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Overexpression of AtSGT1, an Arabidopsis salicylic acid glucosyltransferase, leads to increased susceptibility to *Pseudomonas syringae*

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Abstract

We reported previously that a recombinant salicylic acid (SA) glucosyltransferase1 (AtSGT1) from *Arabidopsis thaliana* catalyzes the formation of both SA 2-*O*-β-D-glucoside (SAG) and the glucose ester of SA (SGE). Here, transgenic Arabidopsis plants overexpressing *AtSGT1* have been constructed, and their phenotypes analyzed. Compared to wild-type plants, transgenic plants showed an increased susceptibility to *Pseudomonas syringae* and reduced the accumulation levels of both free SA and its glucosylated forms (SAG and SGE). On the other hand, the overexpression increased the levels of methyl salicylate (MeSA) and methyl salicylate 2-*O*-β-D-glucoside (MeSAG), and also induced *SA carboxyl methyltransferase1* (*AtBSMT1*) expression, whose products catalyze the conversion of SA to MeSA. Our data indicate that reduced resistance by *AtSGT1* overexpression results from a reduction in SA content, which is at least in part caused by increases in MeSAG and MeSA levels at the expense of SA. Our study also suggests that genetic manipulation of AtSGT1 can be utilized as an important regulatory tool for pathogen control.

Keywords: *Arabidopsis thaliana*; Plant disease resistance; Salicylic Acid (SA); SA glucosyltransferase; SA conjugate; SA metabolite

1. Introduction

Plant defense against microbial attack such as *Pseudomonas syringae* is associated with the production of salicylic acid (SA) (**1**) (Fig. 1) leading to induction of a battery of *pathogenesis-related* (*PR*) genes (Rassmussen et al., 1991). The importance of SA (**1**) as a key component of a signal transduction pathway in local defense and systemic resistance of plants has been well documented. Exogenous application of SA (**1**) can induce resistance to *P. syringae* (Ryals et al., 1996) whereas depletion of endogenous SA (**1**) by employing the bacterial SA hydroxylase (NahG) gene can increase susceptibility to the pathogen (Delaney et al., 1994; Gaffney et al., 1993).

In plants, a portion of SA (**1**) applied either exogenously or produced endogenously during a defense response is metabolized to conjugated forms mainly because of the presence of glucosylation and methylation activities in the

Abbreviations: AtBSMT1, *Arabidopsis thaliana* SA carboxyl methyltransferase1; AtSGT1, *Arabidopsis thaliana* SA glucosyltransferase1; MeSA, methyl salicylate; MeSAG, methyl salicylate 2-O-β-D-glucoside; SA, salicylic acid; SAG, SA 2-O-β-D-glucoside; SGE, glucose ester of SA.

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cell (Lee et al., 1995). Glucose conjugation at either the hydroxyl or the carboxyl group of SA (**1**) forms the SA glucoside (SA 2-*O*-β-D-glucoside, SAG (**3**); Fig. 1) as a major conjugate, or the SA glucose ester (SGE, **2**) as a minor one, respectively (Dean et al., 2005; Edwards, 1994; Enyedi et al., 1992; Lee and Raskin, 1998). These conjugation reactions are catalyzed by SA glucosyltransferases (SA GTs) that are induced by SA (**1**) application or pathogen attacks in tobacco plants (Lee and Raskin, 1999). In addition, SA GT activities have been detected in many plants, including tobacco and soybean (Dean et al., 2003, 2005; Edwards, 1994; Enyedi et al., 1992; Lee and Raskin, 1998; Tanaka et al., 1990). A significant portion of SA (**1**) is also converted to a volatile methyl ester of SA, methyl salicylate (MeSA, **4**), when the leaves are infected by tobacco mosaic virus (TMV) or *P. syringae* (Huang et al., 2003; Shulaev et al., 1997). In Arabidopsis, a SA carboxyl methyltransferase (AtBSMT1) responsible for the conversion of SA (**1**) to MeSA (**4**) is induced by either methyl jasmonate (MeJA) or *P. syringae* infection (Chen et al., 2003; Koo et al., 2007).

Many studies have focused on biological roles of MeSA (**4**) and SAG (**3**) as an airborne signal and a probable storage form of SA (**1**) (Edwards, 1994; Lee and Raskin, 1998; Seskar et al., 1998; Shulaev et al., 1997). Recently, studies on SA (**1**) metabolism in tobacco cell suspension cultures demonstrates that the three major metabolites, MeSA (**4**), SAG (**3**), and MeSA 2-*O*-β-D-glucoside (MeSAG, (**5**); Fig. 1), were derived from added [^{14}C]SA (**1**) (Dean et al., 2005). MeSAG (**5**) has also been detected in soybean cell cultures and the fruits of some *Passiflora* species (Chassagne et al., 1997; Dean et al., 2003). MeSAG (**5**) may serve as a non-volatile storage form that releases MeSA (**4**) during pathogenesis, or that may protect the plant cell from high intracellular levels of MeSA (**4**) in response to pathogen infection as does SAG (**3**) to SA (**1**) (Dean et al., 2005). However, the enzyme responsible for the formation of MeSAG (**5**) has not yet been identified.

Our previous data indicates that a recombinant SA glucosyltransferase from Arabidopsis, AtSGT1, catalyzed the formation of glucosyl SAs (SAG (**3**) and SGE (**2**)) *in vitro* and its corresponding gene expression was rapidly induced by SA or *P. syringae* infection (Song, 2006). In this report, we show that overexpression of *AtSGT1* leads to increased susceptibility to the pathogen which can be attributed to the reduced level of free SA (**1**). Interestingly, glucosyl SA level was also reduced during pathogen infection whereas MeSA (**4**) and MeSAG (**5**) levels were increased, probably due to increased levels of *AtBSMT1* and *AtSGT1*, respectively.

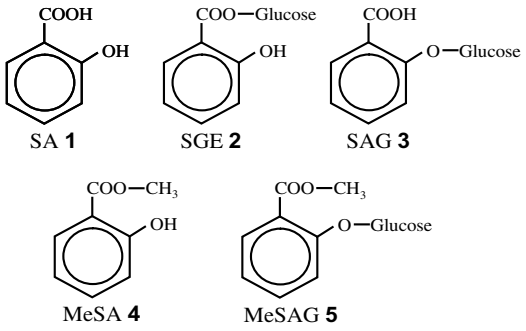

Fig. 1. Structures of salicylic acid (SA (**1**)) and its metabolites. SAG (**2**), SA 2-*O*-β-D-glucoside; SGE (**3**), glucose ester of SA; MeSA (**4**), methyl salicylate; MeSAG (**5**), methyl salicylate 2-*O*-β-D-glucoside.

2. Results

2.1. Overexpression of AtSGT1 in Arabidopsis increases disease susceptibility

To investigate the biological effect of AtSGT1 on both SA (**1**) metabolism and plant defense, transgenic Arabidopsis plants were generated, where expression of *AtSGT1* was under the control of *CaMV 35S* promoter. Northern blot analysis was performed to determine the steady-state levels of *AtSGT1* transcripts in each of fourteen independent lines (Fig. 2A). All the lines showed a much higher level of *AtSGT1* mRNA compared to wild-type plants, and among them two lines were selected as representatives for high (line 7, AtSGT1-7) and low (line 10, AtSGT1-10) expressors. We also determined the relative enzymatic activities of SA glucosyltransferases in AtSGT1-7, AtSGT1-10 and wild-type plants (Fig. 2B). The levels of both

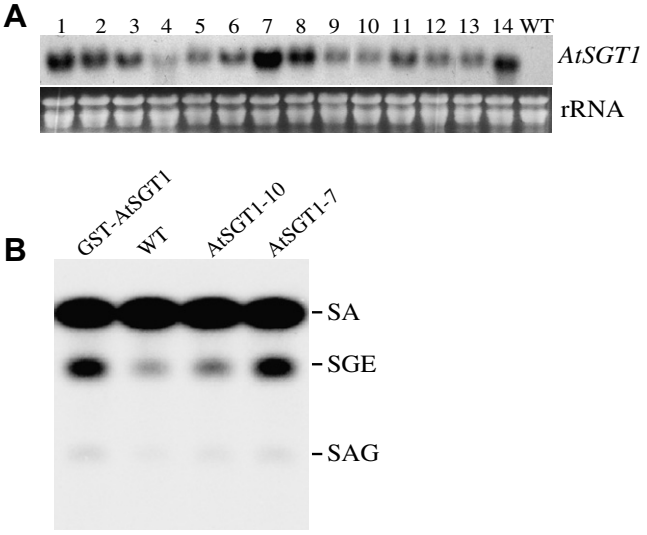

Fig. 2. Levels of *AtSGT1* mRNA and activities of SA glucosyltransferase in transgenic plants. A. Expression of *AtSGT1* in the *AtSGT1*-overexpressing plants. Total RNAs from an untransformed control (WT) and 14 independent transgenic lines containing the *AtSGT1* gene under the control of the *CaMV 35S* promoter were extracted and hybridized using an *AtSGT1* DNA probe. B. Relative activities of SA glucosyltransferases in transgenic plants. Soluble crude extracts from leaves of wild-type, AtSGT1-10 and AtSGT1-7 plants were incubated with [^{14}C]SA (**1**) and UDPG, and the reaction mixtures were analyzed by TLC. As a control, recombinant GST-AtSGT1 protein was purified, incubated with [^{14}C]SA (**1**) and then loaded onto TLC.

SGE (**2**) and SAG (**3**) were increased in transgenic plants, compared to those of wild-type, indicating that the increased level of *AtSGT1* mRNAs indeed resulted in its increased enzymatic activities.

To examine the role of AtSGT1 in disease resistance, wild-type and *AtSGT1*-overexpressing plants were inoculated with either the virulent (DG3) or avirulent (DG6 carrying the *avrRpt2* gene) pathogen *P. syringae* pv. *maculicola* strain. Leaves of transgenic AtSGT1-7 plants showed either more severe water-soaking or chlorotic symptoms than those of wild-type plants did on day 3 after infection (Fig. 3A). AtSGT1-10 plants also showed stronger symptoms than wild-type plants. *P. syringae* DG3 grew better by at least 10- or 5-fold, in the AtSGT1-7 or AtSGT1-10 plants, respectively, than in wild-type plants on days 2 and 3 after infection (Fig. 3B). Similar pattern of growth was also observed with avirulent bacteria (Fig. 3B). All these results indicate that overexpression of *AtSGT1* increases susceptibility to the pathogen *P. syringae*, which is very consistent with the fact that both timing and level of *PR1* gene expression was delayed and reduced in the AtSGT1-7 and AtSGT1-10 plants, compared to those of wild-type plants (Fig. 4).

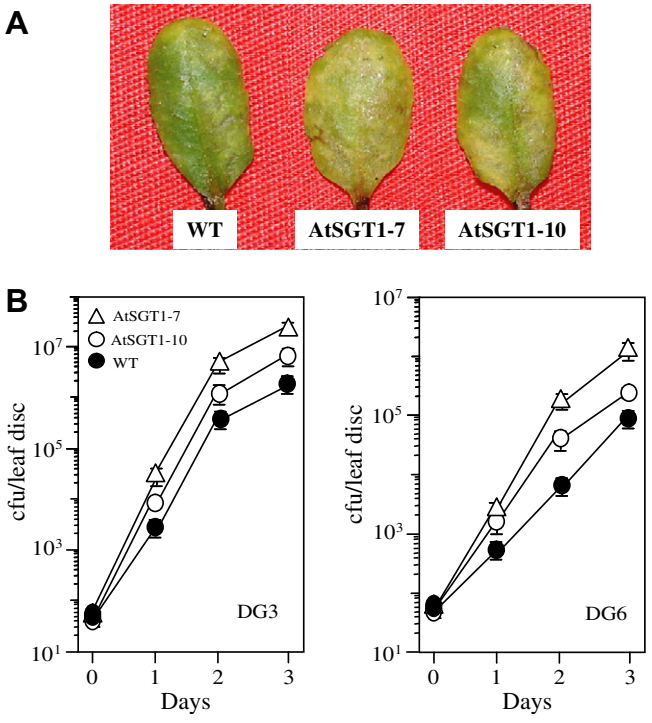

Fig. 3. Bacterial growth of *P. syringae*. A. Enhanced disease susceptibility of *AtSGT1*-overexpressing plants. Wild-type and *AtSGT1*-overexpressing plants were infected with the virulent pathogen *P. syringae* DG3 at $OD_{600} = 0.0001$. Infected leaves were photographed after 3 days of inoculation. B. Bacterial growth in transgenic plants. Plants were infected with *P. syringae* DG3 (virulent, left) or DG6 (avirulent, right) at $OD_{600} = 0.0001$. Growth of *P. syringae* in the AtSGT1-7 (triangles), AtSGT1-10 (open circles) and wild-type (closed circles) plants was significantly different on days 2 and 3 ($P < 0.01$, *t*-test, $n = 8$). cfu, colony-forming units. Bars indicate standard error.

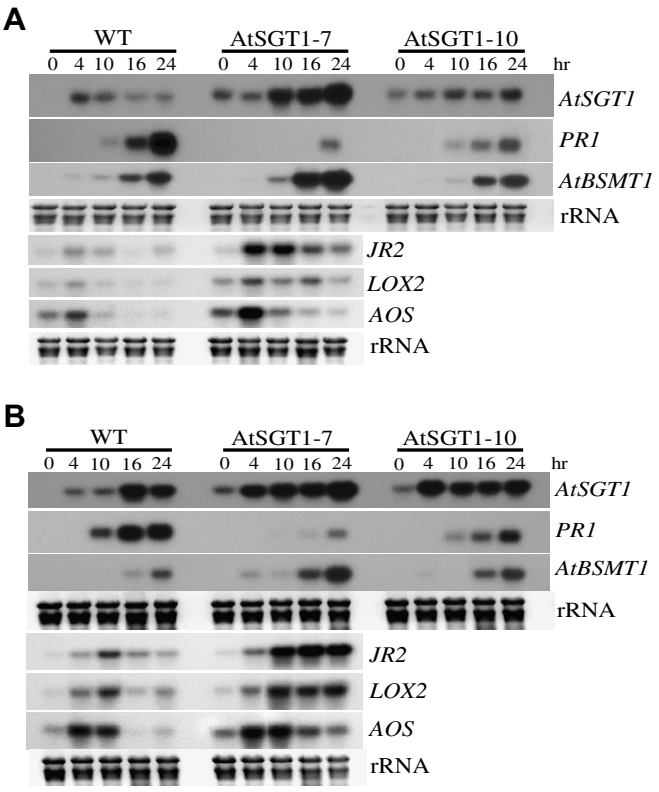

Fig. 4. mRNA expression profiles of *AtSGT1*, *PR1*, *AtBSMT1*, *JR2*, *LOX2* and *AOS*. Leaves (fourth and fifth) from 20-d-old plants were infected with *P. syringae* DG3 (A) or DG6 (B) at $OD_{600} = 0.01$.

2.2. Overexpresion of AtSGT1 reduces SA (*1*) accumulation

We anticipated that the *AtSGT1* overexpressors would lower levels of free SA (**1**) and higher contents of glucosyl SA, since overexpression of the *AtSGT1* gene would stimulate production of glucosyl SA at the expense of free SA (**1**). Indeed, the level of SA (**1**) was markedly reduced in the AtSGT1-7 plants at all time points tested after infection with either *P. syringae* DG3 or DG6 (Fig. 5), indicating that the increased susceptibility to the pathogen is at least partially attributable to decrease in free SA (**1**) content in the transgenic plants. Unexpectedly, the level of glucosyl SA was also greatly decreased in AtSGT1-7 plants. It should be noted that the basal levels of both free and glucosyl SA were decreased by about 2-fold in the AtSGT1-7 plants, compared to those of wild-type. After infection with virulent pathogen DG3, wild-type plants showed about 3-fold higher levels of free and glucosyl SA than those of the AtSGT1-7 plants (Fig. 5A). This observation was more obvious when plants were inoculated with avirulent pathogen DG6 (Fig. 5B).

2.3. Overexpression of AtSGT1 induces accumulation of MeSA (*4*) and MeSAG (*5*)

Recently, Koo et al. (2007) showed that Arabidopsis transgenic plants overexpressing *OsBSMT1*, a SA carboxyl methyltransferase (SA MT) gene isolated from rice,

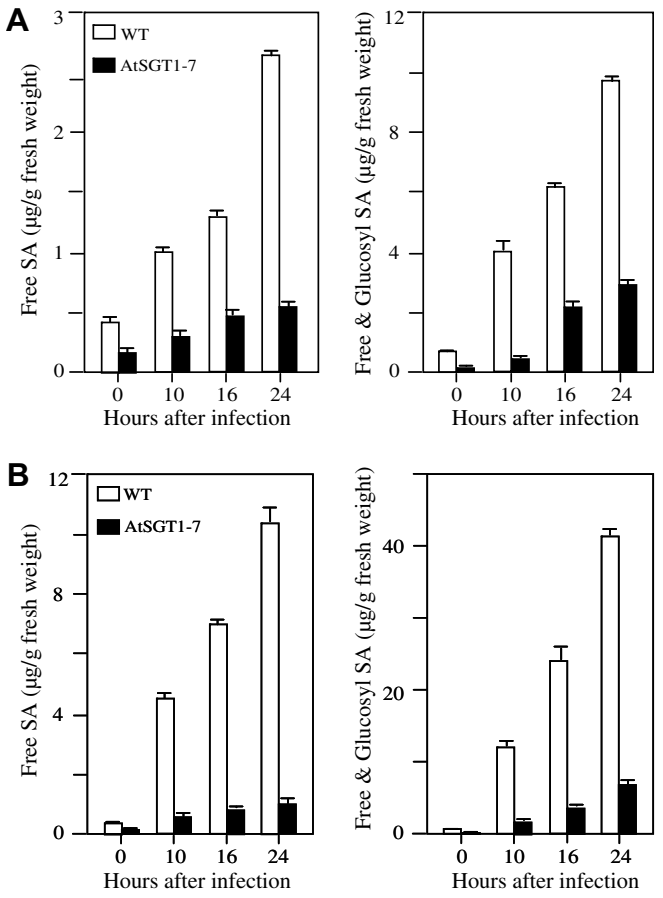

Fig. 5. Free and glucosyl SA levels in transgenic plants during pathogen infection. Leaves (fourth and fifth) from 20-d-old plants were infected with *P. syringae* DG3 (A) or DG6 (B) at $OD_{600} = 0.01$ and were harvested, extracted, and analyzed by HPLC. SA levels in the AtSGT1-7 and wild-type plants were significantly different at all time points tested ($P < 0.01$, *t*-test, $n = 8$). Bars indicate standard errors of three sets of samples.

increased MeSA production and induced expression of JA-responsive genes such as *AtBSMT1, JR2* (*JA-RESPONSIVE GENE2*), *LOX2* (*LIPOXYGENASE2*) *and AOS* (*ALLENE OXIDE CYCLASE*). *LOX2* and *AOS* encode two key enzymes in the octadecanoid pathway leading to JA biosynthesis (Creelman and Mullet, 1997). They also demonstrated that, in *OsBSMT1*-overexpressing Arabidopsis plants, pathogen attacks induced a reduction in SA level with concomitant accumulation of JA-responsive gene expression and JA production, suggesting that SA MT plays a role in the cross-talk between SA and JA signaling pathways.

With respect to the possible cross-talk, it was reasoned that reduction in free SA (**1**) level in *AtSGT1* overexpressors might induce *AtBSMT1, JR2, LOX2* and *AOS* expression. Indeed, northern blot analysis showed that their expressions were induced to a higher level in the *AtSGT1*-overexpressors than in wild-type plants after *P. syringae* infection (Fig. 4). These data indicate that the reduction in free SA (**1**) content in the overexpressors increased JA signaling, probably at least in part by increase in JA production.

To test whether the increased level of *AtBSMT1* expression leads to accumulation of MeSA or not, we determined the levels of MeSA (**4**) and MeSAG (**5**) in transgenic AtSGT1-7 and wild-type plants before and 18 h after inoculation with the avirulent bacterial pathogen *P. syringae* DG6. The basal levels of MeSA (**4**) and MeSAG (**5**) in the AtSGT1-7 plants increased about 2- and 1.5-fold, respectively, compared to wild-type plants (Fig. 6A). MeSA (**4**) and MeSAG (**5**) levels at 18 h after infection were about seven times higher in the AtSGT1-7 plants than in wild-type plants.

Because the *AtSGT1*-overexpressing plants increased MeSAG (**5**) contents during pathogenesis, we wondered whether or not AtSGT1 might use MeSA (**4**) as alternative substrates to synthesize MeSAG (**5**). To test the possibility, recombinant AtSGT1 proteins, fused to glutathione-*S*-transferase (GST), were expressed in *Escherichia coli* cells,

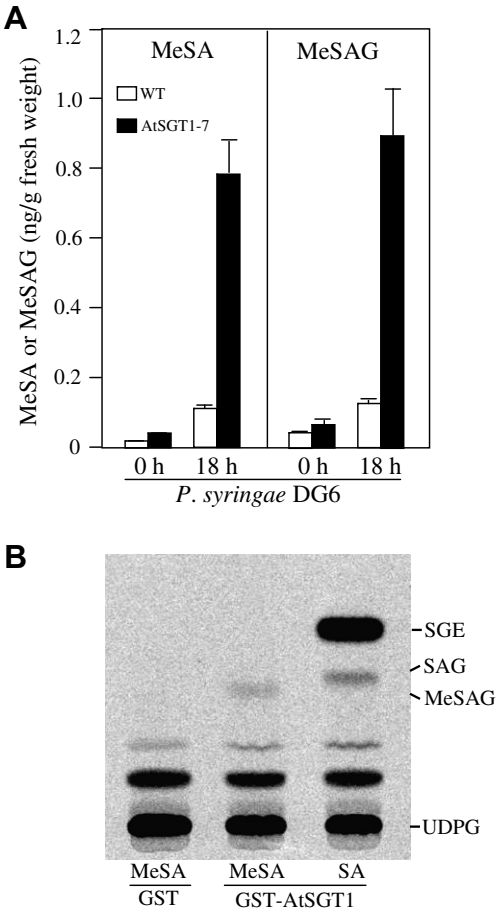

Fig. 6. MeSA (**4**) and MeSAG (**5**) levels in plants. A. MeSA (**4**) and MeSAG (**5**) levels in transgenic plants during pathogen infection. Leaves (fourth and fifth) from 20-d-old plants were infected with avirulent pathogen *P. syringae* DG6 at $OD_{600} = 0.01$ and then were harvested 18 h after inoculation. Bars indicate standard errors of three sets of samples. B. Formation of MeSAG (**5**) by recombinant AtSGT1. The recombinant GST-AtSGT1 construct and pGEX-5T-1 (vector) were introduced into *E. coli* BL21, and GST-AtSGT1 or GST protein expression was induced by IPTG treatment. Purified GST-AtSGT1 or GST protein was incubated with UDP-[^{14}C] glucose and MeSA (**4**) (or SA (**1**)), and the reaction mixtures were analyzed by TLC.

purified, and subjected to glucosyltransferase assays. A substantial amount of [^{14}C] MeSAG (**5**) was produced when GST-AtSGT1 fusion proteins were added into the reaction mixture containing UDP-[^{14}C] glucose and MeSA (**4**) (Fig. 6B). No synthesis of MeSAG (**5**) was detected with GST proteins alone. These results indicate that the recombinant AtSGT1 protein was able to synthesize MeSAG (**5**) by using MeSA (**4**) as a substrate. It seems, however, that the recombinant AtSGT1 protein has greater catalytic activities to SA (**1**) than to MeSA (**4**), as SAG (**3**) was produced to a 3-fold higher level than MeSAG (**5**) (Fig. 6B).

3. Discussion

A complex network of events comprising synthesis, catabolism, transport and inactivation of secondary metabolites or hormones is involved in the regulation of homeostasis in plants. Many enzymes and their corresponding genes that are involved in those metabolic pathways of SA (**1**) have been identified and characterized (Delaney, 2005; Lee et al., 1995; Lim and Bowles, 2004), although a clear understanding of how the processes are regulated on a cellular level has not been achieved yet.

Based on a phylogenetic analysis of sequences in the Arabidopsis genome and biochemical data of their recombinant proteins, two genes, *AtSGT1* (*At2g43820*) and *At2g43840*, were chosen as possible candidates responsible for production of glucosyl SA (Lim et al., 2002; Song, 2006). However, we previously showed that no full-length product for *At2g43840* was obtained from reverse transcriptase (RT)-PCR analysis (Song, 2006). Moreover, we did barely detect its RT-PCR products in response to the *P. syringae* infection (data not shown). Therefore, it is likely that the accumulation of glucosyl SA upon pathogen infection might be attributable to expression of *AtSGT1* but not to the *At2g43840* gene.

Our purpose in this study is to investigate the action of the SA glucosyltransferases and physiological relevancy of their activities in defense response of plants. To address these questions, we constructed and analyzed the *AtSGT1*-overexpressing Arabidopsis. Upon *P. syringae* infection, the *AtSGT1*-overexpressing plants accumulated less amounts of free SA (**1**) than wild-type plants did, resulting in increased susceptibility to the pathogen, whereas, surprisingly, the overexpressors produced less amount of glucosyl SA as well (Fig. 5). One possible interpretation for the discrepancy is that free SA (**1**) might be shunted to other metabolites, including to MeSA (**4**) and MeSAG (**5**), rather than to SAG (**3**). The notion is consistent with the fact that overexpression of *AtSGT1* increased the level of *AtBSMT1* expression, accompanied by an increase in the amount of MeSA (**4**) and MeSAG (**5**) after pathogen infection (Figs. 4 and 6). In fact, affinity of AtBSMT1 enzyme to SA (**1**) is greater than that of AtSGT1. The K_m values of AtSGT1 and AtBSMT1 for SA (**1**) are 190 μM and 16 μM, respectively (Chen et al., 2003; Song, 2006). Furthermore, the increased production of MeSAG (**5**) may be due to constitutive expression of *AtSGT1* because the recombinant AtSGT1 does have glucosyltransferase activities to produce MeSAG (**5**). Another possibility for more MeSAG (**5**) production is that AtBSMT1 or other methylltransferases might catalyze the formation of MeSAG (**5**) from SAG (**1**).

However, in transgenic plants, the increments of MeSA (**4**) and MeSAG (**5**) explain only a minor portion of the decrements of free and glucosyl SA (Figs. 5 and 6). This difference might be due to the conversion of free and glucosyl SA to another metabolite such as benzoic acid (BA), methylbenzoate (MeBA) or glucosyl BA because the recombinant AtSGT1 protein has the highest glucosylating activity to BA *in vitro* (Chen et al., 2003; Song, 2006). This prompted us to compare the endogenous levels of BA, glucosyl BA, and MeBA in the AtSGT1-7 and wild-type plants. We found no significant difference in the levels of those three metabolites under either normal growth conditions or pathogen infection (data not shown), indicating that it was not the case. Another possibility is that, given its high volatility, MeSA (**4**) may evaporate into the air shortly after synthesis by AtBSMT1 during pathogenesis, which was not examined.

Finally, we cannot rule out the possibility that AtSGT1 overexpression may cause disturbances in the regulatory network of SA (**1**) homeostasis in the cell, making it difficult to predict the role of *AtSGT1* in disease response.

4. Concluding remarks

In conclusion, we constructed the *AtSGT1*-overexpressing plants to investigate SA (**1**) metabolism and its role in defense response, and demonstrated that overexpression of *AtSGT1* was able to alter the defense responses to *P. syringae* by changing the free SA level. Our results suggest the possibility that *AtSGT1* could be a target gene for genetic manipulation to regulate defense responses of crops to various pathogens. This report is the first demonstration that MeSAG (**5**) is not only present in Arabidopsis but also accumulated in response to pathogen infection, although MeSAG (**5**) has been detected in soybean cell culture, tobacco cell culture, and fruits of *Passiflora* species (Dean et al., 2003, 2005; Chassagne et al., 1997). Further detailed study will provide new insight into the regulation of SA (**1**) homeostasis as well as the biological importance of MeSAG (**5**) in the aspects of plant development and defense.

5. Experimental

5.1. Construction of plant expression vectors and plant transformation

All Arabidopsis plant materials were in the Columbia background. For the *AtSGT1*-overexpressing plants, the

AtSGT1 cDNA (Genbank accession no. DQ407524) was cloned to the binary vector pAOV containing the *CaMV 35S* promoter for plant transformation (Mylene and Botella, 1998). The *AtSGT1*-overexpressing plants were selected on soil by spraying with BASTA (Bayer, Korea). Transgenic lines overexpressing *AtSGT1* are freely available upon request.

5.2. Pathogen infection

For pathogen infection, plants were grown in soil at 22 °C under 14 h light/10 h dark. During infection, plants were covered with a plastic dome to maintain the relative humidity. Bacterial strains used were derived from *P. syringae* pv. *maculicola* strain ES4326; strains had *avrRpt2* (DG6) or a vector control (DG3) chromosomally intergrated at the *recA* locus (Guttman and Greenberg, 2001; provided by Dr. Jean T. Greenberg, University of Chicago). Bacterial culturing, syringe inoculations and growth curve procedures were performed as previously described (Greenberg et al., 2000).

5.3. Determination of endogenous levels of free SA (*1*) and glucosyl SA

Free SA (**1**) and glucosyl SA were extracted and quantified as previously described (Seskar et al., 1998).

5.4. Determination of endogenous levels of MeSA (*4*) and MeSAG (*5*)

Determination of MeSA (**4**) and MeSAG (**5**) was quantified with minor modification of Engelberth et al. (2003). Leaf tissue samples (1 g fresh weight) were frozen in liquid N_2, ground to a fine powder, and extracted with 50 mM citric acid (4 ml in water/acetone (30/70 [v/v]) containing 100 ng of methyl-*O*-anisate as an internal standard. Samples were sonicated for 15 min at RT and then centrifuged at 4000*g* for 5 min. The acetone of the supernatant was evaporated under air stream. The remaining citric acid phase was extracted with Et_2O (2 × 2 ml) by vortexing and phase separation. The ether phase was transferred to a 4-ml screw-cap glass vial and the volatile compounds were trapped on a Super Q filter under a constant air stream, and then eluted with CH_2Cl_2 (150 μl) from the adsorbent. For determination of MeSAG (**4**), the remaining citric acid phase after extraction with Et_2O was hydrolyzed by adding 5 mM sodium acetate buffer (1 ml, pH 5.5) containing β-glucosidase (40 units/g of fresh weight), and then incubated overnight at 37 °C. After adding 100 ng of methyl-*O*-anisate, extraction was performed with Et_2O (2 × 2 ml) by vortexing and phase separation. The Et_2O phase was transferred and trapped on a Super Q filter under a constant air stream, and then eluted with CH_2Cl_2 (150 μl) from the adsorbent. The eluent was analyzed by GC–MS as described (Engelberth et al., 2003).

5.5. Preparation of crude extract

Leaves were ground in liq N_2 and resuspended in 1.5 volumes of extraction buffer containing 60 mM NaOAc/pH5.3, 1 mM KCl, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin. The extract was cleared by centrifugation at 12,000 rpm for 15 min. The supernatant was assayed for enzyme activity, and protein concentration was determined using the BCA Protein Assay Kit (Pierce, USA).

5.6. Glucosyltransferase activity assay

The enzyme assay for AtSGT1 activity was described previously (Song, 2006). UDP-[^{14}C] glucose (GE Healthcare Bio-Sciences, USA) was added to the reaction mixture containing 2 mM of either SA (**1**) or MeSA (**4**). After reaction, the samples were applied to a K6F thin-layer plate (Whatman Inc., USA) and developed with 1-BuOH/AcOH/H_2O (4/1/1, v/v/v). The chromatogram was autoradiographed using the BAS-1500 system (Fuji, Japan). A labeled spot corresponding to MeSAG (**5**) was scraped off the plate, dissolved in MeOH (500 μl) and dried in a speed vacuum. The identity of the labeled product was confirmed by GC–MS analysis following treatment with β-glucosidase that can hydrolyze MeSAG (**5**) to MeSA (**4**).

5.7. Northern blot analysis

Total RNAs were isolated as described by Kroczek and Siebert (1990). Eight micrograms of the total RNAs were separated by electrophoresis on a 1% agarose gel, transferred on to nylon membrane and then hybridized as previously described (Song, 2006). Gene-specific DNA fragments for *AtSGT1*, *AtBSMT1*, *JR2*, *LOX2*, *AOS* and *PR1* were labeled with [α-^{32}P]dATP and used as probes.

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