing the *MiCpl1* gene by RNA interference (RNAi) resulted in a reduced proteolytic activity in nematode extracts to a similar level to that caused by the addition of oryzacystatin (Shingles *et al.*, 2007), indicating that MiCpl1 is the major target of oryzacystatin. Silencing *MiCpl1* also reduced the number of nematodes infecting plants by 60%, consistent with a reduced feeding efficiency (Shingles *et al.*, 2007).

HgCP-1 (*Heterodera glycines* cysteine protease-1) is a PLCP from the soybean cyst nematode *Heterodera glycines*. Although cyst nematodes are distinct from root-knot nematodes, such as *M. incognita*, both nematodes use PLCPs as digestive enzymes. Homogenates of feeding *H. glycines* females contain cysteine protease activity, and the growth of *H. schachtii* is severely reduced by feeding on *Arabidopsis* roots expressing oryzacystatin (Lilley *et al.*, 1996; Urwin *et al.*, 1997a). *HgCP-1* was isolated from a cDNA library constructed from feeding females of *H. glycines*. HgCP-1 was expressed as a recombinant protein, and was shown to have proteolytic activity that could be inhibited by oryzacystatin (Urwin *et al.*, 1997b). *HgCP-1* silencing, however, did not reduce the number of feeding nematodes, but changed the gender ratio, indicating that HgCP-1 plays a role in development rather than in virulence (Urwin *et al.*, 2002).

There are many more insects and nematodes for which a reduced growth has been observed when feeding on cystatin-overproducing plants (reviewed by Dunaevsky *et al.*, 2005; Haq *et al.*, 2004; Lawrence and Koundal 2002). Oryzacystatin-producing tobacco even showed enhanced resistance to different potyviruses, and the growth of juvenile slugs was reduced when feeding on oryzacystatin-producing *Arabidopsis* (Gutierrez-Campos *et al.*, 1999; Walker *et al.*, 1999). The molecular mechanism of the enhanced resistance to herbivores, however, remains to be elucidated. Apart from the obvious hypothesis of reduced nutrient release during feeding on cystatin-containing diets, cystatins can also prevent the degradation of proteins that are harmful to the herbivore. Soyacystatin, for example, inhibits the degradation of soybean Kunitz inhibitor and wheat α-amylase inhibitor, which are both harmful to seed-feeding insects (Amirhusin *et al.*, 2004, 2007). The stabilization of these two proteins by soyacystatin indicates that these plant proteins are an important target for insect PLCPs.

The enhanced resistance displayed by cystatin-overproducing plants has inspired transgenic crop protection programmes. Unfortunately, insects also quickly adapt to cystatins in their diet. Nutritional stress can induce the overproduction of the protease, and the up-regulation of the expression of cystatin-insensitive or even cystatin-degrading PLCPs (Cloutier *et al.*, 2000; Gruden *et al.*, 2003, 2004; Lecardonnel *et al.*, 1999; Liu *et al.*, 2004; Michaud *et al.*, 1995; Rivard *et al.*, 2004).

CONCLUDING REMARKS

It is becoming evident that PLCPs can no longer be considered as only part of a basal protein degradation system. These proteases have distinct and diverse roles. PLCPs can be highly substrate specific, and their location, activation and inactivation are tightly regulated. Stress-induced plant PLCPs are part of an extracellular defence shield. Bacterial PLCPs are injected into plant cells to manipulate the host, and herbivores use PLCPs as digestive enzymes. The available data, summarized in Fig. 2, raise many intriguing questions.

1 How are PLCPs targeted to subcellular locations? Do they use the targeting machinery of the other organism? Do they interact with other proteins?

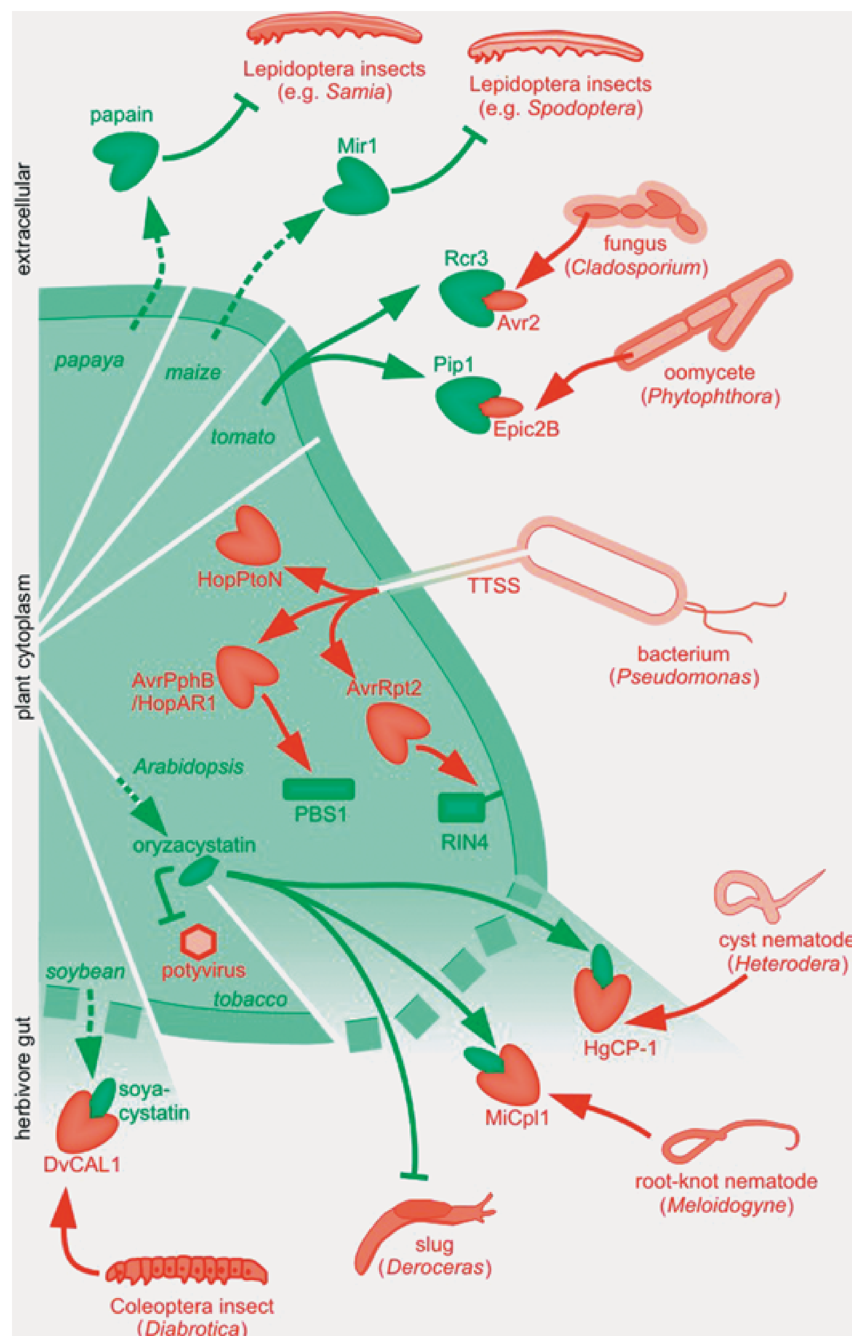

Fig. 2 Summary of the diverse roles of the papain-like cysteine proteases (PLCPs) discussed here. PLCPs (heart shapes) are produced by plants (green) and their pests and pathogens (red). Broken lines indicate the predicted localizations based on the presence or absence of a signal peptide. The broken cell wall (bottom) illustrates that putative cytoplasmic phytocystatins act in the herbivore gut on digestive PLCPs.

2 How are PLCPs activated? Is the prodomain autocatalytically removed? What are the endogenous inhibitors or activators?
3 How specific are PLCPs towards their substrates? How do they discriminate between self and non-self proteins? What are the roles of these substrates?
4 Are PLCPs and their inhibitors involved in co-evolutionary arms races between plants and their invaders, e.g. between Epic2B and Pip1 or soyacystatin and DvCAL1?

These are just a few of the obvious questions. However, PLCPs are not easy to evaluate as their substrates and localization signals are elusive. PLCPs are often encoded by multigene families and can act redundantly, even with proteases of other classes. New technologies, such as protease activity profiling, have been introduced to provide novel insights (Van der Hoorn *et al.*, 2004). Given the role of PLCPs and the importance of the remaining questions, progress in this field will be highly rewarding.

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