Key Roles of Cysteine Protease in Different Plant Pathosystem

Marjohn Niño¹, Joonki Kim¹, Hye Jung Lee¹, Sailila E. Abdula^{1,2}, Ill Sup Nou³, Yong-Gu Cho^{1*}

¹Department of Crop Science, Chungbuk National University, Cheongju 361-763, Republic of Korea

²Current Address, Philippine Rice Research Institute-Midsayap, North Cotabato 9410, Philippines

³Department of Horticulture, Sunchon National University, Sunchon 540-742, Republic of Korea

ABSTRACT Cysteine protease is one of the well-studied proteolytic enzymes in plants. This class of protease has been implicated in various physiological aspects of developmental stages in plants including seed germination, senescence, and disease immunity. A handful of studies assign plants cysteine protease in different molecular battlefield under a few selected pathosystems, and initially extricate complex molecular mechanism of resistance. Interestingly, the same type of proteases released by pathogens have been demonstrated to play a crucial role in establishing disease infection in plants. Most of which target resistance proteins resulting either in an access to the hosts biochemical loot or cascades of signals for defense. As means of enabling and disabling host immunity, this class of protease is subject to diversifying selection, which is an intriguing outcome under natural host pathosystem. This paper summarizes the key roles of cysteine proteases in a few selected plant pathosystems.

Keywords Cysteine protease, Disease immunity, Diversifying selection, Host-effector interaction, Phytopathogen, Plant pathosystem

INTRODUCTION

Cysteine proteases activity comprises majority of the proteolytic activities in plants. It shares approximately 30% of total proteolysis in mature non-senescing organs, and in some cases rises to 90% in response to stimuli (Grudkowska and Zagdanska 2004). Papain-like cysteine protease (PLCP) is equipped with N-terminal auto-inhibitory domain covering the substrate binding groove which needs to be removed proteolytically for activation (Taylor *et al.* 1995). Cysteine proteases use catalytic cysteine residue as a nucleophile to cleave bonds of protein substrates (Shindo and van der Hoorn 2008) which results in an intermediate state where the enzyme is covalently attached to the substrate (van der Hoorn *et al.* 2004). Subsequent hydrolysis cleaves the peptide bond and releases the protease (Powers *et al.* 2002). The catalytic cysteine (C), which is responsible in binding with the substrate, is situated right in the middle of a cleft as part of a catalytic triad including histidine (H), and asparagine (N) (Drenth *et al.* 1971; Shindo and van der Hoorn 2008). Aside from catalytic residues, the protease

domain also contains two disulfide bridges (SS) (Shindo and van der Hoorn 2008).

Apparently, plants utilize the same proteolytic machinery to halt pathogen invasion. There have been reports on papain-like cysteine proteases acting as inhibitory agent against fungus, bacteria, oomycete and nematode such as *Rcr3* (Krüger *et al.* 2002), *PIP1* (Tian *et al.* 2007), *RD19* (Bernoux *et al.* 2008), *RD21* (Shindo *et al.* 2012), *C14* (Kaschani *et al.* 2010) and *Cath B* (Gilroy *et al.* 2007). Each confers unique participation in the induction of host immunity which is evident in the specific interactions with the effectors, involvement in a specific molecular battlefield, and the resulting immunity threshold. Interactions of proteases with their substrates and inhibitors, to some extent provokes selection of the counteracting inhibitors, non-cleavable substrates and other means to evade proteolysis (Shindo and van der Hoorn 2008) which lead to evolutionary diversification.

Interestingly, pathogens are also engaged in proteolysis which either leads to repression or activation of plant defense system. A substantial evidence revealed *Pseudomonas*

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*Corresponding author Yong-Gu Cho, ygcho@cnbu.ac.kr, Tel: +82-43-261-2514, Fax: +82-43-273-2242

syringae as among those which produce more than one family type of cysteine protease (CP) (Axtell and Staskawicz 2003; Mackey *et al.* 2003; Shao *et al.* 2003; Lopez-Solanilla *et al.* 2004; Coaker *et al.* 2005). Some of the reported microbial cysteine proteases are channeled into the apoplasts while others are directly translocated to the plant cytoplasm. Like other classes of effector, activities of PLCP effector also perturb key host resistance protein target.

This review focuses on some members of clans CA and CE that are related to plant disease and resistance. In particular, this paper shows the diverse functions of selected PLCPs such as *Cath B*, *PIP1*, *Rcr3*, *RD19*, *RD21*, *PopP1*, *AvrPphB*, and *AvrRPT2*. Moreover, this paper describes the nuclear trafficking incidence of PLCP during plant-pathogen interaction, and shows extent of evolutionary diversification of PLCP caused by selection pressure.

Major clan of cysteine proteases implicated to disease immunity

There are at least two important clans of cysteine proteases implicated to play crucial role in plant diseases and resistance, these include CA and CE. Both contain proteases with a papain-like fold (van der Hoorn 2008). MEROPS database showed that papain-like cysteine proteases (PLCPs) classified under CA are the most abundant in nature being present in numerous species of bacteria, archaea, protozoa, fungi, plants, animals, and viruses (Rawlings *et al.* 2014). Most members of this clan are pre-proteases containing a signal sequence, an autoinhibitory prodomain, and a mature protease domain (van der Hoorn *et al.* 2004). To date, this clan comprises 32 families, four of which were indicated to be related to plant diseases and resistance including C1 (mainly host plants), C6, C58, and C70 (mainly pathogenic microbes) (Rawlings *et al.* 2014). The C1 family is subdivided into C1A, an extracytoplasmic PLCPs that possess disulfide bridges and accumulate in the vesicles, vacuole, and apoplast, and the subfamily C1B which comprises cytoplasmic PLCPs (subfamily C1B) (Rawlings *et al.* 2006; Shindo and van der Hoorn 2008). The MEROPS database has indicated 169 plants species with CP assigned to this family (Rawlings *et al.* 2014). Of which, 11 species were shown to contain more than 30 homologues in their genome including *Glycine max*, rice,

Arabidopsis lyrata and *Zea mays*, among others.

The other three families under clan CA implicated to plant disease and immunity represent the key effectors important in initiating disease in a specific host plant. The C6 family contains cysteine endopeptidases of RNA viruses such as potato virus Y-type helper component peptidase (potato virus Y) and barley yellow mosaic virus-type helper component peptidase (Rawlings *et al.* 2014). The only known peptidase activity of this group is the cleavage at its own C-terminus, at a preferred sequence site -Tyr-Xaa-Gly+Gly-, which releases it from the viral polyprotein (Carrington and Herndon 1992; Rawlings *et al.* 2014). In the case of C58 family, endopeptidases members act as transamidases, which attach a lipid moiety to the exposed N-terminus of the substrate. This group is further classified to two subfamilies, C58A and C58B, both solely distributed among species of bacteria (Rawlings *et al.* 2014). The *AvrPphB* of *Pseudomonas syringae* is the only member of C58B family and has been implicated to initiate disease in beans (Shao and Dixon 2004). For family C70, putative members are mainly of bacterial origin showing close homology with *AvrRpt2* peptidase in *Pseudomonas syringae* (Rawlings *et al.* 2014). *AvrRpt2* has been demonstrated to induce self-cleavage as means of auto-activation, and inhibition of RIN4 protein in the host cell (Axtell *et al.* 2003). These activities contribute to the virulence of *Pseudomonas syringae*.

Several peptidases in clan CE exhibit preferential cleavage after diglycine due to the presence of aromatic residue such as tryptophan which follows the catalytic histidine in the sequence (Golubtsov *et al.* 2006; Rawlings *et al.* 2014). Proteases in this clan are characterized by a cysteine nucleophile and a catalytic core composed of three amino-acid residues, H/E/C or H/D/C (Hotson and Mudgett 2004). As mentioned in MEROPS database, members of CE contain two structural subdomains with the active site in the middle. One subdomain carries the active site His and Glu (or Asp) and the second subdomain carries the catalytic Cys. CE peptidases structure resembles clan CA except for the reversed order of subdomains. Peptidase family C55 comprises bacterial effector peptidases with restricted specificity, three of which are key factors affecting economically important crops. The members include *PopP1*

(*Ralstonia solanacearum*), AvrXv4 (*Xanthomonas* sp.) and HopZ1 protein (*Pseudomonas syringae*) (Rawlings *et al.* 2014).

Cysteine protease-mediated resistance in different pathosystem

Plants developed a variety of perception system such as pattern recognition receptors (PRRs) and resistance (R) gene products alerting the defense signals once perturbed by activities of pathogen. Classical definition of plant immunity solely involves ligand-receptor model indicating products of R genes as receptors that directly interact with the products of Avr genes (Keen 1990). However, in some R-Avr combinations, direct physical interactions are rather an isolated case as perception is often shown as indirect (van der Biezen and Jones 1998; Dangl and Jones 2001).

Cysteine proteases, particularly the PLCPs have been reported to play an important role either in pathogen perception taking the role as a guard, and in disease resistance signaling (van der Hoorn 2008). Cysteine proteases are also involved in the programmed cell death (PCD) induced by developmental cues or pathogens (Solomon *et al.* 1999). The following are selected members of the PLCP acting in plant disease and resistance (Table 1).

Cath B

Hypersensitive response (HR) is a form of programmed cell death (PCD) that occurs at the site of pathogen entry and is mostly accompanied by the induction of defense responses to wall-off the pathogen (Lam *et al.* 2001). Using virus-induced gene silencing, Gilroy *et al.* (2007) demonstrated the involvement of *cathepsin B* in the plant disease resistance hypersensitive response. Effects of *CathB*-silencing to HR were demonstrated in two different non-host pathosystem involving *N. benthamiana* infiltrated with *Erwinia amylovora* (causal pathogen of fireblight in apple and pears) and *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (causal agent of bacterial speck in tomato). Both *Eam*- and *Pst*-mediated HR in TRV::*NbCathB* plants diminished even at the lower inoculum concentration (10^6 cfu ml⁻¹) and the onset was delayed for 72 hpi at the higher concentration (10^7 cfu ml⁻¹). This suppression leads to the accumulation of higher viable cells (an eight-fold increased recovery of *Eam* and a five-fold increase recovery of *Pst*) in virus-induced gene silencing (VIGS) plants than in control plants transformed with TRV::*gfp*. Using temporal expression analysis, regulation of the HR by *CathB* was validated wherein, expression of HR gene marker *Hs203* was suppressed in VIGS plants. Interestingly, *CathB* also functions in HR triggered by a specific gene-for-gene

Table 1. Selected papain-like cysteine proteases implicated to play in plant disease and immunity.

Protease	Source	Affinity	Function	Reference
<i>Cath B</i>	<i>N. benthamiana</i>	C1A	Regulates HR; involved in nonhost and host-specific disease resistance	Gilroy <i>et al.</i> 2007
<i>RcR3</i>	<i>S. lycopersicon</i>	C1A	Common virulence target of <i>C. fulvum</i> Avr2 and <i>G. rostochiensis</i> VAPI	Krüger <i>et al.</i> 2002; Lozano-Torres <i>et al.</i> 2012
<i>PIP1</i>	<i>S. lycopersicon</i>	C1A	A PR protein and a virulence target of <i>P. infestans</i> EPIC2B	Tian <i>et al.</i> 2007
<i>RD19</i>	<i>A. thaliana</i>	C1A	Virulence target of <i>R. solanacearum</i> PopP2	Bernoux <i>et al.</i> 2008
<i>RD21</i>	<i>A. thaliana</i>	C1A	Renders protection against necrotrophic pathogen <i>Botrytis cinerea</i>	Shindo <i>et al.</i> 2012
<i>PopP1</i>	<i>R. solanacearum</i>	C55	Encodes Avr protein which modulates aggressiveness to the host	Lavie <i>et al.</i> 2002
<i>AvrPphB</i>	<i>P. syringae</i>	C58	Inhibits PBS1 kinase and induces HR in <i>Arabidopsis</i>	Shao <i>et al.</i> 2003
<i>AvrRpt2</i>	<i>P. syringae</i>	C70	Activates RPS2 R-mediated resistance in tomato by inhibiting <i>RIN4</i>	Mackey <i>et al.</i> 2002

interaction. The HR in TRV::gfp coexpressed with potato *R3a* and *Phytophthora infestans Avr3a* was abolished in TRV::NbCathB plants. But such suppression was not observed under *Cladosporium fulvum Avr4*- and tomato *Cf-4*-mediated HR. The specificity of action suggests that *Cath B* is not an obligatory component for all PCD triggered during disease resistance. Detailed mechanism of resistance remains limited, nevertheless, these findings suggest the dynamic role of *Cath B*, being an important component of defense both in non-host and in specific gene-for-gene disease resistance. A detailed information was reported by Gilroy *et al.* 2007.

Rcr3

Recently, it has been shown that the *Cf-2* mediates dual resistance in tomato involving two different pathogens, fungus and nematode, utilizing a common virulence target cysteine protease (Lozano-Torres *et al.* 2012). In the first report, a trans-membrane protein with extracellular leucine-rich repeats (LRRs) and short (23 to 36 amino acid) cytoplasmic domains-encoding *Cf*-genes confer resistance to the fungus *Cladosporium fulvum* in association with the *required for Cladosporium resistance 3 (Rcr3)* (Fig. 1B) (Dang and Jones 2001; Dixon *et al.* 2000; Krüger *et al.* 2002). *Rcr3* encodes an apoplast-secreted papain-like cysteine protease of 344 amino acids with proven proteolytic activity (van der Hoorn 2008). *Avr2* on the other hand encodes a secreted, cysteine-rich protein (Dixon *et al.* 1996). Inoculation of tomato with *C. fulvum* results in a rapid elevation of *Rcr3* transcripts especially in incompatible (resistant) interactions, resembling a pathogenesis-related genes response (de Wit *et al.* 1986; Krüger *et al.* 2002). The resistance response involves HR at the site of inoculation thereby limiting further pathogen invasion (van der Hoorn 2008). Since *Rcr3* is a secreted cysteine protease and has a specific role in *Cf-2*-mediated resistance, it was thought to function upstream of *Cf-2* and was considered as obligatory component required for *Cf-2*-mediated fungal resistance (Krüger *et al.* 2002; Rooney *et al.* 2005). It was proposed that inhibition of *Rcr3* by *Avr2* induces a conformational change in *Rcr3* resulting in *Rcr3-Avr2* complex which activates *Cf-2*-mediated HR (Rooney *et al.* 2005; Bernoux

et al. 2008). The mechanism pattern is consistent with the guard theory where in *Cf-2* guards *Rcr3* and triggers defense response upon perception of an *Rcr3/Avr2* complex. A detailed information was reported by Krüger *et al.* 2002.

Interestingly, a recent study conducted by Lozano-Torres *et al.* (2012) demonstrates that the *Cf-2*-mediated resistance against the nematode *Globodera rostochiensis* requires the same target cysteine protease inhibited by the *Avr2* from *C. fulvum* (Fig. 1C). As the obligate dormant period of the potato cyst nematode *G. rostochiensis* culminates and as soon as the nematode begins to hatch from the egg, its effectors are activated and are ready for translocation to the apoplast and cytoplasm of host cells through the stylet. Using liquid chromatography-mass spectrophotometry, the venom allergen-like effector protein, VAP1, was shown to be secreted to the apoplast of host cells alongside plant cell wall modifying proteins. Based on the yeast two-hybrid analysis which used Gr-VAP1 as bait, this effector was found to interact with the apoplastic cysteine protease *Rcr3^{pim}* of tomato. A subsequent co-immunoprecipitation assay asserts the specificity of the Gr-VAP1 and *Rcr3^{pim}* interaction. Disease screening for resistance to *G. rostochiensis* revealed a 68% lower infection in *Cf-2/Rcr3^{pim}* compared to *Cf-O/Rcr3-3* and *Cf-2/rcr3-3* indicating that both *cf-2* and *Rcr3* are necessary for resistance to the cyst nematode in tomato. Further, transient expression of Gr-VAP1 in the leaves of tomato triggers a *Cf-2/Rcr3^{pim}*-dependent programmed cell death. A detailed information was reported by Lozano-Torres *et al.* 2012.

PIPI

Phytophthora inhibited protease 1 (PIPI), a papain-like extracellular cysteine protease, is closely related to the tomato cysteine protease *Rcr3* in the C1A-5 group (Tian *et al.* 2007). Genetic mapping analysis of the two genes in a population derived from a cross between tomato cv. Sun1642 and *S. pimpinellifolium* accession LA1589 reveals tight linkage of *PIPI* and *Rcr3* near marker TG14 on chromosome 2 (van der Knaap and Tanksley 2001; Tian *et al.* 2007). The oomycete *P. infestans* has been reported to induce late blight in tomato and potato (Birch and Whisson 2001; Smart and Fry 2001; Ristaino 2002). Motif searches

of *P. infestans* unigenes revealed a family of cystatin-like protease inhibitors, two of which EPIC1 and EPIC2 were upregulated during infection of tomato (Tian *et al.* 2007). The Epic-*PIP1* interaction was investigated by Tian *et al.* (2007). By performing coimmunoprecipitation assay on tomato intercellular fluids isolated from two different genotypes: *Rcr3^{prim}* and *rcr3* mutant, both incubated with rEPICs, it was found that EPIC2B interacts with *PIP1* (Fig. 1E). Confirmation of EPIC2B-*PIP1* interaction was done through western blot analysis of the intercellular fluids of *PIP1*-transiently transformed *N. benthamiana*. Results of cysteine protease activity profiling assay corroborated previous findings that EPIC2B inhibits the intercellular-secreted PIP1, a functional cysteine protease. Previously, *PIP1* was shown to correlate with a large number of SA-induced PR protein genes including PR1, PR2, PR3, and PR7 (Zhao *et al.* 2003). Indeed, upregulation of *PIP1* was observed during treatment with SA analog BTH

implying that the gene is a PR protein. A detailed information was reported by Tian *et al.* 2007.

RD19

Most Gram-negative bacteria utilize type-III secretion system either to suppress basal defense or initiate effector-triggered immunity. This group of effectors is mostly translocated directly to the host cell cytoplasm (Jones and Dangl 2006). *Ralstonia solanacearum* is a soil-borne proteobacterium known to cause wilts especially to Solanaceous plants (Hayward 1991). *R. solanacearum* encodes *Pseudomonas* outer protein P2 (*PopP2*) which confers a specific immunity response in *Arabidopsis* mediated by *RESISTANT TO R. SOLANACEARUM I-R* (*RRS1-R*), its cognate R protein (Fig. 1A) (Deslandes *et al.* 2003). *PopP2* is a cysteine protease effector and a member of YopJ/AvrRxv protein group under C55 peptidase family of clan

Fig. 1. Cellular localization of PLCPs during host-pathogen interaction in six selected pathosystems. Genes in red are host's PLCP, the green ones are PLCPs secreted by pathogen, the blue ones are non-cysteine protease effectors that interact with host's PLCP, and the black ones are host's resistance proteins. (A) Secretion of PopP2 to host's nucleus recruits RD19 from lytic vacuoles and form nuclear complex which activates RRS1-R mediated resistance (Deslandes and Rivas 2011); (B,C) *C. fulvum* Avr2 and *G. rostochiensis* VAP1 target the same protein, Rcr3, in the apoplast to form complex ligand which activates common R protein Cf-2 (Shindo and van der Hoorn 2008); (D) AvrRpt2, a PLCP secreted by *P. syringae*, inhibits RIN4 and activates RPS2 (Mackey *et al.* 2003); (E) The Epic2B effector delivered by *P. infestans* targets and inhibits PIP1, a tomato PLCP secreted in apoplast (Shindo and van der Hoorn 2008); and (F) *B. cinerea*, a necrotrophic fungus activates RD21, a PLCP localized in vacuole (Shindo *et al.* 2012).

CE (Barret and Rawlings 2001). *RRS1-R*, on the other hand, is an atypical resistance protein which contains Toll/Interleukin 1 receptor (TIR)-NBS-LRR domain and C-terminal WRKY domain (Deslandes *et al.* 2002; Eulgem and Somssich 2007). To investigate the components of RRS1-R/PopP2-mediated disease resistance signaling, Bernoux *et al.* (2008) performed a yeast two-hybrid with PopP2 as bait which led to the identification of *RESPONSIVE TO DEHYDRATION19* (*RD19*), a predicted PLCP of subfamily C1A (Rawlings *et al.* 2014). Using gene knockout strategy, involvement of *RD19* in *RRS1-R*-mediated resistance signaling was studied. Disease screening test involving mutant line (*rd19/RRS1-R*) showed compromised resistance wherein disease symptoms correlated with an increase of bacterial growth in the mutant. Confirmation of this function was done through genetic complementation of the mutant lines which resulted in a regained resistance phenotype. Accumulation of *RD19* transcripts was found to increase after inoculation with *R. solanacearum*. The quantitative noninvasive FLIM approach reveals the physical association of RD19 with PopP2. Interestingly, it fails to show interaction between RRS1-R and RD19. A detailed information was reported by Bernoux *et al.* 2008.

RD21

The role of C14-like protease in the model pathosystem *Hyaloperonospora arabidopsis* (*Hpa*) and *Arabidopsis thaliana* has been investigated. *Hpa* is an obligate biotrophic oomycete pathogen causing downy mildew in *Arabidopsis*. It proliferates in the leaf apoplast and uses haustoria to gain access for nutrition in the parenchyma cells (Coates and Beynon 2010). Shindo *et al.* (2012) identified RD21 (At1g47128), the closest ortholog of tomato *C14* with 58% prodomain homology, 76% protease domain similarity, and are 61% alike in granulin domain. Accordingly, a search for EPIC orthologs from *Hpa* was done and three EPIC-like proteins, HpaEPIC-A, -B and -C were identified. Phylogenetic analysis determined HpaEPIC1C-B and -C, the most likely ortholog of PinfEPIC1 and -2B, which were then selected for the subsequent analysis. To determine the function of the gene, knockout lines were generated. Subsequent disease evaluation shows that the gene knockout

does not affect interactions with either virulent or avirulent strains of *Hpa*. To demonstrate the proposed role of the gene during incompatible interactions, another pathogen *P. syringae* was used to infect the mutant line, and yet the treatment failed to show changes in the phenotypic response compared with the wild type. The *rd21* mutant was tested against the pathogen *Botrytis cinerea* and surprisingly it exhibits an increased susceptibility suggesting its specific role in immunity against necrotrophic fungal pathogen (Fig. 1F). This sensitivity response of the host proteases is in consistent with the fact that the *B. cinerea* genome does not encode PLCPs inhibitor protein (Rawling *et al.* 2010). A detailed information was reported by Shindo *et al.* 2012.

Cysteine protease secreted by phytopathogen

Pathogens have developed arsenal effectors that target host defense either by inhibiting or manipulating the receptors or host resistance gene products. Phytopathogenic bacteria use the type-III secretion system (TTSS) to directly deliver effector proteins into the plant cells presumably to initiate disease infection (Cornelis and Gijsegem 2000). These effector proteins are host-specific and trigger immunity in plants harboring a corresponding resistance gene (Keen 1990). Most Gram-negative phytobacteria contain TTSS that are encoded by *hrp* (*hypersensitive response and pathogenicity*) genes, proving their crucial role in mediating diverse plant-bacteria interactions (Lindgren *et al.* 1986; Alfano and Collmer 2004; He *et al.* 2004; Buttner and Bonas 2006). Previously, bacterial cysteine proteases have been shown to play important role both in compatible and incompatible host-gene interaction by targeting specific plant substrates (Hotson and Mudgett 2004). Most phytopathogenic bacterial cysteine proteases are classified to two major clans, CA and CE (Rawlings *et al.* 2014). The following are examples of cysteine protease TTSS in phytobacteria.

PopP1

Pseudomonas outer protein P1 (*PopP1*), a cysteine protease secreted by *Ralstonia solanacearum*, is closely related with the YOPP/YOPJ pathogenicity factors from human pathogen *Yersinia* (Orth *et al.* 2000; Orth 2002).

PopP1 encodes a 41-kDa protein and possesses the conserved amino acid triad characteristics for cysteine proteases (Orth *et al.* 2000). Questions regarding its function in plant-bacteria interaction were addressed by Lavie *et al.* (2002). The loss-of-function analysis revealed that *PopP1* acts as a host-specificity factor encoding an Avr protein which modulates aggressiveness of the bacterium toward the host plant. Modulation of virulence is evident in the virulent nature of *popP1* mutant in resistant petunia line St40, and in the restoration of avirulence following genetic complementation. Results in western blot using bacterial cells of wild type and the mutants showed that *PopP1* is secreted by *R. solanacearum* using TTSS. But unlike *PopA*, *PopB*, and *PopC* TTSS-dependent effectors, *PopP1* gene does not colocalized in the vicinity of the *hrp* gene cluster on the megaplasmid but rather on the bacterial chromosome. A detailed information was reported by Lavie *et al.* 2002.

AvrPphB

P. syringae pv. *Phaseolicola* secretes *AvrPphB*, an avirulence protein responsible for initiating halo-blight disease in bean (Jenner *et al.* 1991). *AvrPphB* protein is a cysteine protease member of YopT family, capable of autoproteolytic cleavage of an *AvrPphB* precursor for eliciting HR (Shao *et al.* 2002). Shao *et al.* (2002) demonstrated that proteolytic inactive mutants of *AvrPphB* (induced mutation in the *AvrPphB* catalytic triad C98S, H212A, and D227A) are incapable of inducing HR in *Arabidopsis* suggesting that proteolytic activity is needed for *AvrPphB* to induce HR. *P. syringae* strains carrying *AvrPphB* induce an HR when inoculated into *Arabidopsis* plants carrying the cognate R gene *RPS5*, and also, it induces HR when transiently expressed in tobacco leaves (Simonich and Innes 1995). Since HR cannot proceed without *AvrPphB*, it was proposed that *RPS5* is activated after it binds to a peptide released from the substrate of *AvrPphB* (Shao *et al.* 2002). PBS1 kinase was considered as the potential substrate interacting with *AvrPphB*, as deletion of the *PBS1* from *Arabidopsis* makes *AvrPphB* unrecognizable by *RPS5* (Swiderski and Innes 2001). Further, *AvrPphB* was shown to cleave PBS1 independent

of RAR1 and RPS5 in *Arabidopsis* (Shao *et al.* 2003). Specifically, this team of investigators identified the cleavage sites in PBS1 at a position composed of stretch of three amino acids immediately preceding the *AvrPphB* autocleavage site after K²⁴³. A detailed information was reported by Shao *et al.* 2003.

AvrRpt2

More recently, apart from the two sequence-unrelated TTSS proteins of *P. syringae* targeting *Arabidopsis* protein RIN4 (Mackey *et al.* 2002), a third sequence-unrelated effector called *AvrRpt2* was found to inhibit RIN4 inside the host cell by inducing post transcriptional elimination (Mackey *et al.* 2003). *AvrRpt2*, a *Pseudomonas syringae* pv. *tomato* (*Pst*) TTSS effector protein, is thought to elicit an immunity response in *RPS2*-expressing *Arabidopsis* (KunKel *et al.* 1993). Translocation of *AvrRpt2* to the cell during infection results in its cleavage at amino acids 71 and 72 to liberate a stable terminal 21 kDa product for *RPS2* binding (Mudgett and Staskawicz 1999). Hotson and Mudgett (2004) demonstrate that activation of the *RPS2* R protein solely depends on the *AvrRpt2*-mediated removal of RIN4 (Fig. 1D). In a proposed model by Mackey *et al.* (2003), inhibiting RIN4 disappearance prevents *RPS2* activation, and the lethality associated with RIN4 disruption requires *RPS2*. Even at a barely detectable RIN4 levels, plants still initiate normal *RPS2*-dependent response to *AvrRpt2* implying that only by total elimination of *RIN4* does *AvrRpt2* activate *RPS2* (Mackey *et al.* 2002). Moreover, elaboration of *AvrRpt2* interference mechanism was depicted in the physical interactions of *AvrRpt2*, RIN4, and RPM1 at the plasma membrane (Hotson and Mudgett 2004). A detailed information was reported by Mackey *et al.* 2002.

Nucleocytoplasmic shuttling of PLCP during interaction

Recent studies on subcellular localization of key resistance components situate nucleus at the forefront of the host-pathogen interface (Rivas 2012). With the accumulating evidence highlighting the significance of nuclear localization of effectors and key host resistance components for disease resistance in plants, nuclear dynamics is taken as a key event essential for plant defense (Deslandes and Rivas

2011). Some of the dynamic cellular processes that occur with defense response include plant cell trafficking, cytoskeleton reorganization, organelle positioning, and cytoplasmic-nuclear transport and partitioning (Lipka and Panstruga 2005; Robatzek 2007; Shen *et al.* 2007; Shen and Schulze-Lefert 2007; Wiermer *et al.* 2007; Bernoux *et al.* 2008). Among the interesting consequences of nuclear translocation of effectors is the subcellular localization of their cognate R proteins (Deslandes *et al.* 2003; Shen *et al.* 2007). Having been implicated in different pathosystems, PLCPs were shown to be an important target of microbial effectors resulting in nucleocytoplasmic trafficking during interaction.

PopP2 from *R. solanacearum* presents a nuclear localization signal (NLS) that is required for its nuclear translocation (Deslandes *et al.* 2003). As previously reported, direct translocation of PopP2 effector to the nucleus of host plant cells promotes nuclear accumulation of RRS1-R, an atypical R protein which confers resistance to *R. solanacearum*, possibly by preventing its proteasomal degradation (Deslandes *et al.* 2003; Rivas 2012). RRS1-R has been shown to interact with its cognate effector PopP2 in yeast cells (Deslandes and Rivas 2011). *PopP2* interaction with *RRS1-R* was proposed to regulate defense-related gene expression either directly via the RRS1-R WKRY domain or through the action of additional plant TFs (Deslandes *et al.* 2003; Tasset *et al.* 2010). In their extensive study, Bernoux *et al.* (2008) identified and demonstrated additional important component of *PopP2*-*RRS1-R* mediated resistance. By performing yeast-two hybrid screening using PopP2 as bait, a cysteine protease *RD19* was identified among the targets. Subsequent experiments assert that *RD19* is a crucial host factor for *PopP2*-triggered *RRS1-R*-mediated resistance as exemplified in *rd19* loss of function and genetic complementation studies. As detailed by Bernoux *et al.* (2008), *RD19* localizes to the lytic vacuole and becomes active after proteolysis (Paris *et al.* 1996). Interestingly, when co-expressed in *N. benthamiana*, *RD19* was shown to shuttle to the nucleus with PopP2. Further, the FLIM analysis in living cells confirmed a physical interaction between PopP2 and *RD19* in the nucleus (Fig. 1A). Intriguingly, there was no interaction detected between *Rd19* and RRS1-R. Also, PopP2 and *RD19* association was

not affected in the presence of RRS1-R. Since *RD19* is required for *Arabidopsis* resistance to *Ralstonia*, it was proposed that the *RD19*-PopP2 nuclear complex is the one required for activation of the plant resistance response (Rivas 2012). How *RD19* exactly activates resistance response remains unknown but Bernoux *et al.* (2008) theorized that the nuclear *RD19* may function as transcriptional activator or competes with RRS1-R for similar or overlapping *cis*-elements in the promoters of defense-related genes. Unlike PopP2, *RD19* lacks consensus NLS that would have allow its nuclear trafficking (Rivas 2012). Possibly, the vacuolar membrane collapse during the elicitor-induced membrane permeabilization releasing *RD19* from vacuole-associated compartments into the cytoplasm where they may become available for SUMOylation (Matarasso *et al.* 2005; Bernoux *et al.* 2008), a process which generate signals required for their nuclear translocation (Rivas 2012). A detailed information was reported by Bernoux *et al.* 2003.

Coevolutionary arms race between plants PLCP and effectors

Translocation mechanisms for pathogen effectors are continuously evolving as means of manipulating the host cytoplasm and suppression of host immunity (Jones and Dangl 2006). Selection pressure on host enzyme yields variant residues at the interaction surface of the enzyme-inhibitor interactions (Misas-Villamil and van der Hoorn 2008). An emerging concept in antagonistic host-pathogen interactions states that to escape manipulation, effector/avirulence targets are subject to diversifying selection (Hougenhout *et al.* 2009). Shabab *et al.* (2008) reported that tomato plants create a harmful proteolytic apoplast through secretion of PLCPs during defense to halt pathogen build up. In particular, tomato apoplast is populated with remarkable diverse PLCP activities by seven PLCPs that fall into four different subfamilies, two of which are PIP1 and RCR3. However, Avr2 effector inhibits all apoplastic PLCP activities. Shabab *et al.* (2008) further demonstrate the fate of these two PLCPs (*PIP1* and *RCR3*) upon inhibition by *AVR2*. As indicated in their findings, *PIP1* and *RCR3* are less conserved compared with the other proteases. Sequencing of the region that encodes protease

domain of eight wild tomato relatives revealed variant nucleotides in the seven PLCPs, with *PIP1* and *RCR3* containing variant codons that cause nonsimilar amino acid substitutions. This means that *PIP1* and *RCR3* are under diversifying selection resulting in variant residues around the substrate binding groove of the protease, deemed to match the coevolving substrates (Shabab *et al.* 2008). Diversifying selection at interaction surfaces between inhibitors and their substrates are common in plant-pathogen interactions (Misas-Villamil and van der Hoorn 2008). A detailed information was reported by Shabab *et al.* 2008.

In the study performed by Kaschani *et al.* (2010), C14 was tagged as a new common target of EPIC1 and EPIC2B, the two apoplastic, cystatin-like proteins secreted by potato typified by a carboxyl-terminal granulin domain. These EPICs proteins strongly inhibit C14, as significant inhibition assays proceed even at lower EPIC concentrations and at wide pH value-range (Kaschani *et al.* 2010). C14 is a highly conserved cysteine protease in the plant kingdom characterized by a unique, C-terminal granulin-like domain that shares homology with animal growth hormones (Bateman and Bennett 1998). In tomato, C14 was reported to undergo conservative selection which is evident in the resultant few variant codons causing change in the encoded amino acid (Shabab *et al.* 2008). Possibly, this could be due to the fact that wild tomato is not the natural host of *P. infestans* (Debener *et al.* 1990). This prompted the investigators to examine for natural variation of C14 in the natural hosts of *P. infestans* in the three wild potato species including *S. demissum*, *S. verrucosum*, and *S. stoloniferum* (Debener *et al.* 1990; Grünwald and Flier 2005; Kaschani *et al.* 2010). As demonstrated by Kaschani *et al.* (2010), a total of six new C14 alleles were identified in addition to the C14 allele of cultivated potato deposited in the National Center for Biotechnology Information database. Further, phylogenetic analysis revealed the difference of potato and tomato C14 alleles both at the protein and nucleotide levels marked by expanded branch lengths of potato than in tomato indicating most nucleotide polymorphisms in potato C14s cause amino acid changes (Kaschani *et al.* 2010). Moreover, as shown using phylogenetic analysis of potato C14 sequences (Yang 2007), there is 95% probability that potato C14 is under diversifying selection due to strong

selective pressure from *P. infestans*. A detailed information was reported by Kaschani *et al.* 2010.

General inference

Despite the overwhelming number of cysteine proteases in plants, only a handful were successfully investigated. In *Arabidopsis* alone, functions for only a few of the more than 550 peptidases (Rawlings *et al.* 2014) have been studied genetically (Adam and Clarke 2002; Beers *et al.* 2004; van der Hoorn *et al.* 2004). Introduction of protease activity profiling has been very instrumental in assigning functions for proteases. The current status of cysteine protease research in plant disease immunity features its diverse and overlapping role in basal and effector-triggered immunity. Among the findings, PLCPs are inferred as important target of pathogen effector guarded by other R genes for defense. An intriguing incidence of nucleocytoplasmic trafficking of a few members such as *RD19* provides a notion that PCLPs are involved in the crucial transcriptional regulation of defense-related genes in the host. Yet, our understanding regarding how PLCPs shuttle to the nucleus remains obscure. Another exciting inference is the effect of strong selection pressure in inducing variant codons which leads to coevolutionary arms race between R gene and effector under natural pathosystem.

The limited current data insufficiently benchmark our basic understanding on how PLCP activities modulate host-pathogen interaction. Although most of the demonstrated cysteine proteases are closely related, yet they exhibit different roles thus prompting us to explore more PLCP members in many other pathosystems, and extricate further mechanism of defense regulation in the host cell.

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REFERENCES

- Adam Z, Clarke AK. 2002. Cutting edge of chloroplast proteolysis. *Trends Plant Sci.* 7: 451-456.
- Alfano JR, Collmer A. 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* 42: 385-414.
- Axtell MJ, Chisholm ST, Dahlbeck D, Staskawicz BJ. 2003. Genetic and molecular evidence that the *Pseudomonas syringae* type III effector protein AvrRpt2 is a cysteine protease. *Mol. Microbiol.* 49: 1537-1546.
- Barrett AJ, Rawlings ND. 2001. Evolutionary lines of Cysteine peptidases. *Biol. Chem.* 382: 727-733.
- Bateman A, Bennett HPJ. 1998. Granulins: the structure and function of an emerging family of growth factors. *J. Endocrinol.* 158: 145-151.
- Beers EP, Jones AM, Dickerman AW. 2004. The S8 serine, C1A cysteine and A1 aspartic protease families in *Arabidopsis*. *Phytochemistry* 65: 43-58.
- Bernoux M, Timmers T, Jauneau A, Briere C, de Wit PJ, Marco Y, Deslandes L. 2008. RD19, an *Arabidopsis* cysteine protease required for RRS1-R-mediated resistance, is relocalized to the nucleus by the *Ralstonia solanacearum* PopP2 effector. *Plant Cell* 20: 2252-2264.
- Birch PRJ, Whisson S. 2001. *Phytophthora infestans* enters the genomics era. *Mol. Plant Pathol.* 2: 257-263.
- Büttner D, Bonas U. 2006. Who comes first? How plant pathogenic bacteria orchestrate type III secretion. *Curr. Opin. Microbiol.* 9: 193-200.
- Carrington JC, Herndon KL. 1992. Characterization of the potyviral HC-Pro autoproteolytic cleavage site. *Virology* 187: 308-315.
- Coaker G, Falick A, Staskawicz B. 2005. Activation of a phytopathogenic bacterial effector protein by a eukaryotic cyclophilin. *Science* 308: 548-550.
- Coates ME, Beynon JL. 2010. *Hyaloperonospora arabidopsidis* as a pathogen model. *Annu. Rev. Phytopathol.* 48: 329-345.
- Cornelis G, Van Gijsegem F. 2000. Assembly and function of type III secretory systems. *Annu Rev Microbiol.* 54: 735-74.
- Dangl JL, Jones JDG. 2001. Plant pathogens and integrated defence responses to infection. *Nature* 411: 826-833.
- Debener T, Salamini F, Gebhardt C. 1990. Phylogeny of wild and cultivated *Solanum* species based on nuclear restriction fragment length polymorphisms (RFLPs). *Theor. Appl. Genet.* 79: 360-368.
- Deslandes L, Olivier J, Peeters N, Feng DX, Khounlotham M, Boucher C, Somssich I, Genin S, Marco Y. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc. Natl. Acad. Sci. USA* 100: 8024-8029.
- Deslandes L, Rivas S. 2011. The plant cell nucleus: a true arena for the fight between plants and pathogens. *Plant Signal. Behav.* 6: 42-48.
- Deslandes L, Olivier J, Theulières F, Hirsch J, Feng DX, Bittner-Eddy P, Beynon J, Marco Y. 2002. Resistance to *Ralstonia solanacearum* is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. *Proc. Natl. Acad. Sci. USA* 99: 2404-2409.
- De Wit PJGM, Buurlage MB, Hammond KE. 1986. The occurrence of host, pathogen and interaction-specific proteins in the apoplast of *Cladosporium fulvum* (syn. *Fulvia fulva*) infected tomato leaves. *Physiol. Mol. Plant Pathol.* 29: 159-172.
- Dixon MS, Golstein C, Thomas CM, van der Biezen EA, Jones JDG. 2000. Genetic complexity of pathogen perception by plants; the example of Rcr3, a tomato gene required specifically by cf-2. *PNAS* 97: 8807-8814.
- Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JD. 1996. The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. *Cell* 84: 451-59.
- Drenth J, Jansonius JN, Koekoek R, Wolthers BG. 1971. The crystal structure of papain C. I. Two dimensional fourier synthesis. *Adv. Protein Chem.* 25: 79-115.
- Eulgem T, Somssich IE. 2007. Networks of WRKY transcription factors in defence signalling. *Curr. Opin. Plant Biol.* 10: 366-371.
- Gilroy EM, Hein I, van der Hoorn RAL, Boevink PC, Venter E, McLellan H, Kaffarnik F, Hrubikova K, Shaw J, Holeva M, Lopez EC, Borrás-Hidalgo O, Pritchard L, Loake GJ, Lacomme C, Birch PRJ. 2007. Involvement of cathepsin B in the plant disease resistance hypersensitive response. *Plant J.* 52: 1-13.
- Golubtsov A, Kaariainen L, Caldentey J. 2006. Characterization of the cysteine protease domain of Semliki Forest virus replicase protein nsP2 by in vitro mutagenesis. *FEBS Lett.* 580: 1502-1508.

- Grudkowska M, Zagdanska B. 2004. Multifunctional role of plant cysteine proteinases. *Acta Biochim. Pol.* 51: 609-624.
- Grünwald NJ, Flier WG. 2005. The biology of *Phytophthora infestans* at its center of origin. *Annu. Rev. Phytopathol.* 43: 171-190.
- Hayward AC. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* 29: 65-87.
- He SY, Nomura K, Whittam TS. 2004. Type III protein secretion mechanism in mammalian and plant pathogens. *Biochim. Biophys. Acta* 1694: 181-206.
- Hotson A, Mudgett MB. 2004. Cysteine proteases in phytopathogenic bacteria: Identification of plant targets and activation of innate immunity. *Curr. Opin. Plant Biol.* 7: 384-390.
- Hougenhout SA, van der Hoorn RAL, Terauchi R, Kamoun S. 2009. Emerging concepts in effector biology of plant associated organisms. *Mol. Plant-Microbe Interact.* 22: 115-122.
- Jenner C, Hitchin E, Mansfield J, Walters K, Betteridge P, Teverson D, Taylor J. 1991. Gene-for-gene interactions between *Pseudomonas syringae* pv. *phaseolicola* and *Phaseolus*. *Mol. Plant Microbe Interact.* 4: 553-562.
- Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* 444: 323-329.
- Kaschani F, Shabab M, Bozkurt T, Shindo T, Schornack, Gu C, Ilyas M, Win J, Kamoun S, van der Hoorn RA. 2010. An effector-targeted protease contributes to defense against *Phytophthora infestans* and is under diversifying selection in natural hosts. *Plant physiol.* 154: 1794-804. DOI: 10.1104/pp.110.158030.
- Keen N. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24: 447-463.
- Krüger J, Thomas CM, Golstein C, Dixon MS, Smoker M, Tang S, Mulder L, Jones JDG. 2002. A tomato Cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. *Science* 296: 744-747.
- Kunkel BN, Bent AF, Dahlbeck D, Innes RW, Staskawicz BJ. 1993. RPS2, an Arabidopsis disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2*. *Plant Cell* 5: 865-875.
- Lam E, Naohiro K, Lawton M. 2001. Programmed cell death, mitochondria and the plant Hypersensitive response. *Nature* 411: 848-853.
- Lavie M, Shillington E, Eguiluz C, Grimsley N, Boucher C. 2002. PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. *Mol. Plant Microbe Interact.* 15: 1058-1068.
- Lindgren PB, Peet RC, Panopoulos NJ. 1986. Gene-cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* 168: 512-522.
- Lipka V, Panstruga R. 2005. Dynamic cellular responses in plant-microbe interactions. *Curr. Opin. Plant Biol.* 8: 625-631.
- Lopez-Solanilla E, Bronstein PA, Schneider AR, Collmer A. 2004. HopPtoN is a *Pseudomonas syringae* Hrp (type III secretion system) cysteine protease effector that suppresses pathogen-induced necrosis associated with both compatible and incompatible plant interactions. *Mol. Microbiol.* 54: 353-365.
- Lozano-Torres JL, Wilbers RHP, Gawronski P, Boshoven JC, Finkers-Tomczak A, Cordewener JHG, Americab AHP, Overmars HA, Van 't Kloosterc JW, Baranowskid L, Sobczak M, Ilyase M, van der Hoorn RAL, Schotsa A, de Wit PGJM, Bakker J, Goversea A, Smanta G. 2012. Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode. *PNAS* doi/10.1073/pnas.1202867109.
- Mackey D, Belkadir Y, Alonso JM, Ecker JR, Dangl JL. 2003. Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112: 379-389.
- Mackey D, Holt BF, Wiig A, Dangl JL. 2002. RIN4 interacts with *Pseudomonas syringae* Type III effector molecules and is required for RPM1-mediated disease resistance in Arabidopsis. *Cell* 108: 743-754.
- Matarasso N, Schuster S, Avni A. 2005. A novel plant Cysteine protease has a dual function as a regulator of 1-aminocyclopropane- 1-carboxylic acid synthase gene expression. *Plant Cell* 17: 1205-1216.
- Misas-Villamil JC, van der Hoorn RAL. 2008. Enzyme inhibitor interactions at the plant-pathogen interface. *Curr. Opin. Plant Biol.* 11: 380-388.
- Mudgett MB, Staskawicz BJ. 1999. Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial

- pathogenesis. *Mol. Microbiol.* 32: 927-941.
- Orth K. 2002. Function of the *Yersinia* effector YopJ. *Curr. Opin. Microbiol.* 5: 38-43.
- Orth K, Xu Z, Mudgett MB, Bao ZQ, Palmer LE, Bliska JB, Mangel W, Staskawicz B, Dixon JE. 2000. Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. *Science* 290: 1594-1597.
- Paris N, Stanley CM, Jones RL, Rogers JC. 1996. Plant cells contain two functionally distinct vacuolar compartments. *Cell* 85: 563-572.
- Powers JC, Asgian JL, Ekici OD, James KE. 2002. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem. Rev.* 102: 4639-4750.
- Rawlings ND, Morton FR, Barrett AJ. 2006. MEROPS: the peptidase database. *Nucleic Acids Res.* 34: 270-72.
- Rawlings ND, Barrett AJ, Bateman A. 2010. MEROPS: the peptidase database. *Nucleic Acids Res.* 38: D227-D233.
- Rawlings ND, Waller M, Barrett AJ, Bateman A. 2014. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* 42: D503-D509.
- Ristaino JB. 2002. Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. *Microbes Infect.* 4: 1369-1377.
- Rivas S. 2012. Nuclear dynamics during plant innate immunity. *Plant Physiol.* 158: 87-94.
- Robatzek S. 2007. Vesicle trafficking in plant immune responses. *Cell Microbiol.* 9: 1-8.
- Rooney HC, Van't Klooster JW, van der Hoorn RAL, Joosten MHAJ, Jones JDG, de Wit PJGM. 2005. *Cladosporium Avr2* inhibits tomato *Rcr3* protease required for Cf-2 dependent disease resistance. *Science* 308: 1783-1786.
- Shabab M, Shindo T, Gu C, Kaschani F, Pansuriya T, Chinthra R, Harzen A, Colby T, Kamoun S, van der Hoorn RAL. 2008. Fungal effector protein AVR2 targets diversifying defense-related cysteine proteases of tomato. *Plant Cell* 20: 1169-1183.
- Shao F, Dixon JE. 2004. YopT protease and its homologs. In *Handbook of Proteolytic Enzymes*, 2 ed. p.1257-1259, Elsevier, London.
- Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE, Innes RW. 2003. Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector. *Science* 301: 1230-1233.
- Shao F, Merritt PM, Bao Z, Innes RW, Dixon JE. 2002. A *Yersinia* effector and a *Pseudomonas* avirulence protein define a family of cytosolic proteases functioning in bacterial pathogenesis. *Cell* 109: 575-588.
- Shen QH, Saijo Y, Mauch S, Biskup C, Bieri S, Keller B, Seki H, Ulker B, Somssich IE, Schulze-Lefert P. 2007. Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* 315: 1098-1103.
- Shen QH, Schulze-Lefert P. 2007. Rumble in the nuclear jungle: Compartmentalization, trafficking, and nuclear action of plant immune receptors. *EMBO J.* 26: 4293-4301.
- Shindo T, Misas-Villamil JC, Horger AC, Song J, van der Hoorn RAL. 2012. A role in immunity for *Arabidopsis* cysteine protease RD21, the ortholog of the tomato immune protease C14. *PLoS ONE* 7: e29317. doi:10.1371/journal.pone.0029317.
- Shindo T, van der Hoorn RAL. 2008. Papain-like cysteine proteases: key players at molecular battlefields employed by both plants and their invaders. *Mol. Plant Pathol.* 9: 119-125.
- Simonich MT, Innes RW. 1995. A disease resistance gene in *Arabidopsis* with specificity for the *avrPph3* gene of *Pseudomonas syringae* pv. *phaseolicola*. *Mol. Plant Microbe Interact.* 8: 637-640.
- Smart CD, Fry WE. 2001. Invasions by the late blight pathogen: renewed sex and enhanced fitness. *Biol Invasions* 3: 235-243.
- Solomon M, Belenghi B, Delledonne M, Menachem E, Levine A. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell* 11: 431-443.
- Swiderski MR, Innes RW. 2001. The *Arabidopsis* PBS1 resistance gene encodes a member of a novel protein kinase subfamily. *Plant J.* 26: 101-112.
- Tasset C, Bernoux M, Jauneau A, Pouzet C, Brierre C, Kieffer-Jacquod S, Rivas S, Marco Y, Deslandes L. 2010. Autoacetylation of the *Ralstonia solanacearum* effector PopP2 targets a lysine residue essential for RRS1-R-mediated immunity in *Arabidopsis*. *PLoS Pathog.* 6:e1001202.
- Taylor MA, Baker KC, Briggs GS, Connerton IF, Cummings NJ, Pratt KA, Revell DF, Freedman RB, Goodenough PW. 1995. Recombinant pro-regions from papain and papaya proteinase IV- are selective high affinity inhibitors of the mature papaya enzymes. *Protein Eng.* 8: 59-62.

- Tian M, Win J, Song J, van der Hoorn RAL, van der Knaap E, Kamoun S. 2007. A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. *Plant Physiol.* 143, 364-377.
- van der Biezen EA, Jones JDG. 1998. Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* 23: 454-456.
- van der Hoorn RAL. 2008. Plant proteases: From phenotypes to molecular mechanisms. *Annu. Rev. Plant Biol.* 59: 191-223.
- van der Hoorn RAL, Leeuwenburgh MA, Bogyo M, Joosten MHAJ, Peck SC. 2004. Activity profiling of papain-like cysteine proteases in plants. *Plant physiol.* 135: 1170-1178.
- van der Knaap E, Tanksley SD. 2001. Identification and characterization of a novel locus controlling early fruit development in tomato. *Theor. Appl. Genet.* 103: 353-358.
- Wiermer M, Palma K, Zhang Y, Li X. 2007. Should I stay or should I go? Nucleocytoplasmic trafficking in plant innate immunity. *Cell. Microbiol.* 9: 1880-1890.
- Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24: 1586-1591.
- Zhao Y, Thilmony R, Bender CL, Schaller A, He SY, Howe GA. 2003. Virulence systems of *Pseudomonas syringae* pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *Plant J.* 36: 485-499.