Negative Regulation of Pathogenesis in Pseudomonas syringae pv. tabaci 11528 by ATP-Dependent Lon Protease

Hyun Ju Yang1,2,3, Jun Seung Lee1,2, Ji Young Cha1, and Hyung Suk Baik1,⋆

Pseudomonas syringae pv. tabaci causes wildfire disease in tobacco plants. The hrp pathogenicity island (hrp PAI) of P. syringae pv. tabaci encodes a type III secretion system (TTSS) and its regulatory system, which are required for pathogenesis in plants. Three important regulatory proteins-HrpR, HrpS, and HrpL-have been identified to activate hrp PAI gene expression. The bacterial Lon protease regulates the expression of various genes. To investigate the regulatory mechanism of the Lon protease in P. syringae pv. tabaci 11528, we cloned the lon gene, and then a Δlon mutant was generated by allelic exchange. lon mutants showed increased UV sensitivity, which is a typical feature of such mutants. The Δlon mutant produced higher levels of tabtoxin than the wild-type. The lacZ gene was fused with hrpA promoter and activity of β-galactosidase was measured in hrp-repressing and hrp-inducing media. The Lon protease functioned as a negative regulator of hrp PAI under hrp-repressing conditions. We found that strains with lon disruption elicited the host defense system more rapidly and strongly than the wild-type strain, suggesting that the Lon protease is essential for systemic pathogenesis.

INTRODUCTION

Pseudomonas syringae is an important plant pathogenic bacterium commonly used to study plant-microbe interactions. More than 50 pathovars of P. syringae have been identified based on their virulence and host range. For example, P. syringae pv. tabaci causes wildfire diseases in host tobacco plants but induces a hypersensitive response (HR) in other non-host plants (Hirano and Upper, 2000). The ability of P. syringae to cause disease in its hosts and elicit an HR in non-host plants is controlled by the hypersensitive response and pathogenicity (hrp) hypersensitive response and conserved (hrc) genes residing in a pathogenicity island also known as Hrp PAI (Alfano et al., 2000). Hrp PAI is conserved among many gram-negative plant pathogenic bacteria, including P. syringae,Ralstonia solanacearum, Xanthomonas campestris, and Erwinia amylovora. Hrp PAI can be classified into 3 categories on the basis of function: (i) a regulatory system, (ii) a type III secretion system (TTSS), and (iii) the substrates of TTSS (Collmer et al., 2002). Similar to the TTSS of most pathogenic bacteria, expression of the hrp/hrc gene-encoded TTSS in P. syringae is environmentally regulated. The hrp/hrc genes are expressed at a very low level in a nutrient-rich medium but are induced in plants or in artificial hrp-inducing minimal media that mimic apoplastic conditions. Three intracellular regulatory proteins-HrpR, HrpS, and HrpL-have been identified as activators of hrc/hrp gene expression. HrpR and HrpS are enhancer-binding proteins that belong to the NtrC family of two-component regulatory proteins. HrpL is an alternative sigma factor in the extracytoplasmic factor (ECF) family. HrpR, HrpS, and HrpL appear to function in a regulatory cascade in which HrpS and HrpR synergistically activate the expression of hrl in response to signals from plants or in hrp-inducing minimal medium. HrlP is presumed to induce the expression of hrp and avr genes by recognizing a consensus sequence motif (hrp box) in the upstream regions (Buell et al., 2003; Hutcheson et al., 2001). In hrp-repressing environments, such as nutrient-rich media, the Lon protease has been found to degrade HrpR and repress the expression of the hrl regulon. Under hrp-inducing conditions, however, the hrl regulon is down-regulated by another negative regulator, HrpV (Bretz et al., 2002).

Proteolysis plays a key role in prokaryotic and eukaryotic cells by controlling the availability of critical regulatory proteins and removing abnormal and misfolded proteins. In bacteria, most intracellular proteolysis is initiated by four energy-dependent proteases, namely, Lon, the Clp family (ClpAP and ClpXP), HslUV, and FtsH, which are also known as stress-induced proteins. Among these, Lon is responsible for more than half of all energy-dependent proteolysis in Escherichia coli (Laskowska et al., 1996). Lon consists of four identical 87-kDa subunits, each having a highly charged N-terminal domain, a centrally located ATP-binding domain, and a proteolytically active C-terminal domain (Goldberg, 1992). Lon appears to perform important functions in the bacterial cell through its ability to degrade proteins that regulate gene expression. Recent studies have provided evidence that Lon is responsible for the regula-
tion of various pathways (Takaya et al., 2008). The role of the Lon protease in connection with bacterial pathogenesis in plant has been demonstrated by a recent study in which P. syringae Lon was shown to act as a negative regulator of hrp regulon expression. The mechanisms by which environmental signals are transduced into this apparent regulatory cascade in order to control expression of the hrp regulon during pathogenesis are, however, not obvious from the transcriptional factors mediating hrp regulon expression. Environmental regulation of the hrp regulon occurs primarily at the level of the hrp promoter and involves Lon protease-mediated degradation of HrpR. Lon-associated degradation of HrpR is reduced under conditions inductive to hrp regulon expression (Bretz et al., 2002).

The aims of this study were to determine the effect of the Lon protease, functioning as a negative regulator of the hrp regulon, in the pathogenicity of P. syringae pv. tabaci. In this study, a lon homologue from P. syringae pv. tabaci 11528 was isolated and characterized. A lon-defective mutant strain was constructed by allelic exchange between P. syringae pv. tabaci 11528 and P. syringae pv. tomato DC3000. Our results showed that the Lon protease regulates hrp PAI expression and the production of tabtoxin. By comparing the pathogenic phenotypes of the lon mutant and wild-type, we were able determine the effect of the Lon protease in pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. P. syringae strains were grown at 26°C in King’s medium B (KMB) or M9 minimal medium (M9MM) (Ahn et al., 2011; Cha et al., 2008). When necessary, antibiotics were added to the media at the following concentrations: ampicillin (100 μg ml⁻¹), rifampicin (50 μg ml⁻¹), and tetracycline (20 μg ml⁻¹).

Identification of the lon gene in P. syringae pv. tabaci 11528

Since the domains of the Lon protease are conserved in P. syringae homologues (Bretz et al., 2002), we aligned the lon gene nucleotide sequences of four pathogens. We designed a primer set (Table 2), lon-F(KpnI) and lon-R(Sacl), to amplify the complete lon gene. The 2.3-kb PCR product was cloned into a pGEM-T Easy vector (Promega), resulting in pBL40. We used the same pair of primers to clone the lon gene of P. syringae pv. tomato DC3000 into a pGEM-T Easy vector, resulting in pBL44. DNA sequencing of the lon gene was performed in an ABI model 310 automated DNA sequencer, using a Terminator Cycle Sequencing Kit (Applied Biosystems, USA). The nucleotide sequence of the lon gene of P. syringae pv. tabaci 11528 has been deposited in the GenBank database under accession number AY999716.

Construction of a lon mutant by allelic exchange

DNA isolation and routine manipulations were carried out in accordance with standard protocols as previously described (Sambrook and Russell, 2001) or in keeping with the instructions provided by the reagents’ manufacturers. pBL40 and pBL44 were treated with EcoRI, and then a 2.3 kb fragment was subcloned into pBlueScript II SK(+) (Stratagene), resulting in pBL41 and pBL45 (Fig. 1), respectively. To remove approximately 1.7 kb of the lon gene, pBL41 was treated with NcoI and pBL45 was treated with Styl. Both were then self-ligated in order to construct pBL42 and pBL46, respectively. The 1.2-kb fragment from pBL42 and pBL46 was digested with KpnI and SacI and subcloned into the suicide vector pDM197, resulting in pBL43 and pBL47, respectively. Allelic exchange was used to replace each chromosomal copy of the P. syringae target genes with the internally deleted copies of these genes that were without any integrated plasmid sequences, as previously described (Cha et al., 2008; Edwards et al., 1998).

To complement the deletion mutants, each gene was PCR-amplified using the primer set lon-C(F BamHI) and lon-C(R(HindIII) and cloned into the broad-host-range plasmid pRK415. The constructs were then introduced into the mutant strains by mating.

Construction of an hrpA promoter-lacZ transcriptional fusion protein

The 620-bp hrpA promoter region, encompassing the putative hrpA promoter region, was amplified from P. syringae pv. tabaci 11528, using the primer pair hrpA-pF(PstI) and hrpA-pR(XbaI). The PCR product was digested with PstI and XbaI and cloned into pfC1010, following which it was treated again with the same restriction enzymes, resulting in pBL52. β-galactosidase assays were performed according to the method of Miller (1972).

UV sensitivity test

Cultures were adjusted to an OD₆₀₀ of 1.0. Cells were harvested and resuspended in an equal volume of 0.9% NaCl. Then, 5-ml aliquots of cells were irradiated at 20, 40, 60, 80 erg in uncovered petri dishes. In this study, 1 erg was equivalent to 10⁻¹ J cm⁻². Initial and surviving cell numbers were determined by plate counts. Colonies arising from the surviving cells were counted following 48 h of incubation in the dark.

Tabtoxin assay

We identify the ability to produce tabtoxin by using a microbial assay (Staskawicz et al., 1980). In this assay, a zone of inhibition in Agrobacterium tumefaciens NT1 cultures is caused by the tabtoxin produced by P. syringae strains.

Virulence tests in tobacco leaves

The virulence of P. syringae was evaluated in tobacco (Nicotiana tabacum) leaves as previously described (Cha et al., 2008). Inoculations were performed by gently pin-pricking leaves with a sterilized needle through droplets of inoculum (20 μl) in parallel with 10⁻⁴ to 10⁻⁶ CFU ml⁻¹. To investigate the HR cell death, tobacco leaves were infiltrated with a needle-less syringe at 10⁻³ to 2 × 10⁻⁶ CFU ml⁻¹ respectively. Inoculated leaves were incubated at 26°C and illuminated continuously with a fluorescent light. Infiltrated leaf panels were observed for responses from 24 h after inoculation.

RESULTS

Identification and sequence analysis of a lon homologue of P. syringae pv. tabaci 11528

The complete ORF of the lon homologue from P. syringae pv. tabaci 11528 encoded a 798 a.a., 88.8-kDa deduced product that exhibited 99% identity/100% similarity, 98% identity/99% similarity, and 95% identity/98% similarity across its entire length with the Lon proteases from P. syringae pv. phaseolicola 1448A, P. syringae pv. tomato DC3000, and P. fluorescens, respectively. All known Lon protease domains were conserved in the P. syringae homologues. The ATP-binding domain, catalytically active Ser 674, and the substrate discriminator domain were present (Ebel et al., 1999). As observed in P. fluorescens, E. coli, and Salmonella enterica serovar Typhimurium genomes, the homologues of clpX, which encodes another energy-depen-
We tested cent to the codes a histone-like protein (Delic-Attree et al., 1995), and hupB, which encodes a histone-like protein (Delic-Attree et al., 1995), are adjacent to the P. syringae lon homologues.

**Mutation of lon results in increased UV sensitivity**

We tested *P. syringae pv. tabaci* 11528 and its *lon* deletion mutant, BL42, for their abilities to survive exposure to UV. The survival ratio of BL42 was 10 times lower than that of the wild-type after UV exposure at 20 erg and that difference was maintained up to an exposure of 80 erg (Fig. 2B). In particular, exposure to 40 erg UV light resulted in a 1.3 × 10^2 decrease in BL42 population levels (6.0 × 10^4 versus 0.8 × 10^2 CFU ml^-1). The UV sensitivity of BL43, the complement of BL42, is similar to that of *P. syringae pv. tabaci* 11528. *P. syringae pv. tabaci* 11528 lon mutants exhibited enhanced sensitivity to UV light similar to *E. coli* lon mutants (Schoemaker et al., 1984).

**Effect of lon mutation on tabtoxin production**

Many *P. syringae* strains produce low-molecular mass, non-host-specific phytopotoxins that induce chlorosis or necrosis (Buell et al., 2003). From a previous study (Penaloza-Vazquez et al., 2000), we know that the *hrp/hrc* secretion system is not required for COR production by *P. syringae pv. tomato* DC3000; however, a mutation in this system may have regulatory effects on the production of virulence factors such as COR. COR is a phytotoxin and a virulence factor produced by *P. syringae pv. tomato* DC3000. This result suggests a potential relationship between virulence factors such as COR and TTSS that are encoded by the *hrp/hrc* gene cluster. Our results showed that *P. syringae pv. tabaci* BL42 produces higher levels of tabtoxin than the wild-type. Although the exact quantity was not measured, the same result was obtained

---

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Relevant characteristics</th>
<th>References or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Transformation host for cloning vector</td>
<td>(Sambrook and Russell, 2001)</td>
</tr>
<tr>
<td>7213</td>
<td>Conjugation donor; an <em>E. coli</em> SM10 λ.pr derivative, DAP required (λascd)</td>
<td>(Daigle et al., 2001)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Host for protein overexpression: an <em>E. coli</em> B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and LacI</td>
<td>(Sambrook and Russell, 2001)</td>
</tr>
<tr>
<td><em>P. syringae pv. tabaci</em> 11528</td>
<td>ATCC 11528, wild-type</td>
<td>ATCC, USA</td>
</tr>
<tr>
<td>BL42</td>
<td>ATCC 11528 derivative, <em>lon</em></td>
<td>This study</td>
</tr>
<tr>
<td>BL43</td>
<td>BL42 carrying pBL50, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>BL46</td>
<td>ATCC 11528 carrying pBL52, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>BL47</td>
<td>BL42 carrying pBL52, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. syringae pv. tomato</em> DC3000</td>
<td>ATCC BAA-871, wild-type, spontaneous Rf</td>
<td>ATCC, USA</td>
</tr>
<tr>
<td>BL44</td>
<td>ATCC BAA-871 derivative, <em>lon</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em> NT1</td>
<td><em>Agrobacterium tumefaciens</em> carrying pDC41E33</td>
<td>(Shaw et al., 1997)</td>
</tr>
</tbody>
</table>

**Plasmids**

- pDMS197: Suicide vector, R6K ori, sacB', oriT, Tc'
- pRK415: Broad-host-range vector, RK2 ori, Tc'
- pRKlac290: lacZ transcriptional fusion vector, To' (Gober and Shapiro, 1992)
- pCF1010: lacZ transcriptional fusion vector, RSF1010
- pBL43: pDMS197 derivative, recombinant suicide plasmid for *lon* in *P. syringae pv. tabaci* 11528, Tc'
- pBL47: pDMS197 derivative, recombinant suicide plasmid for *lon* in *P. syringae pv. tomato* DC3000, Tc'
- pBL50: pRK415 derivative, *lon* complementation
- pBL52: pCF1010 derivative, *hrpA* promoter fusion

**Table 2. Primers used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence (5'-3')*</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>lon-F (KpnI)</td>
<td>GGTACCAGAAGACCACTATT</td>
<td>ORF of lon</td>
</tr>
<tr>
<td>lon-R (SacI)</td>
<td>GAGCTCTTAATAGCTGCTATTC</td>
<td>ORF of lon</td>
</tr>
<tr>
<td>lon-cF (BamHI)</td>
<td>AAGCTTGGCTGGAAGCGCAAGA</td>
<td>Complementation of lon</td>
</tr>
<tr>
<td>lon-cR (HindIII)</td>
<td>GGATCCTTAATGCCTGCTATTC</td>
<td>Complementation of lon</td>
</tr>
<tr>
<td>hrpA-pF (PstI)</td>
<td>AGTCTGGATCTGCTCCGTCTG</td>
<td>Promoter region of <em>hrpA</em></td>
</tr>
<tr>
<td>hrpA-pR (XbaI)</td>
<td>GACTCTTGAAGCGTTTCTTCCG</td>
<td>Promoter region of <em>hrpA</em></td>
</tr>
</tbody>
</table>

* Underlines indicate the restriction enzyme sites for the enzymes indicated in the primer names.
Lon is a negative regulator in *P. syringae pv. tabaci* 11528

From a previous report, we know that the Lon protease functions as a negative regulator of TTSS in *P. syringae pv. syringae* 61 by degrading HrpR under hrp-repressing conditions (Bretz et al., 2002). Therefore, we decided to verify that the Lon protease also acts as a negative regulator of TTSS in *P. syringae pv. tabaci* 11528. In order to confirm this, we constructed an HrpL-dependent *hrpA* promoter (*hrpA*-lacZ) transcriptional fusion plasmid, pBL52. *hrpA* encodes the structural protein of the Hrp pilus and is one of the genes comprising the *hrp/hrc* gene cluster.

In the parental strain, the expression of the *hrpA*-lacZ reporter construct used to monitor *hrp* regulon expression resulted in approximately 700 MU of β-galactosidase activity during logarithmic growth in the *hrp*-repressing medium (Fig. 3). In contrast, a *hrp* constitutive mutant, BL42, exhibited more than 2,000 MU activity, consistent with the LacZ+ phenotype observed on X-gal plates. The LacZ+ activity detected in the mutant grown in *hrp*-repressing medium was equivalent to the activities observed in the wild-type strain grown in *hrp*-inducing medium. Growth of BL42 in *hrp*-inducing medium caused a further, but
Fig. 3. β-galactosidase activity of a hrpA promoter-lacZ transcriptional fusion. β-galactosidase activity was measured in P. syringae pv. tabaci 11528 and BL42 grown in hrp-repressing medium (King’s medium B) and hrp-inducing medium (M9MM supplemented with 5 mM mannitol). The LacZ activity detected in the mutant grown in hrp-repressing medium was equivalent to the activity observed in the wild-type grown in hrp-inducing medium. The data are expressed as the average of 3 replicates ± the standard deviation.

The lon mutant is more rapidly recognized by its target plant
We determined that Lon also functions as a negative regulator of TTSS. However, we still do not know if Lon selectively degrades HrpR according to the growth conditions or if the expression of the Lon protease is differently regulated based on the growth conditions. Although the Lon protease degrades HrpR in hrp-repressing medium, we decided to examine the effects of the Lon mutant on TTSS expression under conditions of hrp induction similar to the conditions in planta. BL42 showed slightly increased expression of the hrpA promoter in hrp-inducing medium compared to the wild-type. We found that lon disruption elicits a more rapid and strong response from the host defense system than that seen in the wild-type strain (Fig. 4), suggesting that Lon is essential for systemic P. syringae infection in plants.

DISCUSSION
P. syringae hrp genes are necessary for pathogenicity of the host range variants of P. syringae. Proteolysis by Lon has been implicated in the regulation of hrp gene expression and the activity of this broadly conserved protein secretion system. The ability of P. syringae to elicit defense responses in resistant plants and pathogenesis in susceptible plants has been linked to a TTSS and effectors encoded by the environmentally regulated hrp regulon (Bretz et al., 2002; Collmer et al., 2002). The induction of hrp regulon expression during pathogenesis had been shown previously to be dependent upon the unusual enhancer binding proteins HrpR and HrpS and the alternative
322 Lon Protease in *P. syringae* pv. *tabaci* 11528

Fig. 4. (A) Pathogenicity test of *P. syringae* pv. *tabaci* 11528 (WT) and BL42 on tobacco leaves. *P. syringae* pv. *tabaci* 11528 and BL42 overnight cultures were adjusted to an OD$_{600}$ of 0.5 (approximately $1.0 	imes 10^8$ CFU ml$^{-1}$). After 14 DPI, the leaves showed different levels of necrosis. Those treated with BL42 showed larger necrotic brown lesions than those treated with the wild-type, indicating that BL42 elicited a more rapid and strong host defense response and that Lon protease performs other functions in *hrp*-inducing medium. (B) Population assays of the wild-type (black circles) and BL42 (white circles) strains in tobacco leaves. Each value represents the average of at least three leaf discs. Vertical bars indicate the standard error. (C) HR test of *P. syringae* pv. *tomato* DC3000 (WT) and BL44 on tobacco leaves. *P. syringae* pv. *tomato* DC3000 and BL44 typically elicited a non-host defense response, HR, at a concentration of $2 \times 10^8$ CFU ml$^{-1}$. BL44 elicited an HR at concentrations of $10^7$, and $10^6$ CFU ml$^{-1}$. However, *P. syringae* pv. *tabaci* 11528 and *P. syringae* pv. *tomato* DC3000 strains differed in that the symptoms of the plant defense response were not dispersed in *P. syringae* pv. *tomato* DC3000 strains after 4 DPI. This is because the *P. syringae* pv. *tomato* DC3000 strain elicited only an HR in tobacco, which is regarded as a non-host plant. HR is a rapid cell death response at the site of pathogen infection in resistant plants. All results are representative of 3 independent experiments.

The results presented here indicate that the Lon protease also negatively regulates the TTSS in *P. syringae* pv. *tabaci* 11528, as determined by measuring the expression level of the HrpL-dependent *hrpA* promoter. The HrpRS operon was observed to be constitutively expressed, whereas the HrpR- and HrpS-dependent *hrpL* promoter was environmentally regulated, thereby requiring a mechanism to negatively regulate HrpR and/or HrpS activity. Inactivation of *lon* in *P. syringae* pv. *syringae* 61 resulted in constitutive expression of the *hrpL* promoter and substantially reduced the degradation of HrpR. As both HrpR and HrpS are required for maximal expression of the *hrpL* promoter (Hutcheson et al., 2001), Lon-associated degradation of HrpR would reduce the expression of the *hrpL* promoter, thereby explaining the increased activity observed in the *lon* mutants.

The activity of Lon appears to be regulated in a manner consistent with the observed environmental regulation of the *hrp* regulon. Lon-associated degradation of HrpR was detected during growth in media known to be non-inductive for *hrp* regulon expression, but was minimal during growth of the wild-type strains in inductive media. The reduced degradation of HrpR during growth in *hrp*-inducing media indicates that regulated proteolysis is required for the expression of the TTSS in *P. syringae* strains. Consistent with this conclusion, a representative *hrp* regulon promoter was expressed in *lon* mutants at levels nearly equivalent to that observed in the wild-type strain during growth in *hrp*-inductive media.
Interestingly, lon mutants of \textit{P. syringae} exhibited substantially higher secretion of an effector and induced plant defense responses faster than the wild-type strain. This suggests that Lon has an additional activity in the regulation of TTSS. The role of Lon in the proteolysis of abnormally folded proteins (Gottesman, 1996) raises the possibility that Lon could affect the stability of TTSS effectors, as they are predicted to be in an unfolded state prior to secretion (Feldman et al., 2002; Stebbins and Galan, 2001). As the rate of synthesis of an effector is expected to be equivalent in the wild-type and Lon mutant, there would consequently be a difference in the half-life of the effector. Thus, it is suggested that Lon plays a dual role in the regulation of the hpr TTSS of \textit{P. syringae} strains by (i) regulating the hpr regulation through regulated proteolysis of HrpR (Bretz et al., 2002), and (ii) by controlling the accumulation of effectors prior to secretion (Losada and Hutcheson, 2005).

The data presented here indicate that Lon protease plays a significant role in the regulation of \textit{P. syringae} pathogenesis and TTSS. Lon may also play a similar role in the regulation of the TTSS found in other pathogenic bacteria. A recent report implicating Lon in \textit{Pseudomonas aeruginosa} virulence (Takaya et al., 2008) seems to indicate that Lon-mediated regulation of TTSS may be a common mechanism of regulating virulence factors in gram-negative pathogens. These results, coupled with the results reported in this manuscript, support the conclusion that Lon protease plays a critical role in the TTSS of these bacteria.

**ACKNOWLEDGMENTS**

This work was supported by a Korea Research Foundation Grant funded by the Korean Government (KRF-2008-313-C00799).

**REFERENCES**


Lon Protease in *P. syringae pv. tabaci*