Agrobacteria reprogram virulence gene expression by controlled release of host-conjugated signals

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It is highly intriguing how bacterial pathogens can quickly shut down energy-costly infection machinery once successful infection is established. This study depicts that mutation of repressor SghR increases the expression of hydrolase SghA in Agrobacterium tumefaciens, which releases plant defense signal salicylic acid (SA) from its storage form SA β-glucoside (SAG). Addition of SA substantially reduces gene expression of bacterial virulence. Bacterial vir genes and sghA are differentially transcribed at early and later infection stages, respectively. Plant metabolite sucrose is a signal ligand that inactivates SghR and consequently induces sghA expression. Disruption of sghA leads to increased vir expression in planta and enhances tumor formation whereas mutation of sghR decreases vir expression and tumor formation. These results depict a remarkable mechanism by which A. tumefaciens taps on the reserved pool of plant signal SA to reprogram its virulence upon establishment of infection.

chemical signaling | cost-pathogen interaction | Agrobacterium | sucrose | glucosidase

Bacterial pathogens commonly deploy an array of virulence factors to establish infections in various host organisms. For example, major virulence genes of Agrobacterium tumefaciens are carried by a large plasmid (over 200 kb), and infection requires a range of regulatory and structural proteins and a DNA fragment, which are transported from bacterial cells into host plant cells (1). These virulence factors are energy-costly to synthesize, and therefore bacteria might have evolved mechanisms to reprogram expression of virulence genes to survive in changed environmental conditions. Pseudomonas aeruginosa is known to switch from acute infection to chronic persistence by turning off expression of the genes encoding the type III secretion system (T3SS) through the Gac/Rsm regulatory pathway at the later stage of infection (2). Contrarily, Salmonella enterica escapes from its intracellular niche and spreads to a secondary infection site by inducing the expression of invasion-associated T3SS genes (3). However, how bacterial pathogens sense and perceive environmental changes to reprogram virulence gene expression remains elusive.

A. tumefaciens is a well-studied pathogen for causing crown gall diseases on more than 140 plant species (4). Infection of agrobacteria is modulated by various plant-derived chemical signals (5). Initially, wound-associated acidic conditions induce the expression of the chromosomal ChvG/I 2-component system, which activates the expression of transcriptional regulator VirG. VirA senses acetosyringone in the wounding site, phosphorylates VirG, and activates the expression of vir regulon that encodes the type IV secretion system and accessory proteins for processing and transferring transfer DNA (T-DNA) into plant cells. After integration, T-DNA genes encode biosynthesis of auxin, cytokinin, and opines. Plant hormones auxin and cytokinin promote plant cell proliferation and formation of crown gall tumors whereas opines are utilized by A. tumefaciens as specific nutrients. The bacterial pathogen thus creates an ecological niche that provides a selective advantage over other bacterial species, and this phenomenon is known as genetic colonization (6).

Transformation of host plant cells and building up of competitive advantages in ecological systems make A. tumefaciens an excellent model for exploring various features of pathogen–host interactions (7). A. tumefaciens infection is sensitive to high temperature, and therefore crown gall disease rarely happens in tropical regions. Previous findings suggest that Agrobacterium-mediated infection requires only a short period of time (8). This raises an intriguing question how pathogen can turn down vir expression after the infection to reduce energy cost. In this regard, it is interesting to note that exogenous application of plant defense signal salicylic acid (SA) can inhibit vir gene expression and virulence of A. tumefaciens (9, 10). In plants, a proportion of SA conjugates with glucose to prepare the storage form SA β-glucoside (SAG) (11, 12). Thus, it would be fascinating to explore if Agrobacterium has evolved a mechanism to hijack SA of host plants to switch from infection mode to free living style after the initiation of crown gall formation.

In this study, we report identification and characterization of a gene, sghA, encoding a hydrolytic enzyme. This enzyme releases...
SA from SAG to elevate its levels in planta. Transcription of sghA is tightly repressed by a repressor (SghR), and plant metabolite sucrose specifically releases this repression. Study further revealed that exogenous addition of SA decreased vir gene expression in A. tumefaciens. This phenomenon confirmed that the pathogen could use SA to reset its virulence via SghR/SghA after completing their infection. SA is an essential signal molecule for plant local defense and systemic resistance against numerous plant pathogens. These results indicate a broad implication in controlling crown gall disease and increasing plant transformation efficiency in agriculture. This study also unravels a previously unknown sophisticated strategy of microbial infection evolved during the long history of pathogen–host interaction.

**Results**

**Null Mutation of Repressor SghR Increases the β-Galactosidase Activity of A. tumefaciens.** SAG, a glucose-conjugated salicylic acid in plant hosts, shares a similar structure with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and ortho-nitrophenyl-β-D-galactopyranoside (ONPG) (SI Appendix, Fig. S1). This tempted us to search for potential SAG-hydrolyzing enzymes by using agar plates supplemented with X-gal. Wild-type A. tumefaciens strain A6 did not show obvious β-galactosidase activity as the color of bacterial colonies remained unchanged in the X-gal plate (Fig. 1A). Assuming that the bacterium may contain a repressed gene encoding SAG hydrolytic enzyme, we generated a library of transposon mutants and screened for enhanced β-galactosidase activity. After screening over 20,000 mutants, one mutant was identified that exhibited a dark blue color on basic medium (BM) plates supplemented with X-gal. Sequence analyses showed that Tn5 was inserted in A. tumefaciens strain CS8 homolog atu1522 sharing 92% amino acid sequence identity (GenBank no. KU512833). Its translational product is a 350-amino acid (aa) protein containing a helix-turn-helix (HTH)-type DNA-binding domain at its N terminus and a periplasmic binding protein-like domain (Peripla_BP_3) at its C terminus (Fig. 1B). These findings suggest that atu1522 homolog gene might encode a SAG hydrolase gene repressor and was named as sghR. Fig. 1A shows blue color morphology of sghR mutant (sghR::Tn5) on an X-gal agar plate. ONPG-based quantitative analysis indicated that sghR mutation increased β-galactosidase-like activity by about 10-fold as compared to parental strain A6 (Fig. 1A).

**To identify the SghR-repressed gene encoding putative SAG hydrolase, another round of mutagenesis was conducted with transposon Mariner by using mutant sghR::Tn5 as the parental strain. Screening of the resultant mutant library identified 1 mutant with much reduced blue color on X-gal agar plates than the parental strain.** Sequence analysis showed that Mariner was inserted in an ORF sharing about 94% identity to the atu4485 gene localized on the linear chromosome of A. tumefaciens strain CS8. Sequence analysis showed that homolog atu4485 encodes a 467-aa protein (GenBank no. KU512832), and this putative SAG hydrolase gene was designated as sghA. An X-gal plate assay showed that disruption of sghA by sghR mutant abolished its blue color morphology (Fig. 1A), suggesting that sghA is the sole SghR-repressed gene encoding putative SAG hydrolase. In trans expression of sghA in double mutant sghRA::Tn restored blue color morphology on the X-gal plate and significantly increased β-galactosidase activity against ONPG (Fig. 1A). In silico analysis was conducted with the online tool SMART (http://smart.embl-heidelberg.de), and results revealed that SghA contains a glycoside hydrolase family 1 (Glyco_hydro_1) domain (Fig. 1B). This is a typical feature of the glycoside hydrolase family comprising numerous enzymes, including β-glucosidase, β-galactosidase, 6- phospho-β-glucosidase, 6-phospho-β-galactosidase, and β-mannosidase.

**SghA as SA-Releasing Enzyme.** To test whether SghA could hydrolyze SAG to release SA, SAG was synthesized by using a modified protocol (SI Appendix, Fig. S2). After structural verification with mass spectrometry (MS) and NMR, SAG molecules were incubated with recombinant SghA and boiling-denatured SghA, respectively. High performance liquid chromatography (HPLC) analysis of the denatured SghA reaction mixture detected only 1 peak at a retention time of 5.0 min (Fig. 2A) and was confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis (SI Appendix, Fig. S3A). In contrast, HPLC analysis resolved 2 peaks from the reaction mixture of active SghA: i.e., SAG peak at 5 min and a new peak at a retention time of 9.0 min (Fig. 2A). ESI-MS analysis of the new peak showed a strong quasimolecular (M+H) ion with an m/z of 137.00 (SI Appendix, Fig. S3B) corresponding to the molecular mass of SA. Glucose, another predicted enzymatic reaction product, was not detected in this HPLC analysis as the molecule lacks detectable UV absorbance.
These results unequivocally demonstrated that SghA is a β-glucosidase that hydrolyzes SAG to release SA.

To determine the enzyme specificity of SghA, its catalytic activity was compared against SAG analogs. Results showed that SghA also hydrolyzed salicin to produce a product peak at around 4.0 min (Fig. 2B). ESI-MS analysis of this new peak fraction showed a strong quasi-molecular (M-H) ion at an m/z of 123.00, similar to salicin-hydrolyzed product salicylic alcohol (SI Appendix, Fig. S3C). However, SghA could not hydrolyze other SAG structural analogs coniferin and sucrose (SI Appendix, Fig. S4), indicating the substrate selectivity of SghA. Consistent with this finding, SghA digested SAG at a much faster rate than salicin and ONPG under the same reaction conditions (Fig. 2A and B). Michaelis constant (K_m) of SghA was determined as 0.47 mM, 2.81 mM, and 5.91 mM for SAG, salicin, and ONPG, respectively (SI Appendix, Fig. S5). SghA of agrobacterium cells in culture in medium readily cleaved exogenous SAG to its metabolic product SA, as detected in the culture supernatant (SI Appendix, Fig. S6). These results suggest that SAG entered the bacterial cells and its cleaved product was exported out.

To further understand the molecular bases of SghA substrate specificity and catalytic mechanism, 2 substrates SAG and salicin were used to determine SghA structures in apo and in complex. The apo-SghA structure showed a typical (β/α)_8-barrel fold in a single domain (SI Appendix, Fig. S7A), which is commonly found in β-glycoside hydrolase family 1 (GH1). The asymmetric unit contains 2 SghA molecules and forms a homodimer whereas dimerization does not influence the free access to its active site.
SghR Suppresses sghA Expression by Binding to Its Promoter. Results of RT-PCR and real time RT-PCR analyses revealed that the level of sghA messenger RNA (mRNA) was much higher in sgrR mutant than wild-type strain A6 at different intervals (Fig. 3A and SI Appendix, Fig. S11). Gel shift analysis showed that SghR directly and specifically bound with the sghA promoter to shift it in a dose-dependent manner, but the control promoter (pA0305 from P. aeruginosa PA01) was unaffected by SghR (Fig. 3B). An unrelated IeIR-type regulator (AttJ or BlcR) of A. tumefaciens also failed to shift the sghA promoter (15). Taken together, these results indicate that SgrR suppresses sghA transcription via direct binding to its promoter in A. tumefaciens.

Sucrose Inactivates SgrR and Induces sghA Expression. Given that SghA is tightly suppressed by SgrR under in vitro conditions, we hypothesized that there might be a signal ligand interacting with SgrR to induce sghA expression in planta. An extract of carrot

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SI Appendix, Fig. S7 B and C). Based on the comparison of the apo-SghA structure with other reported homolog structures, we identified that E179 of SghA plays a critical role in its activity. Substitution of E179 with S179 completely abolished enzymatic activity, as reflected by ONPG hydrolysis.

SghA–SAG and SghA–salicin complexes were cocry stallized by generating a hydrolyase-dead mutant SghA(E179S) (SI Appendix, Fig. S8). Similar to other GH1 family proteins (13), SghA also has a bell-shaped binding cavity where glycones (sugar) bury in- side and aglycone is located at the wide entrance gate (Fig. 2 C and D). To form the SghA–SAG complex, highly conserved residues (Q33, H134, N178, E367, and E420) formed hydrogen bonds with hydroxyl groups of sugar. Sugar binding was stabilized by the interacting of SAG with Y307 and W413 side chains on one side and indole groups of W135 and W421 from the other. SghA inter- action with the aglycone group of SAG involved H193 in loop B and W340 in loop C to form a sandwich arrangement. In particu- lar, 2 hydrogen bonds were established between the residue H193 and carboxylic acid group of SAG. In the case of SghA– salicin, salicin orientation was similar to SAG, and structural comparison revealed that there was little change of the active site except for H193. H193 appeared to form a hydrogen bond with the hydroxymethyl group of salicylaglycone; however, its geometrical preference was less perfect than SghA–SAG.

In contrast to the compact and conserved bottom half of (β/α) barrel (β-strand N terminus side), the top half (β-strand C terminus side) of SghA was surrounded by 4 flexible loops that gate the exit of the bell-shaped cavity. It was proposed that these loops are involved in the formation of the aglycone-binding site that determines substrate preference (14). In particular, loops B and C of the 4 loops A to D (Fig. 2 C and D) are mainly responsible for aglycone binding. In line with this notion, residues H193 and W340, respectively, located in loop B and C established direct interactions with the SAG salicylic acid group or salicin. Residue W340 formed an imperfect π–π interaction with the benzene ring, considering that W340 is quite conserved in the binding pocket among SghA homologs whereas H193 is only found in the binding pocket of SghA (SI Appendix, Fig. S9). The unique residue H193 in agrobacterial SghA interacting with SAG only suggests that H193 might play a more crucial role in substrate recognition of SghA than other residues. H193 made strong hydrogen-binding interac- tions with the SAG salicylic acid group that rationalizes our biochemical data about the substrate preference of SghA to SAG, rather than salicin.

Compared with the structure of Neotermes koshunensis β-glucosidase (13), SghA exhibits a conserved substrate-binding site (SI Appendix, Fig. S9). It is common for GH1 family proteins to have subtle differences in substrate specificity. In addition to the native substrate, these proteins could hydrolyze a broad spectrum of artificial substrates. Similarly, SghA hydrolyzed ONPG and X- gal, which are synthetic analogs of SAG. The GH1 protein family hydrolizes glycosidic bonds through a retention mechanism that retains the overall anomeric configuration of the saccharide sub- strate. The combined data of structural and biochemical analysis proposed that a catalytic mechanism of SghA on substrate SAG:E367 makes a nucleophilic attack on the anomeric carbon C1 in SAG and E179 acts as a proton donor to release SA (SI Appendix, Fig. S10).

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Fig. 3. SgrR controls the transcriptional expression of sghA. (A) RT-PCR analysis of the sghA expression patterns in wild-type strain A6 and its sgrR mutant at different time intervals postinoculation in BM medium with mannitol as the sole carbon source. (B) Electrophoretic mobility-shift assay (EMSA) analysis of SgrR binding to the sghA promoter. The unrelated transcriptional regulator AttJ from A. tumefaciens and the promoter Ppa0305 from P. aeruginosa were used as specificity controls. Purified proteins of SgrR and AttJ were mixed with the biotin-labeled probes (1 nM) at various concentra- tions, and reaction mixtures were separated by electrophoresis. Experiments were repeated at least 3 times with similar results, and 1 set of representative data is presented.
tubers was prepared to test its activity in the induction of β-galactosidase encoded by sghA, and results depicted that sghA expression was induced with the addition of the extract (Fig. 4A). To purify the active signal, dry powders of carrot extract were further extracted with methanol, and samples were separated by silica column chromatography. Active fractions were pooled, and a compound was crystallized during the evaporation of solvent. This crystallized molecule displayed a strong activity to induce sghA expression when dissolved in water (SI Appendix, Fig. S12A). MS analysis showed that the molecular weight of this compound is 342.20, and, together with the results of NMR analysis, the active compound was identified as disaccharide sucrose (SI Appendix, Fig. S12 B–F). Commercial sucrose induced β-galactosidase activity in A. tumefaciens A6 on the X-gal plate (Fig. 4B). Consistent with the above results, gel shift analysis demonstrated that sucrose dissociated repressor SghR from the sghA promoter. SghR binding to the sghA promoter was substantially reduced and completely abolished when sucrose was added at a final concentration of 50 μM and 500 μM, respectively (Fig. 4C). This is within the range of extracellular sucrose concentration in planta. Quantitative measurement of intercellular sucrose levels in carrot tissues showed that sucrose concentrations were higher than 40 mM in both uninoculated control and tissues infected with bacterial cells after deletion of sghA and sghR (SI Appendix, Fig. S13). These findings demonstrate that plant metabolite sucrose is a cognate signal deactivating SghR and induces transcriptional expression of sghA.

**SghA Increases Plant SA Level during A. tumefaciens Infection.** To confirm that SghA hydrolyzes SAG to SA during agrobacteria plant interaction, the level of free SA in infected plants was determined by using SA biosensor Acinetobacter sp. strain ADPWH_lux (16). Results revealed that inoculation of sghR mutant significantly enhanced free SA concentration in Arabidopsis at 20 h postinfection. Contrarily, challenge of the sghA/sghR double mutant led to lower SA level than plants inoculated with wild-type strain A6 (Fig. 5A). As expected, overexpression of sghA in the mutant sghA::Tn5 resulted in substantially increased free SA level.

Similar results were also obtained with HPLC by directly measuring free SA concentration in the roots of Arabidopsis infected with A. tumefaciens A6 and its derivatives (Fig. 5B). Data indicated that, during infection, A. tumefaciens used SghA to release SA from SAG in the host plant.

The tumor mass on Arabidopsis stems was measured to examine the impact of SghA-mediated SA increment in the host plant on bacterial infection (17, 18). Results showed that the tumor mass was substantially reduced in wild-type Arabidopsis ecotype WS-2 plants inoculated with the sghR mutant, as compared to those inoculated with wild-type A. tumefaciens A6. In contrast, disruption of sghA from the sghR mutant background led to significantly enhanced tumor growth, and tumor weight was almost 2 times that resulting from strain A6. Consistent with this trend, overexpression of sghA substantially attenuated bacterial tumorigenicity (Fig. 5C).

These results were further confirmed by examining tumor weights on carrot discs (SI Appendix, Fig. S14A) and validated by quantification of tumor numbers after inoculation of carrot discs with serial dilutions of bacterial inoculum (SI Appendix, Fig. S14B).

Tumorigenicity of strain A6 and its derivatives was tested on Arabidopsis Ugt741 as a control. Arabidopsis Ugt741 is a mutant derived from the Arabidopsis thaliana ecotype WS-2. In this mutant, SA glucosyltransferase was mutated to substantially reduce SAG formation, leading to a significantly higher SA level than in wild-type Arabidopsis (19, 20). As shown in Fig. 5D, strain A6 and its mutants caused a similar level of tumor growth on Arabidopsis Ugt741, but the tumor mass was substantially less than that induced by strain A6 on wild-type Arabidopsis. In controls, no significant growth difference was recorded among wild-type and mutants in lysogeny broth (LB) and BM media, or with glucose and sucrose as the sole carbon source (SI Appendix, Fig. S15 A–D). sghR- and sghA-mutants produced similar levels of biofilms, and mRNA levels of virE3 in mutants were comparable with wild-type strain A6 in virulence-inducible or noninducible media (SI Appendix, Fig. S15 E and F). Data are consistent with the notion that an
A6 at different intervals after the inoculation of expression patterns of tested strains whereas SAG affected SA and SAG effects on the expression patterns of sghR were analyzed. With a basal level of gene expression of constitutively expressed at different post-inoculation intervals (Fig. 6). Tumor sizes incited by strain A6 and derivatives in the SAG-defective mutant of A. thaliana (21, 22), but at other stages of infection. Taken together, these results suggest that SghR/SghA-mediated release of SA plays a key role in reprogramming the virulence gene expression in A. tumefaciens.

**Discussion**

At least 2 lines of evidence indicate that the time required for tumor induction by A. tumefaciens is less than 10 h (21, 22), but expression patterns of the bacterial virulence gene after establishing infections is not clear. This study showed that, upon inoculation, vir genes of A. tumefaciens maintained a high level of expression in planta at the early stage of infection but their transcript levels were substantially decreased after 18 h. The key mechanism that reprograms vir gene expression in A. tumefaciens consists of a regulator and enzyme pair SghR/SghA that timely releases SA from SAG in a working model, as presented in Fig. 7. In this model, we proposed that plant SAG enters agrobacterial cells for hydrolysis rather than the secretion of bacterial SghA to act extracellularly. Secretion-specific signal peptide was not identified in the SghA amino acid sequence; however, it is well-known that SAG analogs having high structural similarity with X-gal, ONPG, and salicin can enter bacterial cells. However, further studies using isotope-labeled SAG are required to verify this mechanism. The model suggests that SAG has been reported to exclusively deposit in the vacuole via ATP-dependent transportation (23), but it is currently unknown whether plant cells can also actively export SAG for hydrolysis. Generally, agrobacterium infection at the wound site of the host plant, and SAG could be one of the wound-released chemical signals perceived by bacterial cells.

SghA is known to hydrolyze different compounds, such as X-gal, ONPG, and salicin. Biochemical and structural analyses in this study suggested that SAG can serve as a bona fide substrate of SghA because 1) SghA failed to hydrolyze coniferin and its inducer sucrose, 2) SghA mutant grows readily with sucrose as the only carbon source, and 3) SghA releases SA from SAG in a highly efficient manner, as compared to other substrates. SA is a key signal that activates both local and systemic acquired resistance to regulate disease resistance. Under normal growth conditions, SA conjugates with β-glucosides and is stored in the inactive form to minimize its cytotoxicity, maximize SA stability, and facilitate its transportation. Among SA conjugates, SAG has been identified as a predominant and stable metabolite (24, 25). A previous study showed that plant growth hormone auxin influences A. tumefaciens virulence by inhibiting vir gene induction and bacterial growth (26).

However, this effect was only observed at higher concentrations of auxin (25 to 250 μM), marginally matching with its level in Arabidopsis crown galls (17.3 ± 8.8 μM) and taking multiple days from agrobacteria inoculation to the formation of visible crown galls (27). During this study, inactivation of SghA promoted tumor formation in plants whereas it failed to improve agrobacterial growth in vitro. Future work is required to examine whether specific release of SA from SAG by SghA benefits bacteria during in vivo infection. SA is considered a systemic signal triggering a defense response in uninfected host cells. Initiation of SA synthesis in host cells by SghA lacking pathogens suggests that SghA-based SA release is not the only mechanism to manipulate the host SA signaling pathway during microbial infection. SghA may also metabolize additional substrates, and the fact that SghA releases SA might be coincidental although it exhibits no enzymatic activity toward sucrose and coniferin.

Transcriptional repressor SghR and its cognate signal ligand sucrose control the expression of sghA. Mutation of sghR led to constitutive expression of sghA, suggesting that SghR is the key regulator that governs transcriptional expression of sghA. Our data revealed that sghA is specifically induced by the plant metabolite sucrose, which interacts to inactivate repressor SghR at a concentration lower than 0.5 mM. Sucrose is a major photosynthesis enhancer free SA level in host plants is positively associated with increased disease resistance and SghR/SghA play an important role in the modulation of A. tumefaciens virulence.

**SghA-Released SA Reprograms A. tumefaciens Virulence.** To understand the role of SghR/SghA in A. tumefaciens tumorigenicity, SA and SAG effects on the expression patterns of sghR and vir genes were analyzed. With a basal level of gene expression of virE3 in BM minimal medium, real-time RT-PCR results showed that SA and SAG treatments had no detectable effect on the expression of sghR and sghA in the presence or absence of functional SghA (SI Appendix, Fig. S10). SA significantly inhibited virA expression in all tested strains whereas SAG affected virA expression only in the presence of functional SghA (Fig. 6A). Results suggest an essential role of SghA in the modulation of vir gene expression by releasing SA from the inactive SAG.

RT-PCR and real-time RT-PCR were used to determine in planta expression patterns of sghR and vir genes in wild-type strain A6 at different intervals after the inoculation of A. thaliana. SghA and vir genes displayed differentially overlapping expression patterns while sghR constitutively expressed at different post-inoculation intervals (Fig. 6B and SI Appendix, Fig. S17). The 3 tested vir genes including virA, virD2, and virE3 significantly expressed during early stages of infection at 6, 12, and 18 h post-inoculation, but their expressions were inhibited at the later stages. In contrast, hardly any sghA transcript was found at 6 and 12 h, and maximum expression occurred at 24 h postinoculation. Western blot analysis also showed that VirD2 protein expression in wild-type strain A6 occurred at around 12 h postinoculation, peaked at 24 h, and decreased afterward (Fig. 6C). Contrarily, sghR mutant resulted in a significantly decreased level of VirD2 protein (Fig. 6C), but sghA mutant caused higher expressions of VirD2, even at the later stage of infection. Taken together, these results suggest that SghR/SghA-mediated release of SA plays a key role in reprogramming the virulence gene expression in A. tumefaciens.
product (>95%) in plants that could reach to a concentration of 1 M in conducting vascular cells and 2 to ∼7 mM in extracellular spaces (28). Consistent with this report, soluble sucrose concentration in carrot tissues was noted in the range of 47 to 100 mM, depending on postinoculation intervals. Extracellular concentration of sucrose can dramatically increase after tissue disruption. In addition to a carbon source, sucrose also acts as a signal molecule to regulate plant growth, development, differential gene expression, and stress-related responses (29). Furthermore, evidence supporting sucrose as a potent signal to induce plant defense responses has been reported (30, 31). However, the precise mechanism of sucrose-induced immunity and the related signaling pathway remains a mystery. Results of this study depict that sucrose specifically induces the expression of hydrolyase SghA by deactivating repressor SghR, which degrades the SAG conjugate to increase the cellular level of plant signal SA. Therefore, identification of SghR/SghA depicts a mechanism that corroborates sucrose-induced immunity and explains that the SA level in plant tissues is not elevated by the initiation of agrobacterial infection, but dramatically enhances at the later infection stage (32).

Characterization of SghR/SghA unveils a mechanism that allows the bacterial pathogen to tap on the reserved pool of plant defense signal SA to reprogram its virulence gene expression after establishing infection. Bioinformatics analysis showed that SghA is highly conserved in A. tumefaciens and other members of the Rhizobiaceae family of α-proteobacteria. SghA shares moderate similarities with the homologs of other bacterial species, such as Dickeya and Pectobacterium. It is not clear whether these homologs can hydrolyze SAG or pair with an SghR-like regulator to release SA in a controlled manner. In this regard, it is interesting to note that SA could directly influence virulence gene expression in A. tumefaciens and other bacterial pathogens. At lower concentrations, SA was found to impair bacterial attachment and biofilm formation and down-regulate fitness and production of virulence factor in P. aeruginosa (33). Similarly, SA inhibited biofilm formation, motility, and N-acyl homoserine lactone quorum sensing signal production in Pectobacterium carotovorum and Pseudomonas syringae pv. Syringae (34). Moreover, the amazing sensing abilities of microbes and the response to changing environmental conditions require further investigations to unveil mechanisms that allow pathogens to reprogram virulence gene expression after establishing infections.

**Methods**

**Bacterial Strains, Plasmids, and Growth Conditions.** SI Appendix, Table S1 lists bacterial strains and plasmids used in this study. *A. tumefaciens* strains were grown at 28 °C in BM medium (pH 7.0, 0.2% mannitol) or in VIB medium (NH4Cl, 1 g/L; MgSO4 • 7H2O, 0.3 g/L; KCl, 0.15 g/L; CaCl2, 0.01 g/L; FeSO4 • 7H2O, 2.5 mg/L; K2HPO4, 0.06 g/L; NaH2PO4, 0.023 g/L; pH 5.5, 0.2% arabinose and 100 μM acetosyringone) (10). One liter of BM medium contained K2HPO4 (10.5 g), KH2PO4 (4.5 g), MgSO4 • 7H2O (0.2 g), (NH4)2SO4 (2.0 g), FeSO4 (5 mg), CaCl2 (10 mg), MnCl2 (2 mg), and mannitol (2.0 g). LB medium was used for general cultivation of *Escherichia coli* strains, and antibiotics were added at the following concentrations when required: kanamycin, 100 μg/mL; tetracycline, 5 μg/mL; gentamycin, 100 μg/mL; ampicillin, 200 μg/mL.

**Genetic Manipulation of *A. tumefaciens*.** Transposon mutagenesis was performed in E. coli BW20767 containing a mini Tn5 or E. coli SM10 (pBT20) carrying a mariner transposon, and disrupted genes were identified (35). A. tumefaciens mutants were screened on BM solid plates containing X-gal (50 μg/mL) and relevant antibiotics. To conduct complementation analysis, a DNA fragment containing the putative promoter and full-length sghA was amplified from...
A. tumefaciens A6 by using PCR primers (SI Appendix, Table S2). The PCR product was digested by BamHI and cloned in pLAFR3, and the resultant construct pLAsghA was verified by sequencing before transformation into A. tumefaciens by electroporation (15). Deletion of SgrH, SghA, and its double deletion were performed according to Wang et al. (36).

**Recombinant Proteins.** The coding regions of target genes were amplified from the genomic DNA of A. tumefaciens A6 and fused into 6xHis-tagged expression vector pET11b (Novagen). After verification by DNA sequencing, resultant constructs were separately transformed into strain BL21(DE3), and recombinant proteins were purified (37).

**Enzyme Assay.** SghA β-galactosidase activity was indicated by the appearance of a blue color on BM agar plates supplemented with X-gal (50 μM) according to Zhang et al. (38). Briefly, solidified BM medium in Petri dishes was incubated at 28 °C for 40 h, and color appeared on BM agar plates supplemented with X-gal (50 μM) in 1 mM MgSO4, 50 mM β-ME, pH 7.0 containing 1 mM ONPG or salicin or SAG and 10 mM SghA. The same amount of boiled SghA was added to the reaction mixture as a negative control. Samples were induced onto a symmetry reverse-phase column (4.6 × 250 mm) for HPLC. Fractions were eluted with 50/50 methanol/water (v/v) at a flow rate of 1 ml/min and detected by a Waters 996 photodiode detector. ESI-MS was performed by a Finnigan/MAT ion-trap mass spectrometer following standard procedures.

**RT-PCR and Western Blot Analysis.** To study sgrh and sgha expression levels during bacterial growth, strain A6 and its mutant strain Tn5 were cultivated in BM medium and harvested at specific time intervals. Total RNAs were extracted by using the RNAesy Mini Kit (Qiagen). To treat SAG and SA, specified bacterial cells were grown overnight in LB medium and then subcultured in 5 ml of induction medium (with 100 μM acetylsalicylic acid) at an initial OD600 of 0.1, with or without SAG (10 μM) and SA (5 μM). After 5 h of shaking (rpm 200) at 28 °C, 1 ml of cell cultures was harvested for RNA extraction with the RNeasy Prep Reagent Kit (Qiagen). Residual RNAs were digested by RNase-free DNase I (Promega), and its absence was validated by PCR with purified RNA templates. RNA integrity was assessed by agarose gel electrophoresis, and RNA concentration was measured by Nanodrop ND-1000 (Nanodrop Technologies). To minimize data variation, total RNAs were prepared from 3 independent repeats and pooled together for RT-PCR analysis.

To study the effect of the 1-step strategy (QS) on the OD of the reaction mixture, RT-PCR analysis was performed in Rotor-Gene Q (Qiagen) by using the SYBR Green RT-PCR Kit (Qiagen). Housekeeping gene rpob encoding the β subunit of RNA polymerase was included as an internal control. Results were presented as the ratio of target gene expression versus control gene. Real-time RT-PCR primers are listed in SI Appendix, Table S2.

To determine the expression levels of sgrh, sgha, and vir genes during Agrobacterium infection, strain A6 cells grown in LB medium at late exponential phase were harvested, washed 3 times with PBS (pH 7.4) and resuspended in the same buffer for carot infection. Carrots were washed 3 times with 75% ethanol, sliced into 1.5 cm-thick disks, and inoculated with 200 μl of bacterial cells. Infected carrot disks were placed into Petri dishes moisturized with wet filter papers and incubated at 28 °C. Bacterial cells were collected at specific time intervals by washing with PBS buffer containing Triton X-100 (0.1%), treated with RNase-free DNase (Promega), and kept until RNA extraction. RT-PCR was conducted following the protocol of the 1-step strategy (Qiagen) or 2-step strategy (Promega). An aliquot of 0.2 μg of total RNAs was used as template to amplify a portion of the target genes with primers (SI Appendix, Table S2). A fragment of 16S rRNA (rRNA) was also amplified in each RT-PCR as an internal control.

Western blotting was carried out by harvesting bacterial cells from infected plant tissues as mentioned above and lysed in urea buffer (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M Urea, 1 mM Na2VO4, 20 mM NaF, 0.1 mM β-glycerophosphate, and 20 mM sodium pyrophosphate) supplemented with a complete Protease Inhibitor Mixture tablet (Roche, pH 8.0). Lysates were cleared by centrifugation (14,000 rpm, 10 min, 4 °C), and protein concentration in supernatants was determined with the BCA Protein Assay Kit (Pierce). Equal amounts of total proteins were used for Western blot with anti VirD2-specific (catalog no. MBS5394219; Mybiosource) and RNA polymerase (RNAP)-specific antibodies (catalog no. 663903; Biolegend).

**Electrophoretic Mobility-Shift Assay.** The SghA promoter region (~345 bp to +43 bp) was amplified from genomic DNA of A. tumefaciens strain A6 by using 5′-biotinylated primers (SI Appendix, Table S2). The fragment was designated as PsgHA and used as a probe for gel retardation analysis to determine potential interaction with transcriptional repressor SgrH. As a specificity control, PCR was used to generate the PsgA305 probe (~281 bp to +81 bp) from genomic DNA of P. aeruginosa with 5′-biotinylated primes (SI Appendix, Table S2). A gel retardation assay was performed with the LightShift Chemiluminescent EMSA Kit by following the recommended protocol (Pierce Biotechnology).
The overnight Quantitative Analysis of Soluble Sucrose in Carrot Tissues. Crystallographic data and refinement statistics are listed in (42). Models were refined with Phenix (43) and checked by PROCHECK (44). 1NP2) as a search model for SghA. Automatic model building was performed X06SA (Swiss Light Source). Data were processed in XDS (40), and structures tallization procedure for the apo form. carried out according to Ye et al. (37). SghA derivative E179S was generated Acinetobacter for agrobacterial infection. Root segments were collected at 20 h post- into ∼4 °C for 2 d, and transferred to a growth chamber with a short-day (8 h light) photoperiod at 23 °C for root culture. After 2 wk, roots were axenically cut into ~3-cm segments on a sterile filter paper and transferred to MS medium for abiotic stress. Root segments were collected at 20 h post-infection for SA extraction and quantified with HPLC (39) and biosensor Acinetobacter sp. ADPWH_lux (16).

Protein Preparation, Crystallization, and Structural Determination. The SghA coding region was amplified by PCR and cloned into vector pET-14b. The resultant construct was verified by DNA sequencing and transformed into E. coli BL21 CodonPlus-(DE3) RIL for protein expression. SghA purification was carried out according to Ye et al. (37). SghA derivative E179S was generated by QuikChange (Stratagene) and purified by following the same procedure as SghA.

Crystallization of SghA has been previously reported (37). To crystallize SghA-SAG, –salicin, and –glucose protein–ligand complexes, the ligands were mixed with SghA derivative E179S at a final concentration of 20 mM and incubated for 30 min at room temperature, followed by a similar crystallization procedure for the apo form. Dialysis was performed at 100 K on beam lines I04 (Diamond) or X06SA (Swiss Light Source). Data were processed in XDS (40), and structures were determined with Phaser (41) using homolog structure (PDB ID code 1NP2) as a search model for SghA. Automatic model building was performed in ARP/WARP (40) and improved by building a manual model with COOT (42). Models were refined with Phenix (43) and checked by PROCHECK (44). Crystallographic data and refinement statistics are listed in SI Appendix, Table 53, and figures were generated with Pymol (Delano Scientific).

Quantitative Analysis of Soluble Sucrose in Carrot Tissues. The overnight starter cultures of A. tumefaciens strains were inoculated (1%) in 20 mL of LB medium and grown at 28 °C and 200 rpm until OD590 reached to 1.0. Bacterial cells were collected by centrifuging at 12,000 rpm, 10 min, washed twice with sterile PBS buffer, and resuspended in 2 mL of the same buffer. Carrot samples were sterilized with 80% ethanol and dissected into 1-cm-thick slices. Each sample was evenly spread with 100 μL of bacterial solution, placed on a Petri dish with moisturized filter papers, and incubated at 28 °C for 6 h. Similarly, PBS buffer was added to serve as a control. To measure extracellular sucrose content, carrot samples were collected and washed with sterile water to remove bacterial cells. Slices were dried with tissue paper, and a thin layer (2 mm) was cut from the end of inoculation and weighed. In parallel, cells were collected by centrifuging for 10 min at 12,000 rpm. Supernatants of the same sample were combined, and sterile water was added to a final volume of 11 mL, which was diluted accordingly for sucrose quantification with the SCA20-1KT.

Tumorgenesis Assay. Arabidopsis infection was carried out according to Deeken et al. (46). Briefly, A. thaliana seeds were cultivated in a growth room under a short-day condition at 23 °C. Tumors were induced by applying A. tumefaciens strains to the base of a wounded young inflorescence stalk. Disease symptoms were recorded on the 28th day of infection. Statistical analyses were conducted with the nonparametric 1-way ANOVA test.

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