SHORT COMMUNICATION

Involvement of a FAD-Linked Oxidase RSc0454 for Expression of the Type III Secretion System and Pathogenicity in *Ralstonia solanacearum*

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Ralstonia solanacearum RSc0454 is predicted as a FAD-linked oxidase based on protein homologies, while it contains distinct domains of lactate dehydrogenase and succinate dehydrogenase. A previous study demonstrated that RSc0454 exhibits lactate dehydrogenase activity using pyruvate and NADH as substrates, and is essential for pathogenicity of *R. solanacearum*. Here, we genetically characterized involvement of RSc0454 on bacterial growth and expression of genes for the type III secretion system (T3SS, a pathogenicity determinant) in *R. solanacearum*. The RSc0454 mutant grew normally in rich medium but grew faintly in host plants, and failed to grow in minimal medium. Supplementary succinate but not lactate could substantially restore some phenotypes of RSc0454 mutants, including faint growth in host plants, diminished growth in the minimal medium, and lost pathogenicity toward host plants. Expression of T3SS genes is directly controlled by a master regulator, HrpB, and *hrpB* expression is positively regulated by HrpG and PrhG in parallel ways. Deletion of RSc0454 substantially reduced expression levels of *hrpB* and T3SS both in vitro and in planta. Moreover, RSc0454 is revealed to be required for the T3SS expression via HrpG and PrhG, although through some novel pathway, and impaired expression of these genes was not due to growth deficiency of RSc0454 mutants. RSc0454 is suggested to be important for redox balance inside cells, and

supplementary NADH partially restored diminished growth of the RSc0454 mutant in the minimal medium only in the presence of succinate at some moderate concentrations, indicating that the unbalanced redox in the RSc0454 mutant might be responsible for its diminished growth in the minimal medium. Taken together, these results provide novel insights into the understanding of various biological functions of this FAD-linked oxidase RSc0454 and involvement of the redox balance on expression of the T3SS in *R. solanacearum*.

Keywords: bacterial pathogenesis, bacteria-plant symbiosis, genetics and gene regulation, type-3 secretion

Ralstonia solanacearum is a causal agent of bacterial wilt diseases on a wide variety of plant species and currently is regarded as one of the most destructive plant-pathogenic bacteria worldwide due to its ability to cause severe yield losses in many economically important crops (Genin 2010; Mansfield et al. 2012). *R. solanacearum* is a soilborne, Gram-negative, vascular bacterium that generally invades host xylem vessels through root wounds or natural openings (Vasse et al. 1995). Once bacteria have invaded xylem vessels, they proliferate extensively and produce a huge amount of exopolysaccharide (EPS) slime, which blocks sap flows in xylem vessels, resulting in quick stunting and wilting of host plants and is believed to be one of the main virulence factors (Denny 1995; Roberts et al. 1988). In addition to the EPS, a syringe-like type III secretion system (T3SS) is another pathogenicity determinant of *R. solanacearum* that plays an essential role in the infection process of *R. solanacearum*, especially at early stages, to invade host cells (Angot et al. 2006; Cunnac et al. 2004; Jones and Dangl 2006). The T3SS is conserved in many pathogenic bacteria of animals and plants, and bacteria use this syringe-like system to inject virulence factors (so-called type III effectors [T3Es]) into host cytosol to subvert host defense (Jones and Dangl 2006; Poueymiro and Genin 2009).

The *R. solanacearum* T3SS is encoded by 22 genes forming a hypersensitive response and pathogenicity (*hrp*) gene cluster (Arlat et al. 1992), and is globally regulated by a complex network (Genin and Denny 2012; Hikichi et al. 2017). In general, an AraC family of transcriptional regulator HrpB directly controls

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expression of genes for the T3SS and T3Es (Coll and Valls 2013; Mukaihara et al. 2010). Genes of *hrpB*, T3SS, and T3Es are not expressed in a nutrient-rich medium but are activated in a nutrient-limited medium (the minimal medium) that might mimic plant apoplastic fluids, or conditions getting in contact with host signals (Marenda et al. 1998; Yoshimochi et al. 2009b; Zhang et al. 2013). Two close paralogs, HrpG and PrhG, OmpR/PhoB family of response regulators of the two-component systems, can respond to host signals, or some mimic signals by phosphorylation and positively regulate *hrpB* expression in parallel ways (Plener et al. 2010; Zhang et al. 2013). Although HrpG is well expressed in nutrient-rich medium, its regulation on expression of *hrpB* and the T3SS genes is only activated by host signals or mimic signals (i.e., in the minimal medium). Host signals are presumed to be recognized by PrhA, an outer membrane protein, and transferred to HrpG through the PrhA-PrhR/I-PrhJ signaling cascade or some novel signaling cascades (Aldon et al. 2000; Marenda et al. 1998; Valls et al. 2006; Yoshimochi et al. 2009b). Moreover, a transcriptional regulator of the LysR-family, PhcA, negatively regulates expression of the T3SS, which represses *prhI/R* expression at high cell density by binding to *prhI/R* promoter and, in turn, shuts down expression of *prhJ*, *hrpG*, *hrpB*, and the T3SS (Genin et al. 2005; Yoshimochi et al. 2009a). The *prhG* expression is independent from the PrhA signaling cascade and positively regulated by PhcA, PrhN, and some other regulators (Zhang et al. 2013, 2015).

To further elucidate complex regulation of the *R. solanacearum* T3SS, we previously screened a subset of T3SS-regulating candidates with transposon mutagenesis (Zhang et al. 2013). Among them is RSc0454 (1,345 amino acids), which is predicted to be a FAD-linked oxidase based on protein homologies, while it contains distinct domains of N-terminal DUF3683, lactate dehydrogenase (LDH), and succinate dehydrogenase (SDH). A pioneering study demonstrated that *R. solanacearum* RSc0454 shows catalytic activities of both LDH and SDH using pyruvate and NADH as substrates, and is essential for pathogenicity of *R. solanacearum* toward tomato plants (Hu et al. 2019). It remains unclear why the RSc0454 mutant fails to cause wilt disease on host plants. The T3SS expression was substantially reduced in RSc0454 transposon mutants. It is the first report linking the T3SS with FAD-linked oxidase. Thus, we focused on RSc0454 to investigate its functional contribution to the T3SS expression and pathogenicity in *R. solanacearum*.

The FAD-linked oxidases are known to catalyze vital redox reactions, especially for basic metabolism in many microbes (Gao et al. 2015; Heikal et al. 2014). We first evaluated growth profiles of RSc0454 mutants. Deletion of RSc0454 does not alter growth of *R. solanacearum* in a rich medium (Hu et al. 2019). Here, we shifted to evaluate its growth in a minimal medium (Hoagland medium supplemented with 2% of sucrose), which is used to induce the T3SS expression in some studies. RSc0454 of GMI1000 and OE1-1 are identical in amino acids (data not shown). We generated an RSc0454 mutant (RQ5699) from OE1-1 with methods for the in-frame deletion of target genes (Zhang et al. 2015). The *R. solanacearum* wild-type strain and its derivative mutants used in this study are listed in Table 1. RQ5699 exhibited a growth profile similar to that of parent strain (RK5050), a *popA-lacZYA* reporter strain of OE1-1 in the nutrient-rich medium (broth medium), while it failed to grow in the minimal medium (Fig. 1A). The genetic complementation assay was performed with the Tn7-based site specific chromosomal integration system (Choi et al. 2005; Zhang et al. 2021), and the complementation strain (RQC307) fully restored its diminished growth in the minimal medium (Fig. 1A and B), confirming that RSc0454 is essential for *R. solanacearum* to grow in the minimal medium.

Supplementary succinate and lactate restore some phenotypes of the GMI1000 RSc0454 mutant (i.e., cell aggregation at the

bottom of the rich medium with stationary culture) (Hu et al. 2019). We assessed whether supplementary succinate or lactate could restore diminished growth of RQ5699 in the minimal medium. Growth of RQ5699 was substantially restored only with 40 mM succinate, whereas growth of the wild-type strain was significantly repressed in this medium (Fig. 1C). The addition of 100 mM succinate resulted in no growth of either the wild-type strain or the RSc0454 mutant. Note that supplementary lactate at concentrations of 5 mM or higher completely diminished growth of both the wild-type strain and RSc0454 mutant in the minimal medium (data not shown), while that at 1 mM did not affect growth of the wild-type strain in the minimal medium. Therefore, lactate was added into the minimal medium at concentrations of less than 1 mM for the growth assay. Supplementary lactate at any concentration did not restore cell growth of the RSc0454 mutant in the minimal medium (Fig. 1D; Supplementary Fig. S1). All of these results indicate that high concentration of succinate is generally toxic to *R. solanacearum* cells and RSc0454 could be used to supply succinate from fumarate by reverse reaction of SDH.

RSc0454 is essential for pathogenicity of *R. solanacearum* GMI1000 on tomato plants (Hu et al. 2019). GMI1000 wilts tomato plants but elicits hypersensitive response in tobacco leaves, while OE1-1 is virulent in tomato and tobacco plants (Kanda et al. 2003; Poueymiro et al. 2009; Salanoubat et al. 2002). We assessed whether RSc0454 is required for OE1-1 to wilt tomato and tobacco plants. Tomato plants were challenged with the inoculation method of petiole inoculation, which drops 2 μ l of bacterial suspension at a density of 10^8 CFU ml^{-1} onto the fresh-cut surface of tomato petioles, and tobacco plants were challenged with the inoculation method of leaf infiltration, which infiltrates approximately 50 μ l of bacterial suspension at a density of 10^8 CFU ml^{-1} into tobacco leaves with a needleless syringe (Zhang et al. 2015). RQ5699 failed to cause disease on tomato and tobacco plants, and complementation strain RQC307 fully restored its diminished pathogenicity to that of the parent strain on tomato and tobacco plants (Fig. 2A and B), confirming that the essential role of RSc0454 on pathogenicity is conserved in different *R. solanacearum* strains and different host plants. Supplementary succinate significantly restored growth of the RSc0454 mutant in the minimal medium (Fig. 1C). We assessed whether supplementary succinate could restore diminished pathogenicity of RQ5699 on host plants. Approximately 2- to 3-week-old tomato and tobacco plants were irrigated with one-quarter-diluted Hoagland medium with supplementary succinate at a final concentration of 40 mM for 7 days and subjected to the virulence assay. Supplementary succinate did not alter virulence of the wild-type strain on tobacco and tomato plants, whereas it somehow enabled RQ5699 to wilt tobacco and tomato plants (Fig. 2A and B), indicating that diminished pathogenicity of the RSc0454 mutant is possibly due to insufficient succinate in host plants.

The in planta proliferation is one of the most important pathogenicity determinants of *R. solanacearum* (Genin and Denny 2012). We assessed whether RSc0454 deletion impaired *R. solanacearum* proliferation in host plants. For the growth assay in tobacco leaves, a bacterial suspension at a density of 10^4 CFU ml^{-1} was infiltrated into tobacco leaves and cell discs were punched for quantification of cell densities every other day (Zhang et al. 2015). The wild-type strain RK5050 proliferated extensively in tobacco leaves that reached the maximum of 10^9 CFU cm^{-2} at 6 days postinoculation (dpi). At this point, RK5050-infiltrated leave became withered and dried. Proliferation of RQ5699 in tobacco leaves that reached the maximum of approximately 10^5 CFU cm^{-2} at 4 dpi was substantially impaired, and the leaves did not grow any more (Fig. 3A). For the growth assay in tomato stems, tomato plants were inoculated

by dropping 2 μ l of bacterial suspension at a density of 10^8 CFU ml^{-1} onto fresh-cut surfaces of petioles, and stem pieces were cut for quantification of cells densities from 3 dpi. RK5050 proliferated extensively in tomato stems that reached the maximum of approximately 10^{10} CFU g^{-1} at 5 to 6 dpi (Fig. 3B). At this point, RK5050-inoculated tomato plants became withered and dried. Proliferation of RQ5699 in tomato stems that reached the maximum of approximately 10^4 CFU g^{-1} at 3 to 4 dpi was significantly impaired, and the stems did not grow any more (Fig. 3B). Supplementary succinate significantly restored growth of the *RSc0454* mutant in the minimal medium. We further assessed whether supplementary succinate could restore proliferation of the *RSc0454* mutant in host plants. Tomato and tobacco plants were irrigated with one-quarter-diluted Hoagland medium supplemented with succinate at a final concentration of 40 mM and the in planta growth assay was performed. Proliferation of RQ5699 in succinate-irrigated tobacco leaves was significantly increased and reached the maximum of approximately 10^8 to 10^9 CFU cm^{-2} at 9 dpi (Fig. 3A). At this point, RQ5699-infiltrated tobacco leaves became withered and dried. In succinate-irrigated tomato stems, RQ5699 proliferated to the maximum of approximately 10^9 CFU g^{-1} at 6 to 9 dpi (Fig. 3B). All of these results indicate that *RSc0454* is important for the in planta proliferation of *R. solanacearum*, and impaired

in planta proliferation of the *RSc0454* mutant is possibly due to insufficient succinate in host plants.

The *RSc0454* mutant was originally screened as one of the T3SS-regulating candidates, in which *popA* expression was substantially reduced (Zhang et al. 2013). In our study, expression profiles of the T3SS genes were monitored by a *popA-lacZYA* fusion, which does not alter the infection process of OE1-1 on host plants (Zhang et al. 2013). The T3SS genes are not expressed in rich medium but are induced in the minimal medium (called *hrp*-inducing medium) (Yoshimochi et al. 2009b). We first assessed whether *RSc0454* is required for *popA* expression in *R. solanacearum*. Because *RSc0454* mutants failed to grow in the minimal medium, we added 40 mM succinate to support cell growth in this medium. The *popA* expression was much lower in the *RSc0454* mutant (RQ5699) and the complementation strain RQC307 fully restored *popA* expression to the level of the wild type (Fig. 4A). The T3SS expression can be greatly enhanced in planta or under conditions getting in contact with plant signals compared with that in the minimal medium (Valls et al. 2006; Yoshimochi et al. 2009b). We assessed whether *RSc0454* is required for *popA* expression in host plants. The in planta enzyme assay was performed in tobacco leaves, in which a bacterial suspension at 10^8 CFU ml^{-1} was infiltrated into tobacco leaves and leaf discs were punched for the in planta

Fig. 1. Involvement of *RSc0454* on growth of *Ralstonia solanacearum* in **A**, nutrient-rich broth medium; **B**, nutrient-limited minimal medium; and **C** and **D**, minimal medium supplemented with succinate and L-lactate at different concentrations. RK5050 (OE1-1, *popA-lacZYA*) = the wild-type strain, $\Delta Rsc0454$ = RQ5699 (RK5050 = $\Delta RSc0454$), and $\Delta RSc0454 + RSc0454$ = RQC307 (complementary *RSc0454* in RQ5699). For the growth assay in medium, a cell suspension was washed twice with distilled water, adjusted to an optical density at 600 nm (OD₆₀₀) of 1.0, inoculated into fresh broth medium and minimal medium (Hoagland medium supplemented with 2% of sucrose) with a proportion of 1%, and OD₆₀₀ was measured periodically. Mean values of all three biological replicates with three replicates per trial were averaged with standard deviation (error bars). Statistical significance between *RSc0454* mutants and RK5050 was assessed using a post hoc Dunnett test following analysis of variance. Significance level ** indicates $P < 0.01$.

enzyme assay at 6 to 24 h postinoculation (hpi) (Zhang et al. 2011). The *popA* expression in RQ5699 was significantly impaired in tobacco leaves, and complementary *RSc0454* fully restored its impaired *popA* expression to that of the wild-type strain in tobacco leaves (Fig. 4B). On the other hand, the *popA* expression in RQ5699 and RK5050 was not improved even when succinate was supplied for irrigation of tobacco plants

(Fig. 4B), confirming that *RSc0454* is important for the T3SS expression both in vitro and in planta, and impaired T3SS expression in the *RSc0454* mutant is not because of growth deficiency.

T3SS genes are directly controlled by HrpB and *hrpB* expression is positively regulated by HrpG and PrhG, which are regulated by PrhJ, PhcA, and PrhN, respectively. Involvement of

Fig. 2. Virulence assay of the *RSc0454* mutant on **A**, tomato plants with the petiole inoculation method and **B**, tobacco plants with the leaf infiltration inoculation method. RK5050 = the wild-type strain, $\Delta RSc0454$ = RQ5699 (RK5050 = $\Delta RSc0454$), $\Delta RSc0454 + RSc0454$ = RQC0307 (complementary *RSc0454* in RQ5699), and $\Delta RSc0454$ +succinate = succinate-irrigated plants were subjected to the virulence assay. For the petiole inoculation, 2 μ l of bacterial suspension at 10^8 CFU ml^{-1} was dropped onto the freshly cut surface of tomato petioles. For the leaf infiltration, approximately 50 μ l of bacterial suspension at 10^8 CFU ml^{-1} was infiltrated into tobacco leaves with a blunt-end syringe. For succinate supplementation, approximately 2- to 3-week-old plants were irrigated with one-quarter-diluted Hoagland medium containing succinate (40 mM) for 7 days and subjected to a virulence test. Wilt symptoms were inspected daily and scored on a disease index scale from 0 to 4, where 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 repeated with at least four biological replicates including 12 plants per trial. Mean values of all experiments were averaged with standard deviation (error bars).

Fig. 3. Involvement of *RSc0454* on growth of *Ralstonia solanacearum* in **A**, tobacco leaves (intercellular spaces) and **B**, tomato stems (xylem vessels). RK5050 (OE1-1, *popA-lacZYA*), $\Delta RSc0454$ = RQ5699 (RK5050 = $\Delta RSc0454$), and $\Delta RSc0454 + succinate$ = succinate-irrigated plants were subjected to the growth assay. For the growth assay in tobacco leaves, which was presented in log CFU per square centimeter, tobacco plants were inoculated with the leaf infiltration method and leaf disks (0.38 cm^2) were punched and subjected to quantification of cells number by dilution plating every other day. The RK5050-inoculated tobacco leaves were punched till 6 days postinoculation (dpi), when tobacco leaves became withered and dried, while the RQ5699-inoculated tobacco leaves were assessed till 12 dpi, when tobacco leaves remained healthy but became yellow. For the growth assay in tomato stems, which was presented in log CFU per gram, tomato plants were inoculated with the petiole inoculation method and stem species were cut, weighed, and subjected to quantification of cells number by dilution plating on a daily basis from 3 dpi. The RK5050-inoculated tomato stems were harvested till 6 dpi, when tomato plants died, while the RQ5699-inoculated tomato stems were harvested till 12 dpi. For the succinate supplementation, approximately 2- to 3-week-old tomato and tobacco plants were irrigated with one-quarter-diluted Hoagland medium containing succinate for 7 days and subjected to the in planta growth assay. Each assay was repeated with at least four biological replicates including six replicates per trial. Mean values from all experiments were averaged and presented with standard deviation (error bars). Statistical significance between RQ5699 and RK5050, or that between treatment with supplementary succinate or without, was assessed using a post hoc Dunnett test following analysis of variance. Significance level ** indicates $P < 0.01$.

RSc0454 in expression of these genes was assessed. Expression of *hrpB* and the T3SS was not expressed in the nutrient-rich medium but was activated in the minimal medium. Expressions of *hrpB*, *hrpG*, and *prhG* were reduced in *RSc0454* mutants in the minimal medium and addition of succinate for cell growth did not alter expression of these genes in the minimal medium (Fig. 4C). Expression of *hrpG* and *prhG* in *RSc0454* mutants were very low even in the nutrient-rich medium, in which *RSc0454* mutants grew normally (Supplementary Fig. S2). The complementary *RSc0454* fully restored impaired expression of *hrpG* and *prhG* to those of parent strains in nutrient-rich medium (data not shown). On the other hand, three genes (*prhJ*, *phcA*, and *prhN*) which regulate the T3SS expression indirectly expressed normally in *RSc0454* mutants both in the minimal and rich media (Fig. 4C; Supplementary Fig. S2). Impact of RSc0454 on *hrpB* expression was also assessed in tobacco leaves. Deletion of *RSc0454* significantly reduced *hrpB* expression in tobacco leaves and supplementary succinate for irrigation

of tobacco plants did not alter *hrpB* expression in tobacco leaves (Fig. 4D). All of these results suggested that expressions of both *hrpG* and *prhG* were reduced with *RSc0454* deletion regardless of growth conditions and it, in turn, impaired expression of the T3SS in *RSc0454* mutants, and impaired expression of T3SS genes was not due to growth deficiency of *RSc0454* mutants.

RSc0454 is suggested to be involved in redox balance inside cells that is in line with different living conditions of *R. solanacearum* (Hu et al. 2019). We assessed whether supplementary NADH or NAD⁺ could restore unbalanced redox in the *RSc0454* mutant. Supplementary NADH at concentrations of 0.1 to 5 mM or NAD⁺ at 0.1 mM did not alter growth of the wild-type strain, whereas supplementary NAD⁺ at 5 mM diminished growth of the wild-type strain in minimal medium (Supplementary Fig. S3A), indicating that excess NAD⁺ might disturb redox status inside cells. Supplementary succinate at 40 mM substantially restored diminished growth of the *RSc0454* mutant in the minimal medium (Fig. 1C), indicating that unbalanced redox in

Fig. 4. Involvement of RSc0454 on expression of the type III secretion system (T3SS) and T3SS-regulated genes *popA-lacZYA* in **A**, hypersensitive response and pathogenicity (*hrp*)-inducing medium and **B**, tobacco leaves; **C**, expression of T3SS-regulated genes in *hrp*-inducing medium; and **D**, expression of *hrpB-lacZYA* in tobacco leaves. WT = wild type. RK5050 (OE1-1, *popA-lacZYA*); $\Delta RSc0454$ = deleted *RSc0454* from reporter strains of *hrpB-lacZYA*, *hrpG-lacZYA*, *prhG-lacZYA*, *prhJ-lacZYA*, *phcA-lacZYA*, and *prhN-lacZYA*; $\Delta RSc0454 + 0454$ (complementary *RSc0454* in RQ5699); and *hrp*-inducing medium + succinate = supplemented succinate into the minimal medium (*hrp*-inducing medium) at a final concentration of 40 mM that were subjected to cell growth. For the in vitro enzyme assay, strains were grown in medium to an optical density at 600 nm (OD₆₀₀) of approximately 0.1 and subjected to the enzyme assay, enzymatic activities of which were presented in Miller Units. For the in planta enzyme assay, tobacco leaves were infiltrated with bacterial suspension at OD₆₀₀ of 0.1 and leaf disks were punched for the enzyme assay with the Galacto-Light Plus kit, enzymatic activities of which were presented with luminescence normalized by cells number (relative luminescence units [RLU] per cell). For the in planta enzyme assay with supplementary succinate, succinate-irrigated tobacco leaves were used for cell infiltration. The in planta enzyme assay was carried out from 6 days postinoculation to 24 h postinoculation (hpi), when infiltrated leaves became withered and dried. Cells densities were quantified by dilution plating and luminescence was determined using the GloMax20 luminometer (Promega Corp.). 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. Significance level ** indicates $P < 0.01$.

the *RSc0454* mutant was somehow restored to some extent with supplementary succinate at this concentration. It is striking that supplementary NADH at 5 mM began to diminish growth of the *RSc0454* mutant in the presence of succinate at 40 mM in the minimal medium (Supplementary Fig. S3C). Supplementary NADH or NAD⁺ at 0.1 mM did not restore growth of the *RSc0454* mutant in the minimal medium, whereas they began to partially restore growth of *RSc0454* mutants only in the presence of succinate at 10 mM in the minimal medium at 36 hpi (Supplementary Fig. S3B). Note that supplementary succinate at 10 mM did not restore growth of the *RSc0454* mutant in the minimal medium. This was consistent with the fact that NADH and NAD⁺ work as redox cofactors in reactions. Supplementary succinate and NADH and NAD⁺ together restored redox balance inside *RSc0454* mutant cells. The unbalanced redox in the *RSc0454* mutant might be responsible for its lack of growth in the minimal medium.

In summary, we provided multiple lines of evidence to demonstrate that *RSc0454* is important for *R. solanacearum* to grow under nutrient-limited conditions and required for expression of the T3SS genes both in vitro and in planta. The *RSc0454* mutant is easily aggregated at the bottom of nutrient-rich medium with stationary culture, which is known to be insufficient in oxygen, indicating that the oxidase encoded by *RSc0454* is possibly important for a redox balance inside cells (Hu et al. 2019). Succinate and fumarate are important components in the citric acid cycle and play important roles in the respiratory electron transfer chain in many microbes (Huynen et al. 1999; Park et al. 1999). Succinate and fumarate might restore the redox balance to some extent and, hence, can restore some phenotypes of the *RSc0454* mutant (i.e., cell aggregation) (Hu et al. 2019). Supplementary succinate at a higher concentration significantly impaired growth of the wild-type strain, especially at early stages of growth. It possibly perturbs the redox balance inside cells and, hence, is toxic for *R. solanacearum*. Supplementary succinate at moderate concentrations (i.e., 40 mM) substantially restores phenotypes of the *RSc0454* mutant, including the faint growth in host plants, diminished growth in the minimal medium, and lost pathogenicity toward host plants. It might restore the redox balance inside

RSc0454 mutant cells to some extent. Excess NADH or NAD⁺ might disturb redox status inside cells, and supplementary NADH or NAD⁺ at higher concentration (i.e., 5 mM) diminished growth of *R. solanacearum*. Supplementary succinate at 40 mM might somehow restore redox balance inside the *RSc0454* mutant because it significantly restores diminished growth of the *RSc0454* mutant in the minimal medium. It was consistent with the fact that supplementary NAD⁺ at 5 mM began to diminish growth of the *RSc0454* mutant in the minimal medium in the presence of succinate at 40 mM. Supplementary succinate at 10 mM failed to restore growth of the *RSc0454* mutant in the minimal medium, whereas supplementary NADH or NAD⁺ at 0.1 mM began to restore growth of the *RSc0454* mutant in the minimal medium in the presence of succinate at 10 mM, indicating that supplementary NADH or NAD⁺ works as a redox cofactor to restore the redox balance inside *RSc0454* mutant cells in the presence of succinate at 10 mM. The unbalanced redox in the *RSc0454* mutant might be responsible for its lack of growth in the minimal medium.

Nutrients in xylem sap are believed to be relatively poorer than that in nutrient-rich medium (i.e., broth medium) (Evert and Eichhorn 2006; Lowe-Power et al. 2018a; Pegg 1985). *R. solanacearum* could utilize some compounds in xylem sap as carbon sources (i.e., sugars) to fulfill proliferation in xylem vessels (Lowe-Power et al. 2018a and b). Plants synthesize abundant secondary metabolites and bioactive compounds in xylem vessels, and nutrients in plants are relatively more abundant than in the sucrose medium (minimal medium). It is not striking that the *RSc0454* mutant fails to grow in the minimal medium but can grow faintly in host plants. The condition in xylem vessels is known to be low in oxygen, and *R. solanacearum* uses nitrate, which is rich in xylem sap, as an alternate electron acceptor for cell respiration to fulfill proliferation in the hypoxic xylem environment (Dalsing et al. 2015; Jacobs et al. 2012). In addition to *RSc0454*, *R. solanacearum* has authentic membrane-bound SDHs (i.e., Rsp1195 and Rsp1196) encoding a putative transmembrane SDH anchor subunit and cytochrome B-556 subunit, respectively, and Rsc0028 and Rsp1611 encoding putative succinate-semialdehyde dehydrogenases. Therefore, the *RSc0454*

Table 1. Bacterial strains used in this study

Strain	Relative characteristics ^a	References
<i>Ralstonia solanacearum</i>		
OE1-1	Wild-type, race 1, biovar 3	Kanda et al. 2003
RK5043	OE1-1, <i>phcA-lacZYA</i>	Yoshimochi et al. 2009b
RK5046	OE1-1, <i>hrpB-lacZYA</i>	Yoshimochi et al. 2009b
RK5050	OE1-1, <i>popA-lacZYA</i>	Yoshimochi et al. 2009b
RK5120	OE1-1, <i>hrpG-lacZYA</i>	Yoshimochi et al. 2009b
RK5124	OE1-1, <i>prhJ-lacZYA</i>	Yoshimochi et al. 2009b
RK5212	OE1-1, <i>prhG-lacZYA</i>	Zhang et al. 2013
RK5619	OE1-1, <i>prhN-lacZYA</i>	Zhang et al. 2015
RQ5699	RK5050, $\Delta rsc0454$	This study
RQ5932	RK5046, $\Delta rsc0454$	This study
RQ5987	RK5120, $\Delta rsc0454$	This study
RQ5990	RK5212, $\Delta rsc0454$	This study
RQ6081	RK5124, $\Delta rsc0454$	This study
RQ6081	RK5619, $\Delta rsc0454$	This study
RQ6115	RK5043, $\Delta rsc0454$	This study
RQC0307	Complementary <i>rsc0454</i> in RQ5699	This study
<i>Escherichia coli</i>		
DH12S	Host strain for standard DNA cloning manipulation	Invitrogen
S17-1	Host strain for plasmid mobilization	Simon et al. 1983
Plasmid		
pK18mobsacB	<i>sacB</i> , <i>lacZa</i> , Km ^r ; cloning vector for allelic exchange	Simon et al. 1983
pUC18-miniTn7T-Gm	Gm ^r on mini-Tn7T; mobilizable	Choi et al. 2005
pTNS2	Ap ^r ; helper plasmid encoding the site-specific TnsABCD Tn7 transposition pathway	Choi et al. 2005

^a Km^r, Gm^r, and Ap^r and indicate resistant to kanamycin, gentamicin, and ampicillin, respectively.

mutant is not a simple auxotroph. It was consistent with previous reports that, in addition to catalytic activities for redox reaction directly, LDH and SDH are essential for cell proliferation, membrane generation, and virulence in many microbes (Abdelbaset et al. 2017; Kim et al. 2017; Mercado-Lubo et al. 2008; Pecsai et al. 2014).

RSc0454 was previously screened as one of the T3SS-affecting candidates (Zhang et al. 2013). Here, we experimentally confirmed that RSc0454 is required for expression of the T3SS both in vitro and in planta. The T3SS expression of the RSc0454 mutant in the minimal medium was substantially decreased even in the presence of supplementary succinate at 40 mM that partially restored diminished growth of the RSc0454 mutant in the minimal medium, confirming that impaired expression of the T3SS in the RSc0454 mutant was not due to growth deficiency in the minimal medium. Moreover, supplementary succinate did not restore expression of the T3SS in the RSc0454 mutant inside host plants, confirming that involvement of RSc0454 in T3SS expression is possibly due to the unbalanced redox reaction inside cells but not growth deficiency under nutrient-limited conditions. The *hrpB* expression was significantly impaired in the RSc0454 mutant. It was consistent with the master role of HrpB that directly controls expression of the entire T3SS and T3Es genes (Mukaihara et al. 2004; Valls et al. 2006). Two close paralogues (HrpG and PrhG) positively regulate *hrpB* expression in parallel ways (Plener et al. 2010; Zhang et al. 2013). Deletion of RSc0454 substantially impaired expression of both *hrpG* and *prhG*, whereas deletion of RSc0454 did not alter expression of *prhJ*, *phcA*, and *prhN*, which positively regulate expression of *hrpG* or *prhG*, respectively, indicating that the RSc0454-based redox balance is possibly important for expression of *hrpG* and *prhG* through some novel pathway. It is not a special case and we previously reported that a plant-derived compound, umbelliferone, impairs the T3SS via pathway of HrpG and PrhG to HrpB but is independent of known pathways (Yang et al. 2017). A novel regulator (PrhO) positively regulates expression of *hrpG* and *prhG* to HrpB through some novel pathway (Zhang et al. 2018), supporting our speculation that some novel pathways should be integrated for expression of *hrpG* and *prhG* in *R. solanacearum*.

Taken together, these results provide novel insight into understanding the biological functions of this FAD-linked oxidase and requirement of the RSc0454-based redox balance for expression of the T3SS in *R. solanacearum*.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Ralstonia solanacearum database:
<https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi>

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