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Molecules and Cells

The Expression Patterns of *AtBSMT1* and *AtSAGT1* Encoding a Salicylic Acid (SA) Methyltransferase and a SA Glucosyltransferase, Respectively, in Arabidopsis Plants with Altered Defense Responses

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We reported previously that overexpression of a salicylic acid (SA) methyltransferase1 gene from rice (*OsBSMT1*) or a SA glucosyltransferase1 gene from *Arabidopsis thaliana* (*AtSAGT1*) leads to increased susceptibility to *Pseudomonas syringae* due to reduced SA levels. To further examine their roles in the defense responses, we assayed the transcript levels of *AtBSMT1* or *AtSAGT1* in plants with altered levels of SA and/or other defense components. These data showed that *AtSAGT1* expression is regulated partially by SA, or nonexpressor of pathogenesis related protein1, whereas *AtBSMT1* expression was induced in SA-deficient mutant plants. In addition, we produced the transgenic *Arabidopsis* plants with RNAi-mediated inhibition of *AtSAGT1* and isolated a null mutant of *AtBSMT1,* and then analyzed their phenotypes. A T-DNA insertion mutation in the *AtBSMT1* resulted in reduced methyl salicylate (MeSA) levels upon *P. syringae* infection. However, accumulation of SA and glucosyl SA was similar in both the *atbsmt1* and wild-type plants, indicating the presence of another SA methyltransferase or an alternative pathway for MeSA production. The *AtSAGT1*-RNAi line exhibited no altered phenotypes upon pathogen infection, compared to wild-type plants, suggesting that (an)other SA glucosyltransferase(s) in *Arabidopsis* plants may be important for the pathogenesis of *P. syringae*.

INTRODUCTION

Salicylic acid (SA) is a well-known signaling molecule in plants playing a critical role in regulating local defense and systemic acquired resistance (SAR) against microbial attack such as *Pseudomonas syringae* (Kim et al., 2008). The accumulation of SA is associated with the subsequent induction of genes including those encoding pathogenesis-related (PR) proteins (Loake and Grant, 2007). Direct support for the importance of SA in plant disease resistance has come from the observation that transgenic tobacco plants expressing a bacterial SA hydroxylase (NahG) gene depleted endogenous SA and, consequently, generated susceptibility (Delaney et al., 1994; Gaffney et al., 1993). Moreover, a number of mutants with reduced accumulation or signal transduction of SA are also disease-susceptible. The resistance-compromised mutants include the *npr1* (*nonexpressor of pathogenesis related protein1*) that blocks the SA-dependent signaling pathway (Cao et al., 1994), the *pad4* (*phytoalexin deficient4*) that partially blocks defense signaling and SA accumulation (Zhou et al., 1998) and the *sid2* (*salicylic acid induction-deficien2*) that cannot synthesize SA to respond to pathogen attack (Wildermuth et al., 2001). Further, numerous mutants with constitutively high SA levels displayed increased resistance. In particular, the *acd6* (*accelerated cell death6*) and *agd2* (*aberrant growth and death2*) mutants have phenotypes including spontaneous cell death, constitutive disease resistance and high accumulation of SA without pathogen infection (Rate et al., 1999; 2001). The crossing mutants *acd6-nahG* and *agd2-nahG* caused depletion of SA accumulation and abolished disease-resistance to *P. syringae*.

In plants, modification of SA during a defense response mainly results from glucosylation and methylation (Lee et al., 1995; Fig. 1). Many studies on the SA metabolism have revealed that methyl salicylate (MeSA), a volatile ester, is likely to be present as an airborne signal to activate defense responses in neighboring plants or a long-distance mobile signal in SAR (Baldwin et al., 2006; Park et al., 2007; Shulaev et al., 1997), whereas the glucosyl SAs including SA 2-*O*-β-D-glucoside (SAG) and SA glucose ester (SGE) can exist as a probable

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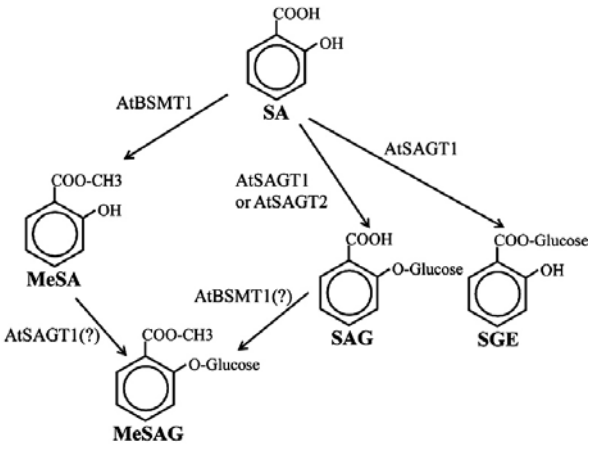

Fig. 1. Structures of salicylic acid (SA) and its metabolites. SAG, SA 2-*O*-β-D-glucoside; SGE, glucose ester of SA; MeSA, methyl salicylate; MeSAG, methyl salicylate 2-*O*-β-D-glucoside. AtSAGT1 catalyzes the biosynthesis of both SAG and SGE whereas AtSAGT2 makes only SAG. AtBSMT1 catalyzes the biosynthesis of MeSA. Formation of MeSAG from SAG or MeSA may be by AtBSMT1 or AtSAGT1, respectively. Recombinant AtSAGT1 can catalyze the in vitro formation of MeSAG from MeSA (Song et al., 2008).

storage form of SA (Edwards, 1994; Lee and Raskin, 1998). Recent data showed that the three major metabolites, MeSA, SAG and MeSA 2-*O*-β-D-glucoside (MeSAG), are present in tobacco cell suspension culture cells by conversion of added [^{14}C]SA (Dean et al., 2005). In Arabidopsis, MeSA is synthesized by a SA carboxyl methyltransferase (AtBSMT1), of which the gene is induced by methyl jasmonate (MeJA) or *P. syringae* infection (Chen et al., 2003; Koo et al., 2007; Song et al., 2008). Arabidopsis plants overexpressing *OsBSMT1* from rice showed that accumulated MeSA and reduced SA in those plants resulted in increased susceptibility to *Golovinomyces orontii* and *P. syringae*. The glucosyl SAs are catalyzed by SA glucosyltransferases (SA GTs). In Arabidopsis, two SA GT enzymes were reported. The first AtSAGT1 [formerly named AtSGT1 by Song (2006); designated UGT74F2 by Lim et al. (2002)] forms SAG and SGE whereas the second AtSAGT2 (UGT74F1) only forms SGE. The AtSAGT1 was induced by SA or *P. syringae* infection in *Arabidopsis* plants, whereas AtSAGT2 was not (Song, 2006). Overexpression of *AtSAGT1* in *Arabidopsis* plants led to reduced SA, increased MeSA and MeSAG, and susceptibility to *P. syringae* (Song et al., 2008).

Here, we report expression patterns of *AtBSMT1* and *AtSAGT1* in disease-resistant or -susceptible plants with altered SA accumulation or resistance signaling. The characteristics of *AtBSMT1*-knockout plants and *AtSAGT1*-RNAi transgenic plants are also presented in the aspects of defense responses as well as expression of the genes.

MATERIALS AND METHODS

Plant materials and construction of transgenic plants

All Arabidopsis plants were in the Columbia background. *sid2-2* was from Frederic M. Ausubel (Harvard Medical School, USA) and *nahG* (a *NahG*-overexpressing line) was from J. Ryals (Ciba-Geigy Agricultural Biotechnology, Research Triangle Park, NC). *pad4-1* was from Jane Glazebrook (University of Minnesota, USA) and *npr1-1* was from the Arabidopsis Biological Resource Center (stock number CS3726, http://www.arabidopsis.org). *agd2* and *acd6-1* were from Jean T. Greenberg (University of Chicago, USA). A T-DNA insertion line of *AtBSMT1* (SALK_140496) was from the Arabidopsis Biological Resource Center. To isolate the homozygous *atbsmt1* mutant of mutant, PCR was performed with two sets of primers. Primers for wild-type plants were salk140496-LB (5′-TGTTCTTT-CTATCTATATGTTGA-3′) and salk140496-RB (5′-TGGTGA-GTTTTTGTTCACATGTTGG-3′). Primers for the T-DNA insertion line were LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′) and salk140496-RB. For the *AtSAGT1*-RNAi plants, the partial coding region (nucleotides 535-1051 of the cDNA clone, Genbank accession no. DQ407524) was cloned, flanking a GUS coding region, and inserted into the binary vector twice, in opposite orientations. The *AtSAGT1*-RNAi transgenic plants were selected on soil by spraying with Basta (Bayer, Korea).

Pathogen infection

Bacterial culturing, syringe inoculations and growth curve procedures were performed as previously described (Song et al., 2008).

Determination of SA metabolites

Free and glucosyl SA were extracted and quantified as previously described (Seskar et al., 1998). MeSA was determined with minor modification of Engelberth et al. (2003) as previously described (Song et al., 2008).

Northern blot analysis

Total RNAs were isolated as described by Kroczek and Siebert (1990). Ten micrograms of the total RNAs were separated by electrophoresis on a 1% agarose gel and hybridized as previously described (Song, 2006). A gene-specific DNA probe for *AtSAGT1*, *AtBSMT1* or *PR1* was labeled with [α-^{32}P]dCTP.

RESULTS AND DISCUSSION

Expression patterns of *AtBSMT1* and *AtSAGT1* in Arabidopsis plants with reduced SA signaling

AtBSMT1 and *AtSAGT1* induced by *P. syringae* infection are two major genes for metabolizing SA in plants, and alter the amount of SA when they are overexpressed in plants (Koo et al., 2007; Song et al., 2008). Therefore it is important to understand the regulation of these two genes to obtain the information about SA metabolism. In order to understand how AtBSMT1 or AtSAGT1 is regulated during a disease response, we examined its transcript levels after *P. syringae* infection in *pad4*, *npr1*, *nahG* and *sid2* plants with defense-signaling defects. *AtBSMT1* expressions in *pad4*, *nahG* and *sid2* plants with reduced SA levels were much higher than those in *npr1* and wild-type plants (Fig. 2). This indicates that low accumulation of SA cause more strong induction of *AtBSMT1*, which is consistent with previous reports that reduction in SA levels resulting from overexpression of *OsBSMT1* or *AtSAGT1* in Arabidopsis plants induced *AtBSMT1* as well as other JA-responsive genes (Koo et al., 2007; Song et al., 2008). *AtBSMT1* expression was induced by JA, wounding or *P. syringae* infection, but not by SA (Chen et al., 2003; Koo et al., 2007). As suggested previously that the elimination of SA makes JA signals strong by antagonistic effects (Koo et al., 2007), inductions of *AtBSMT1* in SA-depleted mutants were much higher than that in wild-type plants. Furthermore, as shown in *npr1* mutants, the blocking of SA signaling slightly decreased *AtBSMT1* induction. Therefore these results indicate that the induction of the *AtBSMT1* gene was regulated by JA signaling and was also antagonistically affected by SA level but not mainly by the SA signaling mediated by *npr1*.

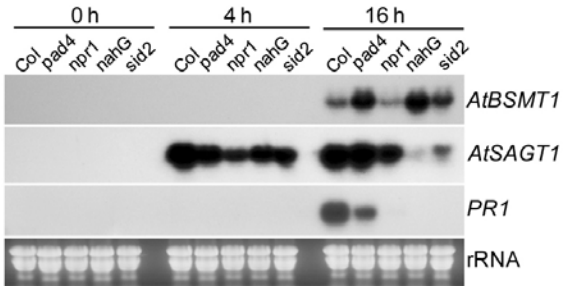

Fig. 2. RNA expression of *AtBSMT1*, *AtSAGT1*. and *PR1* during disease responses to *P. syringae* in plants with defense signaling defects. Leaves (fourth and fifth) from 20-d-old plants of the indicated genotypes were inoculated with *P. syringae* at OD_{600} = 0.01.

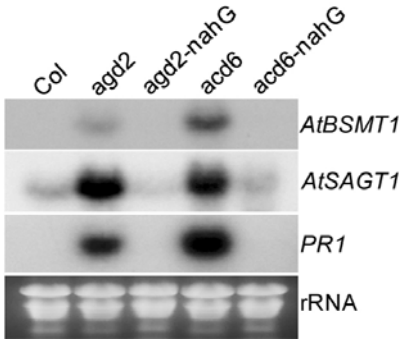

Fig. 3. RNA expression of *AtBSMT1*, *AtSAGT1*, and *PR1* in plants with altered SA levels. Leaves (fourth and fifth) from 20-d-old plants of the indicated genotypes were harvested for RNA analysis.

AtSAGT1 inductions after pathogen infection was reduced in *pad4*, *npr1*, *nahG* and *sid2* plants at all time points tested, compared to that of the wild-type plants. *AtSAGT1* is one of the early responsive genes upon *P. syringae* infection (Song, 2006) as shown in Fig. 2. In response to the bacterial pathogen, transcript levels of *AtSAGT1* 4 h after infection were modestly lower in all plants with defense-signaling defects. At 16 h after infection, the transcripts in *nahG* and *sid2* plants decreased rapidly, compared to those of *pad4*, *npr1* and wild-type plants. These data indicate that *AtSAGT1* expression is regulated partially by SA, PAD4 or NPR1.

Expression patterns of AtBSMT1 and AtSAGT1 in constitutively SA-producing plants

To further define the requirements for AtBSMT1 and AtSAGT1 regulation during a disease response, we examined the expression levels of two genes in plants with elevated glucosyl SA levels such as *agd2* or *acd6* plants (Rate et al., 1999; 2001). Both *AtBSMT1* and *AtSAGT1* were strongly expressed in the constitutively defensive *agd2* and *acd6* plants (Fig. 3). In SA-depleted *agd2-nahG* and *acd6-nahG* plants, their expressions were suppressed at the basal level. This suggests that a high level of glucosyl SA in *agd2* and *acd6* plants may be due to the constitutive expression of *AtSAGT1*.

In addition, *AtBSMT1* expression was also slightly elevated in these mutants. This can be in line with the induction of *AtBSMT1* by *P. syringae* infection. Although there are not any reports about the MeSA levels in these plants, it is possible that elevated levels of MeSA may be present in *agd2* and *acd6* plants due to higher expressions of AtBSMT1, compared to those in wild type plants.

The AtBSMT1-knockout plants cause reduced accumulation of MeSA during a disease response

To examine the role of AtBSMT1 in disease resistance, we isolated a T-DNA insertion mutant of *AtBSMT1* (Fig. 4A). To determine if the T-DNA insertion affected the level of *AtBSMT1* expression, we performed Northern blot analysis with total RNAs of *atbsmt1* homozygotes and those from the wild-type plants (Fig. 4B). We found that the accumulation of *AtBSMT1* transcripts in the wild-type plants was increased after *P. syringae* infection, whereas no detectable up-regulation of *AtBSMT1* expression was observed in the *atbsmt1* mutant. To examine the pathogen resistance of this mutant, the *atbsmt1* and wild-type plants were inoculated with *P. syringae*. However, there were no significant differences in bacterial growth between two plants (Fig. 4C). In addition, *PR1* expression in the *atbsmt1* mutants was similar to those in wild-type plants (Fig. 4B). Finally, we examined SA, MeSA and glucosyl SA levels upon infection. No significant difference was found in the levels of free and glucosyl SA in the *atbsmt1* and wild-type plants under either normal growth condition or pathogen infection (Fig. 4D). However, MeSA levels were different between the *atbsmt1* and wild-type plants. At the point of 24 h after infection, MeSA was highly accumulated in wild-type plants whereas it stayed in a basal level in *atbsmt1* mutants. These data indicate that blocking of MeSA production can not lead the pathogen resistance because the reduced amount of MeSA in *atbsmt1* mutants did not affect to the free or glucosyl SA levels. Very recently, Attaran et al. (2009) showed that MeSA production is not essential for local and systemic defense responses upon *P. syringae* infection in Arabidopsis, consistent with these results.

In addition, AtBSMT1 is not fully responsible for MeSA production, because basal level of MeSA was still remaining in this mutant although AtBSMT1 had a crucial role for MeSA production in pathogen infection. It could be suggested that (an) other SA methyltransferase gene(s) can exist in Arabidopsis plants. Another possibility is that the MeSA production may come from demethylation of MeSAG by an esterase(s), because MeSAG and transcripts of some esterase genes can be accumulated upon pathogen infection (Song et al., 2008; Vlot et al., 2008), even though the specific activities of MeSA formation by the esterase enzyme(s) were not determined.

RNAi-mediated inhibition of AtSAGT1 expression did not alter the phenotype of wild-type plants upon pathogen infection

AtSAGT1 that catalyzes the formation of glucosyl SAs (SAG and SGE) from SA can be important for storage of SA in disease resistance, because *AtSAGT1* expression is early induced by SA treatment and pathogen infection (Song, 2006). To discover the more informative role of AtSAGT1 in plant defense response, RNA interference (RNAi) lines were produced. One (line 6, named AtSAGT1-i6) out of six independent lines was selected due to much lower expression of *AtSGT1* when applied SA exogenously (Fig. 5A). We expected that almost complete silencing of *AtSAGT1* leads to increased resistance to *P. syringae* probably because of blocking to the major metabolites of SA. However, upon infection free and glucosyl SA levels in the AtSAGT1-i6 plants were similar to those in wild-type plants (Fig. 5B), and the AtSAGT1-i6 plants were not resistant or susceptible to the pathogen (Fig. 5C). Consistently, *PR1* expression was also not altered in transgenic plants (Fig. 5D). Our data can imply that glucosyl SA production in Arabidopsis plants in response to the pathogen infection may be due not to

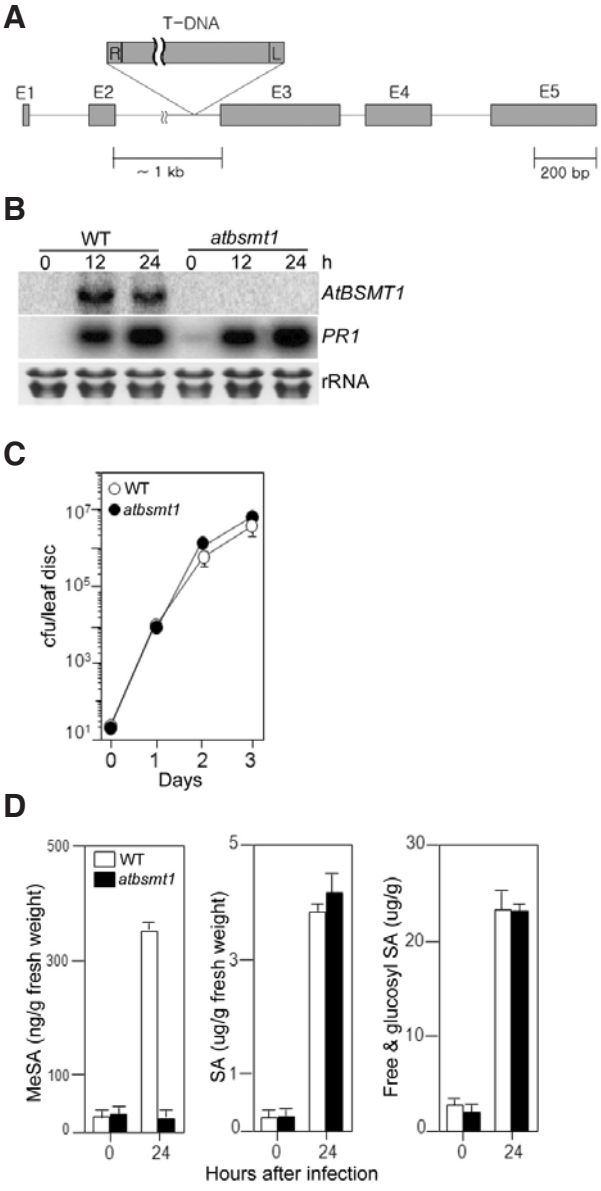

Fig. 4. Isolation and analysis of a T-DNA insertion mutation in the *AtBSMT1*. (A) Diagram of the T-DNA and insertion site in the second intron of the *AtBSMT1* gene in the *atbsmt1* mutants. Boxes represent exons and lines represent introns. R, right border; L, left border. Bar = 200 bp. (B) RNA expression of *AtBSMT1* in the *atbsmt1* and wild-type plants during *P. syringae* infection. Leaves (fourth and fifth) from 20-d-old plants were inoculated with *P. syringae* at OD_{600} = 0.01, and harvested for RNA analysis. (C) Bacterial growth of *P. syringae*. Plants were infected with *P. syringae* at OD_{600} = 0.0001. Growth of *P. syringae* in the *atbsmt1* and wild-type plants was not significantly different on days 1, 2 and 3 (P > 0.1, *t*-test, n = 8). cfu, colony-forming units. Bars indicate standard error. (D) MeSA, free and glucosyl SA levels in the *atbsmt1* and wild-type plants during *P. syringae* infection. Leaves (fourth and fifth) from 20-d-old plants were infected with *P. syringae* at OD_{600} = 0.01 and were harvested, extracted, and analyzed by HPLC. Free and glucosyl SA levels in the *atbsmt1* and wild-type plants were not significantly different at 0 and 24 h time points tested (P > 0.1, *t*-test, n = 8). Bars indicate the standard errors of three sets of samples.

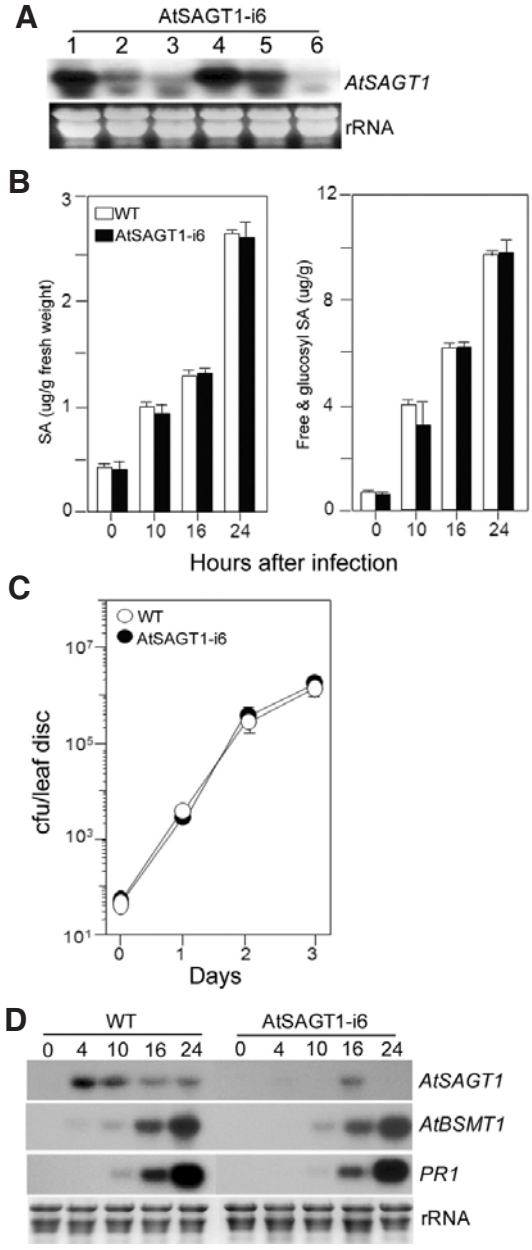

Fig. 5. Isolation and analysis of the *AtSAGT1*-RNAi plants. (A) RNAi-mediated inhibition of *AtSAGT1* expression. Leaves of 6 independent transgenic lines containing the *AtSAGT1*-RNAi construct under the control of the *CaMV 35S* promoter were treated with MeSA (0.1 mM) for 6 hrs, and total RNAs were extracted and hybridized. (B) 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* at OD_{600} = 0.01 and were harvested, extracted, and analyzed by HPLC. SA levels in the *AtSAGT1*-i6 and wild-type plants were not significantly different (P > 0.3, *t*-test, n = 8). Bars indicate the standard errors of three sets of samples. (C) Bacterial growth of *P. syringae*. Plants were infected with *P. syringae* at OD_{600} = 0.0001. Bacterial growth in the *AtSAGT1*-i6 and wild-type plants were not significantly different (P > 0.3, *t*-test, n = 8). cfu, colony-forming units. Bars indicate standard error. (D) Steady-state levels of *AtSAGT1*, *AtBSMT1* and *PR1* mRNAs in transgenic plants. Leaves (fourth and fifth) from 20-d-old plants were infected with *P. syringae* at OD_{600} = 0.01 and were harvested for RNA analysis.

the presence of AtSAGT1 but to that of other SA glucosyltransferase(s). AtSAGT2 may be important for glucosyl SA production, even though the gene cannot be induced by SA or pathogen infection (Song, 2006).

Recently, *AtSAGT1*- and *AtSAGT2*-knockout lines were isolated and characterized using metabolite analysis by conversion of added [^{14}C]SA (Dean et al., 2008). They showed that SA-added *ugt74f2* (*atsagt1*) mutant leaves were able to form SAG and other metabolites such as 2, 5-dihydroxybenzoic acid 2-*O*-β-D-glucoside, not to form SGE. However, SA-added *ugt74f1* (*atsagt2*) mutant leaves were able to make SAG and SGE due to AtSAGT1 activity. The levels of SAG or/and SGE in the mutants were lower than those of wild-type plants. But, they did not examine the levels of SA metabolites during pathogen responses. No differences were showed during defense responses including SA metabolites between the AtSAGT1-i6 and wild-type plants. It may be because *AtSAGT1* expression was not completely suppressed in the AtSAGT1-i6 plants as shown in Fig. 5D. Therefore, it will be interesting to examine changes of SA metabolites and disease resistance after pathogen infection in *atsagt1* and *atsagt2* mutants.

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