Functional analysis of a tomato salicylic acid methyl transferase and its role in synthesis of the flavor volatile methyl salicylate

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SUMMARY

Methyl salicylate (MeSA) is a volatile plant secondary metabolite that is an important contributor to taste and scent of many fruits and flowers. It is synthesized from salicylic acid (SA), a phytohormone that contributes to plant pathogen defense. MeSA is synthesized by members of a family of O-methyltransferases. In order to elaborate the mechanism of MeSA synthesis in tomato, we screened a set of O-methyltransferases for activity against multiple substrates. An enzyme that specifically catalyzes methylation of SA, SlSAMT, as well as enzymes that act upon jasmonic acid and indole-3-acetic acid were identified. Analyses of transgenic over- and under-producing lines validated the function of SlSAMT in vivo.

The SlSAMT gene was mapped to a position near the bottom of chromosome 9. Analysis of MeSA emissions from an introgression population derived from a cross with Solanum pennellii revealed a quantitative trait locus (QTL) linked to higher fruit methyl salicylate emissions. The higher MeSA emissions associate with significantly higher SpSAMT expression, consistent with SAMT gene expression being rate limiting for ripening-associated MeSA emissions. Transgenic plants that constitutively over-produce MeSA exhibited only slightly delayed symptom development following infection with the disease-causing bacterial pathogen, Xanthomonas campestris pv. vesicatoria (Xcv). Unexpectedly, pathogen-challenged leaves accumulated significantly higher levels of SA as well as glycosylated forms of SA and MeSA, indicating a disruption in control of the SA-related metabolite pool. Taken together, the results indicate that SISAMT is critical for methyl salicylate synthesis and methyl salicylate, in turn, likely has an important role in controlling SA synthesis.

Keywords: aroma, phytohormones, volatile synthesis.

INTRODUCTION

Methyl salicylate (MeSA) is a volatile organic compound that is widespread in the plant kingdom. It is often produced by flowers as a scent compound and contributes to the flavor of fruits such as tomato (Buttery and Ling, 1993). It is the major constituent of oil of wintergreen, a common food additive. The gene responsible for MeSA synthesis was first identified in the annual plant Clarkia breweri (Ross et al., 1999). This gene, S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase (SAMT) catalyzes the reaction of salicylic acid (SA) and the methyl donor S-adenosyl-L-methionine (SAM) to MeSA. The discovery of this methyltransferase led to the identification of a new class of O-methyltransferases and N-methyltransferases called the SABATH family, named for the substrates salicylic acid, benzoic acid, and theobromine (D’Auria et al., 2003). The O-methyltransferases in the SABATH family can utilize a wide range of substrates including SA (SAMT), benzoic acid (BAMT), SA and benzoic acid (BSMT; Negre et al., 2003; Underwood et al., 2005), jasmonic acid (JMT; Seo et al., 2001), indole-3-acetic acid (IAMT; Qin et al., 2005; Zubieta et al., 2003), and gibberellic acid (GAMT; Varbanova et al., 2007). Other family members include N-methyltransferases that act on substrates such as 7-methylxanthine and theobromine to produce the methylated products theobromine and caffeine, respectively (Kato
et al., 2000; Ogawa et al., 2001). Interestingly, several plant hormones are substrates of O-methyltransferases (OMT) and methylation may be one mechanism for plants to regulate hormone levels. For example, when AtGAMT1 and AtGAMT2 were over-expressed in Arabidopsis plants, the transgenic plants assumed a dwarf GA-deficient phenotype and the predicted GA substrates were depleted (Varbanova et al., 2007).

MeSA is a key compound influencing many inter-kingdom interactions. Beyond its role in insect attraction and flavor, MeSA and its immediate precursor, SA, also have important roles related to biotic stresses. SA has been implicated in both local and systemic responses to disease-causing microorganisms (Durrant and Dong, 2004; Loake and Grant, 2007). For example, the Arabidopsis sid2 (SA induction-deficient) mutant is more susceptible to local infection by Pseudomonas syringae and Peronospora parasitica (Nawrath and Metraux, 1999). The sid2 mutant fails to accumulate SA because it contains a mutation in isochoris-mate synthase, a step in the SA biosynthesis pathway (Wildermuth et al., 2001). Plants can mount a systemic resistance to pathogens, protecting themselves from subsequent attack. This systemic acquired resistance response (SAR) causes distal tissues not infected by a pathogen to accumulate SA and increase expression of pathogenesis-related genes, providing protection against subsequent infection. While SA is an essential component of SAR (Delaney et al., 1994), it appears not to be the systemically transmitted signal. MeSA has been suggested as a good candidate for the transmitted signal and recent evidence supports this conclusion (Chen et al., 2003; Koo et al., 2007; Park et al. 2007; Schualaev et al., 1997). Following systemic transmission, MeSA can be converted back to SA via an esterase, salicylic acid-binding protein 2 (SABP2) (Forouhar et al., 2005). SABP2-silenced tobacco plants infected with tobacco mosaic virus had larger lesions and were impaired in SAR and their responsiveness to SA, indicating a role for the MeSA pool in conversion back to SA (Kumar and Klessig, 2003; Park et al., 2007).

Here, we identify and characterize the properties of a tomato SAMT. A QTL that positively influences MeSA emissions co-segregates with the gene encoding SAMT. Consistent with an important regulatory role for methyl transferase and MeSA, pathogen challenged transgenic SISAMT over-producing plants were significantly altered in all SA-related metabolites.

RESULTS

Identification of a tomato SAMT

Because of the importance of MeSA to inter-kingdom communications and responses to the biotic environment, we were interested in elaborating factors responsible for its synthesis. As a first step, it was necessary to identify the gene(s) responsible for MeSA synthesis. The TIGR tomato EST database (http://compbio.dfci.harvard.edu/tgi/) was searched for cDNAs with similarity to the petunia BSMT gene (Negre et al., 2003). A total of seven unigenes with significant identity were identified. Alignment of the peptides encoded by each unigene with proteins of known function produced the phylogenetic tree shown in Figure 1. Full length cDNA sequences for each of the genes were isolated and placed into the pDEST15 vector for expression of recombinant proteins in Escherichia coli. Expression of each recombinant protein was validated by protein blotting and immunodetection of fused GST-tags. Each protein was screened for its ability to methylate a set of known OMT substrates. Of the eight recombinant proteins, three had significant activity against the tested substrates (Table 1). One protein, subsequently designated SISAMT1, was highly active and specifically methylated SA. One protein (cLEI13O4) was most active with jasmonic acid (JA), although it also methylated benzoic acid and SA less efficiently. Another (cTOA28E18) was highly specific for indole-3-acetic acid (IAA) and was therefore designated SIIAMT. Identical results were obtained with a set of recombinant His-tagged proteins (data not shown). The lack of activity of

Figure 1. Phylogenetic tree of amino acid sequences of SABATH methyltransferases. Sequences were aligned using the ClustaW multiple sequence alignment program. The phylogenetic tree was generated using the neighbor-joining method. Proteins with activities demonstrated in this paper are in bold (SISAMT1, cTOA28E18 and cLEI13O4). Tomato proteins are cLEI13O4, cLEM109, cTOA14P1, cTOA28E18, cTOD6818, cTOF25N7 and cLEW1K6. AbSAMT is Atropa belladonna (Genbank number: BAB30396), AmBSMT is Antirrhinum majus (AAF98284), AtGAMT1, AtGAMT2, AtBSMT, AtIAMT, and AtJMT are Arabidopsis thaliana (NP_194372, AAV34779, AAF57210, NP_200336, AAG23343), CbSAMT is C. brevior (AAF00108), CnSAMT is Cestrum nocturnum (AAW66830), DsSAMT is Datura stramonium (AAW66827), HcSAMT is Hoya carnosa (CAI05934), PhBSMT1 and PhBSMT2 are Petunia x hybrida (AAO45012 and AAO45013), SaSAMT is Schwennokia americana (AAW66829), and SfSAMT is Stephanitis floribunda (CAC33768).
the remaining five proteins may be either due to inactive E. coli-expressed proteins or specificity for as yet unidentified substrates.

The kinetic properties of purified SlSAMT1 were determined. At 25°C, the $K_m$ for SA was $50 \pm 10$ μM and the $K_m$ for S-adenosylmethionine (SAM) was $14.6 \pm 0.5$ μM. The $V_{max}$ for SA was $140 \pm 13$ pmol mg$^{-1}$ min$^{-1}$ and the $V_{max}$ for SAM was $86 \pm 4$ pmol mg$^{-1}$ min$^{-1}$. The $k_{cat}$ for SA was $0.056 \pm 0.005$ sec$^{-1}$ and $k_{cat}$ for SAM was $0.028 \pm 0.001$ sec$^{-1}$. The $K_m$ values for SA and SAM are within the range of reported values in other characterized methyltransferases, including CbSAMT (Ross et al., 1999), PhBSMT1, PhBSMT2 (Negre et al., 2003), and SfSAMT (Effmert et al., 2005). Since the in vitro data showed that SlSAMT1 specifically converted SA to MeSA, SlSAMT1 was chosen for further analysis in planta.

Validation of SlSAMT function

To validate the in vitro results with recombinant protein, transgenic plants containing either a sense over-expression (OE) vector or an antisense (AS) vector were produced. Transgenes were introduced into the tomato cultivars M82 (a processing-type variety) and Pearson (a larger fruited variety). The presence of transgenes was validated with construct-specific PCR analysis and RNA levels were validated with gene-specific quantitative real-time PCR (TaqMan). Three M82-derived antisense lines with significantly reduced SlSAMT RNA and MeSA emissions from fruits were identified (Table 2). Two over-expressing lines in the M82 background and one line in the Pearson background were also selected for subsequent analyses. The data from the over-expressing lines clearly indicate that SlSAMT does convert SA to MeSA in vivo as levels of MeSA emissions from fruits of the transgenic lines were elevated between five- and 120-fold. MeSA emissions from the AS lines were reduced by 50–70% relative to controls. Failure to completely eliminate MeSA emissions in the AS lines may be due to incomplete shut-off of SlSAMT, but could also be the consequence of additional genes encoding enzymes with SAMT activity. Taken together, the data indicate that SlSAMT encodes the major enzyme for conversion of SA to MeSA. While we cannot exclude post-transcriptional regulation, levels of MeSA production correlated with RNA levels. All of the transgenic lines were morphologically indistinguishable.

Table 1 Substrate specificities of tomato O-methyltransferases expressed in E. coli. Relative activities were calculated as a percentage of substrate with the highest activity. All assays were performed in duplicate as described in Methods

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SlSAMT1 relative activity % SA (100% = 23 pmol/mg/min)</th>
<th>cLEI3014 relative activity % JA (100% = 95 pmol/mg/min)</th>
<th>cTOA28E18 relative activity %IAA (100% = 347 pmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylic acid</td>
<td>100</td>
<td>8</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>2</td>
<td>62</td>
<td>1</td>
</tr>
<tr>
<td>p-Aminosalicylic acid</td>
<td>2</td>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Jasmonic acid</td>
<td>&lt;1</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>Indole3-acetic acid</td>
<td>&lt;1</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Gibberellic acid 3</td>
<td>&lt;1</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

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Tomato transferase and synthesis of methyl salicylate
from controls over multiple generations and seasons of growth in greenhouses and field.

**Expression of SlSAMT1**

We further examined expression of *SlSAMT1* as well as the levels of internal SA and MeSA in various tissues. The levels of RNA were measured by gene-specific TaqMan assays. Outside of fruits, the highest expression of *SlSAMT1* was observed in floral buds with somewhat lower expression in open flowers, young and mature leaves (Figure 2). Analysis of fruit expression indicated that the highest level of expression is early in development with progressively lower levels of expression as fruits mature and ripen (Figure 2). Expression was below the level of detection in fully ripe fruits. The internal pools of SA were relatively constant throughout fruit development (Figure 2). The highest levels of internal MeSA were observed in 15 day old fruits with relatively constant and low levels observed in mature and ripening fruits (Figure 2). While *SlSAMT1* expression drops off to essentially undetectable levels by the time fruits are fully ripened, the level of MeSA inside fruits remains constant during ripening, as does the SA pool. Tomato fruit have a thick cuticle that is essentially impermeable to volatile organic compounds such as ethylene (Klee, 1993). Thus, the MeSA in ripe fruits may be due to earlier synthesis. However, we cannot exclude the possibility that the SlSAMT enzyme is stable and the ripe fruit-associated MeSA is synthesized by enzyme produced much earlier in fruit development. Efforts to quantify SlSAMT protein with an antibody were unsuccessful due to the low level of protein present in fruit tissues.

**Identification of a MeSA QTL**

We have been working to identify genes that affect flavor-associated volatile synthesis (Tieman et al., 2006; Mathieu et al., 2008). The transgene data indicated that expression of *SlSAMT* is highly correlated with MeSA synthesis. Therefore, we examined whether different alleles of *SAMT* would influence MeSA production. The *SlSAMT* gene was mapped to a position near the bottom of chromosome 9 (Figure 3). We then examined whether replacement of the *SlSAMT* allele with the gene from *Solanum pennellii* LA716 (*SpSAMT*), would affect MeSA emissions. This was accomplished by exploiting the introgression line (IL) population developed by Eshed and Zamir (1995). Genomic DNA sequence for the *SpSAMT* and *SlSAMT* genes was isolated and used to develop a cleaved amplified polymorphic sequence (CAPS) marker to track the different *SAMT* alleles. The *SpSAMT* cDNA and its predicted peptide exhibit multiple differences from the *SlSAMT* (Figures S1 and S2). A CAPS marker was developed and used to track alleles. IL9-3, IL9-3-1 and IL9-3-2 were all shown to contain the *SpSAMT* allele. Quantification

### Table 2: MeSA emissions and *SlSAMT1* expression changes in ripe fruits from control (Pearson and M82) as well as transgenic over-expression (OE) and antisense (AS) lines

<table>
<thead>
<tr>
<th>Line</th>
<th>MeSA emission [ng (gfw)⁻¹ h⁻¹] ± SE</th>
<th>Fold MeSA difference</th>
<th>Fold RNA difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson</td>
<td>0.40 ± 0.08</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OE-6841</td>
<td>48.97 ± 5.96</td>
<td>123.0</td>
<td>9.0</td>
</tr>
<tr>
<td>OE-6833</td>
<td>1.83 ± 0.44</td>
<td>4.6</td>
<td>2.1</td>
</tr>
<tr>
<td>M82</td>
<td>0.04 ± 0.01</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OE-5220</td>
<td>1.77 ± 0.76</td>
<td>47.6</td>
<td>3.53</td>
</tr>
<tr>
<td>AS-7001</td>
<td>0.017 ± 0.003</td>
<td>0.47</td>
<td>0.36</td>
</tr>
<tr>
<td>AS-6918</td>
<td>0.02 ± 0.003</td>
<td>0.55</td>
<td>0.08</td>
</tr>
<tr>
<td>AS-6917</td>
<td>0.022 ± 0.005</td>
<td>0.59</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Plants were grown in the field and fruit harvested on multiple days. For Pearson, n = 17; for OE-6841, n = 16; for M82, n = 13; for OE-5220, n = 17; for AS-7001, n = 5; for AS-6918, n = 9; for AS-6917, n = 3. *SlSAMT* RNA levels were quantified from total RNA by TaqMan RT-PCR (n = 2).
of MeSA emissions from these ILs indicated that IL9-3-1 and 9-3-2 but not IL9-3, had significantly higher MeSA emissions from ripe fruits (Figure 4). To further characterize the QTL, internal pools of SA and MeSA were determined (Figure 4b,c). Consistent with the emissions, internal pools of MeSA were higher in IL9-3-1 and IL9-3-2 but not IL9-3. While the free SA was somewhat higher in IL9-3-1, there were no significant differences among the ILs. Finally, SAMT RNA levels were examined in each IL by quantitative RT-PCR. Consistent with the higher MeSA emissions, only IL9-3-1 and 9-3-2 has significantly higher content of SAMT RNA. The data are consistent with a QTL within bin 9-J (Figure 3), i.e., the SAMT gene, being a quantitative determinant for fruit MeSA emissions. That IL9-3 does not exhibit higher MeSA emissions, despite having the SpSAMT allele, suggest that there may be a second negative QTL located outside of bin 9-J that suppresses SAMT gene expression. IL9–2, which does not span the SAMT locus, has extremely low levels of MeSA emissions and SISAMT RNA, suggesting that such a locus could reside within bin 9-H, although we cannot rule out bin 9-I. While we cannot exclude differential protein stability or a more efficient SpSAMT enzyme as the cause of the higher MeSA levels in the lines containing SpSAMT, the IL and transgene analyses indicate a strong correlation between SAMT gene expression and MeSA emissions in fruits.

Altered SISAMT1 expression changes the SA-related metabolite pool

Plants use multiple mechanisms to regulate the internal pools of phytohormones including modification and degradation. SA acts to mediate many aspects of plant defense to pathogenic organisms. Known metabolites include glycoside conjugates and MeSA. The volatility of MeSA makes it unique as a hormone metabolite; volatile MeSA can be readily dissipated. This ability to be rapidly dissipated has led to speculation that it may be the translocated systemic signal mediating systemic acquired resistance (Park et al., 2007). Compatible strains of Xanthomonas campestris pv. vesicatoria (Xcv) normally induce SA synthesis in infected tomato leaves (O’Donnell et al., 2003). We used Xcv as a stimulant to alter SA production in leaves of wild-type and transgenic SISAMT over-producing plants to measure the subsequent effects of SA metabolite pools. Tomato OE line 5220 was chosen for analysis because it emits threefold higher levels of MeSA from leaves than the M82 parental control (Figure 5a). Bacterial growth was unaffected by the presence of the transgene (Figure 5b). Symptom development was slightly delayed but the transgenic leaves did eventually develop full symptoms with complete necrosis (Figure S3). This delay in symptom development was quantified with ion leakage measurements (Figure 5c), a direct measure of cell death. These results indicate that over-expression of SISAMT1 does not provide long term protection against Xcv infection. Expression of the native SISAMT transcript was monitored in the wild type parent, M82 (Figure 5d). There was a small increase in expression during the course of infection but even at the peak of RNA accumulation, this induction was 100-fold lower than SISAMT RNA in the transgenic line (data not shown).

The internal pools of SA-related metabolites were quantified in leaves throughout the course of disease symptom development. Our current method to quantify hormone levels in plant tissue relies upon the derivatization of hormones to methyl esters so that the volatile derivatives can be collected by vapor phase extraction (Schmelz et al., 2004). A new procedure was developed to quantify endogenous SA and MeSA from the same sample (described in Experimental procedures). Over-expression of SISAMT1 did not deplete the SA pool (Figure 6a). Rather, the internal SA pool was higher in the transgenic line (361 versus 202 ng (gfw)$^{-1}$) prior to infection. At 10 dpi, the time point with the greatest difference in
necrotic symptoms between the samples, the internal MeSA pool of the transgenic line was, as expected, sevenfold higher than M82 (Figure 6a). Although there was a slight increase in SA pool of the control M82, the free SA pool was fourfold higher in the transgenic line (Figure 6b) indicating that over-expression of \textit{SlSAMT1} caused an increase in SA as well as MeSA in response to pathogen infection. Both MeSA and SA pools remained high through day 14, the point of complete necrosis of the transgenic and wild type leaves.

As the SA and MeSA levels were significantly higher in the transgenic infected tissues, we determined the levels of SA and MeSA glucosides as well. The conjugated SA and MeSA pools started to increase in the transgenic line after the levels of free metabolites had reached a maximum. Both conjugated SA and conjugated MeSA followed the same
trend. The conjugated pools started to increase at 10 dpi and continued to increase as the disease progressed to 14 dpi (Figure 6c,d). Taken together, over-expression of **SlSAMT1** caused the leaves of transgenic infected plants to accumulate a fivefold higher level of all forms of SA metabolites during pathogen infection (free MeSA, conjugated MeSA, free SA, and conjugated SA). These data indicate that altering MeSA pools through over-expression of **SlSAMT1** profoundly disrupts the regulation of flux through the SA pathway. This unexpected disruption in the SA-derived pools should be cautionary for interpreting results related to hormone perturbations.

**DISCUSSION**

Volatile compounds are important to many aspects of plant growth and development as well as inter-kingdom interactions. They act as attractants and repellents of insects (James, 2003; Zhu and Park, 2005), promote defense against microbial pathogens (Durrant and Dong, 2004; Loake and Grant, 2007) and act as attractants for seed-dispersing organisms. MeSA is one of several phenylpropanoids that significantly contribute to the unique flavor of tomato fruits. It is also widely used as a flavor additive in the form of oil of wintergreen. Because of its importance to tomato flavor, we identified the major gene encoding the enzyme responsible for MeSA biosynthesis. This gene was independently identified by Ament et al. (2010), described in an accompanying paper. Members of the SABATH family are responsible for synthesis of diverse chemicals related to flavor, scent, pigmentation, cell walls and phytohormone signaling (Lam et al., 2007; Zhao et al., 2007). Thus it was not surprising that seven different unigenes with extensive sequence identity to the *C. breweri* BSMT were found in the tomato EST database. Using an in vitro methylation assay with recombinant proteins and a range of substrates, we were able to identify functions for three of these enzymes, all of which modify phytohormones; SA methyl transferase, JA methyl transferase and IAA methyl transferase (Figure 1; Table 1). It will be interesting to examine the biological roles of all of these enzymes that metabolize plant hormones. Here, we have focused on **SlSAMT1**.

Volatile emissions from transgenic **SlSAMT1** over- and under-expressing lines confirmed the in vivo function of SISAMT. Constitutive over-expression of the gene led to greatly increased MeSA emissions in leaves and fruits. Conversely, expression of an antisense RNA significantly reduced MeSA emissions. The failure to abolish MeSA emissions may be due to incomplete gene shut-off in the transgenic antisense lines or SAMT function of one of the other four as yet uncharacterized OMT gene products. **SlSAMT1** has high specificity for SA and is much less effective in methylating BA. Thus, it has somewhat different substrate specificity from the closely related petunia BSMT (Negre et al., 2003). The lack of significant activity on BA is
consistent with the absence of significant emissions of methyl benzoate from tomato tissues.

MeSA emissions from transgenic over- and under-producing SlSAMT plants are consistent with the enzyme being limiting for MeSA synthesis. We therefore hypothesized that the SlSAMT gene might be a QTL for MeSA emissions. To test this hypothesis, we determined the map position of the SlSAMT gene. The gene maps to the lower arm of chromosome 9. ILs containing the S. pennellii gene did produce significantly higher fruit MeSA. The higher MeSA emissions from fruits are correlated with significantly higher levels of SpSAMT gene expression in those ILs. Thus, the most likely explanation for the QTL localized to bin 9-J is increased transcription, although differential protein stability or kinetic properties cannot be excluded. The correlation of transcript abundance and MeSA emissions is consistent with transgenic over- and under-expression of SlSAMT. We cannot, however, rule out a linked gene affecting SAMT gene expression. The data further suggest the existence of another locus on chromosome 9 outside of bin 9-J that negatively influences SAMT gene expression.

Plants have multiple mechanisms for controlling the pools of active hormones such as SA, JA and IAA. Methylation is an effective mechanism of hormone removal. The O-methyl esters of many hormones, including SA, are inactive and volatile (Schulaev et al., 1997). We were interested to determine the effects of altered SlSAMT expression on the levels of SA and its metabolites. We previously showed that SA accumulates in response to bacterial pathogen infection and is essential for disease symptom development (O’Donnell et al., 2003). Therefore, we used Xcv infection to perturb SA metabolism and measure the consequences of SlSAMT1 over-expression on the pool of SA metabolites. We had expected that over-expression of SlSAMT1 would result in conversion of SA to MeSA, depleting the pool of free SA, as has been reported in Arabidopsis (Koo et al., 2007). This situation turned out not to be the case. Prior to infection, MeSA emission from leaves of the transgenic plants was higher than non-transgenic controls (Figure 5a), as was the internal pool of SA. Following infection, the internal pools of MeSA and SA both significantly increased, as did the pools of MeSA and SA glucosides. Over-expression of SlSAMT1 and the consequent increased MeSA synthesis caused a disruption of the normal flux into SA metabolites. Xcv infection exacerbated that difference. Levels of free SA more than doubled while MeSA pools were sixfold higher in the infected leaves of transgenic plants. These altered SA metabolite pools only slightly delayed disease symptom formation. While we did achieve higher levels of SA in all tissues by disrupting the normal mechanism(s) regulating hormone metabolism, these elevations in the transgenic lines did not induce resistance or tolerance to the pathogen.

The results presented here are an important step toward characterizing this family of O-methyltransferases in tomato. The role of methylation of plant hormones in growth and development has only started to be addressed. Over-expression of Arabidopsis GAMT led to depletion of the endogenous GA pool (Varbanova et al., 2007). Conversely, over-expression of AtJMT led to elevated MeJA emissions but no alteration in the endogenous JA pool (Seo et al., 2001). In tomato, SlSAMT1 over-expression had a measurable effect on basal hormone levels and a larger effect following pathogen challenge. Since all of the genes encoding key activities affecting the SA pool have not been identified, we cannot yet identify the point of perturbation. One possible point of intervention, however, may be the SABP2. This enzyme activity is inhibited by the product, SA (Forouhar et al., 2005). Blocking SABP2 action may disrupt the normal mechanism of SA/MeSA balance. Finally we note that in an accompanying paper (Ament et al., 2010), altered SA/MeSA balance affects another inter-kingdom interaction with parasitic insects. Future work examining the role of MeSA in attraction of herbivores and pollinating insects is certainly appropriate.

**EXPERIMENTAL PROCEDURES**

**Cloning of SlSAMT1**

The full-length EST of SlSAMT1 was identified in the TIGR database by identity to the amino acid sequence of Petunia hybrida PhBSMT (Negre et al., 2003) and was amplified with primers (Fwd) 5'-CAC-CATGAAAGTTTGTGGATGTCTTACATGAATGGAGG-3' and (Rev) 5'-TTATTTTTCTTGTGTCAGGAGACATGAACTTATTTATAACTCAGTATCC-3' from Flora-Dade (Solanum lycopersicum) bud cDNA. For SlSAMT (tomato methyltransferase) sequences, full-length clones from the TIGR database were obtained from Jim Giovanni (Boyece Thompson Institute) and sequenced. The coding region of each putative SlSAMT was amplified and cloned into pENTR/D-TOPO Gateway vector followed by recombination into pDEST15 to produce GST-tagged proteins in E. coli BL21-AI (Invitrogen, http://www.invitrogen.com/). Primers used for amplification of the methyl transferases were as follows:

cLEW1K6 forward, CACCATGGATTTGAGAAATGTCCACATGAAGTAGG,
cLEW1K6 reverse, CTATGGTTTTGCAGAAACAGAAGAAAGAATGCAGGG,
cLEM709 forward, CACCATGAATGGAGAGATTGGGAAGATGGTATG,
cLEM709 reverse, CTAATTTATTTTGTAGCAAAAACGAGAAGAAAGAATGGAAG,
cTOA14P1 forward, CACCATGGATTTGAGAAATGTCCACATGAAGTAGG,
cTOA14P1 reverse, CTAATTTATTTTGTAGCAAAAACGAGAAGAAAGAATGGAAG,
cTOD6B16 forward, CACCATGACCATGACCCAGTCTTACAGAAGATGGTATG,
cTOD6B16 reverse, CTAATTTATTTTGTAGCAAAAACGAGAAGAAAGAATGGAAG,
cTOA28E18 forward, CTATGGTTTTGCAGAAACAGAAGAAAGAATGCAGGG,
layer was measured by counting for 5 min in a 3 ml Ready Gel Scintillation Fluid (Beckman Coulter, http://www.beckmancoulter.com). Counts for the no enzyme controls were subtracted from the sample counts, and activity for SA was normalized to 100%. For the $K_m$ of SA, 2.85 $\mu$g of purified GST-SISAMT1 was used. $^{14}$C-SAM was held constant at 75 $\mu$m.

**Volatil collection**

Volatiles were collected from tomato fruits according to Tieman et al. (2006). For leaf volatiles collections, two whole leaves, approximately 4-5 g of fresh tissue, were carefully loaded into glass collection tubes to avoid unnecessary damage. Briefly, air was passed over the samples and volatiles were collected on a SuperQ Resin for 1 h. Volatiles were eluted off the column with methylene chloride and run on a GC for analysis as described in Tieman et al. (2006).

**SISAMT1 and SpSAMT1 RNA expression quantification**

Total RNA was extracted using the Qiagen RNeasy Plant Mini Kit and levels of SISAMT1 mRNA levels were quantified by real-time polymerase chain reaction (RT-PCR) using Taqman one-step RT-PCR reagents (Applied Biosystems, http://www.appliedbiosystems.com/). The pericarp and locular gel from several fruits were pooled for each RNA extraction for the analysis of transgenic plants, and each extraction was run in duplicate. For the tissue-specific expression and pathogen experiments, four biological replicates were analyzed per time point. SISAMT1 expression was determined using the following primer/probe set – Fwd: 5'-TCCAGAAGAATTGATGCTGAT-3'; Rev: 5'-AATGACCTTAACAGTCTGTAACACTAA-3'; Probe: 5'-FAM-6'-FAM) 5'-BHQ_1) Samples were run on a BioRad iCycler PCR detection system and quantified with the MyiQ software. The following PCR conditions were used: 48°C, 30 min; 95°C 10 min; 40 cycles of 95°C, 15 sec; 60°C 1 min. A sense strand was in vitro-transcribed from plasmid DNA with $^{3}$H-UTP (MAXscript; Ambion, http://www.ambion.com) and used to determine the absolute values of RNA in the sample.

**SAMT mapping**

SAMT was initially mapped by determining which introgression lines contain the gene. $S. lycopersicum$, $S. pennelli$ DNA and DNA from 74 $S. pennelli$ introgression lines (Eshed and Zamir, 1995) were amplified with SAMT forward primer 5'-CATGATGCTTAAATGAATTGTG and SAMT reverse primer 5'-TGGAGAGGCA-TAGTTAAAGAACA-3' using 60°C annealing temperature. The 682-bp PCR products were digested with Scal. $S. lycopersicum$ amplification products digested with Scal, whereas $S. pennelli$ products did not. The gene was subsequently mapped by the Tanksley laboratory with a CAPS marker using the F2 2000 tomato population.

**Pathogen inoculations**

Xanthomonas campestris pv. vesicatoria (Xcv) 93–1 inoculations were performed on leaves 3 and 4 of 5–6-week-old plants as previously described (O'Donnell et al., 2001). Leaves of mock-treated plants at 0 dpi were dipped in mock buffer only. For ion leakage measurements, three plants were assayed, and each infected leaf

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was assayed separately \((n=6)\). Measurements in \(\mu\text{mho}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}\) are described in Lund et al. (1998). Bacterial colony counts were performed on two leaves of three plants for each time point. Two 0.5 cm² discs were excised with a number 5 cork borer from representative leaflets for each time point. Discs were ground in 10 mM MgCl₂ and serial dilutions were plated on 0.7% Nutrient Broth, 1.5% Bacto-agar (Difco Laboratories, http://www.bd.com). Plates were incubated at 30°C for 2 days and colonies were counted for each time point.

**SA and MeSA quantification**

Vapor phase extraction of free metabolites and conjugated metabolites was performed according to Engelberth et al. (2003) and Schmelz et al. (2004) with some modifications. In previously reported vapor phase extraction protocols, free SA was derivatized to its methyl ester, MeSA, to quantify the amount of SA in the leaves. However, the goal of this experiment was to quantify the amounts of free SA and free MeSA in the same sample, so a different method of derivatization was developed to analyze both metabolites without interference. Individual leaves were frozen in liquid N\(_2\) and ground to a fine powder. Approximately 100 mg of frozen tissue was weighed into a Fastprep tube containing 1 g ceramic beads (1.1 mm Zirmil Beads; SEPR Ceramic Beads and Powders) and an internal standard of a 2H₄-MeSA standard in methylene chloride. The samples were extracted with 300 \(\mu\text{l}\) Extraction Buffer (2:1:0.005 \(\mu\text{M}\) propanol: H₂O: HCl) and shaken in a Fastprep FP 120 homogenizer (Qbiogene, http://www.qbiogene.com) for 30 sec. Then 1 ml methylene chloride was added and the samples were shaken an additional 10 sec. Samples were mixed and the vapor phase was collected on the same column as described above. After the liquid evaporated, the sample was left on the heat block for an additional 2 min. Columns were rinsed and eluted as described above. The glass vial was sealed with a cap containing a high-flow Septum, followed by a needle carrying a stream of N₂. Then the 2H₆-SA standard was added and dried with a constant temperature of 70°C. Hydrolyzed MeSA and SA were collected by vapor phase extraction in one step at 70°C and kept on the heat block 2 min after drying. Columns were rinsed and eluted as described above. Samples were analyzed as described above.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Nucleotide sequences of Solanum lycopersicum (SISAMT) and Solanum pennellii (SpSAMT) SAMT cDNAs.

**Figure S2.** Predicted amino acid sequences of Solanum lycopersicum (SISAMT) and Solanum pennellii (SpSAMT) SAMT proteins.

**Figure S3.** Symptom development of Xcv-infected leaves.

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