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Effects of *galU* Mutation on *Pseudomonas syringae*–Plant Interactions

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Bacterial *galU* coding for a uridine diphosphate-glucose pyrophosphorylase plays an important role in carbohydrates biosynthesis, including synthesis of lipopolysaccharides (LPS), membrane-derived oligosaccharides, and capsular polysaccharides. In this study, we characterized the *galU* mutant of *Pseudomonas syringae* pv. *syringae* 61 (Psy61), a necrotizing plant pathogen whose pathogenicity depends on a functional type III secretion system (T3SS), and showed that the Psy61 *galU* mutant had reduced biofilm formation ability, was nonmotile, and had an assembled T3SS structure but failed to elicit hypersensitive response in resistant plants and necrotic lesions in susceptible plants. Moreover, the defective LPS and other pathogen-associated molecular patterns (PAMPs) on the surface of the Psy61 *galU* mutant were capable of inducing PAMP-triggered immunity, which severely compromised the ability of the Psy61 *galU* mutant to survive in planta. Our results demonstrated that the complete LPS protected plant-pathogenic bacteria from host innate immunity, similar to what was found in animal pathogens, prior to the translocation of T3S effectors and bacterial multiplication.

Phytopathogenic *Pseudomonas syringae* pv. *syringae* 61 (Psy61) was previously characterized as a potent hypersensitive response (HR)-eliciting bacterium on tobacco, whose HR-eliciting activity depends on a functional type III secretion system (T3SS) and an array of effectors that were encoded by the *hrp*/*hrc*/*hop* genes (Huang et al. 1995; Collmer et al. 2000). Mutations in genes coding for the T3SS led to the incapability of eliciting HR in resistant plants and multiplication in susceptible plants, whereas T3S effectors (e.g., AvrPtoB) (Abramovitch et al. 2003), are known virulence proteins that are involved in promoting parasitism by various mechanisms (Mudgett 2005). Other virulence-associated genes involved in the biosynthesis of exopolysaccharides (EPS), extracellular enzymes, and toxins have differential contributions to the infectivity on susceptible plants without compromising their HR-eliciting ability on resistant plants (Beattie and Lindow 1994). The HR, also known as gene-for-gene defense response or effector-triggered immunity (ETI) (Jones and Dangl 2006), is achieved by successful recognition of the T3S effectors by various plant resistance (R) proteins. Aside from ETI, plant cells recognize pathogen-associated or microbe-associated molecular patterns (PAMPs or MAMPs) by pattern-recognition receptors to launch a quick but less specific PAMP-triggered immunity (PTI) that can restrict bacterial growth (He et al. 2007; Schwessinger and Zipfel 2008). The PTI occurred early upon the invasion of both pathogenic and nonpathogenic bacteria; therefore, it becomes the first active defense encountered by microbial invaders. Successful pathogens must overcome the PTI before they can grow in planta. The bacterial cell wall serves as a shielding armor to protect bacteria against the early defense prior to the expression and translocation of T3S effectors—for example, AvrPto (Hauck et al. 2003) and HopPtoM (DebRoy et al. 2004)—into plant cells to actively suppress the PTI.

In gram-negative bacteria, such as Psy61 used in this study, the cell wall is composed of peptidoglycan and an outer membrane in which lipopolysaccharide (LPS) is a major component. LPS is a tripartite molecule consisting of lipid A, core oligosaccharides, and O-antigen. The O-antigen plays an important role as a virulence factor in many gram-negative, animal-pathogenic bacteria (Finlay and McFadden 2006), whereas the lipid A moiety, recognized by Toll-like receptor (TLR) proteins, induces innate and acquired immunity in mammalian systems (Miller et al. 2005). In plant systems, the oligosaccharide moieties of LPS induce localized defense-related responses that can be readily detected by the suppression of ETI elicited by subsequent pathogen inoculation (Newman et al. 2001; Klement et al. 2003). Similar to the mammalian innate immunity, the perception of bacterial LPS in *Arabidopsis thaliana* induces the expression of nitric oxide (NO) synthase and the accumulation of NO, leading to the expression of defense-related genes and accounting for the resistance to pathogenic bacteria (Zeidler et al. 2004). Purified LPS also induces defense responses associated with programmed cell death in rice (Desaki et al. 2006) and systemic resistance in radish (Leeman et al. 1995), potato (Reitz et al. 2000), and *A. thaliana* (Van Wees et al. 1997), although the critical structures of LPS recognized by individual plant species remain to be elucidated.

The aim of this work was to study the fate of LPS-defective Psy61 in planta by creating a marker-exchanged mutation in *galU*. The *galU* codes for a uridine diphosphate (UDP)-glucose pyrophosphorylase with a well-defined role in the LPS biosynthesis by synthesizing UDP-glucose (UDP-Glc) from glucose 1-phosphate and UTP (Schulman and Kennedy 1977).

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Nucleotide sequence data reported is available in the GenBank database under the accession number U03751.

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The *galU* mutants of animal-pathogenic bacteria—for example, *Klebsiella pneumoniae* (Chang et al. 1996), *P. aeruginosa* (Priebe et al. 2004), and *Shigella flexneri* (Sandlin et al. 1995)—produce an altered LPS structure lacking all sugars beyond the heptose residues, cause a loss of flagellar motility, reduce biofilm formation, and attenuate virulence for host infection. The *galU* mutation also affects the secretion of *Escherichia coli* α-haemolysin and *Dickeya dadantii* (previously known as *Erwinia chrysanthemi*) proteases via the type I secretion system (T1SS) (Wandersman and Letoffe 1993) and the correct trafficking of *S. flexneri* IcsA on the cell surface (Sandlin et al. 1995). Nevertheless, the truncated LPS of the *S. flexneri galU* mutant does not appear to interfere with the formation and function of the T3SS, which was supported by the abundance of T3SS structure emerging from the outer membrane and the potential of bacterial invasion into epithelial cells (West et al. 2005). Based on the traits of the Psy61 *galU* mutant in this study, we reported that the physical barrier of LPS is critical for protecting plant-associated bacteria from PTI. The incomplete LPS of the Psy61 *galU* mutant lacking O-antigen and an outer oligosaccharide core can still mediate the interaction between the bacteria and plant cells to induce PTI which, consequently, arrests bacterial growth in the initial phase of infections before the bacteria translocate T3S effectors into plant cells. As a result, the LPS-deficient mutant cannot survive in susceptible plants or elicit the HR in resistant plants even though it is capable of assembling the T3SS.

RESULTS

Cloning and sequencing of a Psy61 genomic region harboring *galU*.

Escherichia coli FF4001 (Brede et al. 1991) harboring Psy61-derived genomic library plasmids was grown on MacConkey agar plates supplemented with 0.2% galactose, and the transformants that could grow and form red colonies on the plates were selected. One recombinant clone, pNCHU467, was chosen for further characterization. Sequence analysis revealed that pNCHU467 has an insert of 2,742 bp harboring complete *galU* and *gor* genes coding for a putative UDP-Glc pyrophosphorylase and glutathione reductase, respectively (Fig. 1A). The nucleotide sequences of Psy61 *galU* share 80% identity with the *P. aeruginosa* ATCC10145 *galU* gene (GenBank accession no. U03751) that has been biochemically characterized to encode a functional UDP-Glc pyrophosphoylase (Chang et al. 1999). In addition, a number of putative *galU* sequences of *Pseudomonas* spp. were identified from the GenBank database that display high similarities, ranging from 77 to 94%, to the Psy61 *galU*. Comparison of their deduced amino acid sequences shows some substitutions between residues 143 and 153, whereas the rest of the amino acid sequences are identical to one another.

Biochemical characterization of Psy61 *galU* mutant.

Using the cloned plasmid pNCHU467, a deletion in Psy61 *galU* was generated by marker-exchange strategy. The relevant

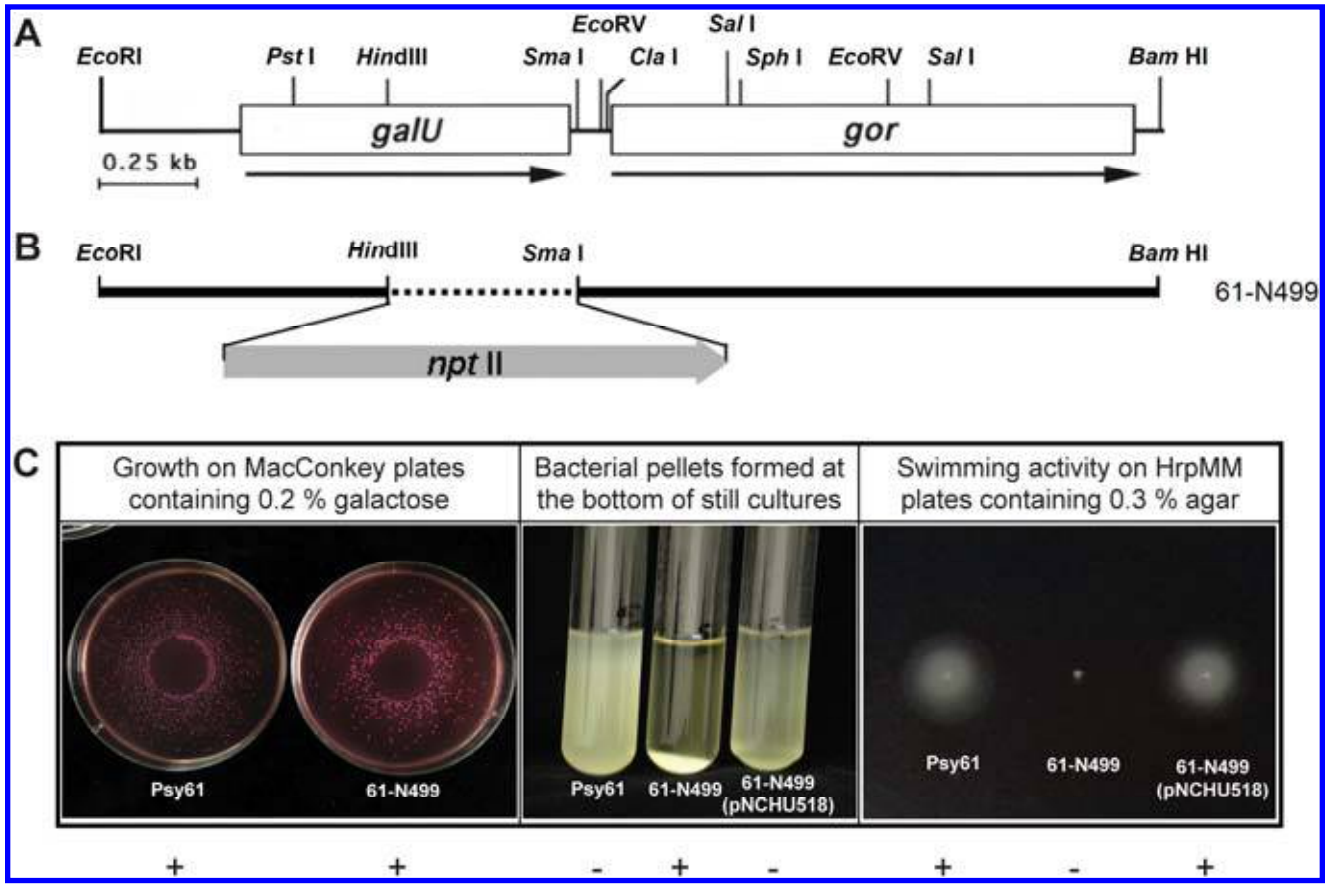

Fig. 1. Construction and phenotypes of the *galU* mutation in *Pseudomonas syringae* pv. *syringae* 61 (Psy61). **A,** Schematic presentation of the cloned 2.7-kb DNA fragment containing *galU* and *gor* genes. Coding regions are shown in open boxes, and arrowed lines indicate the direction of transcription. **B,** Gray arrow in the mutant construction denotes the terminator-lacking *nptII* cassette and its transcriptional direction, and dashed line represents internal deletion of *galU* coding region that is replaced by the *nptII* cassette. **C,** Phenotypes of Psy61 (wild type), 61-N499 (*galU* mutant), and 61-N499 (pNCHU518) (complementing strain) on different medium. Left panel: galactose sensitivity test on MacConkey agar plates supplemented with 0.2% galactose, "+" reaction indicates bacterial growth. The colony-forming and growing phenotypes of the complementing strain are similar to 61-N499 (*data not shown*). Middle panel: sedimentation test in King's B broth; "+" and "–" reaction indicates bacterial sedimentation and no sedimentation in still-liquid cultures, respectively. Right panel: bacterial motility on Hrp-derepressing medium (HrpMM) containing 0.3% agar; "+" and "–" represent bacterial swimming and no-swimming phenotypes, respectively.

gene organization of the resultant mutant strain 61-N499 (Fig. 1B) was confirmed by Southern hybridization and polymerase chain reaction (PCR) (data not shown). The *galU* mutant 61-N499 was characterized for its growth on MacConkey agar plates containing 0.2% galactose, bacterial sedimentation in a still-liquid culture, and motility on 0.3% HrpMM agar medium. The results showed that 61-N499 is drastically different from the wild-type Psy61 in all assays, except that it can grow as well as the wild type on MacConkey plate supplemented with 0.2% galactose (Fig. 1C). LPS prepared from 61-N499 was devoid of O-antigen, and its LPS core migrated slightly faster than that of the wild-type strain (Fig. 2). The 61-N499 had no detectable UDP-Glc pyrophosphorylase activity (<0.1 unit) compared with the specific activity of 35.13 units for Psy61 (Table 1). 61-N499 grown in King's B (KB) broth had reduced capability of forming biofilms on plastic surfaces (determined by crystal violet staining, as described below). The stained biofilms at 595-nm absorbance were 0.114 ± 0.009 and 0.332 ± 0.045 for 61-N499 and Psy61, respectively (Table 1). In addition, H_2O_2 sensitivity assays showed the diameters of the inhibition zone on KB agar plates were 17.5 ± 0.299 for Psy61 and 22.63 ± 0.674 for 61-N499, indicating that 61-N499 is more sensitive to hydrogen peroxide than the wild-type Psy61 (Table 1). The 61-N499 mutant phenotypes could be rescued by introducing the plasmid pNCHU518 containing the complete *galU* coding region, indicating that a single mutation in *galU* leads to multiple alterations in the biological activities of Psy61 (data not shown). The results are in agreement with previously characterized *galU* mutations in other gram-negative bacteria, except for the colony forming capability on MacConkey agar containing 0.2% galactose, on which the growth of enteric bacteria *galU* mutants is inhibited (Brede et al. 1991).

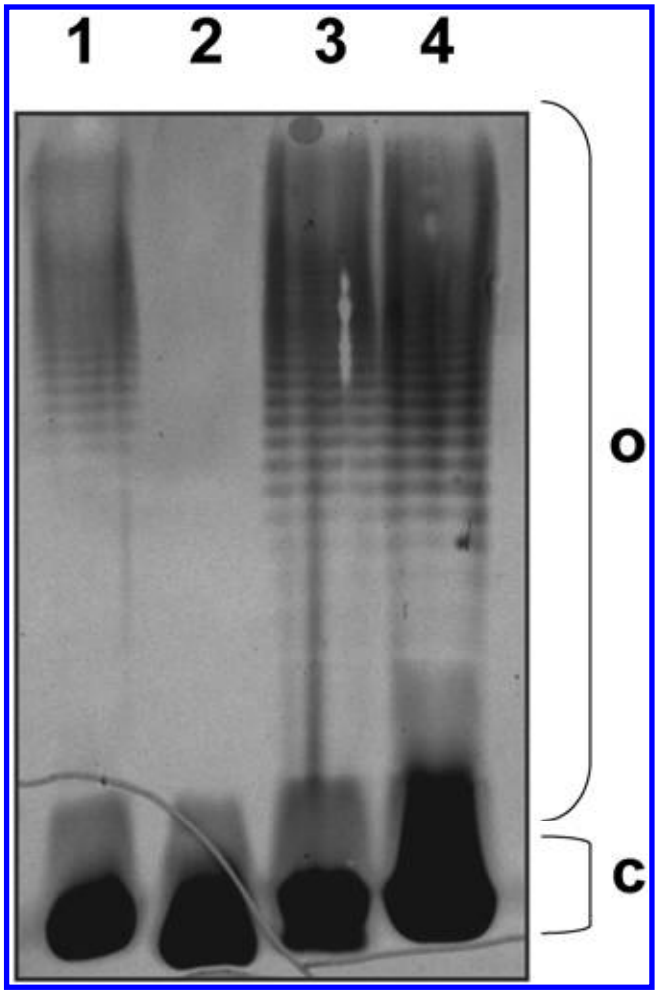

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide) analysis of lipopolysaccharide (LPS) from *Pseudomonas syringae* pv. *syringae* 61 (Psy61) and its derivatives. Lane 1, type III secretion system-defective mutant 61-N393 (*hrcC* mutant); lane 2, 61-N499 (*galU* mutant); lane 3, 61-N499 (pNCHU518) (complementing strain); lane 4, wild-type Psy61. LPS was extracted by phenol-water method, separated by SDS-PAGE, and stained by silver nitrate. O = O-antigen; C = LPS core.

Microscopic observation of flagella on bacterial surface.

The motility assay in Figure 1C showed that the Psy61 *galU* mutant has marked reduction in motility, which prompted us to examine the flagellar biogenesis in the *galU* mutant. The flagellar filaments were collected by shearing flagella off bacterial surfaces, denatured in 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and detected by immunoblotting with FliC antiserum. The result showed that the amount of flagellin collected from the surface of the Psy61 *galU* mutant was much lower than that in the wild-type Psy61 (Fig. 3A, upper left and central panels); nevertheless, the flagellin of the *galU* mutant was as glycosylated as that of its wild type (Fig. 3A, upper right panel). Taguchi and associates (2008) showed that the glycosylation of flagellin stabilizes flagellar filament structure and is required for swarming and adhesion abilities. Mutation of *galU* in Psy61 does not affect flagellin glycosylation (Fig. 3A), indicating that the reduced number of flagella on the surface of 61-N499 is not due to the formation of unstable, nonglycosylated flagellar filament. Concerned with the low recovery of flagellar filaments, we monitored flagellin synthesis in Psy61 and 61-N499 by simultaneously resolving equal amount of total proteins prepared from cultured bacteria (TF fractions) in 12% SDS PAGE for Coomassie blue staining (data not shown) and immu-

Table 1. Phenotypic characterization of *Pseudomonas syringae* pv. *syringae* 61 (Psy61) and its derivative *galU* mutant[a]

Phenotypes, strains	Psy61	*galU* mutant
Biofilm formation (OD_{595} absorbance)[b]	0.332 ± 0.045	0.114 ± 0.009
UDP-Glc pyrophosphorylase activity[c]	35.13	<0.1 (ND)
Bacteria with surface flagella (%)[d]	22.65 ± 0.095	2.27 ± 0.032
H_2O_2 sensitivity (mm)[e]	17.50 ± 0.299	22.63 ± 0.674

[a] All values listed in this table are significantly different based on Duncan's analysis.
[b] Bacteria were cultured in King's B (KB) broth for 70 h before assay for biofilm formation at and optical density at 595 nm (OD_{595}) absorbance (means ± standard deviation [SD] of three replications).
[c] Specific units of uridine diphosphate–glucose (UDP-Glc) pyrophosphorylase was determined by the synthesis of nanomoles NADPH per minute per milligram of total proteins. ND, no detectable activity.
[d] Percentage of surface flagella (means ± SD of nine fields) was calculated from the number of bacteria bearing intact flagella observed by transmission electron microscopy.
[e] H_2O_2 sensitivity, shown in millimeters of diameter (means ± SD of three replications), was assayed by disk diffusion test using 6-mm-diameter paper disk containing 0.1% (vol/vol) hydrogen peroxide on KB agar.

nodetecting with FliC antiserum (Fig. 3A, bottom panel), and the immunoblot revealed that the amount of flagellins in the TF fractions of both bacteria was equal, indicating that the loss of *galU* does not affect flagellin synthesis, either. Furthermore, bacterial flagellation was directly observed by transmission electron microscopy (TEM), and the percentage of flagellated bacteria was calculated using the total number of bacteria seen in at least nine randomly selected fields. The percentage of flagellated bacteria was greatly reduced in the *galU* mutant 61-N499 (2.27 ± 0.032%) compared with 22.65 ± 0.095% in Psy61 (Table 1). Two representative micrographs were shown in Figure 3B, depicting flagellated bacteria of Psy61 (Fig. 3B, top panel) and the *galU* mutant 61-N499 (Fig. 3B, bottom panel).

Mutation in *galU* of Psy61 affects HR elicitation and bacterial survival in tobacco leaves.

To test whether the *galU* mutation has altered the ability of Psy61 to elicit an HR, the *galU* mutant 61-N499 and its complementing strain 61-N499 (pNCHU518) were individually infiltrated into tobacco leaves at a concentration of 10^8 CFU/ml, and the HR was recorded at 24 h postinfiltration (hpi). 61-N499 has lost its HR-eliciting activity (Fig. 4A), which can be restored by the heterologous expression of galU harbored in pNCHU518. The null response was further confirmed by staining the infiltrated leaf panels with Evans blue to show that there were only a few dead cells in the 61-N499-infiltrated area at 2 days postinoculation (dpi) (Fig. 4B, second panel from the left), whereas the wild-type Psy61 and the complementing strain 61-N499 (pNCHU518) caused massive destruction of tobacco parenchyma cells at 1 dpi (Fig. 4B, first and third panels from the left). The number of dead cells elicited by the *galU* mutant 61-N499 was not increased with prolonged inoculation time (6 dpi), demonstrating that the reduction of the HR-eliciting ability of the *galU* mutant could not be restored at a later time. Moreover, the kinetics of bacterial survival in tobacco leaves differed greatly between wild-type Psy61 and *galU* mutant 61-N499 (Fig. 5). Bacteria were infiltrated at concentrations of approximately 10^8 (Fig. 5A) and 10^5 (Fig. 5B) CFU/ml, respectively, and leaf disks were harvested at 0, 2, 4, 6, 8, 12, 24, 45, and 72 hpi for bacterial enumeration. For Psy61, the leaf panels infiltrated with bacteria of 10^8 or 10^5 CFU/ml developed confluent necrosis at 12 to 16 hpi or no discernible phenotype to sporadic necrosis at 24 to 36 hpi, respectively, whereas there was no detectable phenotype in the leaves infiltrated with 61-N499 at the two bacterial densities. The population of *galU* mutant 61-N499 in tobacco leaves declined rapidly after infiltration at both inoculation concentrations, showing that the reduced HR-eliciting ability of *galU* mutant 61-N499 does not enhance its survival in nonhost tobacco plants.

Mutation in *galU* of Psy61 reduces bacterial multiplication in susceptible bean plants.

The multiplication of *galU* mutant 61-N499 in susceptible hosts was assessed by infiltrating bacteria at both low (10^5 CFU/ml) and high (10^8 CFU/ml) concentrations of inocula into bean leaves. Although Psy61 is not strongly virulent to bean, it is the only known plant that is susceptible to Psy61 and has been used previously for testing Psy61 multiplication in planta (Deng et al. 1998). At the initial inoculum of 10^5 CFU/ml (Fig. 6A, right panel), both wild-type Psy61 and the *galU* mutant 61-N499 grew with similar kinetics during the assay period, except the initial population of the *galU* mutant was greatly reduced immediately after infiltration, which was evident by the large deviations of bacterial numbers harvested at 0 dpi (leaf disks were collected approximately 2 hpi and ground immediately for enumeration). Similar reduction of the initial bacterial

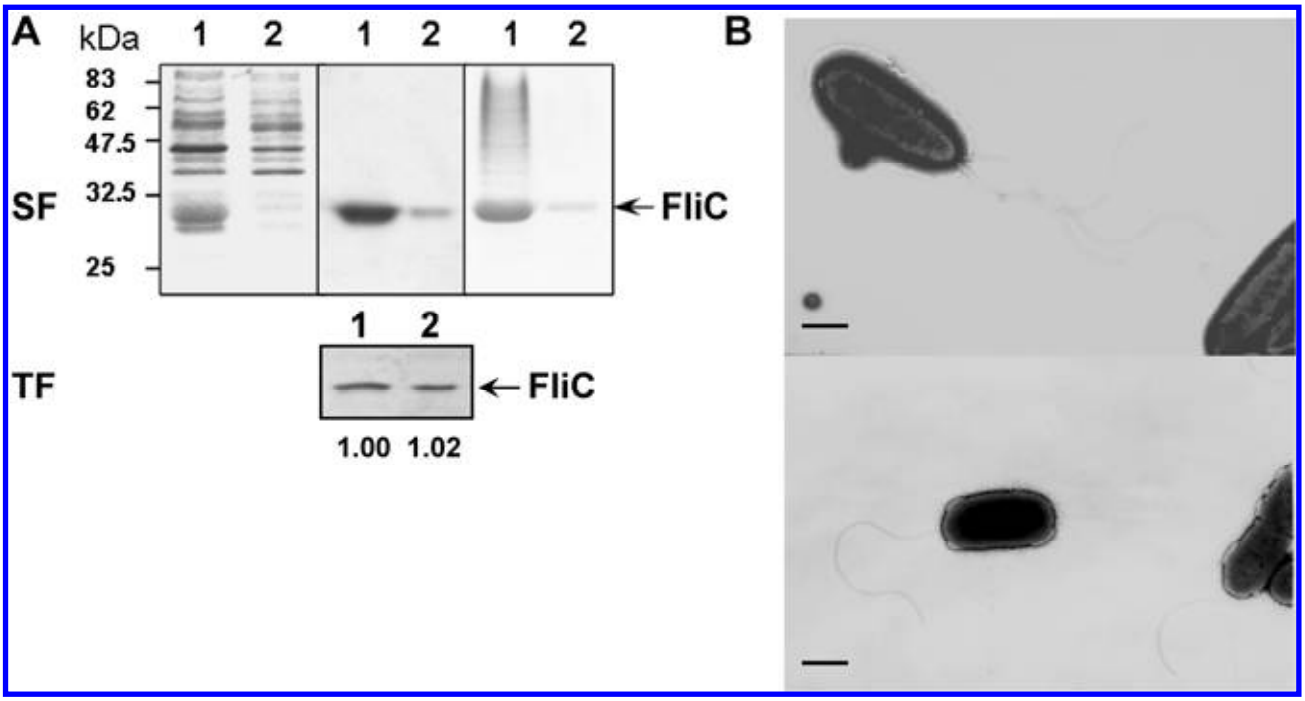

Fig. 3. Purification and detection of flagella from *Pseudomonas syringae* pv. *syringae* 61 (Psy61) and its *galU* mutant 61-N499. **A,** 12%-Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) resolution and immunodetection of flagellins collected from 24-h HrpMM-cultured bacteria. The upper three panels show the analyses of electrophoretic, denatured sedimented flagella (SF) fractions by staining with Coomassie brilliant blue (upper left panel), probing with FliC antiserum (upper central panel), and staining with GelCode glycoprotein staining kit (upper right panel). The bottom panel shows the Western blot analysis of the total flagellins (TF) that were collected from 24-h HrpMM-grown bacterial suspension and probed with FliC antiserum. The relative concentration of antiserum-bound flagellins is quantified by simultaneously staining an SDS-PAGE gel with Coomassie blue to ensure that each lane contains an equal amount of total proteins prior to immunoblotting, and the banding signals of the antiserum-bound FliC were determined by a densitometer (LAS-4000mini; FUJIFILM Co., Tokyo). Lane 1, Psy61 and lane 2, 61-N499. **B,** Electron micrographs of Psy61 (top panel) and its *galU* mutant 61-N499 (bottom panel). Bar = 1 μm.

population was observed when bacteria were inoculated at 10^8 CFU/ml (Fig. 6A, left panel). Both wild-type Psy61 and *galU* mutant 61-N499 reached the highest population at 3 dpi at the two inoculation concentrations, and the population of Psy61 exceeded that of *galU* mutant by at least four orders of magnitude. Moreover, the Psy61-inoculated leaves started to develop necrotic lesions at 4 dpi, whereas the *galU* mutant failed to elicit any symptom in an assay period of 21 days (data not shown). The pathogenicity of the *galU* mutant was also tested on bean pods by toothpick pricking inoculation. No symptom development was observed in the *galU* mutant 61-N499-inoculated pods at 7 dpi (Fig. 6B).

Mutation in *galU* of Psy61 does not eliminate the capability of its T3SS to secrete outer proteins into bacterial milieu.

The observation that the *galU* mutant 61-N499 failed to elicit the HR on tobacco or cause disease on bean led us to examine the function of T3SS by immunodetecting the secretion of T3S substrates HrpZ1 and HopA1. Induced cultures were separated into supernatants (S) and cell pellets (C) by high-speed centrifugation at 15,000 × *g* to obtain secreted and cytoplasmic proteins, respectively. In contrast to the *hrcC* mutation (61-N393), the *galU* mutation did not affect the secretion of HrpZ1 and HopA1-Cya fusion protein, as clearly shown in the immunoblots probed with antibodies against HrpZ1 and Cya (Fig. 7). The result indicates that the *galU* mutant has a functional T3SS to secrete HrpZ1 and HopA1-Cya into the bacterial milieu.

Psy61 *galU* mutant induces PTI.

The HR inhibition test and callose deposition assay were performed to test the capability of the *galU* mutant possessing incomplete LPS structure on eliciting plant innate immunity. The levels of the HR inhibition were recorded at 24 to 36 hpi using the following scale: 4, complete inhibition of the HR; 3, HR developed on approximately 25% of the co-injected area; 2, HR developed on approximately 50% of the co-injected area; 1, HR developed on approximately 75% of the co-injected area;

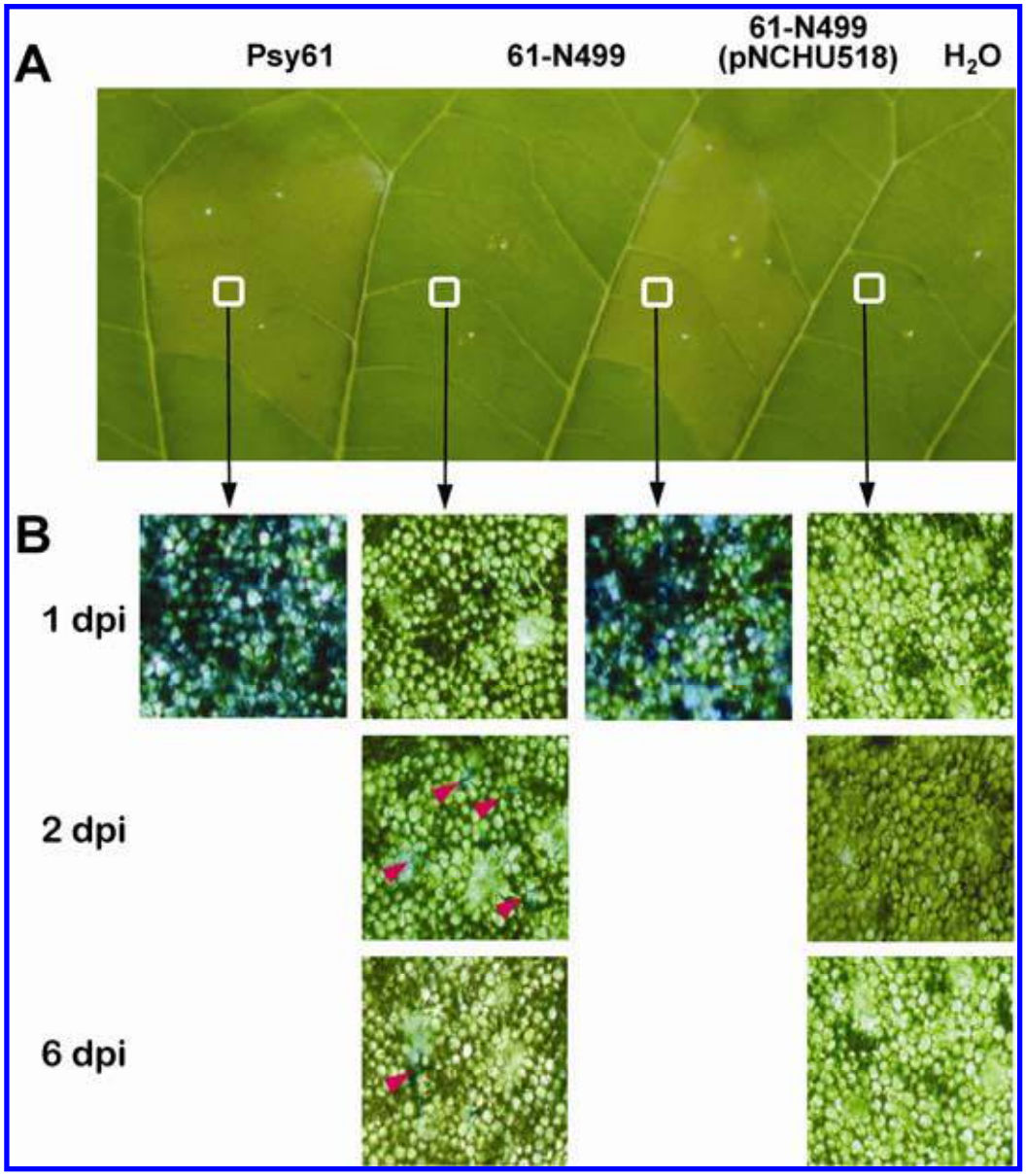

Fig. 4. Development of hypersensitive response (HR) on tobacco leaves. **A,** HR of tobacco induced by *Pseudomonas syringae* pv. *syringae* 61 (Psy61) (wild type), 61-N499 (*galU* mutant), and 61-N499 (pNCHU518) (complementing strain) at an inoculum of 10^8 CFU/ml. The inoculated leaf was photographed 24 h after infiltration. **B,** Microscopic observations of the inoculated leaf stained with Evans blue at 1, 2, and 6 days postinoculation (dpi). White square boxes indicate the area of microscopic observation, and pink arrowheads indicate compromised plant cells stained by Evans blue.

and 0, no inhibition of the HR. Inoculation of fully expanded tobacco leaves with Psy61 at 10^7 CFU/ml led to confluent cellular collapse of the HR within 24 h, which was prevented by preinoculation of the tobacco leaves with serially diluted bacteria, including heat-killed Psy61, T3SS-deficient *hrcC* mutant 61-N393 (Deng et al. 1998), and the *galU* mutant 61-N499, at 10^8, 10^7, and 10^6 CFU/ml 8 h prior to challenging inoculation (Table 2). The result indicates that the LPS-defective *galU* mutant can elicit plant innate immunity, although at a moderate level in comparison with the *hrcC* mutant. The HR inhibition phenotype is positively correlated with the concentrations of pretreated bacteria. The leaf segments where the *galU* mutant and the challenging Psy61 were inoculated started to show chlorosis at 1 dpi and became necrotic at 2 to 3 dpi (data not shown), indicating that the HR inhibition was temporary. The experiment was repeated twice with similar results. In addition, the HR inhibition phenotype was very pronounced when the bacteria were co-inoculated in young leaves (data not shown), an observation similar to the reported case in which the intensity of PTI induced by general bacterial elicitors was high in young leaves and at high temperature (30°C) (Ott et al. 2006). Because PTI could also lead to the deposition of callose, callose deposits in leaves of *Nicotiana benthamiana* were analyzed as described below. After inoculation with the *galU* mutant, *N. benthamiana* leaves exhibited characteristic callose depositions on the cell wall (Fig. 8), and the numbers of callose deposits induced by *galU* mutant and *hrcC* mutant were similar. Taken together, the results indicate that the LPS-defective *galU* mutant, similar to the LPS-intact *hrcC* mutant, can induce PTI in tobacco leaves.

DISCUSSION

In this study, we characterized the biological activities of the Psy61 *galU* mutant and found that it cannot grow in susceptible plants, nor can it elicit the HR in resistant plants even though it has a complete set of T3SS and effector genes. We hypothesize that the null plant responses elicited by the Psy61 *galU* mutant might be due to the loss of intact LPS as a protective structure against plant defenses. The hypothesis was tested by bacterial sensitivity to H_2O_2 and growth kinetics in plants. Our results showed that i) the Psy61 *galU* mutant was more sensitive to H_2O_2 than the wild type, ii) its survival was reduced immediately after infiltration in both susceptible and resistant plants, and iii) the population of the *galU* mutant declined more rapidly when a higher bacterial inoculum (i.e., 10^8 versus 10^5 CFU/ml) was injected into the apoplast. All the mutant phenotypes can be fully restored, except for bacterial survival in plants, by heterologous expression of the wild-type *galU* gene from the P_{lacZ} promoter residing in pRK415, indicating that the multiple defects resulted from a single mutation in the *galU* gene. The partial complementation of bacterial survival in plants is probably due to the instability of pRK415 when plasmid-harbored strains were grown under no-tetracycline-selection conditions (Keen et al. 1988) (i.e., in the apoplast of plants). Knowing that LPS-induced PTI exhibits callose deposition and prevents the HR elicited by a subsequent inoculation of incompatible phytopathogens (Dow et al. 2000; Newman et al. 2001), tobacco leaves infiltrated with the Psy61 *galU* mutant were subjected to aniline blue staining and the HR inhibition assay. Our results showed that the LPS-defective *galU* mutant could induce callose deposition and suppress the HR at the levels comparable with those elicited by the LPS-intact strains (e.g., the heat-killed Psy61 and *hrcC* mutant), indicating that the truncated LPS on the surface of the Psy61 *galU* mutant can still induce PTI.

The growth of *galU* mutants of enteric bacteria (e.g., *E. coli* and *Pectobacterium carotovorum* subsp. *carotovorum*, previously known as *Erwinia carotovora* subsp. *carotovora*) in galactose-containing medium was inhibited due to the accumulation of toxic galactose intermediates (Brede et al. 1991), and the toxic effect rendered *P. carotovorum* subsp. *carotovorum* unable to cause potato tuber decay (Jayaswal et al. 1985). In this work, we showed that the Psy61 *galU* mutant had no detectable UDP-Glc pyrophosphorylase activity but was able to grow on MacConkey agar plates supplemented

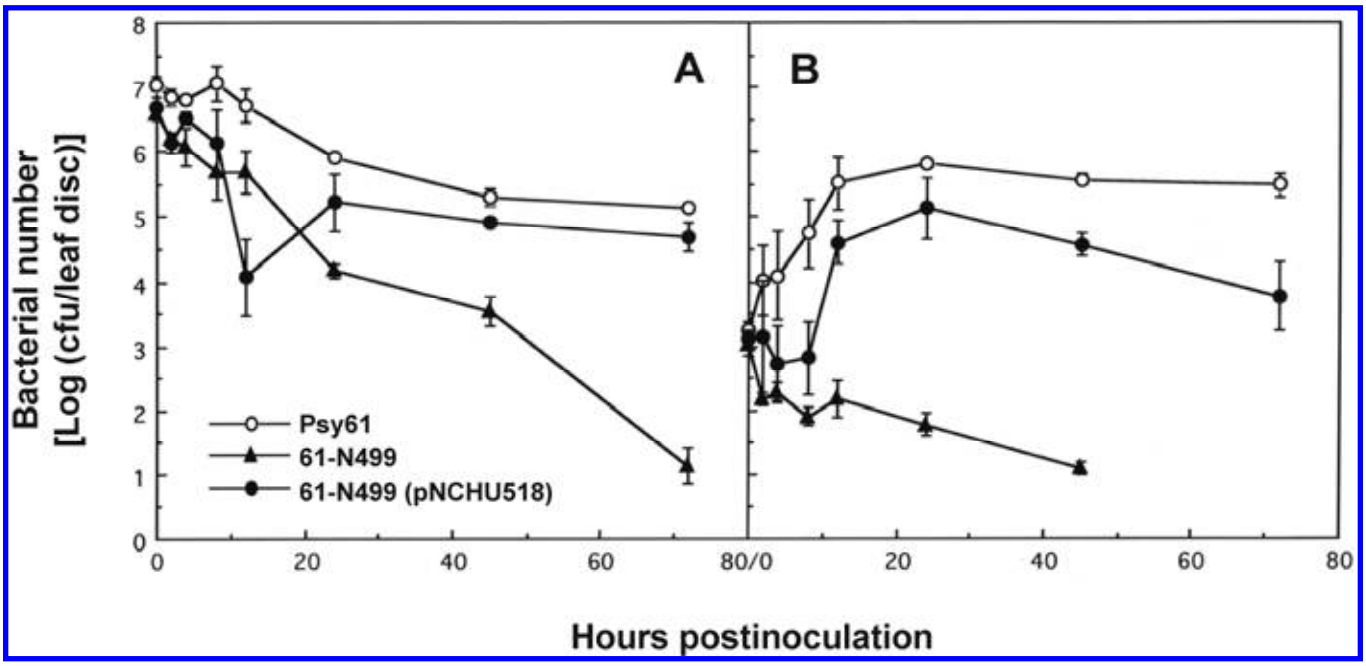

Fig. 5. Bacterial growth kinetics of *Pseudomonas syringae* pv. *syringae* 61 (Psy61), 61-N499 (*galU* mutant), and 61-N499 (pNCHU518) (complementing strain) in tobacco. Tobacco leaves were syringe-infiltrated with bacteria at **A,** 10^8 and **B,** 10^5 CFU/ml. Leaf disks were harvested by a cork borer (diameter = 6 mm), ground in 10 mM $MgCl_2$ buffer, and spread onto King's B plates supplemented with appropriate antibiotics. Symbols represent the mean of three replications and vertical lines indicate standard deviations.

with 0.2% galactose (Fig. 1C). Moreover, a Biolog microtiter plate assay showed that the carbon source utilization pattern of the *galU* mutant is identical to that of wild-type Psy61 (data not shown). Therefore, we ruled out the possibility that the nonpathogenic phenotypes of Psy61 *galU* mutant in planta resulted from the lack of enzymatic activities in metabolizing carbohydrates. The lack of growth inhibition by galactose intermediates was also reported in the *Pseudomonas aeruginosa galU* mutant (Chang et al. 1999), suggesting that there is a GalU-independent pathway for galactose metabolism in *Pseudomonas* spp.

LPS is known to induce defense responses in both mammals and plants. LPS recognition by a receptor complex of TLR4, MD2, and CD14 activates innate immunity in mammalian systems, leading to cytokine production and activation of antigen-presenting cells (Miller et al. 2005). In plant systems, LPS treatment also induces various defense-related responses, including the production of reactive oxygen species (ROS), the deposition of callose-rich papillae on cell walls (Brown et al. 1995), the expression of NO synthase and pathogenesis-related proteins (Newman et al. 2002; Zeidler et al. 2004; Silipo et al. 2005), the induction of systemic resistance in some plant species (Mishina and Zeier 2007), and the activation of programmed cell death in various plant species (Dow et al. 2000; Desaki et al. 2006). Herein, the question regarding the critical structures of LPS responsible for inducing plant defense responses remains to be elucidated. In previously studied LPS-triggered plant innate immunity (PTI), Newman and associates (1997) used complete or truncated forms of LPS purified from different gram-negative bacteria and showed that the core oligosaccharides, but not lipid A, of the purified LPS are potent elicitors of PTI in pepper. Bedini and associates (2005) inferred that the coiled structures of the O-specific oligorhamnans can act as stimulators of PTI in *A. thaliana*, while Zeidler and colleagues (2004) showed that the lipid A moiety is as effective as intact LPS in inducing PTI in *A. thaliana*. The discrepancy in which LPS structures are important for inducing PTI might be due to the different plant species used in those studies, in which different plants may have developed their own LPS recognition systems to perceive LPS, or one plant simply has multiple receptors to recognize different parts of LPS. In this study, the Psy61 *galU* mutant with modified LPS and reduced

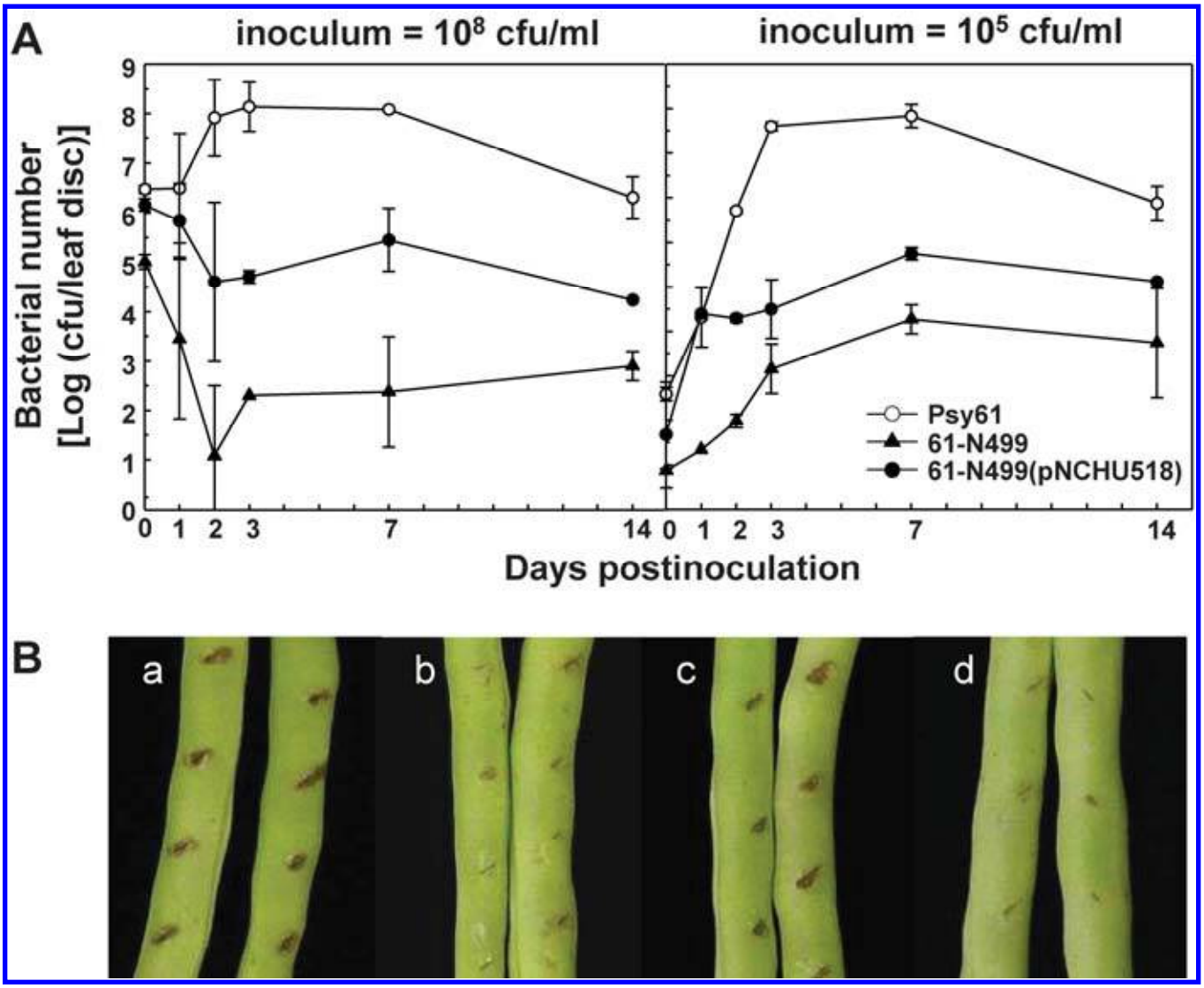

Fig. 6. Plant bioassays on bean **A,** leaves and **B,** pods. **A,** Leaves of 6-week-old bean plants were syringe infiltrated with *Pseudomonas syringae* pv. *syringae* 61 (Psy61), 61-N499 (*galU* mutant), and 61-N499 (pNCHU518) (complementing strain) at 10^8 and 10^5 CFU/ml (left and right panels, respectively). Leaf disks were harvested by a cork borer (diameter = 6 mm), ground in 10 mM $MgCl_2$ buffer, and spread onto King's B plates supplemented with appropriate antibiotics. Bacterial multiplication was enumerated at 0, 1, 2, 3, 7, and 14 days postinoculation. Symbols represent the mean of three replications and vertical lines indicate standard deviations. **B,** Bean pods were inoculated by toothpick pricking method with **a,** Psy61 (wild type); **b,** 61-N499 (*galU* mutant); **c,** 61-N499 (pNCHU518) (complementing strain); and **d,** H_2O. Lesion formation was scored 7 days postinoculation.

flagellation was capable of inducing PTI in tobacco and bean, suggesting that its surface-presenting PAMPs are effective triggers of PTI. The questions of whether the Psy61 *galU* mutation affects the number and structure of other PAMPs presenting on bacterial surfaces and whether its defective LPS could act as potent elicitor of PTI remain to be addressed. Once the issues are resolved, the *galU* mutant generated in this study may serve as a source of truncated LPS for a subsequent search of LPS receptors in plants.

LPS, the principal component of the outer membrane of gram-negative bacteria, is involved in maintaining the integrity of the outer membrane. LPS-deficient *galU* mutants showed aberrant localization of outer membrane proteins in enteric bacteria (Ames et al. 1974; Koplow and Goldfine 1974), leading to reduced secretion of flagellin and some virulence proteins (Komeda et al. 1977; Wandersman and Letoffe 1993; Sandlin et al. 1995). The expression and assembly of T3SS in the Psy61 *galU* mutant were assayed by monitoring the secretions of HrpZ1 and HopA1-Cya, two well-characterized T3S substrates (He et al. 1993; Wei et al. 2005), and the result in Figure 7 clearly showed that the *galU* mutant expressed and secreted HrpZ1 and HopA1-Cya at the level equivalent to the wild type, indicating that the structure of T3SS was not disrupted by the incomplete LPS structure under in vitro inducing conditions. In comparison, the electron micrographs of the *S. flexneri* wild type and its *galU* mutant show that they have similar numbers of T3SS needles emerging from the outer membrane, and the mutant secretes T3S effectors to bacterial milieu and has high invasive potential to cultured HeLa cells (West et al. 2005). In addition, the *galU* mutant of *P. aeruginosa* was not compromised in its ability to inject T3S effectors into cultured bronchial epithelial cells (Priebe et al. 2004). The results obtained from various *galU* mutants indicate that the defective LPS does not affect the assembly of T3SS in the outer membranes or its functions in secretion and translocation of T3S effectors under the in vitro assay conditions. However, in vivo analyses show that the mutations in *galU* exhibit characteristically different phenotypes between animal- and plant-pathogenic bacteria. Upon infection of mice, the *P. aeruginosa galU* mutant showed attenuated virulence in corneal infection and minimal systemic spread in the lung but it can still elicit moderate to severe pneumonia that was confined to the lungs (Priebe et al. 2004). Viable counts of the *P. aeruginosa galU* mutant in mice lungs at 6 h post intranasal inoculation with an initial inoculum of 1×10^8 CFU were approximately 1 log lower than those of the wild-type strain. The *galU* mutation of *Vibrio cholerae* resulted in an approximate 50-fold reduction in colonization of the infant mouse small intestine (Nesper et

Table 2. Comparison of the hypersensitive response (HR)-inhibition level caused by the pretreatment of various agents

	Average inhibition level of the challenged HR per inoculum concentration (CFU/ml)[a]			
Agents used in pretreatment	**10^8**	**10^7**	**10^6**	**0**
Water	NA	NA	NA	0
hrcC mutant 61-N393[b]	4	3.5	0	NA
Heat-killed Psy61	3	2.5	0	NA
galU mutant 61-N499	2.5	1	0	NA

[a] Leaf segments pretreated with bacteria or water were challenged 8 h later with HR-inducing *Pseudomonas syringae* pv. *syringae* 61 (Psy61) at 10^7 CFU/ml. The average level of HR inhibition was the mean of two inoculated leaves recorded from one representative experiment. Scale: 4, complete inhibition of the HR; 3, HR developed on approximately 25% of the co-injected area; 2, HR developed on approximately 50% of the co-injected area; 1, HR developed on approximately 75% of the co-injected area; 0, no inhibition of the HR; NA, nonapplicable. The experiment was repeated twice with similar results.

[b] 61-N393 harbored a nonpolar mutation in *hrcC* coding for the outer membrane secretin protein of the type III secretion system.

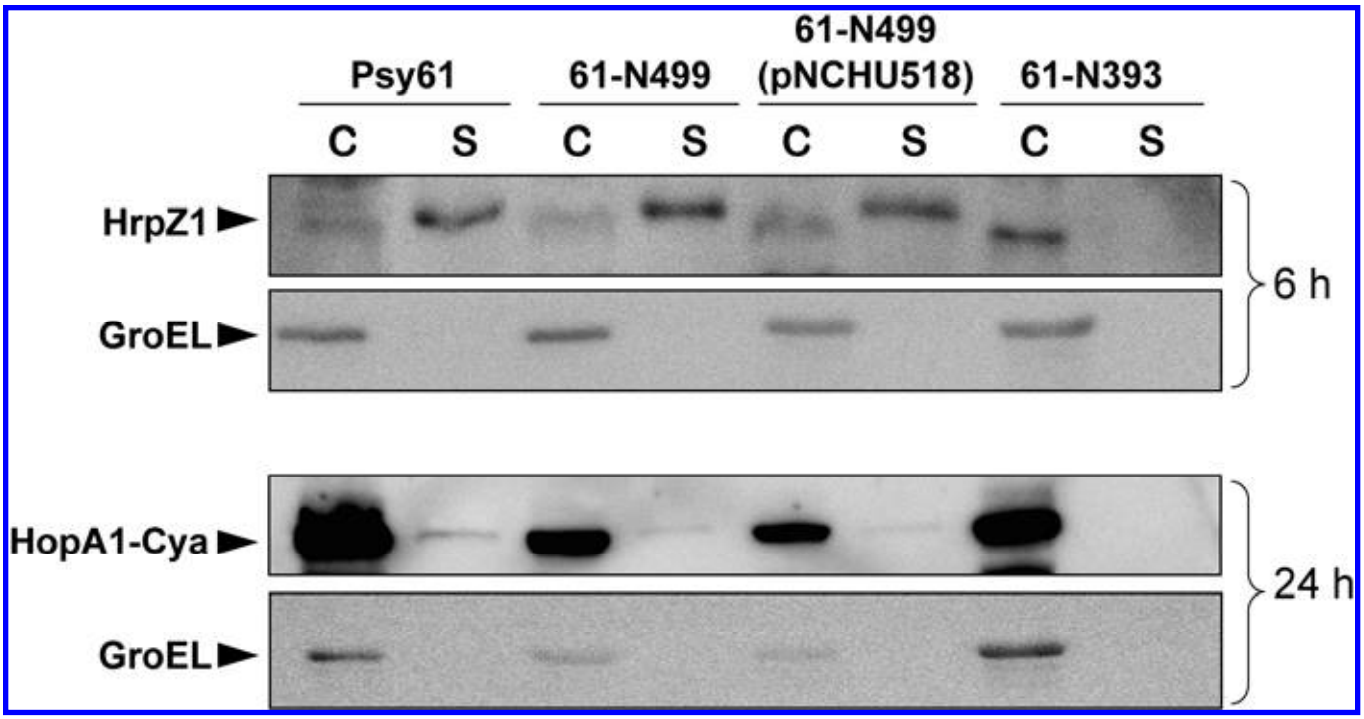

Fig. 7. Type III secretion system-dependent secretion of HrpZ1 and HopA1-Cya by *Pseudomonas syringae* pv. *syringae* 61 (Psy61), 61-N499 (*galU* mutant), and 61-N499 (pNCHU518) (complementing strain). Bacteria were grown in Hrp-derepressing medium (HrpMM) at 25°C for 6 h (for HrpZ1 secretion) to 24 h (for HopA1-Cya secretion) and harvested by centrifugation at 15,000 × *g* for 30 min to obtain cell-bound (C) and supernatant (S) protein fractions. Proteins were resolved in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunodetected by antibodies against HrpZ1, HopA1-Cya, and GroEL. The GroEL serves as a cytoplasmic marker in this experiment.

al. 2001). In contrast, when the Psy61 *galU* mutant was tested in planta, it elicited null responses in both host and nonhost plants. The Psy61 *galU* mutant showed poor survival and no lesion formation during the 14-day incubation in bean leaves, and it lost the ability to trigger the HR in tobacco even though it has a complete set of T3SS and effector genes. The rapid reduction in the numbers of the viable cells of the Psy61 *galU* mutant immediately after infiltration let us hypothesize that, unlike the LPS-defective animal-pathogenic bacteria in their hosts, the metabolic activities of the Psy61 *galU* mutant cells are probably suppressed by host preformed defensive compounds or innate immune factors before they are able to express T3SS genes in planta. Previous researches on the hypersensitive reaction in plants revealed that the presence of living bacteria in the HR-induction period, varying from 0.5 to 4 h among different combinations of bacteria and plant species, is crucial for incompatible bacteria to launch the HR (Goodman and Novacky 1994; Klement et al. 1999), and inactivating bacterial metabolism with antibiotics (Goodman and Novacky 1994) or PTI (also known as early induced resistance) (Klement et al. 1999) during the induction period prevents the development of HR. The result of an in vitro H_2O_2 sensitivity assay (Table 1) shows that the *galU* mutant is more sensitive to hydrogen peroxide than the wild type, suggesting that the ROS-rich conditions induced by bacterial PAMPs in planta might abolish the cellular activities of the Psy61 *galU* mutant during the HR-induction period. Consequently, no HR or diseased responses were elicited in nonhost or host plants, respectively. The differences in host responses elicited by the *galU* mutants and their deleterious effects on bacterial survival suggest that the essence of innate immune mechanisms might be different between plants and animals, yet they are both effective to fight against bacterial infections.

The Psy61 *galU* mutant reproducibly induced a small number of dead cells in tobacco leaves (Fig. 4B), and it regained growth, although at the level of five orders of magnitude lower than that of the wild type, in bean leaves after 2 dpi (Fig. 6A), suggesting that the LPS-defective Psy61 *galU* mutant is not

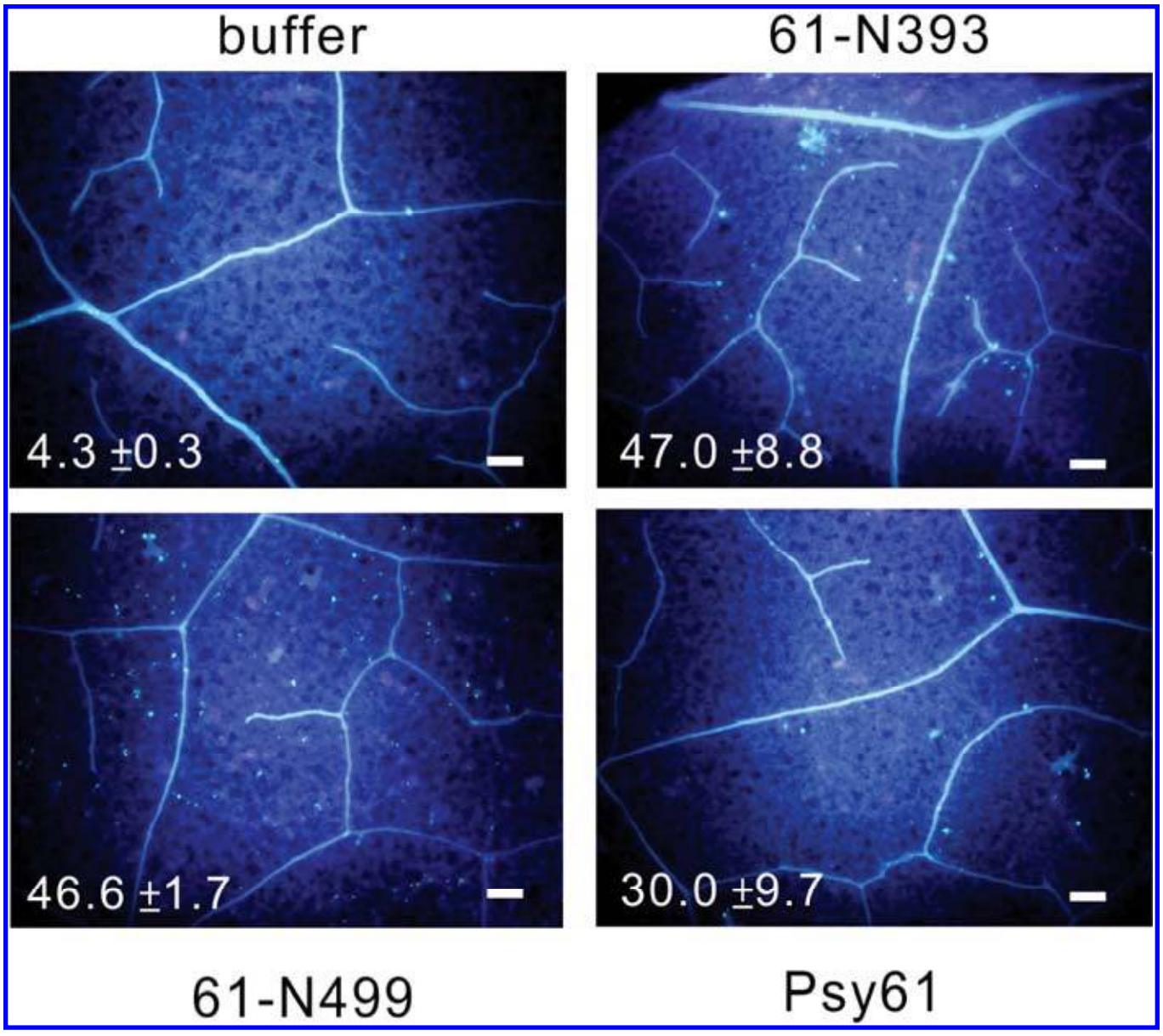

Fig. 8. Elicitation of callose deposition in *Nicotiana benthamiana* by *Pseudomonas syringae* pv. *syringae* 61 (Psy61) and its derivatives. Tobacco leaves were infiltrated with (clockwise) buffer, 61-N393 (*hrcC* mutant), 61-N499 (*galU* mutant), and Psy61 (wild type) at 1×10^8 CFU/ml. Leaf disks were collected at 12 h postinfiltration, stained with aniline blue, and visualized by an epifluorescence microscope. For each treatment, the average number of callose depositions per field of view ± standard deviation ($n = 10$) is shown at the lower left corner of the micrograph. Bar = 100 µm.

impaired in its ability to translocate T3S effectors into plant cells once they can survive the killing effect of PTI. Attempts to use HopA1-Cya fusion protein as a reporter (Schechter et al. 2004; Wei et al. 2005) for a translocation assay in the Psy61 *galU* mutant produced values below detection limits, which is most likely due to the fact that only a few bacterial cells can survive PTI to translocate the HopA1-Cya fusion protein (H.-C. Huang, unpublished data). Taken together, our results reveal that the LPS of plant-pathogenic bacteria, similar to its counterparts in animal-pathogenic bacteria, play important roles in the early stage of pathogen–plant interactions leading to successful recognition and infection.

MATERIALS AND METHODS

Bacterial strains, culture conditions, plasmids, and DNA manipulation techniques.

Bacterial strains and plasmids used in this study are described in the text. Conditions for culturing *Escherichia coli* and *P. syringae* strains have been described (Deng et al. 1998). Cloning and DNA manipulations were done in *E. coli* DH10B using standard procedures (Sambrook et al. 1989). A pUC18-based genomic library was constructed by partially digesting the genomic DNA of Psy61 with *Sau*3A, followed by cloning 4- to 5-kb fragments into pUC18. Constructed plasmids were transformed into *E. coli* FF4001 that has a mutation in *galU* (Brede et al. 1991). For the galactose sensitivity test, MacConkey-galactose agar was prepared with Difco MacConkey agar base (BD Diagnostic Systems, Franklin Lakes, NJ, U.S.A.) supplemented with 0.2% galactose. A motility assay was carried out on a 0.3% Hrp derepressing agar medium (HrpMM) (Huynh et al. 1989) at 28°C for 2 days. In vitro activity of the T3SS was assayed by the secretion of HrpZ1 and HopA1-Cya into culture medium. Bacteria expressing HopA1-Cya chimeric protein was obtained by electroporating pNCHU1623, a pBBR1-MCS5 (Kovach et al. 1995) derivative harboring a 3-kb *Bam*HI-*Sac*I fragment of *hopA1-cya* fusion gene from pNCHU1180 (Wei et al. 2005), into the wild-type Psy61, *galU* mutant 61-N499, complementing strain 61-N499 (pNCHU518), and *hrcC* mutant 61-N393 (Deng et al. 1998). Bacteria were first grown in KB broth to an optical density at 600 nm (OD_{600}) of 0.8 and transferred to HrpMM for inducing the expression of *hrp* genes at 25°C for 6 and 24 h for HrpZ1 and HopA1-Cya secretion assays, respectively (Deng et al. 1998). GroEL was used as a cellular marker to monitor cellular integrity during the secretion assays. Immunodetections of HrpZ1, HopA1-Cya, and GroEL proteins were performed as described (Wei et al. 2005) using HrpZ1 antiserum, mouse monoclonal Cya A (3D1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and rabbit polyclonal GroEL (from *E. coli*) antibody (Assay Designs, Ann Arbor, MI, U.S.A.) at the ratios of 1/500, 1/5,000, and 1/2,000, respectively. Antibiotics were added to the culture medium at the following concentrations: ampicillin at 100 µg/ml, kanamycin at 50 µg/ml, tetracycline at 20 µg/ml, and nalidixic acid at 20 µg/ml. DNA sequences were analyzed with DNAStar software (DNAStar Inc., Madison, WI, U.S.A.). Database searches for homology, computer-aided amino acid sequence prediction, and percent similarity calculation were performed using the BLAST programs (Altschul et al. 1997) at the National Center for Biotechnology Information.

Construction of *galU* mutation in Psy61 by marker-exchange strategy.

A 494-bp deletion at the 3′ end of the 840-bp *galU* gene of Psy61 was constructed by subcloning border fragments into restriction sites on either side of a kanamycin-resistant cassette lacking a transcription terminator in pRK415 to produce pNCHU499 that was conjugated into Psy61 by triparental mating, followed by screening and selecting for marker-exchanged mutant 61-N499, as described (Deng et al. 1998). Mutant construction was confirmed by Southern hybridization using standard procedures (Sambrook et al. 1989). The complementing plasmid, pNCHU518, was generated by cloning a 2.7-kb fragment containing the entire *galU* gene and its flanking regions into pRK415, which was conjugated into the *galU* mutant 61-N499 by triparental mating.

UDP-Glc pyrophosphorylase assay.

The enzymatic activity of UDP-Glc pyrophosphorylase was assayed by quantitative measurement of glucose-1-phosphate (Glc1P) production according to Duggleby and associates (1996). The protein concentration of sonicated cell extracts was determined by Bradford assay according to the manufacturer's instruction (Bio-Rad, Hercules, CA, U.S.A.). Each reaction mixture contained 100 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1 mM UDP-Glc, 8 µM glucose 1,6-diphosphate, 1 mM NADP, 1 mM NaPPi, 5 units each of phosphoglucomutase (PGM) and glucose 6-phosphate dehydrogenase (G6PD), and cell extracts at a final volume of 1 ml. Reactions were performed at 37°C for 15 min, and the reduction of $NADP^+$ was measured at OD_{340}. The synthesis of NADPH was accompanied by the oxidation of Glc1P at the molar ratio of 1:1, following the biochemical reactions of Glc1P (PGM) → Glc6P, Glc6P + $NADP^+$ (G6PD) → 6-phosphogluconate + NADPH (the enzyme for catalyzing the reaction is shown in parentheses). Therefore, the UDP-Glc PPase activity was defined as the amount (in nmole) of NADPH synthesized per minute per milligram of total protein.

Electron microscopy observation.

P. syringae strains grown on KB agar plates were suspended in sterile distilled water. The cell suspension was placed onto a carbon-coated copper grid, stained with 2% uranyl acetate (Sigma-Aldrich, St. Louis), and examined by TEM (JEM-1200CX II; JEOL, Tokyo) at 80 kV.

Biofilm formation.

The biofilm production assay was performed as described by Djordjevic and associates (2002) with a slight modification. Overnight-grown *P. syringae* strains in KB broth at 28°C were harvested by centrifugation, adjusted to an OD_{600} of 0.5 with KB broth, diluted 10-fold with the same medium, and 200 µl of the diluted bacteria was transferred with eight replicates per strain into the wells of polystyrene microtitration plates (Greiner Bio-one GmbH, Solingen, Germany) that were previously rinsed with 70% ethanol for surface sterilization and air dried. Wells containing the assay medium without bacteria were used as negative controls. Plates were sealed with plastic wrap and incubated at 28°C for 70 h without shaking. After incubation, the plate wells were washed five times with sterile distilled water, air dried for 45 min, and stained with 200 µl of 0.5% (wt/vol) crystal violet solution for 45 min. After staining, plates were washed five times with sterile distilled water, air dried, and destained with 95% ethanol (200 µl per well) followed by transferring 150 µl of the destained solution to a new microtitration plate for a spectrophotometric measurement at OD_{595}.

H_2O_2 sensitivity assay.

Sensitivity of Psy61 and the *galU* mutant 61-N499 to hydrogen peroxide was assayed at 28°C by a paper-disk diffusion test. Paper disks (6 mm in diameter) soaked in 0.1% (vol/vol) hydrogen peroxide were placed in the center of KB agar plates that had been spread with 100 µl of 10^7 CFU/ml bacterial sus-

pensions. The diameter (in millimeters) of growth inhibition zone was recorded at 2 dpi.

LPS preparation and silver staining.

LPS was prepared by a modified phenol-water method (Goldman and Leive 1987). All steps were carried out at 4°C unless specified. In brief, 2-ml bacterial cells (OD_{600} = 0.3) in phosphate-buffered saline (PBS, pH 7.2) were centrifuged and washed once in PBS containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The bacterial pellets were resuspended in 300 µl of H_2O and extracted with an equal volume of hot phenol (60 to 70°C) by vigorous stirring at 65 to 70°C for 15 min. Next, the suspension was chilled on ice and centrifuged at 8,500 × *g* for 15 min, and the phenol phase was re-extracted with 300 µl of H_2O. The aqueous phase of two extractions was collected and precipitated with 0.5 M sodium acetate (final concentration) and 10 volumes of 95% ethanol at –20°C overnight. The LPS pellet was obtained by centrifugation at 2,000 × *g* for 10 min, resuspended in 100 µl of H_2O, and precipitated with the ethanol again. The precipitated LPS was dried, resuspended in 50 µl of H_2O, resolved by 12% SDS-PAGE gel, and detected by silver staining as described by Dubray and Bezard (1982). In brief, the SDS-PAGE gel was fixed in acid-alcohol solution (25% isopropyl alcohol and 10% acetic acid) at room temperature overnight, followed by a wash with 7.5% acetic acid for 30 min. The fixed gel was treated with 0.2% periodic acid at 4°C for 1 h, washed three times with distilled water, and silver stained for 30 min at room temperature with a freshly-made staining solution prepared in the following order: i) add 1.4 ml of 25% ammonia solution to 21 ml of 0.36% NaOH, ii) add 4 ml of 20% silver nitrate slowly while agitating vigorously, and iii) add 83.6 ml of H_2O. The stained gel was transferred to a freshly prepared developer solution containing 0.05% citric acid, 0.019% formaldehyde solution, and 10% methanol, and the staining process was terminated when the LPS bandings appeared dark brown by washing the gel with 1% acetic acid for a few minutes, followed by several washes with distilled water.

Flagellin purification and glycosylation detection.

For flagellin purification, all steps were performed according to Taguchi and associates (2003) at 4°C unless specified. *P. syringae* strains were grown in 5 ml of Luria-Bertani medium supplemented with 10 mM $MgCl_2$ for 24 h at 25°C and transferred to 60 ml of HrpMM for 24 h of incubation at 23°C (Huynh et al. 1989). A 200-µl aliquot of bacterial suspension containing bacterial cells and culture medium was saved for subsequent immunodetection of total flagellins (TF) with FilC antiserum. The rest of the bacterial cells were harvested by centrifugation at 7,000 × *g* for 10 min and resuspended in a phosphate buffer (50 mM sodium phosphate, pH 7.0) by vigorous vortex to shear off flagella. Sheared flagella in the supernatant were collected by repeated centrifugation at 10,000 × *g* for 30 min to remove the remaining intact cells and, finally, by ultrahigh-speed centrifugation at 100,000 × *g* for 30 min to obtain sedimented flagella (SF). The SF was suspended in 300 µl of H_2O and stored at –20°C. For immunodetection of flagellins, the fractions of TF and SF were denatured in 2× sample buffer, separated by a 12% SDS-PAGE gel, electrotransferred to Immobilon-P membrane, and probed with FliC antibody (kindly provided by Y. Ichinose, Okayama University, Japan) at a ratio of 1/5,000, followed by alkaline phosphatase-conjugated anti-mouse immunoglobulin G antibody (Boehringer Mannheim GmbH, Mannheim, Germany) for chemiluminescent detection using CDP-*Star* (Applied Biosystems, Bedford, MA, U.S.A.) as a substrate. The detection of glycoprotein was carried out using a Pierce GelCode glycoprotein staining kit (Thermo Fisher Scientific Inc. Rockford, IL, U.S.A.) according to the manufacturer's instruction.

Plant bioassays.

Tobacco (*N. tabacum* L. cv. Van-Hicks and *N. benthamiana*) and snap bean (*Phaseolus vulgaris* L. cv. Eagle) plants were grown under greenhouse conditions at 23 to 25°C with a photoperiod of 16 to 24 h until the six-leaf stage, and transferred to the laboratory for bioassays. For HR elicitation assays, bacteria were grown overnight on KB agar supplemented with appropriate antibiotics, suspended in distilled water at a density of 10^8 CFU/ml, and then infiltrated with a needless syringe into the interveinals of tobacco leaves (Huang et al. 1988). HR development was observed within 24 h at room temperature. The HR inhibition test was performed to test the LPS-induced basal resistance using wild-type Psy61 as the challenging strain (infiltrated at 10^7 CFU/ml) that was inoculated at 8 hpi with serially diluted bacteria (heat-killed wild-type Psy61, *hrcC* mutant 61-N393, and *galU* mutant 61-N499) at 10^8, 10^7, and 10^6 CFU/ml. For bacterial multiplication assays in susceptible bean leaves, the bacteria were suspended in distilled water at 10^5 and 10^8 CFU/ml for syringe infiltration as described above. Bean plants were incubated in a humid growth chamber (relative humidity = 90%) with a light intensity of 150 µE/cm^2 and a photoperiod of 12 h at 25 to 28°C, and leaf disks were sampled 0 to 14 dpi for bacterial enumeration, as previously described (Deng et al. 1998).

Callose deposition assay.

Six-week-old *N. benthamiana* leaves were infiltrated with Psy61 and its derivatives at a concentration of 1×10^8 CFU/ml by a blunt syringe. Leaf disks were harvested at 12 hpi; cleared with alcoholic lactophenol (1:1:1:1:8 phenol/glycerol/lactic acid/water/ethanol); rinsed sequentially in ethanol, then water; and stained with aniline blue solution (0.01% aniline blue [Sigma-Aldrich] in 150 mM K_2HPO_4, pH 9.5) for 60 min as previously described (Underwood et al. 2007). Stained leaf disks were examined by an epifluorescent microscope (Olympus IX71) equipped with a 365-nm excitation filter and a 450-nm emission filter. The number of callose depositions was determined with Image J software (National Institute of Health, Bethesda, MD, U.S.A.). More than 10 microscopic fields (2.28 mm^2 per field of view) taken from three independent leaves were analyzed and calculated to get the means and standard deviations of callose deposits that are shown in Figure 8. The callose deposition assays reported here have been performed three times with similar results.

Nucleotide sequence accession number.

The nucleotide sequence of a 2,742-bp DNA fragment containing *galU* and *gor* genes has been deposited in GenBank database under accession number EU833989.

ACKNOWLEDGMENTS

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