Functional analysis of *Ralstonia solanacearum*
PrhG regulating the *hrp* regulon in host plants

Yong Zhang,¹ Li Chen,² Takeshi Yoshimochi,³ Akinori Kiba,³
Yasufumi Hikichi³ and Kouhei Ohnishi²

1Research Center of Bioenergy and Bioremediation, Southwest University, BeiBei District, Chongqing 400715, China
2Research Institute of Molecular Genetics, Kochi University, Nankoku, Kochi 783-8502, Japan
3Laboratory of Plant Pathology and Biotechnology, Kochi University, Nankoku, Kochi 783-8502, Japan

Genes in the *hrp* regulon encode component proteins of the type III secretion system and are
essential for the pathogenicity of *Ralstonia solanacearum*. The *hrp* regulon is controlled by HrpB.
We isolated several genes regulating *hrpB* expression from the Japanese strain OE1-1 using
minitransposon mutagenesis. Among them, we mainly focused on two genes, *hrpG* and *prhG,*
which are the positive regulators of *hrpB*. Although the global virulence regulator PhcA negatively
regulated *hrpG* expression via *phrIR,* it positively regulated *prhG* expression. We further
investigated the contrasting regulation of *hrpG* and *prhG* by PhcA and speculated that *R.
solanacearum* may switch from HrpG to PrhG for *hrpB* activation in a cell density-dependent
manner. Although the *prhG* mutant proliferated similarly to the wild-type in leaf intercellular spaces
and in xylem vessels of the host plants, it was less virulent than the wild-type. The expression of
the *popA* operon, which belongs to the *hrp* regulon, was significantly reduced in the *prhG*
mutant by more than half in the leaf intercellular spaces and more than two-thirds in the xylem vessels
when compared with the wild-type.

INTRODUCTION

*Ralstonia solanacearum* (Yabuuchi et al., 1995) is a Gram-negative soil-borne vascular phytopathogen. It generally
invades plant vascular tissues through root wounds and natural openings. After invasion, it multiplies extensively in
the intercellular spaces of the root cortex and then invades xylem vessels. Once bacteria have invaded the vessels,
they multiply and travel rapidly throughout the entire plant (Roberts et al., 1988; Vasse et al., 1995). In the xylem, the
bacteria produce large amounts of extracellular polysaccharides (EPS). Such large amounts of EPS slime reduce
the sap flow in the xylem and cause extensive wilting of the plant; thus the polymers are believed to be the main
virulence factor in *R. solanacearum* (Denny, 1995). In addition to EPS, several molecular determinants are
involved in pathogenicity (Genin & Denny, 2012).

Hypersensitive response and pathogenicity (*hrp*) genes encoding component proteins of the type III secretion
system (TTSS) are essential for the pathogenicity of Gram-negative plant pathogens (Lindgren, 1997; Hueck, 1998).
The bacteria use the TTSS to interact with host plants and inject virulence factors, i.e. type III effectors, into the host
cytosol (Hueck, 1998; Van Gijselgem et al., 1995). *hrp* mutants display impaired growth in plants and cannot
cause disease in susceptible plants (Boucher et al., 1987; Kanda et al., 2003a). The *hrp* genes are clustered, and most
effector genes are scattered around the genome. These genes are regulated as part of the *hrp* regulon (Galán &
Collmer, 1999).

The regulatory pathway controlling the *hrp* regulon is well
characterized in *R. solanacearum*. The *hrp* regulon is repressed in nutrient-rich media (Arlat et al., 1992). Nutrient-poor conditions, which may mimic the conditions
in the intercellular spaces of plants, induce a 20-fold increase in the expression of the *hrp* regulon (Genin et al., 1992).
Plant signals increase the expression of operons belonging to the *hrp* regulon by 10- to 20-fold in nutrient-poor
conditions (Marenda et al., 1998). The signals are perceived by the outer membrane receptor PrhA (Aldon et al., 2000)
and are transduced to HrpG through PrhR/PrhI and PrhJ.

HrpG is a two-component response regulator that is the key component of the regulatory cascade and directly
controls the expression of *hrpB* (Brito et al., 1999, 2002),

Abbreviations: EPS, extracellular polysaccharides; p.i., post-inoculation; *phr,* positive regulation of *hrp* regulon; TTSS, type III secretion system.

The GenBank/EMBL/DDBJ accession number for the *prhG* sequence of *Ralstonia solanacearum* strain OE1-1 is AB510918.

Supplementary methods, two supplementary tables and three supplementary figures are available with the online version of this paper.
which encodes an AraC-type transcriptional regulator regulating the hrp regulon (Arlat et al., 1992; Genin et al., 1992; Tamura et al., 2005). HrpG has been shown to integrate three major signals: physical contact with the plant host (Aldon et al., 2000), bacterial metabolic status related to growth conditions (Brito et al., 1999) and a PhcA-dependent quorum-sensing signal (Genin et al., 2005). PhcA is a LysR-type transcriptional activator and was originally identified as a positive regulator of EPS production (Brumbly et al., 1993). The levels of active PhcA are proportional to the density of bacterial cells (Clough et al., 1997) and are controlled by a quorum-sensing system using a unique autoinducer, 3-hydroxy palmitic acid methyl ester (3-OH PAME) (Flavier et al., 1997). In vitro PhcA activity starts to increase at a cell density of 10^7 cfu ml^{-1} and reaches a maximum at 5 × 10^8 cfu ml^{-1}. Mutation of phcA dramatically increases the expression of hrpB and the hrp regulon, even in nutrient-rich medium (Genin et al., 2005). PhcA binds to the promoter region of the prhIR operon and represses the expression of prhIR, which in turn shuts down the expression of all downstream genes (Yoshimochi et al., 2009a).

A close parologue of hrpG, prhG was shown to be a positive regulator of hrpB expression in R. solanacearum strain GMI1000 (Plener et al., 2010). Despite the high level of similarity between hrpG and prhG and their shared function as hrpB activators, prhG expression is activated only in minimal medium, whereas hrpG expression is highly induced during co-cultivation with plant cell suspensions. While HrpG controls the expression of many pathogenicity determinants in addition to hrpB (Valls et al., 2006), PrhG controls very few specific target genes. A prhG deletion mutant was found to be slightly less virulent than the wild-type (Plener et al., 2010), whereas a hrpG mutant showed complete loss of pathogenicity (Brito et al., 1999).

In this study, we investigated new pathogenicity determinants affecting the expression of the hrp regulon. The hrp regulon contains at least six operons in the hrp locus, and five of these, including the popA operon, are controlled by HrpB (Cunnac et al., 2004). We used a popA-lacZYA reporter strain (Yoshimochi et al., 2009b) to monitor the expression of the hrp regulon and screened mutants with reduced expression of popA from the R. solanacearum strain OE1-1 (Kanda et al., 2003a) by using minitransposon mutagenesis (Dennis & Zylstra, 1998). Among the screened genes regulating the expression of the hrp regulon, we focused on hrpG and prhG in this study. We analysed the contribution of hrpG and prhG in planta on hrp regulon expression, and found that PrhG and HrpG both functioned in the intracellular spaces and xylem.

**METHODS**

**Bacterial strains, plasmids, media and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table S1 (available in Microbiology Online). All R. solanacearum strains were derivatives of OE1-1 (phytype 1 race 1 biovar 3) (Kanda et al., 2003a). *Escherichia coli* strains DH125 (Invitrogen) and S17-1 (Simon et al., 1983) were used for plasmid construction and for the conjugal transfer of plasmids, respectively. *E. coli* was grown in LB medium at 37 °C, and *R. solanacearum* was grown at 28 °C in rich B medium (1 % bacto peptone, 0.1 % yeast extract and 0.1 % casamino acids), hydroponic plant culture medium with 2 % sucrose (sucrose medium), or in co-cultivation with *Arabidopsis thaliana* seedlings (Yoshimochi et al., 2009b). Antibiotics were added to the media at the following concentrations: ampicillin, 100 μg ml^{-1}; gentamicin (Gm), 20 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; polymyxin B (PB), 50 μg ml^{-1}; and tetracycline, 10 μg ml^{-1}.

**Minitransposon mutagenesis.** The popA-lacZYA reporter strain RK5050 (Fig. S1) (Yoshimochi et al., 2009b) was mutagenized using the plasposon pTnMod-OGm (Dennis & Zylstra, 1998). The plasmid pTnMod-OGm was electroporated into RK5050 cells using a Gene Pulser II (Bio-Rad). After incubation for 1 h at 28 °C, the cells were spread on B agar plates supplemented with 0.5 % glucose (BG), Gm, and PB, and incubated at 28 °C for 3–4 days. Transformant cells were incubated on sucrose media supplemented with 40 μg ml^{-1} X-Gal at 28 °C for 2 days.

**Mapping of minitransposon-insertion sites.** Chromosomal DNA was purified from the plasmospont mutants and digested with *PstI*. After the enzyme had been inactivated, the digested DNA was self-ligated and put into *E. coli* DH125. Plasmids were purified from colonies grown on LB agar plates supplemented with Gm. The DNA fragment surrounding the transposon insertion site was amplified from the plasmid with the primers OgmA1 (5′-CTTATGCTAATTCG-AGCT-3′) and OgmB1 (5′-TGTACCGTGAACATGCA-3′) using PrimeSTAR HS DNA polymerase (Takara Bio). The nucleotide sequences of the PCR amplified products were determined using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with either the OgmA1 or OgmB1 primer.

**Virulence assays.** Tomato (*Solanum lycopersicum* cv. Moneymaker) and tobacco (*Nicotiana tabacum* cv. Bright Yellow) were grown in plastic pots containing commercial soil under a photoperiod of 16 h light and 8 h dark at 25 °C under 10 000 lx. The virulence of strains in 2–3-week-old plants was quantified by soil inoculation or direct petiole inoculation (Tans-Kersten et al., 2001). Soil inoculation is a natural means of infection, more similar to natural invasion. Petiole inoculation is an artificial route of infection, which directly introduces cells into the xylem. Briefly, we poured a bacterial suspension (10^8 cfu ml^{-1}) onto the soil in which the plants were growing to achieve a final concentration of 10^7 cfu g^{-1} soil. We cut the petiole of the first true leaf 0.5 cm from the stem and placed 2 μl of the bacterial suspension (10^8 cfu ml^{-1}) onto the freshly cut surface. Plants were inspected for wilt symptoms daily for 14 days and more after inoculation.

**Bacterial population in plants.** In order to measure cell growth in the intercellular spaces, bacterial suspension (10^4 cfu ml^{-1}) was inoculated into 4-week-old *N. tabacum* cv. Bright Yellow by leaf infiltration using a 1 ml disposable syringe in a volume of 100 μl. After incubation for several days, a leaf disc (0.38 cm^2) was excised with a 0.7 cm cork borer from the infiltrated leaf and kept in a 2.0 ml tube supplemented with 500 μl of Z buffer (Miller, 1992). The leaf disc was then crushed at 3000 r.p.m. for 60 s using 5 mm zirconia beads (Micro smash MS-100; TOMY SEIKO). For inoculation of cut petioles to measure the cell growth in xylem, 2 μl of the bacterial suspension (10^6 cfu ml^{-1}) was placed on the freshly cut surface of the petioles of tomato plants. Stem pieces (1 cm in length) were removed above the cut petiole, weighed, and crushed similarly to the leaf discs. Diluted suspensions were spread on B agar supplemented with glucose and PB, and the colonies were counted after 2 days of incubation at 28 °C.
**β-Galactosidase assay.** The β-galactosidase assay was performed as previously described (Miller, 1992) with some modifications. Bacterial cells (40 μl) were diluted into 2 ml of B medium or sucrose medium and incubated with shaking at 28 °C for 5 or 8 h. An aliquot (100 or 200 μl) of the cells was used for the β-galactosidase assay. Enzyme activity was measured at least three times and reported as the average with SD.

**Measurement of β-galactosidase activity in planta.** The β-galactosidase activity in planta was determined using the Galacto-Light Plus kit (Applied Biosystems). The activity was evaluated using the GloMax 20/20 luminometer (Promega). The leaf discs or stem pieces inoculated with bacterial suspensions were crushed in 500 μl of Z buffer using zirconia beads as described earlier. After Z buffer was added to a final volume of 1 ml, the bacterial suspension was treated with 10 μl of 0.1% SDS and 20 μl of chloroform. A 70 μl aliquot of reaction buffer (1:100 dilution of Galacto-Light Plus substrate with reaction buffer diluent) was added to each 20 μl of the SDS-chloroform treated sample. After incubation at 25 °C for 30 min, 100 μl of accelerator II solution was added, and the chemiluminescence was measured. Luminescence was normalized by cell number. Statistical significance was determined by the two-tailed Student’s t-test.

**Nucleotide sequence accession numbers.** The sequence of prhG in strain OE1-1 has been deposited in DDBJ under the accession number AB510918.

## Results

### Screening genes affecting the expression of the **hrp regulon**

We screened more than 20,000 minitransposon-insertion mutants of the popA-lacZYA reporter strain RK5050. Colonies of RK5050 turned blue when they grew on sucrose agar plates containing X-Gal (Yoshimochi et al., 2009b). Several mutants could not grow on the sucrose agar plates, which likely indicates that these mutants were auxotrophic. Other mutants formed deep-dark-blue colonies, indicating that the hrp regulon was derepressed in these mutants (data not shown). We did not analyse these mutants in this study. In addition to these mutants, approximately 100 other mutants exhibiting white or a lighter blue colour than RK5050 were picked up at the first qualitative screening. All of these mutant clones were incubated in the liquid sucrose medium and the β-galactosidase activity was measured using ONPG as substrate. The expression of the popA operon in these mutants was measured and compared with that of RK5050 as a second quantitative screening, and we selected 43 mutants. These mutants were designated prh (positive regulation of hrp regulon) as they were associated with substantially reduced expression levels of popA (Table S2).

The minitransposon-insertion sites were mapped on the OE1-1 genome. We did not detect any mutations in the lacZYA genes. We focused on six mutants, i.e. prh2, prh3, prh20, prh23, prh27 and prh28, in this study. Minitransposons were inserted into different positions of hrpG in the prh3, prh23 and prh27 mutants, and hrpG in the prh2, prh20 and prh28 mutants. PrhG was recently identified as regulating the expression of hrpB (Plener et al., 2010) and found to be similar to HrpG (Brito et al., 1999). We determined the 1882 bp nucleotide sequence of the cloned DNA fragment containing the prhG of the OE1-1 strain in p3T1023-3, and the prhG ORF sequence of OE1-1 was 100% identical to that of GMII1000. The average expression levels of popA in the prhG and hrpG mutants were 9% and 15% of that of the wild-type, respectively (Table S2).

**hrp regulon expression in vitro**

We constructed the mutants by deleting the entire prhG gene (RK5185) and the entire hrpG gene (RK5196) in the popA-lacZYA background. Although both deletion mutants grew similarly to the wild-type in rich media and sucrose media (data not shown), the expression of popA in both mutants was considerably diminished in sucrose media and upon co-cultivation with A. thaliana seedlings (Fig. S2a, b). Deletion mutants of the prhG gene (RK5189) and the hrpG gene (RK5198) in the hrpB-lacZYA background were also constructed. Both deletion mutants grew similarly to the wild-type (RK5046) in rich media and sucrose media (data not shown). The expression of hrpB in both mutants was also reduced (Fig. S2c, S2d).

**Virulence of the prhG mutant**

Virulence of the mutant strains was tested by soil-soak inoculation and petiole inoculation (Tans-Kersten et al., 2001). Soil-soak inoculation is an infection method that leads to bacterial entry through the root, and thus better reproduces the natural infection. Petiole inoculation directly injects cells into xylem. The popA mutant killed the tomato plants similarly to the wild-type (Kanda et al., 2003b). Tomato plants inoculated with RK5050 in the roots started wilting at 5 days post-inoculation (p.i.) and died by 12 days p.i. (Fig. 1a). The hrpG mutant was not virulent in tomato plants (Fig. 1a), as shown previously (Aldon et al., 2000). When tomato plants were challenged with the prhG mutant, although the inoculated plants started wilting at the same time p.i., the virulence of the prhG mutant was weaker, and not all of the plants died (Fig. 1a). When we used the petiole inoculation method, the same phenotype was observed. Although the tomato plants inoculated with RK5050 and the prhG mutant started wilting at 3 days p.i., not all of the plants inoculated with the prhG mutant died (Fig. 1b).

The R. solanacearum strain OE1-1 is pathogenic to tobacco (Kanda et al., 2003b). Tobacco inoculated with RK5050 in the roots started wilting at 4 days p.i. and died by 13 days p.i. (Fig. 1c). The hrpG mutant showed no virulence to tobacco at all. Although tobacco plants inoculated with the prhG mutant started wilting at 4 days p.i., the wilting was gradual (Fig. 1c).
Mutuation analysis of the predicted phosphorylation site of PrhG

HrpG and PrhG are the response regulators in two-component systems. An aspartate residue, predicted to be a phosphorylation site, is necessary for HrpG function (Yoshimochi et al., 2009b). The predicted phosphorylation site of PrhG, the aspartate residue at position 51, was mutated to asparagine (PrhGD51N, Fig. S1). The prhGD51N mutant strain (RK5280) grew similarly to the wild-type RK5046 (hrpB-lacZYA) in rich media and sucrose media (data not shown). The expression of hrpB was decreased by two-thirds in RK5280 relative to RK5046 but was still higher than in deletion mutant RK5189 (Table 1), which indicates that the predicted phosphorylation site makes a small contribution to PrhG function.

Tomato plants inoculated with RK5272 (prhGD51N) at the roots started wilting at 5 days p.i. and died by 13 days p.i. (Fig. S3a), which was similar to the pattern observed for OE1-1. When we used the petiole inoculation method, the prhGD51N mutant killed tomato plants similarly to OE1-1 (Fig. S3b).

Regulation of prhG expression

The PrhA-PrhR/PrhI-PrhJ signal cascade activates the expression of hrpG (Brito et al., 2002). We constructed a prhG-lacZYA reporter strain (RK5212, Fig. S1) and hrpG (RK5228), prhJ (RK5230) and prhA (RK5232) mutant strains of RK5212. The expression of prhG was fourfold higher in sucrose media than in rich media (Table 2). The expression of prhG in the hrpG, prhJ and prhA mutants was not different from that in the wild-type in rich and sucrose media (Table 2). Because the hrp regulon is negatively regulated by PhcA in GMI1000 (Genin et al., 2005), we examined the involvement of phcA in the expression of prhG. The expression of prhG was dramatically reduced in the phcA deletion mutant RK5234 (Table 2), which shows that PhcA activates, directly or indirectly, the expression of prhG in R. solanacearum. When the phcA gene was transferred using the pLAFR3 vector into RK5234, the transformant cells showed recovery of prhG expression to half the level observed in the wild-type (Table 2). A prhG deletion mutant in RK5120 (hrpG-lacZYA), RK5192, was

**Table 1. hrpB expression in prhG-phosphorylation mutant**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>β-Galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RK5046</td>
<td>hrpB-lacZYA</td>
<td>175 (16)</td>
</tr>
<tr>
<td>RK5280</td>
<td>hrpB-lacZYA prhGD51N</td>
<td>66 (7)</td>
</tr>
<tr>
<td>RK5189</td>
<td>hrpB-lacZYA ΔprhG</td>
<td>44 (8)</td>
</tr>
</tbody>
</table>

*Cells were grown in sucrose medium to OD600 ~ 0.1, which corresponds to 1.8 × 10^8 c.f.u. ml^{-1}, treated with SDS-chloroform and assayed. Mean values of at least six measurements are in Miller units with SE in parentheses.
constructed, and the expression of hrpG was evaluated. The expression of hrpG did not differ between RK5120 and RK5192 (data not shown), which indicates that PrhG did not affect hrpG expression.

Cell density-dependent expression of prhG and hrpG

PhcA becomes active at high cell densities (Clough et al., 1997). Because the expression of prhG was regulated by PhcA, as shown earlier, we examined the cell density-dependent expression of prhG in sucrose media or under co-cultivation with A. thaliana seedlings (Fig. 2a). The expression of prhG was low at a low cell density (less than $10^5$ c.f.u. ml$^{-1}$) and gradually increased and reached a maximum at a high cell density (more than $10^8$ c.f.u. ml$^{-1}$), which agrees with the activation of prhG by PhcA. In contrast, the expression of hrpG in RK5120 was decreased at a high cell density (Fig. 2b), which is consistent with the negative regulation of the complex multigene regulatory cascade PrhA-PrhR/PrhI-PrhJ-HrpG by PhcA (Genin et al., 2005; Yoshimochi et al., 2009a).

The expression of hrpG was much higher under co-cultivation with A. thaliana seedlings than in sucrose media without plants (Fig. 2b) (Yoshimochi et al., 2009b). In contrast, there was no difference in the expression of prhG between co-cultivation with A. thaliana seedlings and cultivation in sucrose media (Fig. 2a), which is consistent with the observation that prhG expression is specifically induced under minimal medium conditions (Plener et al., 2010).

Expression of hrpG and prhG in planta

The hrpG-lacZYA and prhG-lacZYA reporter strains were infiltrated into tomato leaves at different cell densities. Cells in the intercellular spaces were recovered from the infiltrated leaves after 15 h, and β-galactosidase activity was measured using a chemiluminescent substrate and normalized by cell number. We treated the non-infiltrated leaves in the same way and measured the enzyme activity, which we considered to represent endogenous β-galactosidase activity. In the leaf intercellular spaces, hrpG expression was high at a low cell density (less than $10^5$ c.f.u. cm$^{-2}$).

Table 2. prhG expression in various mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Genotype</th>
<th>β-Galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B medium</td>
</tr>
<tr>
<td>RK5212</td>
<td>none</td>
<td>prhG-lacZYA</td>
<td>781 (39)</td>
</tr>
<tr>
<td>RK5228</td>
<td>none</td>
<td>prhG-lacZYA ΔprhA</td>
<td>783 (34)</td>
</tr>
<tr>
<td>RK5230</td>
<td>none</td>
<td>prhG-lacZYA prhG::Tc'</td>
<td>822 (36)</td>
</tr>
<tr>
<td>RK5232</td>
<td>none</td>
<td>prhG-lacZYA ΔhrpG</td>
<td>832 (20)</td>
</tr>
<tr>
<td>RK5234</td>
<td>none</td>
<td>prhG-lacZYA ΔphcA</td>
<td>27 (1)</td>
</tr>
<tr>
<td>RK5234</td>
<td>pLAFphcA</td>
<td>prhG-lacZYA</td>
<td>421 (59)</td>
</tr>
</tbody>
</table>

*Cells were grown to an OD$_{600}$ ~0.1, which corresponds to $1.8 \times 10^8$ c.f.u. ml$^{-1}$, treated with SDS-chloroform and assayed. Mean values of at least six measurements are in Miller units with ±SE in parentheses.

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and reduced to the basal level at a high cell density (Fig. 3a), whereas prhG became active only at a high cell density (more than $10^6$ c.f.u. cm$^{-2}$, Fig. 3b).

Different numbers of cells of the hrpG-lacZYA and prhG-lacZYA reporter strains were directly inoculated into the cut leaf petioles of tomato. After 3 days, the stem (1 cm) was removed, and bacterial cells in xylem vessels were recovered from the stem tissue. In the stem xylem, hrpG expression was high at a low cell density (less than $10^7$ c.f.u. ml$^{-1}$) and was dramatically reduced at a high cell density (Fig. 3c), whereas prhG expression increased at a high cell density (more than $10^8$ c.f.u. ml$^{-1}$, Fig. 3d).

**Growth of R. solanacearum prhG mutant in host plants**

Three strains, i.e. RK5050, RK5196 (ΔhrpG) and RK5185 (ΔprhG), were infiltrated into tobacco leaves at a low concentration ($10^4$ c.f.u. ml$^{-1}$). At 0 days p.i., the cell density in tobacco leaves was approximately $10^2$ c.f.u. cm$^{-2}$. Multiplication of the hrpG mutant was severely impaired, and the cell density of RK5196 reached only $10^4$ c.f.u. cm$^{-2}$ at 6 days p.i. (Fig. 4a). The wild-type strain proliferated, and the cell density reached $10^8$ c.f.u. cm$^{-2}$ at 6 days p.i. The prhG mutant showed the same growth in tobacco leaves as the wild-type (Fig. 4a).

We inoculated bacteria ($2 \times 10^4$ c.f.u.) directly into the host vascular system via the cut leaf petioles of tomato. At 2, 3 and 4 days p.i., the population size of *R. solanacearum* strains in each plant was measured by grinding the stem tissue and dilution plating on the appropriate selective medium. Under these conditions, the hrpG mutant RK5196 was undetectable. The population of the wild-type increased until 4 days p.i. and decreased at 5 days p.i. (Fig. 4b), when the inoculated plants died (Fig. 1b). The numbers of cells in the stem tissues were similar to those reported previously (Kang et al. 1999). There was no significant difference in stem population size between OE1-1 and the prhG mutant RK5185 over the 5 days of the experiment (Fig. 4b) ($P=0.10$ to 0.96).

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**Fig. 3.** Expression of *hrpG* and *prhG* in planta. Different concentrations of cells ($10^5$, $10^6$, $10^7$, $10^8$, $10^9$, and $10^{10}$ c.f.u. ml$^{-1}$) were infiltrated into tomato leaves (a, b) or inoculated directly onto the freshly cut petioles (c, d). Leaf discs (0.38 cm$^2$) were taken from the infiltrated leaves at 15 h p.i. or stem pieces (1 cm in length) were removed above the cut petiole at 3 days p.i. Bacterial cells were recovered from plant tissues disrupted using zirconia beads, and β-galactosidase activity was measured using Galacto-Light Plus containing chemiluminescent substrates. Luminescent signals were detected using a luminometer and normalized by cell number and shown on y-axes in RLU (Relative Light Unit), (a, c) RK5120 (*hrpG-lacZYA*) and (b, d) RK5212 (*prhG-lacZYA*).
**popA expression in leaf intercellular spaces**

The popA-lacZYA reporter strain was infiltrated into leaves of *N. tabacum* at a concentration of 10^4 c.f.u. ml⁻¹. Leaf discs were taken daily from the infiltrated leaves. At time 0, expression of popA was undetectable. Although the cell density in the leaves remained constant at approximately 2 x 10^8 c.f.u. cm⁻², popA expression in the wild-type strain increased over time up to 24 h p.i. (Fig. 5a). At this time, necrotic lesions were not yet observed on the leaves. popA expression was not detected in the hrpG mutant RK5196 and was substantially reduced in the prhG mutant RK5185 (Fig. 5a).

The reporter strains were infiltrated into tobacco leaves. In intercellular spaces, popA expression in the wild-type strain increased over time (Fig. 5b), and no popA expression was detected in the hrpG mutant. In contrast to tomato leaves, popA expression in the prhG mutant did not show a significant difference from that in the wild-type (Fig. 5b), which may reflect that the prhG mutant is more virulent in tobacco than in tomato (Fig. 1a, c).

**popA expression in xylem vessels**

Cells (2 x 10⁴ c.f.u.) of the reporter strain were directly inoculated into the cut leaf petioles of tomato. The stem was periodically removed, and bacterial cells were recovered from the stem tissue. At 1 day p.i., the cell number in the stem was low, and the expression of popA was undetectable. popA expression in the wild-type strain increased at 2 and 3 days p.i., and then decreased to the basal level at 4 days p.i. (Fig. 6). popA expression in the hrpG mutant was not detected in the xylem. The prhG
HRP at a higher cell density and PrhG regulates different stages of bacterial cell growth. HrpG activates reporter strains RK5050 (wild-type, white bars) and RK5185 (ΔprhG, black bars) were inoculated onto the cut petioles of tomato plants at cell concentrations of 10^7 c.f.u. ml⁻¹. Stem pieces were removed, and bacterial cells were recovered as indicated in Fig. 6. β-Galactosidase activity was measured using Galacto-Light Plus. Each assay was repeated in four successive trials, and four plants were treated within each trial. The average and SE were calculated. *P<0.05.

![Graph](Image)

**Fig. 6.** *popA* expression in xylem vessels. The *popA-lacZYA* reporter strains RK5050 (wild-type, white bars) and RK5185 (ΔprhG, black bars) were inoculated onto the cut petioles of tomato plants at cell concentrations of 10^7 c.f.u. ml⁻¹. Stem pieces were removed, and bacterial cells were recovered as indicated in Fig. 6. β-Galactosidase activity was measured using Galacto-Light Plus. Each assay was repeated in four successive trials, and four plants were treated within each trial. The average and SE were calculated. *P<0.05.

The high cell density of 10^9 c.f.u. g⁻¹ stem (Jacobs et al., 2012). A luminescent reporter fused to *hrpB* was used to confirm that *hrpB* was transcribed throughout plant infection, even at late stages, where cells proliferate and reach a high cell density (Monteiro et al., 2012). In this study, we demonstrated that *hrpG* expression was dramatically decreased in the leaves and in the xylem at high cell density, i.e. 10^6 c.f.u. cm⁻² in leaves and 10^9 c.f.u. g⁻¹ in the xylem. Therefore, sustained expression of *hrpB* and genes in the *hrp* regulon at late stages of infection could not be achieved by HrpG. Indeed, *prhG* expression became higher at high cell density. We can thus explain the high expression of the *hrp* regulon at the late stages of infection (at high cell density) by PrhG.

Under natural conditions, *R. solanacearum* cells invade the roots and proliferate in the intercellular spaces. Cell densities in the intercellular spaces do not become high. At this stage, *hrpB* activation could be achieved mainly by HrpG. The pathogen invades xylem elements and spreads into the stem xylem. Bacterial densities become high in the xylem, and some bacterial cells form biofilms on xylem vessel walls (Tans-Kersten et al., 2001), which further increases the cell density. At higher cell densities, PhcA becomes active, represses *hrpG* expression and activates *prhG* expression. When the pathogen spreads into adjacent uninfected vessels and xylem parenchyma cells, expression of the *hrpB*-regulated *hrp regulon* may be necessary. At this stage, PrhG but not HrpG could activate *hrpB* expression.

Although the expression of *popA* and *hrpB* was greatly reduced in both *hrpG* and *prhG* mutants in vitro, it was not greatly affected by *prhG* mutation at low cell density. *hrpB* expression did not decrease in the GMI1000 *prhG* mutant upon co-culture with tomato or *A. thaliana* cell suspensions (Plener et al., 2010). Because PhcA, which is not regulated by a known regulatory pathway such as PrhA-PrhIR-PrhJ (which senses plant–bacterium interaction) and is not active at low cell density (Flavier et al., 1997), regulates *prhG* expression, PhrG is not assumed to be involved in the regulation of *hrpB* and *popA* at low cell density. *popA* expression in tomato leaves increased more than 10-fold within 24 h p.i. As observed in vitro, *popA* expression in the *hrpG* mutant decreased to the basal level in the leaves, whereas the expression of *popA* in the *prhG* mutant was approximately half to one-third of that in the wild-type. During this period, the number of cells in the leaves was maintained at approximately 2 × 10^7 c.f.u. cm⁻². Considering that the thickness of leaves is less than 1 mm and that the pathogen can occupy, at most, 10% of the leaf volume (Kang et al., 1999), the cell density cannot exceed 10^6 c.f.u. ml⁻¹ in the intercellular spaces. At this cell density, HrpG could contribute more than PrhG to *hrpB* activation in the tomato leaves.

*popA* expression in the *prhG* mutant was not repressed in the tobacco leaves. Although we do not have a clear explanation for the difference, this phenomenon likely supports the virulence of the *prhG* mutant in tobacco.
prhG mutant was less virulent in tomato than in tobacco. The prhG mutant proliferated similarly to the wild-type in the tobacco leaves. Although more than $10^8$ cells were present per cm$^2$, the cell density of the pathogen in the intercellular spaces of tobacco leaves may not have been high enough to activate prhG expression, and HrpG may be sufficient to fully active hrpB.

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REFERENCES


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