FUNCTIONAL ANALYSIS OF RIP36 AND HOPH1
EFFECTORS OF PHYTOPATHOGENIC BACTERIA ON
WILD EGGPLANT SOLANUM TORVUM

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Dedication

This dissertation is dedicated to my little angel Muhtashi Nawar Mahira
Functional Analysis of Rip36 and HopH1 Effectors of Phytopathogenic Bacteria on Wild Eggplant Solanum torvum

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ABSTRACT

Plant diseases cause major crop losses worldwide. Host-plant resistance could be a sustainable strategy to ensure future food security. This thesis describes my research effort to better understand the bacterial virulence and plant defense response in Ralstonia-Solanum torvum and Pseudomonas-Solanum torvum interactions.

The Gram-negative phytopathogenic bacteria Ralstonia solanacearum and Pseudomonas syringae infect many crop species and cause economic losses all over the world. These bacteria deliver the effectors called virulence factors to elicit hypersensitive response (HR) in nonhost or incompatible host plants or to cause disease in compatible host plants via type III secretion system. Recently, in Ralstonia solanacearum RS1000 72 rip (Ralstonia protein injected into plant cells) genes have been identified (Mukaihara et al., 2010). RS1002, a spontaneous nalixidic acid–resistant derivative of RS1000, induced strong HR in the nonhost wild eggplant Solanum torvum in a Hrp-dependent manner. Rip36 effector of R. solanacearum encodes a putative Zn-dependent protease motif with an active domain HExxH, and induces HR on S. torvum when transiently expressed in S. torvum leaves by an Agrobacterium-mediated transient expression system. A mutation in the putative Zn-binding motif (E149A) completely abolished the ability to induce HR. In agreement with this result, the RS1002-derived Δrip36 and rip36E149A mutants had lost the ability to induce HR in S. torvum. An E149A mutation had no effect on the translocation of Rip36 into plant cells. These results indicate that Rip36 is an avirulent factor that induces HR in S. torvum and that a putative Zn-dependent protease motif is essential for this activity.

The rip36 homologues were found in P. syringae, and were called as hopH1. The hopH1 exists in P. syringae pv. tomato (Pto) DC3000 and P. syringae pv. syringae (Psy) B728a but not in P. syringae pv. phaseolicola (Pph) 1448A. Inoculation with Pto DC3000 and Psy B728a WT strains induce strong HR on S. torvum leaves but not with Pph 1448A WT strain. Interestingly, when
hopH1 from Pto DC3000 and Psy B728a was introduced into Pph 1448A, the resultant transformants acquired HR inducing activity. I have generated hopH1-defective mutants in Pto DC3000 and Psy B728a, and found that both mutants induced HR in S. torvum, might be presence of other unknown avirulence factors. HopH1 also possesses a putative Zn-dependent protease motif. Although, E150A mutation in protease motif of HopH1 has no effect on the ability to induce HR on S. torvum, an additional mutation in protease motif E150A•H153A completely abolished the HR inducing activity of HopH1. These results indicate that HopH1 of Pto DC3000 and Psy B728a is also an avirulence determinant to induce HR on S. torvum, and Zn-protease motif is essential for HopH1-induced HR like Rip36. Identification of target protein and demonstration of enzymatic activity will provide insight into the HR triggering mechanism of Rip36 and HopH1 on S. torvum.


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IV. DISCUSSION

V. EXPERIMENTAL PROCEDURES

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REFERENCES
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Avr</td>
<td>Avirulence</td>
</tr>
<tr>
<td>PAMPS/MAMPS</td>
<td>Pathogen/Microbe- Associated Molecular Pattern</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP Triggered Immunity</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector Triggered Immunity</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type III Secretion System</td>
</tr>
<tr>
<td>Hrp</td>
<td>Hypersensitive response and Pathogenicity</td>
</tr>
<tr>
<td>Hop</td>
<td>Hrp Outer Protein</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive Response</td>
</tr>
<tr>
<td>RS</td>
<td><em>Ralstonia solanacearum</em></td>
</tr>
<tr>
<td>Rip</td>
<td><em>Ralstonia</em> protein injected into plant cells</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3’,5’ cyclic monophosphate</td>
</tr>
<tr>
<td>Cya</td>
<td>Calmodulin-dependent adenylate cyclase</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td><em>Pseudomonas syringae</em></td>
</tr>
<tr>
<td>Pto</td>
<td><em>P. syringae</em> pv. <em>tomato</em></td>
</tr>
<tr>
<td>Psy</td>
<td><em>P. syringae</em> pv. <em>syringae</em></td>
</tr>
<tr>
<td>Pph</td>
<td><em>P. syringae</em> pv. <em>phaseolicola</em></td>
</tr>
<tr>
<td>pv</td>
<td>Pathovar</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>R gene</td>
<td>Resistance gene</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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CHAPTER I

GENERAL INTRODUCTION
1.1 Plant innate immunity

Plants are sessile and exposed to myriads of potential microbial pathogens during their lifecycle. To protect themselves, plants have developed a complicated innate immune system. Upon the invasion of microbial pathogens, plants use both preformed barriers and active defense responses towards off potentially dangerous microbes. One of the well-characterized preformed barriers for foliar pathogen is the plant surface structure. Pathogen infection could be hindered by the wax and cuticle layer covering plant epidermal cells, or by the unfavorable size, location and shapes of stomata and lenticels. Besides the physical barriers, plants also produce antimicrobial chemicals such as phytoalexin to restrict pathogen infection. Once the preformed barriers are overcome, plants initiate active defense responses upon pathogen infection. There are two major types of plant induced defense responses, systemically induced resistance (SIR) and localized innate immunity (LII). SIR refers to resistance that is induced in uninfected above-ground part of plants by a chemical treatment or pathogen infection. The well-studied SIR includes systemic acquired resistance (SAR, Durrant and Dong, 2004), induced systemic resistance (ISR, Van Loon et al., 1998) and wound inducible resistance (WIR, Kessler and Baldwin, 2002). SAR is triggered by a group of small signaling molecules, including salicylic acid (SA) and its analogs, or by pathogen infection (Durrant and Dong, 2004). ISR is elicited by nonpathogenic rhizobacteria colonizing roots (Van Loon et al., 1998). WIR is induced upon tissue damage typically caused by feeding insects (Kessler and Baldwin, 2002). The signaling network of SIR involves salicylic acid (SA), jasmonic acid (JA) and ethylene with intertwining crosstalk (Pieterse and Van Loon, 2004). brassinosteroids and abscisic acid were also found to play a role in SIR (Nakashita et al., 2003; Ton and Mauch-Mani, 2004).

Localized innate immunity refers to local defense responses triggered by plant pattern recognition receptors (PRRs) upon recognition of pathogen associated molecular patterns (PAMPs) or by resistance (R) proteins upon recognition of avirulence (Avr) proteins (Ausubel, 2005). Often the plant localized innate immunity is associated with a rapid, localized hypersensitive response (HR) elicited by gene-for-gene resistance, which accounts for most cultivar level resistance and in some cases, species level resistance (Zhao et al., 2005). The species level resistance conferred by an entire plant species to a whole pathogen species or pathovar is also called nonhost resistance (Thordal-Christensen, 2003). PTI, together with effector-triggered immunity (ETI), forms the important layers of plant innate immunity (Thordal-Christensen, 2003; da Cunha et al., 2006;
Chisholm et al., 2006) and shares striking similarity with animal innate immunity (Nurnberger and Brunner, 2002; Buttner and Bonas, 2003; Nurnberger et al., 2004).

1.2 Nonhost resistance

Despite the large number of potential phytopathogenic species, pathogens can successfully infect only a limited number of plant species. Infrequent changes in the host range of phytopathogenic micro-organisms are indicative of the stability of plant species resistance (Heath, 2000). The predominant form of plant resistance is the so-called “nonhost” resistance, whereby an entire plant species is resistant to all isolates of a particular pathogen species (Heath, 2000). Nonhost resistance is conferred by a number of pre-formed and induced defenses that prevent microbial entry and generally provide an unfavorable environment for microbial proliferation. A pathogen that has the ability to infect another plant species but cannot infect a nonhost plant is referred to as a nonhost pathogen. Nonhost pathogens that land on a plant surface are exposed to a wide range of preformed plant defenses (Heath, 2000; Hückelhoven, 2007). Subsequently, some nonhost pathogens are able to enter through natural openings, such as stomata, or through wounds on the plant surface to reach the apoplastic space. The apoplast is the major battleground in plant-bacteria interactions. It is here that the plant defense mechanisms restrict nonhost pathogen multiplication (Alfano and Collmer, 1996). Apart from preformed defenses, several inducible responses are employed in the apoplast (Alfano and Collmer, 1996; Szabo et al., 2012). Inducible nonhost defense responses can occur through PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI) or through effector-triggered immunity (ETI). Such inducible nonhost resistance responses are more efficient than preformed defenses.

Nonhost resistance of plants against pathogens has been described as a three broad layers relevant to bacterial foliar infection strategies and plant counter-defense responses (Senthil-Kumar and Mysore, 2013). The first plant defense layer restricts pathogen entry. This step usually has preformed defenses, such as a wax layer, and inducible defenses, such as stomatal closure (Grimmer et al., 2012; Ham et al., 2007; Melotto et al., 2006). The second layer of defense occurs after the bacterial pathogen reaches the apoplastic region of photosynthetically active mesophyll cells to acquire nutrients available from carbohydrate metabolism (Alfano and Collmer, 1996). This step also involves preformed defenses, such as the presence of antimicrobial compounds, coupled with overall apoplastic physiological incompatibility and induced defenses, such as phytoalexin production (Dixon et al., 2002; Grimmer et al., 2012; Hann and Rathjen,
2007; Hückelhoven, 2007). The third layer involves inducible defense responses triggered in the plant cytosol, nucleus, organelles, plasma membrane, and cell wall, and executed in the cell wall region or apoplast. The induced plant defense responses in all of the above mentioned steps involve perception of bacterial PAMPs, effectors, and other molecular responses (Li et al., 2005; Nicaise et al., 2009; Sohn et al., 2012). Cellular responses triggered by PAMP recognition constitute a plant’s basal defense, in which physiological changes, such as changes in ion fluxes, production of reactive oxygen intermediates (ROI) and other signaling molecules occur to limit pathogen spread. Recognition of PAMPs in nonhost resistance results in activation of signaling cascades that show significant overlap with those associated with cultivar-specific resistance, are discussed below.

1.3 Gene-for-gene resistance

Gene-for-gene resistance is believed to be the last barrier of plant defense. Plants have evolved receptors that specifically recognize the virulence action of pathogen effectors, resulting in avirulence of the pathogen and resistance of the plants. This cultivar-specific recognition is best described by the gene-for-gene concept (Flor, 1971). In Flor’s genetic studies with flax and flax rust, disease resistance of the plant was determined by single (semi)-dominant genes called resistance (R) genes. However these R-genes conferred resistance only if the pathogen contained a matching, dominant “avirulence” (Avr) gene. Therefore, in a given combination of host cultivar and pathogen race, resistance is only observed if both a particular Avr locus and its corresponding R-gene locus are present. The absence of either of the loci leads to the breakdown of resistance. It turned out that the gene-for-gene concept held true for many important plant-pathogen interactions (Flor, 1971). Plant immune system can be represented as a four phased “zig-zag” model (Jones and Dangl, 2006) which describes the interaction between plants and pathogens in an evolutionary perspective. In a first step, plants have evolved pattern recognition receptors to perceive microbe-associated molecular patterns (MAMPs) in general and to mount a general defense response-the pattern-triggered immunity (PTI). In a next step, pathogens have evolved multiple effectors, which suppress PTI and lead to a state of “effector-triggered susceptibility” (ETS). The plants response consists in the evolution of multiple R genes, the products of which recognize, directly or indirectly, the presence of effectors. This recognition leads to “effector-triggered immunity” (ETI). The pathogens response, in a co-evolutionary “arm’s race”, is to “design” effectors that now interfere with ETI or evade recognition by R genes. Both ETI and PTI induce a similar set of defense responses, including the generation of reactive oxygen species
(ROS), mitogen activated protein kinase (MAPK) activation and the induction of defense genes. However, ETI is usually associated with a strong “hypersensitive response” (HR), accompanied by local cell death, which restricts pathogen spread at the site of infection. In contrast, cell death is only induced by a limited number of MAMPs (Kamoun et al., 1998; Shimizu et al., 2003). Despite some similarities in the signaling outcome, ETI and PTI significantly differ in many ways. While MAMPs are generally recognized at the plasmamembrane, ETI is usually induced by an intracellular recognition event. PTI is effective against microbes in general, whether pathogenic or not. In contrast, ETI is effective in a cultivar-specific manner based on its complement of R genes, and only against pathogens that carry corresponding effector (Hann et al., 2010; Oliver and Solomon, 2010).

1.4 Ralstonia solanacearum, a model plant pathogen

*Ralstonia solanacearum*, previously known as *Pseudomonas solanacearum* in the Proteobacteria β subdivision, is a casual agent of bacterial wilt. This Gram-negative, rod-shaped bacterium with polar flagella has a very high impact on economics worldwide, causing dramatic losses in yield. Affected crops range from tomato and potato to banana including more than 200 species in 53 different plant families (Alvarez et al., 2008). Broad host range, species composed of a large group of strains and fast development of disease symptoms probably make *R. solanacearum* one of the most destructive plant pathogens worldwide (Mansfield et al., 2012). *R. solanacearum* is a soil-borne pathogen, which infects plants through roots, especially wounds and smaller cracks, and invades xylem. After infection, the pathogen rapidly colonizes the vascular system of the plant, invading the root xylem first and reaching stem and leaves through vessels then (Alvarez, 2008). There are several external and internal symptoms of the disease. External symptoms include wilting, stunting and yellowing of leaves and stems (Kelman, 1953). Frequently observed internal symptoms include tissue discoloration, xylem discoloration and degradation and cell death of infected areas. Biochemically, *Ralstonia* can block xylem vessels and alter water movement by producing extracellular polysaccharide (EPS1) (Genin and Denny, 2012). EPS1 might also contribute to *Ralstonia* virulence by minimizing contact of bacterial cells surface with the plant cell, therefore avoiding recognition (Schell, 2000).

*Ralstonia* has been extensively studied biochemically and genetically. The complete genomic sequence of one strain was published in 2002 (Salanoubat et al., 2002). The pathogen genome consists of a 3.7 Mb chromosome and a 2.1 Mb megaplasmid, with an average G+C content as
high as 67% (Genin and Boucher, 2002, Salanoubat et al., 2002). The chromosome carries genes necessary for the survival, and the mega-plasmid contains genes required for virulence, including \textit{hrp} genes, along with duplicates of metabolic genes. \textit{Hrp} genes encode type III secretion system pathways and are required in many phytopathogenic bacteria to elicit HR in resistant plants (Lindgren et al., 1986). The fitness of the bacterium and its ability to adjust to environmental changes are also determined by mega-plasmid genes. A well-known phenomenon of \textit{Ralstonia} is its genetic instability; rearrangements have been found in the GMI1000 genome (Genin and Boucher, 2002). These rearrangements have contributed to the evolution of \textit{Ralstonia} strains.

The genes coding for the T3SS are called \textit{hrp} (Hypersensitive response and pathogenicity) because mutations in the genes coding for T3SS lead to an inability to cause the hypersensitive response in nonhost plants and reduce pathogenicity in host plants (Mukaihara and Tamura, 2009). The T3SS injects effector proteins into the plant cell. \textit{Ralstonia} is now a model pathogen for the study of virulence determinants, particularly bacterial effector proteins. The pathogen delivers effectors into the plant cell via the T3SS, similarly to \textit{Pseudomonas} and other Gram-negative plant pathogens (Mukaihara et al., 2010). Effectors share similarities between strains, and most of the known effectors require an Hrp-associated protein, HpaB, for their transfer into the plant cell (Mukaihara and Tamura, 2009).

\textit{R. solanacearum} is defined as “species complex” and according to newest classification (Lebeau et al., 2011), strains of \textit{Ralstonia} belong to four different phylotypes based on accessible genome sequences. This phylogenetic diversity of \textit{Ralstonia} strains provided an opportunity to evaluate the resistance of crops to different phylotypes of the pathogen and, therefore, find potential sources of resistance to use in future breeding or engineering of susceptible crops (Lebeau et al., 2011). High genetic diversity within the \textit{Ralstonia} species complex and the different ability of pathogens belonging to different phylotypes to cause disease in crops may be used as an efficient tool for screening crop breeding lines to reveal new genetic sources of resistance to this pathogen.

1.5 \textit{Pseudomonas syringae}, a model plant pathogen

\textit{Pseudomonas syringae} is a Gram-negative phytopathogenic bacterium that causes leaf spot, stem canker and other symptoms on a wide range of uncultivated plants and important crops, including bean, tobacco and tomato. \textit{P. syringae} represents a group of economically important bacterial pathogens noted for their diverse interactions with plants (Hirano and Upper, 2000). Pathovars of
*P. syringae* vary in their capacity for epiphytic survival, the nature of the symptoms they elicit, and their host range. The molecular basis for these interactions appears complex because it is controlled by the interplay of several classes of virulence factors and each class can have many members with overlapping functions. Classes of virulence factors include phytotoxins, phytohormones, adhesins, cell-wall degrading enzymes, and most importantly effector proteins translocated into plants by the type III secretion system (T3SS) (Buell *et al.* 2003). The T3SS is also known as the Hrp system because it is encoded by *hrp* genes that are required for *P. syringae* strains to elicit the hypersensitive response (HR) in nonhosts or to be pathogenic in hosts. Also modulating bacterium-plant interactions are classes of factors contributing to plant defense, including microbe-associated molecular patterns, which are produced by the bacteria and corresponding pattern-recognition proteins that are produced by the plant, as well as resistance proteins that recognize effectors or their activity within plant cells (Alfano and Collmer, 2004; Chisholm *et al.*, 2006; Grant *et al.*, 2006; Mudgett, 2005; Nomura *et al.*, 2005).

The complete genome sequence of three pathovars, *P. syringae pv. tomato* DC3000 (Buell *et al.*, 2003), *P. syringae pv. syringae* B728a and *P. syringae pv. syringae* 1448A (Feil *et al.*, 2005; Joardar *et al.*, 2005) have been published. These three strains represent an attractive set for functional genomics. They have different host ranges and pathogenic strategies and they represent each of the three major phylogenetic clades that contain the 50 or so pathovars within this species (Sarkar and Guttman, 2004; Sawada *et al.*, 1999). Each strain provides a different window into the complex interactions of *P. syringae* with plants. *P. syringae pv. tomato* DC3000 causes bacterial speck of *Arabidopsis* and tomato and has emerged as a model for studying basic virulence mechanisms (Preston, 2000; Quirino and Bent, 2003). *P. syringae pv. phaseolicola* causes halo blight of bean, an important disease in several developing countries, and is a model for studying the nature and variability of race cultivar interactions (Jackson *et al.*, 1999; Pitman *et al.*, 2005). *P. syringae pv. syringae*, which causes brown spot of bean, is a model for studying bacterial epiphytic growth and behavior in the field (Hirano and Upper, 2000; Marco *et al.*, 2005; Monier and Lindow, 2003). The diverse interactions of *P. syringae* with model plants and crops may provide new tools for improving quantitative resistance and for breeding crops with durable resistance.
1.6 Type III secretion system (T3SS) in pathogenic bacteria

The type III secretion system (T3SS) is a sophisticated molecular machinery of Gram-negative bacteria used to ‘inject’ (translocate) bacterial proteins (effectors) into eukaryotic cells. For this, the T3SS has to assemble into a multiprotein complex, which is constituted of distinct parts; a basal body spanning the two bacterial membranes connected with a cytoplasmic bulb, an attached needle structure resembling a molecular syringe, and a distal needle tip structure that re-organizes into a ‘translocon’, which is a protein complex that inserts into the host cellular membrane. Like most Gram-negative bacterial pathogens of animals studied to date, the bacterial plant pathogens require a functional type III secretion system (T3SS) for pathogenesis (Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000; Staskawicz et al., 2001; Buttner and Bonas, 2003; Jin et al., 2003). Type III secretion systems mediate the transfer of bacterial proteins (also referred to as “effectors”) directly into the cytosol of the host cell, where they interfere with or modulate normal host cell processes to facilitate bacterial invasion, growth and disease production. In animal systems, many of these effectors induce changes in the host cell cytoskeleton, while others modify eukaryotic signal transduction pathways (Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000; Cornelis, 2002; Buttner and Bonas, 2003). In the case of plant pathogenic bacteria, mutants defective in T3SS are usually unable to grow or cause disease on normally susceptible hosts, indicating that the integrity of the T3SS is essential for pathogenesis (Lindgren et al., 1986).

The structural components of the T3SS of Gram negative bacterial pathogens of animal and plants are highly conserved. However, structurally the T3SSs of plant pathogenic bacteria are slightly different from the animal pathogens. For example, the T3SS of several mammalian pathogens, including Salmonella typhimurium and Shigella flexneri, are associated with protruding, needlelike surface structures that are approximately 80 nm in length (Kubori et al., 1998; Blocker et al., 1999) (Fig. 1-1). The TTSS of several plant pathogenic bacteria are associated with relatively longer, pilus-like structures (referred to as “Hrp pili”; He and Jin, 2003). The Hrp pilus of P. syringae pv tomato strain DC3000 is approximately 8 nm in diameter and has been observed to be up to 200 nm in length, which is presumably long enough to span the plant cell wall (Brown et al., 2001; Jin and He, 2001). Several studies suggest (but do not directly demonstrate) that the Hrp pilus serves as the conduit through which bacterial in length (Kubori et al., 1998; Blocker et al., 1999) (Fig. 1-1).
Fig. 1-1. Schematic representation of the T3SS from plant (A) and animal (B) pathogenic bacteria. The secretion apparatus spans both bacterial membranes and is associated with a cytoplasmic ATPase. The T3SS from plant pathogenic bacteria is connected to an extracellular pilus that presumably spans the plant cell wall. The T3SS from plant pathogenic bacteria is associated with a long extracellular needle, which serves as a transport channel for secreted proteins. The needle is linked to the translocon, which forms a proteinaceous channel in the host plasma membrane and allows transport of effector proteins into the host cell cytosol. Evidence for the presence of a tip complex in plant pathogenic bacteria is still missing. IM, Inner membrane; OM, outer membrane; PM, plasma membrane. This Figure is reproduced from Büttner and He, 2009, Plant Physiology.
The TTSS of several plant pathogenic bacteria are associated with relatively longer, pilus-like structures (referred to as “Hrp pili”; He and Jin, 2003). The Hrp pilus of P. syringae pv tomato strain DC3000 is approximately 8 nm in diameter and has been observed to be up to 200 nm in length, which is presumably long enough to span the plant cell wall (Brown et al., 2001; Jin and He, 2001). Several studies suggest (but do not directly demonstrate) that the Hrp pilus serves as the conduit through which bacterial proteins are secreted (Brown et al., 2001; Jin and He, 2001).

T3SSs are highly complex nanomachines that consist of more than 20 components. The T3SS pathway is encoded by *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes (Bogdanove et al., 1996). The Hrc proteins direct secretion of TTSS substrates across the bacterial envelope, whereas a subset of the Hrp proteins (only partially defined) are themselves secreted by the T3SS and direct the translocation of effectors through host cell barriers. The membrane-spanning core apparatus is associated with an extracellular pilus-like appendage that is assumed to serve as a channel for transport of secreted proteins to the host-pathogen interface. The translocation of effector proteins into eukaryotic cells is probably mediated by a bacterial channel-like translocon that inserts into the host plasma membrane.

Several of the best-studied T3SS effectors are designated as Avr proteins because they were detected through gain-of-function avirulence phenotypes (Keen, 1990; Staskawicz et al., 1984). Many more effectors subsequently have been identified by their ability to travel the T3SS pathway, and these are designated Hop (Hrp outer protein) in *Pseudomonas* (Alfano and Collmer, 1997), Xop (*Xanthomonas* outer protein) in *Xanthomonas* (Noel et al., 2001), or Pop (*Pseudomonas* outer protein, as based on a previous genus designation) in *Ralstonia* (Arlat et al., 1994).

### 1.7 Function of T3SS effectors

The majority of T3SS effectors secreted by bacterial plant pathogens are predicted to function inside plant cells, and secretion into the host cell has been demonstrated for several effector proteins (Casper-Lindley et al., 2002; Szurek et al., 2002; Hotson et al., 2003). Type III effector proteins are translocated into the host during infection and these molecules (including those that elicit host defenses) are believed to modulate various aspects of host cell biology and physiology to promote disease. The proposed mode of action of these proteins include suppressing plant defenses, eliciting release of water and nutrients from host cells into the apoplast, promoting disease symptom development, and facilitating bacterial transmission (Alfano and Collmer, 1996;
Greenberg and Vinatzer, 2003; Jin et al., 2003; Ponciano et al., 2003). However, in the majority of cases, neither the mode of action nor the targets of these effector proteins within the plant are known. Various strategies to elucidate the activities of these effectors have been employed. These strategies include protein sequence and structural analyses, biochemical approaches to identify interacting proteins, and the analysis of transgenic plants expressing effector proteins. Such studies have revealed several different potential roles for T3SS effectors, including facilitating type III secretion, suppressing plant defense, enzymatic activity, colocalization within the host cell and acting as a double agent in promoting pathogenesis.

1.7.1 Effectors promote type III secretion

Several type III effectors, including HrpZ and HrpW from P. syringae, and HrpF from X. campestris pv. vesicatoria, are believed to be secreted into the apoplastic space and are proposed to function as “helper proteins” to facilitate type III secretion during pathogenesis. The predicted structural properties of the P. syringae HrpZ protein resemble those of other bacterial proteins believed to interact with host cell membranes, such as YopB from Y. enterocolitica (Lee et al., 2001). Likewise, HrpF from X. campestris has been found to contain two putative transmembrane regions, suggesting its association with membranes (Buttnner et al., 2002). Consistent with these hypotheses, both proteins have been shown to have lipid-binding activity and to form ion-conducting pores in vitro when associated with lipid bilayers (Lee et al., 2001; Buttnner et al., 2002). The pore forming activity of these proteins suggests that they functions either in assisting delivery of virulence factors into the plant cell cytoplasm or by mediating nutrient and water release from host cells. Since HrpF is dispensable for protein secretion in vitro but is required in vivo for the recognition of an effector with elicitor activity by resistant plants, it has been proposed that HrpF may facilitate translocation of one or more effector proteins into the host cell (Rossier et al., 2000).

1.7.2 Effectors suppress plant defense responses

Many type III effectors have been shown to modulate host defense responses. For example, several effectors, including AvrRpt2, AvrPphC and VirPphA, can inhibit host recognition of bacterial strains expressing specific avirulence factors (Ritter and Dangl, 1996; Jackson et al., 1999; Chen et al., 2000; Tsiamis et al., 2000). One of the best-studied cases of this type of defense suppression involves the P. syringae effector AvrRpt2. AvrRpt2 can interfere with the
recognition of *P. syringae* strains carrying avrRpm1 or avrB by plants carrying the corresponding resistance gene, RPM1 (Ritter and Dangl, 1996; Chen *et al*., 2000). The mechanism underlying this interference activity of AvrRpt2 has been recently studied and demonstrating that the T3SS-mediated delivery of AvrRpt2 leads to the disappearance of RIN4, a plant protein required for the stability of RPM1 (Mackey *et al*., 2002; Mackey *et al*., 2003; Axtell and Staskawicz, 2003). This finding is consistent with the hypothesis that AvrRpt2 is a sequence divergent cysteine protease whose activity is required for elimination of RIN4 during infection (Axtell *et al*., 2003). The suppression of host defenses by type III effector proteins can also occur downstream of pathogen recognition. For instance, the type III effectors AvrPtoB and HopPtoD2 suppress programmed cell death (e.g., the Hypersensitive Response or HR). HopPtoD2 also suppresses production of reactive oxygen species, and AvrPto suppresses cell wall-based extracellular defenses that are normally induced following pathogen recognition (Abramovitch *et al*., 2003; Bretz *et al*., 2003; Espinosa *et al*., 2003; Hauck *et al*., 2003; Jamir *et al*., 2004). HopN1 effector suppresses defense associated cell death, production of reactive oxygen species (ROS) and callose deposition (Rodríguez-Herva *et al*., 2012). These observations indicate that plant pathogenic bacteria can use type III effectors to suppress plant defenses at multiple steps in the plant defense signaling pathway.

1.7.3 Proteolytic activity of effectors

Amino acid sequence alignment and structural analyses have suggested that several type III effectors have proteolytic activity. AvrPphB from *P. syringae* pv. *phaseolicola* has similarity to *Yersinia* YopT, a cysteine proteinase (Shao *et al*., 2002). Consistent with the hypothesis that AvrPphB is a protease, AvrPphB has been shown to proteolytically cleave both itself and PBS1, an *Arabidopsis thaliana* protein kinase required for AvrPphB avirulence activity (Shao *et al*., 2003). Other cystein protease family members are AvrPpiC2 (Puri *et al*., 1997), HopPtoC (Petnicki-Ocwieja *et al*., 2002), and HopPtoN (López-Solanilla *et al*., 2004) in *P. syringae* and one in *R. solanacearum* (RSc3212) (Salanoubat *et al*., 2002). AvrRpt2 from *P. syringae* pv. *tomato* is also predicted to encode a cysteine protease. Although AvrRpt2 protease activity has not been demonstrated biochemically, the amino acid residues predicted to make up the catalytic core of this protease are required for the defense-inducing activity of AvrRpt2 (Axtell *et al*., 2003). Two possible substrates for AvrRpt2 have been identified: AvrRpt2 itself (Axtell *et al*., 2003) and the *A. thaliana* RIN4 protein, which is required for RPM1-mediated resistance (Axtell and Staskawicz, 2003; Mackey *et al*., 2003). The cysteine protease effector HopN1 of *P. syringae*
pv tomato DC3000 inhibited photosystem II activity by degrading PbsQ protein (Rodríguez-Herva et al., 2012).

The C-terminal portion of the XopD protein from X. campestris pv. vesicatoria has a high degree of similarity with the C-terminal catalytic domain of the Ulp1 ubiquitin-like protease protein family and has been shown to have cysteine protease activity specific for small ubiquitin-like modifier (SUMO)-lated substrates found specifically in plants (Hotson et al., 2003). On the basis of amino acid sequence similarity, three additional effectors from X. campestris pv. vesicatoria, AvrRxx, AvrBsT and AvrXv4 (Noel et al., 2003; Noel et al., 2001; Orth et al., 2000; Whalen et al., 1993) as well as PopP1 and PopP2 (Deslandes et al., 2003; Lavie et al., 2002) from R. solanacearum and AvrPpiG1, HopPmaD and HopPsyV (Alfano et al., 2000; Arnold et al., 2001; Deng et al., 2003; Guttman et al., 2002) appear to belong to the YopJ family of ubiquitin-like protein proteases. Interestingly, like XopD, YopJ exhibits specificity for SUMO-lated proteins (Orth et al., 2000; Lavie et al., 2002).

1.7.4 Localization of effectors in plant cells

The function of T3SS effectors is to involve colocalization with targets within the host cell. Several P. syringae effectors (AvrB, AvrPphB, AvrPto, AvrRpm1, and AvrRpt2) have been shown to carry myristoylation signals and/or to localize to the plasma membrane (Axtell and Staskawicz, 2003; Nimchuk et al., 2000; Shan et al., 2000). Putative myristoylation sites have been noted in other P. syringae effectors (e.g., HopPtoS1, HopPtoF, and AvrC) (Guttman et al., 2002; Maurer-Stroh and Eisenhaber, 2004; Petnicki-Ocwieja et al., 2002). In fact, based on their putative cleavage sites, it appears that the whole YopT/AvrPphB family of effectors in bacterial plant pathogens have putative myristoylation sites. Therefore, it is clear that the plasma membrane is a major site of action for effectors. In the cases of AvrRpt2 and AvrPphB, their respective targets, RIN4 and PBS1, also are localized to the plasma membrane (Axtell and Staskawicz, 2003; Shao et al., 2003).

Some phytopathogen T3SS effectors are localized to the plant nucleus. The AvrBs3 and YopJ/P/AvrBsT families have members that localize to the nucleus (Deslandes et al., 2003; Hotson et al., 2003; Van den Ackerveken et al., 1996; Yang and Gabriel, 1995). The mechanisms for nuclear transport may differ among effectors. For example, members of the AvrBs3 family carry nuclear localization signals (NLS) (Van den Ackerveken et al., 1996; Yang and Gabriel,
Pepper importin α interacted with the NLS sequences of AvrBs3 in a yeast two-hybrid analysis (Szurek et al., 2001), and this host protein, along with importin β, is involved in directing eukaryotic proteins to the nucleus. In contrast, the PopP2 effector appears to help direct the R protein RRS-1 to the nucleus because RRS-1 is only nuclear-localized in the presence of PopP2 (Deslandes et al., 2003). HopN1 and other T3SS effectors have been predicted to localize to the chloroplast of plant cells (Rodríguez-Herva et al., 2012; Guttman et al., 2002).

1.7.5 Effectors as double agent

Some effectors can promote lesion formation in susceptible hosts without a commensurate effect on bacterial growth. These include members of the AvrBs3/PthA family and HopPtoM (Badel et al., 2003; Yang et al., 1994). HopPtoM is encoded in the conserved effector locus of P. syringae, which is linked to the hrp/hrc cluster. HopPtoN is another effector encoded in this region in P. syringae pv. tomato DC3000, which has the opposite effect on lesion formation in host tomato: a hopPtoN mutant produces more necrotic lesions than does wild-type DC3000 (López-Solanilla et al., 2004). But in neither the hopPtoM nor the hopPtoN mutant has a commensurate effect on bacterial growth. In planta bacterial growth is approximately same for the two mutants and the wild-type, regardless of the severity of the lesion symptoms. One interpretation is that disease lesions represent a “delayed HR” resulting from delayed (or suppressed) recognition of HopPtoM and that suppressors like HopPtoN can further delay such defensive recognition. This scenario is consistent with the double agent abilities of many effectors and with the observed similarity in gene expression profiles of Arabidopsis plants at the time plant cells are dying in both compatible and incompatible P. syringae interactions (Tao et al., 2003).

AvrPtoB in P. syringae pv. tomato DC3000 is a novel example of an effector acting as a double agent in promoting pathogenesis. AvrPtoB is able to suppress programmed cell death in tomato plants lacking the Pto resistance gene (Abramovitch et al., 2003). However, AvrPtoB possesses an N-terminal domain that can elicit the HR in these otherwise susceptible tomato plants and a C-terminal domain that is required to suppress the HR triggered by the N-terminal domain. Thus, a DC3000 mutant that produces only the N-terminal domain of AvrPtoB is avirulent on tomato unless complemented with full-length AvrPtoB (Abramovitch et al., 2003). It is noteworthy that several of the effectors recently demonstrated to have HR suppressor activity are members of effector families that were previously shown to have avirulence activity (Jamir et al., 2004). Thus, many effectors may be double agents, and whether their elicitor or suppressor activity prevails
will depend on the complement of R proteins in the host, on the complement of effectors in the bacterium, and most likely also on quantitative factors associated with the timing and level of delivery relative to other effectors.

1.8 Recognition of Avr effectors and activation of R proteins

Many R genes have been identified in diverse plant species. To date, 73 R genes (Sanseverino et al., 2009) and more than 40 AVR genes (Gabriel, 1999) have been identified. Most of these R proteins have a conserved nucleotide binding domain (NBS) and a C-terminal leucine rich repeat (LRR). Based on the structural characteristics of their N-terminus domain, NBS-LRR type of R proteins can be classified into TIR (Toll and Interleukin 1 Receptor)- and CC (Coiled-coil)-NBS-LRR proteins (Baker et al., 1997; Staskawicz et al., 2001; Martin et al., 2003). Other R proteins include transmembrane receptor like proteins, such as Xa21 from rice and Cf family proteins from tomato (Romeis, 2001; Rivas and Thomas, 2005), variants of NBS-LRR proteins, such as Pita from rice, RRS1 and SLH1 from Arabidopsis (Bryan et al., 2000; Lahaye, 2004; Noutoshi et al., 2005), cytosolic kinase like proteins such as Pto from tomato and Rpg1 from maize, or R proteins that do not fall into any of the above classes (reviewed in Martin et al., 2003; McDowell and Woffenden, 2003). An inducible R gene, Xa27, has been characterized in rice-Xanthomonas oryzae interaction (Gu et al., 2005). Xa27 shares identical sequence in the coding region with its susceptible allele. However, only the resistant allele is directly induced by Xanthomonas oryzae pv. oryzae strains containing avrXa27, a AvrBs3/PthA family effector protein with conserved nucleotide binding motifs and transcription activation domain. The differential expression of Xa27 resistant and susceptible alleles is caused by the sequence variation in their promoter region that is responsible for the disease outcome (Gu et al., 2005). The identification and characterization of Xa27 and its avirulence effector AvrXa27 provide a new model of AVR-R interaction in which avirulence effector directly binds to the promoter region of R genes and induces R gene expression.

Most R genes rely on their protein products to carry out AVR recognition except Xa27. There are two widely discussed models explaining how R proteins recognize AVR proteins: the ligand-receptor model and the guard-guardee model (Fig. 1-2). In the ligand-receptor model, R proteins act as receptors for AVR effectors. The binding of the AVR protein triggers R protein activation and disease resistance. The most likely candidate R proteins that fit in this model should have been the typical transmembrane receptor like R proteins because they structurally mimic known
**Ligand-Receptor model**

R protein directly recognize Avr protein and activate defense response

**Guard-Guardee model**

Effector modify host target and this modification is recognized by R protein and activate defense response

**Fig. 1-2.** Models to explain gene-for-gene recognition
receptor proteins. However, direct binding between these R proteins and their AVR effectors has never been demonstrated (Luderer et al., 2001). At present, there are only three examples of direct AVR-R interaction: The AvrPita-Pita interaction in Magnaporthe grisea-rice pathosystem (Bryan et al., 2000), the PopP2-RRS1 interaction in R. solanacearum-Arabidopsis pathosystem (Deslandes et al., 2003) and the AvrL567-L5, 6, 7 interactions in Melampsora lini-flax pathosystem (Dodds et al., 2006). Both Pita and RRS1 are NBS-LRR proteins. Pita has a typical NBS domain, a N-terminal CC domain and a C-terminal LRR domain (Bryan et al., 2000). RRS1 is a TIR-NBS-LRR type of R protein with unique features. RRS1 has two tandem TIRs at its N terminus and a WRKY domain at its C terminus. Upon binding PopP2, RRS1 translocates to the nucleus and regulates gene expression (Deslandes et al., 2003). L5, 6, 7 are flax rust resistance TIR-NBS-LRR proteins recognizing the secreted, 127 amino acid Avr567 protein variants encoded in the highly polymorphic Avr567 locus (Dodds et al., 2004). There were 12 Avr567 variants identified and named from A to L. Each individual variant has been tested for its interaction with L5, L6 and L7 respectively in the yeast two-hybrid system. The specificity and strength of the interactions correlate well with the HR eliciting ability of Avr567 variants and with the virulence level of rust strains carrying those variants. For example, Avr567-D interacts with L6 but not L5. Consistently, AvrL567-D specifically induces a necrotic response in L6 and is not able to infect L6 plants. Another Avr567 variant, Avr567-B slightly interacts with L6 and only triggers a weak chlorotic response and weak resistance in L6 plants (Dodds et al., 2006).

Compared to the ligand-receptor model, the guard-guardee model seems to explain the activity of a greater number of R proteins. The central idea of guard-guardee model, or the guard hypothesis, is that AVR effectors mediate virulence activity by targeting and modifying host component(s). R proteins detect the modification and trigger disease resistance. Based on this hypothesis, the host targets should interact with the AVR effectors in planta and enhance pathogen virulence in the absence of the corresponding R gene. To date, there are four plant proteins identified as targets of AVR effectors. They are Arabidopsis RIN4 (RPM1 interacting protein 4) targeted by P. syringae effectors AvrB, AvrRpm1 and AvrRpt2 (Mackey et al., 2002; Mackey et al., 2003; Axtell et al., 2003), Arabidopsis PBS1 (required for AvrPphB/RPS5-mediated resistance) by P. syringae effector AvrPphB (Shao et al., 2003), tomato Pto (resistance to P. syringae pv. tomato) by P. syringae effector AvrPto (Tang et al., 1996; Pedley and Martin, 2003) and tomato Rcr3 (required for Cf-2 mediated resistance) by Cladosporium fulvum effector Avr2 (Kruger et al., 2002; Rooney et al., 2005). However, none of these plant targets have been shown to assist virulence
activity of AVR effectors, jeopardizing the virulence criterion for host targets in guard-guardee model. To explain this, the guard hypothesis has been modified, and a guard-multiple guardee model has been proposed (Lim and Kunkel, 2004; Belkhadir et al., 2004). Supporting the modified guard hypothesis, a second host target for AvrB has recently been identified (Shang et al., 2006). RAR1 (required for Mla12 resistance), a well-studied signaling component required for R gene activity, is found to negatively regulate cell wall defense and mediate AvrB triggered leaf chlorosis and bacterial growth enhancement. A point mutation at the end of RAR1 CHORII domain abolished its interaction with SGT1b (suppressor of the G2 allele of Skp1), a cochaperone of HSP90 (heat shock protein), and the AvrB virulence activity in Arabidopsis (Shang et al., 2006), suggesting the involvement of SGT1 in AvrB virulence activity. Like RIN4, RAR1 could be an ancient basal defense regulator that is exploited by bacterial effectors to carry out virulence activity. Further exploration of how AvrB modifies RAR1 and how R proteins guard the AvrB-RAR1 complex will shed light on the molecular mechanism of AVR recognition. Besides these two models, it is noteworthy that some effectors are processed by host proteins before coming into contact with host targets. A good example is AvrRpt2. AvrRpt2 is secreted as a 28 kD protein into plant cell. Inside the plant cell, AvrRpt2 is able to cleave its own N terminus 71 amino acid peptide (Mudgett and Staskawicz, 1999). The self-process and the HR eliciting ability of AvrRpt2 require AtROC1, an Arabidopsis cyclophilin peptidyl-prolyl cis/trans isomerase (Coaker et al., 2005; Coaker et al., 2006). Cyclophilin is a well-known chaperone functioning in protein folding. Refolding and processing of type III effectors in the host might be a general mechanism for their activation (Joosten and de Wilt, 1999; Coaker et al., 2006).

1.9 PAMPs and their recognition by the host

PAMPs, also called MAMPs (microbe associated molecular patterns), refer to pathogen structures or components, usually indispensable for the microbial lifestyle, that are not found in potential hosts (Nurnberger et al., 2004). Some PAMPs are not critical for bacteria survival but mediate bacterial virulence activity and are required for bacteria pathogenesis. For example, flagellum is dispensable for bacteria viability whereas critical for bacteria motility. A bacterium lacking functional flagellin synthesis or flagellum assembly genes is unable to exert full virulence on its hosts (Takeuchi et al., 2003; Tans-Kersten et al., 2001). Flagellin has been identified as a general elicitor in boiled P. syringae pv. tabaci crude extracts which induce strong medium alkalization in tomato cell cultures (Felix et al., 1999). A conserved N-terminal 22 amino acid peptide of flagellin (flg22) has full elicitor activity and triggers strong growth inhibition in A. thaliana
seedlings (Gomez-Gomez et al., 1999). The fls2 gene controlling the flg22-induced growth inhibition was identified in a genetic screen. FLS2 is an LRR-RLK sharing high sequence similarity with the resistance protein Xa21 (Gomez-Gomez and Boller, 2000). Flg22 directly binds to FLS2 (Chinchilla et al., 2006). Upon flagellin binding, FLS2 rapidly activates a downstream MAPK (mitogen-activated protein kinase) cascade, WRKY family transcription factors (Asai et al., 2002), ion channels, the NADPH oxidase complex (Gomez-Gomez and Boller, 2002), defense gene expression (Zipfel et al., 2004) and callose deposition (Gomez-Gomez et al., 1999; Kim et al., 2005).

The cytosolic PAMPs, cold shock protein (CSP) and the elongation factor EF-Tu has been characterized recently and found to elicit innate immune responses in plants (Felix and Boller, 2003; Kunze et al., 2004). The PRR recognizing CSP has not been identified. The Cold shock protein was identified in an experiment initially designed to characterize peptidoglycan, a surface exposed PAMP. Peptidoglycan from Gram-positive bacteria activates innate immunity in animal system (Michel et al., 2001). Commercially available peptidoglycan from Micrococcus lysodeikticus elicits strong medium alkalinization in cultured cells of Solanales species such as tobacco, potato and L. peruvianum. Another cytosolic PAMP, EF-Tu triggers medium alkalinization in Arabidopsis and other Brassicaceae family plants. Peptides containing the N-terminus 18-26 amino acids, elf18 to elf26, retain the full elicitor activity. Arabidopsis plants treated with elf18 exhibit enhanced oxidative burst, ethylene production and resistance to bacterial pathogens (Kunze et al., 2004). EF-Tu and flagellin induce a common set of responses and are perceived by closely related LRR-RLKs (Zipfel et al., 2006).

The perception of PAMPs is mediated by a group of pattern recognition receptors (PRRs), including transmembrane Toll-like receptors (TLRs) and cytosolic Nod proteins in animals as well as receptor like kinases (RLKs) and NBS-LRR proteins in plants (Ausubel, 2005). However, a group of RLKs and RLPs (receptor like proteins) have been identified as important players in plant growth, development and plant-microbe interactions (Torii, 2004). In the Arabidopsis genome, there are more than 600 RLK family members (Morillo and Tax, 2006). About 200 of them, including the well-characterized RLKs such as CLV1 (CLAVATA1, Clark et al., 1997), BRI1 (brassinosteroid-insensitive 1, Li and Chory, 1997), FLS2 (flagellin sensing 2, Gomez-Gomez and Boller, 2002) and the recently identified EF-Tu receptor EFR1 (elongation factor Tu receptor 1, Zipfel et al., 2006), belong to the LRR-RLK subfamily (Torii, 2004).
1.10 Aim of the work

One of the major goals of studying plant-pathogen interaction is to develop strategies to protect crop plants from various diseases and reduce the loss in crop production. As of today, many effector genes have been identified in model pathogen *R. solanacearum* and *P. syringae*. Effectors are virulence factors that promote disease in compatible interactions, but function as avirulence factors that induce resistance responses in incompatible interactions. To date, very few *avr* genes have been identified in these pathogens and their corresponding *R* genes are rarely known. With the discovery of more *Avr-R* pairs, there will be more cultivar specific resistance genes available for disease management. The main objectives of this dissertation are to:

(i) identify *Avr* effector in *R. solanacearum* RS1002.
(ii) study the functions of *Avr* effector of *R. solanacearum* RS1002 on *Solanum torvum* and
(iii) elucidate the role of HopH1 effector of *P. syringae* on *S. torvum*
(iv) elucidate the mechanism of *Avr* effector induced HR on *S. torvum*. 
CHAPTER II

RALSTONIA SOLANACEARUM TYPE III SECRETION SYSTEM
EFFECTOR RIP36 INDUCES HYPERSENSITIVE RESPONSE IN
THE NONHOST WILD EGGPLANT SOLANUM TORVUM

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secretion system effector Rip36 induces a hypersensitive response in the nonhost wild
I. SUMMARY

*Ralstonia solanacearum* is a Gram-negative soilborne bacterium that causes bacterial wilt disease in more than 200 plant species, including economically important *Solanaceae* species. In *R. solanacearum*, the Hrp type III secretion system is required for both the ability to induce hypersensitive response (HR) in nonhost plants and pathogenicity in host plants. Recently, a total of 72 effector genes called *rip* (*Ralstonia* protein injected into plant cells) were identified in *R. solanacearum* RS1000. RS1002, a spontaneous nalixidic acid–resistant derivative of RS1000, induced strong HR in the nonhost wild eggplant *Solanum torvum* in a Hrp-dependent manner. An *Agrobacterium*-mediated transient expression system revealed that Rip36, a putative Zn-dependent protease effector of *R. solanacearum*, induces HR in *S. torvum*. A mutation in the putative Zn-binding motif (E149A) completely abolished the ability to induce HR. In agreement with this result, the RS1002-derived Δrip36 and rip36E149A mutants had lost the ability to induce HR in *S. torvum*. An E149A mutation had no effect on the translocation of Rip36 into plant cells. These results indicate that Rip36 is an avirulent factor that induces HR in *S. torvum* and that a putative Zn-dependent protease motif is essential for this activity.

II. INTRODUCTION

*Ralstonia solanacearum* is a Gram-negative soilborne bacterium that causes bacterial wilt disease in more than 200 plant species in over 50 plant families, including many economically important crops, such as potato, tomato, tobacco, banana, and eggplant, worldwide (Hayward, 1991; Lebeau et al., 2010). The pathogen enters host plants through natural openings or wounds in the root system, penetrates the xylem vessels, colonizes the root cortex and vascular parenchyma, proliferates and spreads throughout the vascular system, and produces a large amount of exopolysaccharide (EPS) that blocks water transport, resulting in the wilting and death of infected plants (Genin and Denny, 2012; Schell, 2000). In addition, *R. solanacearum* secretes plant cell wall-degrading enzymes such as endopolygalacturonase and endoglucanase that break down pectins and cellulosic glucans in the cell wall (Gonzalez and Allen, 2003; Liu et al., 2005).

Numerous Gram-negative plant pathogenic bacteria use the Hrp type III secretion system (T3SS) encoded by a cluster of approximately 20 hypersensitive response (HR) and pathogenicity (*hrp*) genes (Alfano and Collmer, 1997; Galan and Wolf-Watz, 2006; Tang et al., 2006; Van Gijsegem
et al., 2000). Basically, Hrp T3SS acts as a specialized system for injection of virulence factors, the so-called effector proteins, from pathogens into host plant cytoplasm. Generally, effector proteins modulate host cellular functions to establish infection and promote propagation in plants. Recent studies have unveiled biochemical activities of several T3SS effectors, including proteases, phosphatases, ubiquitin ligases, ribosyltransferases, transcriptional activators, and phosphothreonine lyases (Dean, 2011; Deslandes and Rivas, 2012; Grant et al., 2006; Hann et al., 2010; Staskawicz et al., 2001; Zhou and Chai, 2008). Like many other plant pathogenic bacteria, Hrp T3SS is essential for the growth of R. solanacearum in host plants because Hrp T3SS-deficient mutants completely lose pathogenicity, and effector proteins collectively contribute to virulence because mutation of a single effector gene produces little or no effect in most cases (Boucher et al., 1987; Cunnac et al., 2004; Kanda et al., 2003; Mukaihara et al., 2004; Zolobowska and Van Gijssegem, 2006). In R. solanacearum strain GMI1000, 74 T3SS effector proteins have been identified by in silico analysis of whole genome sequence and functional genomic approaches (Cunnac et al., 2004; Poueymiro and Genin, 2009). To date, three effector proteins, namely, AvrA, PopP1, and PopP2, have been shown to act as avirulence factors in nonhost and incompatible host plants. For example, AvrA is responsible for HR elicitation in Nicotiana tabacum and N. benthamiana (Carney and Denny, 1990; Poueymiro et al., 2009; Robertson et al., 2004). PopP1, a member of the YopJ/AvrRxv (C55) family of cysteine proteases, is the major HR elicitor in Petunia St40 (Lavie et al., 2002) and N. glutinosa (Poueymiro et al., 2009). PopP2, another member of the YopJ-like family, confers RRS1-R-mediated resistance on Arabidopsis (Deslandes et al., 2003). Mutation of the conserved cysteine residue in the catalytic triad of PopP2 abolishes its ability to induce RRS1-R-mediated resistance in Arabidopsis (Tasset et al., 2010). Some of the abovementioned effector proteins have been shown to contribute to virulence in host plants. For example, AvrA is required for the early stage of root infection of the legume Medicago truncatula (Turner et al., 2009) and bacterial fitness on tomato leaves (Macho et al., 2010). Macho and his associates (2010) also showed that PopP2 is required for bacterial fitness on eggplant or bean. Thus, effector proteins play important roles in the interaction between R. solanacearum and its host/nonhost plants.

Plants have evolved the ability to recognize potential pathogens by detecting conserved microbial molecules, such as bacterial flagellin, elongation factor, and peptidoglycans, called PAMPs/MAMPs (pathogen/microbe-associated molecular patterns), leading to activation of the basal defense response, called PTI (PAMP-triggered immunity) (Monaghan and Zipfel, 2012). Subsequently, pathogens developed effector proteins to suppress PTI for successful infection.
Furthermore, plants developed a system for recognition of bacterial effectors through sensing their virulence by the product of the resistance (R) gene. Therefore, effectors are virulence factors that promote disease in compatible interactions, but function as avirulence factors that induce resistance responses in incompatible interactions. Understanding the mechanism by which an Avr protein is recognized by the corresponding R protein may lead to identification of the virulence function of the effector protein and the detection system in plants, and contribute to engineering more-durable resistance in crops.

III. RESULTS

1. Identification of Rip36 as the major determinant responsible for HR elicitation on Solanum torvum

Infiltration of R. solanacearum RS1002, a spontaneous nalixidic acid–resistant derivative of RS1000 (Table 1), into the leaves of S. torvum Sw. cv. Torubamubiga induced HR, a rapid cell death in the infiltrated area (Fig. 2-1A). It has been shown that the wild eggplant S. torvum is highly tolerant of most of the R. solanacearum strains (Clain et al. 2004; Gousset et al. 2005). Hence, this plant species is widely used as rootstock for eggplant or tomato cultivation. In R. solanacearum strain RS1000, 72 T3SS effector proteins, the so-called Rip (Ralstonia protein injected into plant cells) have been identified using a calmodulin-dependent adenylate cyclase (Cya) reporter system, and their translocations into plant cells have been demonstrated (Mukaihara et al., 2010). To identify the RS1000 effector protein eliciting HR, I cloned the entire coding region of each rip gene into a binary vector pEl2Ω-MCS (Fig. 2-2A, Ohtsubo et al., 1999) under the control of the high expression promoter 35S-Ω. Agrobacterium tumefaciens strain GV3101 was transformed by the resultant plasmids, and then each Rip was transiently expressed in leaves of S. torvum and tobacco (N. tabacum L. cv. Xanthi NC) via agroinfiltration. I tested sixty-four RS1000 effectors and found that Rip36 specifically induced HR in S. torvum leaves, but not in tobacco leaves (Fig. 2-2B, -2C, and -2D). Transient expression of AvrA, an avirulence protein in tobacco leaves (Carney and Denny, 1990; Robertson et al., 2004; Poueymiro et al., 2009), specifically induced HR only in tobacco leaves, indicating that the system worked effectively.

To confirm the results obtained from the transient expression analysis in R. solanacearum, I constructed rip36 and avrA mutant derivatives of strain RS1002. In S. torvum, HR was induced by inoculation of strains RS1002 (parental wild-type, WT) and RS1650 (avrA::Gm+) but not of
strains RS1662 (∆rip36) (Fig. 2-1A). HR in tobacco leaves was induced by inoculation of the WT, and ∆rip36 strains, but not by that of the avrA::Gm' strain (Fig. 2-1B). This result clearly indicates that Rip36 and AvrA of RS1002 are required for HR in S. torvum and tobacco, respectively.

To confirm that Rip36 is a crucial avirulence factor in the induction of HR in S. torvum, I introduced the WT rip36 gene into a ∆rip36 mutant strain, and generated complemented strain. The leaves of S. torvum were infiltrated with the resultant complemented strain, RS1672 (∆rip36 Tnrip36'). As expected, HR in S. torvum leaves was restored by the introduction of Tnrip36' (Fig. 2-1C). This result further confirmed that Rip36 is an avirulent factor that induces HR in S. torvum.

2. Mutation in Zn-dependent protease domain of Rip36 abolishes the HR inducing activity on S. torvum

Rip36 is a Zn-dependent protease-like T3SS effector with a putative Zn-binding motif (HExxH). Therefore, we next investigated the molecular mechanism of Rip36-induced HR in S. torvum. To examine whether the HExxH motif of Rip36 is required to induce HR, we changed the essential glutamic acid of position 149 to alanine (HELIH to HALIH) using a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the gene designated as rip36E149A. WT rip36 and rip36E149A genes were cloned into a binary vector pEl2Ω-MCS (Ohtsubo et al., 1999) and transiently expressed in leaves of S. torvum and tobacco (N. tabacum L. cv. Xanthi NC) via a transient Agrobacterium-mediated expression system. I found that Rip36E149A no longer induced HR in S. torvum (Fig. 2-2). This result indicates that the putative Zn-dependent protease motif of Rip36 is required for HR elicitation in S. torvum.

Furthermore, Rip36 abolished the ability to induce HR in S. torvum, when the Zn-binding motif HELIH was changed to HALIH, indicating that the putative Zn-dependent protease motif is required for HR elicitation in S. torvum. To confirm that the Zn-protease motif is essential for the induction of HR in S. torvum, I have generated rip36E149A mutant strain RS1668 and also introduced the rip36E149A gene into a ∆rip36 mutant strain, and generated complemented strain.
Fig. 2-1. Hypersensitive responses induced by inoculation of *Ralstonia solanacearum* wild-type (WT) RS1002 and its derivatives in *Solanum torvum* Torubamubiga (A, C) and tobacco leaves (B). Leaves were infiltrated with bacterial suspensions (OD$_{600}$ of 0.3) of *R. solanacearum* RS1002 (WT), RS1662 (Δrip36), RS1668 (rip36E149A), RS1650 (avrA::Gmr), RS1273 (ΔhrpY), or the complemented strains RS1672 (Δrip36 Tnrip36+) or RS1673 (Δrip36 Tnrip36E149A), and incubated under a 16 h light/8 h dark cycle at 28°C. Photographs show representative results of *S. torvum* at 1 dpi (A, C) or tobacco at 2 dpi (B) from three independent experiments, which gave similar results. The dotted lines represent the infiltrated area. (D) Summary of the plant responses caused by various strains in *S. torvum* and tobacco leaves. NT: not tested.
Fig. 2-2. Effects of *Agrobacterium*-mediated transient expression of Rip36, Rip36E149A, and AvrA in *Solanum torvum* Torubamubiga and tobacco leaves. (A) Construction of a series of pEl2Ω-MCS plasmids expressing the Rip effector. Leaves were infiltrated with *Agrobacterium tumefaciens* GV3101 harboring no plasmid, an empty plasmid, a plasmid expressing Rip36, Rip36E149A, or AvrA at OD$_{600}$ of 0.5, and incubated under a 16 h light/8 h dark cycle at 28°C. Photographs show representative results of *S. torvum* at 2 dpi (B) or tobacco at 4 dpi (C) from three independent experiments, which gave similar results. The dotted lines represent the infiltrated area. (D). Summary of plant responses.

<table>
<thead>
<tr>
<th>Test plants</th>
<th>rip36</th>
<th>rip36E149A</th>
<th>avrA</th>
<th>Empty vector</th>
<th>No vector</th>
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</thead>
<tbody>
<tr>
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<td>No HR</td>
<td>No HR</td>
<td>No HR</td>
<td>No HR</td>
</tr>
<tr>
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<td>No HR</td>
<td>No HR</td>
<td>HR</td>
<td>No HR</td>
<td>No HR</td>
</tr>
</tbody>
</table>
The leaves of *S. torvum* were infiltrated with the resultant mutant RS1668 (rip36E149A) and complemented strain RS1673 (Δrip36 Tnrip36E149A). As expected, HR was not induced by the inoculation of rip36E149A mutant strain (Fig. 2-1A) and HR in *S. torvum* leaves was not restored by the introduction of Tnrip36E149A (Fig. 2-1C). This result further confirmed that the Zn-protease motif is indispensible for the induction of HR.

3. Translocation and stability of Rip36 into plant cells is independent of Zn-protease motif

To exclude the possibility that the E149A mutation of Rip36 affects the ability to translocate into plant cells via the Hrp T3SS, we constructed *R. solanacearum* strains expressing the Rip36-HA-′Cya or Rip36E149A-HA-′Cya fusion proteins. An in planta adenylate cyclase assay was carried out as previously described (Murata *et al.*, 2006). RS1273 (ΔhrpY) was used as a negative control because this strain lacks the ability to translocate effectors into plant cells (Mukaihara *et al.*, 2009). The cAMP in *S. torvum* leaves inoculated with the WT strain expressing Rip36E149A-HA-′Cya was increased to a level similar to that in leaves inoculated with the strain expressing Rip36-HA-′Cya (Fig. 2-4A). As predicted, the increase in the cAMP level was completely abolished by the ΔhrpY mutation (Fig. 2-4A). This result indicates that the E149A mutation in the putative Zn-binding motif has no effect on the translocation of Rip36 into plant cells.

I further tested the stability of Rip36E149A protein by immunoblot analysis. The leaves of 5- to 6-wk-old *S. torvum* were infiltrated with *R. solanacearum* strains RS1002 (WT), RS1662 (Δrip36), and RS1668 (rip36E149A). After 15 h, total protein was prepared from the infiltrated parts of leaves and investigated by Western blot analysis after SDS-PAGE as described previously (Nguyen *et al.*, 2012). Bands corresponding to the expected size of Rip36 protein were detected in WT and rip36E149A mutant strains, whereas no band was detected in the Δrip36 strain (Fig. 2-4B). On the basis of these results, it is clear that the point mutation protein Rip36E149A is as stable as WT Rip36 and that the E149A mutation in the Zn-protease motif has no effect on the translocation and stability of Rip36 in plant cells.
Fig. 2-3. Schematic diagram of generation of *Ralstonia solanacearum* strains expressing Rip36-’Cya and Rip36E149A-’Cya fusion protein.
**Fig. 2-4.** Translocation and stability of Rip36 and Rip36E149A. (A) The cAMP levels of *Solanum torvum* leaves inoculated with *Ralstonia solanacearum* RS1002 (*hrp*) or RS1273 (*ΔhrpY*) strains expressing the calmodulin-dependent adenylate cyclase (Cya) fusion with Rip36 or Rip36E149A. cAMP level is shown as an average of three replications with standard deviation in brackets. (B) The stability of Rip36 and Rip36E149A in *S. torvum* leaves after infiltration. Leaves of *S. torvum* were infiltrated with RS1002 (lane 1) and the *rip36*E149A (lane 2) and *Δrip36* (lane 3) mutants, and total proteins were prepared from the inoculated leaves at 15 h post inoculation. The Rip36 and Rip36E149A proteins were detected by an anti-Rip36 peptide antibody after an SDS-PAGE analysis. Photograph shows a representative result of Western blotting from two independent experiments. An arrow indicates the position of the 20 kDa marker protein.
4. Role of rip36 in pathogenicity of *R. solanacearum* on host and nonhost egg plant

To examine whether Rip36 contributes to the virulence of *R. solanacearum* on host eggplant (*Solanum melongena* cultivar Senryo-nigou), RS1002 (WT) and the Δrip36 and rip36E149A mutants (5 × 10⁴ cfu/ml) were infiltrated into eggplant leaves and bacterial growth was measured at 2 and 5 days post inoculation (dpi). This quantitative assay is reported to be more sensitive than the disease scoring method (Macho *et al.*, 2010). However, no significant difference in bacterial multiplication was observed between RS1002, Δrip36, and rip36E149A mutant strains at either 2 or 5 dpi (Fig. 2-5A). This result suggests that Rip36 has no or little effect on the virulence of *R. solanacearum* in host eggplant, probably due to the functional redundancy of a large effector repertoire, as reported (Mukaihara *et al.*, 2010).

We further measured the bacterial growth on nonhost *S. torvum*. The leaves of *S. torvum* were infiltrated with RS1002 (WT) and the Δrip36 and rip36E149A mutants (5 × 10⁴ cfu/ml), and bacterial growth was measured at 2 and 5 dpi. The Δrip36 and rip36E149A mutants significantly increased in *S. torvum* leaves as compared with the WT RS1002 both at 2 and 5 dpi (Fig. 2-5B). This indicates that Rip36 is the major avirulence factor in RS1002 that elicits resistance and restricts bacterial growth in *S. torvum* leaves.

IV. DISCUSSION

In this study, we showed that the *R. solanacearum* T3SS effector Rip36 functions as an avirulence protein to induce HR in *S. torvum*. We also showed that the putative Zn-dependent protease motif of Rip36 is essential for its avirulence function. In the fungal pathogen *Magnaporthe grisea*, the causal agent of rice blast disease, an AVR-Pita avirulence protein is known to contain a Zn-dependent protease motif HExxH. Three important conserved residues in this motif are two histidines (His-176 and His-180) acting as ligands of the catalytic zinc and the glutamate (Glu-177) which is the active site residue. The protease motif is essential for the avirulence function of AVR-Pita because a point mutation in the conserved motif completely abolished the ability to induce rice blast resistance mediated by the corresponding disease resistance (R) protein Pi-ta in rice (Orbach *et al.*, 2000).
Fig. 2-5. Effect of *rip36* mutation on the growth of *R. solanacearum* RS1002 on host eggplant (A) and nonhost *S. torvum* (B). Leaves were infiltrated with wild-type (WT) RS1002 or *Δrip36* and *rip36E149A* mutants at 5 × 10^4 cfu/ml. Bacterial growth were measured 2 and 5 days after inoculation. Error bars indicate the standard deviation measured from six biological replicates. Means of three independent experiments are presented. Asterisks indicate a significant difference from the growth of the RS1002 WT strain in a *t* test (***, *P*<0.001).
It is also known that AVR-Pita interacts directly with Pi-ta and that a mutation in the protease motif results in the loss of its avirulence and disrupts the physical interaction between them. This direct interaction raises the possibility that the Pi-ta protein may sense the cleavage by AVR-Pita (Jia et al., 2000). Our results also indicate that the Zn-protease activity of Rip36, like Avr-Pita, is required for the induction of HR, although the enzymatic activity has not been tested yet.

It was reported that the Rip36 homologue NleD from enteropathogenic Escherichia coli cleaves c-JUN N-terminal kinase by its Zn-dependent protease activity and inhibits the inflammatory reaction, an animal innate immunity response (Baruch et al., 2011). In R. solanacearum, Rip36 might cleave a particular S. torvum protein to exhibit its virulence function, and then the resultant peptide fragment might be specifically recognized by a resistance protein, leading to HR. The Rip36 homologue HopH1 from P. syringae pv. tomato DC3000 may contribute to virulence on host plants because deletion of both hopH1 and hopC1 from this pathogen reduced both lesion formation and growth in Arabidopsis and tomato (Wei et al., 2007). It is interesting that the putative protease motif of HopH1 is required for its virulence function. The mechanism of HR induction by Rip36 might be similar to the cysteine protease effectors AvrRpt2 from P. syringae pv. tomato DC3000 (Axtell and Staskawicz, 2003) and AvrPphB from P. syringae pv. phaseolicola (Shao et al., 2002). AvrRpt2 and AvrPphB effectors respectively cleave Arabidopsis RIN4 and PBS1 proteins, whose cleavage is monitored by the corresponding R proteins RPS2 and RPS5, respectively (Mackey et al., 2003, Shao et al., 2003). To reveal the mechanism of Rip36-induced HR in S. torvum, identification of the target protein is now under investigation.

V. EXPERIMENTAL PROCEDURE

1. Bacterial strains, media and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Each strain of Ralstonia solanacearum was maintained in BG medium with 50 µg/ml polymixin B (Boucher et al. 1985) at 28°C, and Escherichia coli strains were grown at 37°C in Luria-Bertani (LB) medium (Miller, 1992). When required, antibiotics were added to the following final concentrations: nalidixic acid 30-50 µg/ml and kanamycin 20 µg/ml.
2. Plant materials and inoculation experiment

Eggplants (Solanum torvum Sw. cv. Torubamubiga and Solanum melongena cultivar senryo-nigou) were grown at 30°C in a growth chamber. Tobacco plants (Nicotiana tabacum L. cv. Xanthi NC) were grown at 26°C with a 16 h photoperiod, and leaves of 2- to 3-month-old plants were used for inoculation experiments. For HR test, bacterial strains cultured overnight in BG broth medium were harvested after centrifugation. The cell pellets were washed with distilled water (DW), then suspended in distilled water at a density of OD$_{600}$=0.3 and infiltrated into 5 to 6 weeks old Torubamubiga and 2 to 3 months old tobacco leaves with a needleless syringe as described (Sol et al., 2012). Bacterial growth was examined as described (Macho et al., 2010), 4 to 5 weeks old eggplant (senryo-nigou) and S. torvum leaves were infiltrated with bacterial suspension at a density of 5×10$^4$ cfu/ml. For each strain three biological replicates were taken and each contains four leaf disks of 8 mm in diameter. Leaf disks were soaked in 15% H$_2$O$_2$ for 1 min to sterilize the leaf surfaces and were then washed with sterile distilled water. Then, leaf disks were ground with a mortar and pestle. The homogenates in distilled water were plated on BG plates after serial dilutions. After 48 h incubation at 28°C, the colonies were counted and the bacterial populations were calculated.

3. Agrobacterium-mediated transient expression of proteins in plant cells

The entire open reading frame of each rip gene was cloned into XbaI and SpeI site of pEl2Ω-MCS vector under the control of the high expression promoter 35S-Ω. Agrobacterium tumefaciens strain GV3101 was transformed by the resultant plasmids. Agrobacteria were grown overnight at 30°C in LB medium. Cells were pelleted at 4,000 rpm, then resuspended in infiltration medium (10 mM MgCl$_2$, 10 mM morpholineethanesulfonic acid, and 150 µM acetosyringone) and were incubated at 22°C for 3 h. Cells were harvested at 4,000 rpm and resuspended in infiltration medium and adjusted OD$_{600}$=0.5, and infiltrated into S. torvum and tobacco leaves with a 1 ml needle-free syringe as described (Poueymiro et al., 2009). The infiltrated leaves were incubated in a growth chamber for a 16 h photoperiod at 30°C.

4. Generation of mutant strains of Ralstonia solanacearum RS1002

For the construction of a Δrip36 mutant, a 0.8-kb fragment upstream of rip36 was amplified from RS1000 genomic DNA using the primers P2639 (5’-gggaattccgtacctactcgtcgtgtatatgg-3’) with an EcoRI site (underlined) and P2640 (5’-gaccctgacatgctgctgttattgctgcaagag-3’), and
similarly, a 0.8-kb fragment downstream of *rip36* was amplified using the primers P2641 (5′-ttggagcaataacgacacgtaacccattgtca agctcgcc-3′) and P2642 (5′-ggaagcttagaggacggcggcggcggag-3′) with a *Hind*III site (underlined). The two PCR fragments were purified, mixed, and further amplified using the primers P2639 and P2642 to connect the two fragments. The resultant 1.6-kb *Eco*RI-*Hind*III fragment containing a Δ*rip36* mutation was inserted into plasmid pK18*mobsacB* (Schäfer *et al.*, 1994) to produce pK18-Δ*rip36*. For the construction of a *rip36*E149A mutant, a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used. The *rip36* fragment was inserted into pHSG vector at *Eco*RI site. This double stranded DNA vector was used as the template. Two complementary oligonucleotides containing the mutation: Rip36E149A/S: 5′-gtgctctgaaagctgatccatgccggtcgcg-3′ and Rip36E149A/AS: 3′-acaggagcgggtagtga ctaggtacggccggtcgcg-5′ were synthesized. Temperature cycling, *Dpn*I digestion and transformation were performed according to the manufacturer’s instructions. The desired mutation was confirmed by DNA sequence analysis, and the *rip36*E149A fragment was inserted into the mobilizable vector pK18*mobsacB* (Schäfer *et al.*, 1994) to produce pK18-rip36E149A. The resulting plasmids were introduced into *E. coli* S17-1 by electroporation and were integrated into wild type *R. solanacearum* RS1002 by conjugation. After excised of the plasmid on BG agar plate containing 10% sucrose, mutation in the bacteria was confirmed by sequencing and designated as RS1668 (RS1002rip36E149A).

For construction of an *avrA*::Gm′ mutant, a 2.8-kb fragment containing *avrA* was amplified using the primers P2493 (5′-agatcaaggcgctgatccac-3′) and P2498 (5′-tccttgaactgcgggccgat-3′). The 2.8-kb fragment was inserted into pK18*mobsacB*, and then the Gm′ cassette was inserted into *avrA* to produce pK18-avrA::Gm′. The pK18*mobsacB* derivatives were inserted into *E. coli* S17-1 and used for the marker-exchange method (Schäfer *et al.*, 1994).

5. Generation of complemented strains of *R. solanacearum* RS1662

A set of primers, P2639 and P2642, was used to clone a 2090-bp region containing the promoter and the entire open reading frames of the WT *rip36* and *rip36*E149A genes by PCR using genomic DNA from RS1002 and RS1668 as a template, respectively. The amplified DNA fragments were inserted into the *Eco*RI site of pBSL118, a transposon vector (Alexeyev *et al.* 1995), to produce pBSL-rip36 and pBSL-rip36E149A, respectively. Each transposon vector was introduced into the Δ*rip36* mutant through conjugation with *E. coli* S17-1 λpir (Simon *et al.* 1983) to generate RS1672 and RS1673 (rip36 and rip36E149A complement).
6. Generation of *R. solanacearum* strains expressing Rip36-HA-´Cya and Rip36E149A-HA-´Cya fusion protein

To construct the Cya fusion protein, each 654-bp DNA fragment from *rip36* and *rip36E149A* open reading frames without a stop codon was amplified with primers M13 forward and Rip36-3’*Xho*I (5’-ccgtcgcgcgcgttgtgttgtactt-3’) with an *Xho*I site (underlined) and then inserted into pARO-HA-´Cya at *Xba*I and *Xho*I sites (Murata *et al.*, 2006; Fig. 2-3). The plasmids pARO-rip36-HA-´Cya and pARO-rip36E149A-HA-´Cya were integrated into *R. solanacearum* WT (RS1002, Mukaihara *et al.*, 2004) and its Δ*hrpY* derivative (RS1273, Mukaihara *et al.*, 2004) through conjugation with *E. coli* S17-1 to yield strains expressing the Rip36-´Cya and Rip36E149A-´Cya fusion protein.

7. CyaA’-based translocation assay

The procedures for the adenylate cyclase assay were previously described in Murata and associates (2006) with some modifications. Briefly, *R. solanacearum* RS1002 (WT) and RS1273 (Δ*hrpY*) strains expressing the Cya fusion protein were grown overnight in BG medium at 28°C, pelleted, washed with distilled water, then resuspended at an OD$_{600}$ of 0.5. The bacterial suspension was infiltrated into 5- to 6-week-old *S. torvum* leaves, then the leaves were incubated at 28°C in a growth chamber. After 15 h, three leaf disks (8 mm in diameter) from each sample were collected, frozen with liquid nitrogen, then ground with mortar and pestle. The leaf homogenate was boiled in 200 µl of 0.05 M sodium acetate buffer containing 0.02% bovine serum albumin for 5 min. Cell debris was removed by centrifugation twice for 10 min each, and the supernatant was collected in new tubes and stored at -80°C until assayed for cAMP. Fifty microliters of the supernatant was used to measure the cAMP with a Cyclic AMP EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA).

8. Protein purification

*R. solanacearum* strains RS1002 (WT), RS1662 (Δ*rip36*), and RS1668 (*rip36E149A*) were grown overnight in BG medium at 28°C, pelleted, washed with distilled water, then resuspended at an OD$_{600}$ of 0.3. The leaves of 5- to 6-wk-old *S. torvum* were infiltrated with the bacterial suspensions and incubated at 28°C in a growth chamber. After 15 h, the infiltrated parts of leaves were collected, frozen with liquid nitrogen, and ground with a mortar and pestle. Then, 1.5 ml of 50 mM Tris-HCl buffer (pH 7.5) was added per 0.5 mg leaf tissue. Cell debris was removed by
centrifugation twice for 10 min at 12,000 rpm, and the supernatant was collected in new tubes. Proteins were then precipitated from the supernatant by adding 5% trichloroacetic acid and incubating 1 h at 4°C. Precipitated proteins were pelleted by centrifugation and washed with 70% ethanol, dried, resuspended in 50 µl PBST buffer (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, and Tween-20, pH 7.4), and subjected to Western blot analysis after SDS-PAGE as described (Nguyen et al., 2012).

9. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot analysis

Samples were mixed 5:1 with 6x SDS sample buffer and boiled for three minutes, loaded on 15% acrylamide gel, and migrated for 1 to 3 hrs at 20 mA. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), and Western blot analysis was performed using a rabbit polyclonal anti-Rip36-specific peptide antibody with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (Bio-Rad, Hercules, CA, U.S.A.) and an alkaline phosphatase-based chemiluminescent detection system (CPD-Star Reagent, New England Biolabs, Ipswich, MA, U.S.A.) according to the manufacturer’s instructions. The synthetic peptide (peptide sequence: QGLQARSKYKPRNA, 205-218 amino acids in Rip36) and the peptide antibody were generated by Operon Biotechnologies (Tokyo, Japan) and the serum from the rabbit was collected 42 days after the initial immunization. The polyclonal antibody was affinity-purified from 10 ml of antiserum. The anti-Rip36-specific peptide antibody was used after 5,000 fold dilution with phosphate-buffered saline Tween buffer (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, and 0.1% Tween-20, pH 7.4).

10. Statistical analysis

The results of the bacterial populations in host and non-host eggplant leaves are expressed as means with the SD. The two-tailed t-test was performed for comparisons between the quantitative measurement of the WT, and each mutant strains. Values of P<0.05 were considered statistically significant.
Table 2-1. Bacterial strains and plasmids used in this study

<table>
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<th>Strains</th>
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</tr>
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</tr>
<tr>
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<tr>
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<td>RS1662 chr::mini-Tn5rip36E149A</td>
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<td></td>
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<tr>
<td>GV3101</td>
<td></td>
<td>Lamblin et al., 2001</td>
</tr>
<tr>
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<td>DH5α</td>
<td>F-∼−o80dLacZDM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK mK') supE44 thi-1gyrA relA1</td>
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<tr>
<td>S17-1</td>
<td>thi pro hsdR−hsdM + recA [chr::RP4-2-Tc::Mu-Km::Tn7]</td>
<td>Sch fer et al. (1994)</td>
</tr>
<tr>
<td>S17-1 λpir</td>
<td>λ pir lysogen of S17 (Tpr Smr thi pro hsdR−M + recA RP::2-Tc::Mu-Km::Tn7)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
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</tr>
<tr>
<td>pEl2Ω-MCS</td>
<td>Binary vector that can replicate in both E.coli and A. tumefaciens, used for transient expression analysis</td>
<td>Ohtsubo et al., 1999</td>
</tr>
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<td>Murata et al., 2006</td>
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<td>pARO-rip36-HA'-Cya</td>
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<td>Mukaihara et al., 2010</td>
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<td>pARO-HA'-Cya Cya carrying rip36E149A at XbaI and XhoI sites</td>
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<td>Small mobilizable vector, Km', sucrose sensitive (sacB)</td>
<td>Sch fer et al. (1994)</td>
</tr>
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<td>pK18-avrA::Gm'</td>
<td>pK18mobsacB-derived that contains disrupted avrA by the insertion of Gm' cassette into pK18-avrA, Gm', Km'</td>
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<td>Mini-Tn5 derived plasmid vector for insertion mutagenesis, Amp', Km'</td>
<td>Alexeyev et al., 1995</td>
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<td>pBSL118-derived that contains a 2.09-kb rip36E149A, Amp', Km'</td>
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</tbody>
</table>

† = Resistance, Amp = Ampicillin, Km = Kanamycin, NaI = Nalidixic acid, Rif = Rifampicin, Gm = Gentamycin
CHAPTER III

HOPH1 EFFECTORS OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000 AND PV. *SYRINGAE* B728A INDUCE HR ON NONHOST EGGPLANT *SOLANUM TORVUM*
I. SUMMARY

The Gram-negative phytopathogenic bacteria *Pseudomonas syringae* infects a broad range of plant species and causes significant economic losses worldwide. *P. syringae* deliver the effectors to elicit hypersensitive response (HR) in nonhost or incompatible host plants or to cause disease in compatible host plants via type III secretion system. Rip36, a putative Zn-dependent protease effectors in RS1000 induces hypersensitive response (HR) in nonhost Solanaceae, *Solanum torvum* Sw. cv. (St) Torubamubiga, and Zn-protease motif is required for the induction of HR. *HopH1*, a Rip36 ortholog was found in *P. syringae* pv. *tomato* (Pto) DC3000 and *P. syringae* pv. *syringae* (Psy) B728a but not in *P. syringae* pv. *phaseolicola* (Pph) 1448A. Both wild type (WT) and Δ*hopH1* mutant induced HR in *S. torvum*, whereas WT of Pph 1448A did not. Interestingly, when *hopH1* from Pto DC3000 and Psy B728a were introduced into Pph 1448A, the resultant strains acquired the HR inducing activity. Mutation in the putative Zn-binding motif of HopH1 (E150A) did not abolish the ability to induce HR. *P. syringae* pv. *phaseolicola* 1448A possessing truncated peptides of *hopH1*E150A,Pto did not induce HR on *S. torvum*. These results indicate that putative Zn-protease HopH1 of *P. syringae* is an avirulent factor to induce HR in *S. torvum* and the full length *hopH1* is required for this activity and E150A mutation in Zn-dependent protease motif may not inactivate the HopH1 to induce HR.

II. INTRODUCTION

*Pseudomonas syringae* is a Gram-negative host-specific pathogen and encompasses 50 or more pathovars that cause leaf spot, leaf blight, leaf speck or bacterial canker disease from the majority of cultivated crop and ornamental plant species all over the world (Agrios, 1997). This pathogen depends on the Hrp type III secretion system (T3SS) to secrete and translocate effector proteins into the plant cell, thus causing disease in compatible host plants, and triggering a hypersensitive response (HR) in nonhost and incompatible host plants (Alfano and Collmer, 1997). *P. syringae* type III effectors are designated Hrp outer protein (Hop) and avirulence (Avr) protein depending on the phenotype by which they were discovered (Lindeberg et al., 2005). Using comparative genomics and bioinformatics approach based on draft genome sequences and amino acid features in the N termini of proteins, 58 effectors were predicted in *P. syringae* pv. *tomato* DC3000 (Collmer et al., 2002, Greenberg and Vinatzer, 2003) and 29 effectors in *P. syringae* pv. *syringae* B728a (Guttmann et al., 2002, Greenberg and Vinatzer, 2003). To date 40 of the predicted
effectors in *P. syringae pv. tomato* DC3000 (Schetcher et al., 2004), 22 in *P. syringae pv. syringae* B728A (Vinatzer et al., 2006) have been confirmed. Furthermore, 44 high-probability putative Hrp promoters upstream of genes encoding the core T3SS machinery, and 27 candidate effectors and related T3SS substrates have been identified in *P. syringae pv. phaseolicola* 1448A (Vencato et al., 2006). Only 13 effectors are shared between the above three *P. syringae* strains and the remaining effectors are either unique to one of these strains or shared between two of them (based on data available at http://www.pseudomonas-syringae.org). These differences in effector repertoires between strains determine the host range in *P. syringae* (Alfano and Collmer, 2004). In *P. syringae*, T3SS effectors are associated with *htrp* promoters which responded to the HrpL alternative sigma factor (Lan et al., 2006, Ferreira et al., 2006).

A large repertoire of effectors identified in *Pseudomonas* genome. A few of them have been ascribed a role in basic pathogenicity as well as avirulence, for example HopQ1 also known as HopQ1-1 in *P. syringae pv. tomato* DC3000 enhances bacterial virulence and associates with tomato 14-3-3 proteins in a phosphorylation dependent manner and transgenic tomato plants expressing HopQ1 exhibited enhanced susceptibility to virulent *Pto* as well as the *Pto ΔhrcC* mutant (Li et al., 2013). HopQ1 also acts as an avirulence determinant in *Nicotiana benthamiana* for *P. syringae pv. tomato* DC3000 and *P. syringae pv. tabaci* 11528 and deletion of this single effector gene enables DC3000 to cause disease in *N. benthamiana* (Wei et al., 2007). Furthermore, HopQ1 was demonstrated to enhance the bacterial virulence on bean when expressed from *P. syringae pv. tabaci* (Ferrante et al., 2009). HopK from *Pto* DC3000 elicited HR in *N. benthamiana* (Schechter et al., 2004). The *Pto* DC3000 type III secretion effector HopU1 is a mono-ADP-RTs required for full virulence in Arabidopsis (Fu et al., 2007). The effector HopF1/AvrPphF from *P. syringae pv. phaseolicola* race 7 shows weak homology to mono-ADP-RTs and is required for virulence and avirulence functions in different bean and soybean cultivars (Jackson et al., 1999; Singer et al., 2004; Tsiamis et al., 2000). HopF2 from *Pto* DC3000 is a homologue of AvrPphF and possesses a putative myristoylation site that is important for its membrane localization and avirulence and virulence functions in tobacco and tomato (Robert-Seilanianz et al., 2006). AvrPto from *P. syringae pv. tomato* strains JL1065 and DC3000 elicit hypersensitive response when expressed in tomato leaves containing *Pto*. Deletion of *avrPto* from these two tomato strains did not eliminate the ability of these bacteria to trigger *Pto*-specific plant resistance (Ronald et al., 1992). A later study found that a second *Pseudomonas* effector AvrPtoB (also known as HopAB2) interacts with the *Pto* kinase and elicits *Pto*-specific and Prf-dependent disease resistance in tomato leaves similar to AvrPto (Kim et al. 2002). In susceptible tomato and
*Arabidopsis* plants, AvrPto and AvrPtoB are important virulence determinants (Abramovitch *et al*., 2003, Shan *et al*., 2000) and E3 ubiquitin ligase activity of AvrPtoB is required for full enhancement of *Pto* DC3000 virulence in *Arabidopsis* (Göhre *et al*., 2008). AvrPtoB homologs from diverse *P. syringae* pathovars designated avrPtoT1, avrPtoBPT23, avrPtoB3L1065, avrPtoBB728a interact with *Pto* in a yeast two-hybrid system and confer avirulence activity to DC3000ΔavrPtoΔavrPtoB in tomato leaves expressing *Pto* and promote bacterial growth in susceptible tomato leaves like AvrPtoB (Lin *et al*., 2006). In addition, avrPtoBB728a elicits nonhost resistance in tomato cultivars VFNT Cherry and Moneymaker that lack *Pto* but express *Pto* family members like *SlFen* and *SlPtoC* due to a defective E3 ubiquitin ligase activity (Chien *et al*., 2013). The AvrB protein from *P. syringae* pv. *glycinea* induces resistance responses in soybean that contain resistance gene *Rpg1-b* and *Arabidopsis* that carry *RPM1* (Ashfield *et al*., 2004, Grant *et al*., 1995). In *Arabidopsis*, AvrB is targeted plant plasma membrane via myristoylation (Nimchuk *et al*., 2000), where it binds the plant RIN4 protein and mediates its phosphorylation via an unknown mechanism (Mackey *et al*., 2002). In addition, AvrB enhances bacterial virulence on susceptible soybean plants (Ashfield *et al*., 1995) and induces a chlorotic phenotype on *RPM1*-null *Arabidopsis* ecotypes (Nimchuk *et al*., 2000). AvrPphE and AvrPphB from *P. syringae* pv. *phaseolicola* causes hypersensitive reaction on bean cultivars carrying the corresponding *R2* and *R3* resistance gene respectively (Jenner *et al*., 1991, Mansfield *et al*., 1994). AvrPphB also confers avirulence to pea, soybean, and *Arabidopsis* after transfer to appropriate bacterial pathogens that are virulent on these hosts (Fillingham *et al*., 1992, Pirhonen *et al*., 1996, Simonich and Innes, 1995). HopN1 effector of *P. syringae* pv. *tomato* DC3000 suppresses cell death, ROS generation and callose deposition by interacting with host tomato and nonhost *N. benthamiana* protein PsbQ (Rodríguez-Herva *et al*., 2012). Thus, effectors secreted by the bacterial type III secretion system play a crucial role in the interaction between Gram-negative bacterial pathogens and their host and nonhost plants.

Recently, we have reported that Rip36, a putative Zn-dependent protease effector in RS1000 induces hypersensitive response (HR) in nonhost Solanaceae, *Solanum torvum* Sw. cv. (St) Torubamubiga, and Zn-protease motif is required for the induction of HR (Nahar *et al*., 2014). HopH1, a Rip36 ortholog was found in *P. syringae* pv. *tomato* (Pto) DC3000 and *P. syringae* pv. *syringae* (Psy) B728a. In this study, I investigated whether HopH1 induces HR in *S. torvum*. 

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III. RESULTS

1. HopH1 is an elicitor that induces hypersensitive response on nonhost *S. torvum*

Rip36 effector in *Ralstonia solanacearum* RS1000 induces HR in nonhost *Solanaceae, Solanum torvum* Sw. cv. (St) Torubamubiga (Nahar *et al.*, 2014). Rip36 conserves Zn-protease motif, and the protease activity seems to be required for the induction of HR, because the mutation of this protease motif abolished the ability to induce HR (Nahar *et al.*, 2014). Whole genome analysis of *Pto* DC3000, *Psy* B728a and *Pph* 1448A revealed that *Pto* DC3000 and *Psy* B728a possess a hopH1, a rip36 ortholog, but *Pph* 1448A does not. Similar to Rip36, both HopH1 from *Pto* DC3000 and *Psy* B728a also conserve Zn-protease motif HExxH as shown in Fig. 3-1. I first examined whether wild-type (WT) of *Pto* DC3000, *Psy* B728a and *Pph* 1448A induces HR in *S. torvum* by infiltration, and found that both hopH1possessing strains, *Pto* DC3000 and *Psy* B728a induced HR, but hopH1-nonpossessing strain *Pph* 1448A did not (Fig. 3-7A). However, when I introduced hopH1 of *Pto* DC3000 and *Psy* B728a into *Pph* 1448A, the resultant transformants 1448A-H01 and 1448A-H02, that possess hopH1Psy or hopH1Psy, respectively, acquired HR inducing activity (Fig. 3-7B) suggesting both hopH1 of *Pto* DC3000 and *Psy* B728a is an avirulence determinant to induce HR on *S. torvum*.

To examine whether hopH1 is indispensable avirulence factor on *S. torvum*, I generated hopH1-defective mutants in *Pto* DC3000 and *Psy* B728a. The infiltration of *S. torvum* leaves with each resultant mutant, *Pto* DC3000-ΔH and *Psy* B728a-ΔH induced typical HR as WT strain did (Fig. 3-7B). These results indicate that *Pto* DC3000 and *Psy* B728a possess other unknown avirulence factor that induce HR on *S. torvum*.

2. Mutation in Zn-dependent protease motif of HopH1 had no effect on the ability to induce HR on *S. torvum*

As mentioned above Rip36 requires Zn-protease HExxH motif is essential for HR on *S. torvum* (Nahar *et al.*, 2014). Therefore I examined whether HopH1 effectors of *Pto* DC3000 and *Psy* B728a also require HExxH motif in its HR-inducing activity. The glutamic acid (E) at 150 amino acid from the N-terminus of the HExxH motif of HopH1Psy and HopH1Psy effectors was replaced by alanine (A, E150A) using a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Then the mutated hopH1E150A from *Pto* DC3000 and *Psy* B728a was
Fig. 3-1. Multiple Alignment of RsRip36 and HopH1 of Pto DC3000 and Psy B728a and AVR-Pita of Megnaporthe grisea. The HExxH Zn metalloprotease motif is shown in red colour. Conserved amino acids are denoted by asterisks.
Fig. 3-2. Schematic diagram of generation of PtoΔHopH1 mutant strain.
PsyHopH1 amplified by PCR with primers
• Digestion of PCR product with NheI
• Self ligation and digestion with DpnI
• Transformation into E. coli

Selection of PsyΔHopH1 mutant in KB medium containing 10% sucrose

Conjugation withPsy B728a

Fig. 3-3. Schematic diagram of generation of PsyΔHopH1 mutant strain
Fig. 3-4. Schematic diagram of generation of Pph 1448A-HopH1_pwo complementation strain
Fig. 3-5. Schematic diagram of generation of *Pph* 1448A-*Psy*HopH1 complementation strain
Fig. 3-6. Schematic diagram of generation of \textit{Pph} 1448A-\textit{HopH1E150A}_{Psy/Psy} complementation strain
introduced into *Pph* 1448A to generate *Pph* 1448A-H03 and 1448A-H04, and *S. torvum* leaves were infiltrated with these resultant strains. Unexpectedly, both transformants, 1448A-H03 and _H04, induced HR on *S. torvum* leaves (Fig. 3-7B). Namely the mutation in Zn-protease motif did not abolish the activity of HopH1<sub>Pto</sub> and HopH1<sub>Psy</sub> to induce HR on *S. torvum*. This result indicates that the mutation HAxH motif was not sufficient to lose protease activity in HopH1 in *P. syringae*, otherwise protease activity is not required for HopH1-induced HR.

In the later case, some part of peptide fragments of HopH1 might be essential to induce HR. To identify the peptide fragment that induce HR on *S. torvum* if exists, I generated a series of clones that express truncated peptides of HopH1<sub>E150A</sub><sub>Pto</sub>. First efficiently manipulate hopH1<sub>E150A</sub> gene, I reamplify short DNA fragment (1260 bp) containing entire promoter sequence and *hopH1*<sub>E150A</sub> ORF from pGEM-H03 by PCR using a set of primers, HopH1<sub>-13</sub> and -14, and cloned into pCR-Blunt II-TOPO plasmid as pCR-H05. To express truncated HopH1<sub>E150A</sub> polypeptides, stop codon was introduced into the codons at K56, S119 and E179 of HopH1<sub>E150A</sub><sub>Pto</sub> using a QuickChange XL site-directed mutagenesis kit and pCR-H05 as a template shown in Fig. 3-8. In order to construct the truncated HopH1<sub>E150A</sub><sub>Pto</sub> as shown in Fig 3-9, inverse PCR was carried out using pCR-H05 as a template. Each truncated peptide was introduced into *Pph* 1448A to generate 1448A-H06, _H07, _H08, _H09, _H10, _H11 and _H12 strains. *S. torvum* leaves were infiltrated with *Pph* 1448A expressing each truncated peptide and found that none of these strains induced HR (Fig. 3-10B and 3-10C). These results indicated that the full length HopH1, probably Zn-protease activity is required for HR induction and E150A mutation may not be sufficient to abolish the protease activity of HopH1.

**3. An additional mutation H153A in Zn-protease motif completely abolished the HR inducing activity of HopH1**

To further examine the necessity of Zn-protease motif in HR inducing activity, additional mutation was introduced into Zn-protease motif in hopH1<sub>Pto</sub>. PCR was carried out to generate hopH1<sub>E150A.H153A</sub><sub>Pto</sub> using a pCR-hopH1<sub>E150A</sub><sub>Pto</sub> as a template and HopH1-27 and HopH1-28 as primers. The resultant plasmid was introduced into *Pph* 1448A by conjugation, then HR-inducing activity was examined. As shown in Fig. 3-11 Pph-hopH1<sub>E150A.H153A</sub><sub>Pto</sub> no longer induced HR on *S. torvum*, indicating that Zn-protease motif is essential for HopH1-induced HR.
IV. DISCUSSION

We report here that a putative Zn-dependent protease HopH1 from Pto DC3000 and Psy B728a acts as an avirulence factor to elicit HR on S. torvum when heterologously expressed in Pph 1448A and mutation in Zn-dependent protease motif (E150A•H153A) completely abolished the HR inducing activity. The HopH1 homologue Rip36 in R. solanacearum RS1000 is an avirulence factor to induce HR on S. torvum and the putative Zn-dependent protease motif is essential for its avirulence function because mutation in protease motif completely eliminates the avirulence activity (Nahar et al., 2014). The Zn-dependent protease motif is also conserved in rice blast avirulence factor Avr-Pita of fungal pathogen Magnaporthe grisea. The protease motif of Avr-Pita (HExxH) is known to be essential for its avirulence function because a point mutation in protease motif (E177G) completely abolished the ability to induce rice blast resistance mediated by corresponding R protein Pi-ta in rice (Orbach et al., 2000). Although Avr-Pita directly interacts with Pi-ta, a mutation in the protease motif caused failure of interaction with Pi-ta and abolished Pi-ta-mediated defense response. The direct interaction between the Pi-ta and Avr-Pita proteins raises the possibility that the putative Avr-Pita metalloprotease may directly cleave the Pi-ta (Jia et al., 2000).

The Rip36 homologue NleD in enteropathogenic E. coli is reported to cleave JNK MAP kinase and to inhibit the inflammatory reaction by its Zn-dependent protease activity (Baruch et al., 2011). Therefore Rip36 might cleave a particular S. torvum protein, then the resultant peptide fragment might be specifically recognized by R protein to induce HR. The Rip36 function might be similar to the cysteine proteases AvrRpt2 from Pto DC3000 (Axtell et al., 2003) and AvrPphB from Pph 1448A (Shao et al., 2002). Each AvrRpt2 and AvrPphB cleaves Arabidopsis RIN4 and PBS1, respectively, and the cleavages result in the activation of respective R proteins RPS2 and RPS5, respectively (Mackey et al., 2003, Shao et al., 2003). It was reported that HopH1 of Pto DC3000 might contribute to virulence in host Arabidopsis and tomato, because inoculation of the hopH1 and hopC1 polymutant reduced both lesion formation and growth (Wei et al., 2007). Heterologous expression of five effectors HopH1, HopX1, HopG1, HopT1-1 and AvrPtoB from Pto DC3000 inhibit yeast growth under certain stress condition and one of these effectors HopX1 attenuates the activation of high osmolarity glycerol (HOG) MAPK pathway in yeast (Salomon et al., 2012). MAPK pathways are also conserved in plants and several bacterial T3SS effectors were shown to target plant MAPK pathways (Feng and Zhou, 2012; Shan et al., 2007). Our results indicate that the mechanism of HR induction by HopH1 might be similar to that by Rip36.
Fig. 3-7. Hypersensitive response induced by the inoculation of different *Pseudomonas syringae* strains. *Solanum torvum* Torubamubiga leaves were infiltrated with bacterial suspensions (OD600 of 0.3) of *P. syringae* pv. *tomato* DC3000, pv. *syringae* B728a and pv. *phaseolicola* 1448A wild type (A), and DC3000-ΔH and B728a-ΔH mutants and the complemented strains of *Pph* 1448A with *hopH1Pto* (1448A-H01), *hopH1Psy* (1448A-H02), *hopH1E150APto* (1448A-H03) or *hopH1E150APsy* (1448A-H04) (B), and incubated under a 16 h light/8 h dark cycle at 28°C. Photographs show representative results of *S. torvum* at 1 dpi from three independent experiments, which gave similar results. The dotted lines represent the infiltrated area. C) Summary of the plant responses caused by various strains in *S. torvum* leaves.

<table>
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<th>DC3000</th>
<th>B728a</th>
<th>1448A</th>
<th>DC3000 ΔH</th>
<th>B728a ΔH</th>
<th>1448A -H01</th>
<th>1448A -H02</th>
<th>1448A -H03</th>
<th>1448A -H04</th>
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<td>HR</td>
<td>HR</td>
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Insertion of stop codon

**Fig. 3-8.** Schematic diagram of generation of truncated peptides of PtoHopH1E150A and introduction into P. syringae pv phaseolicola 1448A
Fig. 3-9. Schematic Diagram of generation of truncated peptides of PtoHopH1E150A and introduction into P. syringae pv phaseolicola 1448A
Fig. 3-10. Response of *Solanum torvum* leaves by the inoculation of *Pseudomonas syringae* pv. *phaseolicola* 1448A expressing HopH1E150APto and its truncated peptides. (A) Schematic representation of HopH1E150APto and its derivatives with each name of strain at the left. M and each number indicate N-terminal methionine and the position of amino acids from N-terminus. (B and C) *S. torvum* Torubamubiga leaves were infiltrated with bacterial suspensions (OD600 of 0.3) of *P. syringae* pv. *phaseolicola* 1448A strains expressing HopH1E150APto or its truncated polypeptides, and incubated under a 16 h light/8 h dark cycle at 28°C. Photographs show representative results of *S. torvum* at 1 dpi from three independent experiments, which gave similar results. The dotted lines represent the infiltrated area. D) Summary of the plant responses caused by various strains in *S. torvum* leaves.
Fig. 3-11. Response of *Solanum torvum* leaves by the inoculation of *Pseudomonas syringae* pv. *phaseolicola* 1448A expressing HopH1E150A<sub>Pto</sub> and HopH1E150A•H153A<sub>Pto</sub>. (A) Schematic representation of HopH1E150A•H153A<sub>Pto</sub>. (B) *S. torvum* Torubamubiga leaves were infiltrated with bacterial suspensions (OD600 of 0.3) of *P. syringae* pv. *phaseolicola* 1448A strains expressing HopH1E150A<sub>Pto</sub> and HopH1E150A•H153A<sub>Pto</sub>, and incubated under a 16 h light/8 h dark cycle at 28°C. Photographs show representative results of *S. torvum* at 1 dpi from three independent experiments, which gave similar results. The dotted lines represent the infiltrated area.
Figure 3-12. Models for Rip36/HopH1 induced HR in *S. torvum*
There were three possible mechanisms of Rip36/HopH1 induced HR in \textit{S. torvum} (Fig. 3-12): firstly, Rip36/HopH1 might be directly recognized by R protein and induced HR. Secondly, Rip36/HopH1 might cleaved host target protein and the cleaved peptide was recognized by R protein and induced HR. Thirdly, Rip36/HopH1 might cleaved host target protein results in dissociation and activation of R protein and thus induced HR. On the basis of results second or third model is the possible mechanism of Rip36/HopH1 in \textit{duced HR in S. torvum}. Identification of target protein and demonstration of enzymatic activity will provide insight into the HR triggering mechanism of HopH1.

V. EXPERIMENTAL PROCEDURE

1. Bacterial strains, media and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 3-1. Each strain of \textit{Pseudomonas syringae} was maintained in King’s B medium (KB) at 27°C, and \textit{Escherichia coli} strains were grown at 37°C in Luria-Bertani (LB) medium (Miller, 1992). When required, antibiotics were added to the following final concentrations: nalidixic acid 30-50 µg/ml and kanamycin 20 µg/ml.

2. Plant materials and inoculation experiment

Eggplants (\textit{Solanum torvum} Sw. cv. Torubamubiga) were grown at 30°C in a growth chamber. For HR test, bacterial strains cultured overnight in KB broth medium were harvested after centrifugation. The cell pellets were washed with distilled water (DW), then suspended in distilled water at a density of \(OD_{600}=0.3\) and infiltrated into 5 to 6 weeks old Torubamubiga leaves with a needleless syringe as described (Sol et al., 2012).

3. Construction of deletion mutant of \textit{hopH1} gene

In order to clone the \textit{hopH1} gene from \textit{P. syringae} pv. \textit{tomato} DC3000 and \textit{P. syringae} pv. B728a, a 2.6 kb fragment and 2.2 kb fragment containing the promoter and entire open reading frame (ORF) of the \textit{hopH1} genes were amplified from chromosomal DNA by PCR and cloned into a
pGEM-T Easy vector by using the pair of primers HopH1-01: 5’-CTTCCGCAACCCCGAGTA-3’ and HopH1-02: 5’-CCCCATGAGGTGTACACGA-3’, and HopH1-03: 5’-CCCCATGAGGTGTACACGA-3’, and HopH1-04: 5’-CCCCATGAGGTGTACACGA-3’, respectively to produce pGEM-H01 and pGEM-H02 that possess hopH1_Pto and hopH1_Psy, respectively. The PCR primers were designed based on the published sequences of the hopH1 gene of P. syringae pv. tomato DC3000 (GenBank accession no. AE016853) and P. syringae pv. B728a (GenBank accession no. CP000075). After cloning DNA sequencing was performed using an ABI PRISM 3130xl (Applied biosystems, Chiba, Japan) and a BigDye Terminator Cycle Sequencing Kit.

In order to delete whole open reading frame (ORF) of hopH1 gene in Pto DC3000 and Psy B728a, inverse PCR was carried out using respective primers HopH1-05: 5’-cgggatccAGTTATCCTGAAAGCTAATTGAGTTGATC-3’ and HopH1-06: 5’-cgggatccGGCAACCTACTGTAAGTTATTTTTTAGCG-3’ (small letters indicate sequence of BamHI enzyme) to delete hopH1_Pto and HopH1-07: 5’-ctagctagcGAATTGCAACTATTGG-3’) and HopH1-08: 5’-ctagctagcAGGCCACATCAATA GGACAA-3’ (small letters indicate sequence of NheI enzyme) to delete hopH1_Psy and pGEM-hopH1_Pto and pGEM-hopH1_Psy as templates. Each PCR product was digested with BamHI for hopH1_Pto and NheI for hopH1_Psy, and self-ligated for the generation of hopH1-deleted plasmids, pGEM-ΔhopH1_Pto and pGEM-ΔhopH1_Psy, respectively as shown in Fig. 3-2 and Fig. 3-3. From the resultant plasmids the hopH1-deleted DNA fragments were excised with EcoRI digestion and introduced into an EcoRI site of the pK18mobsacB plasmid (Schäfer et al., 1994). The resulting plasmids, pK18-ΔhopH1_Pto and pK18-ΔhopH1_Psy were transformed into E. coli S17-1 and introduced into Pto DC3000 and Psy B728a by conjugal transfer. Then the plasmids were excised on a KB agar plate containing 10% sucrose, and deletion of hopH1 was confirmed by PCR using the pairs of primers described above and also by sequencing.

4. Introduction of hopH1 gene into P. syringae pv. phaseolicola 1448A

pGEM-H01 and pGEM-H02 plasmids produced above was digested with EcoRI and then each insert DNA was transferred into a pBSL118, a transposon vector (Alexeyev et al.,1995) and produced pBSL_H01 and pBSL_H02 (Fig. 3-4 and Fig. 3-5). Each plasmid was introduced into the Pph 1448A genome through conjugation with Escherichia coli S17-1/λpir (Simon et al., 1983) and obtained Pph 1448A transformants, 1448A-H01 and 1448A-H02, that possess hopH1_Pto or hopH1_Psy, respectively.
To introduce the hopH1E150A point mutation, a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used. Using pGEM-H01 and pGEM-H02 produced above as templates, two complementary oligonucleotides containing the mutation (HopH1-09: 5’-CACGACCGCTCATGCTCGACGCTCGAC-3’ and HopH1-10: 5’-GTCGAGCATGAA CGAGCATGACTAGGCTCGG-3’ (small letters indicate exchanged nucleotides and underline sequences are codon for Alanine) for hopH1E150APto and HopH1-11: 5’-CACGACCGCTCATGCTCGACGCTCGAC-3’ and HopH1-12: 5’-TCGAACATGAA CGAGCATGACTAGGCTCGG-3’ (small letters indicate exchanged nucleotides and underline sequences are codon for Alanine) for hopH1E150APsy were used to mutagenize Zn-protease HEexH motif according to the manufacturer’s protocol. The E150A mutation of the resultant plasmid, pGEM-H03 and pGEM-H04 was confirmed by DNA sequencing. The DNA fragments for hopH1E150APto and hopH1E150APsy were inserted into the EcoRI site of pBSL118 to produce pBSL_H03 and pBSL_H04, respectively (Fig. 3-6). Each plasmid was introduced into the Pph 1448A genome through conjugation with E. coli S17-1 λpir to generate Pph 1448A-H03 and 1448A-H04.

5. Generation of truncated peptides of hopH1E150APto and introduction into P. syringae pv. phaseolicola 1448A

To introduce the stop codon in different position of hopH1E150APto First efficiently manipulate hopH1E150A gene, a short DNA fragment (1260 bp) containing entire promoter sequence and hopH1E150A ORF from pGEM-H03 was reamplified by PCR using a set of primers, HopH1-13: 5’-TTTGCGCATCTGCGCATCGA-3’ and HopH1-14: 5’-AATCATGGGCGTCCTAATTCGG-3’, and cloned into pCR-Blunt II-TOPO plasmid as pCR-H05. DNA sequencing was performed using an ABI PRISM 3130xl (Applied biosystems, Chiba, Japan) and a Big Dye Terminator Cycle Sequencing Kit. To introduce stop codon at the position K56, S119 and E179 of HopH1E150APto using a QuickChange XL site-directed mutagenesis kit and pCR-H05 as a template. Sets of primers, HopH1-15: 5’-CCACTCTTTGCCAGTCAATAGAGATAGAAGAAGTCACGC-3’ and HopH1-16: 5’-GCGTGACTTTTCTATTTTTATGACTGGCAAGAGTGG-3’ (small and bold letter indicates exchanged nucleotide and underline sequences indicate the stop codon) for the introduction of stop codon at K56, HopH1-17: 5’-GCGATTATTGCTGGGTAGCAGACAAAGCAAGCAT-3’ and HopH1-18: 5’-TGCTTTGCTTTGTCTGGCTAC CAGCAGCAATAATCGC-3’ (small and bold letter indicates exchanged nucleotide and underline...
Table 3-1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5a</td>
<td>( F^{-\lambda-a80d\text{LacZDM15}} \Delta(\text{lacZYA-argF})U169) recA1 endA1 hsdR17 (rK mK(^+)) supE44 thi-1 gyrA relA1</td>
<td>Takara, Kyoto, Japan</td>
</tr>
<tr>
<td>S17-1</td>
<td>( \lambda) pro hsdR–hsdM + recA [chr::RP4-2-Tc::Mu-Km::Tn7]</td>
<td>Schifer et al. (1994)</td>
</tr>
<tr>
<td>S17-1 ( \lambda )pir</td>
<td>( \lambda ) pir lysogen of S17 (Tpr Smr thi pro hsdR-M + recA RP::2-Tc::Mu-Km::Tn7)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>P. syringae pv. tomato</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC3000</td>
<td>Race 0 wild-type, Rif(^{\text{f}})</td>
<td>Wei et al. (2007)</td>
</tr>
<tr>
<td>DC3000-AH</td>
<td>Isolate DC3000 ( \Delta)hopH1, Rif(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td><strong>P. syringae pv. syringae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B728a</td>
<td>Wild-type, Rif(^{\text{f}})</td>
<td>Loper and Lindow (1987)</td>
</tr>
<tr>
<td>B728a-( \Delta)H</td>
<td>Isolate B728a ( \Delta)hopH1</td>
<td>This study</td>
</tr>
<tr>
<td><strong>P. syringae pv. phaseolicola</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1448A</td>
<td>Race 6 wild-type</td>
<td>Mansfield et al. (1994)</td>
</tr>
<tr>
<td>1448A-H01</td>
<td>1448A chr::mini-Tn5hopH1(_{\text{Psy}}) (2683 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H02</td>
<td>1448A chr::mini-Tn5hopH1(_{\text{Psy}}) (2240 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H03</td>
<td>1448A chr::mini-Tn5hopH1/E150A(_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H04</td>
<td>1448A chr::mini-Tn5hopH1/E150A(_{\text{Psy}}) (1260 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H06</td>
<td>1448A chr::mini-Tn5hopH1 (1/55) (_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H07</td>
<td>1448A chr::mini-Tn5hopH1 (1/118) (_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H08</td>
<td>1448A chr::mini-Tn5hopH1 (1/178) (_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H19</td>
<td>1448A chr::mini-Tn5hopH1 (M+57/218) (_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H10</td>
<td>1448A chr::mini-Tn5hopH1 (M+120/218) (_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H11</td>
<td>1448A chr::mini-Tn5hopH1 (M+180/218) (_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H12</td>
<td>1448A chr::mini-Tn5hopH1 (57/180) (_{\text{Psy}})</td>
<td>This study</td>
</tr>
<tr>
<td>1448A-H13</td>
<td>1448A chr::mini-Tn5hopH1/E150A(<em>{\text{Psy}})+H153A(</em>{\text{Psy}}) (1260bp)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Cloning vector, Amp(^{\text{f}})</td>
<td>Promega Madison, WI, USA</td>
</tr>
<tr>
<td>pGEM-H01</td>
<td>pGEM-T Easy carrying 2682-bp PCR product with ( \text{hopH1} ) from DC3000, Amp(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-H02</td>
<td>pGEM-T Easy carrying 2240-bp PCR product with ( \text{hopH1} ) from B728a, Amp(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-H03</td>
<td>pGEM-( \text{hopH1})(_{\text{Psy}})-derived plasmid that contains ( \text{hopH1}/\text{E150A} ) from DC3000, Amp(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-H04</td>
<td>pGEM-( \text{hopH1})(_{\text{Psy}})-derived plasmid that contains ( \text{hopH1}/\text{E150A} ) from B728a, Amp(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-( \Delta)hopH1(_{\text{Psy}})</td>
<td>Whole ( \text{hopH1})(<em>{\text{Psy}}) ORF-deleted pGEM-( \text{hopH1})(</em>{\text{Psy}}), Amp(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-( \Delta)hopH1(_{\text{Psy}})</td>
<td>Whole ( \text{hopH1})(<em>{\text{Psy}}) ORF-deleted pGEM-( \text{hopH1})(</em>{\text{Psy}}), Amp(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pCR-Blunt II TOPO</td>
<td>Cloning vector for PCR product, Km(^{\text{f}})</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCR-H05</td>
<td>pCR-Blunt II-TOPO carrying 1260-bp PCR product with ( \text{hopH1}/\text{E150A} ) from DC3000, Km(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pCR-H06</td>
<td>pCR-H05-derived plasmid that was introduced stop codon at K56, Km(^{\text{f}})</td>
<td>This study</td>
</tr>
<tr>
<td>pCR-H07</td>
<td>pCR-H05-derived plasmid that was introduced stop codon at S119, Km(^{\text{f}})</td>
<td>This study</td>
</tr>
</tbody>
</table>
### pCR-H08
pCR-H05-derived plasmid that was introduced stop codon at E179, Km\(^r\)

This study

### pCR-H9
pCR-H05-derived plasmid that possessing the region for D57 to Q218, Km\(^f\)

This study

### pCR-H10
pCR-H05-derived plasmid that possessing the region for P120 to Q218, Km\(^f\)

This study

### pCR-H11
pCR-H05-derived plasmid that possessing the region for L180 to Q218, Km\(^f\)

This study

### pCR-H12
pCR-H05-derived plasmid that possessing the regions for L57 to E180, Km\(^f\)

This study

### pCR-H13
pCR-H05-derived plasmid that contains hopH1\(^E150A\)•H153A from DC3000, Km\(^r\)

This study

### pBSL118
Mini-Tn5-derived plasmid vector for insertion mutagenesis, Amp\(^r\), Km\(^r\)

Alexeyev et al. (1995)

### pK18mobsacB
Small mobilizable vector, sucrose-sensitive (sacB), Km\(^r\)

Schäfer et al. (1994)

### pK18-ΔhopH1\(_{pno}\)
pK18mobsacB containing ΔhopH1-DNA fragment from pGEM-ΔhopH1\(_{pno}\), Km\(^r\)

This study

### pK18-ΔhopH1\(_{psy}\)
pK18mobsacB containing ΔhopH1-DNA fragment from pGEM-ΔhopH1\(_{psy}\), Km\(^r\)

This study

### pBSL-H01
pBSL118 carrying 2682-bp with hopH1\(_{pno}\), Amp\(^r\), Km\(^f\)

This study

### pBSL-H02
pBSL118 carrying 2240-bp with hopH1\(_{psy}\), Amp\(^r\), Km\(^f\)

This study

### pBSL-H03
pBSL118 carrying 2682-bp with hopH1\(_{E150A}A_{pno}\), Amp\(^r\), Km\(^f\)

This study

### pBSL-H04
pBSL118 carrying 2240-bp with hopH1\(_{E150A}A_{psy}\), Amp\(^r\), Km\(^f\)

This study

### pBSL-H05
pBSL118 carrying 1260-bp with hopH1\(_{E150A}A_{pno}\), Amp\(^r\), Km\(^f\)

This study

### pBSL-H06
pBSL118 carrying 1260-bp with hopH1\(_{E150A}A_{psy}\), with stop codon at K56, Amp\(^r\), Km\(^f\)

This study

### pBSL-H07
pBSL118 carrying 1260-bp with hopH1\(_{E150A}A_{pno}\), with stop codon at S119, Amp\(^r\), Km\(^f\)

This study

### pBSL-H08
pBSL118 carrying 1260-bp with hopH1\(_{E150A}A_{psy}\), with stop codon at E179, Amp\(^r\), Km\(^f\)

This study

### pBSL-H09
pBSL118 that possessing the region for D57 to Q218, Amp\(^r\), Km\(^f\)

This study

### pBSL-H10
pBSL118 that possessing the region for P120 to Q218, Amp\(^r\), Km\(^f\)

This study

### pBSL-H11
pBSL118 that possessing the region for L180 to Q218, Amp\(^r\), Km\(^f\)

This study

### pBSL-H12
pBSL118 that possessing the regions for L57 to E180, Amp\(^r\), Km\(^f\)

This study

### pBSL-H13
pBSL118 carrying 1260-bp with hopH1\(_{E150A}A_{pno}\), Amp\(^r\), Km\(^f\)

This study

### pBSL-H14
pBSL118 carrying 1260-bp with hopH1\(_{E150A}A_{psy}\), Amp\(^r\), Km\(^f\)

This study

Amp\(^r\) ampicillin resistance, Km\(^r\) kanamycin resistance, Nal\(^r\) nalidixic acid resistance
### Table 3-2. List of primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>HopH1-01</td>
<td>CTTCGCATAACCCCGGAGTA</td>
<td>Amplification of hopH1&lt;sub&gt;Pto&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-02</td>
<td>CCCATGAGGTTGATACAGA</td>
<td>Amplification of hopH1&lt;sub&gt;Pto&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-03</td>
<td>CCCGAATTCTGTGGCGCTC</td>
<td>Amplification of hopH1&lt;sub&gt;Psy&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-04</td>
<td>TGGCGAATTCGAGACTTCGAC</td>
<td>Amplification of hopH1&lt;sub&gt;Psy&lt;/sub&gt;</td>
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<tr>
<td>HopH1-05</td>
<td>cggataccAGTTATCTGAAAGCTAATTGAGTTAG</td>
<td>Deletion of hopH1&lt;sub&gt;Pto&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-06</td>
<td>cggataccGGCAACCTACTGTTAAGTTTTTATAGCG</td>
<td>Deletion of hopH1&lt;sub&gt;Pto&lt;/sub&gt;</td>
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<tr>
<td>HopH1-07</td>
<td>ctagctagGAAATTGCAACTATTGG</td>
<td>Deletion of hopH1&lt;sub&gt;Psy&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-08</td>
<td>ctagctagAGGCAACATCATAGGACAA</td>
<td>Deletion of hopH1&lt;sub&gt;Psy&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-09</td>
<td>CACGACCTAGCTCATGCTGTCATGCTGAC</td>
<td>Generation of hopH1&lt;sub&gt;E150A_Pto&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-10</td>
<td>GTCGAGCATGAACGAGC</td>
<td>Construction of pCR-hopH1&lt;sub&gt;E150A_Pto&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-11</td>
<td>GTCGAGCATGAACGAG</td>
<td>Construction of pCR-hopH1&lt;sub&gt;E150A_Pto&lt;/sub&gt;</td>
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<td>HopH1-12</td>
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<td>HopH1-13</td>
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<td>Construction of pCR&lt;sub&gt;_09&lt;/sub&gt;</td>
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<tr>
<td>HopH1-14</td>
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<td>Construction of pCR&lt;sub&gt;_09&lt;/sub&gt;</td>
</tr>
<tr>
<td>HopH1-15</td>
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<td>Generation of hopH1&lt;sub&gt;E150A_Psy&lt;/sub&gt;</td>
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<tr>
<td>HopH1-16</td>
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<td>Construction of pCR&lt;sub&gt;_09&lt;/sub&gt;</td>
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<tr>
<td>HopH1-17</td>
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<td>Construction of pCR&lt;sub&gt;_09&lt;/sub&gt;</td>
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<tr>
<td>HopH1-18</td>
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<td>HopH1-19</td>
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<tr>
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<tr>
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<td>HopH1-24</td>
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<td>Construction of pCR&lt;sub&gt;_09&lt;/sub&gt;</td>
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<tr>
<td>HopH1-25</td>
<td>GTCGAGCATGAACGAGC</td>
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<tr>
<td>HopH1-26</td>
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</tr>
<tr>
<td>HopH1-27</td>
<td>GTCGAGCATGAACGAGC</td>
<td>Construction of pCR&lt;sub&gt;_09&lt;/sub&gt;</td>
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<tr>
<td>HopH1-28</td>
<td>GTCGAGCATGAACGAGC</td>
<td>Construction of pCR&lt;sub&gt;_09&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

The small letters indicate artificial nucleotides. The underlined sequences in HopH1-05 to -08 and HopH1-21 to -26 indicate one of BamHI-, NheI- or BglII-recognition sequences. The underlines in HopH1-09 to -12 and HopH1-15 to -20 indicate the substituted codons by site-directed mutagenesis. The small and bold letters are substituted nucleotides.
sequences indicate the stop codon) for S119, and HopH1-19: 5’-TACGGGATCTGG CAAATAGGACTTAGGGCCGTGG-3’ and HopH1-20: 5’-CAACG GCCCTAAGTTCgTATT TGCCAGATCCCCGA-3’ (small and bold letter indicates exchanged nucleotide and underline sequences indicate the stop codon) for E179 were used to construct pCR-H06, -H07 and -H08, respectively. In order to construct the truncated HopH1E150A by inversePCR was carried out using pCR-H05 as a template and primer pairs HopH1-21: 5’-gaagatctAGTTATCTGAAAGCTAATTGA-3’ and HopH1-22: 5’-gcaatctATGGATAGAAGTCACGCTAA-3’ (small letters indicate artificial nucleotides and underlined sequences indicate BglII recognition sequences) for pCR_H09, HopH1-21 and HopH1-23: 5’-gcaatctATGCCAGACAAAGCAAGCATAC-3’ (small letters indicate artificial nucleotides and underlined sequences indicate BglII recognition sequences) for pCR_H09, HopH1-21 and HopH1-24: 5’-gcaatctATGGAACCTAGGGCCGTGGAT-3’ (small letters indicate artificial nucleotides and underlined sequences indicate BglII recognition sequences) for pCR_H11 to amplify promoter and part of hopH1E150A region with vector sequence. Furthermore to construct HopH1M+57/180, PCR was carried out using pCR_H09 as a template and HopH1-25: 5’-ctagctagTTCCCTTTGCCAGATCCG-3’ and HopH1-26: 5’-ctagctagTAGGGCAACCTAGTGAAGTT-3’ (small letters indicate artificial nucleotides and underlined sequences indicate NheI recognition sequences) as a set of primers. All PCR products were digested with respective restriction enzymes designed in the primer sequences and self-ligated to generate each mutant plasmid, then mutated DNA fragment was introduced into pBSL118 to generate pBSL_H06 to_H12 (Fig. 3-8 and Fig. 3-9). Each plasmid DNA constructed in pBSL118 was introduced into Pph 1448A through conjugation with E. coli S17-1 λpir to generate 1448A-H05, 1448A-H06, 1448A-H07, 1448A-H08, 1448A-H09, 1448A-H10, 1448A-H11 and 1448A-H12 strains.

6. Generation of hopH1E150A•H153A and introduction into P. syringae pv. phaseolicola 1448A

To introduce an additional point mutation H153A in hopH1Pto, a QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used. Using pCR-H05 produced above as template, two complementary oligonucleotides containing the mutation (HopH1-27: 5’-AGCTCATGcGCTGTTGCTCGACATGTGTG-3’ and HopH1-28: 5’-CTAAACATGTCGAGCgeAACGAGCg CATGAGCT-3’ (small letters indicate exchanged nucleotides and underline sequences are codon for Alanine) for hopH1E150A•H153A were used to mutagenize Zn-protease HExxH motif according to the manufacturer’s protocol. The
H153A mutation of the resultant plasmid, pCR-H13 was confirmed by DNA sequencing. The DNA fragments for hopH1E150A•H153A\textsubscript{Pto} was inserted into the EcoRI site of pBSL118 to produce pBSL_H13. The resultant plasmid was introduced into the Pph 1448A genome through conjugation with \textit{E. coli} S17-1 \lambda pir to generate Pph 1448A-H13.
REFERENCES


**López-Solanilla E., Bronstein, P.A., Schneider, A.R. and Collmer, A.** (2004) HopPtoN is a *Pseudomonas syringae* TTSS effector and cysteine protease that suppresses pathogen-


