Salicylic Acid and Systemic Acquired Resistance Play a Role in Attenuating Crown Gall Disease caused by Agrobacterium tumefaciens

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We investigated the effects of salicylic acid (SA) and systemic acquired resistance (SAR) on crown gall disease caused by Agrobacterium tumefaciens. Nicotiana benthamiana plants treated with SA showed decreased susceptibility to Agrobacterium infection. Exogenous application of SA to Agrobacterium cultures decreased its growth, virulence, and attachment to plant cells. Using Agrobacterium whole-genome microarrays, we characterized the direct effects of SA on bacterial gene expression and showed that SA inhibits induction of virulence (vir) genes and the repABC operon, and differentially regulates the expression of many other sets of genes. Using virus-induced gene silencing, we further demonstrate that plant genes involved in SA biosynthesis and signaling are important determinants for Agrobacterium infectivity on plants. Silencing of ICS (isochorismate synthase), NPR1 (nonexpresser of pathogenesis-related gene 1), and SABP2 (SA-binding protein 2) in N. benthamiana enhanced Agrobacterium infection. Moreover, plants treated with benzo-(1,2,3)-thiadiazole-7-carboxothioic acid, a potent inducer of SAR, showed reduced disease symptoms. Our data suggest that SA and SAR both play a major role in retarding Agrobacterium infectivity.

**Agrobacterium tumefaciens**, a soil-borne phytopathogen, is the causal agent of crown gall disease in plants. Agrobacterium has a very broad host range that includes about 600 characterized plant species (DeCleene and DeLey, 1976). The process of tumor formation involves the transfer and integration of a specific segment of the Ti (tumor-inducing) plasmid, the T-DNA (transferred DNA), from the bacterium into the plant genome (Chilton et al., 1977). Crown gall disease causes significant economic losses worldwide, mostly pertaining to perennial horticulture plants, by reducing crop yield and increasing susceptibility to opportunistic pathogens (Agrios, 1997; Burr et al., 1998). Attempts to control crown gall disease have largely failed, with the exception of biological control by Agrobacterium radiobacter strain K84 (a nonpathogenic bacterium that produces agrocin 84, which is toxic to most A. tumefaciens strains that are able to utilize agrocinopine-type opines; Burr and Otten, 1999). However, this cross-protection is limited to only certain Agrobacterium strains. Alternate methods based on RNA interference to down-regulate auxin biosynthetic genes, including *niaD* and *ipt* oncogenes (Escober et al., 2001; Lee et al., 2003; Viss et al., 2003), were proposed for controlling the disease. However, these approaches are faced with limited practical application due to the need for developing transgenic lines. Therefore, there is a need to develop and adopt durable disease control measures for combating crown gall disease.

Plants are constantly attacked by pathogens, and as a result plants have evolved a plethora of constitutive and induced basal defenses to defend against pathogens. The phytohormones salicylic acid (SA), jasmonic acid, and ethylene are known to participate in regulating defenses in plants (Pieterse and Van Loon, 1999; van Wees et al., 2000; Glazebrook, 2001; Spoel et al., 2003; Thaler et al., 2004). SA is predominantly associated with resistance against biotrophic and hemibiotrophic pathogens, and triggering systemic acquired resistance (SAR; Grant and Lamb, 2006). Although the complete mechanism of SA-mediated plant defense is not understood, the central role of SA in plant defense is universally accepted (for review, see Pieterse and Van Loon, 1999; Shah, 2003; Grant and Lamb, 2006). Exogenous application of SA or its functional analogs, such as 2,6-dichloroisonicotinic acid and benzo-(1,2,3)-thiadiazole-7-carboxothioic acid S-methyl ester (BTH), induces SAR in plants, resulting in resistance to certain pathogens (Ryals et al., 1996; Achuo et al., 2004; Wang et al., 2005). Conversely, plants expressing the bacterial NahG gene (encoding salicylate hydroxylase, which...
converts SA to catechol) are more susceptible to several pathogens (Gaffney et al., 1993). Direct evidence for the role of SA in plant defense comes from the identification and characterization of an Arabidopsis (Arabidopsis thaliana) isochorismate synthase (ICS) mutant (sid2-2) that is defective in SA biosynthesis (Wildermuth et al., 2001). Endogenous SA levels in plants can also affect their interaction with symbiotic microorganisms, as demonstrated by increased root nodulation and infection upon inoculation with Mesorhizobium loti, in transgenic Lotus japonicus and Medicago truncatula expressing NahG (Stacey et al., 2006). Only a limited number of studies has demonstrated the direct effects of SA on microbes including Pseudomonas aeruginosa (Prithiviraj et al., 2005b), Staphylococcus aureus (Prithiviraj et al., 2005a), Sinorhizobium meliloti (Martinez-Abarca et al., 1998), and, more recently, A. tumefaciens (Yuan et al., 2007).

Plants mount defense responses against Agrobacterium infection similar to those triggered by other bacterial pathogens (Veena et al., 2003; Zipfel et al., 2006). Furthermore, a virulent strain of Agrobacterium was shown to suppress host defenses at later times after infection (Veena et al., 2003). Using several approaches, we demonstrate the role of SA-mediated plant defense responses against Agrobacterium. Nicotiana benthamiana plants treated with SA were less susceptible to Agrobacterium infection, whereas N. benthamiana plants silenced for genes involved in SA biosynthesis and signaling were hypersusceptible to Agrobacterium infection. Exogenous application of SA to bacterial cultures impeded the growth and virulence of Agrobacterium. Furthermore, we show that SA interferes with the transcription of a set of Agrobacterium genes, including the vir regulon, the repABC operon, and genes associated with quorum sensing. We also show that SAR has a role in mitigating susceptibility to crown gall disease.

RESULTS

SA Application on Plants Decreases Agrobacterium Infection

We analyzed the direct effects of the phytohormone SA on Agrobacterium infectivity by exogenously applying SA to N. benthamiana through soil drenching. Exogenous application of SA or its analogs on plants has been shown to induce SA-mediated plant defenses to a broad range of pathogens (for review, see Vallad and Goodman, 2004). Our preliminary experiments suggested that exogenous application of SA at concentrations of 7.5 mM and above resulted in chlorosis, stunting, and cell death (data not shown). Therefore, SA was applied at lower concentrations (0–5 mM). Leaves from the SA-treated and mock-treated plants were collected 7 d posttreatment and subjected to stable and transient transformation assays as described (Anand et al., 2007b). The transient transformation assays were performed using strain A. tumefaciens GV2260 (Deblaere et al., 1985) harboring the binary vector pBISN1 (carrying a uidA-intron on its T-DNA enabling the characterization of transient and stable expression of the reporter gene in plants; Nam et al., 1999). Leaves derived from plants treated with SA (5 mM) showed a significant reduction in GUS activity at 2, 5, and 10 d postinfection (dpi) when compared with mock-inoculated plants (Fig. 1, A and B). Stable transformation assays were performed on leaf discs of mock- and SA-treated plants using tumorigenic strain A348 (pCCT133; pTiA6NC; Garfinkel et al., 1981; bacterial concentration 10^7 cfu) as described (Anand et al., 2007b). A significant reduction in the biomass of the tumors (fresh and dry weight of the tumors incited on leaf discs infected with A348) was observed in the leaf discs derived from 5 mM SA-treated plants as compared with mock-treated plants (F test; P < 0.05; mock treatment, mean weight = 3.07/0.23 g; 5 mM SA, mean weight = 2.02/0.14 g; fresh/dry weights; n = 125). We therefore conclude that SA application partially blocks Agrobacterium-mediated plant transformation.

We quantified the free SA levels in the leaf tissues collected from SA-treated N. benthamiana plants using a quadrupole mass spectrometry system as described (Schmelz et al., 2004). SA levels in SA-treated (5 mM) plants showed 6.5-fold increase in endogenous free SA levels when compared with mock-treated plants (Fig. 1C). We also observed induction of PRIa (a marker for SA-mediated plant defense) following SA treatment (data not shown). Based on these results, we speculate that endogenous levels of SA may play a role in antagonizing Agrobacterium infection.

Transgenic Plants Expressing NahG Are Hypersusceptible to Agrobacterium Infection

The SA-dependent pathway has been analyzed in detail using transgenic plants expressing salicylate hydroxylase (NahG), which degrades SA to catechol (Gaffney et al., 1993; Delaney et al., 1994). NahG-expressing tomato (Solanum lycopersicum) plants show an increased susceptibility to viral and bacterial pathogens that is correlated with a block in expression of PRI (pathogenesis-related 1; Gaffney et al., 1993; Mur et al., 1997). To provide further evidence for the role of SA in Agrobacterium infectivity, wild-type and NahG-expressing tomato plants (Brading et al., 2000) were vacuum infiltrated with a low concentration (10^3 cfu) of the nonmutorigenic strain A. tumefaciens GV2260 carrying the binary vector pBISN1. We performed GUS activity assays at 2 dpi. NahG-expressing plants were more susceptible to Agrobacterium infection, as indicated by the increased X-Gluc staining and higher GUS activity when compared with the wild-type tomato plants (Fig. 1, D and E). These results, together with the reduced Agrobacterium infectivity on SA-treated plants, further suggest that SA plays a role in protecting plants against Agrobacterium infection.
SA Inhibits Agrobacterium Growth in Vitro and Affects Its Virulence

On the basis of our observation that the endogenous SA levels in plants affect Agrobacterium infectivity (Fig. 1), we hypothesized that SA may be an important determinant of Agrobacterium pathogenicity. Although direct effects of SA have been proposed on pathogens and symbiotic microorganisms, only a few case studies have indicated the direct role of SA on bacterial growth and virulence (Martínez-Abarca et al., 1998; Prithiviraj et al., 2005a, 2005b; Yuan et al., 2007). To test whether SA affects Agrobacterium growth or virulence, we carried out in vitro experiments in which SA was added into the culture media at physiologically relevant concentrations (Delaney et al., 1994; Prithiviraj et al., 2005b; Huang et al., 2006), and growth of A. tumefaciens A348, A208 (pCNL65; pTiT37C; Chilton et al., 1977) and Kat153 (GV2260 harboring pDSKGFPuv; Wang et al., 2007) was monitored in both the minimal and rich media. Recently, Yuan et al., (2007) reported that SA at lower concentrations (5–15 μM) did not affect the growth of the bacteria in the rich media (Supplemental Fig. S1). However, SA at low concentrations (5–15 μM) did not affect the growth of the bacteria in the rich media (Supplemental Fig. S2). SA at relatively higher concentrations (200 μM) impeded Agrobacterium growth in the rich media (Supplemental Fig. S2).

It has been suggested that salicylate plays a role in siderophore biosynthesis in bacteria (Neilands, 1995; Crosa and Walsh, 2002). However, the role of salicylate in bacterial iron metabolism is not clear. We tested whether exogenous application of iron sulfate had an effect on SA-mediated Agrobacterium growth inhibition. SA-mediated Agrobacterium growth inhibition was not significantly affected by addition of iron sulfate (0–150 mM) in the minimal media with SA (10 μM; Supplemental Fig. S3). However, agrobacteria grew more vigorously in the minimal media supplemented with iron sulfate (50 mM) when compared with the growth observed in the minimal media without iron, in the absence of SA (Supplemental Fig. S3). The effect of BTH (SA analog) and coronatine, a compound structurally similar to jasmonic acid, was also

Agrobacterium growth inhibition was observed when SA was supplemented in the minimal media (Supplemental Fig. S1). However, SA at low concentrations (5–15 μM) did not affect the growth of the bacteria in the rich media (Supplemental Fig. S2). SA at relatively higher concentrations (200 μM) impeded Agrobacterium growth in the rich media (Supplemental Fig. S2).

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tested. Neither BTH (up to 1 mM) nor coronatine (13.7–54.8 nM) affected bacterial growth and multiplication (data not shown).

To test further if SA directly affects Agrobacterium virulence, we carried out leaf disc infection assays (Anand et al., 2007a, 2007b) with A. tumefaciens A348 treated with SA. SA was incorporated into the induction media (50 μM and 100 μM plus acetosyringone [AS]) for 4 h, and the virulence of the SA-treated bacteria was compared with the uninduced (minus AS) and induced cultures (AS, 100 μM), respectively. The biomass and size of tumors incited by A. tumefaciens A348 (uninduced or induced with AS) were more than those of the tumors incited by A384 treated with SA (Fig. 2A; Supplemental Fig. S4). The tumor biomass of the infected leaf discs was at least 1.5- to 2-fold higher with untreated A. tumefaciens A348 when compared with the leaf discs infected with SA-treated A348 (Supplemental Fig. S4). Based on these results, we conclude that exogenous application of SA, at a concentration of 50 μM, is sufficient to reduce the virulence of Agrobacterium.

**Agrobacterium Treated with SA Is Defective in Attaching to Plant Cells**

The effects of SA on early events of transformation were further investigated using the bacterial attachment assay described previously (Anand et al., 2007b; Wang et al., 2007). We treated A. tumefaciens KAt153 with SA (50–100 μM) for 2 h, and washed the bacterial cells free of residual SA and used it to infect N. benthamiana leaf discs by cocultivating for 12 h in a nonselective basal medium. As detailed earlier, treatment of Agrobacterium with SA (50–100 μM) for 2 h had no effect on bacterial multiplication and viability in the nutrient-rich medium (Supplemental Fig. S2). SA at 100 μM affected Agrobacterium attachment (Fig. 2, B and C), whereas SA at 50 μM concentration did not (Fig. 2C). More GFP fluorescence was observed along the cut surface of leaf discs inoculated with Agrobacterium that was not treated with SA compared with GFP fluorescence observed with Agrobacterium treated with 100 μM SA (Fig. 2B). Consistent with these observations, 20- to 30-fold fewer bacteria were detected on leaf discs inoculated with KAt153 treated with SA (100 μM) when compared with the assays with untreated KAt153 (Fig. 2C). Agrobacteria treated with AS plus SA were also defective in attaching to the leaf discs (data not shown). These findings suggest that SA may affect the virulence of A. tumefaciens by interfering with the attachment of Agrobacterium to plant cells.

**SA Affects Agrobacterium Gene Expression**

To study the effect of SA on genome-wide changes in Agrobacterium gene expression, we compared the transcriptome of strain A208 cultured in the induction medium (without AS) with the transcriptome of strain A208 following AS or AS plus SA (50 μM) using the custom-made whole-genome Affymetrix microarrays. The microarray data suggest that SA treatment significantly affected the expression of the Ti plasmid genes (36 of the 37 genes were induced by AS at 4 h and 103 of the 172 genes were induced by AS at 24 h, respectively; Fig. 3; Supplemental Table S1). SA repressed the expression of the bacterial virulence (vir genes), the conjugal transfer (tra genes), and plasmid replication genes (repABC operon; Fig. 3), which is in accord with a recent report (Yuan et al., 2007). Using real-time quantitative reverse transcription-PCR (qRT-PCR), we confirmed the differential expression of few selected genes (Table I). The results were in accordance with the microarray data, except that the fold changes of all the genes tested were much higher in qRT-PCR when compared with the microarray data. We also confirmed the induction of two chromosomal encoded genes, Atu1525 and Atu0377, upon SA treatment. The Atu0972, Atu1550, and Atu3610 genes did not show any differential expression upon AS and SA treatments and were used as controls for qRT-PCR. Taken together, these results suggest that SA has multiple effects on Agrobacterium resulting in reduced virulence.

**Silencing of SA Biosynthetic and Signaling Genes in N. benthamiana Increases Susceptibility to Crown Gall Disease**

To investigate the role of SA in limiting Agrobacterium infectivity in planta, we performed in planta tumor assays on the stems of N. benthamiana silenced for NbNPR1 (nonexresser of PR gene 1; Cao et al., 1997), NbSABP2 (SA-binding protein 2; Kumar and Klessig, 2003), SIC5 (Wildermuth et al., 2001; Uppalapati et al., 2007), and GFP (control; GFP sequence does not have any homology to plant DNA and therefore will not cause gene silencing) as described (Anand et al., 2007b) using A. tumefaciens strain A348. We observed relatively larger tumors on the shoots of ICS-, NPR1-, and SABP2-silenced plants compared with the tumors on Tobacco rattle virus (TRV)::GFP-inoculated and wild-type plants (Fig. 4A). The down-regulation of the ICS, NPR1, and SABP2 genes in gene-silenced plants of N. benthamiana was confirmed by semi quantitative RT-PCR (Supplemental Fig. S5) and was in accord with the observations made by earlier workers (Ekengren et al., 2003; Kumar and Klessig, 2003). We also confirmed that the SA-mediated plant defense pathway was impaired in ICS-, NPR1-, and SABP2-silenced plants by monitoring the expression of PR1a (Supplemental Fig. S6).

The increased susceptibility of ICS-, NPR1-, and SABP2-silenced plants to Agrobacterium infection was further confirmed by performing leaf disc and transient transformation assays as described (Anand et al., 2007b). Two different bacterial concentrations (107 and 108 cfu of the tumorigenic strain A348) were used to detect the differences in the tumors incited on leaf discs of gene-silenced and control plants. The lower bacterial concentration (107 cfu) yielded larger tumors
and correspondingly higher tumor biomass in ICS-, NPR1-, and SABP2-silenced plants as compared with control plants (Fig. 4, B and C; Supplemental Fig. S7A). A similar trend was observed even with a higher bacterial concentration (10^9 cfu; data not shown). In the transient transformation experiments, we observed significantly higher levels of GUS activity at 2 and 5 dpi in the leaf discs derived from ICS-, NPR1-, and SABP2-silenced plants (Fig. 4, D and E). These results indicate that SA biosynthetic and signaling genes also play a significant role in antagonizing Agrobacterium infection. To determine the effect of gene silencing on cell division, leaf discs (uninoculated with Agrobacterium) of the silenced plants (3 weeks postsilencing) were cultured on a nonselective callus-inducing medium (CIM) for 4 weeks. Uninfected leaf discs of ICS-, NPR1-, and SABP2-silenced plants formed calli on CIM, similar to leaf discs of nonsilenced control plants (Supplemental Fig. S7B). These data suggest that gene silencing of ICS, NPR1, and SABP2 had no observable effect on the cell division and cell proliferation potential of these plant cells.

BTH-Induced SAR Impairs Agrobacterium Infectivity

We characterized the involvement of SAR in imparting resistance against crown gall disease by exogenously applying BTH to N. benthamiana and tomato plants prior to inoculation with tumorigenic strain A348. BTH can mimic SA and induces disease resistance in various host-pathogen systems (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996; Aehou et al., 2004). Our preliminary experiments suggested that N. benthamiana and tomato differed in their ability to tolerate BTH. Tomato plants treated with 1 mM BTH were stunted, whereas no growth defects were observed at a similar concentration in N. benthamiana plants (data not shown). Therefore, BTH was exogenously supplied at different concentrations: 1 mM on N. benthamiana and 0.1 to 0.33 mM on tomato plants. BTH did not induce SA accumulation in N. benthamiana in three independent experiments (Fig. 5A). Upon inoculation with A. tumefaciens +348, considerably smaller tumors were observed on shoots of BTH-treated N. benthamiana and tomato plants when compared with tumors observed on mock-treated plants (Fig. 5, B and C). In N. benthamiana, the average size of tumors (length and width) was 7.8 ± 1.2 mm (0.1 mM, BTH) and 18.3 ± 2.3 (mock-treated plants), while in tomato the tumors averaged 6.8 ± 1.0 mm (0.1 mM) to 4.0 ± 0.7 mm (0.3 mM) in the BTH-treated plants as compared with the larger tumors (23.5 ± 6.9 mm) on the mock-treated plants, respectively.

Figure 2. Effect of SA on Agrobacterium virulence and attachment to plant cells. A, Exogenous application of SA to Agrobacterium attenuates its capacity to incite tumors on leaf discs of N. benthamiana. The leaf disc tumorigenesis assays, as described (Anand et al., 2007a, 2007b), were performed with strain A348 induced with AS in the presence or absence of SA (50 μM). The virulence of A348 treated with SA was attenuated as seen from the reduced number of tumors incited when compared with the tumors produced by strain A348 induced in the SA minus medium. Pictures were taken 4 weeks postinfection. B and C, Agrobacterium attachment assay was performed as described (Anand et al., 2007b) with the disarmed strain A. tumefaciens KAt153 (carrying the binary vector pDSKGFPuv) that was mock or SA treated. Leaf discs derived from N. benthamiana plants were incubated with Agrobacterium and the fluorescent bacteria expressing GFPuv attached to the leaf tissues were visualized, as bacterial colonies, along the cut surfaces after 12 h of cocultivation, using a Leica TCS SP2 AOBS confocal system (left panel: GFP fluorescence; right panel: epifluorescence image). C, Quantification of attached bacteria. SA-treated (50 or 100 μM) agrobacteria that were attached to leaf discs were quantified using serial dilution plating as described (Anand et al., 2007b). Bacterial numbers are mean values for three independent experiments with five replicates each.
The effects of BTH on Agrobacterium-mediated plant transformation were further assessed by performing stable and transient transformation assays as described (Anand et al., 2007b) on wild-type, TRV::GFP-inoculated, and ICS-silenced plants following mock or BTH treatment. ICS-silenced plants were partially impaired in SA biosynthesis and did not show a significant increase in endogenous SA levels following BTH treatment (data not shown). We further confirmed that the SAR pathway is activated upon BTH treatment by monitoring the expression of PR1a (Supplemental Fig. S6). Tumors produced on leaf discs derived from both silenced and wild-type plants were smaller in BTH-treated plants as compared with tumors seen on leaf discs derived from mock-treated plants (Fig. 5, D and E). Leaves treated with BTH showed a significant reduction in GUS activity at 2 and 5 dpi in both silenced and wild-type plants (Fig. 5, F and G). On the basis of the above results, we suggest that BTH application partially blocks transient transformation and SAR plays a role in protecting plants from Agrobacterium infection.

**DISCUSSION**

The major goal of this study was to characterize the role of plant defenses against Agrobacterium infection. The broader implication of this study is to manipulate plant genes for effectively combating crown gall disease and to increase transformation efficiency in recalcitrant crops. We provide direct and indirect evidence to support that both SA and SA-mediated plant defenses play a key role in determining Agrobacterium infectivity on plants. The direct effects of SA on Agrobacterium-mediated plant transformation were assessed by exogenous application of SA to plants. SA application on plants induces endogenous SA accumulation accompanied by activation of SAR genes, which play an important role in conferring resistance to different pathogens (for review, see Ryals et al., 1996; Vallad and Goodman, 2004). The larger crown galls produced in N. benthamiana plants silenced for ICS, NPR1, and SABP2 (only partially knocked down for gene expression) further suggest that SA-mediated plant defenses (Ross, 1961) are important determinants for Agrobacterium infection. Molecularly, SA-mediated plant defense signaling requires NPR1, which interacts with many TGA transcriptional factors.

The Ti plasmid and a few chromosomal genes that were differentially regulated at 4 and 24 h by AS (A4 and A24) and AS plus SA (A+SA4 and A+SA24) for cluster analyses. Data for selected genes were transformed into log2 and gene tree was generated by hierarchical clustering using TMEV (http://www.tm4.org/mev.html). Color codes represent the differential gene expression values, wherein red and green represent the up- and down-regulation of genes, respectively. The genes highlighted in red were selected for validation by quantitative real-time PCR.

Figure 3. Clustering of the differentially expressed Agrobacterium genes upon treatment with AS and SA. We selected all the genes on the Ti plasmid and a few chromosomal genes that were differentially regulated at 4 and 24 h by AS (A4 and A24) and AS plus SA (A+SA4 and A+SA24) for cluster analyses. Data for selected genes were transformed into log2 and gene tree was generated by hierarchical clustering using TMEV (http://www.tm4.org/mev.html). Color codes represent the differential gene expression values, wherein red and green represent the up- and down-regulation of genes, respectively. The genes highlighted in red were selected for validation by quantitative real-time PCR.
to modulate the expression of SAR-dependent genes, including PR proteins (for review, see Shah, 2003). We observed larger tumors on the NPR1-silenced plants due to the increased Agrobacterium infectivity in these plants, which further suggests a role for SAR signaling in Agrobacterium-mediated pathogenesis. The above data contradict a recent observation reported by Yuan et al. (2007) but are in accord with the results obtained by another group (S. Gelvin, personal communication). The differences in plant species and plant tissues used in the infection assays could have contributed to this discrepancy. SABP2 has strong affinity to conjugated SA, such as methyl salicylate, and is crucial for plant innate immunity (Kumar and Klessig, 2003; Forouhar et al., 2005; Park et al., 2007). Furthermore, ICS has been shown to be required for SA accumulation, PRI induction, and SAR (Wildermuth et al., 2001; Uppalapati et al., 2007). In accord with the above findings, we observed that NahG-expressing tomato plants were more susceptible to transformation by Agrobacterium. Interestingly, Gaspar and colleagues (Gaspar et al., 2004) observed significant reduction in free SA levels in Arabidopsis roots upon Agrobacterium infection. It was further hypothesized that the plant response to Agrobacterium appears to be dependent on relative SA levels following infection rather than the absolute levels in the uninfected plants (Gaspar et al., 2004). The increased genetic transformation in the ICS-silenced plants, which are partially impaired in SA biosynthesis, led us to conclude that SA plays an important role in Agrobacterium infectivity. Furthermore, we showed that, independent of SA, SAR is an important determinant in Agrobacterium infectivity on plants through the reduced crown gall disease incidence in the BTH-treated Nicotiana benthamiana and tomato plants. In Nicotiana tabacum, Arabidopsis, and wheat (Triticum aestivum), BTH does not increase SA biosynthesis but induces the same set of SAR genes as induced by SA (Gaffney et al., 1993; Friedlich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996). We therefore conclude that both endogenous SA levels and SAR are critical determinants of Agrobacterium pathogenicity in plants.

Recently, it was demonstrated that application of pathogen-associated molecular patterns (PAMPs) such as flagellin and EF-Tu, which are key activators of plant innate immunity (Zipfel and Felix, 2005; Ingle et al., 2006; Jones and Dangl, 2006), suppresses Agrobacterium-mediated plant transformation, suggesting that PAMPs play an important role in orchestrating plant innate immunity against Agrobacterium (Zipfel et al., 2006). Although Agrobacterium-derived flagellin is inactive as an elicitor in plants, we cannot rule out that other Agrobacterium PAMPs, such as EF-Tu, could activate plant defenses restricting Agrobacterium-mediated plant transformation (Zipfel et al., 2006). Moreover, PAMPs were shown to contribute significantly to SAR initiation and activation in Arabidopsis (Mishina and Zeier, 2007). This provides a scenario wherein SA is likely induced in plants in response to and recognition of Agrobacterium PAMPs, leading to activation of SAR and induced defenses.

Accumulation of SA is associated with many physiological and immune responses in plants (Raskin, 1992; Shah, 2003). There are several studies suggesting that endogenous SA in plants plays a critical role in pathogenesis by its direct effects on the pathogen (for review, see Shah, 2003). However, only limited studies have demonstrated the direct effects of SA on phytopathogens in vitro. SA at concentrations of 150 μM and above showed strong effect on M. loti growth at 4 dpi (Stacey et al., 2006), whereas concentrations of 25 μM did not significantly affect growth of S. meliloti over prolonged incubation periods (Martinez-Abarca et al., 1998). Furthermore, in the S. meliloti-alfalfa (Medicago sativa) interaction, 25 μM SA inhibited nodulation (Martinez-Abarca et al., 1998), whereas 100 μM SA had no apparent effect on root nodulation by M. loti on L. japonicus (van Spronsen et al., 2003). Yuan and coworkers (Yuan et al., 2007) recently demonstrated that SA at very low concentrations (8 μM and above) significantly affects the growth of Agrobacterium in acid-
ified minimal media, but had no growth effects under neutral conditions. We further showed that the growth effects seen on Agrobacterium in the presence of SA are not due the indirect effects resulting from alterations in iron metabolism. A. tumefaciens is known to synthesize a number of siderophores (Ong et al., 1979; Hiroyuki et al., 2002), and recently a siderophore biosynthetic gene cluster was identified from C58 allowing them to grow under low iron conditions (Rondon et al., 2004).

In this study, we show a correlation between endogenous SA levels in SA-treated plants and reduced susceptibility to Agrobacterium infection. Besides triggering the defense responses, SA had direct effects on Agrobacterium fitness and virulence and therefore plays a central role in Agrobacterium-plant interactions. Consistent with these results are the observations that exogenously applied SA retards the infectivity of S. meliloti on alfalfa (Martínez-Abarca et al., 1998) and down-regulates virulence factors of P. aeruginosa (Prithiviraj et al., 2005b). SA is also shown to have an effect on attachment of P. aeruginosa and S. aureus to glass surfaces and their virulence on Arabidopsis (Prithiviraj et al., 2005a, 2005b). However, we cannot rule out the possibility that the reduced Agrobacterium virulence and attachment on SA-treated plants are a result of elicitation of plant defense responses by SA.

The Affymetrix Agrobacterium whole-genome arrays facilitated us to characterize the effects of SA on Agrobacterium virulence under laboratory conditions. We used two different time points (4 and 24 h) to monitor the effects of SA on the transcriptome of Agrobacterium and this is different from the recently reported study (Yuan et al., 2007). Our transcript profiling experiment validated the data recently published (Cho and Winans, 2005; Yuan et al., 2007). We believe that the interference in the induction of the bacterial sensory system, virulence, plasmid replication, and conjugal transfer could have significantly

**Figure 4.** In planta tumorigenesis and leaf disc transformation assays in the gene-silenced plants of N. benthamiana. A, The in planta tumor assay was performed as described (Anand et al., 2007b) on ICS-, NPR1-, and SABP2-silenced and control (TRV: GFP) N. benthamiana plants. Shoots of control and silenced plants were inoculated with the tumorigenic strain A. tumefaciens A348 and the tumors were photographed 6 weeks after inoculation. B and C, Quantification of stable transformation. Axenic leaf discs derived from control and gene-silenced plants were inoculated with A. tumefaciens A348 and were incubated on hormone-free Murashige and Skoog medium. Four weeks after inoculation, the fresh and dry weights of infected leaves were measured. D and E, Quantification of transient transformation. The leaf discs derived from gene-silenced plants were inoculated with the disarmed strain A. tumefaciens GV2260 (carrying the binary vector pBISN1) and GUS activity was determined as described (Anand et al., 2007b) at 2 and 5 dpi. The experiments were replicated three times with a minimum of 100 leaf discs for each gene-silenced plant and the data indicate the average with st values. Letters indicate significant difference using Fisher's LSD test at $P < 0.05$. 

TRV: GFP  TRV: ICS  TRV: NPR1  TRV: SABP2

TRV: GFP  TRV: ICS  TRV: NPR1  TRV: SABP2

TRV: GFP  TRV: ICS  TRV: NPR1  TRV: SABP2

TRV: GFP  TRV: ICS  TRV: NPR1  TRV: SABP2

TRV: GFP  TRV: ICS  TRV: NPR1  TRV: SABP2
contributed to reduced Agrobacterium infectivity in plants inoculated with SA-treated Agrobacterium.

SA also shares structural similarity with other natural inhibitors of vir gene induction, such as indole-3-acetic acid (Liu and Nester, 2006). We speculate that SA, like other natural inhibitors of vir gene induction, namely, indole-3-acetic acid and other benzoxazinones, competes with AS (Zhang et al., 2000; Liu and Nester, 2006). It has been proposed that the simplest way to down-regulate vir gene expression is to com-
pette physically with the phenolic inducer (Liu and Nester, 2006). Based on our transcript profiling data, we speculate that SA competes with AS for direct or indirect interaction with VirA. One issue we have not directly answered in this study relates to the concentration of SA in planta or in the crown galls to which Agrobacterium would be exposed. However, we demonstrated that the exogenous application of SA results in increased endogenous SA levels and retards Agrobacterium infectivity in SA-treated plants. It is likely that the SA levels reached in microenvironments around an infected cell could be much higher than the total measurable SA levels detected in whole infected or systemic leaves (Huang et al., 2006). There are also reports suggesting that SA can accumulate to >70 μM at the site of infection in other host-pathogen systems (Shirasu et al., 1997). It still warrants further investigation to determine if the SA concentrations used in this study are biologically relevant during Agrobacterium—plant interactions in nature. Nevertheless, it is still interesting to note that SA had a direct effect, under laboratory conditions, on Agrobacterium growth and virulence. The results presented in this article support the hypothesis that a direct role for SA in plant defense is possibly articulated by Agrobacterium through down-regulation of the de novo biosynthesis of SA or by modulating plant basal defenses (Gaspar et al., 2004). Therefore, exogenous application of SA or its analogs before the onset of crown gall disease presents a possible means for achieving durable disease control.

**MATERIALS AND METHODS**

**Plant Treatment, Virus-Induced Gene Silencing, and Transformation Assays**

Nicotiana benthamiana, NahG-expressing tomato (Solanum lycopersicum; Brading et al., 2000), and wild-type tomato (‘Moneymaker’) plants were used in the experiments. Details for plant growth and maintenance were as described (Anand et al., 2007a, 2007b). SA (sodium salt; Sigma-Aldrich) was used in the experiments. Details for plant growth and maintenance were as described (Anand et al., 2007a, 2007b). Briefly, Agroinoculations for VIGS were performed using the leaf infiltration method (Orton et al., 2008). For virus-induced gene silencing (VIGS), TRV-based VIGS vectors containing the nicafeica gene (SlICS1, GenBank accession no. EH386450) and tomato corresponding homologs of various plant defense-related genes were obtained from Dr. Dinesh-Kumar, Virginia Bioinformatics Institute (http://agro.vbi.vt.edu/public/). A. tumefaciens strain A208 (pCLN65; pTiT7; napaline-type Ti plasmid) was grown in LB medium, pelleted, washed with saline (0.9% NaCl), and resuspended in induction medium (Gelvin, 2006) supplemented with or without BTH treatment (72 hpi) to perform the leaf disc tumorigenesis assays.

**RNA Extraction and PCR**

RNA extraction, first-strand cDNA synthesis, and semiquantitative RT-PCR on plant samples (N. benthamiana) were performed using standard protocols as described (Anand et al., 2007b). The effectiveness of VIGS and BTH application was tested in the gene-silenced plants of ICS, SABP2, and NPR1 by semiquantitative RT-PCR. The primers used for detecting the relative transcripts of NPR1 and SABP2 are detailed elsewhere (Eckgren et al., 2003; Kumar and Klessig, 2003), while the primer combination IC5F 5′-ATCTTAAACTCATATCCACCAGC-3′ and ICSR 5′-GCAGGCTTCGCCGGCATT-CATTGGC-3′ was used for detecting the relative transcripts of ICS in the gene-silenced plants. The BTH- and SA-induced expression of SAR genes was monitored by profiling the expression of PR1a at 0 h, 48, 72, and 7 dpi using the primer combination NbPR1aF 5′-GTTCTCTTTTCAACATTGCC-3′ and NbPR1aR 5′-CGTAGGGTGGTTAATTGCC-3′.

**SA Quantification**

N. benthamiana leaves (approximately 300 mg) were used to extract SA as described earlier (Schmelz et al., 2004) using a quadrupole mass spectrometry system (5890 GC; Agilent) connected to a 5989B mass selective detector (Agilent) with selective-ion monitoring (selected ion ± 0.5 mass unit) in electron spray ionization mode. SA was separated on a RTx-5 column (30 m × 0.25 mm × 0.25 mm; Restek) using the conditions described earlier (Schmelz et al., 2004; Uppalapati et al., 2007). The pH of the plant tissue in the extraction reagent was carefully adjusted to pH 2 to 3 with concentrated hydrochloric acid to recover the acidic phytohormones. The retention times and mass units of the methyl esters analyzed were: SA-ME, 8.35 min, 152; and [2H6]SA-ME, 9.18 min, 156. Isotopically labeled SA was purchased from CDN Isotopes.

**Effect of Exogenous Application of SA on A. tumefaciens**

For determining the effect of SA on bacterial multiplication, three different strains of A. tumefaciens (A348, A208, and KAt153), Sinorhizobium meliloti AB57, Escherichia coli DH5a, and Pseudomonas syringae pv. glycinea 718 were selected for the study. The Agrobacterium strains were grown in either AB medium; S. meliloti in tryptone yeast medium; P. syringae in King’s B medium), unless otherwise stated. For the growth experiments, all the bacterial cultures were grown to OD600 approximately 1, diluted 1:10 (OD600 approximately 0.1) into fresh media in the presence or absence of SA (5–200 μM), and grown in 20-mL culture tubes, respectively. The possible effect of SA on bacterial multiplication was tested by taking 100-μL aliquots, measuring the OD600 at regular intervals with four replicates up to 48 h posttreatment. For studying the effects of iron on Agrobacterium growth, Fe(SO4)2·4H2O salts were dissolved in water, filter sterilized, and incorporated into AB minimal media with or without SA at concentrations ranging from 0 to 150 mg/L. Agrobacterium virulence and attachment assays were performed as described (Anand et al., 2007b). Briefly, Agrobacterium suspensions (106 cfu/mL) were treated with or without SA (50–100 μM) in the induction media containing or lacking AS (100 μM), washed free of SA, and allowed to infect leaf tissues.

**Agrobacterium Arrays and Transcript Profiling**

The whole Agrobacterium C58 genome arrays were custom designed from Affymetrix containing 7,862 probes representing all the known or predicted genes, intergenic regions, and controls. The probe sequences were also annotated using genome annotation information provided by Virginia Bioinformatics Institute (http://agro.vbi.vt.edu/public/). A. tumefaciens strain A208 (pCLN65; pTiT7; napaline-type Ti plasmid) was grown in LB medium, pelleted, washed with saline (0.9% NaCl), and resuspended in induction medium (Gelvin, 2006) supplemented with or without AS (100 μM) or AS plus SA (50 μM), and were allowed to grow for 4 and 24 h, respectively. The initial transient transformation assays, axenic leaf discs derived from control and gene-silenced plants were inoculated with A. tumefaciens A348 or GV2260 (carrying the binary vector pBI121), and incubated on hormone-free Murashige and Skoog medium or CIM, respectively, as described (Anand et al., 2007b). Leaves were collected from the gene-silenced and control plants with or without BTH treatment (72 hpi) to perform the leaf disc tumorigenesis assays.

**Arrays and Transcript Profiling**

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Validation of the Agrobacterium Arrays

The Agrobacterium array data were validated for a set of differentially expressed genes identified from the transcriptome analysis representing genes on different chromosomes, namely, Atu0377, Atu0972, Atu1550, Atu1525, Atu2022, and Atu2283 (circular chromosome); Atu3610 and Atu3707 (linear chromosome); and Atu6164, Atu6168, Atu6182, Atu6188, and Atu6190 (Ti plasmid) using qRT-PCR. Random primed first-strand cDNA was generated from 3 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen). Each reaction was performed in triplicate plus a negative control using a 7900HT fast real-time PCR system (Applied Biosystems). Power SYBR Green PCR Master Mix (Applied Biosystems) was used for the PCR reaction according to the manufacturer’s protocol, except that 0.1 µL primers were used in a final volume of 10 µL. The primer details are provided as supporting data (Supplemental Table S2). The data from two of the biological replicates used for microarray analysis and an independent third biological replicate each with three technical replicates (n = 9) were normalized to endogenous control Atu0972 (Anand et al., 2007a). Atu0972 was not differentially expressed in response to AS and/or SA treatment (data not shown). The comparative CT method, as described (Pfaffl, 2001), was used for quantifying the relative expression ratio in the wild-type strain of A. tumefaciens A208 in the presence or absence of AS or AS plus SA.

Data Analysis and Gene Clustering

Leaf disc transformation data were subjected to ANOVA using JMP software Version 4.0.4 (SAS Institute) or by ANOVA. When significant results were obtained at P < 0.05, separation of treatment means was determined by Fisher’s protected LSD. For microarray analysis, the CEL file for each sample was exported from the Genechip Operating System program (Affymetrix). Between-chip normalization was conducted using robust multichip average (Bolstad et al., 2003). Differentially expressed genes were selected using Associative Analysis as described (Dozmorov and Centola, 2003). Type I family-wise error rate was reduced using the Bonferroni corrected P-value threshold of 0.05/N, where N represents the number of genes present on the chip. The false discovery rate was reduced using the Bonferroni corrected P-value threshold of 0.05/N, where N represents the number of genes present on the chip. The false discovery rate was reduced using the Bonferroni corrected P-value threshold of 0.05/N, where N represents the number of genes present on the chip. The false discovery rate was reduced using the Bonferroni corrected P-value threshold of 0.05/N, where N represents the number of genes present on the chip. The false discovery rate was reduced using the Bonferroni corrected P-value threshold of 0.05/N, where N represents the number of genes present on the chip.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effect of SA on Agrobacterium growth in different media and various concentrations of SA.

Supplemental Figure S2. Effect of SA on Agrobacterium viability.

Supplemental Figure S3. Exogenous incorporation of SA (50–100 µM) in agroinduction medium with AS attenuates the tumors incited on the leaf discs of N. benthamiana plants.

Supplemental Figure S4. Semiquantitative RT-PCR analyses confirms the down-regulation of ICS, NPR1, and SAB2 gene transcripts in the gene-silenced plants of N. benthamiana.

Supplemental Figure S5. Semiquantitative RT-PCR analyses confirm the induced expression of PR1a upon BTH treatment in the gene-silenced plants.

Supplemental Figure S6. Leaf disc assays to characterize the effect of gene silencing on leaf tumorigenesis by Agrobacterium and on cell division.

Supplemental Table S1. The differential expression of selected genes from Agrobacterium in response to SA.

Supplemental Table S2. The primer combinations used for validating the microarray data by qRT-PCR.

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