Molecular dissection of the Prf-mediated immune signaling in tomato

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Authorization to Submit Dissertation

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Abstract

Tomato, one of the important crops for human consumption (United States Department of Agriculture, 2014), can be infected by *Pseudomonas syringae* pv. *tomato* (*Pst*) and develop speck symptoms on leaf, stem and fruit.

Pst utilizes the Type III secrection system (TTSS) to inject an array of effector proteins into plant cells and these effectors act collectively to trigger diseases. Among the effectors, AvrPtoB is recognized by Pto, a serine/threonine kinase, which is complexed with a NB-LRR immune receptor Prf. Even though a few components involved in the signaling pathway have bee identified, the details of signaling mechanisms remain elusive. Here we report several important findings in the dynamic intracellular events involving the Pto/Prf signaling pathway against *Pst* infection. The Leucine-rich repeat (LRR) domain of Prf suppresses the HR-like cell death triggered by an auto-active Prf mutant (Prf^{D1416V}) by promoting degradation of the Prf^{D1416V} protein. We also found that LRR also suppresses the cell death involved in the Prf immune signaling pathway versus Rpi-blb1 immune signaling pathway. Taken together, we hypothesize that the Prf LRR domain plays a negative regulatory role in the Prf-mediated immune signaling by decreasing Prf protein accumulation via an unknown mechanism.

Abnormal accumulation of immune receptors in plants frequently causes cell death. Therefore, the protein level of immune receptors has to be finely regulated. It has been shown previously that the *Pst* effector AvrPtoB, which acts as a functional ubiquitin E3 ligase, promotes Prf degradation *in planta* (Ntoukakis et al., 2009). Since no interaction between AvrPtoB and Prf has been detected *in vivo*, it appears

that AvrPtoB may ubiquitinate Prf indirectly in plant cells. In our lab, a group of SINA (SEVEN IN ABSENTIA) ubiquitin E3 ligases have been identified that target Prf for ubiquitination and subsequent degradation. Remarkably, AvrPtoB-promoted Prf degradation is severely impaired in *SINA*-silenced plants, indicating the pivotal roles of SINA E3 ligases in this degradation event. Overall, our data suggests a hypothesis that AvrPtoB manipulates the host ubiquitination machinery to promote degradation of the NB-LRR immune receptor Prf thus suppressing plant immunity.

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Chapter 1
Introduction

Plants, like animals, also contract diseases. Even though plants cannot directly communicate with us, we still have a way to define plant diseases. Generally, when a pathogenic organism or an adverse environmental factor stresses plants, the most essential physiological activities are affected and the plants become diseased. A variety of adverse environmental factors can trigger abiotic stress and cause diseases on plants, including heat/cold stress (Chinnusamy et al., 2010; von Koskull-Doring et al., 2007), drought/flood stress (Jackson and Colmer, 2005; Moffat, 2002), salt/acid stress (Chinnusamy et al., 2010; Young and Galston, 1983), nutritional stress (Lynch and Brown, 1997), and so on. However, many plant diseases are caused by pathogens, including fungi, bacteria, viruses, nematodes, and protozoa. My Ph.D. research focuses on studying the molecular basis of bacterial pathogenicity and disease immunity in tomato.

The scientific name of tomato is "*Solanum lycopersicum*". The tomato species originated from South America and were later cultivated as vegetable. Nowadays, tomato has been grown all over the world in fields or greenhouses due to high demand for tomato fruits. Tomato fruits are rich in biotin, vitamin A, vitamin K, vitamin C, copper and potassium (Foundation, 2014) and frequently consumed in our daily life in many different ways, such as tomato juice, ketchup sources and so on. However, tomato plants are also vulnerable to many pathogens that may cause significantly economical loss.

Common diseases in tomato

Typically, tomato plants can be infected by fungal, oomycete, viral, or bacterial pathogens. The representatives of most common and economically important tomato diseases will be described below.

Fungal diseases

Fungal diseases are normally caused by soil-born fungi and transmitted under high humidity and appropriate temperature. In the past decades, the fungal disease outbreak has led to significantly economical loss worldwide as much as millions of dollars (Ajilogba and Babalola, 2013; Judelson, 1997).

Tomato early blight disease is caused by the fungus *Alternaria solani* (Foolad et al., 2000). Phenotypically, older leaves at the bottom of the plants develop some small, brown lesions. In the center of the infected areas, spotted lesions grow and exhibit ring patterns (Barksdal.Th, 1971; Foolad et al., 2000; Nash and Gardner, 1988). If the disease is severe enough, it will result in the complete crop loss (Kalloo and Banerjee, 1993).

Southern blight of tomato is caused by the fungus *Sclerotium rolfsii*. When the young plants are infected, the whole plant droops quickly and permanently. For older plants, stem lesions start appearing near the area of plants in contact with soil (Bulluck and Ristaino, 2002).

Septoria leaf spot, a destructive disease on tomato leaves, petioles, and stems, is caused by the fungus *Septoria lycopersici* (Martin-Hernandez et al., 2000). Right after plants begin to fruit, the fungus initiates the infection on the lower leaves close to the ground. A dark edge surrounds a number of small and round spots in

the infected, old leaves. When the disease becomes severe, spotted leaves will die and fall off the plant (Panthee and Chen, 2010).

Fusarium wilt is caused by the fungus *Fusarium oxysporum* (Prabhukarthikeyan et al., 2013). The infected tomato leaves normally turn yellow, wilt, and eventually die when the disease becomes severe. The infected stems also show a yellow color (Ajilogba and Babalola, 2013).

Oomycete diseases

Oomycetes are a type of fungus-like eukaryote microbe, which aggressively infect plants and can be transmitted by wind or rain. Typically, different groups of plant oomycete pathogens cause distinct diseases including the notorious potato late blight, sudden oak death, downy mildew and white blister rusts.

Potato late blight, a more serious disease than early blight on both tomato and potato plants, is caused by the oomycete *Phytophthora infestans* (Nowicki et al., 2012). The oomycete attacks all parts of the tomato plants above the ground and causes lesions. Lesions in the young leaves appear as dark, water-soaked spots, which quickly spread followed by the formation of a white mycellium at the border of the infected areas on the lower surfaces of the leaves. When the disease progresses to a serious stage, the leaves turn brown/black and eventually die (Foolad et al., 2014). The pathogen can also attack tomato fruits in all developmental stages. The infected fruits are usually covered with greasy spots that eventually lead to brown and leathery rot (Blandon-Diaz et al., 2012).

Buckeye rot is another fruit disease caused by the oomycete *Phytophthora parasitica.* Initially the infected fruits exhibit symptomatic brown spots at the contact

point between the fruit and the soil. Dark ring patterns at the center of the infection can be visible as the spots enlarge (Le Berre et al., 2008).

Pythium fruit rot is caused by certain species of *Pythium*. The infected fruits have small, water-soaked lesions. When such disease symptom proceeds, the whole fruit may eventually collapse. Under humid weather, cotton growth may appear on the lesion surface (Schroeder et al., 2012).

Viral diseases

In addition to the aforementioned fungal and oomycete diseases, viral pathogens potentially transmitted by the intermediate insect vectors also bring about diseases to tomato plants. Severe viral diseases sometimes can cause 100% loss of tomato crops.

The “spotted wilt” disease of tomato is caused by the tomato spotted wilt virus (Crescenzi et al., 2013). Thrips feed on the infected tomato and take up the virus. Later, the virus-harboring thrips feed on healthy tomatoes thereby transmitting the viruses. The growth of the infected tomato plants are retarded and the upper leaves are twisted, with the spreading of bronze spots (Best and Gallus, 1953).

Tomato leaf curl disease is caused by the tomato leaf curl virus (Navot et al., 1991). Whiteflies, arthropod vectors, transmit the viruses from infected plants to nearby cultivated tomato plants. Infected plants typically exhibit curling leaves with yellow borders. Importantly, the plants become fruitless if the infection occurs at early developmental stages. As a result, the virus can cause a loss of fruit yield (Ber et al., 1990).

Tomato plants that are infected by tomato aspermy virus exhibit stunted and bushy phenotypes. Due to the infection, the fruits are small and have few seeds. Additionally, leaf malformation is also a typical symptom of infection (Blencowe and Caldwell, 1949). The viruses can be transmitted by the green peach aphid or grafting.

Golden mosaic of tomato is caused by tomato golden mosaic virus. The viruses are transmitted to healthy tomato or tobacco plants by whiteflies that previously fed on infected tomato plants (Hamilton et al., 1982). Infected tomato plants exhibit stunting and severely deformed young leaves and shoots.

Bacterial diseases

Bacterial diseases can occur at the aboveground tissues, especially the leaves, of tomato plants, and in some cases can be very destructive to tomato crops under humid and cool weather. In general, plant bacterial pathogens enter the plant tissues through natural openings such as stomata and wounding.

Bacterial wilt disease is caused by the bacterium *Ralstonia solanacearum* (Dudman, 1959). The bacteria are soil-born and enter the host plants through the wounds. One significant phenotype of the infected plant is quick wilting of the whole plant where the leaves seem to be healthy (Hayward, 1991).

Bacterial spot of tomato is caused by the bacterium *Xanthomonas vesicatoria* (Matyshevskaia, 1961). The bacterium attacks all the aboveground parts of the plant. Infected leaves are covered with abnormal dark lesions that can coalesce and turn the leaf yellow (Cook, 1969).

Bacterial canker of tomato is caused by the bacterium *Clavibacter michiganense* pv. *michiganense* (Walker and Kendrick, 1948). The bacterium

attacks plants at all growth stages. Early symptoms involve wilting and curling leaves. When the leaves die, the petioles are still green and remain attached to the stem (Hvozdiak et al., 2009).

Bacterial speck disease, known as a globally important disease since the mid-1970s, is caused by the bacterium *Pseudomonas syringae* pv. *tomato* (Bryan, 1933). Patterns of lesions are different between green fruit and ripe fruit. On green fruit, the specks are small, sunken, black, and surrounded by darker green haloes. On ripe fruit, the specks seem to be more superficial with a darkened color. The black lesions on leaves appear as specks with a surrounding yellow halo. Enough specks tend to curl the plant leaves. Specks also occur on the stems as oval-shaped lesions (Pohronezny, 1980). Economically, bacterial speck disease did not lead to serious losses of tomatoes, but it affects the sale value of tomatoes. Several ways have been applied to control bacterial speck disease including a preventive spray of a solution containing copper and mancozeb, and inoculation the tomato seeds with the plant growth-promoting bacterium *Azospirillum brasilense* (Yaov Bashan and de-Bashan, 2002).

Molecular basis of plant-pathogen interactions

Tomato, as an important crop, is not only important for economy, but also serves as a model organism to study plant disease resistance (Pedley and Martin, 2003; Yunis et al., 1980). The molecular basis of the tomato-*Pseudomonas syringae* interaction has been intensively investigated, especially because the genomes of both species have been sequenced (Buell et al., 2003; Tomato Genome, 2012). Now it's generally thought that *Pseudomonas*-secreted effector proteins collectively

aim to suppress the plant defense system and cause diseases in susceptible plants (Cunnac et al., 2009). Yet, resistant plants have evolved specific protein complexes that can recognize effector proteins and activate downstream defense responses eventually leading to arrested bacterial growth (Oh and Martin, 2011).

The causal agent of bacterial speck disease: *Pseudomonas syringae*

The bacterial speck disease in tomato is caused by *Pseudomonas syringae* pv. *tomato* (*Pst*), belonging to the genus of *Pseudomonas* that consists of roughly 191 recorded species, all of which are Gram-negative bacteria (Euzeby, 1997; Harding and Stewart, 1904). Due to the ease of *in vitro* culture (Goldberg and Ohman, 1984) and the availability of the genomic sequences of different strains (Buell et al., 2003; Nelson et al., 2002; Paulsen et al., 2005; Stover et al., 2000), *Pseudomonas* has become widely studied. The well-studied species include *Psudomonas aeruginosa* as an opportunistic human pathogen (Elrod, 1942), the plant pathogen *Pseudomonas syringae* (Staskawicz et al., 1984), the soil-born bacterium *Pseudomonas putida* (Hug et al., 1968), and the plant commensal bacterium *Pseudomonas fluorescens* (Lewis, 1929).

Infection of *Pst* starts at penetration into plants. *Pst* enters the plant's intercellular spaces and proliferates extracellularly (Xin and He, 2013). When the number of bacteria in the infected zone reaches a high enough number, the zone will collapse and visibly exhibit specks on the leaves, releasing bacteria into the environment where the wind and rain will transmit (Buell et al., 2003).

There are three main reasons that attract molecular plant pathologists to focus their research on *Pst*. Firstly, the pathogen is easy to culture and genetically

modify (Kim et al., 2010; Kvitko and Collmer, 2011). Secondly, genetic transformation has been well established in tomato, facilitating the identification of important host defense-related components (Koul et al., 2014; Zhang et al., 2014). Lastly, many *Pst* strains are pathogenic on *Arabidopsis*, a model organism in plant research, allowing verification of the bacterial pathogenesis (Tao et al., 2003; Yao et al., 2013).

Compatible and incompatible interactions are two distinct outcomes when *Pst* infiltrates into the tomato tissues. The compatible interaction results in small black lesions, followed by bacterial multiplication and disease development. In contrast, the incompatible interaction is characterized by the hypersensitive response (HR), a form of localized programmed cell death (PCD) in the infected area, which efficiently arrests bacterial growth (Tao et al., 2003).

The structural basis of bacterial pathogenicity: The type III secretion system

The pathogenicity of *Pst* relies heavily on a molecular syringe termed the type III secretion system, one of several well-studied bacterial secretion systems. Bacteria may possess seven different types of secretion systems. The type I secretion system (TOSS) is responsible for a single-step transportation of proteases and toxins to the exterior space of bacteria (Linhartova et al., 2010). The type II secretion system (T2SS), which is widely conserved in Gram-negative bacteria, employs a two-step secretion mechanism. The initial step is the export of proteins to the periplasm of bacteria, followed by a further export to the exterior through a pore-like structure consisting of different proteins (Filloux, 2004). The type III secretion system (TTSS) consists of 15 to 20 membrane-associated proteins, which are

assembled together as an injection apparatus to deliver host-specific effector proteins into the host cells. These effector proteins collectively interfere with host signal transduction and metabolic pathways; thereby causing diseases in susceptible hosts (Cornelis and Van Gijsegem, 2000). The type IV secretion system (TFSS) is distinctively different from other types of secretion systems regarding the translocated substrate types. Functionally, TFSS can be categorized into three kinds, two of which are involved in macromolecule secretion. The first type involves DNA transfer through conjugation, which helps maintain the genomic plasticity and diversity of bacteria. The second type is mainly responsible for translocation of proteins such as effector proteins harboring ankyrin repeat domains (ANKs) (Backert, 2006). The type V secretion pathway includes autotransporter proteins and two-component secretion system (Henderson et al., 2004). The type VI secretion system has a bacteriophage-like structure for the toxin delivery into both eukaryotic and prokaryotic cells (Zoued et al., 2014). These toxins contribute to both pathogenesis and interbacterial competition (Zoued et al., 2014). The type VII secretion system (T7SS) is very specific to mycobacteria. This system transports extracellular proteins to the exterior all the way through the highly hydrophobic and impermeable cell wall of the mycobacteria (Abdallah, 2007).

The type III secretion system of phytobacteria is made of a series of proteins encoded by *hypersensitive response and pathogenicity* (*hrp*) genes (Lindgren, 1997; Lindgren, 1986). The *hrp* genes are grouped into three classes. The first class is widely conserved in animal and plant bacterial pathogens; For this reason, this class of *hrp* genes was renamed *hrp conserved* (*hrc*). The second class of genes encode

the transcriptional regulators of TTSS regulon genes clusters. The third class of genes encodes a variety of proteins including the components of the pilus, proteins involved in the TTSS assembly and some other secreted proteins, such as chaperones, that are responsible for protein folding (Alfano and Collmer, 1997; Bogdanove et al., 1996; Tang et al., 2006).

The Hrp core components in *P. syringae* include a series of proteins termed HrcJ, HrcU, HrcV, HrcR, HrcT, HrcS, and HrcC, which altogether are assembled as the basal body of the injection apparatus and inserted in between the outer and inner membranes of *P. syringae* (Collmer et al., 2000). HrcC and HrpT are located in the outer membrane and proposed to contribute collaboratively to the formation of a ring structure (Lin, 2006). HrcU and HrcV possess a special N-terminus that orients the proteins in the inner membrane with an exposed cytoplasmic C-terminus. HrcN is required for unfolding the chaperone-substrate complexes (Lorenz and Buttner, 2009). HrpE interacts with HrpO, a structurally flexible protein, through a coiled-coil interaction (Gazi et al., 2008). A cytoplasmic ring structure composed of $HrcQ_A$ and $HrcQ_B$ forms the base of the TTSS(Fadouloglou, 2009). In addition to the Hrp core components, the extracellular components are also required for effective secretion. Typically, the Hrp pilus is the main TTSS appendage for the plant bacterial pathogens and ultimately channels the secreted proteins through itself (Jin et al., 2001; Li et al., 2002; Roine et al., 1997).

Not all TTSS-secreted plant bacterial proteins function in the cytoplasm of host cells. Harpins are a group of glycine-rich and thermally stable proteins also secreted by TTSS. So far, most experimental data indicate that harpins exert their

functions extracellularly. The first identified harpin was HrpN from *Erwinia amylovora* as a HR elicitor when expressed in tobacco plants (Wei et al., 1992). Since then, many other harpins have been identified, including HrpW (Gaudriault et al., 1998), HrpJ (Fu et al., 2006), HrpZ (He et al., 1993), HrpK1 (Kvitko et al., 2007).

Harpins have some unique features. First, the primary structure of harpin consists of a relatively high amount of glycine and serine residues and a very low amount of cysteine and aromatic amino acid residues (Wei et al., 1992). Second, several regions of harpins tend to form α-helix structures (Gaudriault et al., 1998; Kvitko et al., 2007; Wei et al., 1992). Third, harpins are acidic according to their predicted isoelectric points (Gaudriault et al., 1998; Kvitko et al., 2007; Wei et al., 1992). Fourth, harpins are thermally stable probably due to the flexible protein structures, which make it easier for protein purification from *E.coli* (He et al., 1993; Wei et al., 1992).

Even though harpins can trigger the HR defense response in plants, emerging evidence indicates that they also have virulence activity in plants (Barny, 1995; Kim et al., 2003; Noel et al., 2002; Sgro et al., 2012; Sinn et al., 2008). Mutant strains deficient in harpin expression produce fewer virulence-associated symptoms in plants compared to wild-type strains (Barny, 1995; Sinn et al., 2008). Some evidence indicates that the virulence activity of harpins attribute to the ability to deliver effector proteins. For example, the translocation of the *Erwinia amylovora* effector protein DspA/E was severely impaired in the an *Erwinia amylovora hrpN* mutant (Bocsanczy et al., 2008). Moreover, the translocation of a well-studied *Pst*

effector protein AvrPto, was seriously impaired in the *Pst* polymutant in which *hrpK1*, *hrpZ1*, *hrpW1*, *hopP1* and *hopAK1* were knocked out (Kvitko et al., 2007).

The immunity determinant in *Pseudomonas syringae*: Avirulence effector proteins

On one hand, the aforementioned TTSS determines the pathogenicity of *Pseudomonas syringae*; on the other hand, the recognition of certain specific TTSS-secreted virulence effectors by the host immune receptors determines the immunity against the bacterium. Thus these effectors are termed avirulence effectors. The molecular basis of such plant-microbe interactions was first stated formally in the "gene-for-gene" resistance theory, in which immunity is governed by a pair of genes: the resistant gene from the host and the avirulence gene from the corresponding pathogen (Flor, 1942; Flor, 1947; Flor, 1955; Flor, 1971). Diseases susceptibility results if either the resistance (*R*) gene or the corresponding avirulence gene is lacking from the corresponding organisms.

The TTSS effector proteins that trigger immunity were originally termed "avirulence effectors". They have dual function regarding avirulent and virulent activities that is dependent on the genotype of host plants. If the host plants possess the resistance gene and intact immune signaling pathway, these effector proteins are recognized by the plant's immune system and thereby trigger immune responses. On the other hand, in susceptible plants lacking a resistance gene or a functional immune signaling pathway, the effector proteins target host proteins for pathogenesis. Even though many bacterial effector proteins have been identified, only a few of the best-studied ones will be discussed regarding to their biochemical activities in host targets in the following paragraphs.

The immunity determinant in plants: NB-LRR immune receptors

In the case of the incompatible interaction, the effector proteins are sensed by cognate immune receptors in plants, followed by the activation of a chain of cellular events that eventually induce plant defenses. The majority of plant immune receptors are typically multi-domain-containing proteins with a central nucleotide binding (NB) domain and a C-terminal leucine rich repeat (LRR) domain, which can be further divided into two groups based on the identity of the N-terminus. NB-LRR proteins with an N-terminal domain sharing homology with the cytoplasmic domain of the animal Toll and interleukin-1 receptors are termed TIR-NB-LRR proteins, whereas those without a TIR domain generally carry a coiled-coil domain and therefore are termed CC-NB-LRR proteins (Lukasik and Takken, 2009).

It was generally thought that the N-terminal domain of NB-LRR protein is responsible for signal transduction based on the strong conservation of TIR domains between animal NLR (Nod-like Receptor) proteins and plant TIR-NB-LRR proteins (Whitham et al., 1994). This was supported by the experimental findings that a truncated flax rust immune receptor L10 consisting of the TIR domain and the following 39 amino acids from the NB domain triggered effector-independent HR-like cell death when transiently expressed in tobacco leaves (Frost et al., 2004). In addition, self-association of TIR domain of another flax immune receptor L6 is both necessary and sufficient to trigger the L6-mediated immune response (Bernoux et al., 2011).

Despite harboring a conserved EDVID motif mediating the intramolecular interaction, the CC domain, unlike TIR domain, is not highly conserved (Rairdan et

al., 2008). Nevertheless, like the TIR domain, the CC domain is also thought to function in immune sinaling transduction. Transient expression of the CC domain of MLA10, a barley CC-NB-LRR protein, caused HR-like cell death, indicating the importance of the CC domain in MLA10-mediated immune signaling (Bai et al., 2012). A similar case has been found in the CC domain identified from two other CC-NB-LRR proteins, NRG1 and ADR1 (Collier et al., 2011). Moreover, CC-mediated MLA10 dimerization is required for HR cell death signaling (Maekawa et al., 2011).

Among all domains of the NB-LRR type immune receptors, the central NB domain is the most conserved, suggesting its pivotal role in immune receptor function (Tameling et al., 2002). Typically, the NB domain and LRR domain are separated by an ARC (APAF-1, R proteins, and CED-4) domain, which can be further divided into two subdomains termed ARC1 and ARC2 (Albrecht and Takken, 2006; McHale et al., 2006; Rairdan and Moffett, 2006). It was reported previously that the NB domain of two tomato CC-NB-LRR proteins I-2 and Mi-1 are capable of binding and hydrolyzing ATP *in vitro* when purified from *Escherichia coli* (Tameling et al., 2002), suggesting the ADP-ATP exchange of the NB domain could be important for immune signal transduction. Interestingly, when a green fluorescence protein (GFP)-tagged NB domain of potato CC-NB-LRR protein Rx was transiently over-expressed in tobacco leaves, the NB-GFP alone was stable and able to trigger effector-independent HR-like cell death (Rairdan et al., 2008), suggesting this NB domain might have a signaling function unlike the equivalent domain in previously studied TIR-NB-LRR proteins.

The LRR domain is widely present in receptor proteins in different organisms. Generally, the plant LRR domain contains 2-42 tandem repeats and each repeat consists of 24-28 amino acid residues (Padmanabhan et al., 2009; van Ooijen et al., 2007). Numerous studies have shown the essential roles of LRR domain in pathogen recognition. The first case was demonstrated by direct interaction between the LRR domain of rice NB-LRR protein Pi-ta and its cognate effector protein Avr-Pita (Jia et al., 2000). Although the LRR domain was thought to mediate pathogen recognition, emerging evidence shows it also plays an important role in immune signaling. Expression of several NB-LRR proteins without their LRR domains led to effector-independent HR cell death, suggesting that the LRR domain may play a negative role in defense signaling in the absence of pathogen elicitation (Bendahmane et al., 2002).

In addition to functional roles in pathogen recognition and immune signaling transduction, the LRR domain also mediates intramolecular interactions within NB-LRR proteins. Intramolecular interactions are not only important for correct protein folding but also essential for maintaining the protein in the appropriate state. It is currently believed that the intramolecular interactions between the ARC domain and the LRR domain hold NB-LRR immune receptors in an autoinhibited state (Moffett et al., 2002; Rairdan et al., 2008; Rairdan and Moffett, 2006). Deletion of ARC and/or LRR domains in NB-LRR proteins, or NB-LRR proteins carrying point mutations within these domains have been reported to cause effector-independent HR-like cell death (Bendahmane et al., 2002; Howles et al., 2005; Hwang et al., 2000; Kud et al., 2013; Shirano et al., 2002; Tameling et al., 2006; Zhang et al., 2003). Amino acid

substitutions causing autoactivation of immune receptors are normally located in ARC2 and LRR domains. In the resting state, a conserved triple amino acid motif (MHD) within the ARC2 domain contributes to the auto-inhibited status of immune receptor, since substitutions of the histidine residue in the MHD motif of L6, NRC1, Rx, I-2, Mi-1, and Prf results in effector-independent HR-like cell death (Bendahmane et al., 2002; de la Fuente van Bentem et al., 2005; Gabriels et al., 2007; Howles et al., 2005; van Ooijen et al., 2008). Homology modeling of the NB-ARC domain of I-2 based on the structure of Apaf-1 suggests that MHD motif functions as the sensor II motif in AAA+ (ATPases associated with a variety of cellular activities) family proteins and can coordinate nucleotide binding and intramolecular interactions (van Ooijen et al., 2008). In addition, the ARC2 domain contains another conserved motif termed RNBS-D. Mutations located at this motif can cause either inactivation or autoactivation of immune receptors (Bendahmane et al., 2002), suggesting the ARC2 domain could serve as a molecular switch for NB-LRR immune receptors activation (Rairdan and Moffett, 2006).

The aforementioned mutations within the conserved motifs lock NB-LRR proteins in an active form that initiates immune signaling in the absence of effectors. However, in the natural condition it is the appropriate recognition of the effector proteins by the cognate NB-LRR immune receptors that triggers the activation of these receptors. It was previously thought that NB-LRR immune receptors directly recognize the avirulence elicitors for triggering immunity-related responses (Keen, 1990). However, only a few cases supported the direct recognition model, including the direct interaction between the flax rust AvrL567 protein and flax L protein,

Ralstonia solanacearum PopP2 protein and Arabidopsis RRS1-R protein, rice blast fungus AvrPi-ta protein and rice Pi-ta protein, and the P50 subunit of the tobacco mosaic virus replicase and the tobacco N protein (Deslandes et al., 2003; Dodds et al., 2006; Jia et al., 2000; Ueda et al., 2006). In most cases, the recognition of avirulence elicitors by the cognate NB-LRR R proteins is indirect, indicating the presence of co-factors that complex with NB-LRR proteins to function properly (Dangl and Jones, 2001).

Indirect perception of elicitors by plant immune receptors has been modeled in two ways. One hypothesis has been described as the Guard model (Dangl and Jones, 2001) while the other has been called the Decoy model (van der Hoorn and Kamoun, 2008). Both have been under debate for more than one decade. In the Guard model, NB-LRR proteins monitor the modification of a host protein targeted by the effector resulting in activation of the NB-LRR proteins and disease immunity in host plants (Dangl and Jones, 2001; Van der Biezen and Jones, 1998). Moreover, the guard model suggests that the guarded host protein is essential for the virulence activity of the effector when the cognate NB-LRR protein is absent (Dangl and Jones, 2001). However, considerable experimental data has emerged against the guard model (van der Hoorn and Kamoun, 2008). Now it is believed that virulence effectors may have multiple host targets that have been shown to be dispensable for the virulence activity of the effector proteins when the cognate NB-LRR proteins are absent (van der Hoorn and Kamoun, 2008). Based on extensive studies of effector targets (Zhou and Chai, 2008; Zipfel and Rathjen, 2008), the “decoy model” has been proposed: Guarded effector targets are subjected to two opposite selection

forces, which could be evasion from the manipulations of effectors leading to weaker interaction in the absence of the *R* gene or perception improvement resulting in stronger interaction in the presence of the *R* gene (van der Hoorn and Kamoun, 2008). These two opposing selection forces together promote the formation of the evolutionary constraints that can be released via duplication of the effector target gene or independent evolution of a target mimic, eventually leading to a decoy dedicated to effector recognition (van der Hoorn and Kamoun, 2008).

The activation of immune receptors following effector recognition depends on conformational change, which relies largely on the aid of chaperones and partner proteins (Shirasu, 2009). In the absence of pathogen challenge, the NB-LRR immune remains in the resting state not able to induce a defense response. Upon pathogen invasion, immune receptors are activated promptly to trigger a defense response. Therefore, immune receptors have to undergo a maturation process with the aid of chaperones and, on the other hand, be degraded to quench the signal to protect plants from excessive immunity-related side effects. A series of molecular chaperones and accessory co-chaperones have been identified that play essential roles in immune receptor maturation. Extensive studies have focused on the molecular chaperone Heat shock protein 90 (HSP90), together with the co-chaperone Required for MLA12 Resistance 1 (RAR1), Suppressor of the G2 allele of *Skp1* (SGT1) and Protein Phosphatase 5 (PP5) (Heise et al., 2007; Hubert et al., 2003; Kitagawa et al., 1999; Park et al., 2012). HSP90 consists of three domains: an N-terminal domain containing an ATP-binding pocket, a middle domain involved in client protein binding and a C-terminal domain involved in dimerization (Pearl and

Prodromou, 2000). In plants, HSP90 has been shown to regulate the functions of many NB-LRR proteins, supported by the impairment of disease resistance conferred by NB-LRR proteins (Kadota and Shirasu, 2012; Kadota et al., 2010) and reduced accumulation of NB-LRR proteins in the absence of HSP90 (H.R. and A.C., 1998; Holt et al., 2005; Hubert et al., 2003; Lu et al., 2003; Shirasu, 2009). Co-chaperones such as RAR1, PP5 and SGT1 function in concert with HSP90. The steady state level of many NB-LRR proteins dramatically decreases in the absence of RAR1 (Bieri et al., 2004; Holt et al., 2005; Muskett et al., 2002). PP5 brings NB-LRR proteins and HSP90 into close proximity by interacting with the C-terminal LRR domain of NB-LRR proteins and the C-terminal dimerization domain of HSP90 (de la Fuente van Bentem et al., 2005; Golden et al., 2008). Silencing of *PP5* in *Nicotiana benthamiana* compromised the I-2-mediated disease resistance and caused decreased accumulation of I-2 protein (Van Ooijen et al., 2010). Collectively, the experimental data support the positive regulatory roles of HSP90, RAR1, and PP5 in stabilizing the steady state levels of NB-LRR proteins.

More complicated than other co-chaperones, SGT1 has a dual function in regulating NB-LRR protein accumulation. On one hand, SGT1 can stabilize immune receptors as a co-chaperone therefore playing a positive role in immune signaling, supported by the indispensability of SGT1 in the accumulation of several R proteins such as Rx, I2, Mi, and N (Azevedo et al., 2006; Mestre and Baulcombe, 2006; Van Ooijen et al., 2010). On the other hand, SGT1 is a component of the Skp1-Cullin-F-box (SCF) complex, a multi-protein E3 ubiquitin ligase complex that targets the substrate proteins for proteasome-dependent degradation (Petroski and Deshaies,

2005). In this way, SGT1 functions in concert with other components of the SCF complex (Cullins and F-box proteins) to downregulate immune receptor accumulation thereby negatively regulating defense signaling. For example, the Arabidopsis F-box protein Constitutive expresser of *PR*1 (CPR1) was demonstrated to regulate the protein accumulation of two TIR-NB-LRR immune receptors negatively, Suppressor of npr1-1, Constitutive 1 (SNC1) and Resistance to *P. syringae* (RPS2) (Cheng et al., 2011; Gou et al., 2012).

The hypersensitive response, a type of programmed cell death (PCD) in plants, is a cellular hallmark for immune signaling

As described above, plants can undergo a localized cell death termed HR to arrest pathogen growth. HR cell death is PCD that is featured with a cellular self-destroying process triggered by extracellular and/or intracellular stimuli. This process is well organized and tightly controlled to determine development and survival in many organisms (Leopold, 1961). PCD was implicated in both compatible (disease susceptibility) and incompatible (disease resistance) plant-pathogen interactions, resulting in macroscopic necrosis in plants (Devarenne and Martin, 2007; Greenberg, 1997; Greenberg and Yao, 2004; Mittler and Lam, 1996). HR is typically an indication of disease resistance (Heath, 2000; Pontier et al., 1998). One early event of the HR is cytoskeleton rearrangement and sequential destruction (Skalamera and Heath, 1998). Other early events of HR include the production of reactive oxygen species (ROS), nitric oxide (NO), the defense hormone salicylic acid (SA), and calcium fluxes, as well as activation of a mitogen-activated protein kinase (Group) cascade (Meng and Zhang, 2013; Mur et al., 2008).

Remarkably, the MAPK cascade plays a pivotal role in HR signaling by regulating biosynthesis of SA and generation of reactive oxygen and nitrogen species (Meng and Zhang, 2013; Zhang and Klessig, 2001). Three interconnected protein kinases, a MAPK, a MAPK kinase (MEK), and a MAPKK kinase (MAPKKK) constitute the chain of the cascade (Seger and Krebs, 1995). MAPKKK, the first kinase in the cascade, phosphorylates a MEK, followed by another round of phosphorylation upon a MAPK (Seger and Krebs, 1995). Multiple members exist in each class of kinases, altogether shaping a complex phosphorylation web (Group, 2002). For example, *Arabidopsis* has roughly 60 genes encoding MAPKKKs, 10 genes encoding MAPKKs, and 20 genes encoding MAPKs (Group, 2002; Hamel et al., 2006).

Tomato-*Pst* interaction as a model system for studying bacterial pathogenesis and disease immunity

Two avirulence effectors AvrPto and AvrPtoB

In total, *Pst* secretes into plant cells 30 effector proteins, which collectively cause disease. Among them, functional studies have been performed in-depth on two structurally distinct effectors, the membrane-anchored AvrPto and a bacterially-derived E3 ubiquitin ligase AvrPtoB, which trigger disease resistance in tomato plants expressing resistance to *Pseudomonas syringae* pv. *tomato* (*Pto*) and *Pseudomonas* resistance and fenthion sensitivity (*Prf*) genes. These bacterial genes are termed *Avr* genes that encode the avirulence effectors.

AvrPto was first cloned from *Pst* race 0 and later showed to confer an avirulence phenotype after introduction into the normally virulent strain

Pseudomonas syringae pv. *maculicola* ES4326. The ES4326 strain expressing AvrPto triggers resistance on a tomato cultivar expressing *Pto* and *Prf* (Pitblado, 1980; Ronald et al., 1992). Later, the same group found that AvrPto is a small hydrophilic protein sharing no sequence homology with other known proteins in the databanks (Salmeron and Staskawicz, 1993), and they also demonstrated that the tomato *Prf* resistance gene is required for AvrPto-triggered disease immunity. Following the logic raised by the "gene-for-gene" hypothesis, Pto, a serine/threonine kinase, was proven to interact with AvrPto in a yeast-two hybrid assay (Martin et al., 1993; Scofield et al., 1996; Tang et al., 1996). The interaction between AvrPto and Pto is detected by Prf, which results in activation of Prf protein to trigger immune signaling (Mucyn et al., 2006).

Before describing AvrPto and AvrPto-interaction partners, it is necessary to mention briefly the biological background related to Pto and Prf. The *Pto* gene and its orthologues encode a serine/threonine kinase (Martin et al., 1993), which has been found in wild and cultivated varieties of potato and tomato, as well as rice, *Arabidopsis* and *Nicotiana tabacum* (Martin et al., 1993; Rose et al., 2005; Vleeshouwers et al., 2001). The *Prf* gene encodes a CC-NB-LRR immune receptor with homologs in some other plant species (Salmeron et al., 1996). Both *Pto* and *Prf* belong to a tightly linked gene cluster obtained by introgression from the wild tomato *Solanum pimpinellifolium* into a tomato cultivar *Solanum lycopersicum* cv. Rio Grande, by means of which a tomato line expressing both Pto and Prf was created and termed as RG-*PtoR* (Pedley and Martin, 2003). Based on the same genetic

background, two Pto mutant lines (RG-*pto11* or RG-*ptoS*) and one Prf mutant line (RG-*prf3L*) have also been generated (Salmeron et al., 1994).

In addition to the genetic and biochemical studies, structural biology has been recently used to characterize how AvrPto interacts with Pto (Xing et al., 2007). The AvrPto-Pto complex has been crystalized and two key molecular interfaces were identified to mediate the interaction (Xing et al., 2007). The first one is between the Pto loop and one end of an AvrPto helical bundle and the second one is between the Pto P+1 loop and the AvrPto GINP (Gly-Ile-Asn-Pro) motif (Xing et al., 2007). In the absence of AvrPto, these two AvrPto interaction loops inhibit the activation of Prf. In the presence of AvrPto, binding AvrPto to Pto releases the inhibition of Prf conferred by Pto, thereby turning on Prf-mediated defense responses (Rathjen et al., 1999; Wu et al., 2004; Xing et al., 2007). It has been shown that T204 of the Pto P+1 loop mediates the Pto-AvrPto interaction and amino acid substitution of this threonine residue for an asparagine residue causes AvrPto-independent HR-like cell death (Frederick et al., 1998; Rathjen et al., 1999). The phosphorylation status of Pto T199 determines both AvrPto-Pto interaction and AvrPto-trigger HR cell death, presumably by stabilizing the P+1 loop of Pto (Xing et al., 2007). The Pto T199A amino acid substitution mutant interferes with the Pto-AvrPto interaction and AvrPto-triggered HR cell death (Sessa and Martin, 2000; Xing et al., 2007). A group of Pto mutants were generated that can trigger AvrPto-independent and Prf-dependent HR cell death (Rathjen et al., 1999; Sessa and Martin, 2000; Wu et al., 2004). Based on the location of these phenotype-altering sites, destabilization of the P+1 loop appears to interefere with kinase activity and AvrPto interaction (Rathjen et al., 1999;

Wu et al., 2004). In contrast, a group of putatively stable Pto P+1 mutants still trigger HR cell death despite of deficiency in the Pto-AvrPto interaction and kinase activity (Rathjen et al., 1999; Xing et al., 2007), which indicates Pto phosphorylation, instead of its kinase activity, is indispensible for the AvrPto interaction.

Beyond the avirulent activities of AvrPto, the virulent activity of AvrPto has also been well studied. In tomato lines lacking Pto or Prf, AvrPto promotes necrotic symptoms and slightly enhances the bacterial growth of *Pst* T1 (Chang et al., 2000). Several host targets of AvrPto have been identified, including Arabidopsis FLS2 and EFR, as well as tomato LeFLS2 (Xiang et al., 2008). An autophosphorylation event activates both FLS2 and EFR receptor kinases, and this event can be blocked by AvrPto which eventually results in the inhibition of downstream signaling cascades such as callose deposition (Hauck et al., 2003; Xiang et al., 2008), oxidative burst (Xiang et al., 2008) and MAPK activation (He et al., 2006). It has been shown that Pto and FLS2 bind to similar sequence regions of AvrPto (Xiang et al., 2008). The same group showed that Pto competes with FLS2 for AvrPto binding *in vitro* and that Pto interfers with FLS2-AvrPto binding *in vivo* (Xiang et al., 2008). Altogether, it is reasonable to propose that Pto is a newly evolved host protein protecting the membrane-anchored FLS2 receptor kinase from AvrPto (Zipfel and Rathjen, 2008).

Phosphorylation of AvrPto is important for its virulence activity (Anderson et al., 2006). But neither Pto nor Prf is necessary for the phosphorylation of AvrPto *in vivo* (Anderson et al., 2006), suggesting that neither Pto nor Prf is related to the virulence target of AvrPto. Two AvrPto mutants, with amino acid substitutions at the

phosphorylation sites S147 and S149, caused less disease symptoms compared to controls in the susceptible tomato line RG-*prf3* (Anderson et al., 2006).

AvrPtoB is the second effector protein triggering disease immunity to *Pst* in tomato expressing *Pto* and *Prf* genes (Kim et al., 2002). Structurally distinct from AvrPto, AvrPtoB is a functionally modular protein in which the N-terminal region ($AvrPtoB_{1-307}$) is responsible for host recognition and triggering of the HR (Abramovitch et al., 2003), while the C-terminal region $AvrPtoB_{308-553}$ encodes a ubiquitin E3 ligase that mediates immunity-related cell death suppression (Janjusevic et al., 2006).

Disease immunity against *Pst* is induced by bacteria secreting full-length AvrPtoB, but only the first 307 amino acids are needed (Abramovitch et al., 2003). Consistent with this, the avirulence activity of $AvrPtoB_{1-307}$ was confirmed in RG-*Pto*R tomato plants (Xiao et al., 2007). To test whether $AvrPtoB_{1-307}$ carries virulence activity, RG-*prf3* tomato plants were infected with *Pst* secreting $AvrPtoB_{1-307}$ (Xiao et al., 2007). Bacterial scoring results showed enhanced disease symptoms compared to the empty vector control strain, proving the virulence activity of $AvrPtoB_{1-307}$ in the absence of tomato Prf (Xiao et al., 2007). A series of $AvrPtoB_{1-307}$ truncation mutants was generated to test for interactions with Pto and only a small portion of AvrPtoB spanning amino acids 121-200 was found to sufficient to interact with Pto (Kim et al., 2002; Xiao et al., 2007). In particular, F173 was found to be indispensable for $AvrPtoB_{1-307}$/Pto interaction and the avirulence activity of AvrPtoB (Xiao et al., 2007). Moreover, a slightly longer N-terminal region of AvrPtoB, $AvrPtoB_{1-387}$, can trigger Pto-independent disease resistance termed as resistance suppressed by AvrPtoB

C-terminus (Rsb). Notably, the Rsb phenotype is still *Prf*-dependent (Abramovitch et al., 2003). In fact, AvrPtoB$_{1-387}$ interacts with tomato Fen, a Pto homolog in the yeast-two hybrid assay (Rosebrock et al., 2007), to trigger this *Prf*-dependent Rsb. Together, these data indicate Prf governs defense signaling activated by two host proteins Pto and Fen (sensitivity to fenthion). Structural studies of the C-terminus of AvrPtoB showed surprising homology to the eukaryote U-box and RING-finger type E3 ubiquitin ligases (Janjusevic et al., 2006). An *in vitro* ubiquitination assay demonstrated the E3 ubiquitin ligase activity of AvrPtoB C-terminus (Janjusevic et al., 2006). AvrPtoB mutants deficient in E2-binding ability lost ubiquitination activity and the ability to suppress HR-related cell death (Janjusevic et al., 2006). Two key lysine residues (K512, K529) of AvrPtoB are responsible for the AvrPtoB-ubiquitin association in the yeast-two hybrid assay and are required for AvrPtoB virulence activity in tomato (Abramovitch et al., 2006). Therefore, it is reasonable to postulate that AvrPtoB has acquired the C-terminal E3 ligase domain to suppress plant immunity, presumably by targeting host proteins for degradation during co-evolution of *Pst* with tomato.

Rsb triggered by AvrPtoB$_{1-387}$ is mediated through Fen, a serine/threonine kinase like Pto (Abramovitch et al., 2003; Loh and Martin, 1995; Martin et al., 1994). Fen interacts with AvrPtoB$_{1-387}$ in yeast and is ubiquitinated and degraded by full-length AvrPtoB with functional E3 ubiquitin ligase activity (Rosebrock et al., 2007). AvrPtoB mutants (Quad and E2BS) deficient in E3 ubiquitin ligase activity are unable to bind and degrade Fen (Rosebrock et al., 2007). Degradation of tomato Fen results in the suppression of Fen-mediated AvrPtoB recognition. It is tempting to

speculate that Fen arose first and that after AvrPtoB acquires the C-terminal E3 ligase domain, Pto evolved. This theory is supported by the fact that AvrPtoB truncations lacking the C-terminal E3 ligase domain exist in some *P. syringae* strains (Lin et al., 2006).

Unlike Fen, Pto can recognize wild-type AvrPtoB with a functional E3 ligase domain and evade AvrPtoB-mediated degradation, which suppresses a Fen-triggered defense response (Rosebrock et al., 2007). Even though both Fen and Pto are kinases, it was recently reported that Pto possesses higher kinase activity than Fen, which results in successful phosphorylation of AvrPtoB at threonine-450 to abolish its E3 ubiquitin ligase activity (Ntoukakis et al., 2009). Therefore, Pto, rather than Fen, evades the AvrPtoB-mediated degradation (Ntoukakis et al., 2009). $AvrPtoB_{1-307}$ was reported to be sufficient for Pto interaction, but not sufficient for interacting with Fen (Rosebrock et al., 2007). To ensure the AvrPtoB-Fen interaction, the presence of amino acids spanning 307-387 is required, indicating Pto and Fen bind to different AvrPtoB domains even though they are highly homologous (Rosebrock et al., 2007). Interestingly, it was recently reported that Pto can bind two distinct AvrPtoB subdomains ($AvrPtoB_{1-307}$ and $AvrPtoB_{307-387}$) while Fen can only bind the closer subdomain neighboring the C-terminal E3 ligase domain ($AvrPtoB_{307-387}$) (Mathieu et al., 2014). If Pto is obliged to bind to the Fen-interacting domain of AvrPtoB, Pto degradation promoted by AvrPtoB occurs via the same mechanism as Fen degradation (Mathieu et al., 2014). Moreover, Pto binding to an artificial AvrPtoB mutant generated by fusing $AvrPtoB_{1-307}$ with C-terminal E3 ligase domain $AvrPtoB_{388-533}$ is also susceptible to proteasome-mediated degradation (Mathieu et

al., 2014). Pto binding to the Fen-interacting domain of AvrPtoB results in HR-like cell death in both tomato and *N. benthamiana* when the C-terminal E3 ligase activity of AvrPtoB is inactivated (Mathieu et al., 2014).

Beyond avirulence activity, AvrPtoB also has significant virulence function in *Arabidopsis* and tomato lacking Pto or Prf (Gimenez-Ibanez et al., 2009; Gohre et al., 2008; Xiao et al., 2007). *Pst* DC3000Δ*avrPto*Δ*avrPtoB* expressing AvrPtoB or $AvrPtoB_{1-307}$ causes more severe disease symptoms in susceptible tomato lines RG-*pto11* or RG-*prf3* compared to the empty vector control (Lin and Martin, 2005; Xiao et al., 2007). Full-length AvrPtoB was previously reported to induce ethylene biosynthesis and the expression of ethylene biosynthetic genes (Cohn and Martin, 2005). Later, the truncation mutant $AvrPtoB_{1-307}$ was demonstrated to be sufficient to induce this pathway (Xiao et al., 2007). Ethylene sensitivity is essential for the successful infection of *Pst* in susceptible tomato (Lund et al., 1998) and responsive to both AvrPto and AvrPtoB virulence activity (Cohn and Martin, 2005). After necrosis begins, there is an ethylene-dependent phase of the disease. *Pst* also manipulates host abscisic acid (ABA) signaling to trigger pathogenesis (de Torres-Zabala et al., 2007). ABA levels increased when AvrPtoB was conditionally expressed (de Torres-Zabala et al., 2007). However, the mechanism for regulating the ABA level by AvrPtoB remains obscure.

In addition to the manipulation of phytohormones, AvrPtoB efficiently suppresses pathogen-associated molecular patterns triggered immunity (PTI) by targeting pattern recognition receptors (PRRs) that are typically anchored to the plasma membrane (Gimenez-Ibanez et al., 2009; Gohre et al., 2008; He et al., 2006;

Zeng et al., 2012). AvrPtoB expressed from *Pst*DC3000 caused significant loss of FLAGELLIN SENSITIVE 2 (FLS2) accumulation on the plasma membrane (Gohre et al., 2008). Additionally, an *AvrPtoB* transgene promoted FLS2 degradation and AvrPtoB directly bound to and ubiquitinated FLS2 *in vitro* (Gohre et al., 2008).

In addition to the suppression roles in FLS2-mediated defense signaling, AvrPtoB targets the *Arabidopsis* lysine motif (LysM) receptor-like kinase CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) and the tomato CERK1 ortholog Bti9 to suppress chitin-triggered host immunity (Gimenez-Ibanez et al., 2009; Zeng et al., 2012). The N-terminal truncation mutant $AvrPtoB_{1-307}$ is sufficient to bind to the kinase domain of CERK1 and Bti9 (Gimenez-Ibanez et al., 2009; Zeng et al., 2012), indicating it might be involved in chitin-elicited immune signaling. Phenotypically, AvrPtoB suppresses the chitin-induced PTI response in *Arabidopsis* and the PTI response mediated by *Pst*-derived pathogen-associated molecular patterns (PAMPs) other than flagellin in tomato (Gimenez-Ibanez et al., 2009; Zeng et al., 2012). The C-terminal E3 ubiquitin ligase domain of AvrPtoB is dispensable for the interaction with CERK1 or Bti9 (Gimenez-Ibanez et al., 2009; Zeng et al., 2012). Surprisingly, AvrPtoB promotes CERK1 degradation *in planta* in a vacuole-dependent manner other than a proteasome-dependent manner (Gimenez-Ibanez et al., 2009; Zeng et al., 2012). Contrary to the interaction dispensability of the AvrPtoB C-terminal E3 ligase domain, the E3 ligase activity is required to promote CERK1 degradation when transiently expressed in *N. benthamiana* (Gimenez-Ibanez et al., 2009). However, the E3 ligase activity of AvrPtoB is dispensable for its virulence function against Bti9-mediated immunity (Zeng et al., 2012).

In summary, *Pst* evolved effectors AvrPto and AvrPtoB to suppress key plant receptor-like kinases such as FLS2 and CERK1 to block the PTI response (Gimenez-Ibanez et al., 2009; Gohre et al., 2008; Xiang et al., 2008; Zeng et al., 2012). To counter this, the plant host employs Fen and Pto kinases in concert with a NB-LRR protein Prf to trigger an advanced layer of immunity termed as ETI (Effector-triggered immunity), which is typically associated with localized HR cell death (Ntoukakis et al., 2014).

The tomato CC-NB-LRR protein Prf

Pto was first identified in 1993 as a gene in tomato conferring resistance to *Pst*. Three years later, the *Prf* gene was cloned and determined to be required for Pto-mediated resistance to *Pst* and sensitivity to the organothiophosphate insecticide fenthion (Salmeron et al., 1994; Salmeron et al., 1996). *Prf* was originally cloned from a resistant tomato line (*L. pimpinellifolium*), but Pto-mediate disease resistance can be introduced by transformation of *Prf* into a susceptible tomato line (*L. esculentum*) (Martin et al., 1993) or into tomato *prf* mutants (Salmeron et al., 1994). Additionally, virus-induced gene silencing of the *Prf* homolog in *N. benthamiana* compromised the AvrPto/Pto mediated gene-for-gene resistance, indicating an analogous *Prf* existed in *N. benthamiana* (Peart et al., 2002). *Prf*-like sequences have also been found in a variety of plant species including *Arabidopsis*, tobacco, pepper, bean, oat, and maize (Pedley and Martin, 2003). *Prf* was reported to be indispensable for both Pto-mediated and Fen-mediated immunity signaling (Salmeron et al., 1994). *Prf* lies in the middle of the *Pto* gene cluster and is 24 kilo base pairs (kbs) from *Pto* and 500 base pairs (bps) from *Fen* (Salmeron et al., 1996).

In addition, other *Pto* homologs (*Pth*) *Pth2*, *Pth3*, *Pth4*, and *Pth5* are also located together with *Prf* on chromosome 5 (Abramovitch et al., 2006; Chang et al., 2002; Riely and Martin, 2001; Vleeshouwers et al., 2001). Significantly, overexpression of *Prf* mRNA in transgenic tomato plants activated defense signaling transduction pathways in the absence of *Pst*, ultimately leading to the activation of systemic acquired resistance (SAR) (Oldroyd and Staskawicz, 1998; Ryals et al., 1996), which conferred resistance against a variety of pathogens (Oldroyd and Staskawicz, 1998). Transient expression of a Pto autoactive mutant (Pto^{Y207D}) in the RG-*prf3* tomato line, in which *Prf* is lacking, failed to cause HR cell death, suggesting Prf functions downstream of Pto (Rathjen et al., 1999). Nevertheless, overexpression of Prf together with Pto in *N. benthamiana* caused effector-independent HR-like cell death, whereas replacement of Pto by a Pto kinase-deficient mutant (Pto^{D164N}) or a Pto *N*-myristoylation-deficient mutant (Pto^{G2A}) failed to trigger cell death, suggesting Pto can also regulate Prf during HR-like cell death signaling (Mucyn et al., 2006). Collectively, the experimental data indicate that Pto and Prf act coordinately in the immune signaling pathway but Prf acts as the actual immune signal transducer. Furthermore, extreme overexpression of Prf alone by the estradiol-inducible promoter elicited HR-like cell death independent of *Pto* or *Pto* homologs. This again suggests the role of Prf as the key signaling determinant, even though Pto and Prf function together (Mucyn et al., 2006). In addition, Pto and Prf interact and mutually contribute to protein accumulation *in vivo* (Mucyn et al., 2006). Notably, the interaction between Pto and Prf were not disrupted in the presence of the cognate effector AvrPto, suggesting that the AvrPto-activated Pto still associates with Prf

(Mucyn et al., 2006). To determine if the the Pto-Prf interaction recruits more than just these polypeptide chains, size-exclusion chromatography (SEC) was performed on protein extracts from RG-*Pto*R and the results indicated that Pto and Prf coeluted in a high molecular weight fraction, suggesting other signaling component(s) also associate with Prf *in planta* (Mucyn et al., 2006).

Prf is a multi-domain protein, with a canonical CC-NB-LRR domain structure spanning the C-terminus of Prf (Mucyn et al., 2006). The N-terminus of Prf is divided into the N-terminal domain and the Solanaceae domain (SD) (Mucyn et al., 2006). The N-terminal domain (NPrf) spans amino acids 1 to 546, without any identified similarities to known proteins (Mucyn et al., 2006). The SD domain spans amino acids 546 to 900, and shows weak homology with some other Solanaceous NB-LRR proteins such as tomato Mi and Sw-5, as well as potato Hero and R1 (Ballvora et al., 2002; Brommonschenkel et al., 2000; Ernst et al., 2002; Milligan et al., 1998). To narrow down the region in Prf responsible for Pto interaction, a co-immunoprecipitation experiment was carried out between Pto and Prf fragments containing either NPrf, SD-CC-NB-LRR or CC-NB-LRR (Mucyn et al., 2006). Pto was pulled down only by NPrf, indicating that the N-terminal region of Prf is responsible for Pto interaction (Mucyn et al., 2006).

Interestingly, NPrf self-associates as a dimer whether or not Pto is present (Gutierrez et al., 2010). Prf needs both NPrf and SD-CC-NB-LRR domains for its full function, as demonstrated by the failure of both single fragments triggering the effector-independent HR-like cell death (Mucyn et al., 2006). Furthermore, transient overexpression of NPrf and SD-CC-NB-LRR reconstituted both effector-dependent

and effector-independent HR-like cell death (Gutierrez et al., 2010). The functional reconstitution of Prf relies on the presence of Pto (Gutierrez et al., 2010). Pto, like Prf, was found to self-oligomerize when transiently expressed in *N. benthamiana* (Gutierrez et al., 2010). Interestingly, Prf mediates the interaction of Pto and other Pto homologues such as Fen, Pth2 and Pth3 *in vivo* (Gutierrez et al., 2010).

Overall hypothesis

Previous findings that were centered on AvrPto(B)/Pto/Prf signaling collectively indicated the pivotal role of NB-LRR protein Prf in immunity to against *Pst*. Therefore, both the protein accumulation and activation status of Prf have to be delicately regulated. Here we hypothesize that the C-terminal LRR domain of Prf plays a negative role in regulating the HR-like cell death involved in the Pto/Prf signaling pathway, and the Prf protein accumulation is negatively regulated by a group of SEVEN IN ABSENTIA (SINA) E3 ubiquitin ligases *in planta*, which can be usurped by the *Pst*-secreted effector protein AvrPtoB to suppress host immunity in plants lacking Pto.

Common experimental procedures for studying plant-*Pseudomonas syringae* interactions

Several plant-based experimental procedures are frequently used in studying plant disease immunity. Two of them will be extensively used in our tomato-*Pst* interaction system to test the model above.

Agrobacterium-mediated transient expression

Agrobacterium tumefaciens is a soil-born Gram-negative bacterium. It can infect many dicots and induce the formation of tumors or crown galls at the invasion

site. These tumors produce opines that are used nitrogen and carbon sources by bacteria (Zupan et al., 2000). Genes encoding for oncogenesis and opine production are transferred from *Agrobacterium* into plant cells. Formation of tumors caused by *Agrobacterium* relies on the tumor-inducing (Ti) plasmid that contains the T-DNA region transferable into plant cells, the virulence region mediating the conjugative transfer of T-DNA, and the regions responsible for opine biosynthesis and catabolism (Gelvin, 2000). The T-DNA region is flanked by two 25 bp boarder sequences termed the left border and the right border. Genetic removal of the genes within the T-DNA, while keeping the border sequences intact, simply prevents the formation of tumors rather than impeding the transfer of T-DNA of *Agrobacterium*, resulting in so-called “disarmed” Ti plasmids. A series of binary Ti plasmid vectors have been generated that work in conjunction with these disarmed plasmids (Gelvin, 2000). *Agrobacterium* harboring a gene-containing vector and the helper cultured and prepared in an appropriate buffer for infiltration into plant tissues where the encoded proteins are expressed. During the *Agrobacterium*-mediated transient assay, unlike when stable transgenic lines are selected, the T-DNA region is not integrated into plant chromosomes. Instead, gene transcription and translation is transient, not inherited from generation to generation. Transient expression is rapid and less costly, making it an ideal approach for transgenic complementation (Bendahmane et al., 2000), promoter analysis (Yang et al., 2000), protein production (Vaquero et al., 1999), and protein localization studies (Heidrich et al., 2011).

Virus-induced gene silencing (VIGS)

RNA interference (RNAi) is a common molecular phenomenon involved in plant development (Carrington and Ambros, 2003) and in the defense response against viruses (Brigneti et al., 1998). RNAi is triggered by dsRNAs that can be further categorized into microRNAs (miRNAs) and small interfering RNAs (siRNAs).

VIGS is a method derived from the plant RNAi response against viral infections. In nature, RNAi targets the viral genome and induces the degradation of viral transcripts depending on a multiprotein complex termed the RNA-induced silencing complex (RISC). However, viral vectors have been built that allow us to exploit this system to direct RNAi against any transcript in the host itself. VIGS has been widely used for functional genomics in different plant species by taking advantage of *Agrobacterium* to deliver a genetically engineered virus carrying part of the targeted host DNA sequence and thereby triggering RNAi to silence the specific host gene (Burch-Smith et al., 2004). After infiltration of *Agrobacterium*, plants start to replicate the virus genome leading to systemic spread of recombinant viruses that are sensed by the RNAi machinery inside the plants. The dsRNAs transcribed from the inserted gene fragments are cleaved by the Dicer proteins into siRNAs with 21 to 24 nucleotides in length. The siRNAs then direct the RISC to induce the degradation of the targeted mRNA transcripts (Baulcombe, 1999).

A variety of viral vectors have been used for VIGS. Two of the most commonly used vectors are pTRV1 and pTRV2, representing the two essential modules of tobacco rattle virus (TRV) (Liu et al.). pTRV1 harbors genes encoding viral replication and mobile ability, whereas pTRV2 contains the coat protein-

encoding genes and the DNA fragment for VIGS. pTRV1 and pTRV2 have to be transformed into two *Agrobacterium* strains, which are co-infiltrated into plant seedlings to trigger gene silencing. The outcome of silencing needs to be strictly monitored to ensure silencing efficiency (Liu et al., 2002).

Although RNA interference was initially exploited by generating stably transformed plants, VIGS outperforms RNA interference in several aspects. VIGS takes only three to four weeks whereas generation of stable RNAi lines takes several months or longer. In addition, though not always, VIGS could avoid lethality in the early developmental stages of plants. Moreover, VIGS can be used to silence either an individual or a family of genes that could be functionally redundant.

Chapter 2

Materials and Methods

Prf cloning and mutagenesis

The *Prf* gene was PCR amplified from the cosmid pSOR2-7 (Salmeron et al., 1996) and cloned into the pCR2.1 vector using the TOPO TA Cloning Method (Invitrogen, Carlsbad, CA) to generate pTOPO:*Prf* construct with the primer pair: 5'-ATGGCTAAAGAATGCAGAGACGCGATAGGTACTATAAACCT-3' and 5'-CTCAGCTGAGAGTCAAGGGGCTGTTCTTTAGA-3'. The Prf mutants were generated with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) on the pTOPO:Prf template using the following primers: I1414A mutation: forward 5'-GGTGAAAACGTGCCGCGCTCATGATTTGTTGCAT-3' and 5'-ATGCAACAAATCATGAGCGCGGCACGTTTTCACC-3'; H1415D mutation: 5'-GAAAACGTGCCGCATTGATGATTTGTTGCATAAA-3' and 5'-TTTATGCAACAAATCATCAATGCGGCACGTTTTC-3'; H1415V mutation: 5'-GAAAACGTGCCGCATTGTTGATTTGTTGCATAAA-3' and 5'-TTTATGCAACAAATCAACAATGCGGCACGTTTTC-3'; D1416A mutation: 5'-CGTGCCGCATTCATGCTTTGTTGCATAAATTCT-3' and 5'-AGAATTTATGCAACAAAGCATGAATGCGGCACG-3'; D1416V mutation: 5'-CGTGCCGCATTCATGTTTTGTTGCATAAATTCT-3' and 5'-AGAATTTATGCAACAAAACATGAATGCGGCACG-3'; L1417A mutation: 5'-GCCGCATTCATGATGCGTTGCATAAATTCTGCA-3' and 5'-TGCAGAATTTATGCAACGCATCATGAATGCGGC-3'. For making the HA-tagged

Prf and derived mutant constructs, WT and mutant Prf fragments were PCR amplified from the appropriate pTOPO::Prf constructs. Forward primers used for PCR amplifying full-length Prf, ΔNPrf, or LRR were: 5'-GTGGTACCATGGCTAAAGAATGCAGAGACGCGATAGGTACTATAAACCT-3', 5'-AAGGTACCATGAAGAGGTTTCATGAATATATTCTT-3', 5'-AAGGTACCATGCTTCTCCAGATCAATAGTGGAGAAGGT-3', respectively. Reverse primers used for PCR amplifying full-length Prf, ΔLRR, or NPrf were: 5'-CTCAGCTGAGAGTCAAGGGGCTGTTCTTTAGA-3', 5'-CTCAGCTGGATTTGGAGAAGAAAATCCTCTTGTTTGGCCTTTTC-3', 5'-AAGCAGCTGGACAAGAATATATTCATGAAACCT-3', respectively. All PCR products were digested with *Kpn*I and *Pvu*II and inserted into the *Kpn*I and *Stu*I sites of pBTEX:Pto–HA (Xiao et al., 2007) to replace the Pto fragment. The resulting constructs were under the control of CaMV 35S promoter. All constructs were verified by DNA sequencing.

Yeast two-hybrid assay

A LexA yeast two-hybrid (Y2H) system was used to test protein-protein interactions. The C-terminal LRR domain of Prf (PrfLRR) or AvrPtoB$_{1-307}$ (Abramovitch et al., 2003) were cloned into a bait vector pEG202 at the *EcoR*I and *Sal*I sites respectively, whereas the SINA1-6 (The NCBI accession numbers for tomato SINA1-6 genes are AK324518, BT013026, AK322153, AK320390, AK321160 and XM_004248034 respectively) were cloned into a prey vector pJG4-5 at *EcoR*I and *Xho*I sites, respectively. The resulting bait and prey constructs were introduced into yeast (*Saccharomyces cerevisiae*) strain EGY48 and transformed

yeast cells were streaked onto X-Gal plates to assess the interactions between SINA1-6 and PrfLRR or AvrPtoB. Photographs were taken at two days after incubation at 30^{o}C.

Agrobacterium-mediated transient expression

The *Agrobacterium*-mediated transient expression assay was performed as described previously (Xiao et al., 2007). *Agrobacterium* strain GV2260 carrying appropriate constructs was syringe-infiltrated into *Nicotiana benthamiana* leaves. The boundary of the infiltrated area is marked with a pen. The inoculum concentration of the *Agrobacterium* varied depending on the genes to be expressed as indicated in the figure legends. After agroinfiltration, the plants were kept on a lab bench at room temperature. For the expression of genes controlled by the estradiol-inducible promoter, 5 μM estradiol (Sigma-Aldrich, St Louis, Missouri) was sprayed onto leaves 1 d after agroinfiltration. For consistent cell death development, plants were illuminated by fluorescent light continuously. Photographs were taken 7 d after infiltration.

Protein extraction from *Nicotiana benthamiana* leaf tissue and Western blotting

A 2 cm^2 leaf disc of *N. benthamiana* was collected 40 hours after agroinfiltration or 20 hours after estradiol spray and ground in liquid nitrogen. The protein was extracted with 300-μl extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM DTT, 1.5% polyvinylpolypyrolidone, plant protease inhibitor cocktail (Sigma-Aldrich, St Louis, Missouri) and centrifuged at 15,000 g for 10 min at 4°C. 40 μl supernatant was subjected to standard Western blotting using

an α-HA antibody (Roche Applied Science, www.roche.com). Detection of proteins was carried out using HRP-conjugated secondary antibody and the ECL Plus detection system (Amersham-Phamacia, www. amershambiosciences.com). After exposure, the membrane was stained with Coomassie blue to visualize the Rubisco subunits to confirm equal loading of the protein extracts.

In vitro ubiquitination assay

SINA1-6 were PCR amplified from tomato leaf cDNA and cloned into the pMAL-C2 vector (NEW ENGLAND Biolabs, USA) at *Eco*RI and *Sal*I to generate the MBP-SINAs fusion proteins. The resulting constructs were introduced into *Escherichia coli* strain BL21 where the recombinant proteins were expressed in the presence of 0.5μM IPTG. The *in vitro* self-ubiquitination assay was performed as described previously (Abramovitch et al., 2006) with minor adjustments. The ubiquitination reaction mixture (30μL) contained 40ng GST-E1 (AtUBA1), 100ng GST-E2 (AtUBC8), 1μg MBP-SINAs, 2μg FLAG-Ub (Boston Biochem, USA) in the ubiquitination buffer (50mM Tris HCl, pH7.5, 2mM ATP, 5mM MgCl2, 30mM creatine phosphate (Sigma-Aldrich) and 50ng/μL cretine phosphokinase (Sigma-Aldrich, USA)). Lack of GST-E1, GST-E2, FLAG-Ub or MBP-fusion protein served as negative controls. The reaction mixture was incubated at 30°C for 2 hours and stopped by adding 20μl SDS sample buffer. Proteins were separated with 7.5% SDS-PAGE and identified by western blotting using the α-FLAG (Sigma-Aldrich, USA) antibody.

Prf-FLAG transiently expressed from 4-week old *N. benthamiana* was extracted and immunoprecipitated with 15μl anti-FLAG beads (Sigma-Aldrich, USA).

Then the Prf-FLAG-conjugated beads were divided into three aliquots and added to the incubation mixture containing GST-E1, GST-E2, Ub-HA and MBP, MBP-SINA1 or GST-AvrPtoB. After washing three times with the washing buffer (20mM Tris HCl, pH7.5, 0.1M NaCl, 0.1mM EDTA, 0.05% Tween 20), proteins were separated by 7.5% SDS-PAGE and the ubiquitinated Prf-FLAG was determined by Western blotting using the α-FLAG (Sigma-Aldrich, USA).

In vitro **pull-down assay**

Prf-FLAG transiently expressed from 4-week old *N. benthamiana* was extracted and immunoprecipitated with 15μl anti-FLAG beads (Sigma-Aldrich, USA). Then the Prf-FLAG-conjugated beads were divided into two aliquots and incubated with 2μl MBP or MBP-SINA1 respectively, followed by the addition of PBS (Bannasch et al., 2001) supplemented with 0.5% Triton X-100 until the total volume reached 300μl. Tubes containing the reaction mixtures were shaken at 4°C for 2 hours, followed by centrifugation at 4°C to spin down the FLAG beads, which were washed five times afterwards with PBS supplemented with 0.5% Triton X-100. Proteins were separated by 7.5% SDS-PAGE and pulled-down proteins were determined by Western blotting using the α-MBP antibody (Sigma-Aldrich, USA).

Virus-induced gene silencing (VIGS)

VIGS was performed as described previously (Ekengren et al., 2003). The pTRV2::MEK2 construct used for VIGS was described by del Pozo et al. (2004). To generate pTRV2::*NbSINA* construct, a 285bp DNA fragment conserved in all *N. benthamiana SINA* genes was PCR amplified and cloned into the TRV2 vector at the *Eco*RI and *Xba*I sites. pTRV2 and pTRV2::MEK2, pTRV2::*Nb*SINA or empty vector

were transformed into *Agrobacterium tumefaciens* GV3101 strain. The *Agrobacterium* culture containing TRV constructs was syringe-infiltrated into the first two leaves of two week-old *N. benthamiana* seedlings (Ekengren et al., 2003). The silenced *N. benthamiana* plants were maintained in a greenhouse with a 16-h day length, 22°C daytime temperature and 18°C nighttime temperature.

RT–PCR assay

Total RNA was extracted from *N. benthamiana* leaf tissue using Trizol (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, Madison, WI). 1 µg of total RNA was used to generate first strand cDNA using 0.5 µg oligo dT primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The abundance of the Prf^{D1416V}*–HA* or *MAPKKKα* transcript was monitored by PCR using a program of 35 cycles with each cycle including denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 45 s. Primers used for Prf^{D1416V}*–HA* transcript were forward primer: 5'-GTAGTGATGGCCATGGAGAAGAGA-3' and reverse primer 5'-CTAAGCGTAGTCTGGAACGTCGTATGGGTAAGAGTCAAGGGGCTGT TCTTTAGACC-3'. Note that the forward primer was designed to be located 340 bps upstream of the LRR domain and the reverse primer is specific to HA epitope tag to avoid amplifying the native *LRR* or *NbPrf* gene. Primers used for *MAPKKKα* transcript were forward primer: 5'-CCTACAGCTTCTAAACTA-3' and reverse primer: 5'-GGGGAAATATTCCTTCCC-3'.

Chapter 3

Plant programmed cell death caused by an autoactive form of Prf is suppressed by co-expression of Prf LRR domain

Abstract

In tomato, the NBARC-LRR resistance (R) protein Prf acts in concert with the Pto or Fen kinase to initiate immunity against *Pseudomonas syringae* pv. *tomato* (*Pst*). Prf-mediated defense signaling is initiated by the recognition of two dissimilar *Pst*-secreted effector proteins, AvrPto and AvrPtoB, by tomato Pto or Fen. Prf detects these interactions and activates signaling leading to host defense responses including localized programmed cell death (PCD) that is associated with the arrest of *Pst* growth. We found that Prf variants with single amino acid substitutions at D1416 in the IHD motif (isoleucine-histidine-aspartic acid) in the NBARC domain cause effector-independent PCD when transiently expressed in leaves of *Nicotiana benthamiana*, suggesting D1416 plays an important role in activation of Prf. The N-terminal region of Prf (NPrf) and the LRR domain are required for this autoactive Prf cell death signaling but dispensable for accumulation of the Prf^{D1416V} protein. Significantly, co-expression of the Prf LRR but not NPrf, with Prf^{D1416V}, AvrPto/Pto, AvrPtoB/Pto, an autoactive form of Pto (Pto^{Y207D}), or Fen completely suppresses PCD. However, the Prf LRR does not interfere with PCD caused by Rpi-blb1^{D475V}, a distinct R protein-mediated PCD signaling event, or that caused by overexpression of MAPKKKα, a protein acting downstream of Prf. Furthermore, we found the Prf^{D1416V} protein is unable to accumulate in plant cells when co-expressed with the Prf LRR domain, likely explaining the cell death suppression. The mechanism for the

LRR-induced degradation of Prf^{D1416V} is unknown but may involve interference in the intramolecular interactions of Prf or to binding of the unattached LRR to other host proteins that are needed for Prf stability.

Key words: NBARC-LRR resistance protein; Prf; Pto; Fen; AvrPto; AvrPtoB; cell death.

Introduction

Plants use immune receptors to directly or indirectly detect effector proteins secreted from invading pathogens to initiate defense responses. This immune receptor-mediated disease resistance is typically initiated inside the plant cell and associated with a strong localized PCD, termed hypersensitive response (HR), in the area of attempted infection. A number of defense-related cellular events are activated during immune receptor-mediated responses, including generation of reactive oxygen species, activation of MAPK signaling, deposition of callose, and induction of many defense-related genes (Dangl and Jones, 2001). Working together, these host responses arrest pathogen growth and inhibit development of disease symptoms. Despite many advances in our understanding of plant immunity, we still lack detailed knowledge of the underlying mechanisms of how the immune receptors are activated to initiate immunity-associated signaling pathways.

Members of the largest class of immune receptors contain an NB domain, an ARC domain, and a LRR region. The NBARC-LRR proteins belong to the STAND (signal transduction ATPase with numerous domains) family of NTPases, which can bind and hydrolyze NTPs (Lukasik and Takken, 2009). In fact, two NBARC-LRR proteins conferring resistance to *Fusarium oxysporum* and root-knot nematodes, I-2

and Mi-1, respectively, have been demonstrated to have ATPase activity *in vitro* (Tameling et al., 2002). This suggests that an energy-dependent conformational change of R proteins plays a significant role in defense signal activation. A current mechanistic model implicates the ATP-bound form as the active state of NBARC-LRR proteins whereas the ADP-bound form is the inactive state (Lukasik and Takken, 2009). In support of this model, a recent report showed that an autoactive form of the M protein, which confers rust (*Melampsora lini*) resistance in flax, is associated with ATP when purified from a yeast expression system, whereas the wild-type M protein is coupled with ADP (Williams et al., 2011).

The ATP/ADP binding pocket of immune receptors involves NB and ARC domains: the N-terminus of the NB domain provides the catalytic core for ATPase activity and the C-terminus of ARC domain serves as a direct or indirect nucleotide-binding site (Lukasik and Takken, 2009; van Ooijen et al., 2008). In particular, the MHD (methionine-histidine-aspartic acid) motif in the C-terminus of the ARC domain is highly conserved among NBARC-LRR proteins although some variations occur as exemplified by Prf, conferring resistance to *Pst*, which has an IHD sequence (Salmeron et al., 1996). In all cases, the motif is believed to play a role in ADP/ATP binding. Structural modeling of the I-2 protein predicts histidine residue binds the β-phosphate of ADP and the aspartic acid residue facilities the binding, collectively holding I-2 in a closed inactive conformation (van Ooijen et al., 2008), which probably involves negative intramolecular interactions between the LRR and NBARC domains (see model proposed by Collier and Moffett below). Amino acid substitutions in the MHD (particularly at the histidine and aspartic acid residues) may

attenuate the binding of ADP resulting in destabilization of the ADP-bound conformation of the immune receptor. This destabilization consequently facilitates ATP binding that presumably has less influence on the negative intramolecular interactions between domains of the immune receptor and eventually leads to an open, and therefore active, conformation (van Ooijen et al., 2008). Supporting this scenario, mutations in the MHD motif of numerous immune receptors, including I-2, Mi-1, Rx, Rpi-blb1, Rpm1, M, Pit, and L6, have been found resulting in immune receptor autoactivation that elicits HR cell death in the absence of pathogen effectors (Bendahmane et al., 2002; de la Fuente van Bentem et al., 2005; Gao et al., 2011; Howles et al., 2005; Kawano et al., 2010; van Ooijen et al., 2008; Williams et al., 2011).

The LRR domain of NBARC-LRR immune receptors forms a horseshoe-like β-sheet structure that provides a platform for protein–protein interactions (Padmanabhan et al., 2009). It is generally thought that the LRR domain plays a role in direct or indirect recognition of pathogen effectors (Ellis et al., 2007; Jia et al., 2000). However, there is emerging evidence pointing to the involvement of the LRR domain in R protein activation and defense signal transduction through its intramolecular interaction with the NBARC domain and/or intermolecular interaction with other host factors (Collier and Moffett, 2009; Padmanabhan et al., 2009). For example, in the Rx resistance protein that confers resistance to potato virus X, the LRR domain mediates intramolecular interactions with both the NB and ARC domains. Significantly, these LRR-mediated intramolecular interactions are disrupted by the cognate effector, Potato Virus X coat protein, suggesting the LRR applies a

negatively regulatory action on the Rx protein in the absence of the elicitor (Moffett et al., 2002; Rairdan and Moffett, 2006). Based on studies of the Rx protein, a working model for NBARC-LRR immune receptor activation has been proposed recently by Collier and Moffett (2009): the NBARC-LRR protein is normally locked in an inactive state through auto-inhibitive intramolecular interactions between the LRR and NBARC domains (*cis* repression) and/or intermolecular interaction between the N-terminus of the immune receptor and its recognition partner (*trans* repression). Upon binding of the host recognition partner to a pathogen effector, which may involve both LRR and the N-terminus of the immune receptor in some cases, the interaction between LRR and NBARC is perturbed, which alters the nucleotide-binding status (from ADP-bound to ATP-bound) of the immune receptor. This, in turn, triggers further conformation changes to expose the initiation motif in the NB domain to interact with downstream signaling components for activation of immune signaling.

In tomato, resistance to bacterial speck disease caused by *Pst* involves a complex interaction of several proteins: two *Pst* dissimilar effector proteins AvrPto and AvrPtoB, delivered into the host cell via the type III secretion system; two highly homologous tomato protein kinases, Pto and Fen, and one NBARC-LRR-type immune receptor Prf (Oh and Martin, 2011; Pedley and Martin, 2003). Pto and Fen act as recognition-specificity partners for Prf, with Pto interacting with either AvrPto or AvrPtoB and Fen interacting with the N-terminal region (amino acids 1–387) of AvrPtoB (Kim et al., 2002; Rosebrock et al., 2007; Tang et al., 1996). Significantly, Prf can dimerize and physically associate with both Pto and Fen *in vivo* (Mucyn et al., 2006; Mucyn et al., 2009), likely responding to Pto or Fen perception of

AvrPto/AvrPtoB and consequently activating immunity-associated responses. The N-terminal region of Prf (amino acids 1–537, designated NPrf hereafter) appears to serve as a platform for Prf dimerization and interaction with Pto or Fen, suggesting that NPrf may have a role in recognition since Pto and Fen directly interact with AvrPto and/or AvrPtoB (Mucyn et al., 2006; Mucyn et al., 2009). Recently solved co-crystal structures of AvrPto–Pto and AvrPtoB–Pto suggest the AvrPto/AvrPtoB-interacting loops in Pto negatively regulate Prf activation (Dong et al., 2009; Xing et al., 2007). Based on the structural and functional analysis of the AvrPto–Pto and AvrPtoB–Pto complexes, a mechanism of activation of the Prf-mediated defense signaling has been postulated that suggests binding of AvrPto or AvrPtoB to Pto releases Pto inhibition of Prf. In accordance with this idea, several autoactive Pto forms have been generated, all of which carry substitutions in the region involved in AvrPto or AvrPtoB interaction. These mutants trigger HR cell death independently of AvrPto or AvrPtoB when expressed in leaves of *Nicotiana benthamiana*, which contains a conserved Prf-mediated defense signaling pathway (Dong et al., 2009; Xing et al., 2007).

Extensive studies have been done on the interaction of Pto with AvrPto/AvrPtoB in relation to initiation of Prf-mediated defense responses and on the mutual regulation between Pto and Prf (Chang et al., 2002; Kim et al., 2002; Rathjen et al., 1999; Rosebrock et al., 2007; Shan et al., 2000; Tang et al., 1996; Wu et al., 2004; Xiao et al., 2007). However, very limited functional analysis has been done on the Prf protein itself, especially the possible role of the subdomains in defense signal activation and/or transduction. In this study, we report evidence supporting a role for

the IHD motif in activating the Prf protein. Proteins harboring mutations at D1416 triggered HR cell death when overexpressed in *N. benthamiana*. We also show that both NPrf and LRR domains (amino acids 1432–1824) are dispensable for Prf accumulation but essential for cell death signaling. Moreover, expression of the unattached Prf LRR domain suppresses all cell death signaling mediated by Prf in *N. benthamiana*, apparently by causing degradation of Prf protein by means of an unknown mechanism.

Results

Single amino acid substitutions in the IHD motif of Prf activate PCD signaling

Prf has an IHD sequence instead of the more common MHD sequence in the C-terminus of the NBARC domain (Salmeron et al., 1996). To examine the role of the IHD motif in the activation of Prf, we developed five substitutions (I1414A, H1415D, H1415V, D1416A, and D1416V) with a C-terminally fused human influenza hemagglutinin (HA) tag and used these in functional assays. The adjacent L1417 of the IHD motif was also substituted with alanine. The mutated sequences were cloned into a plant expression vector under control of the Cauliflower Mosaic Virus (CaMV) 35S promoter and assessed for their ability to trigger PCD in *Nicotiana benthamiana* leaves upon *Agrobacterium*-mediated transient expression.

As shown in Figure 3-1A, Prf variants with the substitutions D1416V and D1416A triggered cell death in *N. benthamiana* leaves, whereas Prf proteins with substitutions at I1414, H1415, or L1417 did not cause this phenotype. The cell death elicited by Prf^{D1416V} appeared 2 d after *Agrobacterium* infiltration, whereas Prf^{D1416A}-mediated cell death did not appear until 3 d in the same experimental conditions (not

shown). Protein of each of the Prf variants accumulated to similar levels in the plant cell (Figure 3-1A), suggesting the cell death caused by D1416A and D1416V is not due simply to overaccumulation of protein. Thus, the IHD motif appears to play an important role in regulating the activity of Prf. However, unlike I2 or Mi-1, in which substitutions at any of the three amino acids in the MHD motif resulted in activation of these immune receptors (van Ooijen et al., 2008), only mutations in D of the Prf IHD led to its autoactivation.

The cell death elicited by Prf^{D1416V} is due to downstream signaling events

The cell death triggered by the Prf-autoactive proteins could be due to defense-relevant signaling or to general cellular perturbation due to simple overexpression of the variant proteins. To distinguish between these possibilities, we tested whether the Prf-mediated cell death is dependent on a MAPK kinase, MEK2, a known defense signaling component essential for Prf-mediated PCD signaling in *Nicotiana benthamiana* (del Pozo et al., 2004). Since the Prf^{D1416V} variant caused slightly stronger cell death compared to Prf^{D1416A}, we used the former for further functional assays. *Agrobacterium tumefaciens* carrying a *35S::Prf^{D1416V}–HA* construct was infiltrated into *N. benthamiana* leaves in which expression of the *MEK2* gene was suppressed by VIGS. Control plants were infected with a tobacco rattle virus (TRV) empty vector. The effectiveness of *MEK2* silencing was confirmed by the abolishment of cell death caused by Pto^{Y207D} (Figure 3-1B), an autoactive Pto mutant that can trigger Prf-dependent cell death in *N. benthamiana* (Rathjen et al.,

1999). We observed that expression of Prf^{D1416V} did not cause cell death on *MEK2*-silenced *N. benthamiana* leaves although Prf^{D1416V} protein accumulated to similar levels in *MEK2*-silenced plants and control plants (Figure 3-1B). This result indicates that cell death caused by Prf^{D1416V} is due to activation of immunity-associated signaling.

The N-Terminal region of Prf and its LRR domain are required for Prf-autoactive cell death signaling

To determine the possible role of the N-terminal region (NPrf) and LRR in Prf-mediated cell death signaling, we generated Prf^{D1416V} variants with deletions of the NPrf or LRR and carrying a C-terminal HA tag. When transiently expressed in *N. benthamiana*, both Prf^{D1416V}-ΔNPrf and Prf^{D1416V}-ΔLRR variant proteins accumulated as well as Prf^{D1416V} but neither one triggered cell death (Figure 3-2).

Co-expression of the Prf LRR domain with Prf^{D1416V} suppresses cell death and triggers degradation of the Prf^{D1416V} protein

The results above suggested both the NPrf and LRR domains are dispensable for Prf stability but are required for cell death signaling. Based on earlier studies of intramolecular interactions of immune receptors (Moffett et al., 2002; Rairdan et al., 2008; Rairdan and Moffett, 2006), it is possible these domains make contact with other Prf domains. We therefore co-expressed the NPrf or Prf LRR with Prf^{D1416V} in *N. benthamiana* leaves using *Agrobacterium* inocula at an empirically determined 5:2 ratio. We observed that the Prf LRR, but not NPrf, suppressed the Prf^{D1416V}-triggered cell death (Figure 3-3A). To examine whether co-expression of

these domains affected Prf protein accumulation, we performed Western blotting using α-HA. Remarkably, we found that the Prf^{D1416V} protein was barely detectable when co-expressed with Prf LRR, whereas NPrf did not influence Prf^{D1416V} accumulation (Figure 3-3A). Possible explanations for this are that the LRR domain binds to Prf^{D1416V} or other host proteins essential for Prf stability, causing Prf destabilization and subsequent degradation. However, it is also possible that co-expression of the *Prf LRR* and *Prf*^{D1416V} genes caused post-transcriptional gene silencing (PTGS) leading to a less protein production and loss of Prf^{D1416V}-induced cell death. To rule out this possibility, we measured the mRNA level of *Prf*^{D1416V} in the presence of the *Prf LRR* by RT–PCR. As shown in Figure 3-3B, co-expression of the Prf LRR domain did not affect the Prf^{D1416V} transcript level. Taken together, our data suggest that both NPrf and LRR have a role in Prf^{D1416V}-triggered cell death signaling and co-expression of the isolated Prf LRR induces degradation of Prf and abolishes cell death.

Co-expression of the Prf LRR domain with an autoactive form of Pto suppresses its cell death-inducing activity

To further investigate the effect of the LRR domain on Prf-mediated defense signaling, we examined whether this domain influences Pto protein stability or suppresses cell death signaling activated by a constitutively active form of Pto, Pto^{Y207D} (Rathjen et al., 1999). For these experiments, we took the advantage of the fact that *N. benthamiana* has a functional endogenous Prf gene, with a conserved Pto/Prf signaling pathway (Tang et al., 1996). We co-expressed Prf LRR with

Pto^{Y207D} in *N. benthamiana* leaves and found that the LRR region prevented the induction of cell death triggered by this constitutively active form of Pto (Figure 3-4A). Western blot analysis showed the Pto^{Y207D} protein accumulated at a similar level *in planta* with or without the presence of Prf LRR protein (Figure 3-4A), indicating Prf LRR has no influence on Pto protein expression or stability. We also tested whether the Prf LRR domain can inhibit cell death elicited by MAPKKKα protein, a MAPK kinase kinase required for Pto/Prf signaling (del Pozo et al., 2004). When the Prf LRR and MAPKKKα were co-expressed in *Nicotiana benthamiana*, MAPKKKα still triggered cell death despite normal accumulation of the Prf LRR protein and *MAPKKKα* transcripts (Figure 3-4B). Thus, the Prf LRR interferes with the cell death signaling at an early step, likely at the point of Pto/Prf in the signaling pathway.

The LRR domain of Prf protein suppresses cell death elicited by the Pto–AvrPto/AvrPtoB interaction

We next examined the possible effect of the Prf LRR on the PCD caused by recognition of AvrPto or AvrPtoB by Pto in *N. benthamiana*. To do this, Pto and AvrPto or AvrPtoB$_{1–307}$ were expressed in *N. benthamiana* leaves with or without co-expression of the Prf LRR. Note that AvrPtoB$_{1–307}$ is the N-terminal 307 amino acid segment of AvrPtoB that is sufficient to elicit Prf-dependent cell death when co-expressed with Pto in *N. benthamiana* (Abramovitch et al., 2003; Xiao et al., 2007). All proteins were expressed from the CaMV 35S promoter except for AvrPto, which was controlled by an estradiol-inducible promoter. As shown in Figure 5A, the cell death triggered by the AvrPto–Pto or AvrPtoB$_{1–307}$–Pto interaction was suppressed by co-expression of the Prf LRR domain. The accumulation of the Pto and Prf LRR

proteins was confirmed by Western blotting using an α-HA antibody.

The cell death suppression activity of the Prf LRR could be specific to Prf-mediated immunity signaling or it might act at an early step in some other way as a general cell death inhibitor for programmed cell death signaling in *N. benthamiana*. To distinguish between these possibilities, we assessed the ability of the Prf LRR to suppress programmed cell death mediated by the Rpi-blb1signaling pathway. Rpi-blb1 is also an NBARC-LRR type immune receptor conferring resistance in potato to oomycete pathogen *Phytophthora infestans* and a similar D475V mutation results in autoactivation when transiently expressed in *N. benthamiana* (van der Vossen et al., 2003; van Ooijen et al., 2008). Under the same experimental conditions, the Prf LRR was co-expressed with Rpi-blb1^{D475V} in *N. benthamiana* leaves for assessment of Rpi-blb1^{D475V}-mediated cell death. As shown in Figure 3-5A, Prf LRR did not affect PCD elicited by Rpi-blb1^{D475V}, suggesting the cell death suppression function of the Prf LRR is probably specific to Prf-mediated programmed cell death signaling.

The Prf LRR interferes with cell death elicited by overexpression of Fen kinase in *Nicotiana benthamiana*

Fen kinase is a Pto homolog that recognizes the N-terminus of AvrPtoB (AvrPtoB$_{1-387}$) (Rosebrock et al., 2007). As with the Pto-mediated disease immunity, this Fen-mediated defense signaling requires Prf (Rosebrock et al., 2007). In addition, overexpression of Fen from the CaMV 35S promoter in *Nicotiana benthamiana* leaves triggers AvrPtoB-independent programmed cell death that is also dependent on Prf (Chang et al., 2002). Recently, Mucyn et al. (2009) reported that a large Prf fragment (amino acids 960–1824) containing the LRR domain

inhibits this Fen-triggered cell death but did not define which domain is responsible for this cell death suppression. To further examine the effect of the Prf LRR on this Fen-triggered cell death, we infiltrated *Agrobacterium* carrying *35S::Fen–HA, 35S::LRR–HA*, or empty vector into *N. benthamiana* leaves for the cell death suppression assay. As shown in Figure 3-5B, Fen-triggered cell death was abolished in the presence of Prf LRR protein, whereas an empty vector control had no effect. Western blotting indicated the Fen protein was expressed and accumulated well in both cases. These data further indicate the Prf LRR interferes specifically with the Prf-dependent cell death signaling.

Discussion

Although the precise mechanisms for activation of NBARC-LRR immune receptors remain elusive, it is generally thought, in the absence of the pathogen, immune receptors exist in an inactive state brought about through inhibitory intramolecular interactions between functional domains (i.e. interaction between LRR and NBARC in Rx) and/or an intermolecular interaction with a host partner (i.e. Pto as a inhibitory partner for Prf) (Collier and Moffett, 2009; Lukasik and Takken, 2009). Upon recognition of pathogen avirulence effectors, the immune receptor undergoes conformational changes to release this auto-inhibited state, which presumably involves a switch of bound nucleotide occurring at the MHD motif of the NBARC domain (Collier and Moffett, 2009; Lukasik and Takken, 2009). Thus, there are at least three ways to activate immunity signaling: activation of the immune receptor directly or indirectly by pathogen effectors; activation of the immune receptor via mutation mimicking the effector-induced active conformation; and

extreme overexpression of the immune receptor to titrate out the inhibition from its interacting partner. In the system studied here, the Prf protein does not trigger cell death signaling when expressed from CaMV 35S promoter (Figure 3-1;(Mucyn et al., 2006). However, by using an estradiol-inducible system able to promote eightfold higher gene expression than using an 35S promoter (Zuo et al., 2000), the overexpressed Prf protein is able to elicit programmed cell death on *N.benthamiana* leaves (Mucyn et al., 2006). We show in this study that mutations in the IHD motif of Prf also lead to activation of immune signaling, presumably by mimicking the signaling-competent conformation.

The nucleotide-binding state of the NBARC domain appears to be critical for the activation of NBARC-LRR immune receptors (Lukasik and Takken, 2009). Based on structural modeling and functional analysis of Mi-1 and I-2, van Ooijen and colleagues (2008) hypothesized that the MHD motif is responsible for directly binding to ADP and that mutations in this motif disturb this inactive ADP-bound conformation, leading to nucleotide exchange and resulting in the active ATP-bound state. Extensive mutagenesis analysis of all three residues of the MHD motif resulted in autoactivation of Mi-1 or I-2 to different extents (van Ooijen et al., 2008). Importantly, at least in the case of Mi-1, the elicitation of defense signaling was not due to extreme accumulation of the variant proteins *in planta*, suggesting mutations in the MHD motif lead to the active conformation of Mi-1 (van Ooijen et al., 2008). In our study of the IHD motif of Prf, several substitutions were made to each amino acid of the IHD sequence but only D1416A and D1416V variants activated cell death signaling when expressed from the CaMV 35S promoter. Although the histidine

residue is conserved in all 50 NBARC-LRR immune receptors and structural modeling predictions suggest H494 in I-2 might directly contact the nucleotide, substitution of H1415 of Prf with D or V did not result in autoactivation. This result differs from some previous reports in which I-2$^{H494D/V}$ or Mi-1^{H840V} variants did cause constitutive-active phenotypes (van Ooijen et al., 2008).Thus, it appears that, for Prf, the aspartic acid in this IHD motif plays the pivotal role in activation of the Prf protein. Mutations in D1416, such as D1416A or D1416V, may change the Prf protein from an inactive conformation to an active conformation. However, this hypothesis awaits verification by structural analysis of the Prf protein. Nevertheless, van Ooijen and others have found that a D-to-V substitution in the MHD motif of eight different NBARC-LRR proteins results in the constitutive-active phenotype (Bendahmane et al., 2002; de la Fuente van Bentem et al., 2005; Gao et al., 2011; Howles et al., 2005; Kawano et al., 2010; Williams et al., 2011). These seven NBARC-LRR proteins control immunity to various pathogens: Rx to a virus (Bendahmane et al., 1999), I-2, L6, M and Pit to fungi (Anderson et al., 1997; Hayashi and Yoshida, 2009; Lawrence et al., 1995; Simons et al., 1998), Rpi-blb1 to an oomycete (van der Vossen et al., 2003), Rpm1 to a bacterium (Kunkel et al., 1993), and Mi-1 to an insect/nematode (Vos et al., 1998). Here, we add Prf, another NBARC-LRR protein, to this list of D-to-V autoactivation mutants, which controls immunity to *Pst*, a bacterium (Salmeron et al., 1996).

In the Rx protein, a minimal NB domain fused with GFP protein is sufficient to initiate programmed cell death when overexpressed in *N. benthamiana* (Rairdan et al., 2008). In contrast, we found that both NPrf and LRR are required for Prf^{D1416V} -

triggered cell death signaling in *N. benthamiana* (Figure 3-2). However, only expression of the Prf LRR domain caused degradation of Prf^{D1416V} protein and consequently abolished the cell death signaling (Figure 3-3). In fact, Prf LRR can suppress Prf-dependent cell death signaling triggered by all relevant initiating factors, including AvrPto–Pto, AvrPtoB–Pto, the constitutive-active Pto^{Y207D} protein, and Fen (Figures 3-4 and 3-5). However, the cell death suppression activity of Prf LRR is probably specific to Prf-mediated immune signaling as it had no effect on cell death caused by the autoactive form of Rpi-blb1 (Figure 3-5), which determines immunity to *Phytophthora infestans* (van der Vossen et al., 2003).

The ability of the Prf LRR to cause degradation of Prf^{D1416V} protein is also specific because Pto, Pto^{Y207D}, and Fen all accumulated to normal levels when co-expressed with Prf LRR. Co-expression of the Prf LRR did not abolish cell death caused by MAPKKKα (Figure 3-4) that functions at a point downstream of the Prf (del Pozo et al., 2004). Since *N. benthamiana* has an endogenous *Prf* gene, *NbPrf*, that determines Pto-mediated (including AvrPto–Pto, AvrPtoB–Pto, and Pto^{Y207D}) and Fen-mediated programmed cell death, the LRR domain of tomato Prf probably also causes degradation of *Nb*Prf, subsequently interfering with cell death signaling dependent on that protein.

Our current data do not provide insight into the mechanism by which co-expression of the LRR domain causes degradation of Prf. One possibility is that the unattached Prf LRR interacts with full-length protein interfering with intramolecular interactions of Prf. This might destabilize Prf leading to its degradation by normal protein turnover processes. However, it is also possible that the unattached LRR

might interferes with the Pto–Prf interaction to destabilize Prf protein, based on previous work showing that Pto can stabilize Prf when co-expressed (Mucyn et al., 2006). This seems less likely since the Pto–Prf interaction is mediated through the NPrf domain and there is no evidence that the Pto–Prf interaction is critical for Prf stability (Mucyn et al., 2006). Moreover, we found the Prf LRR has no influence on Pto–Prf interaction (Du and Xiao, unpublished data) thus the requirement of Pto for Prf stability might result from Pto kinase activity affecting another protein in the cell. Another possibility is that the unattached Prf LRR binds to and therefore interferes with other host proteins needed for Prf stability. In this regard, it is known that stability of many NBARC-LRR immune receptors relies on molecular chaperones, such as HSP90, SGT1, and RAR1 (Shirasu, 2009). Yeast two-hybrid and coimmunoprecipitation assays have demonstrated these chaperones interact with the LRR domain of several NBARC-LRR proteins, including Bs2, N, I-2, and MLAs (Padmanabhan et al., 2009). Genetically impairing the function of these chaperones resulted in loss of immunity conferred by some NBARC-LRR proteins (Shirasu, 2009), which suggests a positive role for them in stabilizing immune receptor structure and/or maintaining the immune receptor in a signaling-competent state. Silencing of *SGT1* in *N. benthamiana* destabilized the N and Rx proteins, although it is unknown whether the LRR domain of Rx interacts with SGT1 (Azevedo et al., 2006). Recently, van Ooijen et al. have shown that a second small heat shock protein, HSP20, interacts with the LRR domain of I-2 and repression of *HSP20* in *N. benthamiana* by VIGS compromises I-2 protein accumulation and the cell death caused by the autoactive I-2^{D495V} mutant. Therefore, it is also possible that, when

overexpressed, the isolated LRR domain binds to certain chaperones to outcompete their interaction with the Prf^{D1416V} leading to its instability. However, this titration-out feature could still be Prf LRR-biased or -specific because the Prf LRR has no effect on cell death signaling activated by the analogous Rpi-blb1^{D475V} (Figure 3-5).

Conclusions

The present study was an attempt to identify the suppression function of LRR upon the HR cell death triggered by an auto-active Prf mutant Prf^{D1416V} and other Prf-dependent HR elicitors. Here we conclude that the unattached Prf LRR domain specifically suppresses Prf-related HR cell death pathway, in particular, the LRR domain suppresses HR cell death triggered by Prf^{D1416V} via degradation of the Prf^{D1416V} protein. Consistently, the LRR domain suppresses HR cell death triggered by Pto^{Y207D}, AvrPto-Pto/ AvrPtoB$_{1-307}$–Pto and Fen presumably by promoting degradation of endogenous Prf protein in *Nicotiana benthamiana*.

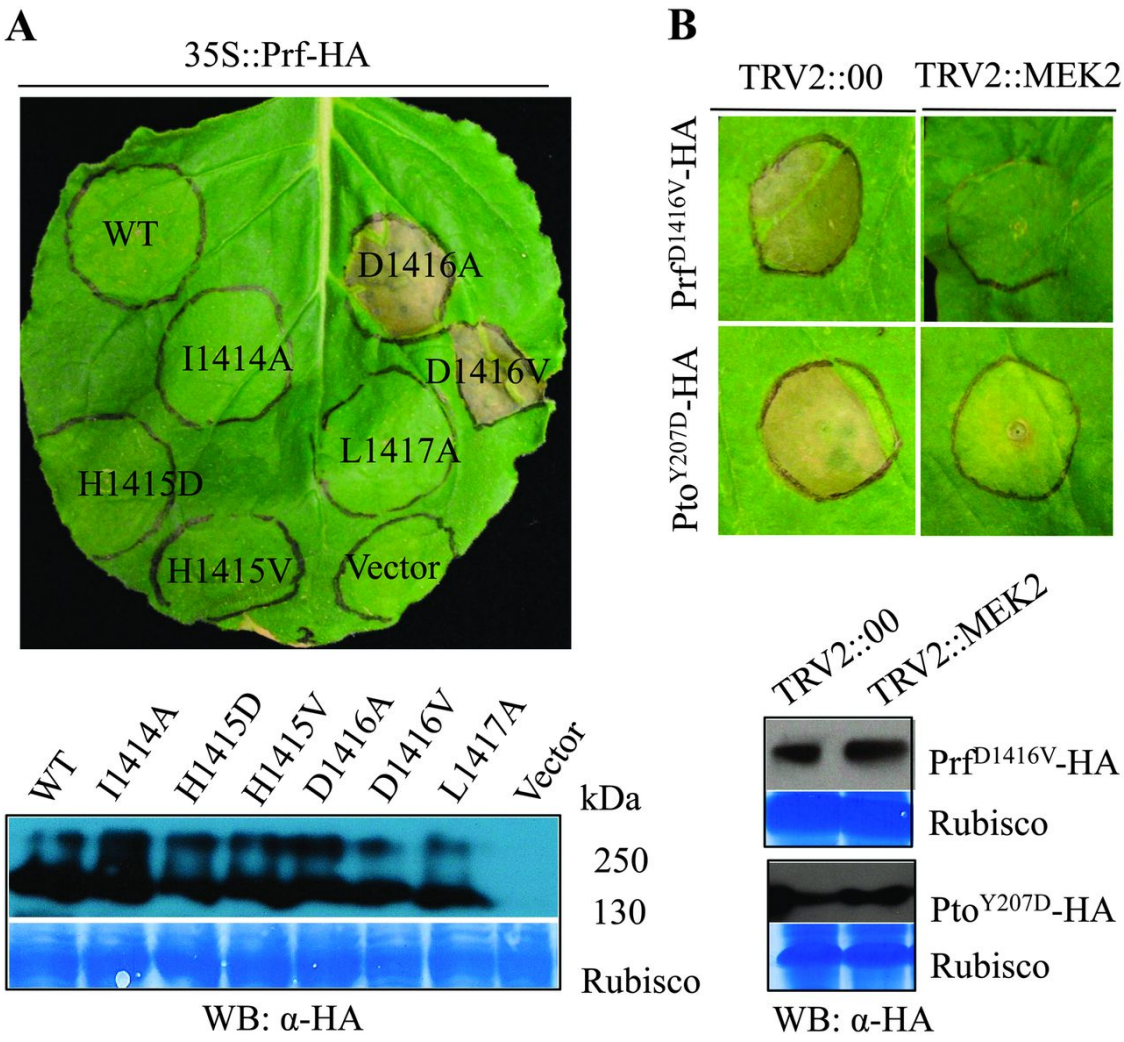

Figure 3-1. Substitutions at Prf D1416 trigger programmed cell death when expressed in leaves of *Nicotiana benthamiana*.

Agrobacterium tumefaciens strains (OD_{600} = 0.4) carrying variant HA-tagged Prf mutants or Pto^{Y207D} expressed from the CaMV 35S promoter were syringe-infiltrated into *N. benthamiana* leaves. Boundaries of the injected zone are marked with a pen. The plants were kept on the lab bench with continuous illumination by fluorescent light and photographs were taken 7 d after infiltration. Protein expression and accumulation in planta were confirmed by α-HA Western blotting (WB). Visualization of Rubisco by Coomassie blue staining confirmed equal loading of the

protein extracts.

(A) Cell death caused by the D1416A and D1416V substitutions at the lHD motif of Prf and the accumulation of WT and mutant Prf proteins *in planta*.

(B) Cell death triggered by Prf^{D1416V} is dependent on MEK2 (del Pozo et al., 2004). *N. benthamiana* plants were subjected to VIGS using a tobacco rattle virus (TRV2) construct carrying a fragment of MEK2 (del Pozo et al., 2004). The abolishment of Pto^{Y207D}-triggered cell death in the *MEK2*-silenced *Nicotiana benthamiana* leaves served as a positive control of gene silencing.

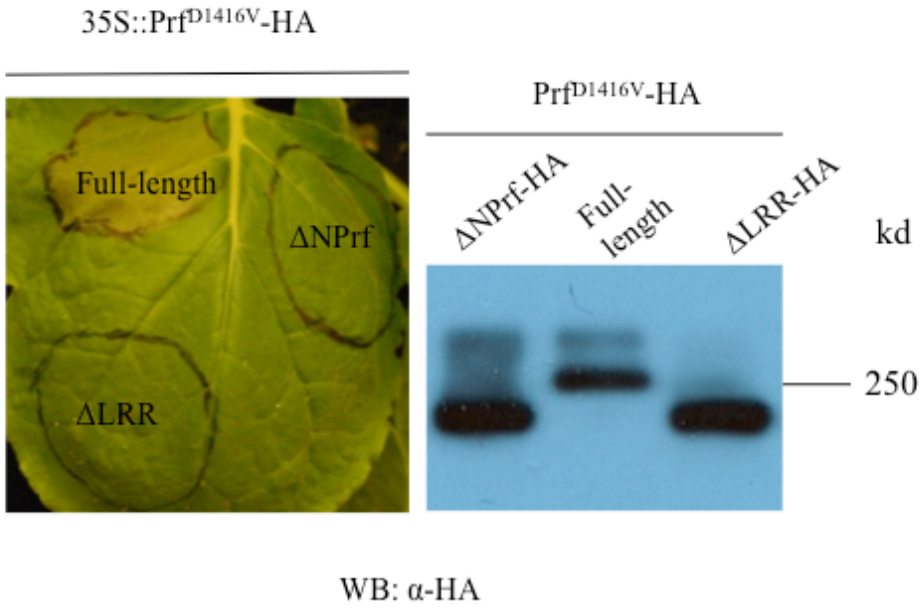

Figure 3-2. The N-Terminal region of Prf (NPrf) and LRR domain are required for cell death signaling.

A. tumefaciens expressing the CaMV 35S promoter-driven Prf^{D1416V}–HA (Full-length), Prf^{D1416V}-ΔNPrf–HA, or Prf^{D1416V}-ΔLRR–HA were syringe-infiltrated into *N. benthamiana* leaves at OD_{600} = 0.8. Plants were kept on the lab bench with continuous illumination by fluorescent light and photographs were taken 7 d after infiltration.

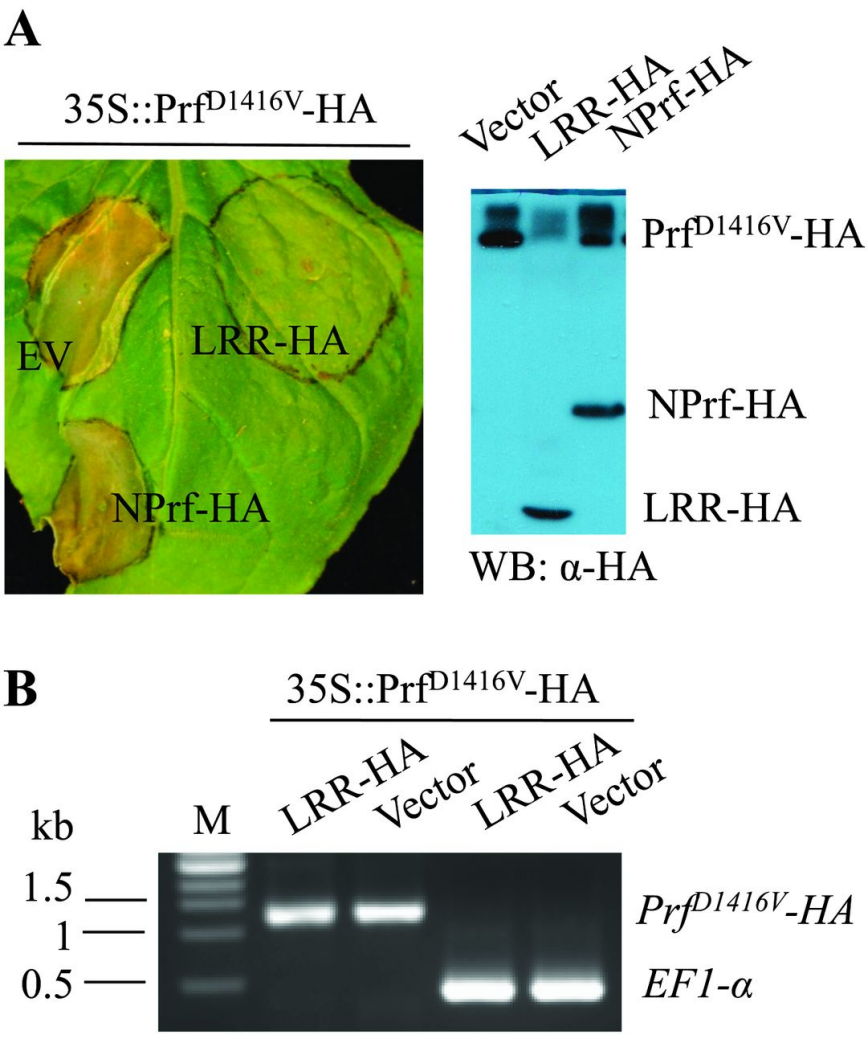

Figure 3-3. Unattached Prf LRR suppresses Prf^{D1416V}-elicited cell death.

A. tumefaciens expressing the CaMV 35S promoter-driven Prf^{D1416V}–HA, NPrf–HA, LRR–HA, or empty vector (EV) were syringe-infiltrated into *N. benthamiana* leaves at OD_{600} = 0.2 for Prf^{D1416V}–HA and OD_{600} = 0.5 for NPrf–HA, LRR–HA, or vector control. Plants were kept on the lab bench with continuous illumination by fluorescent light and photographs were taken 7 d after infiltration.

(A) Suppression of the Prf^{D1416V}-elicited cell death by the Prf LRR. The α-HA WB (right panel) shows the disappearance of Prf^{D1416V}–HA when co-expressed with

the LRR–HA.

(B) RT–PCR shows the *Prf*^{D1416V}*–HA* transcripts were present at similar levels in *Nicotiana benthamiana* leaves with or without the co-expression of Prf LRR. *Agrobacterium*-mediated transient expression was conducted separately from experiments in (A) but with similar results. Primers specific to Prf^{D1416V}–HA (described in 'Chapter 2') were used for PCR with the cDNA generated from tissues in which Prf^{D1416V}–HA was co-expressed with the Prf LRR or the vector control. M, 1-kb DNA ladder.

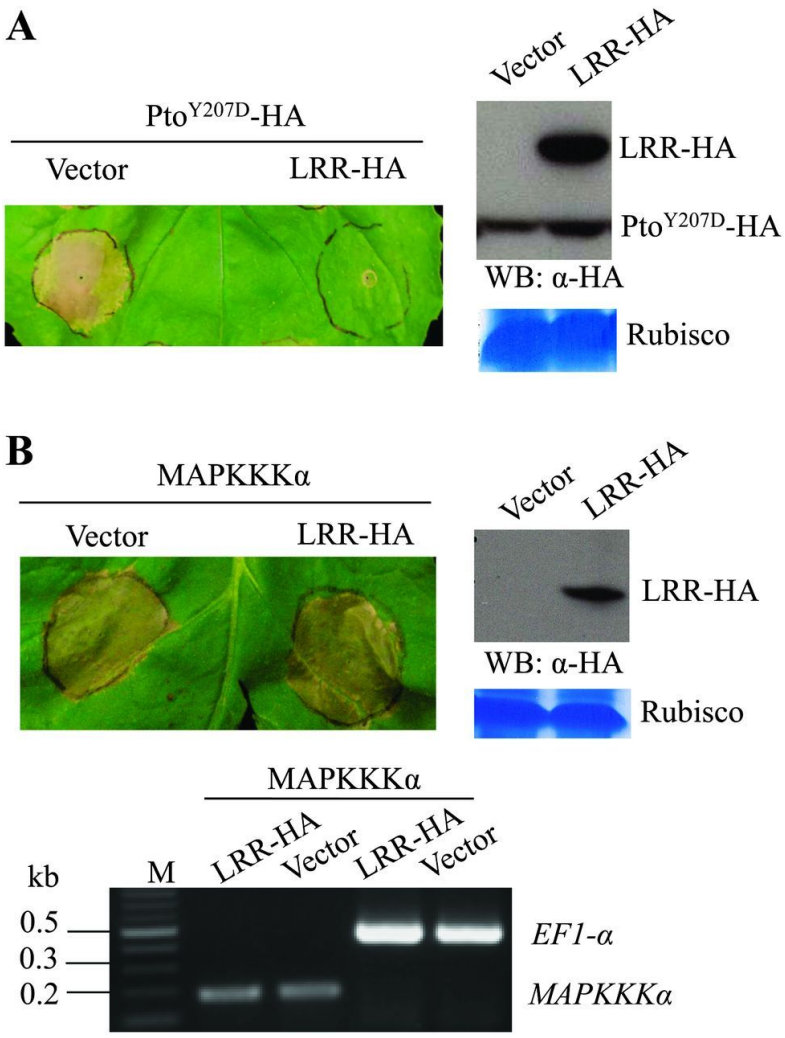

Figure 3-4**.** The LRR domain of Prf suppresses programmed cell death triggered by Pto^{Y207D}.

A. tumefaciens strains carrying the constructs indicated were syringe-infiltrated into *N. benthamiana* leaves at OD_{600} = 0.2 for Pto^{Y207D}–HA or MAPKKKα (del Pozo et al., 2004) and OD_{600} = 0.4 for LRR–HA or vector control. The Pto^{Y207D}–HA was expressed from the CaMV 35S promoter. The expression of MAPKKKα was controlled by the estradiol-inducible system and the *Nicotiana benthamiana* leaf was sprayed with 5 μM estradiol to induce the expression of MAPKKKα 1 d after

agroinfiltration. The plants were kept on the lab bench with continuous illumination by fluorescent light and photographs were taken 7 d after infiltration. Protein expression and accumulation were confirmed by α-HA WB. Visualization of Rubisco by Coomassie blue staining confirmed equal loading.

(A) The Prf LRR suppresses Pto^{Y207D}–HA-triggered cell death.

(B) The Prf LRR does not interfere with the cell death caused by overexpression of MAPKKKα. The bottom panel of the RT–PCR assay shows the *MAPKKKα* transcripts were present at similar levels in *N. benthamiana* leaves with or without the co-expression of Prf LRR. M, 100-bp DNA ladder.

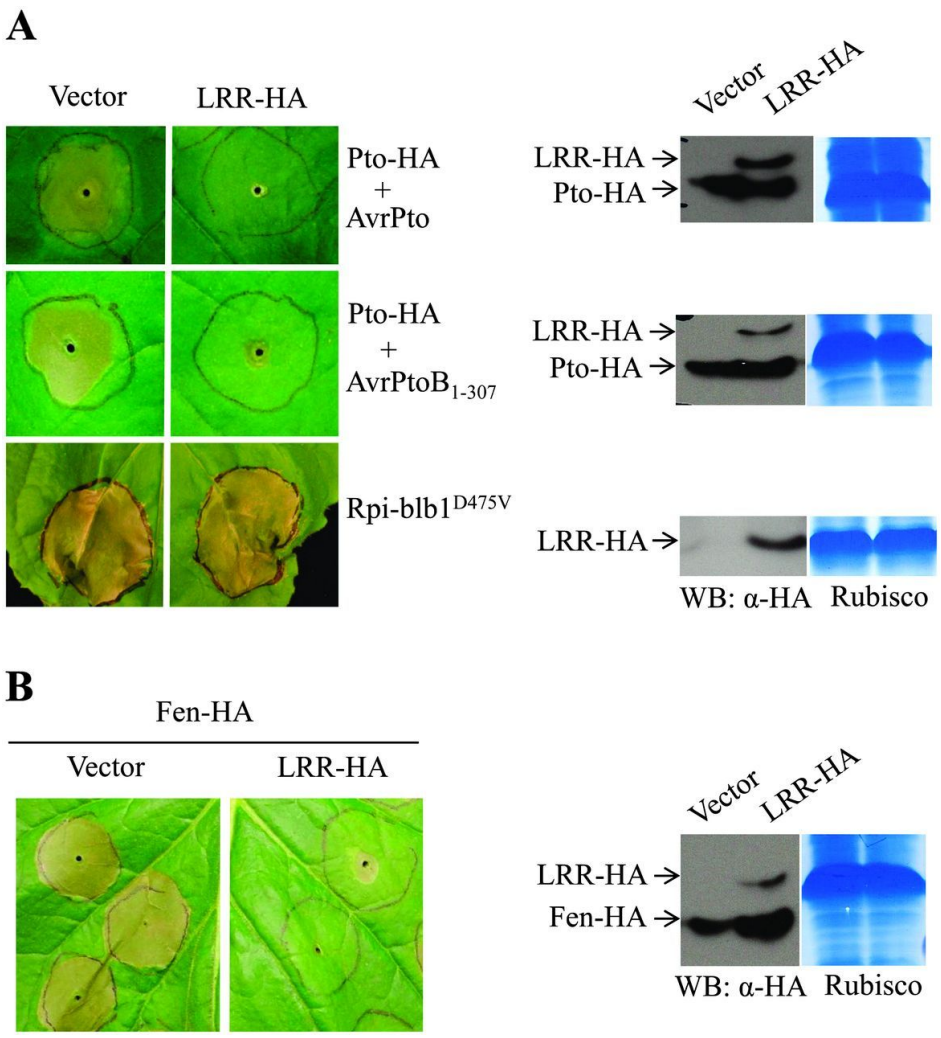

Figure 3-5. The cell death suppression activity of the Prf LRR domain is specific to the Prf pathway.

The inocula of *A. tumefaciens* carrying constructs used for transient expression were: OD_{600} = 0.1 for AvrPto; OD_{600} = 0.2 for Pto–HA, Fen–HA, $AvrPtoB_{1–307}$, Rpi-blb1^{D475V}; and OD_{600} = 0.4 for LRR–HA or vector control. All genes were expressed from the 35S CaMV promoter except avrPto, which was expressed by using an estradiol-inducible system. In the case of cell death induced by Pto-AvrPto

interaction, the *N. benthamiana* leaf was sprayed with 5 μM estradiol to induce the expression of AvrPto 2 d after agroinfiltration. Plants were kept on the lab bench with continuous illumination by fluorescent light and photographs were taken 7 d after infiltration. Protein expression and accumulation was confirmed by α-HA WB. Visualization of Rubisco by Coomassie blue staining confirmed the equal loading.

(A) The Prf LRR interferes with cell death triggered by Pto-AvrPto or Pto-AvrPtoB interaction but does not affect the Rpi-blb1^{D475V}-mediated cell death signaling.

(B) The Prf LRR suppresses cell death caused by overexpression of Fen kinase in *N. benthamiana*.

Chapter 4

Pseudomonas effector AvrPtoB promotes Prf degradation via manipulation of host ubiquitination machinery

Abstract

Bacterial pathogens utilize a variety of strategies to evade or suppress host immunity. AvrPtoB, an effector secreted by *Pst*, is a modular protein containing an N-terminal region that triggers the Prf-mediated immunity and a C-terminal domain with a ubiquitin ligase activity. Prf is an NB-LRR type immune receptor that indirectly detect the effectors AvrPto and AvrPtoB through a recognition partner Pto. It is logical to speculate that the NB-LRR proteins must be tightly controlled to prevent autoimmunity. In contrast, pathogens could use effector proteins to negatively regulate NB-LRR protein levels to suppress immunity. It has been recently demonstrated that AvrPtoB relies on its ubiquitin ligase activity to promote degradation of the tomato NB-LRR immune receptor Prf, but the underlying mechanism is unknown. Here we report that a tomato SINA (SEVEN IN ABSENTIA) E3 ubiquitin ligase acts as an endogenous negative regulator of Prf. Significantly, SINA1 interacts with both Prf and AvrPtoB and AvrPtoB-promoted Prf degradation is dependent on *SINA* gene expression. Thus, our results support a hypothesis that SINA1 is an endogenous ubiquitin ligase controlling the immune receptor Prf and *Pseudomonas* has evolved the AvrPtoB effector to interact with SINA1, and thereby targeting Prf for ubiquitination and degradation.

Key words: E3 ubiquitin ligases; SINA; AvrPtoB; Prf; Fen; immunity; HR-like cell death.

Introduction

Plants, lacking specific immune cells, have evolved two layers of immune response termed PTI (pattern recognition receptor-triggered immunity) and ETI (effector-triggered immunity) against pathogen infection (Jones and Dangl, 2006). PTI is dependent on the plasma membrane-anchored pattern recognition receptors (PRRs), which recognize the microbe-associated molecular patterns (MAMPs) derived from phytopathogens. To overcome PTI, successful pathogens have evolved effector proteins to target components of PTI thereby suppressing the first layer of plant immunity, leading to so-called ETS (effector-triggered susceptibility) (Dodds and Rathjen, 2010). Nevertheless, resistant plants have evolved intracellular NB-LRR immune receptors to recognize effectors thereby counteracting ETS (Bonardi et al., 2012).

Ubiquitination is one of the most important post-translational modifications in eukaryotic cells. It determines the half-life and ultimate fate of proteins involved in a variety of physiological and cellular processes. In recent years, ubiquitination has been implicated in the plant-microbe interactions where it plays significant roles in both pathogenesis and plant immunity (Cheng et al., 2011; Jeong et al., 2010; Lu et al., 2011; van den Burg et al., 2008). On one hand, microbial pathogens can render hosts susceptible by targeting their host ubiquitination system. For example, AVR3a, an effector secreted by *Phytophthora infestans*, stabilizes an essential component of the host ubiquitination machinery thereby suppressing cell death signaling during the biotrophic infection (Bos et al., 2010). On the other hand, the plant ubiquitination system establishes efficient resistance through its contribution to systemic acquired

resistance (SAR) against pathogens (Kim and Delaney, 2002). In general, the ubiquitin-proteasome system contains three essential enzymes, E1, E2 and E3. First, ubiquitin is activated by E1 ubiquitin-activating enzyme. Second, E2 ubiquitin-conjugating enzyme binds to the activated ubiquitin and transfers it to the substrate. Last, E3 ubiquitin ligase binds to its substrate protein and transfers the ubiquitin moiety from E2 to the target substrate. Poly-ubiquitinated proteins are finally delivered into the proteasome for degradation (Ciechanover, 1998; Smalle and Vierstra, 2004).

Bacterial pathogens have diverse strategies to subvert host immunity, one of which is “molecular mimicry” (Ashida et al., 2014). One remarkable example is the effector protein AvrPtoB secreted by the type III secretion system of *Pst*, which causes tomato speck disease (Janjusevic et al., 2006). AvrPtoB is a modular protein with an N-terminus recognized by Pto and a C-terminus encoding an E3 ubiquitin ligase (Janjusevic et al., 2006). The N-terminal region of AvrPtoB consists of two virulent determinants, $AvrPtoB_{1\text{-}307}$ and $AvrPtoB_{308\text{-}387}$ (Xiao et al., 2007). On one hand, $AvrPtoB_{1\text{-}307}$ is able to promote production of ethylene (Xiao et al., 2007), which has been previously shown to enhance bacterial speck disease (Cohn and Martin, 2005). On the other hand, $AvrPtoB_{308\text{-}387}$ is able to suppress PTI signaling events such as the MAPK signaling cascade (Xiao et al., 2007).

Several PRRs have been identified as the substrates of the AvrPtoB E3 ligase, including FLS2 (Gohre et al., 2008) and CERK1 (Gimenez-Ibanez et al., 2009). Moreover, tomato Fen kinase was demonstrated as another substrate of AvrPtoB (Abramovitch et al., 2006). Most recently, AvrPtoB was found to trigger

degradation of Prf resistance protein *in vivo* when the functional Pto is absent. Although no *in vivo* interaction was detected between AvrPtoB and Prf, the AvrPtoB-triggered Prf degradation is dependent on the E3 ubiquitin ligase activity of AvrPtoB. However, the underlying mechanism is unknown (Ntoukakis et al., 2009).

The SINA E3 ubiquitin ligase families were firstly found to be critical for the eye development in Drosophila (Carthew and Rubin, 1990). SINA E3 ligase consists of three modular parts: a variable N-terminal region, a C3HC4 RING domain, a SINA domain (Hu and Fearon, 1999) which can be divided into two zinc-finger motifs and a substrate binding and dimerization (SBD) domain (Depaux et al., 2006). SINA homologs were identified later on to be involved in tumor suppression and response to hypoxia in humans (House et al., 2009; Matsuzawa et al., 1998). In plants, SINA homologs have been demonstrated to be involved in drought stress response and symbiosis regulation (Den Herder et al., 2012; Ning et al., 2011).

Since plant immune receptors can trigger rapid, localized programmed cell death (Bai et al., 2012; Gao et al., 2011; Tameling et al., 2010), termed the hypersensitive response (HR) (Gohre et al., 2008; Pontier et al., 1998) and over-accumulation of these immune receptors often results in cell death (H.R. and A.C., 1998; Shirano et al., 2002; Stokes et al., 2002; Zhang et al., 2003), the cumulative level of immune receptors must be fine-tuned in plant cells. To date, very little has been reported on the mechanistic basis of the regulation of immune receptor level (Cheng et al., 2011). In this study, we report that the accumulation of Prf is controlled by tomato SINA E3 ubiquitin ligases. Significantly, the *Pst*-secreted effector AvrPtoB, a pathogen-derived E3 ubiquitin ligase (Rosebrock et al., 2007),

may manipulate the endogenous SINA E3 ligase-based ubiquitin machinery to promote Prf degradation thereby suppressing host immunity.

Results

SINA1 interacts with the N-terminal region of AvrPtoB (AvrPtoB$_{1-307}$) and the C-terminal region of Prf (PrfLRR) in yeast

To investigate the regulation of Prf, particularly Prf stability in plant cells, we examined whether Prf can interact with several SINA E3 ubiquitin ligases. Recently, a group of tomato SINA E3 ligases have been cloned in our lab and they can ubiquitinate an immunity-related transcriptional factor *Sl*NAC1 (Huang et al., 2013). Given the fact that the *Pst* effector AvrPtoB promotes Prf degradation indirectly *in vivo* (Ntoukakis et al., 2009), it is logical to hypothesize that SINA E3 ligases may serve as a bridge for this effector-promoted immune receptor degradation. To test this possibility, the LexA-based yeast-two hybrid (Y2H) assay (Fields and Song, 1989) was performed by co-expressing six SINA family members in a prey vector and the Prf LRR domain in a bait vector. The possible protein-protein interactions were determined by growing yeast colonies harboring both prey and bait construct on X-Gal-containing medium. We found yeast colonies containing both SINA1 and Prf LRR construct exhibited blue coloration on X-Gal plates (Figure 4-1A), indicating SINA1 interacts with Prf. As described above, indirect degradation of Prf by AvrPtoB may involve tomato host protein partners, and we suspect SINAs might be the candidates. To test this idea, we took advantage of Y2H again by expressing SINA1-6 in a prey vector and AvrPtoB$_{1-307}$ in a bait vector. Consistently, SINA1 showed strong interaction with AvrPtoB$_{1-307}$ (Figure 4-1B). Taken together, our Y2H data

suggest that SINA1 may act as a bridging partner between the effector protein AvrPtoB and tomato immune receptor Prf.

SINAs are functional E3 ubiquitin ligases

To further verify the E3 ligase activity of SINAs, we cloned six tomato SINA homologs (SINA1-6) into an *E.coli* expression vector pMAL-C2 to generate recombinant proteins. The self-ubiquitination assay was conducted in the presence of recombinant E1 enzyme (GST-AtUBA1), E2 enzyme (AtUBC8) and FLAG-tagged ubiquitin (FLAG-Ub). Poly-ubiquitinated SINAs were detected by Western blotting using anti-FLAG antibody. As shown in Fig 4-2, all SINAs exhibited different levels of ubiquitin ligase activity (lanes 1-6), whereas control reactions missing any essential component did not show any activity (lanes 7-9). The six tomato SINA family members can be categorized into two groups based on the strength of such self-ubiquitination. One group consisting of SINA1, SINA2 and SINA6 exhibited strong self-ubiquitination activity, whereas the other group consisting of SINA3, SINA4 and SINA5 exhibited relatively weak self-ubiquitination activity.

SINA1 promotes Prf degradation *in vivo*

Among the six tomato SINA ligases, SINA1 was demonstrated to interact with both Prf and AvrPtoB as well as possess strong self-ubiquitination activity. Therefore, we focused on SINA1 in the following research. If SINA1 is an endogenous ubiquitin E3 ligase regulating the protein level of Prf in the plant cell, it should be able to promote Prf degradation *in planta*. To test this, FLAG-tagged Prf (Prf-FLAG) and HA-tagged SINA1 (SINA1-HA), or empty vector were transiently co-expressed in the *N. benthamiana* leaves. Two days after *Agrobacterium* infiltration, total protein was

extracted from leaf tissues and the Prf level was determined by Western blotting. As shown in Figure 4-3A, the Prf accumulation dramatically decreased in the presence of SINA1, but not the empty vector control, indicating degradation of Prf is promoted by the SINA1 E3 ubiquitin ligase.

To determine the specificity of SINA1-promoted degradation of Prf, we tested whether SINA1 could promote degradation of a tomato Pto kinase homolog Fen, which was reported to be degraded via the E3 ubiquitin ligase activity of AvrPtoB (Rosebrock et al., 2007). Similar *Agrobacterium*-mediated transient expression was carried out in *N. benthamiana* leaves, followed by protein extraction and Western blotting using the α-FLAG antibody to determine the effect of SINA1 or AvrPtoB on Fen accumulation. As expected, Fen protein was totally eliminated when co-expressed with AvrPtoB, which is consistent with previous research (Rosebrock et al., 2007). However, the accumulation of Fen kinase did not significantly change in the presence of SINA1 (Figure 4-3B), indicating the inability of SINA1 E3 ligase to promote degradation of the Fen kinase. Taken together, our data suggest SINA1 E3 ubiquitin ligase can promote degradation of Prf versus Fen *in planta*.

SINA1 binds to Prf *in vitro*

The *in vivo* degradation of Prf promoted by SINA1 ubiquitin ligase prompted us to verify the direct interaction between SINA1 and Prf, which reflects a typical enzyme-substrate relationship. To this end, we carried out an *in vitro* pull-down assay (Den Herder et al., 2012). Prf-FLAG protein was transiently expressed in *N. benthamiana* leaves and isolated by immunoprecipitation with the anti-FLAG antibody matrix, followed by incubation with recombinant MBP or MBP-SINA1 *in*

vitro respectively (Figure 4-4). After incubation, protein samples were subjected to immunoprecipitation with anti-FLAG antibody matrix and the binding of SINA1 to Prf was determined by Western blotting using α-MBP antibody. We found that MBP-SINA1, but not the MBP control, was pulled down by Prf-FLAG, indicating the interaction between Prf and SINA1 *in vitro* (Figure 4-4).

SINA1 promotes Prf ubiquitination *in vivo* and ubiquitinates Prf *in vitro*

We next sought to test the potential ubiquitination of Prf by SINA1 *in vivo* and *in vitro*. Prf-FLAG and HA-tagged ubiquitin (Ub-HA) were co-expressed with or without the presence of SINA1-HA in *N. benthamiana* leaves. Note that in order to observe the possible ubiquitination of Prf, the proteasome-specific inhibitor MG-132 was included in the agrobacterial inoculum to prevent Prf degradation by SINA1. Total protein was extracted at two time points (36 and 48 hours post agroinfiltration) followed by Western blotting using α-FLAG antibody to detect Prf protein. No smearing bands were detected at 36 hours post agroinfiltration in the presence or absence of SINA1, whereas the polyubiquitinated Prf moieties, indicated as the smear banding pattern on the top of Prf, showed up at 48 hours post agroinfiltration only in the presence of SINA1 (Figure 4-5), suggesting that SINA1 may ubiquitinate Prf *in vivo*.

To further determine whether Prf is a substrate of SINA1, an *in vitro* ubiquitination assay (Abramovitch et al., 2006) was performed by co-incubating recombinant E1 (GST-AtUBA1) and E2 (GST-AtUBC8) enzymes, HA-tagged ubiquitin (Ub-HA), MBP-SINA1, GST-AvrPtoB or MBP, as well as the Prf-FLAG protein immunoprecipitated from the leaf tissue (Figure 4-6). As expected, self-

ubiquitination of SINA1 and AvrPtoB were detected using α-HA antibody (the second lane and the third lane in Figure 4-6A), whereas the negative control MBP showed no polyubiquitination (the first lane in Figure 4-6A). Using α-FLAG antibody, trans-ubiquitination of Prf was detected in the presence of MBP-SINA1 or GST-AvrPtoB, but not MBP (Figure 4-6B), suggesting Prf is a substrate protein that can be polyubiquitinated by SINA1 and AvrPtoB *in vitro*.

SINA E3 ubiquitin ligases regulate Prf abundance *in planta*

Given the fact that our gain-of-function assays indicate SINA1 promotes Prf degradation *in vivo* (Figure 4-3A) and directly ubiquitinates Prf *in vitro* (Figure 4-6B), it is necessary to determine whether this E3 ubiquitin ligase is responsible for Prf degradation by loss-of-function approach. To this end, we first adopted VIGS (Burch-Smith et al., 2004) to silence *SINA1* gene in tomato. Unfortunately, the VIGS experiment failed due to the low efficiency of silencing in tomato (Liu et al., 2002). We thus took advantage of *N. benthamiana* that is more readily amenable to VIGS. *N. benthamiana* possesses six tomato *SINA* orthologs (*NbSINA*s) and we could not identify a unique sequence region among the *NbSINA*s due to high homology among them. Thus, we employed a 285-bp fragment conserved region that could potentially silence all six *NbSINA* genes. The *SINA*-silenced *N. benthamiana* plants did not show abnormal growth or morphological alternations. Prf-FLAG, Fen-FLAG or GFP-FLAG was transiently expressed in non-silenced and *SINA*-silenced *N. benthamiana* leaves respectively and the protein accumulation was evaluated by Western blotting using the α-FLAG antibody (Figure 4-7). Remarkably, Prf accumulation dramatically increased in *SINA*-silenced *N. benthamiana* compared to

non-silenced *N. benthamiana*, whereas the accumulation of Fen kinase and GFP did not change in either plant (Figure 4-7). This indicates that suggesting at least one SINA E3 ligase is specifically responsible for Prf degradation *in planta*. Moreover, no altered Fen accumulation in the *SINA*-silenced *Nicotiana benthamiana* (Figure 4-7) was detected, which is consistent with the observation that tomato SINA1 does not promote Fen degradation (Figure 4-3B).

AvrPtoB-promoted degradation of Prf was impaired in *SINA*-silenced *N. benthamiana*

It has been reported that the *Pst*–secreted E3 ubiquitin ligase AvrPtoB promotes Prf degradation in both tomato and *N. benthamiana* (Ntoukakis et al., 2009). However, no interaction between AvrPtoB and Prf has been detected (Ntoukakis et al., 2009). Based on our observation that SINA1 interacts with both AvrPtoB and Prf, we hypothesized that AvrPtoB may take advantage of this SINA1 ubiquitination machinery in the host to promote Prf degradation. To test this hypothesis, we co-expressed Prf-FLAG and either AvrPtoB-FLAG or an empty vector in non-silenced or *SINA*-silenced *N. benthamiana* leaves, respectively (Figure 4-8A). The protein accumulation of Prf-FLAG and AvrPtoB-FLAG was determined by using α-FLAG antibody. As reported previously (Ntoukakis et al., 2009), Prf accumulation was abolished in the presence of AvrPtoB in non-silenced *N. benthamiana* (Figure 4-8A, lane 1 ad lane 2). However, similar Prf protein levels were detected in the *SINA*-silenced *N. benthamiana*, regardless of whether AvrPtoB was present or not (Figure 4-8 A, land 3 and lane 4), suggesting that AvrPtoB is no longer able to promote Prf degradation when *SINA* is silenced.

Since it was reported that AvrPtoB targets some other host plant proteins, including CERK1, FLS2 and Fen, for degradation (Gimenez-Ibanez et al., 2009; Gohre et al., 2008; Rosebrock et al., 2007), we next determined the specificity of this *SINA*-dependent Prf degradation by AvrPtoB. To this end, the Flag-tagged tomato Fen kinase (Fen-FLAG) was co-expressed with AvrPtoB-FLAG or empty vector in *SINA*-silenced or non-silenced *N. benthamiana* leaves, followed by protein extraction and Western blotting using the α-FLAG antibody to determine Fen kinase accumulation (Figure 4-8B). As reported previously, Fen protein accumulation was eliminated in the presence of AvrPtoB in the non-silenced *N. benthamiana* (Figure 4-8B, lane 1 and lane 2). Significantly, this specific AvrPtoB-mediated Fen degradation was not affected in the *SINA*-silenced *N. benthamiana* leaves (Figure 4-8 B, lane 3 and lane 4), suggesting that AvrPtoB-mediated Fen degradation is not dependent on the SINA E3 ubiquitin ligase. Taken together, our data support a hypothesis that AvrPtoB promotes Prf degradation in a SINA E3 ligase-dependent manner.

Discussion

The accumulation of immune receptors in plant cells needs to be tightly controlled to prevent indiscriminate cell death caused by auto-activation of the plant immune signaling (Shirano et al., 2002; Stokes et al., 2002; Zhang et al., 2003). As such, several negative regulators of immune receptors have been identified. Unsurprisingly, most these negative regulators are E3 ubiquitin ligases and that directly mediate degradation of immune receptors (Cheng et al., 2011; Gou et al., 2012). For example, the protein level of SNC1 and RPS2, two *Arabidopsis* immune

receptors, were negatively regulated by CPR1, an F-box protein from the SCF complex (Cheng et al., 2011).

In this study, we found tomato E3 ubiquitin ligase SINA1, previously shown to downregulate a defense-related transcription factor, *Sl*NAC1 (Huang et al., 2013), can ubiquitinate Prf *in vitro* and promote its ubiquitination and degradation *in vivo* (Figure 4-3 A). Prf accumulation dramatically increases when the expression of the *SINA* genes are repressed (Figure 4-7). Thus, we conclude that the SINA ubiquitin ligases are endogenously negative regulators controlling the steady state level of Prf.

It is notable that tomato possesses 6 distinct functional SINA ubiquitin ligases (Figure 4-2). However, when we used yeast-two hybrid assay to test the interaction between Prf and these SINA family members, only SINA1 was found to interact with Prf (Figure 4-1 A), which was further verified by the *in vitro* binding assay (Figure 4-4). In fact, we also found other SINA ubiquitin ligases can promote Prf degradation even though they don't directly interact with Prf (Figure 4-1A, Figure 4-4 and unpublished data). It is possible that SINAs interact with each other to exert their functions: extensive studies have shown that homo- or/and hetero-dimerization of E3 ligases are required for their functional activities (Den Herder et al., 2008; Den Herder et al., 2012; Hu and Fearon, 1999; Xie et al., 2002). Indeed, homo- and hetero-dimerization of tomato SINA E3 ligases were also detected in yeast (Kud, unpublished data), suggesting that other SINAs may interact with SINA1 to promote Prf degradation.

AvrPtoB is conserved in many plant bacteria such as *Pseudomonas* spp., *Erwinia* spp. and *Xanthomonas* spp. (Jackson et al., 1999; Jackson et al., 2002; Lin

et al., 2006; Lin and Martin, 2007; Oguiza and Asensio, 2005; Sarkar et al., 2006) and it is the first identified bacterial E3 ligase targeting the plant immune system (Janjusevic et al., 2006). For years, efforts have been focused on seeking the endogenous targets of AvrPtoB in plants. So far, FLS2 (Zipfel et al., 2004) and CERK1 (Wan et al., 2008), two membrane-anchored PRRs able to trigger PTI against *Pst* and *Erysiphe cichoracearum* respectively, have been demonstrated to be AvrPtoB host targets. Both FLS2 and CERK1 interact with AvrPtoB which then ubiquitinates and targets them for degradation (Gimenez-Ibanez et al., 2009; Gohre et al., 2008). Interestingly, AvrPtoB also promoted degradation of the tomato NB-LRR protein Prf in an E3 ligase-dependent manner (Ntoukakis et al., 2009), yet no evidence of direct interaction between Prf and AvrPtoB has been found so far (Ntoukakis et al., 2009; our unpublished data). Nevertheless, these results prompted us to investigate whether there are functional connections between AvrPtoB and SINA E3 ligase. We found SINA E3 ubiquitin ligases play a significant role in the AvrPtoB-promoted Prf degradation. We first verified that SINA1 interacts strongly with the N-terminal region of AvrPtoB in a Y2H assay (Figure 4-1B). In addition, other Y2H data has shown the homo- and hetero-dimerization of SINA family members (Kud, unpublished data), suggesting the possibility of indirect interactions between AvrPtoB and other SINA E3 ligases. We further determined the dependence of AvrPtoB-mediated Prf degradation on SINA E3 ligases. We transiently co-expressed AvrPtoB and Prf in the non-silenced and *SINA*-silenced *N. benthamiana* leaves (Figure 4-8A). As expected, AvrPtoB promoted Prf degradation when the expression of *SINA* genes was not suppressed (Figure 4-8A, lane 1 and

lane 2). In contrast, Prf protein accumulation in the presence of AvrPtoB remained at the normal level when the expression of *SINA* genes was repressed (Figure 3-8 A, lane 3 and lane 4). Taken together, our data not only indicate that AvrPtoB can target endogenous SINA ubiquitin E3 ligase but also suggest that *SINA* is required for AvrPtoB-promoted degradation of Prf.

It is generally thought that many animal and plant bacterial effectors subvert host immune system by molecularly mimicking some essential endogenous components involved in a variety of cellular events in host. For example, it was reported previously that animal bacterial pathogen *Salmonella* secrets more than 30 TTSS effectors, the majority of which can mimic host-derived GTPase-activating proteins that potentially alter the operation of host immunity-related Rho family GTPases (Dean, 2011; Figueira and Holden, 2012; McGhie et al., 2009). However, there has been little evidence for comparable mimics in plants. Our results provide an example of such a scenario. Firstly, SINA1 is an endogenous E3 ligase regulating Prf accumulation in plants (Figure 4-3A, Figure 4-7), implicating the negative role of SINA1 with regards to the regulation of Prf. Secondly, SINA1 interacts with both Prf and AvrPtoB (Figure 4-1), indicating SINA1 may function as a molecular link between AvrPtoB and Prf. Thirdly, in an *in vitro* reconstruction experiment, AvrPtoB is able to ubiquitinate Prf (Figure 4-6), suggesting Prf is a potential substrate of AvrPtoB *in vivo*. However, AvrPtoB is not able to interact with Prf in plant or yeast (Niu and Kud, unpublished data), suggesting AvrPtoB requires a third partner to select Prf as a target for ubiquitination in the plant cell. Lastly, our gene silencing data also supports the hypothesis that AvrPtoB-promoted degradation of Prf relies

on SINA E3 ligases (Figure 4-8). To our knowledge, this is the first line of evidence showing that a host endogenous regulatory machinery as a mimicry target of a bacterial E3 ubiquitin ligase effector for the pathogenesis.

Our data indicated the manipulation of SINA E3 ligases by *Pst* effector AvrPtoB, however, the nature of this manipulation remains elusive. To further identify the relationships between AvrPtoB and SINAs, we will focus on several issues in future investigations. First, can SINAs and AvrPtoB mutually enhance their E3 ligase activity when they associate together? Second, is the E3 ligase activity of SINAs required for AvrPtoB-promoted Prf degradation if we block the normal E3 ligase function of SINAs? Third, are *SINA*s required for AvrPtoB-dependent virulence activity of *Pst* if we repress *SINA* expression in tomato plants? Fourth, since a stress-related transcription factor *Sl*NAC1 was identified by our lab as a target of SINAs, can AvrPtoB also hijack SINAs thereby decreasing the accumulation of *Sl*NAC1? At last, since the biochemical experiments were conducted in *N. benthamiana*, a close relative of tomato, we need to verify our data in tomato plants to reinstate our hypothesis that AvrPtoB indeed hijacks host ubiquitin machinery to suppress immunity. For example, we will examine the change of native Prf accumulation after repressing the expression of *SINA*s in tomato. In addition, degradation of Prf will be determined in *SINA*-silenced *pto11* (containing functional *Prf* and mutant *pto* allel) tomato plants upon inoculation of *Pst* expressing AvrPtoB.

Conclusions

Based on our experimental data, we conclude that the E3 ubiquitin ligase-encoding *Pst* effector AvrPtoB manipulate host endogenous SINA E3 ubiquitin ligases to promote degradation of the host NB-LRR protein Prf.

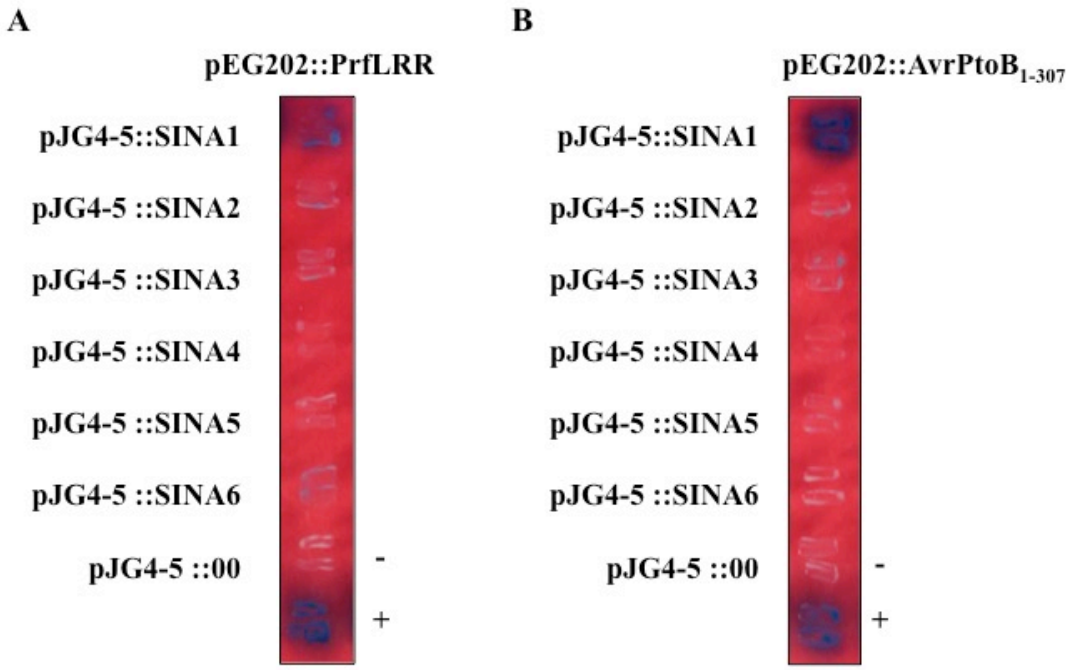

Figure 4-1. Tomato SINA1 interacts with both Prf and AvrPtoB in the yeast-two hybrid assay.

The N-terminal region of AvrPtoB ($AvrPtoB_{1-307}$, A) or the LRR domain of Prf (B) was expressed from the pEG202 bait vector, while six SINA family members were expressed from the pJG4-5 prey vector. Empty pJG4-5 vector serves as the negative (-) control and the interaction between $AvrPtoB_{1-307}$ and Pto serves as the positive (+) control.

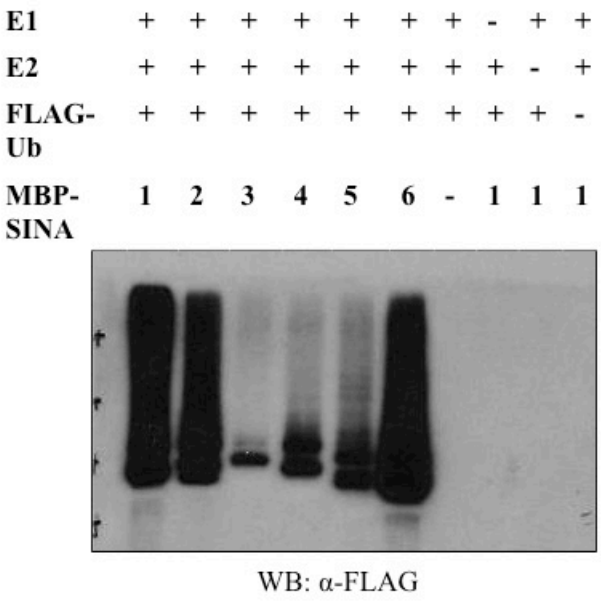

Figure 4-2. Tomato SINA family members are functional E3 ubiquitin ligases. Six MBP-fused SINA proteins were assayed for E3 ligase activity in the presence of GST-E1, GST-E2, and FLAG-Ub. Four lanes on the right represent different negative controls lacking E3, E1, E2 or ubiquitin respectively. α-FLAG antibody was used to detect Ub-FLAG.

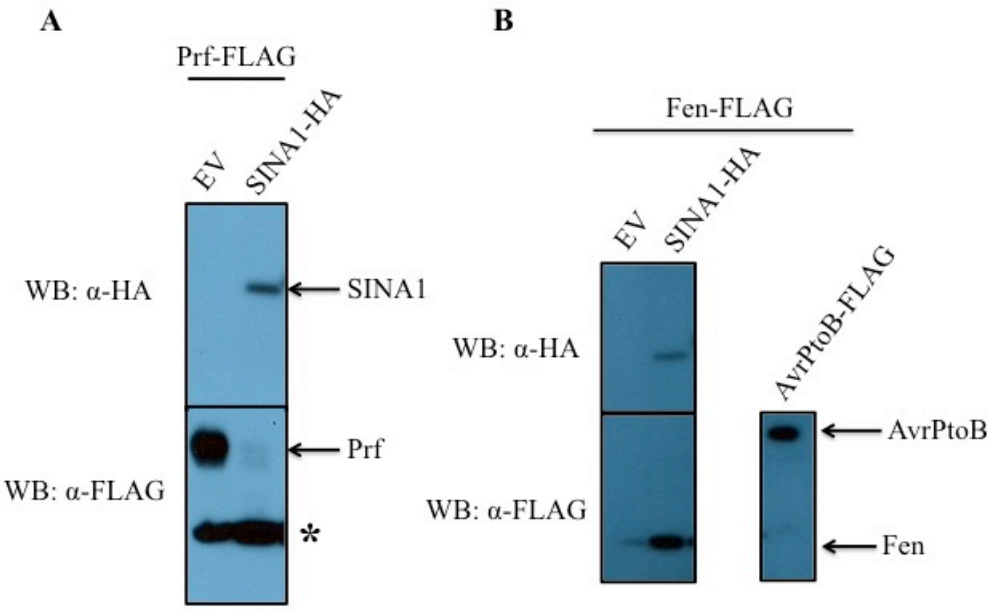

Figure 4-3. SINA1 promotes degradation of Prf, not Fen.

Prf (A) or Fen (B) was co-expressed with SINA1, AvrPtoB or empty vector (EV) in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression. Infiltrated tissues were collected 36 hours post agroinfiltration. Proteins were extracted and verified by using α-HA or α-FLAG antibody. The asterisk indicates a cross-reacting band.

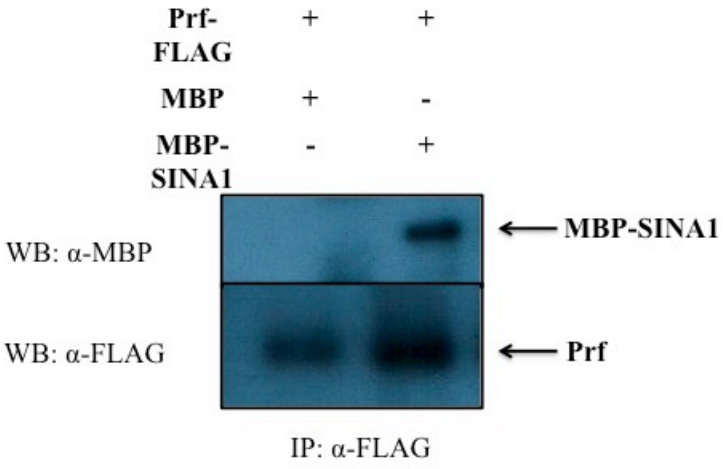

Figure 4-4. SINA1 binds to Prf *in vitro*.

In vitro pull-down assay was performed with FLAG-coated beads on reactions containing Prf-FLAG and MBP or MBP-SINA1. Reactions were subjected to Western Blot using α-MBP or α-FLAG antibody to detect MBP-fused proteins or Prf respectively. MBP-SINA1, not MBP, could be coimmunoprecipitated with Prf-FLAG.

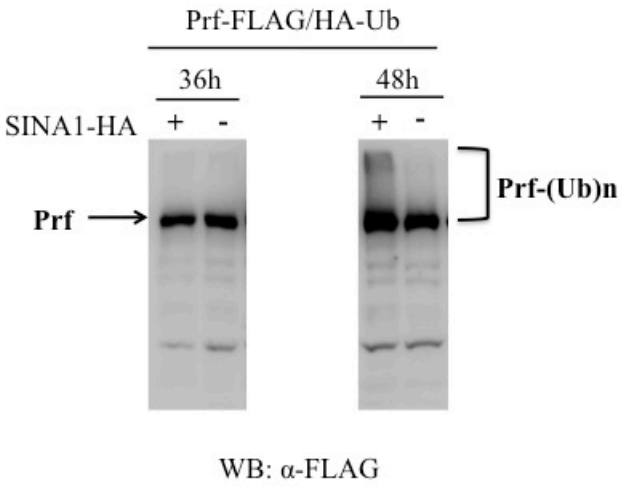

Figure 4-5. SINA1 promotes Prf ubiquitination *in vivo*.

Prf-FLAG and HA-Ub were co-expressed in the presence or absence of SINA1 by *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves. MG-132 was added in all infiltration mixtures to prevent Prf degradation. Inoculated leaf tissues were collected 36 hours post agroinfiltration and proteins were extracted. α-FLAG antibody was used to detect Prf and its polyubiquitinated form, Prf-(Ub)n.

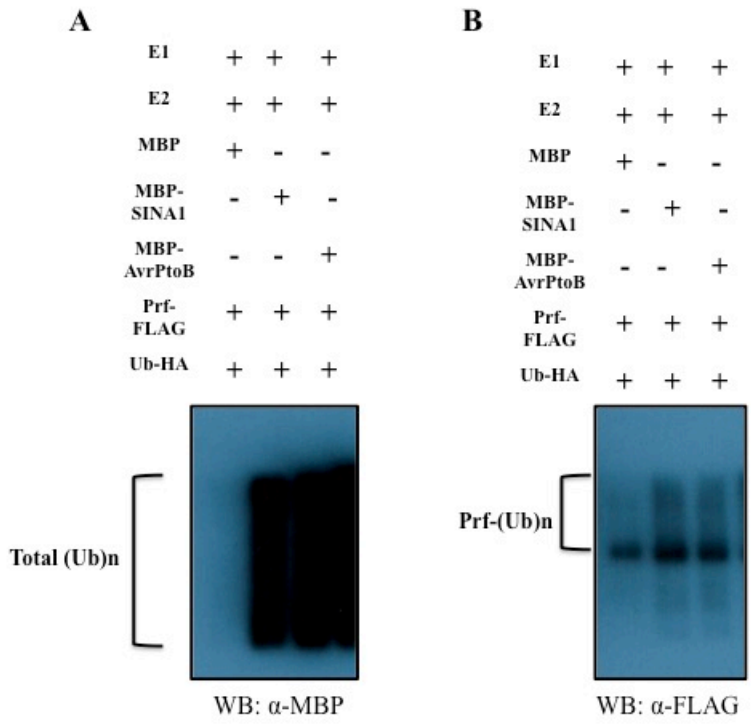

Figure 4-6. SINA1 and AvrPtoB ubiquitinate Prf *in vitro*.

FLAG-tagged Prf (Prf-FLAG) expressed from *N. benthamiana* leaves was immunoprecipitated with anti-FLAG beads, followed by co-incubation with E1 (GST-AtUBA1), E2 (GST-AtUBC8), HA-tagged Ub and MBP, MBP-SINA1 or GST-AvrPtoB. Ubiquitinated forms of Prf (B) were detected using α-FLAG antibody while total ubiquitinated proteins (A) were detected by α-MBP antibody.

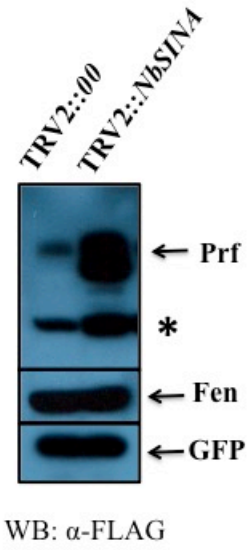

Figure 4-7. SINA E3 ubiquitin ligases regulate Prf abundance *in planta*. *Agrobacterium* strains harboring FLAG-tagged Prf (Prf-FLAG), Fen (Fen-FLAG) or GFP (GFP-FLAG) were infiltrated into non-silenced (TRV2::*00*) or *SINA*-silenced (TRV2::*NbSINA*) *N. benthamiana* leaves. Inoculated leaf tissues were collected 36 hours post agroinfiltration. Protein expression levels were determined by Western Blot using α-FLAG antibody. Arrows indicated specific proteins and the non-specific band was indicated by asterisk.

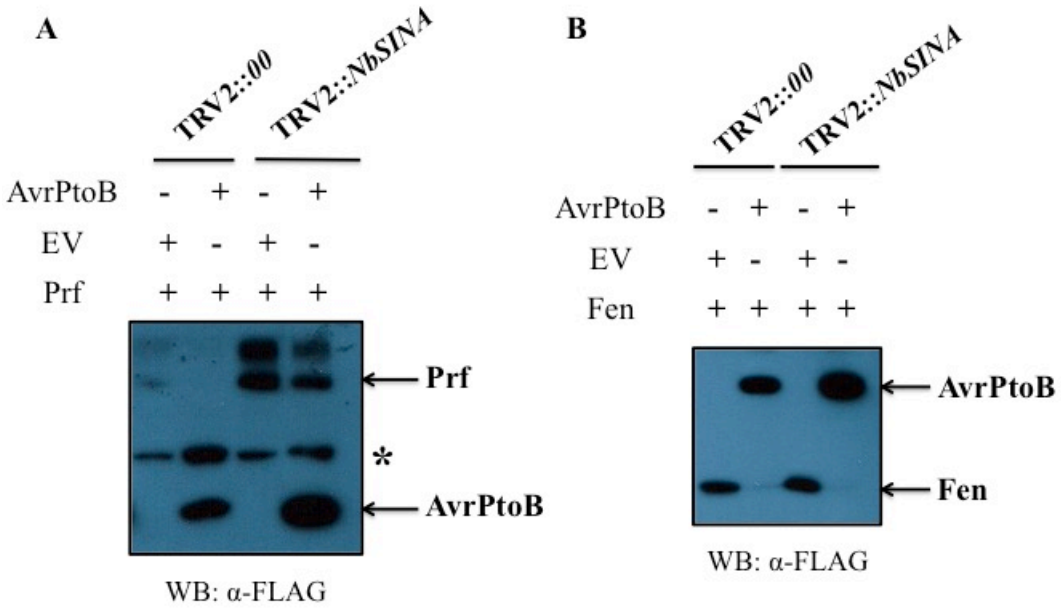

Figure 4-8. AvrPtoB-promoted Prf degradation was impaired in *SINA*-silenced *Nicotiana benthamiana*.

(A) FLAG-tagged Prf (Prf-FLAG) was transiently co-expressed with FLAG-tagged AvrPtoB (AvrPtoB-FLAG) or empty vector (EV) in non-silenced (TRV2::*00*) or *SINA*-silenced (TRV2::*NbSINA*) *N. benthamiana* leaves. (B) FLAG-tagged Fen (Fen-FLAG) was transiently co-expressed with FLAG-tagged AvrPtoB (AvrPtoB-FLAG) or empty vector (EV) in non-silenced (TRV2::*00*) or *SINA*-silenced (TRV2::*NbSINA*) *Nicotiana benthamiana* leaves. Inoculated leaf tissues were collected 36 hours post agroinfiltration. Proteins were extracted and protein levels of relevant proteins were evaluated by Western Blot using α-FLAG antibody. Arrows indicated specific proteins and the non-specific band was indicated by asterisk.

Chapter 5

Dissertation summary/Future direction

Tomato bacterial speck disease is caused by *Pst*, which secrets an array of TTSS effector proteins to suppress host plant immunity. Among these effector proteins, AvrPto and AvrPtoB, two structurally unrelated effectors, can be recognized by tomato Pto kinase leading to the formation of an immune receptor complex with a CC-NB-LRR protein Prf. The molecular basis of this AvrPto/AvrPtoB-Pto-Prf-dependent immunity has been proposed as: AvrPto or AvrPtoB is recognized by Pto kinase, which triggers the activation of Pto. Activated Pto releases the inhibition upon Prf, therefore Prf is activated and transduces the immune signal to downstream components, eventually resulting in disease immunity (Oh and Martin, 2011). Even though the biochemical relationship between Pto and Prf has been studied to some extent, the domain-related functional analysis of Prf itself remains incomplete. In the present study, we demonstrated that the C-terminal LRR domain of Prf could suppress HR-like cell death triggered by a Prf autoactive mutant (Prf^{D1416V}) by promoting its degradation (Figure 3-3). In addition, the LRR-mediated suppression of HR cell death signaling is specific to the Pto/Prf signaling pathway (Figure 3-5). Consistently, LRR suppresses all tested HR-like cell death triggered by elicitors functioning in concert with the Prf (Figure 3-4A and Figure 5) in *Nicotiana benthamiana*. We hypothesize that Prf LRR domain could potentially promote degradation of the endogenous *N. benthamiana* Prf (*Nb*Prf) via an as yet unknown mechanism. To test this hypothesis, we need to clone the *NbPrf* gene and then co-express LRR and *Nb*Prf to verify whether LRR could promote degradation of *Nb*Prf.

Significantly, we have already found that LRR promotes degradation of tomato Prf when they are expressed together in *N. benthamiana* (unpublished data).

As mentioned above, the mechanistic basis of LRR-promoted degradation of Prf^{D1416V} is not known. However, the LRR domain of Prf is able to interact with the rest part of Prf protein, indicating the presence of an intramolecular interaction within Prf (unpublished data). Based on this finding, we hypothesize that over-expression of LRR may interfere with intramolecular interaction within Prf^{D1416V} thus promoting degradation of Prf^{D1416V} protein. To test this hypothesis, generation of a LRR mutant that no longer interacts with the rest of Prf is needed. We can co-express such a LRR mutant with Prf^{D1416V} and determine its ability to suppress HR-like cell death and promote Prf^{D1416V} degradation. On the other hand, given the findings from our lab that a molecular co-chaperone SGT1 is required for Prf stability and the interaction between SGT1 and Prf is mediated through the C-terminal LRR domain of Prf (Kud et al., 2013), it is also possible that over-expression of LRR may titrate out the endogenous SGT1 thereby preventing the correct folding of Prf^{D1416V} protein, resulting in it degradation. To verify this hypothesis, we need to generate a LRR mutant that no longer interacts with SGT1, and then we can co-express such a LRR mutant with Prf^{D1416V} to determine its ability to suppress HR-like cell death and promote Prf^{D1416V} degradation.

Molecular mimicry has been frequently used by animal and human bacterial pathogens to subvert host immunity (Hospenthal et al., 2013; Lin et al., 2011; Piscatelli et al., 2011). However, evidence for this scenario is still lacking in the case of plant bacterial pathogens. In the present study we demonstrated that the *Pst*-

derived E3 ubiquitin ligase AvrPtoB could functionally mimic the tomato SINA E3 ubiquitin ligase thus promoting degradation of the immune receptor Prf (see figures in Chapter 4). However, the exquisite mechanisms underlying this manipulation remain obscure. So far, no studies have revealed how such ligase-ligase interactions might work. We hypothesize that AvrPtoB and SINA1 may mutually enhance their E3 ligase activities upon Prf. This can be tested via the *in vitro* ubiquitination assay. Since no direct interaction of AvrPtoB and Prf has been detected previously (Ntoukakis et al., 2009), we also hypothesize that SINA1 may serve as a helper ubiquitin ligase that directs AvrPtoB to Prf thereby promoting Prf degradation. To verify this hypothesis, we need to test whether the interaction between AvrPtoB and SINA1 is required for AvrPtoB-promoted Prf degradation. Also, we need to test the requirement of the E3 ligase activity of SINA1 in this degradation event. In addition to the studies to elucidate the AvrPtoB-SINA1 relationship, it would be intriguing to identify the degradation determinant within the Prf protein. In particular, the SD domain of Prf contains a PEST (Proline-glutamic acid-serine-threonine) motif that is generally thought as a degron for ubiquitin/proteasome-mediated degradation, thus the SD domain might serve as a recognition sequence for AvrPtoB or/and SINA1.

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