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Molecules and Cells

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

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***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 *hrpL* in response to signals from plants or in *hrp*-inducing minimal medium. HrpL 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 *hrp* regulon. Under *hrp*-inducing conditions, however, the *hrp* 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-

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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 *hrpL* promoter and involves Lon protease-mediated degradation of HrpR. Lon-associated degradation of HrpR is reduced under conditions inducive 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 pathovars. We designed a primer set (Table 2), *lon*-F(*Kpn*I) and *lon*-R(*Sac*I), 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 Δ*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 *Eco*RI, 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 *Nco*I and pBL45 was treated with *Sty*I. 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 *Kpn*I and *Sac*I 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*-cF(*Bam*HI) and *lon*-cR(*Hin*dIII) 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(*Pst*I) and *hrpA*-pR(*Xba*I). The PCR product was digested with *Pst*I and *Xba*I and cloned into pCF1010, 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 OD_{600} 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^{-4} 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 10^8 CFU ml^{-1}. To investigate the HR cell death, tobacco leaves were infiltrated with a needle-less syringe at 10^5 to 2×10^8 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-

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	References or source
Bacterial strains		
E. coli		
DH5α	Transformation host for cloning vector	(Sambrook and Russell, 2001)
χ7213	Conjugation donor: an *E. coli* SM10 λpir derivative, DAP required (*Δasd)*	(Daigle et al., 2001)
BL21(DE3)	Host for protein overexpression: an *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and LacI^q	(Sambrook and Russell, 2001)
P. syringae pv. *tabaci* 11528	ATCC 11528, wild-type	ATCC, USA
BL42	ATCC 11528 derivative, *Δlon*	This study
BL43	BL42 carrying pBL50, Tc^r	This study
BL46	ATCC 11528 carrying pBL52, Tc^r	This study
BL47	BL42 carrying pBL52, Tc^r	This study
P. syringae pv. *tomato* DC3000	ATCC BAA-871, wild-type, spontaneous Rf^r	ATCC, USA
BL44	ATCC BAA-871 derivative, *Δlon*	This study
Agrobacterium tumefaciens NT1	*Agrobacterium tumefaciens* carrying pDCI41E33	(Shaw et al., 1997)
Plasmids		
pGEM-T Easy	TA-Cloning Vector, Ap^r	Promega
pBlueScript II SK(+)	Cloning Vector, Ap^r	Invitrogen
pDMS197	Suicide vector, R6K *ori*, *sacB*$^+$, *oriT*, Tc^r	(Edwards et al., 1998)
pRK415	Broad-host-range vector, RK2 *ori*, Tc^r	(Keen et al., 1988)
pRKlac290	*lacZ* transcriptional fusion vector, Tc^r	(Gober and Shapiro, 1992)
pCF1010	*lacZ* transcriptional fusion vector, RSF1010 *ori*, *oriT*, Tc^r	(Lee and Kaplan, 1995)
pBL43	pDMS197 derivative, recombinant suicide plasmid for *Δlon* in *P. syringae* pv. *tabaci* 11528, Tc^r	This Study
pBL47	pDMS197 derivative, recombinant suicide plasmid for *Δlon* in *P. syringae* pv. *tomato* DC3000, Tc^r	This Study
pBL50	pRK415 derivative, *lon* complementation	This study
pBL52	pCF1010 derivative, *hrpA* promoter fusion	This study

[a] Abbreviations: Ap^r, Ampicillin resistance; Tc^r, Tetracycline resistance; Rf^r, Rifampicin resistance

Table 2. Primers used in this study

Name	Oligonucleotide sequence (5′-3′)*	Characteristics
lon-F (*Kpn*I)	GGTACCATGAAGACCACTATT	ORF of *lon*
lon-R (*Sac*I)	GAGCTCTTAATGCGTGCTAATTC	ORF of *lon*
lon-cF (*Bam*HI)	AAGCTTGCGCTGGAGCGCAAGA	Complementation of *lon*
lon-cR (*Hin*dIII)	GGATCCTTAATGCGTGCTAATTC	Complementation of *lon*
hrpA-pF (*Pst*I)	AGTCCTGCAGTCTGTCCGCTGCT	Promoter region of *hrpA*
hrpA-pR (*Xba*I)	GACTTCTAGAGCCGTTCTCTTCG TTCGC	Promoter region of *hrpA*

*Underlines indicate the restriction enzyme sites for the enzymes indicated in the primer names

dent protease (Gottesman et al., 1993), 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 (6.0×10^6 versus 0.8×10^9 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 phytotoxins 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

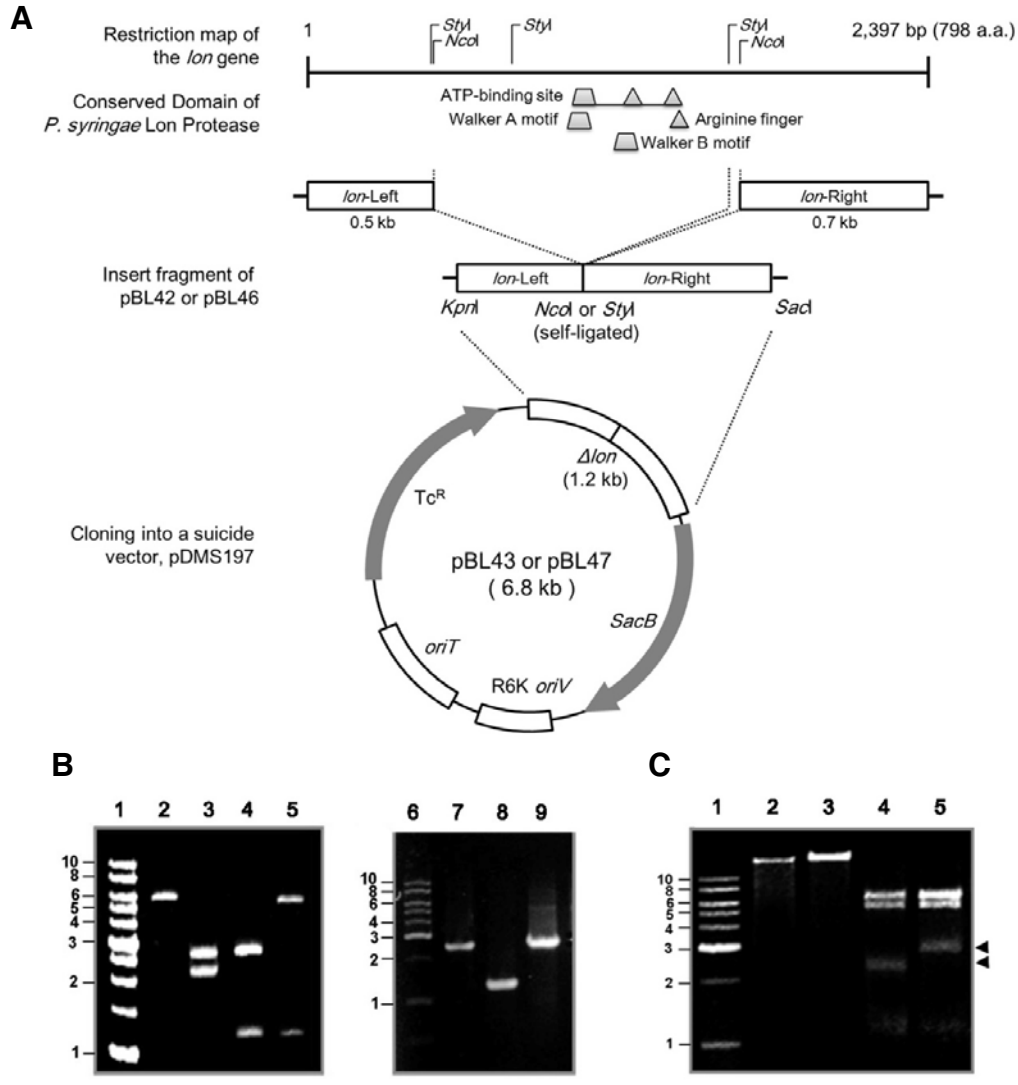

Fig. 1. (A) Physical map of the recombinant suicide plasmid pBL43 (or pBL47). Recombinant suicide plasmid pBL43 (or pBL47) carries a 1.2 kb DNA fragment that was restricted with *Nco*I inside the *lon* gene and self-ligated from pBL42 (or pBL46). The plasmid carries R6K *ori* for its replication and the *sacB* gene for counterselection for sucrose resistance. (B) Genetic confirmation of *lon* deletion. Lanes 1 and 6, DNA ladder; lane 2, pDMS197; lane 3, pBL41 restricted with *Kpn*I and *Sac*I; lane 4, pBL42 restricted with *Kpn*I and *Sac*I; lane 5, pBL43 restricted with *Kpn*I; lanes 7, 8, and 9, PCR product amplified with *lon*-F(*Kpn*I) and *lon*-R(*Sac*I) using the chromosomal DNAs of *P. syringae* pv. *tabaci* 11528, BL42, and BL43, respectively, as templates. (C) Genetic confirmation of pBL52. Lane 1, DNA ladder; lane 2, pCF1010 restricted with *Pst*I; lane 3, pBL52 restricted with *Pst*I; lane 4, pCF1010 restricted with *Bam*HI; lane 5, pBL52 restricted with *Bam*HI. The arrows indicate that pBL52 contains a 620-bp fragment compared with pCF1010.

from 5 repeat experiments. Since tabtoxin is a virulence factor and phytotoxin produced by *P. syringae* pv. *tabaci* 11528, it may also have a potential relationship with TTSS (Fig. 2C). The coordinated regulation of the *hrp/hrc* cluster, which is required for pathogenicity and virulence factors such as COR and tabtoxin, seems logical but has not been established.

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

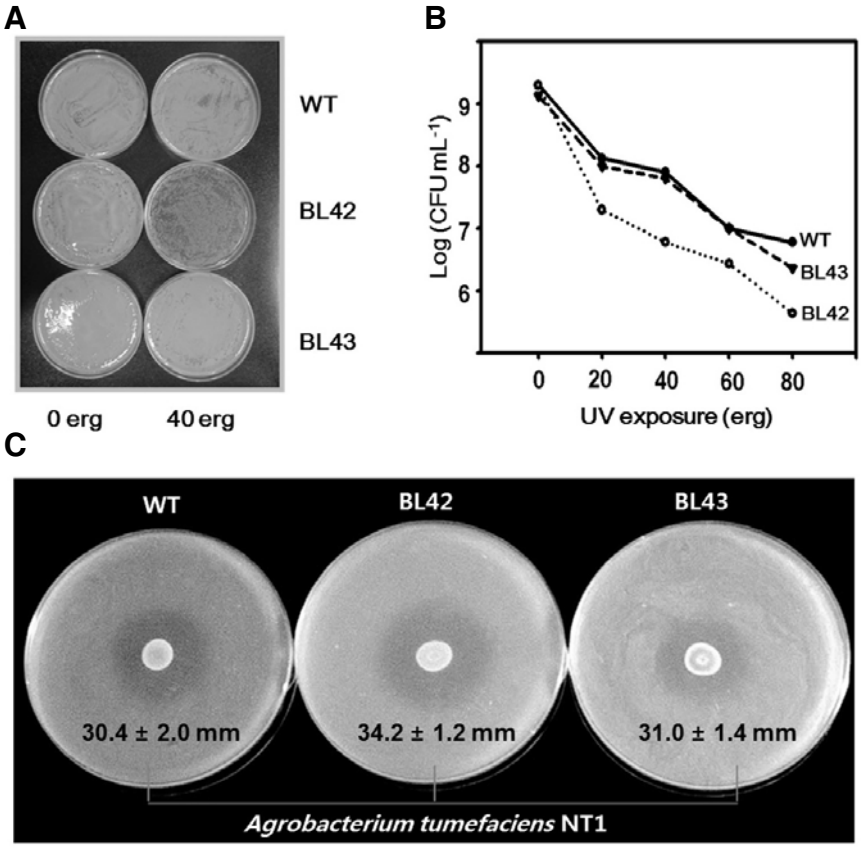

Fig. 2. Phenotype test. (A) Sensitivity of cells to UV irradiation. Stationary phase cells of *P. syringae* pv. *tabaci* 11528, BL42, and BL43 were exposed to UV radiation, expressed as erg (10^{-2} cm^2), for various durations. Colonies arising from surviving cells were counted following 48 h of incubation in the dark. (B) Data were transformed as a log survival ratio. The Lon mutant strain, BL42 showed an increased UV sensitivity compared to the wild-type. BL43 showed a pattern of UV sensitivity similar to the wild type. (C) A comparison of the ability of *P. syringae* strains to produce tabtoxin. As judged from a comparison of the inhibitory zones, BL42 produced more tabtoxin than wild-type and BL43, indicating that there is a potential relationship between Lon protease and tabtoxin. The results are representative of 5 independent experiments. The diameter of clear zone was measured in millimeter. The values indicate the averages of the diameter of clear zones and the standard errors of the each means.

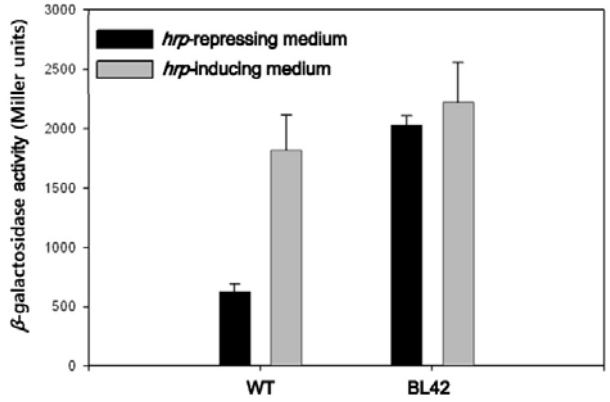

Fig. 3. β-galactosidase activity of a *hrpA* promoter-*lacZ* transcriptional fusion. β-galactosidase activity was measured in *P. syringae* pv. *tabaci* 11528 and BL42 grown in *hrp*-repressing medium (King's medium B) and *hrp*-inducing medium (M9MM supplemented with 5 mM mannitol). The LacZ activity detected in the mutant grown in *hrp*-repressing medium was equivalent to the activity observed in the wild-type grown in *hrp*-inducing medium. This indicates that the Lon protease also serves as a negative regulator in *P. syringae* pv. *tabaci* 11528. The data are expressed as the average of 3 replicates ± the standard deviation.

modest, stimulation of reporter expression.

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

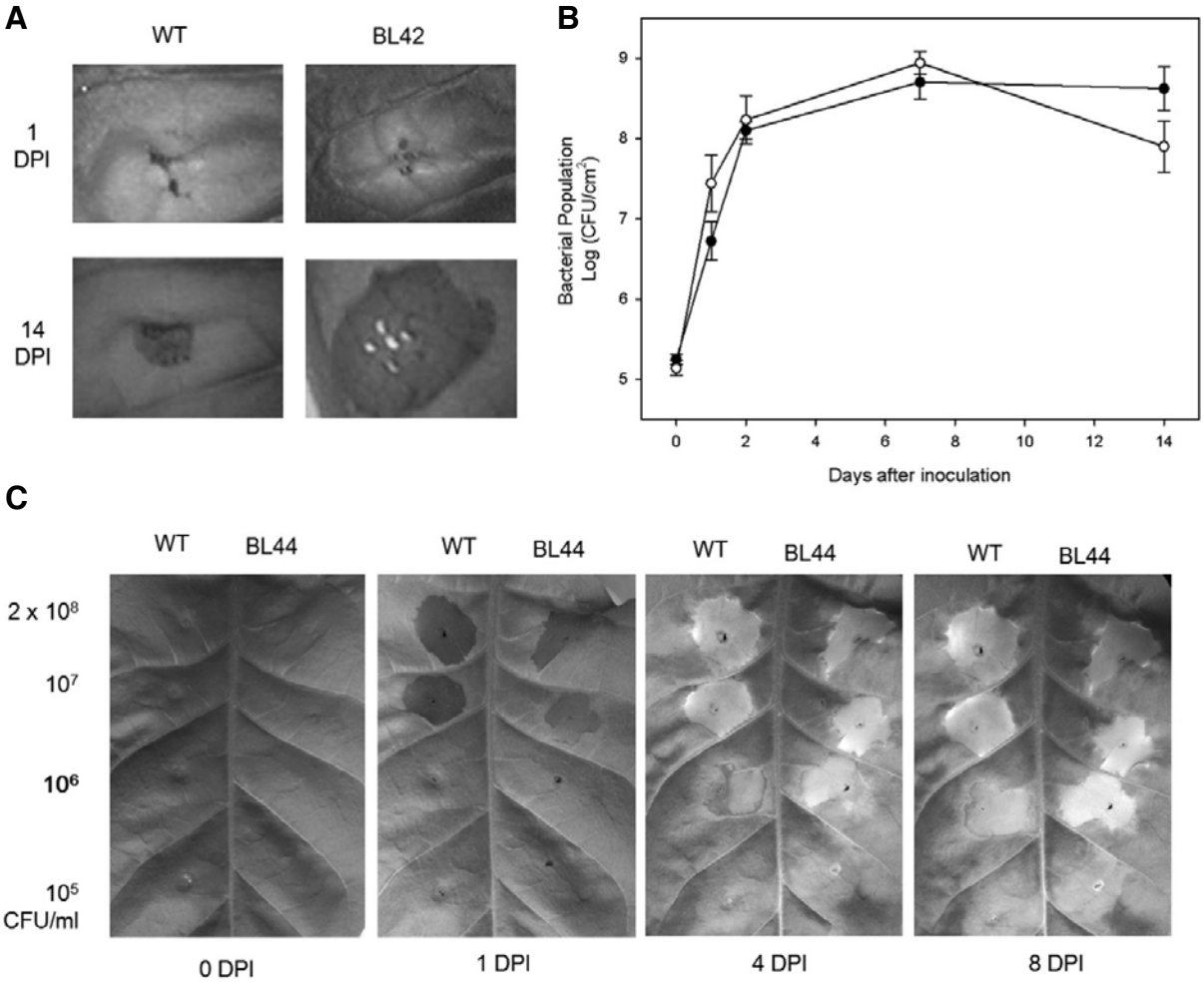

Fig. 4. (A) Pathogenicity test of *P. syringae* pv. *tabaci* 11528 (WT) and BL42 on tobacco leaves. *P. syringae* pv. *tabaci* 11528 and BL42 overnight cultures were adjusted to an OD_{600} of 0.5 (approximately 1.0×10^8 CFU ml^{-1}). After 14 DPI, the leaves showed different levels of necrosis. Those 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 2×10^8 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.

sigma factor HrpL (Hutcheson et al., 2001); however, the mechanism for modulating the activity of these proteins under *hrp*-repressing conditions had not been established.

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 *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 *hrp* TTSS of *P. syringae* strains by (i) regulating the *hrp* regulon 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 *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 *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 the Lon protease plays a critical role in the TTSS of these bacteria.

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