Papain-like Cys proteases as hubs in plant immunity

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Summary

Plants deploy a sophisticated immune system to cope with different microbial pathogens and other invaders. Recent research provides an increasing body of evidence for papain-like cysteine proteases (PLCPs) being central hubs in plant immunity. PLCPs are required for full resistance of plants to various pathogens. At the same time, PLCPs are targeted by secreted pathogen effectors to suppress immune responses. Consequently, they are subject to co-evolutionary host-pathogen arms race. When activated, PLCPs induce a broad spectrum of defense responses including plant cell death. While the important role of PLCPs in plant immunity has become more evident, it remains largely elusive how these enzymes are activated and which signaling pathways are triggered to orchestrate different downstream responses.

Key words: cysteine proteases, PLCPs, apoplast, plant immune signaling, pathogen effectors

Introduction

Plants are continuously challenged by microbes and have developed different mechanisms to defeat pathogens and other invaders. The first contact with microbes usually takes place in extracellular compartments, namely on the epidermal surface and in the apoplast, including cell walls. Processes in this extracellular battleground determine the primary outcome in the majority of plant-microbe interactions. After this first contact, different signaling pathways in the cell are activated to orchestrate downstream responses such as modulation of various enzymatic activities. In this article, we will emphasize the role of papain-like cysteine proteases (PLCPs), which control key processes at different levels of plant defense. PLCPs are prominent enzymes in the plant apoplast and belong to MEROPS (https://merops.sanger.ac.uk/) protease family

C1A of clan CA, of which papain is the type member. Animal PLCPs are often called cathepsins whereas PLCPs in plants fall into nine subfamilies exhibiting a distinctive domain structure (Figure 1, Richau et al., 2011). PLCPs are produced as pre-proproteases, containing an N-terminal signal peptide for secretion and an auto-inhibitory pro-domain that needs to be removed for protein activation, releasing a mature 25-35 kDa active protease. The protease domain contains the catalytic triad formed by the amino acids Cys, His and Asn. Some PLCPs also carry a C-terminal granulin domain with unknown function. Based on recent research, we present here five observations demonstrating that PLCPs are essential and central hubs of plant immunity:

1. Depletion of PLCPs hampers plant immunity

Many cases of protease depletion (e.g. by knock-out or RNAi) indicate important roles for PLCPs in plant immunity. Arabidopsis null mutants for the PLCP RD21 are more susceptible to the necrotrophic fungal pathogen *Botrytis cinerea* (Shindo et al., 2012), although these lines were more resistant for the same pathogen in detached leaf assays (Lampl et al., 2013). Silencing of *Nicotiana benthamiana C14* leads to increased susceptibility for the oomycete pathogen *Phytophthora infestans* (Kaschani et al. 2010; Bozkurt et al., 2011). Likewise, tomato *rcr3* null mutants have lost resistance based on the *Cf-2* resistance gene against both the fungus *Cladosporium fulvum* and the nematode *Globodera rostochiensis* (Dixon et al., 2000; Lozano-Torres et al., 2012). The *rcr3* null mutants are also more susceptible for *P. infestans* (Song et al., 2009), even in the absence of *Cf-2* (Ilyas et al., 2015). Antisense lines depleted for the Pip1 protease of tomato are hyper-susceptible to *C. fulvum*, *Pseudomonas syringae* and *P. infestans* (Ilyas et al., 2015). Interestingly, silencing *NbPip1* in *N. benthamiana* blocks Avr4/Cf-4 induced hypersensitive response (HR) (Xu et al., 2011) whereas silencing *NbCYP1* or *NbCYP2* in *N. benthamiana* increases susceptibility to the necrotrophic fungal pathogen *Colletotrichum destructivum* (Hao et al., 2006). Furthermore, Arabidopsis *rd19* null mutants are impaired in resistance to the bacterial pathogen *Ralstonia solanacearum* (Bernoux et al., 2008). Resistance to herbivore attack is also tightly linked to protease expression. Most prominent example is papain from Papaya, which is present in wound-exuding latex and is activated during wounding (El Moussaoui et al., 2001; Azarkan et al., 2006). Papain is also responsible for the strong toxicity of papaya leaves to insects (Konno et al., 2004). In maize leaves, Mir1~~, a PLCP containing a granulin domain,~~ accumulates at wounding sites and confers enhanced resistance against caterpillars by degrading the peritrophic matrix of the insect gut (Pechan et al., 2000;

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2002). Accumulation of Mir1 also enhances resistance to root-feeding herbivores (Gill et al., 2011) and Mir1 itself acts as an ethylene-dependent, long-distance transport signal that confers resistance to corn leaf aphids (Louis et al., 2015). In summary, PLCPs are found to be required for plant defense to various kinds of biotic stresses in unrelated species.

2. PLCPs are common targets of pathogen effectors

PLCPs representing different subfamilies are targeted by a variety of unrelated pathogen-derived effectors (Table 1). C14 of tomato and potato is inhibited by the cystatin-like effectors EpiC1 and EpiC2B, which are secreted by *P. infestans* (Kaschani et al., 2010). The C14 protease of tomato is also targeted by the *P. infestans* effector AvrBlb2, which prevents C14 secretion into the apoplast presumably by blocking its function in defense (Bozkurt et al., 2011). Closely related to C14 are maize proteases CP1A and CP1B, which are inhibited by the Pit2 effector from the fungal pathogen *Ustilago maydis* (Müller et al., 2013). Pit2 also suppresses the activity of maize proteases XCP2 and CP2, respectively (Müller et al., 2013). Likewise, tomato CYP1, is targeted and inhibited by the RNA-silencing suppressor V2 from the tomato yellow leaf curl geminivirus (Bar-Ziv et al., 2012; 2015). A striking example for a PLCP being targeted by unrelated plant pathogens is tomato Rcr3. At first, it was found to be required for fungal resistance (Krüger et al., 2002). The fungal pathogen *C. fulvum* secretes the effector Avr2, which inhibits Rcr3 (Rooney et al., 2005). In addition, Rcr3 is inhibited by EpiC1 and EpiC2B from *P. infestans* (Song et al., 2009) as well as by Gr-VAP1, an allergen-like effector secreted by the nematode *G. rostochiensis* (Lozano-Torres et al., 2012). Notably, Avr2, EpiC1/2B and Gr-VAP1, although all inhibiting Rcr3, are unrelated proteins. A PLCP closely related to Rcr3 is tomato Pip1, which is also inhibited by EpiC2B (Tian et al., 2007) and Avr2 (Shabab et al., 2008). Another example is Arabidopsis RD19, which is targeted and miss located to the host cell nucleus by the bacterial type III effector PopP2 from *R. solanacearum* (Bernoux et al., 2008). In summary, a growing body of literature demonstrates that evolutionary unrelated plant pathogens including fungi, oomycete, nematodes, bacteria and viruses actively interfere with the activity and subcellular location of plant PLCPs.

3. PLCPs induce defense responses and cell death

One of the first indications that apoplastic cysteine proteases may act in immune-signalling was the finding that E-64, a well-known inhibitor of cysteine proteases, can delay hypersensitive response in the cowpea – cowpea rust fungus system (D'Silva et al., 1998). Later on, it was discovered that *N. benthamiana* Cathepsin B (*Nb*CathB) is required for the hypersensitive

response and disease resistance induced by non-host bacterial pathogens (Gilroy et al., 2007; McLellan et al., 2009). Furthermore, Arabidopsis Catheptin B (*ctb*) mutants show reduced programmed cell death (PCD) induced by abiotic stresses (Ge et al., 2016). Arabidopsis RD21 has been identified as a "pro-death" signal activated during elicitation of cell death. The serpin protease inhibitor, AtSerpin1, exhibits a pro-survival function by covalently inhibiting RD21 and causing a change in compartmentalization (Lampl et al., 2013). Gene expression analysis on barley have shown upregulation of PLCPs during senescence, a form of PCD, for almost all members of different subfamilies (Diaz-Mendoza et al., 2014) but a role during disease resistance still remains to be elucidated.

Besides the contribution of PLCPs in PCD, direct evidence for the importance of apoplastic cysteine proteases during defense responses came from the finding that salicylic acid (SA) treatment activates PLCPs in maize, and that PLCPs themselves activate SA-related gene expression (Van der Linde et al., 2012a, b). Remarkably, inhibition of maize apoplastic cysteine proteases by the endogenous cystatin CC9 is essential to suppress host immunity during infection with the biotrophic pathogen *U. maydis* (van der Linde et al., 2012a, b). Furthermore, Arabidopsis PIRIN2, a member of the cupin protein subfamily, stabilizes the protease XCP2 and increases susceptibility to the vascular pathogen *R. solanacearum* (Zhang et al., 2014). Recently, a 9-lipoxygenase-derived cyclopentanone in maize, 10-oxo-11-phytoenoic acid (10-OPEA), was found to act as a potent cell death signal in multiple organs present during biotic stresses and developmental conditions (Christensen et al., 2015; 2016). The cell death inducing activity of 10-OPEA was characterized by ion leakage and apoptotic-like DNA fragmentation in maize treated leaves (Christensen et al., 2015). Interestingly, the cell-death inducing activity of 10-OPEA requires induction of PLCPs. Consequently, maize plants overexpressing the cystatin CC9 were partially insensitive to 10-OPEA providing further evidence for the importance of PLCPs during immunity.

4. PLCPs can act as co- receptors

Tomato Rcr3 is required for the function of the receptor-like protein Cf-2, which confers resistance against *C. fulvum* secreting Avr2 (Krüger et al., 2002). Avr2 binds to and inhibits Rcr3 and this complex is sensed by Cf-2, consistent with the Guard and Decoy Models (Rooney et al., 2005; Van der Hoorn & Kamoun, 2008). Remarkably, Rcr3 is also required for the perception of nematode effector Gr-VAP1, which also inhibits Rcr3 and triggers immune responses in the presence of Cf-2 (Lozano-Torres et al., 2012). Interestingly, VAP proteins from different nematodes can suppress PCD mediated by surface-localized immune receptors in

Arabidopsis (Lozano – Torres et al., 2014). The molecular mechanism of Avr2/Gr-VAP1 perception is not yet fully understood, but one emerging hypothesis is that Rcr3 is constitutively bound to Cf-2 protein, acting as a co-receptor to perceive the presence of protease inhibitors (Ilyas et al., 2015). Rcr3 of cultivated tomato ($Rcr3^{lyc}$) triggers auto-necrosis in combination with Cf-2, which originates from *S. pimpinellifolium* (Krüger et al., 2002). However, the allelic $Rcr3^{pim}$ protein suppress this necrotic response in the $Rcr3^{pim}$/$Rcr3^{lyc}$ hybrid, suggesting that $Rcr3^{pim}$ protein can outcompete $Rcr3^{lyc}$ and consistent with the preexisting co-receptor model (Ilyas et al., 2015). These data illustrate that PLCPs can operate as co-receptors, sensing perturbations of receptor proteins thus activating defense responses.

5. Natural variation in PLCPs is caused by arms races and host adaptation

Antagonistic protease-inhibitor interactions cause an arms-race that has left its traces in the natural variation of proteases. This was first observed for Rcr3 and Pip1 (Shabab et al., 2008). Natural variation of Rcr3 in wild tomato species resides on the surface of Rcr3, surrounding the active site, and likely represents the footprints of pathogen-derived inhibitors. Indeed, the variant N194D residue in Rcr3 locates close the catalytic Cys and reduces its interaction with Avr2 (Shabab et al., 2008). Interestingly, N194D is also the only variant residue that exclusively prevents inhibition by Avr2 in natural Rcr3 variants (Hörger et al., 2012). The N194D mutation also abolished HR-inducing activity in plants carrying *Cf-2* resistance genes. Other variant residues affect the strength of the HR response, presumably because of the interaction of Rcr3 with Cf-2 (Hörger et al., 2012). Natural variation within Rcr3 also affects its interaction with Gr-VAP1 of the nematode *G. rostochiensis*, which interacts with $Rcr3^{pim}$ but not $Rcr3^{lyc}$, even though these proteases only differ in a few amino acids (Lozano-Torres et al., 2012). The other apoplastic proteases of tomato do not accumulate many variant residues on the surface, consistent with not being targeted by pathogen-derived inhibitors (Shabab et al., 2008). This includes C14 of wild tomato, which is inhibited by cystatin-like EpiC of *P. infestans*. However, *P. infestans* has co-evolved with wild potato, and C14 in wild potato carries variant residues at its surface, illustrating that traces of arms races can only be found in co-evolving host-pathogen interactions (Kaschani et al., 2012). Interestingly, the cystatin-like *Pm*EpiC inhibitor of *P. mirabilis*, which has only recently jumped onto a different host plant, carries an adaptation that facilitates inhibition of the proteases of the new host, but causes reduced affinity to the proteases of the presumed former host (Dong et al., 2014). Taken together, PLCP inhibitor arms-races strengthen the notion that PLCPs are an important part of extracellular defense.

Conclusion: How do PLCPs activate immunity?

In light of the increasing evidence of PLCPs being crucial components of plant immunity, one of the most intriguing questions is how their activity actually results in defense stimulation (Figure 2). Interestingly, their capability to induce immune responses is not restricted to plants, which may suggest activation of highly conserved pathways in the innate immune system. Known plant-derived allergens are cysteine proteases, such as the ragweed (*Ambrosia artemisiifolia*) allergen Amba11 (Bouley et al., 2015), papain or bromelain (Stewart and Thompson, 1996). For Papain it has been found that its proteolytic activity is required for triggering immune responses including MAPK signaling in human cells (Rosenstein et al., 2014). Besides the well-known mechanism that proteases break down the barrier in lungs against allergens, a recently discovered mechanism of PLCPs to induce immune responses is the activation of protease-activated G-protein coupled receptors (Reddy et al., 2015). Interestingly, not only endogenous Cathepsin S, but also the plant-derived proteases papain and mucunan (from tropical bean) were found to induce protease-activated receptors in mammals (Reddy et al., 2015). Controlled proteolysis of receptor proteins, also referred as ectodomain shedding is well known in animal systems, nevertheless there is little known in plants. First evidence for this mechanism comes from the *A. thaliana* chitin receptor CERK1, however the protease involved in this process remains elusive (Petutschnig et al., 2014). Besides activation of receptors, proteases can release small peptides that are perceived as DAMPs to induce immunity. A fascinating mechanism was found for a soybean subtilisin-like protease, which releases an embedded cryptic 12aa signal that triggers defense gene activation (Pearce et al., 2010). However, for PLCPs this kind of mechanism has not been identified so far. It is challenging to deepen our understanding of the involvement of PLCPs in plant immunity since many open questions still have to be addressed. For example, to which extent is proteolytic activity of PLCPs required for triggering plant immunity? How is activation of PLCPs orchestrated? Salicylic acid treatment in maize triggers activation of PLCPs but there is still the possibility that it acts as a feed-back loop since PLCPs themselves induce *PR*-gene expression (van der Linde et al., 2012a). Additionally, the substrates of PLCPs are still unknown. Plants contain a plethora of PLCPs localized in different compartments, but how is specificity achieved? Interestingly, E-64d has been extensively used to suppress autophagy but also apoplastic cysteine proteases. Is it a strategy that pathogens like *P. infestans* deploy to prevent secretion of PLCPs into the apoplast by AvrBlb2 (Bozkurt et al., 2011), or to antagonize host autophagy cargo receptors to counteract host defenses (Dagdas et al., 2016)? Furthermore, activation of PLCPs in the cell might induce a massive proteolytic activity provoking clearance of cell contents and cell death. In this case

PLCPs may need little specificity whilst still releasing signaling molecules. –In light of all the different immune responses involving PLCP activity, it will be a striking challenge to elucidate how target-specificity of PLCPs is regulated and how they discriminate between pathogen and host proteins.

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Figure Legends

Figure 1: Schematic representation of PLCPs found in plants. In general, PLCPs contain a signal peptide (SP, light grey), an auto-inhibitory pro-domain (Pro-, grey) and a protease domain (blue). The mature protease domain holds the catalytic triad Cys-His-Asn. Some members of subfamily 1 and subfamily 4 ~~Some proteases~~ also have at the C- terminus a proline rich domain (P, grey) and a granulin domain (Gran., purple). Subfamily 8 proteases have at the N-terminus a vacuolar targeting signal (NPIR, green) and a minichain that remains after cleavage of the pro-domain (light blue). Family 9 proteases contain the C-terminus motif ECGIE (red). Disulphide bridges, common to most PLCPs (red thin lines) and, subfamily specific disulphide bridges (orange thin lines) are indicated. SF: subfamily classification. Arab.: Arabidopsis.

Figure 2: Tentative model summarizing known and hypothetical functions of PLCPs during plant immune-signaling. (1) PLCPs might release DAMPs (Damage Associated Molecular Patterns) or PAMPS (Pathogen Associated Molecular Patterns) that are recognized by receptors activating signaling cascades and consequently immune responses. (2) Likewise, induction of defense responses e.g. by SA signaling, may lead to an activation of PLCPs, establishing a feed-back loop. (3) PLCPs act as co-receptors and "decoys" that evolve during an evolutionary arms-race to avoid pathogen colonization. (4) To overcome immunity, pathogens produce effector molecules inhibiting PLCPS activity. Since PLCPs are mainly activated by post-transcriptional processing, endogenous inhibitors such as cystatins or serpins may control the outcome in different signaling pathways leading to activation or deactivation of immune responses including PCD (5).

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Table 1: Plant PLCPs involved in biotic interactions.

PLCP	Species	SF*	Function / Phenotype	Reference
RD21	Arabidopsis	1	KO-lines susceptible to *B.cinerea*	Shindo et al., 2012
			KO-lines resistant to *B. cinerea* in detached leaves and *S. sclerotiorum*. Inhibited by AtSerpin1	Lampl et al., 2013
Mir1	Maize	1	accumulates at wounding sites	Pechan et al., 2000; 2002
			enhanced resistance against caterpillars / root-feeding herbivores	Gill et al., 2011
			acts as ethylene signal conferring resistance to aphids	Louis et al., 2015
C14	Potato	1	inhibited by *P. infestans* effectors EPIC1 and EPIC2B . Protease under diversifying selection.	Kaschani et al., 2010; 2011
	Tomato		targeted by *P. infestans* effectors EPIC1, EPIC2B and AvrBlb2	Kaschani et al., 2010 Bozkurt et al., 2011
Papain	Papaya	3	activated during wounding	Azarkan et al., 2006
			involved in defense against polyphagous pests	Konno et al., 2004
XCP2	Arabidopsis	3	increases susceptibility to *R.solanacearum*	Zhang et al., 2014
	Maize		inhibited by *U. maydis* effector Pit2 and maize cystatin CC9	Müller et al., 2013, van der Linde et al., 2012
C14	*N. benthamiana*	4	silenced plants resistant to *P.infestans*	Kaschani et al. 2010; Bozkurt et al., 2011
CP1A/ CP1B	Maize	4	inhibited by *U. maydis* effector Pit2 and maize cystatin CC9	Müller et al., 2013, Van der Linde et al., 2012
Rcr3	Tomato	6	Resistance to *C. fulvum*, *G. rostochiensis* and *P. infestans*	Dixon et al., 2000; Lozano-Torres et al., 2012, Song et al., 2009
			required for the function of Cf2 conferring fungal resistance	Krüger et al., 2002
			inhibited by effectors Avr2, EPIC1, EPIC2B and GrVAP1	Rooney et al., 2005, Song et al., 2009, Lozano-Torres et al., 2012
Pip1	Tomato	6	mutants are hypersusceptible to *C. fulvum*, *P. infestans* and P. syringae	Ilyas et al., 2015
			inhibited by P. infestans EPIC2B and *C. fulvum* Avr2	Tian et al., 2007, Shabab et al., 2008,
	N. benthamiana		silencing blocks HR induced by Avr4/Cf4 recognition	Xu et al., 2011
RD19	Arabidopsis	7	mutants are impaired in resistance to *R. solanacearum*	Bernoux et al., 2008
			targeted by PopP2 from *R. solanacearum*	Bernoux et al., 2008
CYP1/ CYP2	*N. benthamiana*	8	silencing enhanced susceptibility to *C. destructivum*	Hao et al., 2006
CYP1	Tomato	8	inhibited by V2 from tomato yellow leaf curl geminivirus	Bar-Ziv et al., 2012; 2015
CP2	Maize	8	inhibited by *U. maydis* effector Pit2 and maize cystatin CC9	Müller et al., 2013, Van der Linde et al., 2012
CathB	Arabidopsis	9	required for HR induced by non-host bacterial pathogens	Gilroy et al., 2007; McLellan et al., 2009
			mutants show reduced programmed cell death during abiotic stress	Ge et al., 2016

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Insert symbols to SF and DS
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*SF: phylogenetic classification of PLCPs into subfamilies according to Richau et al., 2012~~; †DS: Domain structure as depicted in Figure 1~~

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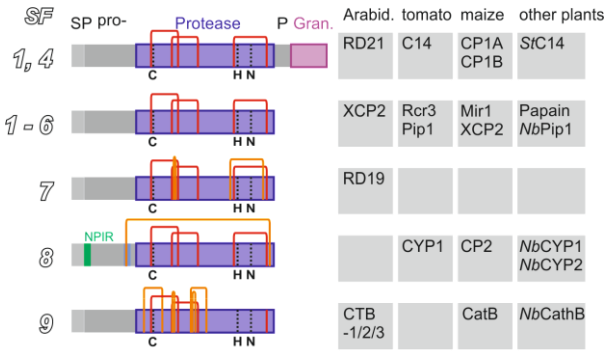

Figure 1. Schematic representation of PLCPs found in plants~~Schematic representation of PLCP domain structure types found in plants~~

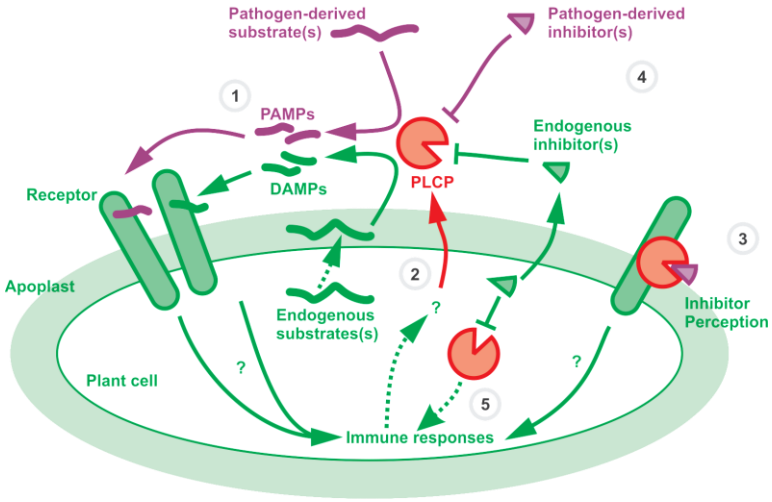

Figure 2. Tentative model summarizing known and hypothetical functions of PLCPs during plant immune-signaling.