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.

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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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 frugiperda) (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 Mr1 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
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 prolyl isomeric bonds of AvrRpt2 (Coaker et al., 2004, 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. 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.

### Table 1

<table>
<thead>
<tr>
<th>Protease</th>
<th>(Sub)family</th>
<th>Origin</th>
<th>Remarks</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain</td>
<td>C1A</td>
<td>Papaya</td>
<td>Present in wound-induced latex</td>
<td>El Moussaoui et al. (2001)</td>
</tr>
<tr>
<td>Mir1</td>
<td>C1A</td>
<td>Maize</td>
<td>Activation during wounding</td>
<td>Azarkan et al. (2006)</td>
</tr>
<tr>
<td>Rcr3</td>
<td>C1A</td>
<td>Tomato</td>
<td>Requires fungal resistance</td>
<td>Shao et al. (2003)</td>
</tr>
<tr>
<td>Pip1</td>
<td>C1A</td>
<td>Papaya</td>
<td>Cleaved by oryzacystatin</td>
<td>El Moussaoui et al. (2001)</td>
</tr>
<tr>
<td>AvrRpt2</td>
<td>C70</td>
<td>Pseudomonas syringae</td>
<td>Cleaved by oryzacystatin</td>
<td>Axtell and Staskawicz (2003)</td>
</tr>
<tr>
<td>HopPtoN</td>
<td>C72</td>
<td>Pseudomonas syringae</td>
<td>Activated by cyclophilin ROC1</td>
<td>Coaker et al. (2005)</td>
</tr>
<tr>
<td>AvrPphB</td>
<td>C58</td>
<td>Pseudomonas syringae</td>
<td>Cleaved by PBS1 and its orthologues</td>
<td>Shao et al. (2003)</td>
</tr>
<tr>
<td>DvCAL1</td>
<td>C1A</td>
<td>Diabrotica virgifera</td>
<td>X-ray explains high specificity</td>
<td>Zhu et al. (2004)</td>
</tr>
<tr>
<td>MiCpl1</td>
<td>C1A</td>
<td>Heterodera glycines</td>
<td>One of many digestive PLCPs</td>
<td>Siegfried et al. (2005)</td>
</tr>
</tbody>
</table>

INSECT AND NEMATODE DIGESTIVE PLCPs ARE INHIBITED BY PLANT CYSTATINS

Although seemingaely 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) (Koïwa 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 soya-cystatin of soybean, but not by a constitutively expressed soya-cystatin (Zhao et al., 1996). Affinity purification of WCR gut proteases binding to immobilized soya-cystatin revealed N-terminal protein sequences of five different PLCPs, one of which, DvCAL1, was cloned (Koïwa 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., 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. Soya-cystatin, 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 soya-cystatin 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?
How are PLCPs activated? Is the prodomain autocatalytically removed? What are the endogenous inhibitors or activators?

How specific are PLCPs towards their substrates? How do they discriminate between self and non-self proteins? What are the roles of these substrates?

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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REFERENCES


