Functional Characterization of RsRsgA for Ribosome Biosynthesis and Expression of the Type III Secretion System in *Ralstonia solanacearum*

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RsgA plays an important role in maturation of 30S subunit in many bacteria that assists in the release of RbfA from the 30S subunit during a late stage of ribosome biosynthesis. Here, we genetically characterized functional roles of RsgA in *Ralstonia solanacearum*, hereafter designated RsRsgA. Deletion of *R. solanacearum* *rsgA* or *rbfA* resulted in distinct deficiency of 16S ribosomal RNA, significantly slowed growth in broth medium, and diminished growth in nutrient-limited medium, which are similar as phenotypes of *rsgA* mutants and *rbfA* mutants of *Escherichia coli* and other bacteria. Our gene-expression studies revealed that RsRsgA is important for expression of genes encoding the type III secretion system (T3SS) (a pathogenicity determinant of *R. solanacearum*) both in vitro and in planta. Compared with the wild-type *R. solanacearum* strain, proliferation of the *rsgA* and *rbfA* mutants in tobacco leaves was significantly impaired, while they failed to migrate into tobacco xylem vessels from infiltrated leaves, and hence, these two mutants failed to cause any bacterial wilt disease in tobacco plants. It was further revealed that *rsgA* expression was highly enhanced under nutrient-limited conditions compared with that in broth medium and RsRsgA affects T3SS expression through the PrhN-PrhG-HrpB pathway. Moreover, expression of a subset of type III effectors was substantially impaired in the *rsgA* mutant, some of which are responsible for *R. solanacearum* GMI1000 elicitation of a hypersensitive response (HR) in tobacco leaves, while RsRsgA is not required for

HR elicitation of GMI1000 in tobacco leaves. All these results provide novel insights into understanding various biological functions of RsgA proteins and complex regulation on the T3SS in *R. solanacearum*.

Keywords: *Ralstonia solanacearum*, RsgA, ribosome biosynthesis, type III secretion system, pathogenesis

As in many pathogenic bacteria of animals and plants, a syringe-like type III secretion system (T3SS) is a pathogenicity determinant in *Ralstonia solanacearum* that the bacteria uses to inject virulence factors (type III effectors [T3Es]) into host cytosol to subvert host defense (Galán and Wolf-Watz 2006; Genin and Denny 2012; Zhou and Chai 2008). *R. solanacearum* causes bacterial wilt disease on many plant species and has been regarded as one of the most destructive plant-pathogenic bacteria worldwide (Genin 2010; Mansfield et al. 2012). Due to extreme genetic diversity, this bacterium is currently referred to as a *Ralstonia solanacearum* species complex (RSSC), while the T3SS is greatly conserved among the RSSC, which is encoded by 22 genes forming a *hrp* regulon (Coll and Valls 2013; Peeters et al. 2013). To date, the T3SS has been well-demonstrated to be globally regulated by a complex network (Genin 2010; Hikichi et al. 2017). In general, an AraC-family of transcriptional regulator HrpB plays a key regulatory role that directly controls expression of the T3SS and entire T3Es (Mukaihara et al. 2004, 2010; Valls et al. 2006). Expression of *hrpB* and the T3SS is not activated until the bacterium comes in contact with host signals or some mimic signals, such as some of the signals in nutrient-limited medium, which might mimic plant apoplastic fluids (Marenda et al. 1998; Yoshimochi et al. 2009). Two close paralogs of HrpG and PrhG, which are response regulators of the two-component system, respond to host signals by phosphorylation and positively regulate *hrpB* expression in a parallel way (Plener et al. 2010; Zhang et al. 2013). Some host signals or mimic signals are presumed to be recognized by an outer membrane PrhA and transferred to HrpG via a signaling cascade of PrhA-PrhR/I-PrhJ-HrpG or some unknown cascades (Genin and Denny 2012; Hikichi et al. 2017). Moreover, *R. solanacearum* integrates numerous regulators, such as PhcA, PrhN, PrhO, and PrhP to globally regulate expression of the T3SS (Zhang et al. 2018; 2019; Genin and Denny 2012; Hikichi et al. 2017).

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In order to further elucidate complex regulation of the T3SS in *R. solanacearum*, we previously screened several T3SS-regulating candidates with transposon mutagenesis, in which a *popA-lacZYA* fusion was used to monitor expression profiles of the T3SS (Zhang et al. 2013). Among them is Rsc0940, which is predicated as a putative ribosome small subunit-dependent GTPase A, RsgA, hereafter designated as RsRsgA (*R. solanacearum* RsgA). Accumulated evidence suggests that at least four factors of RsgA (previously known as YjeQ), RbfA, RimM, and Era function together to ensure maturation of 30S subunit in many bacteria species (Thurlow et al. 2016; Wilson and Nierhaus 2007). RsgA and Era are GTPases, while RbfA and RimM do not exhibit the enzymatic activity of GTPase (Bunner et al. 2010; Shajani et al. 2011). The *rbfA* mutants and *rsgA* mutants exhibit similar phenotypes of slow growth, deficiency of 16S ribosomal RNA (rRNA) biosynthesis in many *Escherichia coli* strains (Bylund et al. 1998; Dammel and Noller 1995; Inoue et al. 2003, 2006). Although both RsgA and RbfA bind to 30S subunit, binding of RbfA to 30S subunit results in destabilization of the 50' end helix of 16S rRNA in 30S subunit, and one of the functions of RsgA is assisting to release RbfA from 30S subunit during a late stage of ribosome biosynthesis, which ensures biosynthesis of the 16S subunit in many bacteria species (Daigle and Brown 2004; Goto et al. 2011; Himeno et al. 2004). The expression level of the T3SS was substantially reduced in *rsgA* transposon mutants, indicating that RsRsgA has a novel effect on the T3SS expression in *R. solanacearum*. This is the first report linking RsgA proteins with the T3SS. Given the fact that both RsgA and RbfA are essential for maturation of 30S subunit in many bacteria, *R. solanacearum* RbfA (RsRbfA) was selected as a system control to confirm the contribution of RsRsgA on maturation of 30S subunit in *R. solanacearum*. We therefore focused on RsRsgA and RsRbfA to investigate their functional role on ribosomal biosynthesis, novel effect on the T3SS expression, and contribution to pathogenicity of *R. solanacearum*.

RESULTS

Structural feature of RsRsgA with amino-acid sequence.

Blast search at the National Center for Biotechnology Information suggests that RsRsgA (Rsc0940 in *R. solanacearum* GMI1000, a standard strain, 318 amino acids) is a putative RsgA, which represents a protein family whose members are broadly conserved and play an important role on ribosome biosynthesis in many bacteria species (Arigoni et al. 1998; Brown 2005). Proteins of the RsgA family are well characterized, displaying a unique architecture including a N-terminal OB-fold RNA-binding domain, a central permuted GTPase domain, and a C-terminal zinc knuckle-like cysteine domain (Supplementary Fig. S1A) (Daigle et al. 2002; Nichols et al. 2007). *Ralstonia* strains are extremely heterogeneous and are currently referred as the RSSC, including a variety of strains from *R. solanacearum*, *R. syzygii*, *R. picketti*, and banana blood disease bacterium (BDB) (Fegan and Piror 2005; Genin and Denny 2012). Whereas RsRsgA was greatly conserved among the RSSC, it shares more than 93% amino acid identity with those of different *R. solanacearum* strains (Supplementary Fig. S1D) and more than 85% amino acid identity with those of closely related RSSC strains from *R. pickettii*, *R. insidiosa*, *R. syzygii*, and BDB, but 69% amino acid identity with those from *R. eutroph* (Supplementary Fig. S1B). RSSC strains belong to class β -proteobacteria and family Burkholderiaceae, and we compared amino acid sequence of RsRsgA with seven different RsgA proteins from strains of the same family, including members of genera *Cupriavidus*, *Burkholderia*,

Massilia, *Bordetella*, and *Acidovorax*, which share about 60% amino acid identity (Supplementary Fig. S1B). We further compared the amino-acid sequence of RsRsgA with 10 well-characterized RsgA proteins from strains of genera *Escherichia*, *Yersinia*, *Shewanella*, *Salmonella*, *Pseudomonas*, and *Bacillus* that belong to class γ -proteobacteria (Daigle et al. 2002; Nichols et al. 2007). The alignment revealed three discrete regions in RsRsgA that correspond to the N-terminal OB-fold RNA-binding domain (residues 11 to 65), central permuted GTPase domain (residues 86 to 251), and the C-terminal zinc knuckle-like cysteine domain (residues 273 to 285), respectively (Supplementary Fig. S1B and C). The GTPase domain of RsgA proteins is highly similar to several P loop-containing GTPases, which have a circular permutation with a G4-G1-G2-G3 pattern of motifs as opposed to the regular G1-G3-G4 pattern (Daigle et al. 2002; Nichols et al. 2007). It was noteworthy that residues in motifs of G4-G1-G2-G3 are identical among RsRsgA of the RSSC strains (Supplementary Fig. S1D) and are extremely conserved among RsRsgA and those from strains belonging to the *Burkholderiaceae* family (Fig. S1B). Although RsRsgA exhibit less than 40% global identity to more than 10 well-characterized RsgA from strains of the γ -proteobacteria class, they share the typical circular permutation of the GTPase domain and residues in their motifs of G4-G1-G2-G3 are greatly conserved (Supplementary Fig. S1C), indicating that RsRsgA belongs to the RsgA family and plays some important role in ribosome biosynthesis. In consideration of the fact that residues in motifs with typical circular permutation of GTPase domain are extremely similar among RsRsgA and those belonging to the *Burkholderiaceae* family, RsRsgA should be much conserved during evolution among the RSSC and closely related bacteria. We therefore focused on RsRsgA to investigate its functional role on ribosome biosynthesis, novel effect on the T3SS expression, and contribution to pathogenicity in *R. solanacearum*.

Deletion of *rsgA* or *rbfA* impaired biosynthesis of 16S rRNA in *R. solanacearum*.

A genome search revealed Rsc1290 (123 amino acids in GMI1000) is annotated as a RbfA homolog, hereafter designated RsRbfA. RsgA and RbfA have been well-demonstrated to be important for maturation of 30S subunit in many bacteria species and biosynthesis of 16S rRNA is impaired in *rsgA* mutants and *rbfA* mutants of *E. coli* (Goto et al. 2011; Thurlow et al. 2016). We first generated *rsgA* mutant RQ5872 (OE1-1, *popA-lacZYA*, $\Delta rsgA$) and *rbfA* mutant RQ6101 (OE1-1, *popA-lacZYA*, $\Delta rbfA$) to ascertain whether RsRsgA and RsRbfA are involved in biosynthesis of 16S rRNA in *R. solanacearum*. Total RNAs of the wild-type strain and the above two mutants were isolated and were analyzed with 5% polyacrylamide gel electrophoresis (PAGE). A clear band appointed to 16S rRNA was detected in the wild-type strain RK5050 (WT) but not in *rsgA* mutant and *rbfA* mutant (Fig. 1A), confirming that RsRsgA and RsRbfA are important for biosynthesis of 16S rRNA in *R. solanacearum*.

Deletion of *rsgA* or *rbfA* significantly slowed growth of *R. solanacearum* in broth medium but diminished growth in nutrient-limited medium.

The *rsgA* and *rbfA* mutants of *E. coli* usually suffer a growth defect (Goto et al. 2011; Himeno et al. 2004). We therefore ascertained their effect on growth of *R. solanacearum* by determining their growth rates in broth medium and nutrient-limited medium (sucrose medium, *hrp*-inducing medium). In broth medium, growth of *rsgA* mutant and *rbfA* mutant was significantly slowed compared with that of the WT, although

these two mutants could eventually grow to the same maximum optical density at 600 nm (OD₆₀₀) as the wild-type strain at about 21 h postinfiltration (hpi) (Fig. 1B).

The T3SS expression of *R. solanacearum* is not activated in broth medium but is highly induced in sucrose medium, a kind

Fig. 1. Involvement of *Ralstonia solanacearum* RsgA and RbfA (RsRsgA and RsRbfA) on typical RsgA-dependent properties of **A**, biosynthesis of 16S ribosomal RNA, **B**, growth in broth medium, and **C**, growth in nutrient-limited medium (sucrose medium). WT = the wild-type strain RK5050 (OE1-1, *popA-lacZ*), ΔrsgA = RQ5872 (RK5050, ΔrsgA), ΔrbfA = RQ6101 (RK5050, ΔrbfA), ΔrsgA+rsgA = RQC0224 (RQ5872 with complementary rsgA), and ΔrbfA+rbfA = RQC0225 (RQ6101 with complementary rbfA). For RNA electrophoresis, *Ralstonia solanacearum* strains were grown in broth medium to an optical density at 600 nm (OD₆₀₀) of about 1.0 and total RNAs were isolated with the TRIzol reagent (Life). A total of 1 μg of RNAs was separated with 5% polyacrylamide gel electrophoresis and were visualized with GoldView II nuclear staining dyes (Solarbio). Each assay was performed with three biological replicates and representative results are presented. For the growth assay, inocula of overnight-cultivated *R. solanacearum* strains were adjusted to an OD₆₀₀ of 1.0 and were inoculated into fresh broth medium and nutrient-limited medium (sucrose medium) with a proportion of 1%, and OD₆₀₀ was measured periodically. Mean values of all three biological replicates were averaged with standard deviation (error bars). Statistical significance between RK5050 and mutants was assessed using a post hoc Dunnett test following analysis of variance. Two asterisks (**) indicate significance level $P < 0.01$.

of nutrient-limited medium that might mimic plant apoplastic fluids, a so-called *hrp*-inducing medium (Yoshimochi et al. 2009). We thus ascertained growth of *R. solanacearum* rsgA and rbfA mutants in this nutrient-limited sucrose medium. Unlike that in broth medium, growth of rsgA and rbfA mutants was severely repressed in sucrose medium, which remained an OD₆₀₀ of 0.1 to 0.2 up to 36 hpi (Fig. 1C). Complementary rsgA and rbfA fully restored their slowed or diminished growth to that of the WT in nutrient-rich and nutrient-limited media, respectively (Fig. 1B and C). It was noteworthy that rsgA and rbfA mutants exhibited similar growth patterns in nutrient-limited sucrose medium or nutrient-rich broth medium (Fig. 1B and C). All these results confirmed that RsRsgA and RsRbfA are important for *R. solanacearum* to grow under different nutrient conditions.

Expression of rsgA was highly induced under nutrient-limited conditions.

Growth of rsgA and rbfA mutants was slowed in broth medium but was diminished in nutrient-limited medium, indicating that RsRsgA and RsRbfA play a more important role on growth under nutrient-limited conditions compared with that under nutrient-rich conditions. In order to assess rsgA expression under different nutrient conditions, we fused promoter-less *lacZ*YA with rsgA promoter and integrated it into a chromosome of *R. solanacearum* with the Tn7T-based site-specific chromosomal integration system, to generate reporter strain RQC0341 (OE1-1, rsgA-lacZYA). With the in-vitro enzyme assay, rsgA expression was induced to 169 Miller units in nutrient-limited sucrose medium, which was 5.0-fold higher than that in nutrient-rich broth medium (Fig. 2A). Xylem sap of plants is a kind of nutrient-limited medium that can induce T3SS expression to much higher levels than that in *hrp*-inducing sucrose medium (Zhang et al. 2013). We collected xylem sap from tomato stems by centrifugation and assessed rsgA expression in tomato xylem sap with the in-vitro enzyme assay. The rsgA expression in nutrient-limited sucrose medium was increased to about a 7.1-fold higher level than that in nutrient-rich broth medium (Fig. 2B), which was consistent with the above result, assessed by the in-vitro enzyme assay, that rsgA expression in sucrose medium was about 5.0-fold greater than that in nutrient-rich broth medium. The rsgA expression in tomato xylem sap was increased to about 3.6-fold greater than that in broth medium (Fig. 2B). All these results confirmed that *R. solanacearum* will produce more RsRsgA to ensure its growth under nutrient-limited conditions.

RsRsgA and RsRbfA are important for expression of genes encoding the T3SS both in vitro and in planta.

Plant signals can enhance T3SS expression to RsRsgA was originally screened as one of the T3SS-regulating candidates by transposon mutagenesis, in which a *popA-lacZ*YA was used to monitor expression profiles of the T3SS in *R. solanacearum* (Zhang et al. 2013). The *popA* gene is located on the left side of the *hrp* regulon and belongs to the T3Es. Expression of *popA* is directly controlled by the key regulator HrpB and *popA-lacZ*YA exhibits identical expression profiles as a *hrp* regulon that is not activated in broth medium but is induced in *hrp*-inducing sucrose medium or by contact with host signals (Cunnac et al. 2004; Yoshimochi et al. 2009). Moreover, *popA-lacZ*YA reporter strain (RK5050) exhibits an identical infection process as parent strain OE1-1 toward host plants (Zhang et al. 2013). We therefore assessed *popA* expression in the rsgA and rbfA mutants to validate their effect on expression of the T3SS. Consistent with that in transposon mutants, *popA* expression in sucrose medium

(*hrp*-inducing medium) was substantially reduced in the *rsgA* mutant (13 versus 315 Miller units of the WT) and in *rbfA* mutant (14 versus 315 Miller units of RK5050) (Fig. 3A). Complementary *rsgA* or *rbfA* fully restored their substantially reduced *popA* expressions to that of the WT (Fig. 3A).

Plant signals can enhance T3SS expression to much higher levels than that in *hrp*-inducing medium (Zhang et al. 2013). We assessed the in-planta expression of *popA* in *rsgA* and *rbfA* mutants. Cell suspensions were infiltrated into tobacco leaves and cells were recovered for the in-planta enzyme assay, which was carried out every 6 h up to 24 hpi, since the wild-type strain caused tobacco leaves to wither and dry after 24 hpi. Both *rsgA* and *rbfA* mutants exhibited substantially reduced *popA* expression in tobacco leaves from 6 to 24 hpi (Fig. 3B). All these results confirmed that RsRsgA and RsRbfA have a novel effect on T3SS expression both in vitro and in planta. It is noteworthy that *popA* expression in *rsgA* and *rbfA* mutants in tobacco leaves remained at about half the level of the wild-type strain, which is different from that in *hrp*-inducing medium, since the *popA* expression in *hrp*-inducing medium was diminished to the degree it was nearly nonexistent in *rsgA* and *rbfA* mutants.

Fig. 2. Expression of *rsgA-lacZYA* under different nutrient conditions assessed with in-vitro enzyme and in-planta enzyme assays. **A**, RQC0341 (OE1-1, *rsgA-lacZYA*) was grown in broth medium and sucrose medium (nutrient-limited medium) to an optical density at 600 nm (OD_{600}) of about 0.1 and was subjected to the in-vitro enzyme assay, enzymatic activities of which are presented in Miller units. **B**, RQC0341 was grown in tomato xylem sap, broth medium, and sucrose medium and was subjected to the in-planta enzyme assay, enzymatic activities of which are presented with luminescence normalized by cell number (relative luminescence units [per cell [RLU cell⁻¹]]). Each assay was performed with four biological replicates including six replications per trial. Mean values of all experiments were averaged with standard deviation, and statistical significance was assessed using a post hoc Dunnett test following analysis of variance. Two asterisks (**) indicate significance level $P < 0.01$.

RsRsgA and RsRbfA are essential for pathogenicity of *R. solanacearum*.

RsRsgA and RsRbfA are important for bacterial growth and expression of the T3SS, both of which are essential for pathogenicity of *R. solanacearum* toward host plants (Genin and Denny 2012), and we assessed virulence of *rsgA* and *rbfA* mutants in tobacco plants. These two mutants were used to invade tobacco plants by two inoculation methods, i.e., soil soaking and leaf infiltration, respectively. RK5050 (WT) could eventually wilt all test tobacco plants with each of the inoculation methods, while the *rsgA* and *rbfA* mutants failed to cause any bacterial wilt disease in test tobacco plants, regardless of inoculation method (Fig. 4A and B). Complementary *rsgA* and *rbfA* fully restored their diminished pathogenicity to that of the wild-type strain (RK5050) in tobacco plants,

Fig. 3. **A**, Expression of *popA* in *Ralstonia solanacearum* strains in sucrose medium (*hrp*-inducing medium) and **B**, tobacco leaves (in planta). WT = the wild-type strain RK5050 (OE1-1, *popA-lacZYA*), $\Delta rsgA$ = RQ5872 (RK5050, $\Delta rsgA$), $\Delta rbfA$ = RQ6101 (RK5050, $\Delta rbfA$), $\Delta rsgA+C$ = RQC0224 (RQ5872 with complementary *rsgA*), and $\Delta rbfA+C$ = RQC0225 (RQ6101 with complementary *rbfA*). For the in-vitro enzyme assay, strains were grown in *hrp*-inducing medium to an optical density at 600 nm (OD_{600}) of about 0.1 and were subjected to the enzyme assay, enzymatic activities of which are presented in Miller units. For the in-planta enzyme assay, tobacco leaves were infiltrated with bacterial suspension of 0.1 OD_{600} and leaf disks were punched for the enzyme assay with Galacto-Light Plus kit, enzymatic activities of which are presented with luminescence normalized by cell number (relative luminescence units [per cell [RLU cell⁻¹]]). The in-planta enzyme assay was carried out to 24 h post-infiltration, when infiltrated leaves became withered and dried. Cell densities were quantified by dilution plating, and luminescence was determined using a GloMax20 luminometer (Promega). Each assay was repeated with four biological replicates including six replications per trial. Mean values of all experiments were averaged with standard deviation and statistical significance between RK5050 and mutants was assessed using a post hoc Dunnett test following analysis of variance. Two asterisks (**) indicate significance level $P < 0.01$.

respectively (Fig. 4A and B), confirming that RsRsgA and RsRbfA are essential for *R. solanacearum* to wilt host plants.

RsRsgA affects T3SS expression through the PrhN-PrhG-HrpB pathway.

R. solanacearum T3SS is directly controlled by the master regulator HrpB, and *hrpB* expression is positively regulated by HrpG and PrhG in a parallel way (Plener et al. 2010; Zhang et al. 2013). We therefore generated *rsgA* mutants from reporter strains of RK5046 (OE1-1, *hrpB-lacZAY*), RK5120 (OE1-1, *hrpG-lacZAY*), and RK5212 (OE1-1, *prhG-lacZAY*) to ascertain how RsRsgA affects T3SS expression. The *hrpB* expression was substantially reduced in *hrp*-inducing medium with deletion of *rsgA* (19 versus 178 Miller units of RK5046), confirming that impact of RsRsgA on T3SS is mediated through the master regulator HrpB (Fig. 5). Furthermore, deletion of *rsgA* diminished *prhG* expression to the degree it was nearly nonexistent in *hrp*-inducing medium (24 versus 2,724 Miller

units of RK5212) but there was no alteration on *hrpG* expression (119 versus 146 Miller units of RK5120), suggesting that impact of RsRsgA on T3SS is mediated through PrhG but is independent of the HrpG. Expression of *hrpG* and *prhG* was also assessed in broth medium with *rsgA* deletion, and the same result was obtained. Deletion of *rsgA* diminished *prhG* expression to the degree it was nearly nonexistent in broth medium but there was no alteration on *hrpG* expression (data not shown). We previously reported that MarR-type regulator PrhN positively regulates *prhG* expression (Zhang et al. 2015), and we generated a *rsgA* mutant from RQ5613 (OE1-1, *prhN-lacZAY*) to ascertain the effect of RsRsgA on *prhG* expression. Expression of *prhN* was substantially impaired with *rsgA* deletion in *hrp*-inducing medium (469 versus 1,605 Miller units of RQ5613) (Fig. 5). All these results suggested that the impact of RsRsgA on the T3SS is mediated through the PrhN-PrhG-HrpB pathway.

RsRsgA and RsRbfA are important for proliferation in the intracellular spaces but essential for migration from intracellular spaces into xylem vessels.

Proliferation and colonization in host plants are essential for *R. solanacearum* to wilt host plants (Genin and Denny 2012), and growth of *rsgA* or *rbfA* mutants was diminished to the degree it was nearly nonexistent in nutrient-limited sucrose medium (Fig. 1C). We thus assessed whether RsRsgA or RsRbfA is required for *R. solanacearum* to proliferate in tobacco leaves (intercellular space). A bacterial suspension at a low concentration of 10^4 CFU ml⁻¹ was infiltrated into tobacco leaves and cell densities in tobacco leaves were quantified every 2 days up to 6 dpi, when RK5050-infiltrated tobacco leaves became withered and dried. The *rsgA* mutant and *rbfA* mutant exhibited similar cell densities as RK5050 (WT) in tobacco leaves up to 2 dpi, which started from approximately 10^2 CFU cm⁻² and reached to approximately 10^5 CFU cm⁻² at 2 dpi (Fig. 6A). RK5050 proliferated extensively in tobacco leaves, which reached to cell densities of approximately 10^7 CFU cm⁻² at 4 dpi and 10^9 CFU cm⁻² at 6 dpi, while cell densities of *rsgA* and *rbfA* mutants were increased to approximately 10^7 CFU cm⁻² in tobacco leaves at 6 dpi, which was lower than that of RK5050 by approximately two orders of magnitude (Fig. 6A), indicating that RsRsgA and RsRbfA are important

Fig. 4. Virulence assay of *Ralstonia solanacearum* strains in tobacco plants with **A**, soil-soaking and **B**, leaf infiltration inoculation methods. WT = the wild-type RK5050 (OE1-1, *popA-lacZAY*), $\Delta rsgA$ = RQ5872 (RK5050, $\Delta rsgA$), $\Delta rbfA$ = RQ6101 (RK5050, $\Delta rbfA$), $\Delta rsgA+rsgA$ = RQC0224 (RQ5872 with complementary *rsgA*), and $\Delta rbfA+rbfA$ = RQC0225 (RQ6101 with complementary *rbfA*). For the soil-soak inoculation method, a bacterial suspension was poured into pot soil of plants at a concentration of 10^7 CFU per gram of soil. For the leaf infiltration inoculation method, about 50 μ l of bacterial suspension at 0.1 optical density at 600 nm (OD_{600}) was infiltrated into tobacco leaves with a blunt-end syringe. Wilt symptoms were inspected daily and were scored on a 0 to 4 disease index scale (0 = no wilting, 1 = 1 to 25% wilting, 2 = 26 to 50% wilting, 3 = 51 to 75% wilting, and 4 = 76 to 100% wilted or dead). Each assay was performed with four biological replicates including 12 plants per trial. Mean values of all experiments were averaged and statistical significance between RK5050 and mutants was assessed using a post hoc Dunnett test following analysis of variance. Two asterisks (**) indicate significance level $P < 0.01$.

Fig. 5. Expression of *hrpB-lacZAY*, *hrpG-lacZAY*, *prhG-lacZAY*, and *prhN-lacZAY* with *rsgA* deletion. *Ralstonia solanacearum* strains were grown in *hrp*-inducing medium to an optical density at 600 nm (OD_{600}) of about 0.1 and were subjected to an in-vitro enzyme assay, enzymatic activities of which are presented in Miller units. Each assay was repeated with four biological replicates, including three replications per trial. Mean values of all experiments were averaged with standard deviation and statistical significance between parent strains (WT) and *rsgA* mutants ($\Delta rsgA$) was assessed using a post hoc Dunnett test following analysis of variance. Two asterisks (**) indicate significance level $P < 0.01$.

for *R. solanacearum* to proliferate in tobacco leaves especially at the late stage of proliferation.

Migration is essential for *R. solanacearum* to colonize throughout the host plants, and we assessed migration of *rsgA* and *rbfA* mutants from the infiltrated tobacco leaves into petioles. A bacterial suspension at a concentration of 10^8 CFU ml⁻¹ was infiltrated into tobacco leaves and cell densities in petioles of infiltrated leaves were determined every 2 days. RK5050 could not be detected in petioles of infiltrated leaves until about 6 dpi, which started from cell densities of approximately 10^4 CFU g⁻¹ at about 6 dpi and proliferated extensively to maximum cell densities of approximately 10^{10} CFU g⁻¹ at 12 dpi, when petioles of RK5050-infiltrated leaves became withered and dried (Fig. 6B). Unlike RK5050, *rsgA* and *rbfA* mutants were rarely detected in petioles of the infiltrated leaves until up to 12 dpi (Fig. 6B) or even up to 24 dpi (data not shown), confirming that *rsgA* and *rbfA* mutants failed to migrate into petioles from infiltrated leaves. It is noteworthy that the *rsgA* mutant and the *rbfA* mutant exhibited similar phenotypes of proliferation in tobacco leaves and migration from the infiltrated tobacco leaves into petioles. All these results suggest that RsRsgA and RsRbfA are essential for *R. solanacearum* to migrate from intracellular space (tobacco leaves) into xylem vessels.

RsRsgA is important for expression of a subset of T3Es but not for elicitation of HR in tobacco leaves.

The master regulator HrpB directly controls expression of the T3SS and all the T3Es in *R. solanacearum*, while *hrpB* expression was substantially impaired with deletion of *rsgA*. We thus assessed whether expression of abundant T3Es was impaired in the *rsgA* mutant. In this study, a total of 11 T3Es, including RipAA, RipAR, RipB, RipD, RipE1, RipO, RipP1, RipR, RipTAL, RipW, and RipX (PopA, positive control) were subjected to quantification of mRNA levels with quantitative reverse transcription PCR (qRT-PCR). Consistent with the above results from the enzyme assay, mRNA levels of *ripX* (*popA*) were significantly decreased in the *rsgA* mutant compared with the wild-type strain (Fig. 7A). The mRNA level of the other 10 T3Es was also significantly decreased in the *rsgA* mutant, indicating that RsRsgA is also important for expression of a subset of T3Es in *R. solanacearum*.

Several T3Es have been experimentally validated to be responsible for HR elicitation of some *R. solanacearum* strains in nonhost plants (Peeters et al. 2013). For instance, GMI1000 and RS1000 cause HR elicitation in tobacco leaves, while inactivation of both *ripAA* and *ripP1* enables GMI1000 to cause wilt symptoms in tobacco plants (Poueymiro et al. 2009). Deletion of *ripB* enables RS1000 to cause wilt symptoms in tobacco plants and two well-known T3Es of RipAA and RipP1 contribute, in part, to the avirulence of RS1002 in tobacco plants (Nakano and Mukaiyama 2019). We thus generated *rsgA* mutant GF0058 (GMI1000, $\Delta rsgA$) and *rbfA* mutant GF0060 (GMI1000, $\Delta rbfA$) from the GMI1000 strain to ascertain whether RsRsgA or RsRbfA was required for HR elicitation of GMI1000 in tobacco leaves. Tobacco leaves were infiltrated with bacterial suspension at an OD₆₀₀ of 0.1, and symptom development of necrotic lesions, which is a representative symptom for development of HR in tobacco leaves, was investigated periodically. Intriguingly, both the *rsgA* mutant and the *rbfA* mutant exhibited formation of necrotic lesions similar to that of GMI1000 in tobacco leaves (Fig. 7B), indicating that RsRsgA and RsRbfA are not required for GMI1000 to elicit HR in tobacco leaves.

DISCUSSION

In the present study, we provided multiple lines of evidence to characterize functional roles of RsRsgA in *R. solanacearum*,

which was originally screened as one of the T3SS-regulating candidates in *R. solanacearum* by transposon mutagenesis (Zhang et al. 2013). RsgA represents a protein family whose members are broadly conserved in many bacteria and have been experimentally validated to be functional for ribosome biosynthesis in many bacteria (Arigoni et al. 1998; Brown 2005). RsgA, RbfA, Era, and RimM function together to ensure maturation of 30S subunit in many bacteria, and the function of RsgA proteins is to assist in the release of RbfA from 30S subunit during a late stage of ribosome biosynthesis (Goto et al. 2011; Thurlow et al. 2016; Wilson and Nierhaus 2007). *R. solanacearum* *rsgA* and *rbfA* mutants exhibited distinct deficiency in biosynthesis of 16S rRNA and slowed growth in broth medium, which are consistent with properties of *rbfA* and *rsgA* mutants in other bacteria species, supporting the prediction that

Fig. 6. Bacterial proliferation and migration of *rsgA* mutant and *rbfA* mutant in tobacco plants. **A**, Proliferation in tobacco leaves (intracellular spaces). A bacterial suspension at 10^4 CFU ml⁻¹ was infiltrated into tobacco leaves with a blunt-end syringe and leaf disks were punched every 2 days for quantification of cell densities by dilution plating. The proliferation assay was carried out up to 6 days postinfiltration (dpi), when infiltrated leaves became withered and dried. **B**, Migration into petioles of the infiltrated leaves. About 50 μ l of bacterial suspension at 10^8 CFU ml⁻¹ was infiltrated into tobacco leaves and petioles of the infiltrated leaves were harvested every 2 days for quantification of cell densities. The wild-type strain RK5050-inoculated petioles were harvested up to 12 dpi, when tobacco plants became withered and died. Each assay was repeated with four biological replicates including six plants per trial. Mean values of all experiments were averaged with standard deviation and statistical significance between RK5050 and mutants, assessed using a post hoc Dunnett test following analysis of variance. Two asterisks (**) indicate significance level $P < 0.01$.

RsRsgA is a putative RsgA protein and implies an important role on ribosome biosynthesis in *R. solanacearum*.

The overall structure of RsRsgA displays the unique architecture of RsgA proteins with three discrete domains of a N-terminal OB-fold RNA-binding domain, a central permuted GTPase domain, and a C-terminal zinc knuckle-like domain. RsRsgA was extremely conserved among the RSSC, which includes strains of *R. solanacearum*, *R. syzygii*, *R. picketti*, and BDB, and their RsgA proteins share more than 93% of identity at amino acids, indicating that RsRsgA are extremely conserved during evolution. Amino acids of RsRsgA and other RsgA proteins from a subset of bacteria belonging to classes of β -proteobacteria and γ -proteobacteria share high similarities in the central GTPase domain and the zinc knuckle-like domain but are greatly variable in the OB-fold RNA-binding domain (Supplementary Fig. S1C), which is consistent with characteristics of several well-characterized RsgA proteins (Daigle et al. 2002; Nichols et al. 2007). Given the fact that amino acid sequences of RsRsgA and those from different classes is extremely conserved in motifs of G4-G1-G2-G3 of the GTPase domain, it is not striking that RsRsgA was supported here as a member of RsgA proteins, although it exhibits less than

40% global identity of amino acids with those well-known RsgA proteins from a subset of bacteria belonging to different classes. The N-terminal OB-fold domain and the C-terminal zinc finger domain exhibit great variation that might enable RsgA to interact with one or more partners and then couple biochemical activities for different cellular metabolism (Nichols et al. 2007; Shin et al. 2004). For instance, some *E. coli* *rsgA* mutants exhibit enhanced resistance to high salt stress (Hase et al. 2009), while some *E. coli* *rsgA* mutants exhibit increased susceptibility to antibiotics (Campbell et al. 2005).

Growth of *R. solanacearum* *rbfA* mutants and *rsgA* mutants was significantly slowed in broth medium but was diminished to the degree it was nearly nonexistent in nutrient-limited sucrose medium (Fig. 1). Plants produce and accumulate abundant secondary metabolites, including phenolic acids, saccharides, and amino acids, while nutrients in the intracellular spaces and xylem vessels are believed to be less limited than those in broth medium but more abundant than those in sucrose medium (Lowe-Power et al. 2018; Zuluaga et al. 2013). Proliferation of *rsgA* and *rbfA* mutants does indeed match well with different nutrient conditions that were significantly impaired in tobacco leaves to a two to three order of magnitude compared with the wild-type strain at the late stage of proliferation but failed to grow in sucrose medium (Fig. 1). The expression level of *rsgA* in sucrose medium was about twofold higher than that in xylem sap (Fig. 2), confirming that *R. solanacearum* will produce more RsRsgA to ensure its growth in sucrose medium compared with that in xylem sap. Moreover, the expression level of *rsgA* in xylem sap was about fourfold higher than that in broth medium. It was consistent with the fact that nutrients in host plants are more abundant than those in sucrose medium but less limited than those in broth medium. Although *rsgA* and *rbfA* mutants could eventually grow to much greater cell densities in tobacco leaves, it was much less than that of the wild-type strain. Moreover, these two mutants failed to migrate into xylem vessels from the infiltrated tobacco leaves. This might be a crucial reason why *rsgA* and *rbfA* mutants failed to cause any wilt disease in host plants, since extensive colonization and proliferation throughout host plants is believed to be one of the most important pathogenicity determinants of *R. solanacearum* (Genin and Denny 2012; Roberts et al. 1988).

Moreover, RsRsgA and RsRbfA were characterized to be important for expression of the T3SS both in vitro and in planta, which is another pathogenicity determinant of *R. solanacearum* (Genin 2010). This is the first report linking RsgA proteins with T3SS in pathogenic bacteria. The *rsgA* and *rbfA* mutants exhibited similar phenotypes of impaired proliferation in tobacco leaves, disabled migration from leaves into xylem vessels, substantially impaired expression of the T3SS, and diminished pathogenicity in host plants. Given the fact that both RsgA and RbfA function together to ensure ribosome biosynthesis in many bacteria and that *R. solanacearum* *rsgA* and *rbfA* mutants failed to grow in source mediums, the effect of RsRsgA and RsRbfA on the T3SS is possibly due to ribosome deficiency. This abnormal status of ribosome biosynthesis results in substantially impaired expression of abundant genes in *R. solanacearum*, which should not be limited in genes of the *hrp* regulon, the T3SS, and T3Es. Deletion of *rsgA* or *rbfA* substantially impaired expression of *hrpB* and a subset of T3Es, which is consistent with the fact that the master regulator HrpB directly controls expression of the T3SS and entire T3Es (Mukaihara et al. 2010; Valls et al. 2006). Both HrpG and PrhG, which exhibit about 72% global amino-acid identity, positively activate *hrpB* expression in a parallel way (Plener et al. 2010;

Fig. 7. Impact of *Ralstonia solanacearum* RsgA (RsRsgA) on expression of type III effectors (T3Es) and hypersensitive response (HR) elicitation. **A**, Relative expression of a subset of T3E genes in *rsgA* mutant. Strains were grown in *hrp*-inducing medium to an optical density at 600 nm (OD_{600}) of about 0.1 and total RNA was isolated. The cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser, and mRNA levels of representative T3E genes were determined by quantitative reverse transcription PCR with reference gene *serC* for normalization. Normalized gene expression was calculated by the $\Delta\Delta$ quantification cycle method and normalized values of *rsgA* mutant were divided with those of the wild-type strain (WT) to present relative values (relative expression). Mean values from four biological replicates including three replications per trial were averaged with standard deviation. Statistical significance was assessed between WT and *rsgA* mutants. Two asterisks (**) indicate significance level $P < 0.01$. **B**, HR test of *rsgA* mutant and *rbfA* mutant in tobacco leaves. Approximate 50 μ l of bacterial suspension at 10^8 CFU ml^{-1} was infiltrated into tobacco leaves with a blunt-end syringe. **A**, GMI1000; **B**, GF0058 (GMI1000, $\Delta rsgA$); **C**, GF0060 (GMI1000, $\Delta rbfA$); and **D**, distilled water. Development of necrotic lesions was observed periodically. Each experiment was performed with four biological replicates including four plants per trial. Representative results are presented and similar results were obtained in all experiments.

Zhang et al. 2013), while only *prhG* expression was substantially impaired in the *rsgA* mutant, indicating that the abnormal status of ribosome biosynthesis in *R. solanacearum* does not impair expression of all genes that regulate expression of the T3SS. In consideration of the fact that many GTPases, especially those that are highly conserved during evolution, could function in translation and some RsgA proteins, indeed, function as specific translation factors in many bacteria (Daigle et al. 2002). Expression of some unknown factors might be severely impaired with deletion of *rsgA* or *rbfA* that results in substantially impaired expression of the T3SS in *R. solanacearum*, and the impact of RsRsgA on the T3SS is mediated through the PrhN-PrhG-HrpB pathway but independent of the PrhA-HrpG-HrpB pathway. HrpG is presumed to respond to host signals or some mimic signals, while PrhG seems to respond to metabolic signals when the bacterium was grown in minimal medium (*hrp*-inducing medium) (Plener et al. 2010). Deletion of *rsgA* or *rbfA* could also possibly interfere with signal recognition and transduction that is mediated by HrpG or PrhG and then results in substantially impaired expression of *hrpB* and the T3SS.

R. solanacearum does not activate T3SS expression until it comes in contact with host signals or mimic signals, since assembly of T3SS and secretion of T3Es require a lot of energy (Aldon et al. 2000; Monteiro et al. 2012). *R. solanacearum rsgA* and *rbfA* mutants should stop all unessential activities in sucrose medium, including the T3SS, at least coordinating for energy conservation, since these two mutants failed to grow in sucrose medium. We recently reported that the T3SS possibly plays some novel function on survival of *R. solanacearum* at extreme starvation in this nutrient-limited sucrose medium. For instance, some auxotrophic mutants with aromatic amino acids failed to grow in sucrose medium but retained expression levels of the T3SS of a quarter of the wild-type strain (Zhang et al. 2019). Expression of T3SS in sucrose medium was diminished in *rsgA* and *rbfA* mutants, indicating that the T3SS is not involved in survival of *R. solanacearum* at this extreme condition caused by ribosome deficiency. *R. solanacearum rsgA* and *rbfA* mutants failed to grow in sucrose medium, while they proliferated slowly in the intracellular spaces of tobacco leaves, and hence, these two mutants could express the T3SS to some extent, which is about one-third to one-half the level of the wild-type strain in tobacco leaves (Fig. 3B). Some T3Es, such as RipAA, RipB, and RipPI are responsible for HR elicitation of some *R. solanacearum* strains in nonhost plants (Nakano and Mukaiharu 2019; Peeters et al. 2013). Note that expression of *ripX* (*popA*) in *rsgA* and *rbfA* mutants was not diminished in tobacco leaves but remained at about half the level of the wild-type strain in tobacco leaves, especially from 6 to 18 hpi (Fig. 3B). Expression of a subset of T3Es in sucrose medium was substantially impaired in *rsgA* and *rbfA* mutants, while expression of *ripAA*, *ripB*, and *ripPI* in tobacco leaves should be retained to some extent in *rsgA* and *rbfA* mutants. These weakly expressed T3Es seem to be enough to trigger plant immunity, which might explain why *rsgA* and *rbfA* mutants exhibited similar HR elicitation as the parent strain in tobacco leaves. It was consistent with our previous reports that several mutants with substantially reduced expression of T3Es exhibited identical HR elicitation as GMI1000 in tobacco leaves (Zhang et al. 2018).

Taken together, our results demonstrated that RsRsgA and RsRbfA play important roles on ribosomal biosynthesis, cell proliferation in medium and in host plants, and migration throughout host plants. Moreover, RsRsgA positively affects the T3SS expression both in vitro and in planta through the PrhN-PrhG-HrpB pathway. This is the first report linking RsgA proteins with the T3SS in pathogenic bacteria, providing novel

insight into understanding various biological functions of RsgA proteins and complex regulatory pathway on the T3SS in *R. solanacearum*.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

R. solanacearum strains used in this study were derivatives of two representative *R. solanacearum* strains, GMI1000 and OE1-1 (Table 1). GMI1000 is virulent in tomato plants but elicits HR in tobacco leaves (Salanoubat et al. 2002), while OE1-1 is virulent in both tomato and tobacco plants (Kanda et al. 2003). *R. solanacearum* strains were grown at 28°C in broth medium (nutrient-rich medium) or *hrp*-inducing medium (sucrose medium, nutrient-limited medium) (Yoshimochi et al. 2009). *E. coli* strains DH12S and S17-1 were grown at 37°C in Luria Bertani medium and were used for plasmid construction and conjugational transfer, respectively.

Mutants generation with in-frame deletion of *rsgA* (*rsc0940*) and *rbfA* (*rsc1290*).

In the present study, mutants with in-frame deletion of target genes were generated with pK18mobsacB-based homolog recombination as previously described (Zhang et al. 2015). In brief, two DNA fragments flanking target gene were conjugated with joint PCR and were cloned into pK18mobsacB to obtain pK18d0940 and pK18d1290, respectively. After validating the sequence, these plasmids were transferred into *R. solanacearum* strains by conjugation with *E. coli* S17-1. After confirmation by colony PCR with respective primer pairs (Supplementary Table S1), desired mutants were generated and are listed in Table 1.

Complementation analyses.

In the present study, complementation analysis was performed with the Tn7T-based site-specific chromosomal integration system as previously described, i.e., the target exogenous gene was integrated into a chromosome of *R. solanacearum* strains at 25-bp downstream of *glmS* (a single *attTn7* site) (Choi et al. 2005; Zhang et al. 2015). In brief, an approximately 600-bp DNA fragment containing the coding sequence of the target gene and its upstream region, empirically harboring its native promoter, was PCR-amplified and was cloned into pUC18-mini-Tn7T-Gm to obtain the plasmid for complementation. After validating the sequence, complementary *rsgA* or *rbfA* was integrated into a chromosome of the

Table 1. Bacterial strains used in this study

Strain	Relative characteristics	References
OE1-1	Wild-type, race 1, biovar 3	Kanda et al. 2003
RK5046	OE1-1, <i>hrpB-lacZYA</i>	Yoshimochi et al. 2009
RK5050	OE1-1, <i>popA-lacZYA</i>	Yoshimochi et al. 2009
RK5120	OE1-1, <i>hrpG-lacZYA</i>	Yoshimochi et al. 2009
RK5212	OE1-1, <i>prhG-lacZYA</i>	Zhang et al. 2013
RQ5613	OE1-1, <i>prhN-lacZYA</i>	Zhang et al. 2015
RQ5872	RK5050, $\Delta rsgA$	This study
RQ6101	RK5050, $\Delta rbfA$	This study
RQ5999	RK5046, $\Delta rsgA$	This study
RQ6100	RK5120, $\Delta rsgA$	This study
RQ6079	RK5212, $\Delta rsgA$	This study
RQ6118	RK5613, $\Delta rsgA$	This study
RQC0341	OE1-1, <i>rsgA-lacZYA</i>	This study
RQC0224	RQ5872, <i>rsgA</i> complementation	This study
RQC0225	RQ6101, <i>rbfA</i> complementation	This study
GMI1000	Wild-type, race 1, biovar 4	Salanoubat et al. 2002
GF0058	GMI1000, $\Delta rsgA$	This study
GF0060	GMI1000, $\Delta rbfA$	This study

R. solanacearum mutants at a single *attTn7* site (25-bp downstream of *glmS*) to obtain desired complementary strains, which were confirmed by colony PCR with the primers of *glmsdown-Tn7R* (Zhang et al. 2015).

Generation of *rsgA-lacZYA* reporter fusion.

In the present study, a reporter strain with *rsgA-lacZYA* fusion was generated with the site-specific chromosomal integration system as described above. In general, promoter-less *lacZYA* was fused to the *rsgA* promoter at 54 bp after the start codon, in which 6 bp of nucleotide acids were replaced into *KpnI* by PCR and were used for *lacZYA* insertion. DNA fragment containing a putative promoter (upstream region of about 600 bp) and *KpnI* site was first cloned into pUC18-mini-Tn7T-Gm and promoter-less *lacZYA* was then inserted to generate pUCrsgA-lacZYA. After validating the sequence, *rsgA-lacZYA* was integrated into chromosome of OE1-1 to generate the reporter strain RQC0341, which was confirmed by colony PCR with primer pair 0940A1B and lacZR1 (Table 1).

Bacterial growth assay.

In the present study, bacterial growth assays were performed both in medium and in planta. Growth in medium was assessed with OD₆₀₀, and that in planta (tobacco leaves and petioles) was quantified by dilution plating, as previously described (Zhang et al. 2013). Cell densities in tobacco leaves and petioles were expressed in log₁₀ CFU cm⁻² and log₁₀ CFU g⁻¹, respectively. Growth in tobacco petioles was determined to monitor migration of *rsgA* and *rbfA* mutants from infiltrated tobacco leaves into petioles. In brief, approximately 50 µl of a bacterial suspension at OD₆₀₀ of 0.1 was infiltrated into tobacco leaves, and petioles of the infiltrated leaves were periodically harvested for quantification of cell densities. Each assay was performed with four biological replicates including six plants per trial. Mean values of all experiments were averaged with standard deviation (SD), and statistical significance was assessed using a post hoc Dunnett test following analysis of variance (ANOVA).

β-Galactosidase assay.

In this study, a β-galactosidase assay was performed, to evaluate expression levels of *lacZYA*-fused genes, as previously described (Zhang et al. 2013). The in-vitro enzyme assay was expressed in Miller units (Miller 1992) and that in planta was normalized with luminescence divided by cell number (relative luminescence units per cell) (Zhang et al. 2013). Each experiment was performed with at least four biological replicates including at least four replications per trial. Mean values of all experiments were averaged with SD and statistical significance was assessed using a post hoc Dunnett test following ANOVA.

Virulence assay and HR test.

The virulence assay was performed in wilt-susceptible tobacco plants (*Nicotiana tabacum* Bright Yellow) as previously described (Zhang et al. 2013). In brief, tobacco plants were challenged with two kinds of inoculation methods, i.e., soil soaking, which mimics natural invasion through roots, and leaf infiltration, which enables bacteria to directly invade intracellular spaces of leaves. Each assay was performed with four biological replicates including 12 plants per trial. Wilt symptoms of plants were daily inspected, using a disease index from 1 to 4, and mean values of all experiments were averaged. Statistical significance was assessed using a post hoc Dunnett test following ANOVA.

The HR test was performed in tobacco leaves with leaf infiltration. In brief, approximately 50 µl of bacterial suspension at an OD₆₀₀ of 0.1 was infiltrated into tobacco leaves and

development of necrotic lesions in tobacco leaves was recorded periodically. Each test was performed with four biological replicates including four plants per trial, and representative results are presented.

RNA extraction, electrophoresis, and qRT-PCR analysis.

In the present study, RNA isolation was carried out with the TRIzol reagent according to manufacturer instructions (Life). In general, *R. solanacearum* strains were grown to an OD₆₀₀ of about 1.0 in broth medium and 0.1 in sucrose medium (nutrient-limited medium) and were harvested for RNA isolation. For RNA electrophoresis, a total of 1 µg of RNAs isolated from cells cultivated in broth medium were separated with 5% PAGE and were visualized with GoldView II nuclear staining dyes (Solarbio). The qRT-PCR was carried out on total RNAs isolated from cells cultivated in sucrose medium. The cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (Takara) according to manufacturer instructions (contaminated genomic DNA is removed by the gDNA Eraser in this kit), and the One Step SYBR PrimeScript PLUS RT-PCR kit (Takara) was used for qRT-PCR reactions with the Applied Biosystems 7500 Real-Time PCR system. Normalized gene expression was calculated by the $\Delta\Delta$ quantification cycle method, and each assay was performed with three biological replicates including four replications per trial. Mean values of all experiments were averaged with SD, and statistical significance between the wild-type strain and mutants was assessed using a post hoc Dunnett test following ANOVA.

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