

The *In Planta* Transcriptome of *Ralstonia solanacearum*: Conserved Physiological and Virulence Strategies during Bacterial Wilt of Tomato

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ABSTRACT Plant xylem fluid is considered a nutrient-poor environment, but the bacterial wilt pathogen *Ralstonia solanacearum* is well adapted to it, growing to 10^8 to 10^9 CFU/g tomato stem. To better understand how *R. solanacearum* succeeds in this habitat, we analyzed the transcriptomes of two phylogenetically distinct *R. solanacearum* strains that both wilt tomato, strains UW551 (phylotype II) and GMI1000 (phylotype I). We profiled bacterial gene expression at $\sim 6 \times 10^8$ CFU/ml in culture or in plant xylem during early tomato bacterial wilt pathogenesis. Despite phylogenetic differences, these two strains expressed their 3,477 common orthologous genes in generally similar patterns, with about 12% of their transcriptomes significantly altered *in planta* versus in rich medium. Several primary metabolic pathways were highly expressed during pathogenesis. These pathways included sucrose uptake and catabolism, and components of these pathways were encoded by genes in the *scrABY* cluster. A UW551 *scrA* mutant was significantly reduced in virulence on resistant and susceptible tomato as well as on potato and the epidemiologically important weed host *Solanum dulcamara*. Functional *scrA* contributed to pathogen competitive fitness during colonization of tomato xylem, which contained $\sim 300 \mu\text{M}$ sucrose. *scrA* expression was induced by sucrose, but to a much greater degree by growth *in planta*. Unexpectedly, 45% of the genes directly regulated by HrpB, the transcriptional activator of the type 3 secretion system (T3SS), were upregulated *in planta* at high cell densities. This result modifies a regulatory model based on bacterial behavior in culture, where this key virulence factor is repressed at high cell densities. The active transcription of these genes in wilting plants suggests that T3SS has a biological role throughout the disease cycle.

IMPORTANCE *Ralstonia solanacearum* is a widespread plant pathogen that causes bacterial wilt disease. It inflicts serious crop losses on tropical farmers, with major economic and human consequences. It is also a model for the many destructive microbes that colonize the water-conducting plant xylem tissue, which is low in nutrients and oxygen. We extracted bacteria from infected tomato plants and globally identified the biological functions that *R. solanacearum* expresses during plant pathogenesis. This revealed the unexpected presence of sucrose in tomato xylem fluid and the pathogen's dependence on host sucrose for virulence on tomato, potato, and the common weed bittersweet nightshade. Further, *R. solanacearum* was highly responsive to the plant environment, expressing several metabolic and virulence functions quite differently in the plant than in pure culture. These results reinforce the utility of studying pathogens in interaction with hosts and suggest that selecting for reduced sucrose levels could generate wilt-resistant crops.

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We know very little about the conditions and opportunities confronting xylem-dwelling pathogens such as *Ralstonia solanacearum*, the causal agent of bacterial wilt disease of tomato. This soilborne bacterial pathogen typically enters hosts through root wounds and colonizes the water-transporting xylem tissue, through which it spreads up into the plant stem. *R. solanacearum* infection reduces water transport, which eventually leads to plant wilting and death. Although xylem is considered a nutrient-limiting, low-oxygen environment (1), *R. solanacearum* is well adapted to it, growing to cell den-

sities of 10^8 to 10^9 CFU/g stem while still remaining limited to xylem (2).

R. solanacearum forms a species complex, a heterogeneous group of xylem-colonizing strains with various host ranges, aggressiveness levels, and ecological traits. Genomic analyses have subclassified strains into four phylotypes that correlate with geographic origin (3). Two well-studied *R. solanacearum* strains, GMI1000, a broad-host-range tropical isolate, and UW551, a temperate strain with a narrower host range, both cause bacterial wilt of tomato (4-6). Strain GMI1000 belongs to phylotype I sequevar

18 and likely originated in Asia, where similar strains cause major losses to tomato growers. Strain UW551 belongs to phyloptype II sequevar 1 (historically and for regulatory purposes known as race 3 biovar 2), a group that originated in the Andes and causes brown rot, a destructive disease of potato in cool tropical highlands. *R. solanacearum* race 3 biovar 2 is a high-concern quarantine pest in Europe and North America and is a U.S. Select Agent pathogen (7). The two strains are not closely related, with a whole-genome average nucleotide identity (ANI) around 91%, below the 95% cutoff separating bacterial species (8). Nonetheless, at tropical temperatures, strains UW551 and GMI1000 are equally aggressive on wilt-susceptible tomato plants (5).

Several quantitative virulence factors contribute to bacterial wilt disease development. These virulence factors include production of extracellular polysaccharide (EPS), a consortium of plant cell wall-degrading enzymes, twitching and swimming motility, taxis, and several dozen effectors secreted by a type 3 secretion system (T3SS) (2). *R. solanacearum* virulence factors are regulated by a complex interlocking cascade involving quorum sensing and undefined plant signals (9, 10). Two major virulence factors, EPS and T3SS, are controlled by a global regulator, PhcA, via the quorum-sensing molecule 3-OH-palmitic acid methyl ester (3-OH-PAME) (11, 12). At high cell densities ($>5 \times 10^8$ CFU/ml) *in planta* and in culture, accumulated 3-OH-PAME triggers PhcA-mediated activation of EPS biosynthesis (13, 14). When the bacterium grows in culture, PhcA indirectly represses expression of *hrpB*, which encodes the transcriptional activator of the T3SS (12, 15, 16). It has been assumed from this *in vitro* result that the T3SS is active only in the early stages of host plant infection and is not needed at higher pathogen cell densities, when wilt symptoms develop (9, 12, 16). However, this model had not been validated *in planta*.

To better understand behavior of *R. solanacearum* strains and to identify the conserved traits required to wilt plants, we used microarrays to measure expression of the *R. solanacearum* UW551 and GMI1000 genomes at comparable cell densities *in planta* and in culture. This defined the *R. solanacearum in planta* transcriptome, the set of orthologous pathogen genes expressed during bacterial wilt of tomato. Bacteria growing in tomato stems strongly expressed many T3SS genes, even at high cell densities. Genes encoding proteins involved in metabolism of sucrose, which was not previously known to be present in tomato xylem, were expressed by both strains *in planta*. Functional analysis of this conserved metabolic trait revealed that *R. solanacearum* requires sucrose for full virulence and to succeed in tomato xylem.

RESULTS

Orthologous genes had similar expression patterns in two bacterial wilt pathogens. We captured the transcriptional profiles of *R. solanacearum* strains UW551 and GMI1000 under two conditions, as summarized in Fig. 1. RNA was harvested from bacteria growing at comparable cell densities ($\sim 6 \times 10^8$ CFU per ml or per gram of stem) in rich medium and from tomato plants showing early wilt symptoms. Gene transcription was measured using microarray chips designed for each strain, with four biological replicates per strain under each condition. The resulting data had strong cross-chip correlation ($R^2 > 0.95$) and good interarray reproducibility. Microarray expression results for selected genes were confirmed with quantitative PCR (qPCR) (see below).

Comparing expression of the genes shared between the two

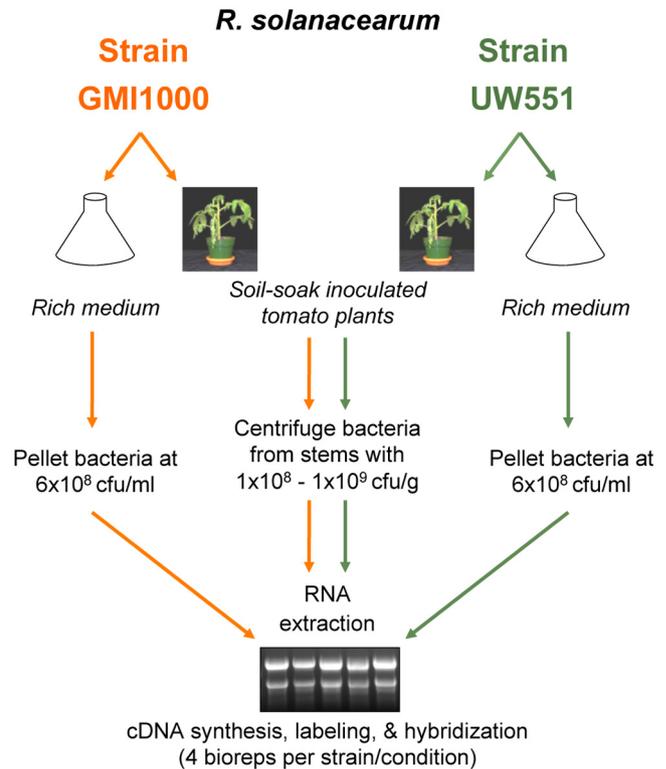


FIG 1 Microarray experimental design and flowchart. We compared transcriptional profiles of phylogenetically and ecologically distinct *R. solanacearum* strains GMI1000 (orange) and UW551 (green) during growth in tomato stems and in rich medium (CPG) at comparable cell densities. Bacteria grown in culture were treated with a transcriptional stop solution and pelleted. Bacteria were harvested from infected tomato xylem by centrifugation from cut stems into a transcriptional stop solution. Bacterial RNA was isolated from four biological replicates per strain per condition (16 replicates total).

strains across the two conditions revealed that these orthologs were regulated similarly *in planta* compared to in rich medium in each strain ($P < 0.001$, hypergeometric distribution). About 70% (3,477) of the coding sequences in the two strains are orthologous, defined as pairwise best BLAST hits with $>70\%$ amino acid identity over $>70\%$ of the protein. About 11.6% (402 genes) of the orthologous genes were differentially expressed in the same direction (up or down) in both strains (see Table S1A and B in the supplemental material). A gene was considered differentially expressed in the two conditions if its expression level was >2 -fold different with an adjusted P value of <0.05 . Only 0.1% (4 genes) of the GMI1000-UW551 orthologs were differentially expressed in opposite directions in the two strains; these are all hypothetical genes and may be artifacts. COG (clusters of orthologous groups) analysis revealed that for both strains, genes of unknown function were over-represented among those upregulated *in planta* ($P < 0.001$, hypergeometric distribution) (see Fig. S1 in the supplemental material).

Eighty-three percent of the GMI1000-UW551 orthologs were expressed during tomato pathogenesis. Direct fold change comparisons of the two strains were not possible, because the technical differences between the GMI1000 and UW551 arrays prohibited normalization across chips. We therefore determined each gene's absolute expression (AE) value, which is the \log_2 of the normalized mean probe intensity of a coding sequence. This allowed us to define the *R. solanacearum in planta* transcriptome, a set of 2,898

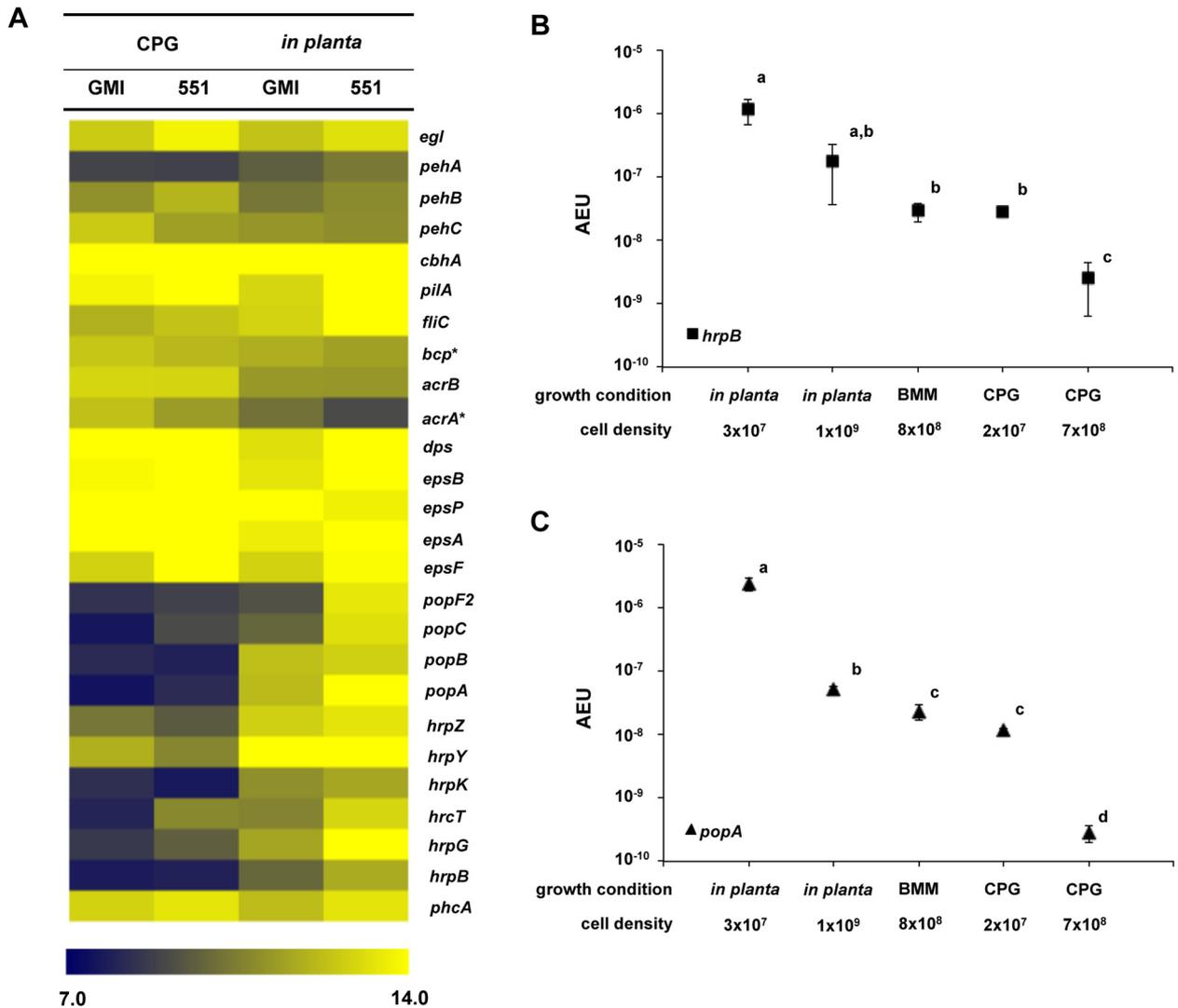


FIG 2 Gene expression heat map of experimentally determined bacterial wilt virulence and fitness factors. (A) Absolute \log_2 gene expression levels of known virulence genes in *R. solanacearum* UW551 (551) and GMI1000 (GMI) was artificially clustered with The Institute for Genomic Research (TIGR) multiple experiment viewer (MeV). Asterisks (*) designate genes previously identified in an IVET screen of *R. solanacearum* genes induced in tomato (28). The color indicates low (7.0 [blue]) to high (14.0 [yellow]) absolute \log_2 expression levels. (B and C) Absolute expression per cell (AEU) of *hrpB* (B) and *popA* (C) was quantified using qPCR at low and high cell densities both *in planta* and in culture. For gene expression in culture, *R. solanacearum* strain GMI1000 was grown in rich medium (CPG) to cell densities of 2×10^7 CFU/ml and 7×10^8 CFU/ml. The strain was also grown in minimal medium to a final cell density of 8×10^8 CFU/ml (BMM). For *in planta* gene expression, susceptible Bonny Best tomato plants were inoculated with strain GMI1000 via soil soak. RNA was isolated from plant stems showing early wilt symptoms and containing 1×10^9 CFU/g stem (*in planta* high) and from asymptomatic plants containing 3×10^7 CFU/g stem (*in planta* low). Purified RNA was reverse transcribed into cDNA and measured by quantitative real-time PCR. The transcript cycle threshold (C_T) values of *hrpB* and *popA* were plotted on their respective cDNA standard curves, and the amount of transcript per cell was used to determine the arbitrary absolute expression unit (AEU). Each point represents the mean absolute expression value of three biological replicates; the error bars indicate the standard error ($P < 0.05$ by ANOVA).

orthologous genes that were expressed during pathogenesis in both strains (see Table S1 in the supplemental material). Expressed genes were conservatively defined as those with AE values of >8.0 , based on a genome-wide survey of expression levels of core metabolic genes. The *in planta* transcriptome includes known virulence factors, conserved metabolic pathways, and many hypothetical genes (Table S1).

Type 3 secretion system and effector genes were expressed during early wilt symptom development. As expected, many genes encoding known virulence traits such as stress tolerance (*bcp*, *acrA*, *acrB*, and *dps*), motility (*pilA* and *fliC*), cell wall-

degrading enzymes (*pehA*, *pehB*, *pehC*, *egl*, and *cbhA*), and EPS biosynthesis (*epsABCDEF*) were expressed during host infection (Fig. 2A; see also Table S1 in the supplemental material), consistent with previous functional analyses (2, 17, 18). Unexpectedly, many genes encoding components of the *R. solanacearum* T3SS were strongly expressed at high cell densities *in planta*, which was inconsistent with the current model of virulence regulation in this pathogen (9, 12, 16). T3SS genes upregulated in wilting plants included those encoding components of the secretion system structure (*hrpK* and *hrpY*), type 3-dependent effectors (*popA*, *popB*, and *popC*), and regulators (*hrpB* and *hrpG*) (Fig. 2A).

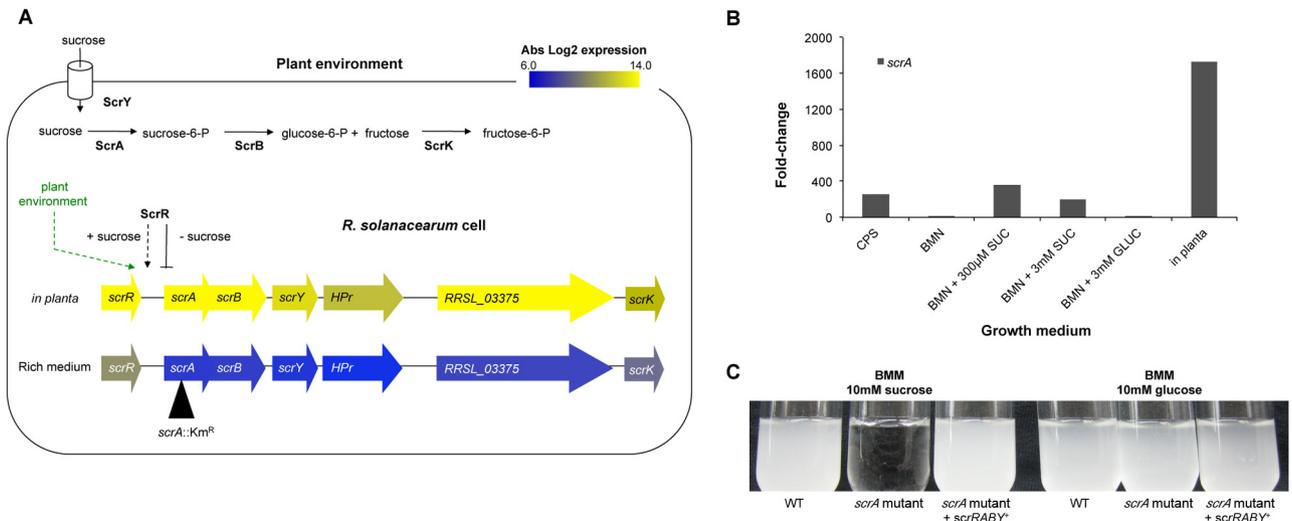


FIG 3 The *scrRABY* sucrose metabolic cluster is induced *in planta* and encodes a sucrose-specific PTS. (A) *R. solanacearum* converts sucrose to glucose-6-phosphate (glucose-6-P) and fructose-6-P with the components of a sucrose-specific PTS encoded by the *scr* cluster, which was highly expressed *in planta*. The absolute log₂ gene expression from bacteria growing *in planta* or in rich medium is shown in a heat map layered on the *scr* gene cluster. The location of the insertional mutation in *scrA* (*scrA::Km^r* or *scrA*) is indicated by a black triangle. (B) Relative fold change of *scrA* expression in *R. solanacearum* UW551 cells growing in culture media (CPS, broth containing Casamino Acids, peptone, and 55 mM sucrose; BMM, Boucher's minimal medium; SUC, sucrose; GLUC, glucose) or in Bonny Best tomato stems. Gene expression was measured at a cell density of 1×10^9 CFU/ml broth or 1×10^9 CFU/g tomato stem and determined on a per-cell basis. The black bars represent the mean *scrA* expression fold change for each growth condition compared to the level in CPG medium, with three biological replicates for culture and four biological replicates for *in planta* expression. *R. solanacearum* UW551 *scrA* expression fold changes *in planta* were significantly different from expression levels in all culture media tested ($P < 0.03$ by Mann-Whitney test). (C) A UW551 *scrA* mutant grew as well as the wild type (WT) on minimal medium (BMM) plus glucose but failed to grow on sucrose as the sole carbon source. Growth on sucrose was restored by *scrRABY* in *trans* on plasmid pUF10.

To validate these microarray findings and to assess the effects of cell density on gene expression *in planta*, we quantified expression of the major T3SS regulator gene *hrpB* and the T3-secreted effector gene *popA* during growth of strain GMI1000 in tomato plants and in culture media. We used qPCR to measure transcripts from bacteria grown to different cell densities in rich medium (rich medium containing Casamino Acids, peptone, and glucose [CPG]), minimal medium (Boucher's minimal medium [BMM]), and tomato stems. The qPCR data were wholly consistent with the array results. In rich culture, both *hrpB* and *popA* were expressed at low cell density (3×10^7 CFU/ml), but at high cell density (7×10^8 CFU/ml), *hrpB* expression dropped 71-fold and *popA* expression dropped 189-fold to nearly undetectable levels (Fig. 2B and C). These results are consistent with the previous observation that PhcA represses T3SS at cell densities of $>5 \times 10^8$ CFU/ml (12). Also consistent with previous reports, *hrpB* and *popA* were expressed at higher levels in minimal medium than in rich medium at high cell densities (Fig. 2B and C) (12). However, during tomato wilt pathogenesis, these two genes were even more highly expressed at both cell densities, although expression was lower at high cell density. Nonetheless, the maximal expression of the two genes *in planta* was more than 70-fold higher than the maximal expression observed in culture (Fig. 2B and C).

Many genes in the HrpB regulon were also significantly upregulated *in planta* (see Table S2 in the supplemental material). In strain GMI1000, 92 of 206 predicted HrpB-regulated genes were differentially expressed *in planta* (Table S2) (19, 20). Of 109 genes known to be directly positively regulated by HrpB, 60 were significantly upregulated *in planta*. These included genes encoding

parts of the secretion apparatus and 21 effectors (Table S2). Thirteen of the 40 genes positively but indirectly controlled by HrpB were also upregulated *in planta*; these encode hypothetical proteins as well as the major virulence regulator HrpG and HrpB itself (21) (Table S2). Strain UW551 had similar expression patterns (Table S1). Together, these results demonstrate that the T3SS and a substantial portion of the HrpB regulon are actively expressed at mid-stage bacterial wilt disease.

***R. solanacearum* uses a sucrose-specific PTS for sucrose uptake and catabolism.** A COG functional analysis revealed that a significant portion of the bacterial *in planta* transcriptome is dedicated to primary physiology, notably uptake and metabolism of carbohydrates (4.2%), amino acids (8.9%), lipids (3.6%), and ions (3.9%) (see Fig. S1 in the supplemental material). Both strains highly expressed *scrRABY* and *scrK*, which encode components of a putative sucrose-specific phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS), suggesting that *R. solanacearum* depends on sucrose as a carbon source during xylem colonization (Fig. 3A). To test this hypothesis, we generated a UW551 mutant lacking a functional *scrA* gene, which encodes a predicted sucrose-6-phosphotransferase (Fig. 3). The UW551 *scrA* mutant multiplied as well as the wild-type (WT) strain did on glucose, but it could not grow on sucrose as the sole carbon source (Fig. 3C). Complementation UW551 *scrA* mutant with *scrRABY* in *trans* restored growth on sucrose. Deletion of an adjacent hypothetical T3 effector gene, *RRSL_03375*, did not alter UW551's ability to metabolize sucrose (data not shown). These results confirm that this cluster plays its predicted role in sucrose metabolism (Fig. 3C).

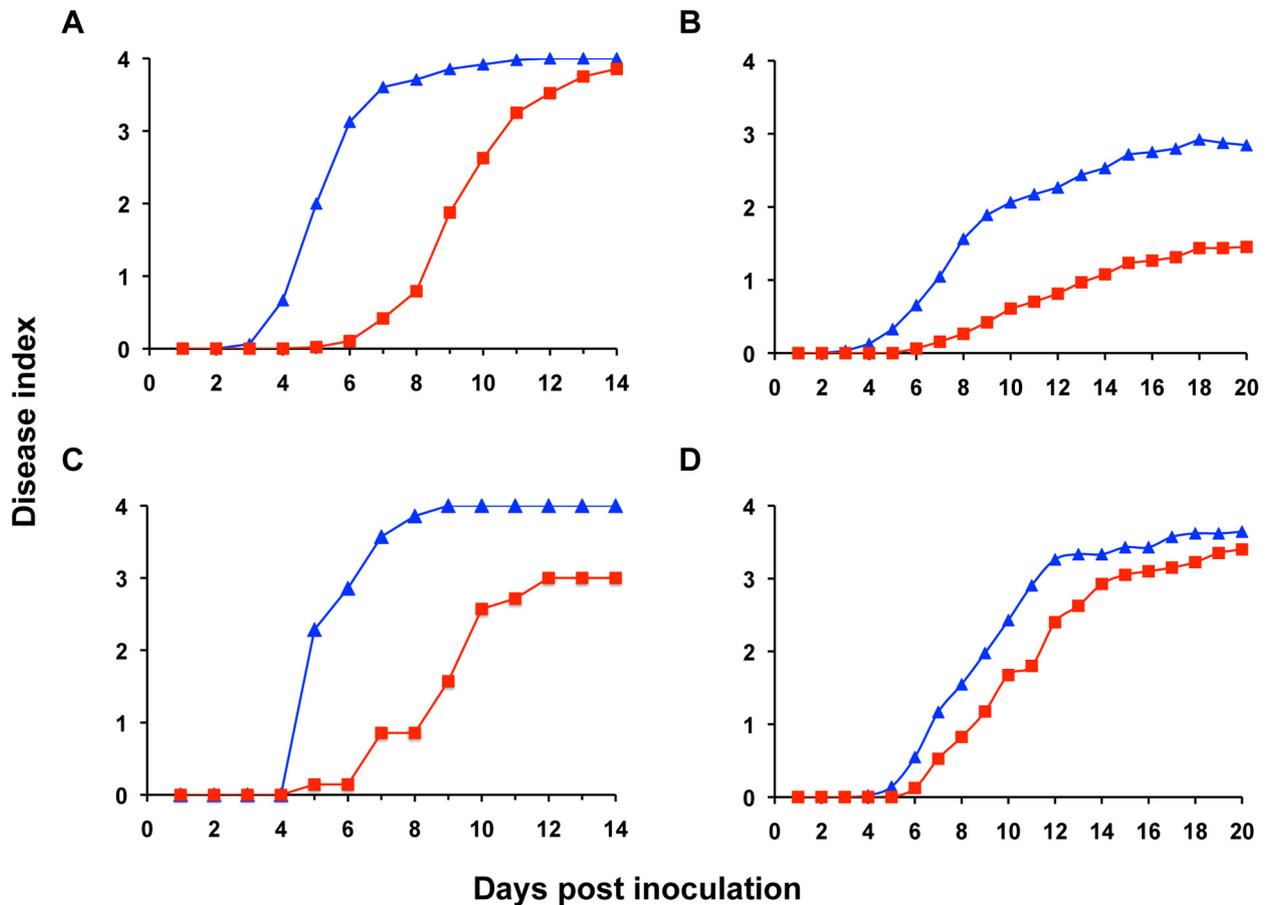


FIG 4 Differential virulence of the *R. solanacearum* UW551 *scrA* mutant on susceptible and resistant hosts. (A to D) The wild-type *R. solanacearum* UW551 (WT) (blue triangles) and *scrA* mutant (red squares) were inoculated by soil soaking onto the unwounded roots of susceptible tomato cultivar Bonny Best (A), quantitatively resistant tomato cultivar Hawaii7996 (B), potato cultivar Russet Norkotah (C), and solanaceous nightshade (D). Plant symptoms were rated daily on a disease index scale from 0 to 4 (0 for healthy or no leaf area wilted, 1 for 1 to 25% of the leaf area wilted, 2 for 26 to 50% of the leaf area wilted, 3 for 51 to 75% of the leaf area wilted, and 4 for 76 to 100% of the leaf area wilted). Each point indicates the mean disease index for three independent inoculations (A, B, and D) or one experiment (C); the *scrA* mutant was significantly less virulent than the wild type on each host ($P < 0.001$ by repeated measures ANOVA).

Expression of *scrA* is induced by sucrose and especially by growth *in planta*. In other bacteria, sucrose induces expression of sucrose-specific PTS gene clusters via the sucrose-binding regulator ScrR (22-24). We used qPCR to measure *scrA* expression by *R. solanacearum* cells growing in different levels of sucrose and in tomato plants. Sucrose induced *scrA* expression 254-fold in broth containing Casamino Acids and peptone plus 55 mM sucrose relative to the same broth plus 55 mM glucose (Fig. 3B). The addition of 300 μ M sucrose to minimal medium increased *scrA* expression 361-fold. Increasing the sucrose concentration to 3 mM did not significantly change *scrA* expression (Fig. 3B). *scrA* expression was not affected by the presence of glucose in minimal medium or by the abundant amino acids and other complex organic nutrients present in CPG (Fig. 3B), demonstrating that this locus is specifically induced by sucrose, independent of the cell's nutrient status. However, *scrA* expression *in planta* was significantly greater than in all culture media tested ($P < 0.03$ by Mann-Whitney test). Growth *in planta* increased *scrA* expression 1,724-fold, indicating that in addition to sucrose itself, a factor or condition present in tomato xylem is a strong inducer of the *R. solanacearum* sucrose metabolic pathway (Fig. 3B).

***R. solanacearum* requires a sucrose-specific PTS for virulence on multiple hosts.** To determine whether this metabolic trait contributes to bacterial wilt disease development, we used a naturalistic soil soak inoculation to measure the virulence of UW551 *scrA* mutant on various plant hosts: wilt-susceptible tomato and potato plants; moderately resistant tomato line Hawaii7996 (H7996); and bittersweet nightshade (*Solanum dulcamara*), an epidemiologically important weed host of *R. solanacearum* (25). The UW551 *scrA* mutant was delayed in disease progress on susceptible tomato plants and on bittersweet nightshade, but it did ultimately wilt all plants to wild-type levels (Fig. 4A and D) ($P < 0.001$ by repeated measures analysis of variance [ANOVA]). However, on resistant H7996 tomato and potato plants, the *scrA* mutant strain was significantly reduced in virulence and never caused wild-type wilt levels ($P < 0.001$) (Fig. 4B and C). An unmarked *scrA* mutant had similarly reduced virulence on susceptible tomato plants, showing that the kanamycin resistance gene in UW551 *scrA* mutant did not by itself lower strain virulence (data not shown). These findings demonstrate that the ability to use sucrose makes a quantitative contribution to bacterial wilt virulence.

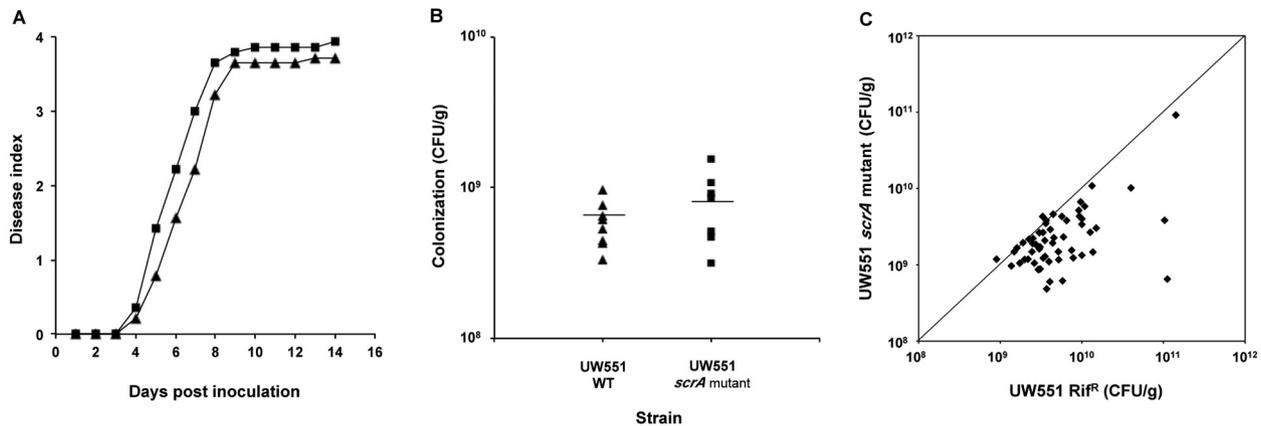


FIG 5 Sucrose metabolism provides *R. solanacearum* with a competitive advantage during xylem colonization. (A to C) Twenty-two-day-old wilt-resistant H7996 tomato plants were inoculated through a cut petiole with 4 μ l of wild-type *R. solanacearum* UW551 (WT) or UW551 *scrA::Km^r* (UW551 *scrA*) (A and B) or with a 1:1 mixture of wild-type *R. solanacearum* UW551 Rif^R and *scrA* mutant (~2,000 CFU total) (C). (A) Wilt disease progress of wild-type UW551 and *scrA* mutant strains on stem-inoculated tomato plants that were rated as described in the legend to Fig. 4. (B) Stem colonization ability of wild-type UW551 and *scrA* mutant cells stem inoculated separately on tomato plants. Ten plants displaying a disease index of 1 (corresponding to 5 days after inoculation) were sampled per treatment (UW551 WT or UW551 *scrA* mutant), and the bacterial population sizes in the stems were quantified by dilution plating. Each symbol represents one plant; short horizontal lines represent the mean bacterial colonization in tomato stems. (C) Competitive fitness of wild-type and *scrA* mutant strains. The tomato plants were sampled 5 days postinoculation, and *in planta* population sizes of wild-type and *scrA* mutant strains were quantified by dilution plating. Data represent two biological replicates containing 30 plants each. The WT strain significantly outcompeted the *scrA* mutant *in planta* ($P < 0.006$ by paired Student's *t* test).

Sucrose in xylem contributes to *R. solanacearum* success *in planta*. These results suggested that *R. solanacearum* encounters sucrose during pathogenesis. To test this hypothesis, we measured sucrose concentrations in xylem fluid from infected tomato plants containing $\sim 7 \times 10^8$ CFU/ml of either strain UW551 or UW551 *scrA* mutant, as well as in xylem fluid from water-inoculated control plants. Fluid from control plants contained 318 ± 35 μ M sucrose. Xylem fluid from plants infected by the wild-type bacterium contained slightly less sucrose, 268 ± 14 μ M, possibly because it was being consumed by the many bacteria growing in these plants. In contrast, xylem from plants infected with comparable populations of UW551 *scrA* mutant contained sucrose levels similar to those in uninfected plants, 312 ± 12 μ M, consistent with the fact that these bacteria cannot utilize sucrose. No glucose was detected in xylem fluid (detection limit < 2 nM), suggesting that it is not an available carbon source in this habitat. These results indicate that sucrose could be a significant nutrient for *R. solanacearum in planta*.

Carbohydrate-rich EPS is a major *R. solanacearum* virulence factor (2), so we tested the hypothesis that a *scrA* mutant strain had reduced virulence because it produced smaller amounts of EPS. However, strain UW551 and UW551 *scrA* mutant produced similar amounts of EPS as determined by an enzyme-linked immunosorbent assay (ELISA) (data not shown), suggesting that catabolism is the principal function of this cluster in bacterial wilt virulence.

When cells of *R. solanacearum* UW551 *scrA* mutant and UW551 were separately introduced directly into tomato vascular systems via a cut leaf petiole, they reached similar final population sizes ($P < 0.2$ by Student's *t* test) and caused comparable levels of disease ($P < 0.2$ by repeated measures ANOVA) (Fig. 5A and B), indicating that loss of sucrose metabolism did not reduce *R. solanacearum*'s fundamental competence to colonize the host stem. This raised the possibility that sucrose is an important nutrient for *R. solanacearum* only in the host rhizosphere. To specifically mea-

sure the contribution of xylem sucrose to bacterial success, we used an *in planta* competition assay to determine whether the UW551 *scrA* mutant was subtly reduced in fitness. Tomato plants were inoculated with a 1:1 mixture of wild-type UW551 and UW551 *scrA* mutant cells directly into the xylem through cut petioles, and the population size of each strain was quantified in each plant after wilting symptoms appeared. Under these conditions, wild-type UW551 consistently outcompeted the UW551 *scrA* mutant ($P < 0.006$ by paired Student's *t* test) (Fig. 5C), indicating that the ability to metabolize xylem sucrose provides *R. solanacearum* with a significant fitness advantage during bacterial wilt pathogenesis.

DISCUSSION

Technical barriers have delayed our understanding of how xylem-dwelling pathogenic bacteria regulate their virulence genes during host infection. Efforts to duplicate the host environment *in vitro* with "hrp-inducing" media, plant cell coculture or plant extracts (19, 21, 26) do not recreate the dynamic physical and biochemical conditions experienced by bacteria during infection and pathogenesis of a living host. Useful glimpses of this complex biological reality have come from *in vivo* expression technology (IVET) (27–29) and reporter gene experiments (30, 31), but whole-genome expression analysis offers a broader picture.

We profiled transcription of *R. solanacearum* cells at early bacterial wilt development, when the pathogen is largely confined to the xylem. Because RNA was extracted from bacteria centrifuged from xylem vessels, our transcriptomic data likely represent the planktonic subset of the bacterial population rather than bacteria tightly attached to vessel walls. However, we obtained similar numbers of CFU/g from ground stem segments and in pellets from centrifuged stem segments, suggesting that the centrifugal extraction was reasonably efficient (data not shown). In addition, the *in planta* transcription profile generated by our analysis is consistent with the pathogen's biology, since the transcriptome

includes essentially all genes encoding known bacterial wilt virulence factors. Finally, qPCR analyses of bacteria extracted by centrifugation and from ground whole-stem samples yielded similar gene expression trends. Together, these results indicate that bacteria harvested by the centrifugation method are biologically representative of the bacterial populations in the stem.

Microarray and qPCR analyses demonstrated that the xylem environment overrides the cell density-dependent repression of *hrpB* expression observed in culture. In culture media, the PhcA quorum-sensing system represses *hrpB* at high cell densities, and this finding generated the idea that the T3SS plays no role after the initial stages of plant infection (9, 12, 16). However, we found that the T3SS was strongly expressed during wilt disease, when pathogen cell densities are high. Similar results were observed using microscopy coupled with *in planta hrpB::reporter* assays (F. Monteiro and M. Valls, personal communication). Increasing cell density did reduce transcription of *popA* and *hrpB* *in planta*, but their mean absolute expression levels were nonetheless higher than in culture; the lowest *popA* expression level *in planta* was 5-fold greater than the highest expression levels in culture. Further, 55% of genes directly positively regulated by HrpB, the transcriptional activator of the T3SS genes, were upregulated *in planta* versus culture medium. Together, these results suggest that signals and/or conditions in the plant environment interact with and largely override the cell density-dependent repression observed in culture. This is consistent with the observation that coculture with plant cells induces *hrpB* expression, although induction levels cannot be directly compared because of methodological differences (26). We speculate that the T3SS may inject effectors into the living xylem parenchyma cells immediately adjacent to the dead xylem vessels. *R. solanacearum* could access these parenchyma cells through the pits in xylem tracheid walls. Additional studies are needed to determine whether these effectors suppress host defenses, increase nutrient availability, or otherwise improve success of *R. solanacearum* at mid-stage bacterial wilt.

Genes involved in sucrose catabolism also displayed significantly different expression profiles *in planta* than in culture, regardless of the presence of sucrose itself. Sucrose did induce *scrA* expression, probably via the LacI-type repressor, ScrR (32), but the regulatory cues that further augment *scrA* induction *in planta* remain to be identified. Similarly, studies are needed to define the plant signal(s) that induce *hrpB* and *popA* and likely many other genes in the *R. solanacearum* *in planta* transcriptome. More broadly, our results suggest that studies of plant pathogen gene expression in artificial media or biologically irrelevant host tissue (e.g., leaf tissue for xylem pathogens) should be validated under biologically relevant conditions in the host.

It has been proposed that the *R. solanacearum* core genome encodes the minimal set of traits needed for success as a bacterial wilt pathogen (33). These 2,820 genes are present in all *R. solanacearum* strains, as determined by genome sequencing and comparative genome hybridization studies (8, 33, 34). Sixty-five percent (2,250) of the 3,477 UW551-GMI1000 orthologs belong to the *R. solanacearum* core genome, and core genes were significantly overrepresented in the *in planta* transcriptome ($P < 0.001$, hypergeometric distribution).

During tomato pathogenesis, 2,898 orthologs were expressed (AE value of >8) by both strains; these include 2,020 core genes. The 597 orthologs that were highly expressed (AE value of >12.0) *in planta* in both strains included 455 core genes. The analyses

presented here focused on shared orthologs rather than strain-specific genes, which will be considered separately. However, orthologs in both strains were more often differentially expressed *in planta* than strain-specific genes ($P < 0.001$, hypergeometric distribution; data not shown). Thus, *R. solanacearum* orthologs were more likely to respond to host environmental signals than the strain-specific traits, as would be predicted by the *R. solanacearum* core genome hypothesis (8, 33). Although suggestive, gene expression level is not a singular measure of relevance. We note that a transcriptomic analysis cannot account for genes that are expressed at very low levels, and some weakly expressed genes are almost certain to be biologically important.

Cell proliferation is essential for bacterial disease development, but little is known about how vascular plant pathogens like *R. solanacearum* grow in the carbon-limited xylem environment. As expected, the *R. solanacearum* *in planta* transcriptome included genes encoding transporters of anions, tricarboxylic acid (TCA) cycle intermediates, and amino acids known to be present in tomato xylem fluid (Fig. 6; see also Fig. S1 in the supplemental material) (2). Unexpectedly, genes for sucrose uptake and catabolism were also expressed at high levels *in planta*, although sucrose was not thought to be a significant component of tomato xylem fluid. Organic and amino acids, relatively abundant in xylem fluid (1, 28, 35), were considered primary carbon sources for *R. solanacearum* during xylem infection, but the reduced competitive fitness of a *scrA* mutant in tomato stems demonstrates that the pathogen exploits host sucrose as a carbon source during growth in xylem. Sucrose metabolism may also be important early during infection in the plant rhizosphere where sucrose is abundant (36).

Diverse animal- and plant-associated bacteria use a sucrose-specific PTS to catabolize sucrose (22, 32, 37), but *R. solanacearum* is the only species in the betaproteobacteria with a sucrose-specific PTS (data not shown). Phylogenetic analysis revealed that *R. solanacearum* ScrA is most closely related to proteins in *Methylobacterium* sp. strain 4-45 (alphaproteobacteria) and Gram-positive *Firmicutes* (amino acid identity of 63% and 52 to 53%, respectively) (data not shown); the sucrose catabolic gene cluster may have been acquired by lateral gene transfer, which has played a major role in evolution of *R. solanacearum* (8). We hypothesize that an ancient transfer event endowed a progenitor of *R. solanacearum* with the fitness-increasing ability to exploit a scarce carbon resource in its plant niche, as has been speculated in another system (38). Our finding that the plant environment induces expression of this gene substantially more than the presence of sucrose suggests that additional adaptation at the regulatory level has optimized *R. solanacearum*'s metabolism to host conditions.

All sequenced *R. solanacearum* strains possess a sucrose-specific PTS, and this core metabolic trait provides the pathogen with a significant physiological advantage *in planta* on several hosts. Bacterial pathogens of *Arabidopsis*, rice, and bean manipulate host sugar transport to their benefit, suggesting that access to host sucrose is broadly important for successful pathogenesis (39, 40). We suspect that many other *R. solanacearum* core metabolic traits expressed in the *in planta* transcriptome also increase fitness during pathogenesis (Fig. 6). For example, the highly expressed cellulose-degrading enzymes endoglucanase (Egl) and cellobiohydrolase (CbhA) are known virulence factors (2); the specific mechanism by which they promote virulence may be nutritional, via release of sucrose from host cell walls. Further studies focused

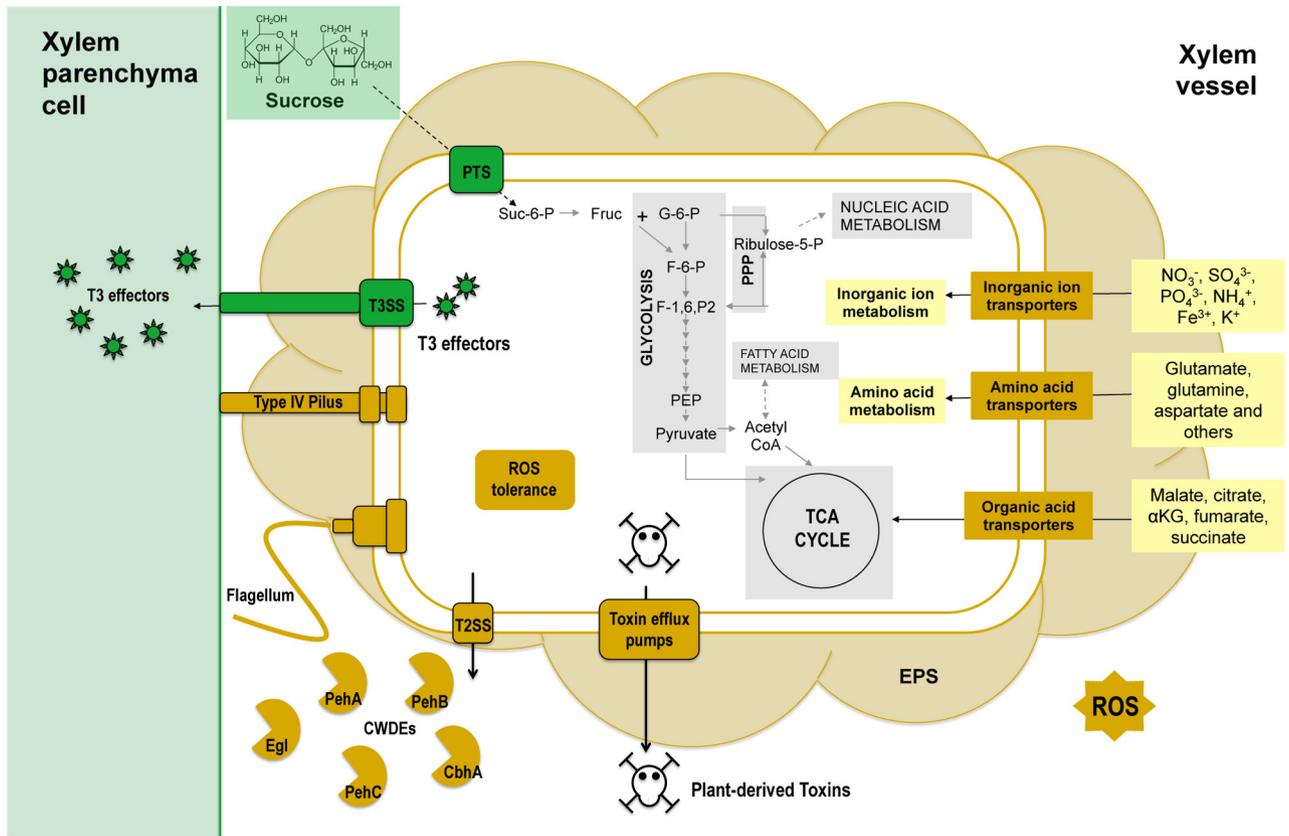


FIG 6 Select biochemical pathways of the *R. solanacearum* core genome represented in the *R. solanacearum in planta* transcriptome. Bacterial processes previously not known to be active during tomato xylem colonization are shown in green. Abbreviations: PTS, phosphoenolpyruvate-carbohydrate phosphotransferase system; Suc-6-P, sucrose-6-phosphate; Fru, fructose; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P2, fructose-1,6-bisphosphate; PEP, phosphoenolpyruvate; CoA, coenzyme A; TCA, tricarboxylic acid; α KG, α -ketoglutarate; Ribulose-5-P, ribulose-5-phosphate; PPP, pentose phosphate pathway; EPS, exopolysaccharide; ROS, reactive oxygen species; T2SS, type 2 secretion system; CWDEs, cell wall-degrading enzymes; T3, type 3; T3SS, type 3 secretion system.

on pathogen primary metabolism may provide additional insights into specific growth requirements for success in the xylem and thus, successful pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table S3 in the supplemental material. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. *R. solanacearum* cells were grown at 28°C on morpholineethanesulfonic acid (MES)-buffered Boucher's minimal medium (BMM) (pH 7.0), BMM with 40 mM nitrate (similar to plant xylem levels) substituted for ammonium (BMN) (pH 7.0), rich medium containing Casamino Acids, peptone, and glucose (CPG), or on tetrazolium chloride (TZC) plate (41). To determine effects of sucrose on gene expression, CPG was modified to replace the 55 mM glucose in CPG with 55 mM sucrose (CPS). When necessary, media were supplemented with gentamicin (Gm) (15 μ g/ml), kanamycin (Km) (25 μ g/ml), or tetracycline (Tc) (15 μ g/ml).

Recombinant DNA techniques and mutagenesis. Total genomic and plasmid DNA were isolated by standard protocols. *E. coli* and *R. solanacearum* cells were transformed as previously described (42). Primer sequences are given in Table S4 in the supplemental material. To construct an *R. solanacearum* UW551 *scrA* mutant (*scrA::Km^r*), the regions flanking *scrA* were amplified with Phusion high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland), and a kanamycin resistance (Km^r) cassette amplified from pSTBlue-1 (Novagen, Madison, WI) was inserted between the two fragments via splice by overlap extension (SOE) PCR (43). The

resulting SOE construct was gel purified, phosphorylated with T4 polynucleotide kinase (Promega, Madison, WI), and ligated into the EcoRV site of cloning vector pSUP202 (Cm^r Tc^r Ap^r), which cannot replicate in *R. solanacearum*, creating pSUP202-*scrA::Km^r* (44). This plasmid was transformed into wild-type (WT) *R. solanacearum* strain UW551 via electroporation. Candidate double-recombined transformants were screened for Km^r and Tc^s to confirm proper allelic exchange and *scrA* disruption. Notably, double recombinations were 98% more frequent than single recombination in strain UW551 (Km^r Tc^r) with pSUP202::*scrA::Km^r*.

RNA extraction for microarrays. To measure gene expression of bacteria growing in culture, RNA was extracted from 30-ml cultures of *R. solanacearum* strain GMI1000 or UW551 grown in CPG to log phase ($A_{600} = 0.6$ to 0.7 ; 6×10^8 to 7×10^8 CFU/ml). To minimize RNA degradation, each sample was immediately mixed with 3.75 ml of an ice-cold transcriptional stop solution (5% [vol/vol] water-saturated phenol in ethanol). Samples were centrifuged at $20,800 \times g$ at 4°C. The supernatant was removed, and the pellets were frozen in liquid nitrogen and stored at -80°C . Each experiment was repeated four times for strains UW551 and GMI1000, with four biological replicates per strain.

RNA was extracted using the modified hot-phenol method (44), followed by four DNase treatments. Briefly, precipitated RNA was resuspended in 400 μ l DNase solution (all reagents from Ambion, Foster City, CA) containing 0.5 μ l RNase inhibitor solution, 40 μ l of $10\times$ DNase I buffer, 15 μ l DNase I, and 344.5 μ l nuclease-free water and incubated at 37°C for 1 h. DNase was inactivated and removed by extracting first with phenol, then with phenol-chloroform, and finally twice with chloroform.

RNA was precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of cold ethanol. After the final DNase treatment, RNA pellets were resuspended in 13 μ l water and stored at -80°C . RNA concentration and purity was determined via microspectrophotometry (NanoDrop; Thermo Fisher Scientific, Wilmington, DE). The quality of RNA samples was assessed with the Agilent bioanalyzer 2100 nanochip system (Agilent Technologies, Santa Clara, CA). The absence of genomic DNA was verified by PCR with RS-universal primers, 759/760-specific or R3bv2-specific primers (see Table S4 in the supplemental material) (45, 46).

To measure gene expression of bacteria growing *in planta*, unwounded 25-day-old tomato plants (wilt-susceptible cv. Bonny Best) were inoculated by pouring a suspension of *R. solanacearum* strain UW551 or strain GMI1000 into the soil to a final density of 1×10^8 CFU/g soil. Bacteria were extracted from plants showing the first signs of wilting (disease index of 1 [$<25\%$ of the leaf area wilted]) 5 to 10 days postinoculation. To enrich for bacterial RNA, bacteria were centrifuged directly from cut stem segments. Individual tomato midstems (7 cm spanning the petiole) were centrifuged at 4°C and $18,500 \times g$ for 6 min in 15-ml conical tubes containing 3 ml of 30% transcriptional stop solution in water. The resulting bacterial pellets were frozen immediately in liquid nitrogen for later extraction. Before centrifugation, a 0.1-g transverse slice from the middle of each stem segment was ground and dilution plated to determine the bacterial cell density *in planta* for each sample, and RNA was extracted and pooled only from the stems containing $\sim 6 \times 10^8$ CFU/g. Each experiment was repeated four times for strains UW551 and GMI1000, for four biological replicates per strain. Each bioreplicate consisted of 10 to 15 bacterial pellets pooled from plants colonized with 1×10^8 to 1×10^9 CFU/g stem tissue. RNA was extracted from pellets and analyzed as described above, but with only three DNase treatments.

Array design. DNA microarrays for *R. solanacearum* strains GMI1000 and UW551 were designed using the genome sequences available from Integrated Microbial Genomes (IMG [<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>]). They were custom designed at Roche Nimblegen with a target melting temperature of 78°C , and a target probe length of 40 to 70 nucleotides (nt). Each array consisted of a total of about 72,000 probes, including controls, and each 4-plex chip contained four identical arrays.

The GMI1000 gene chip consisted of 60,608 gene-specific probes representing 5,061 out of 5,206 open reading frames (ORFs), plus 9,549 probes covering the 2,213 unique intergenic regions at 50-bp intervals. The UW551 gene chip consisted of 51,664 gene-specific probes to represent 4,315 out of 4,421 ORFs and 20,036 probes covering 2,641 unique intergenic regions at 50-bp intervals. Each ORF was represented by 2 to 6 gene-specific probes, duplicated in two blocks on the array. The probes for the intergenic regions were not duplicated, and analysis from the intergenic regions is not presented here.

cDNA synthesis, labeling, and hybridization. We followed NimbleGen's standard protocols to process total prokaryotic RNAs for cDNA preparation, labeling, and hybridization. Double-stranded cDNA was synthesized from 10 or 5 μ g bacterial RNA from RNA derived from bacteria grown in culture or *in planta*, respectively, by reverse transcription (Invitrogen SuperScript II double-stranded cDNA synthesis kit; Invitrogen, Carlsbad, CA), followed by an RNase treatment and subsequent cDNA precipitation according to NimbleGen's protocol. After quantity and quality assessment, 10 μ g of cDNA was labeled with Cy3 fluorescent dye (TriLink BioTechnologies, San Diego, CA) according to the manufacturer's recommendations. Hybridization was carried out for 17 h at 42°C , using the NimbleGen hybridization station at the University of Wisconsin—Madison Biotechnology Center. Following hybridization, microarrays were washed and dried following recommended protocols. The arrays were scanned with a GenePix 4000B scanner at 532-nm wavelength with a photomultiplier tube (PMT) setting of 520 to achieve 1×10^5 normalized counts at the saturation (65,000-intensity) level. We imported the scanned image with NimbleScan 2.4 software to extract the raw data.

Data normalization and bioinformatic statistical analyses and MI-AME (minimum information about a microarray experiment) compli-

ance. NimbleScan 2.4 software was used to compute expression values (gene calls) according to the robust multichip average (RMA) algorithm (45, 46). Signals from the four replicates of each hybridization experiment were normalized using quantile normalization (47) with the RMA procedure incorporated in NimbleScan 2.4. Expression data were analyzed statistically with the ArrayStar software package (DNAStar, Madison, WI). Probabilistic statistical analysis (Student's *t* test) was used to identify differentially expressed (DE) genes. After selection of candidate differentially expressed genes, a statistic (referred to as "adjusted *P* value") was calculated for each gene. If the adjusted *P* value was lower than the desired cutoff (adjusted $P < 0.05$), then the gene was classed as differentially expressed. The false discovery rate (FDR) (Benjamini Hochberg) adjustment for multiple testing correction was applied to the *P* value to reduce the number of false-positive results and to increase the chances of identifying all the differentially expressed genes. All genes with adjusted *P* values less than the desired cutoff were listed as differentially expressed for that data set. If necessary, the significantly differentially expressed gene list was filtered further based on fold changes or expression levels in the treatments, using the options in ArrayStar. Data were imported into a custom Microsoft Access database and analyzed further using Microsoft Excel; scatter plots and heat maps of expression profiles were generated using ArrayStar to visualize expression data within and across treatments.

Validation of microarray results. Expression of several *R. solanacearum* GMI1000 genes was measured via absolute mRNA quantification to validate microarray results. To quantify gene expression of *hrpB* and *popA*, cells were grown under the following conditions: (i) in CPG at a lower cell density (2×10^7 CFU/ml), (ii) in CPG at a high cell density (7×10^8 CFU/ml), (iii) in BMM at a high cell density (8×10^8 CFU/ml), (iv) *in planta* at a lower cell density (3×10^7 CFU/g), and (v) *in planta* at a high cell density (1×10^9 CFU/g). RNA was extracted from equivalent numbers of cells grown in culture as described above but treated with at least 4 DNase steps. The plants were inoculated with strain GMI1000 by soil soak as described above. Stem tissue samples (150 mg) from asymptomatic and symptomatic (disease index of 1) tomato plants (cv. Bonny Best) colonized with $\sim 2 \times 10^7$ CFU/g and $\sim 1 \times 10^9$ CFU/g, respectively, were placed in Ambion RNeasy lysis buffer to preserve RNA integrity before storage at -80°C . Adjacent stem slice samples were dilution plated to determine bacterial cell numbers in each sample. Total RNA was extracted from ground infected stem tissue containing the target cell densities using the RNeasy plant minikit (Qiagen, Valencia, CA), followed by two DNase treatments.

For absolute quantification of *scrA* mRNA levels, *R. solanacearum* UW551 cells were grown to 1×10^9 CFU/ml in CPG or CPS. For minimal medium experiments, overnight CPG cultures were rinsed with BMN, transferred to BMN or BMN supplemented with either 300 μ M or 3 mM sucrose or 3 mM glucose, and incubated at 28°C for 2.5 h. RNA was extracted as described above, with at least three DNase treatments. RNA from tomato (cv. Bonny Best) plant tissue was collected from the stems of infected tomato plants as described above.

cDNA was synthesized from this RNA by reverse transcription with SuperScript III VILO (Invitrogen) using 2.0 μ g of total RNA and 50 ng of random hexamer primers, according to the manufacturer's protocol. Absolute quantification using quantitative PCR (qPCR) reactions were performed in duplicate 25- μ l reactions with Power SYBR green master mix (Applied Biosystems, Carlsbad, CA) and included $1 \times$ master mix, 400 nM forward and reverse primers for *hrpB*, *popA*, or *scrA*, and 50 ng template cDNA. Reactions were carried out on an ABI PRISM 7300 real-time PCR system (Applied Biosystems) with reaction parameters of 10-min polymerase activation, followed by 40 cycles, with 1 cycle consisting of 15 s at 95°C and 1 min at 57°C . Standard curves were made for absolute quantification using 10-fold dilutions (2.5 to 0.0025 μ g cDNA) from RNA collected and then reverse transcribed from GMI1000 cells grown to 7×10^8 CFU/ml on BMM for *hrpB* and *popA* expression. cDNA synthesis was carried out as described above but with 2.5 μ g template RNA. The standard curve for *scrA* absolute quantification was created using 10-fold di-

lutions from 2.0 μg to 0.002 ng cDNA synthesized from RNA from UW551 cells grown in BMN plus 300 μM sucrose.

qPCR via absolute quantification was used to define *scrA*, *hrpB*, and *popA* gene expression (48). Absolute expression was quantified in arbitrary absolute expression units (AEU) per cell by determining the amount of transcript based on each gene's respective cDNA standard curve (described above). The fold change in expression was determined by dividing the absolute expression values on a per-cell basis for bacteria grown under the various conditions by the absolute expression on a per-cell basis of bacteria growing in CPG medium. The statistical program SAS or JMP was used to analyze the raw data.

Comparative genomics. BLAST-based methods were used to identify homologous proteins between genomes. Orthology assignments were limited to those genes whose predicted bidirectional best hits from reciprocal BLASTP searches were filtered to eliminate incomplete and low scoring matches and retain those which shared a common backbone sequence residing in local collinear blocks (LCBs) in pairwise whole-genome alignments using the progressive alignment algorithm Progressive Mauve (<http://asap.ahabs.wisc.edu/mauve/>). Genes were considered orthologous if both their similarity scores and percent identity fell within cutoff values of >70% for pairwise BLASTP searches. Whole-genome alignments using Mauve allowed us to use gene context as an arbiter in cases where multiple paralogs were possible and to designate orthologs with improved specificity and precision. Downstream analyses were limited to high-confidence data sets when true orthology was critical.

Plant assays. To measure pathogen virulence, plants were inoculated with *R. solanacearum* strains and mutants via a naturalistic soil soak inoculation method as previously described (49). Hosts were unwounded 17-day-old susceptible tomato (cv. Bonny Best) plants, 14- to 16-day-old quantitatively resistant tomato (cv. Hawaii7996) plants, 21-day-old susceptible potato (cv. Russet Norkotah) plants grown from minitubers, or 18- to 19-day-old *Solanum dulcamara* [bittersweet nightshade] plants grown from seeds collected in Madison, WI, in 2009. Each plant's pot was soaked with a 50-ml suspension of wild-type UW551 or UW551 *scrA::Km^r* or *popS::Km^r* strain for a final inoculum density of 1×10^8 CFU/g soil. The plants were incubated at 28°C with a 12-h light cycle and were rated daily on a disease index scale from 1 to 4 as follows: disease index of 1 for 1 to 25% of the leaf area wilted; disease index of 2 for 26 to 50% of the leaf area wilted; disease index of 3 for 51 to 75% of the leaf area wilted; and disease index of 4 for 76 to 100% of the leaf area wilted. Virulence assays contained 16 plants per treatment and were replicated three times. To measure strain competitive fitness, 22-day-old wilt-resistant H7996 tomato plants were inoculated with 4 μl of a 1:1 mixture of *R. solanacearum* UW551-Rif^r, a spontaneous rifampin-resistant strain with wild-type fitness (WT) and *scrA::Km^r* (*scrA* mutant) (~2,000 CFU total). The plants were sampled 5 days postinoculation, and *in planta* population sizes of WT and *scrA* mutant strains were quantified by dilution plating on CPG plates with either Rif or Km. Data represent two biological replicates containing 30 plants each.

To quantify bacterial colonization in stem tissue and measure xylem fluid from mock-inoculated or infected plants, 22-day-old H7996 tomato plants were inoculated with water, *R. solanacearum* UW551, or UW551 *scrA* mutant via inoculation with ~1,000 cells per plant through a cut petiole. The plants were sampled at the onset of wilt (disease index of 1) 5 days postinoculation, and stem bacterial colonization was quantified. Plants were decapitated about 20 cm above the crown, and pooled xylem fluid was collected from plants for ~20 min. The first 100 μl of xylem fluid was discarded to remove any nonxylem metabolites released by wounding the plant. We quantified sucrose concentrations in xylem fluid by using a BioVision sucrose assay kit (Mountain View, CA) following the manufacturer's recommendations for detecting sucrose from biological samples. Glucose concentrations were used to check for metabolite contamination from nonxylem cells; there was no detectable glucose present, suggesting little to no cellular contamination beyond xylem fluid.

Microarray data accession number. The data from the microarray experiments described here are available at Gene Expression Omnibus (GEO [<http://www.ncbi.nlm.nih.gov/geo/>]) under the accession number GSE33662.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00114-12/-/DCSupplemental>.

- Figure S1, TIF file, 0.3 MB.
- Table S1A, PDF file, 0.7 MB.
- Table S1B, PDF file, 0.8 MB.
- Table S2, DOCX file, 0.1 MB.
- Table S3, DOCX file, 0.1 MB.
- Table S4, DOCX file, 0.1 MB.

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