ROLE OF METHYLSALICYLATE IN TOMATO FLAVOR AND RESPONSE TO BACTERIAL PATHOGEN INFECTION

By

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This thesis is dedicated to my Mom, who has always supported and encouraged me.

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LIST OF ABBREVIATIONS

3, 7-DMXMT	3, 7-Dimethylxanthine *N*-methyltransferase
ADH	Alcohol dehydrogenase
B	Flower bud
BA	Benzoic acid
BAMT	*S*-Adenosyl-$_L$-methionine: benzoic acid carboxyl methyltransferase
BLAST	Basic Local Alignment Search Tool
Br	Breaker stage
BSMT	*S*-Adenosyl-$_L$-methionine: benzoic acid/salicylic acid carboxyl methyltransferase
cfu	Colony forming units
dap	Days after pollination
dpi	Days post inoculation
EtOH	Ethanol
FAMT	Farnesoic acid carboxyl methyltransferase
Fl	Flower
FMV	Figwort mosaic virus
GAMT	Gibberellin carboxyl methyltransferase
gfw	Gram fresh weight
GST	Glutathione-*S*-transferase
IAMT	Indole-acetic acid carboxyl methyltransferase
ICS	Isochorismate synthase
JA	Jasmonic acid
JMT	Jasmonic acid carboxyl methyltransferase
MeBA	Methylbenzoate

MeSA	Methylsalicylate
MG	Mature green
ML	Mature leaf
MXMT	7-Methylxanthine *N*-methyltransferase
PAL	Phenylalanine ammonia lyase
PBS	Phosphate buffered saline
PL	Pyruvate lyase
PR	Pathogenesis related
RT-PCR	Real-time polymerase chain reaction
SA	Salicylic acid
SABATH	Salicylic acid (SA), benzoic acid (BA), and theobromine (TH)
SABP2	Salicylic acid binding protein 2
SAH	*S*-Adenosyl-homocysteine
SAM	*S*-Adenosyl-$_L$-methionine
SAMT	*S*-Adenosyl-$_L$-methionine: salicylic acid carboxyl methyltransferase
SAR	Systemic acquired resistance
SE	Standard error
SGN	Solanaceae Genomics Network
STDEV	Standard deviation
TCS	Caffeine synthase
TIGR	The Institute for Genomic Research
TMV	Tobacco mosaic virus
Tu	Turning stage
Xcv	*Xanthomonas campestris* pv. *vesicatoria*
YL	Young leaf

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ROLE OF METHYLSALICYLATE IN TOMATO FLAVOR AND RESPONSE TO BACTERIAL PATHOGEN INFECTION

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Volatiles are aroma compounds with low molecular weight and high vapor pressure that evaporate at room temperature. Plants have evolved the use of volatiles for the attraction of pollinators, seed dispersing organisms, and to aid in the response to herbivory and pathogen attack. These plant volatiles are components of floral scent and the flavors of foods, and may even possess medicinal properties. Tomato (*Solanum lycopersicum*) flavor is due to a complex interaction between sugars, acids, and volatile compounds. Methylsalicylate (MeSA), or oil of wintergreen, is an important volatile component of tomato flavor. The purpose of this study was to characterize the contribution of MeSA to tomato flavor as well as any other biological functions in tomato. *S*-adenosyl-$_{L}$-methionine: salicylic acid carboxyl methyltransferase (*LeSAMT1*) is the gene responsible for MeSA synthesis in tomato, and LeSAMT1 specifically converts SA to MeSA. Plants overexpressing *LeSAMT1* were analyzed with respect to MeSA emissions and flavor. Tomato fruits overexpressing *LeSAMT1* tasted different than the controls but were preferred equally to the controls by an untrained consumer panel. In addition, one of these transgenic lines overexpressing *LeSAMT1* was used as a tool to examine the contribution of MeSA overproduction to infection by the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* 93-1. The results indicated that MeSA is a key metabolite that affects the

accumulation of SA during bacterial pathogen stress as well as the progression of disease symptoms.

CHAPTER 1
INTRODUCTION

Tomato (*Solanum lycopersicum*) is a member of the Solanaceae, or nightshade family. Due to their role as food sources, the Solanaceae are the most valuable family of vegetable crops and are economically ranked third among plant taxa (Mueller *et al.,* 2005). Consuming fruits and vegetables such as tomato is highly correlated with disease prevention. However, consumers are often dissatisfied with the flavor of tomatoes due to the quality of available genetic material as well as postharvest handling and storage practices (Baldwin *et al.*, 2000). Breeders and growers have focused on traits such as yield, fruit size, color, and disease resistance while flavor has largely been ignored. Since tomatoes contain a variety of vitamins and health-promoting phytochemicals, including Vitamin A, Vitamin C, β-carotene, lycopene, and fiber, improving the flavor of tomatoes would encourage more consumers to buy tomatoes and improve their overall health. An ongoing project in this lab is to identify the biosynthetic and regulatory genes responsible for the synthesis of compounds linked to tomato flavor and to determine any additional biological functions of these flavor compounds. Once these genes have been identified, their sequences can be used to develop molecular markers for breeders to aid in flavor selection.

The focus of this study was on methylsalicylate (MeSA), or oil of wintergreen, which is a major component of tomato flavor and is also used commercially as a flavoring agent and as an ingredient in topical ointments for muscle pain. MeSA is also known to be involved in pollinator attraction and the gene responsible for MeSA synthesis was first identified in the California 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 and the methyl

donor *S*-adenosyl-$_L$-methionine (SAM) to MeSA. The tomato homolog, *LeSAMT1*, was cloned and overexpressed in tomato plants to determine if overproducing MeSA affected tomato flavor.

In addition, MeSA is emitted in response to biotic stress. For example, MeSA is emitted from the leaves of tobacco mosaic virus-infected tobacco (Schulaev *et al.*, 1997), spider mite-infested tomatoes (Ament *et al.*, 2004), and bacterial-inoculated pepper (Cardoza and Tumlinson, 2006). The current study examined the disease progression of the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) 93-1 in transgenic tomato plants overexpressing *LeSAMT1*. The purpose of this study was to characterize the gene responsible for the biosynthesis of MeSA in tomato, *S*-adenosyl-$_L$-methionine: salicylic acid carboxyl methyltransferase (*LeSAMT1*), and to identify any other biological roles of MeSA in response to *Xcv* infection in tomato.

CHAPTER 2
LITERATURE REVIEW

Flavor Perception

Humans perceive flavor as a combination of taste and smell. Taste receptors in the taste buds of the mouth contain microvilli that bind to food dissolved in the saliva. There are five classes of taste receptors—sour, salty, sweet, bitter, and umami, which in Japanese means "delicious." There is no unique flavor identified with umami—instead it is a flavor enhancer and is associated with the amino acid glutamate and food additives such as monosodium glutamate (MSG). Each taste receptor can respond to the presence of specific chemicals, but receptors in different regions in the mouth are more sensitive to specific tastes (Germann and Stanfield, 2005). For example, sweetness is perceived by the binding of organic molecules to the receptor and is most sensitive at the anterior tip of the tongue. In tomato these organic molecules include fructose and glucose. Nitrogenous compounds are responsible for bitter taste, which is perceived at the back of the tongue. Bitterness is often associated with an avoidance response. Saltiness and sourness are perceived on the sides of the tongue, and the salty-sensitive receptors are located closer to the anterior portion of the tongue. Sodium ions are responsible for salty taste and hydrogen ions are responsible for sourness. In tomato, sourness is primarily due to citric acid and malic acid (Mahakun *et al.*, 1979). There are several mechanisms for the taste receptors to transmit signals to the brain (Germann and Stanfield, 2005). Salty and sour receptors open voltage-gated channels, sweet receptors utilize a G-protein-signaling cascade, and bitter receptors can utilize either voltage-gated channels or G-protein signaling cascades. However, flavor perception is complex, and the sense of smell must also be considered.

Molecules known as odorants or volatiles are responsible for the aroma component of flavor, which gives each food its unique flavor. A volatile compound is a low molecular weight

molecule with high vapor pressure, so it evaporates at room temperature. Once inhaled, volatiles become dissolved in mucus and are carried by olfactory binding proteins to the olfactory receptor cells located in the nasal cavity. The volatiles bind to olfactory receptors, proteins with seven transmembrane domains, which then activate a G-protein signaling cascade (Mombaerts, 1999). These olfactory receptor cells are actually neurons and connect to the olfactory bulb in the brain. Two regions of the brain eventually receive the signals transmitted by the olfactory neurons via second-order neurons—the olfactory cortex and the limbic system. The olfactory cortex perceives and discriminates smells, while the limbic system is responsible for emotions associated with smells. In contrast to taste receptors, in which individual receptors can respond to all classes of taste, each class of olfactory receptor responds to a unique set of volatile compounds (Hallem *et al.,* 2004). To add to the complexity, one volatile compound can activate more than one type of receptor. This flexibility allows an organism to detect a vast range of aromatic cues from the environment, including food sources and mates. For example, the olfactory neuron responsible for MeSA recognition has been identified in the fruit fly *Drosophila melanogaster* (Hallem *et al.,* 2004). This receptor, Or10a, responds strongly to MeSA as well as acetophenone, isoamyl acetate, and benzaldehyde. The complexity of aroma perception is due in part to the large number of genes encoding olfactory receptors. It is estimated that 500 to 750 genes encode for the olfactory receptors in humans, while 1000 genes are estimated in mice, which is a larger class than immunoglobulin and T-cell receptor genes (Moembarts, 1999). This variability allows organisms to detect a broad range of sensory cues and is beneficial for animals and insects, who rely heavily on their sense of smell for survival.

Volatiles and Tomato Flavor

Based on their chemical structures, tomato fruit volatiles are believed to be derived from amino acids, carotenoids, and lipids. It has been suggested that volatile compounds originating

from these essential nutrients can serve as a cue linking the food to its nutritional components (Goff and Klee, 2006). Over 400 volatile compounds have been identified in tomato, and no single compound has been directly linked to a unique "tomato" flavor (Buttery and Ling, 1993). Instead, it is a unique balance and blend of specific volatiles that contribute to the flavor of tomato. These volatile compounds are mostly concentrated in the pulp and locular gel of tomatoes rather than the skin or seeds (Buttery *et al.*, 1988). This localization makes sense since the locular gel houses the seeds, and concentrating the volatiles in this area will facilitate seed dispersal. In addition, the majority of volatiles are not produced until the fruits reach the ripe stage (Figure 2-1). This is either due to substrate availability or the localization of volatile-producing enzymes in different compartments that cannot act on their substrates until the tissue is disrupted (Buttery and Ling, 1993). The carotenoid-derived volatiles, such as β-ionone, geranylacetone, and 6-methyl-5-heptene-2-one, show this correlation because the pigmented substrates, such as lycopene, would not be available until the fruits ripen and the seeds are mature. The change in color along with the increase in aroma volatiles at the ripe stage would attract seed-dispersing organisms to eat the fruit and disperse the mature seeds. Tomatoes also produce higher levels of sugars when they are allowed to fully ripen on the vine as opposed to being picked at an earlier stage of ripeness (Kader *et al.*, 1977). Allowing tomatoes to ripen off the vine resulted in "off" flavor and decreased sweetness, considered negative attributes to flavor. Therefore, plants have evolved survival mechanisms by orchestrating the necessary events for seed maturity with an increase in flavor compounds.

The contribution of individual volatile compounds to a particular flavor can be ranked according to their log odor units. A log odor unit is a ratio measurement comparing the concentration of a particular volatile to its detection threshold. Generally speaking, a compound

with a lower detection threshold is more easily recognized by smell. However, detection limits can vary depending on the solution used during the evaluation (Tandon *et al.*, 2000). For example, when tomato volatiles were evaluated in water, a methanol/ethanol/water solution, and a deactivated tomato homogenate, the detection threshold increased from water to the alcohol solution to the homogenate, except for the branched-chain volatile 3-methylbutanal. In other words, the volatiles became more difficult to detect as the viscosity and polarity of the solutions changed and the solution medium had higher affinity for the volatile compounds. If the log of the ratio between the concentration of a compound and its detection limit is greater than one, that volatile is said to have a positive log odor unit and positively contributes to flavor.

Seventeen volatile compounds, shown in Figure 2-2, have been identified as being important for tomato flavor (Buttery and Ling, 1993). These volatiles are ranked by log odor units and the structures of the volatiles along with their precursors are listed. In addition, taste descriptors are included. In general, lipid-derived volatiles are generally described as "green" or "grassy," ketones are denoted as "sweet, floral and fruity," and the volatiles derived from the branched amino acids leucine and isoleucine are "earthy, stale, or musty." Aromatic compounds are often "flowery." The focus of this study is on methylsalicylate (MeSA), a phenolic compound that is a major component of oil of wintergreen and has a "fragrant, sweet, and root beer-like" aroma (Heath, 1981). The also lists representative values of these compounds from two cultivars of tomato used in this study—M82, a commercial processing tomato and Pearson, a larger variety with more locules. Both varieties are open-pollinated. The majority of the volatile compounds are higher in the Pearson variety, including MeSA. However, it is common to see varying volatile profiles between varieties (Baldwin *et al.*, 1991).

Since tomatoes have not generally been bred for flavor, identifying the genetic components of flavor has been of interest to improve the quality of tomatoes for consumers. A comparison between volatile profiles of a wild species of tomato and a commercial cultivar has shown that the wild species contains higher levels of most of the important tomato volatiles, indicating that selection by breeding has generally led to reduced flavor (Goff and Klee, 2006). An ongoing focus of this lab is to identify the genetic components of volatile synthesis since this is the flavor constituent that gives tomato its unique flavor. The availability of public genomics databases and germplasm has made it possible to begin to link quantitative traits, such as flavor, to positions of candidate genes. For example, using an introgression line population between the wild species *Lycopersicon pennellii* and the commercial processing tomato M82, Tieman *et al*. (2006) mapped important tomato volatiles to specific regions of the tomato genome. The focus of this study is on the volatile MeSA.

Methylsalicylate Is a Ubiquitous Compound Involved in Tomato Flavor

Methylsalicylate (MeSA), or oil of wintergreen, has been identified as an important volatile in tomato flavor (Buttery and Ling, 1993). In a metabolomics study of 94 tomato cultivars, Tikunov *et al.* (2005) found that MeSA and other phenolic-derived volatiles such as guaiacol, eugenol, ethylsalicylate, and salicylaldehyde, were some of the most variable volatiles in tomato flavor and were largely responsible for differences in volatile profiles between cultivars. Figure 2-1 shows that MeSA emission gradually increases during ripening in M82. MeSA is also used commercially in a variety of flavor and cosmetic products and is Generally Recognized as Safe (GRAS) by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) (Adams *et al.*, 2005). In addition to tomato flavor, MeSA is a flavor constituent of strawberry, currant, root beer and various fruit juices—apple, cherry, and raspberry (Heath, 1981; Burdock, 1995). MeSA is also present in black tea, and a study by

Abraham *et al.* (1976) showed that varying the concentrations of MeSA added to tea leaves changed the flavor quality. MeSA levels up to 20 ppm imparted a desirable fragrant and flowery flavor. However, when MeSA was present above 25 ppm, the wintergreen flavor was more pronounced and the tea was described as bitter. In the pharmaceutical industry, MeSA is often used to mask unpleasant odors and flavors and is a constituent of toothpaste and chewing gum. It is also found as an active ingredient in topical ointments to relieve muscle pain and symptoms of osteoarthritis (Hansen and Elliot, 2005). MeSA is abundant in *Gaultheria procumbens*, or the wintergreen plant. In the mid 1800s, it was shown that MeSA could be hydrolyzed to salicylic acid, a compound present in the extract of willow tree (*Salix* sp) used for centuries as a pain reliever (Mahdi *et al.*, 2005). Therefore, plants that synthesize MeSA have been used by humans due to this volatile's medicinal and desirable fragrant properties and its presence in tomato suggests it is an important component for the balance of tomato flavor.

SABATH Family of Methyltransferases

MeSA is known to be involved in pollinator attraction and the gene responsible for MeSA synthesis was first identified in the California 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 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 of methyltransferases can utilize substrates such as salicylic acid (SAMT), benzoic acid (BAMT), jasmonic acid (JMT), indole-acetic acid (IAMT), and gibberellic acid (GAMT). Floral SAMTs have been characterized from *Stephanotis floribunda*—SfSAMT (Pott *et al.*, 2004) and *Atropa belladonna*—AbSAMT (Fukami *et al.*,

2002). Methyltransferases that recognize both benzoic acid (BA) and salicylic acid (SA), or BSMTs, have been identified in *Petunia x hybrida* – PhBSMT (Negre *et al.*, 2003; Underwood *et al.*, 2005) and *Arabidopsis thaliana* – AtBSMT (Chen *et al.*, 2003). So far only the Arabidopsis AtJMT (Seo *et al.*, 2001), AtIAMT (Zubieta *et al.*, 2003; Qin *et al.*, 2005) and AtGAMT (Varbanova *et al.*, 2007) have been identified. Other family members include *N*-methyltransferases that can act on substrates such as 7-methylxanthine (CaMXMT1) and theobromine (TCS1) to produce the methylated products theobromine and caffeine, respectively. CaMXMT1 is theobromine synthase from *Coffea arabica* (Ogawa *et al.*, 2001) and TCS1 is caffeine synthase from *Camellia sinensis* (Kato *et al.*, 2000). Both the *O*- and *N*-methyltransferases use the methyl donor SAM to methylate the substrates into methyl esters. Interestingly, the substrates of the *O*-methyltransferases include several plant hormones, and methylation may serve as a means for plants to regulate hormone levels. When *AtGAMT1* and *AtGAMT2* were overexpressed in Arabidopsis plants, the transgenic plants assumed a dwarf GA-deficient phenotype and the predicted GA substrates were depleted (Varbanova *et al.*, 2007). This finding suggests that methylation may serve as an additional mode of hormone regulation.

Salicylic Acid—the Bridge between Methylsalicylate and Plant Defense

Salicylic acid (SA), the precursor to MeSA, is a plant hormone known to be involved in defense responses by inducing the transcription of pathogenesis-related genes (*PR* genes) and is involved in establishing local resistance and systemic acquired resistance (SAR) (Dempsey *et al*., 1999; Durrant and Dong, 2004). SA biosynthesis remains unclear, and studies have shown that SA can be synthesized from phenylalanine *via* the phenylpropanoid pathway or isochorismate *via* the shikimate pathway (Yalpani *et al.*, 1993; Wildermuth *et al.*, 2001; Strawn *et al.*, 2007). The shikimate pathway precedes the phenylpropanoid pathway, and chorismate is the last common intermediate. The branch-point chorismate can be converted to isochorismate or can be

converted to phenylalanine *via* several intermediates to initiate the phenylpropanoid pathway (Figure 2-3). Isochorismate synthase (AtICS1) was identified in Arabidopsis as an enzyme necessary for SA biosynthesis in response to the pathogen *Erysiphe orontii* (Wildermuth *et al.*, 2001). A follow-up study showed that AtICS1 is located in the stroma of the chloroplast, where the precursor chorismate is localized (Strawn *et al.*, 2007). On the other hand, SA biosynthesis *via* phenylalanine has also been studied. Phenylalanine ammonia lyase (PAL) is the first committed step of this pathway and it catalyzes the conversion of phenylalanine to *trans*-cinnamic acid. Transcriptional activation of key enzymes as well as subcelluar compartmentalization contribute to the regulation of this pathway (Samanani and Facchini, 2006). In Arabidopsis, *PAL* expression is rapidly induced after pathogen inoculation (Dong *et al.*, 1991). Yalpani *et al.* (1993) showed that in tobacco culture cells, ^{14}C-*trans*-cinnamic acid was converted to radiolabeled BA and SA. In addition, tobacco leaves pre-inoculated with the precursors phenylalanine, *trans*-cinnamic acid, BA, or SA had smaller lesions after inoculation with tobacco mosaic virus (TMV), indicating that the increased levels of SA increased the resistance. In a complementary study, Leon *et al.* (1993) showed that a plant extract could convert BA to SA. A follow-up study suggested that this conversion of BA to SA was due to an oxygenase called benzoic acid 2-hydroxylase (BA2H) (Leon *et al.*, 1995). However, few genes involved in the route from phenylalanine to SA have been identified despite the evidence for its existence (Wildermuth *et al.*, 2006). Plants most likely have several routes to synthesize SA, given its importance in plant defense, but conclusive studies have not been done to identify all the enzymes involved.

In addition to *de novo* synthesis, it has been shown that SA can also be produced by the action of an esterase that demethylates MeSA to SA. A MeSA esterase from tobacco, salicylic

acid-binding protein 2 (SABP2), has been identified, crystallized, and silenced in transgenic plants (Kumar and Klessig, 2003; Forouhar *et al.*, 2005). This esterase converts MeSA to SA and is inhibited by its product, SA (Forouhar *et al.*, 2005). SABP2-silenced tobacco plants infected with TMV had larger lesions in the local infection and were impaired in SAR and their responsiveness to SA (Kumar and Klessig, 2003). This result suggests that MeSA may function as an important pool for conversion back to SA following pathogen infection. It also suggests that SABP2 is involved in plant innate immunity. In addition, a methyljasmonate esterase has been identified from tomato (Stuhlfelder *et al.*, 2004), so this mode of regulation is not unique to SA. It has been suggested that methylation via methyltransferases may allow the hormones to move through cell membranes as a more nonpolar molecule and demethylation via esterases allows the bioactive molecule to act in the appropriate tissue (Yang *et al.,* 2006a). From work in tobacco infected with TMV, it has been suggested that MeSA also participates in plant-to-plant signaling or as a signal within the plant after pathogen attack (Schulaev *et al.,* 1997). However, the exact role of these methylated conjugates is not fully understood and has not yet been proven experimentally.

Volatiles Are Involved in Plant Defense

Plant volatiles also function as chemical defenses for protection against feeding insects and herbivores. These volatiles can either directly deter the feeding herbivore or attract predatory insects to dispose of the feeding insect, also known as indirect defense. In addition, plants respond differently to insect-feeding and general wounding, and the volatile profiles between these two types of damage differ (Pare and Tumlinson, 1999). In contrast, insects possess olfactory receptors that can recognize host plants that have been attacked. For example, the strawberry weevil *Anthonomus rubi* has five neural receptors that recognize five classes of induced volatile compounds, including MeSA (Bichao *et al.*, 2005). This sensitivity to the

volatile profiles of infested plants may help the insects find their food source, find mates, or it may deter the insects from laying eggs on occupied plants. MeSA is often identified in the volatile profiles of plant defense studies, which is most likely a consequence of the role of its precursor, SA, in plant defense. There have been numerous studies examining the volatile profiles of damaged plants, but relatively few have linked volatile emissions to the responsible plant genes. As mentioned before, volatiles can have a role in the indirect defense response, and MeSA has been implicated in the attraction of the predatory mite *Phytoseiulus persimilis* to lima bean in response to feeding by the herbivorous mite *Tetranychus urticae* (De Boer *et al.*, 2004). In a dual fungal infection/insect feeding study, Cardoza *et al.* (2002) found that peanut plants infected with the white mold *Sclerotium rolfsii* emitted MeSA in addition to other volatile compounds, but insect feeding alone did not induce MeSA emission. In a separate dual bacterial infection/insect-feeding study, Cardoza and Tumlinson (2006) found that biochemical changes in the plant during bacterial infection affect the plant's response against insect feeding. Insects had a lower survival rate on plants emitting volatiles in response to bacterial infection. Each plant-pathogen interaction is unique, but MeSA emission is repeatedly induced from different hosts and pathogens. This suggests that MeSA may also be important in tomato defense against bacterial pathogens, which will be another focus of the current study.

Directly related to the current project, the *SAMT* from tomato (*LeSAMT1*) has been implicated in cross-talk with jasmonic acid (JA) in defense against spider mite feeding (Ament *et al.*, 2004). The JA-synthesis mutant *def-1* did not accumulate MeSA upon spider mite feeding in tomato, and the *LeSAMT1* transcript did not accumulate in the *def-1* mutant unless exogenous JA was applied. This suggests that JA signaling is upstream of SA signaling in this indirect defense response. *AtBSMT* has been implicated in defense against a fungal elicitor and herbivory (Chen

et al., 2003). A *SAMT* from rice (*Oryza sativa*), *OsBSMT*, has also been implicated in wounding and defense (Xu *et al.*, 2005, Koo *et al.*, 2007). To date, the role of MeSA and *LeSAMT1* in response to a bacterial infection in tomato has not been studied, but this role will be examined in Chapter 4.

Objectives of This Study

Plants have evolved to use volatiles for protection and reproduction. Humans have taken advantage of these plant survival mechanisms for commercial and medicinal means—to sell fragrances, improve flavors, and develop medicines. The development of public genomic and expression databases has greatly increased the accessibility of information to identify the genes involved in secondary metabolism. The purpose of this study was to identify and characterize the tomato gene responsible for MeSA synthesis, *S*-adenosyl-$_L$-methionine: salicylic acid carboxyl methyltransferase (*LeSAMT1*) and to determine its biochemical properties. An additional goal of this project was to use transgenic tomato plants overexpressing *LeSAMT1* to determine the role of MeSA in tomato flavor and defense against the bacterial pathogen, *Xanthomonas campestris* pv. *vesicatoria*.

A

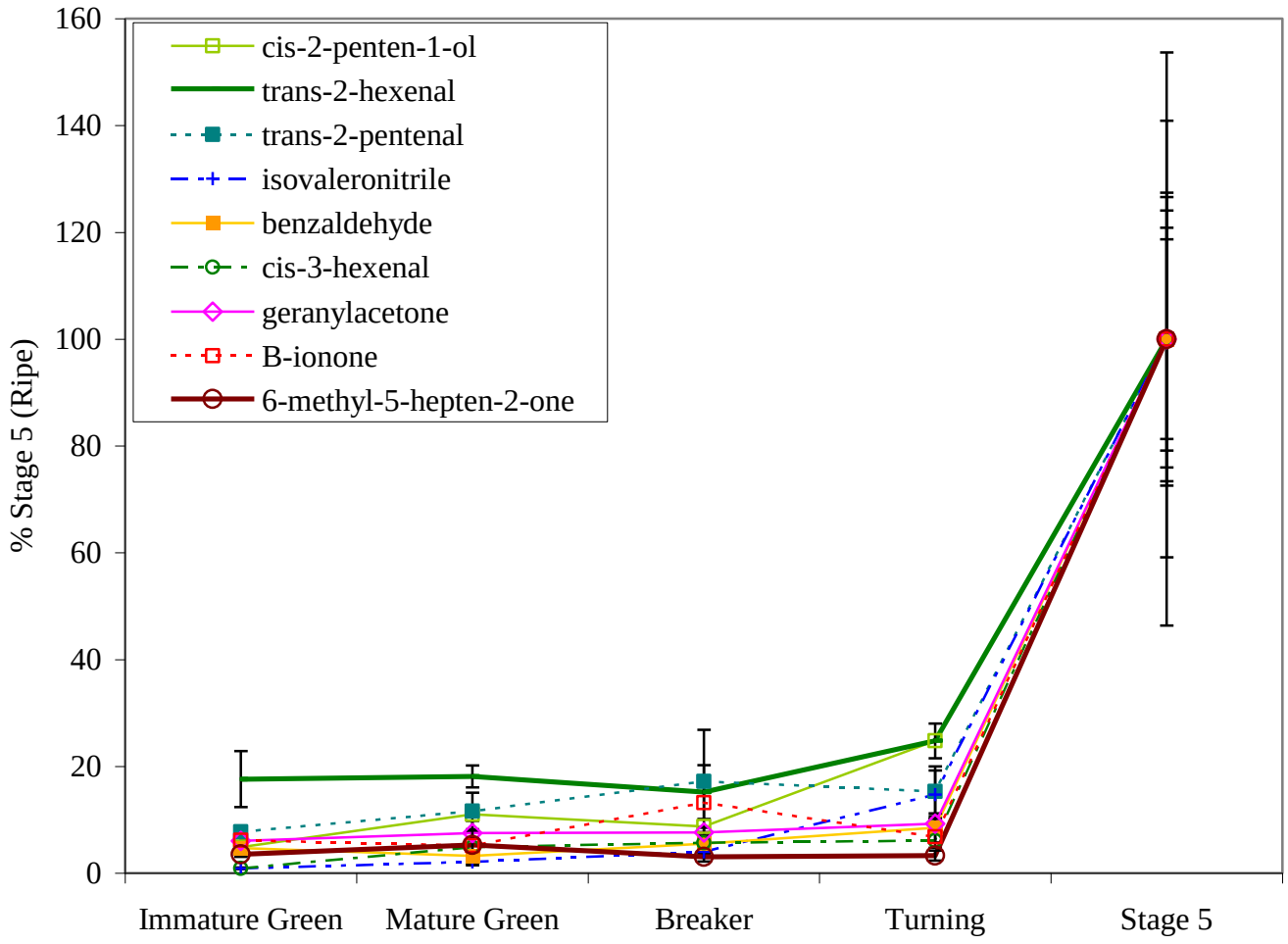
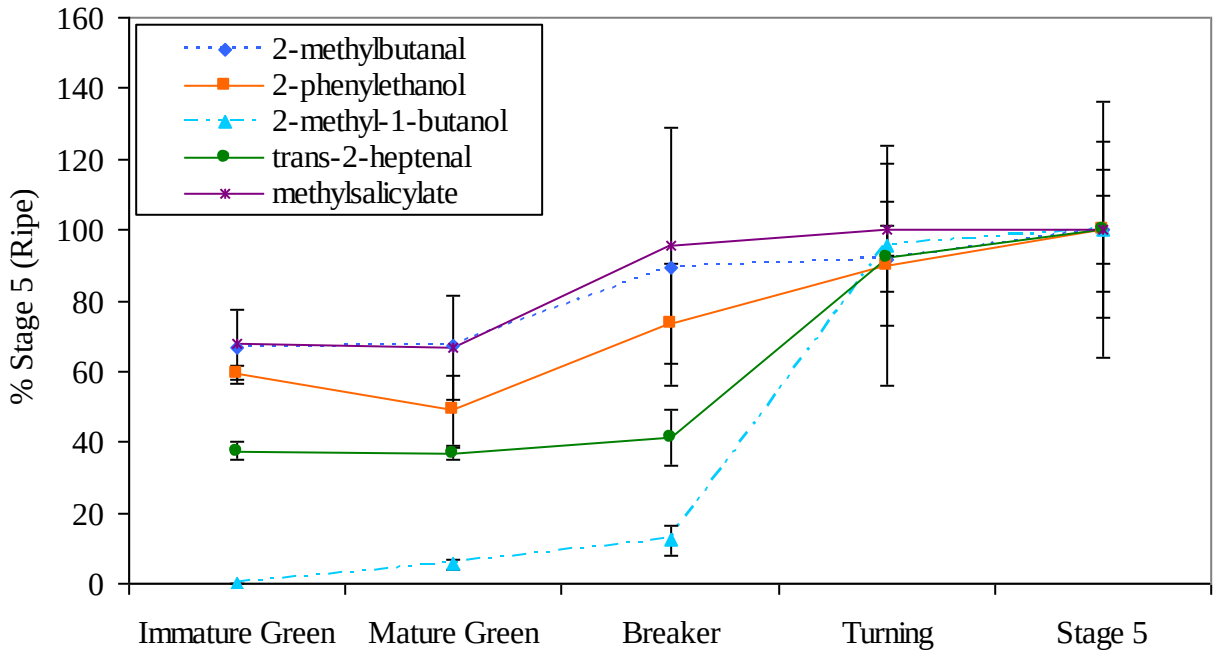


Figure 2-1. Ripening patterns of tomato volatiles in the commercial processing tomato M82. A) Maximum emission at Stage 5, B) Increase at breaker, C) Maximum emission at turning, D) Decrease during ripening, E) No specific ripening pattern. Methylsalicylate appears in B) and shows a slight increase during ripening. Immature green, mature green, and breaker stage fruits were staged by cutting the fruits in half lengthwise. The locular gel of immature green fruits was not fully developed and the immature seeds could be cut with a knife. Mature green fruits had a developed locular gel with a jelly-like consistency and seeds were not cut with a knife. Breaker fruits showed the first signs of pink color on the blossom end of the fruit and showed signs of red color in the locular gel. Turning and Stage 5 fruits were staged by the exterior color. Turning fruits contained approximately 30% red color and Stage 5 fruits were 95% red. Data are presented as a percentage of volatile levels at Stage 5 from field-grown fruits. Error bars represent $\pm$ SE. n = 3.

B



C

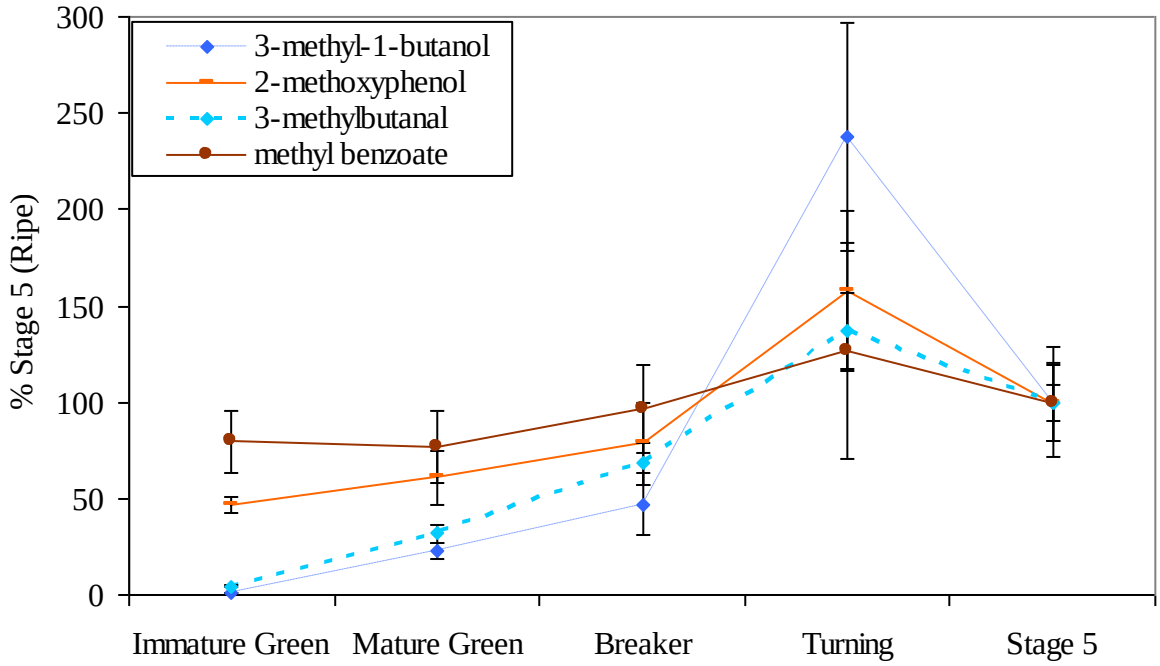
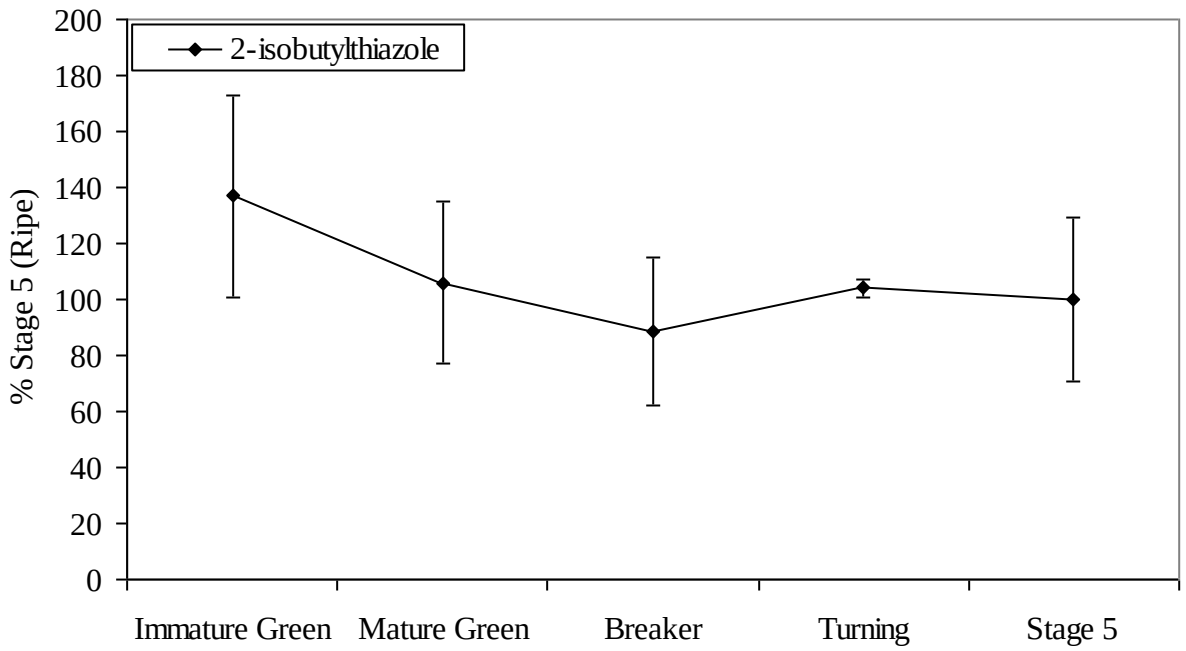


Figure 2-1. Continued

D



E

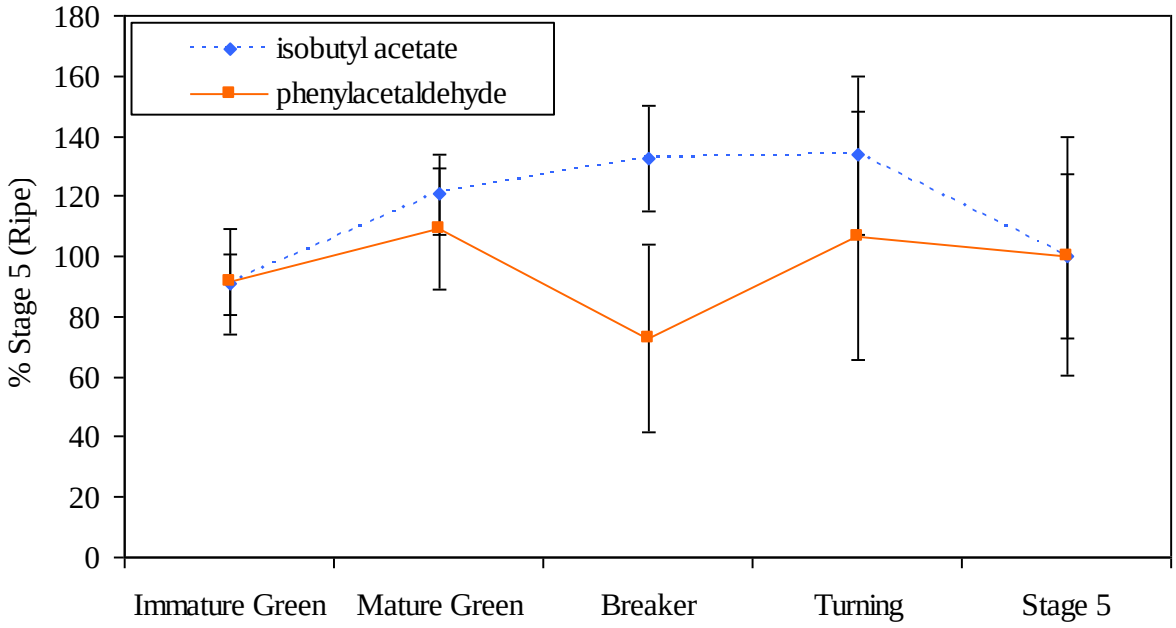


Figure 2-1. Continued

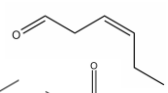
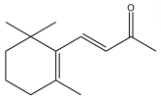
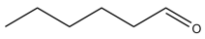
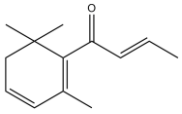
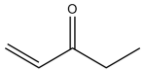
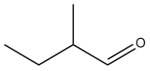
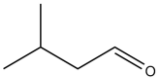
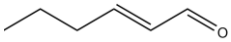
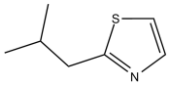
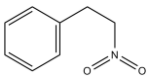
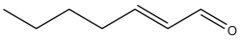
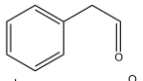
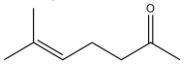
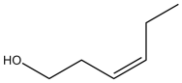
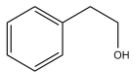
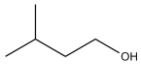
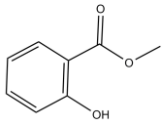
Compound	Structure	Precursor	Log odor unit	M82 (ng/gfw/hr)	Pearson (ng/gfw/hr)	Odor Threshold (ppb)	Flavor Descriptors
cis-3-Hexenal		Lipid	3.7	16.94	19.13	0.25	Green, grass, tomato-like
β-Ionone		Carotenoid	2.8	0.003	0.02	0.007	Floral, sweet
Hexanal		Lipid	2.8	25.02	37.24	5	Green, grass
β-Damascenone		Carotenoid	2.7	0.001	0.001	0.002	Fruity, floral
1-Penten-3-one		Lipid	2.7	0.10	0.38	1	Fresh, sweet, fruity taste, grassy
2-Methylbutanal		Isoleucine	2.1	1.17	1.98	1	Pungent
3-Methylbutanal		Leucine	2.1	2.03	4.32	0.2	Pungent
trans-2-Hexenal		Lipid	1.2	0.33	0.93	17	Green, grassy
Isobutylthiazole		Leucine	1	0.88	1.72	3.5	Pungent, medicinal, tomato leaf
1-Nitro-2-phenylethane		Phenylalanine	0.9	0.03	0.38	2	Musty, earthy
trans-2-Heptenal		Lipid	0.7	0.10	0.34	13	Green
Phenylacetaldehyde		Phenylalanine	0.6	0.01	0.02	4	Floral/alcohol
6-Methyl-5-hepten-2-one		Carotenoid	0.4	0.69	2.20	2000	Sweet, floral, green
cis-3-Hexenol		Lipid	0.3	14.19	44.75	70	Green, leafy
2-Phenylethanol		Phenylalanine	0.3	0.01	0.06	750	Floral, roses
3-Methylbutanol		Leucine	0.2	7.85	23.68	120	Earthy, musty
Methylsalicylate		Phenylalanine	0.08	0.01	0.33	40	Wintergreen

Figure 2-2. Important volatiles in tomato flavor. Compounds are arranged by decreasing log odor unit. Levels of volatile emissions from field-grown ripe Pearson and M82 fruits are also shown.

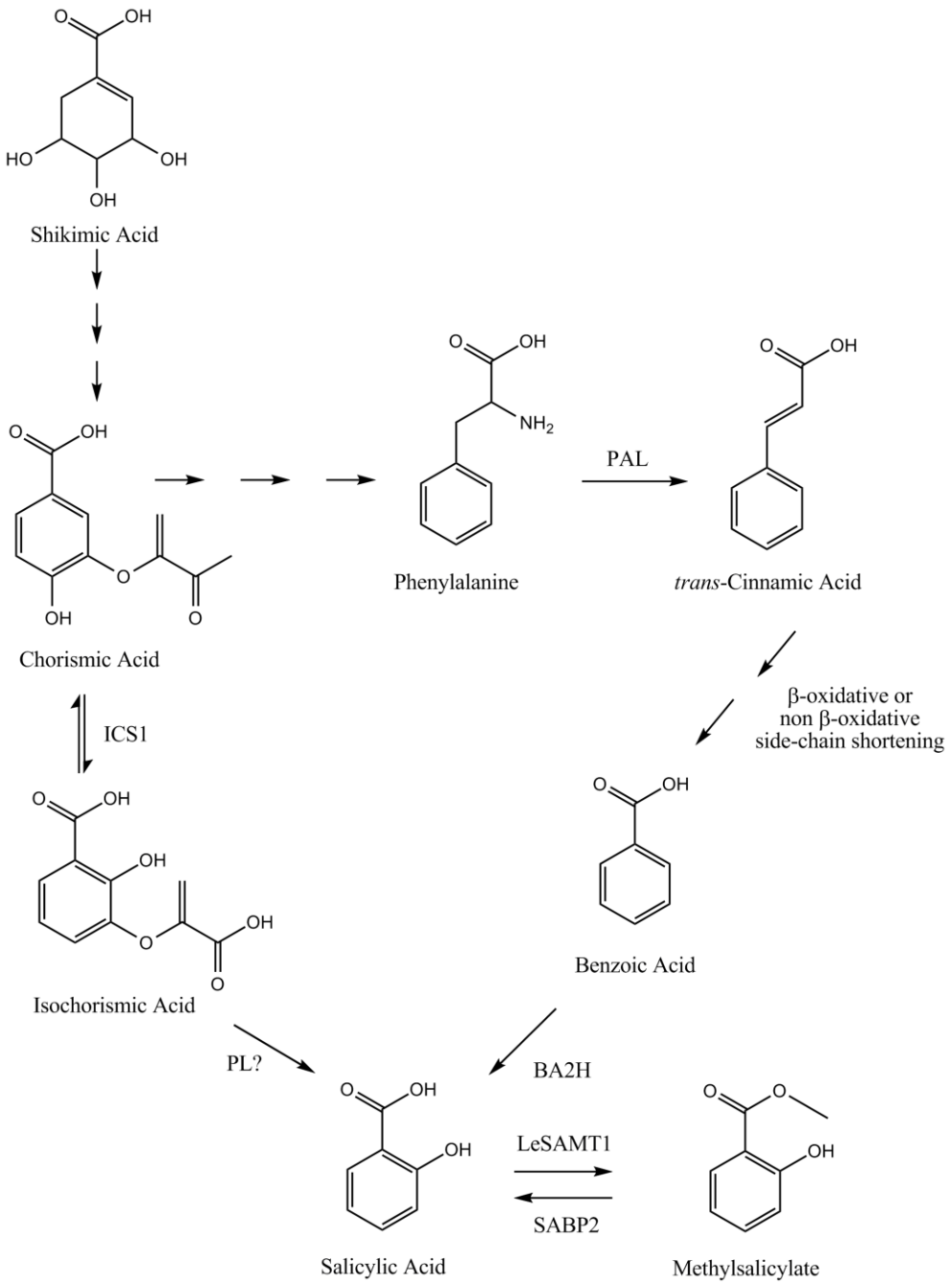


Figure 2-3. Routes of salicylic acid and methylsalicylate synthesis in plants. Abbreviations are as follows: PAL, phenylalanine ammonia lyase; ICS1, isochorismate synthase; BA2H, benzoic acid 2-hydroxylase; LeSAMT1, *S*-adenosyl-$_L$-methionine: salicylic acid carboxyl methyltransferase; SABP2, salicylic acid binding protein; PL, pyruvate lyase, which has not yet been shown in plants but was identified in *Pseudomonas aeruginosa* (Serino *et al.*, 1995).

CHAPTER 3
CHARACTERIZATION OF A TOMATO *S*-ADENOSYL-$_L$-METHIONINE CARBOXYL METHYLTRANSFERASE (*LESAMT1*) IN METHYLSALICYLATE SYNTHESIS AND TOMATO FLAVOR

Introduction

Plants contain a variety of scent compounds in their floral, fruit, and vegetative tissues. These aroma compounds, also called volatiles, are organic compounds of low molecular weight and high vapor pressure that evaporate at room temperature. Plant volatiles serve a variety of functions, including protection, pollination, and seed dispersal. Methylsalicylate (MeSA), or oil of wintergreen, is one such volatile compound found in a variety of flowers, fruits, and leaves. For example, MeSA is a component of floral scent in approximately 80 species of plants represented by approximately 25 families (Effmert *et al.*, 2005). In addition, it is a flavor component of fruits such as apple, strawberry, raspberry, and tomato (Burdock, 1995; Buttery and Ling, 1993). MeSA is also emitted in response to biotic stress. For example, MeSA is emitted from the leaves of tobacco mosaic virus-infected tobacco (Schulaev *et al.*, 1997), spider mite-infested tomatoes (Ament *et al.*, 2004), and bacterial-inoculated pepper (Cardoza and Tumlinson, 2006). MeSA has also been shown to have antioxidant activity in mouthwash (Battino *et al.*, 2002). Therefore, MeSA serves a variety of functions relating to reproduction and protection in plants and in commercial products.

MeSA is synthesized from the plant hormone salicylic acid (SA) via *S*-adenosyl-$_L$-methionine: salicylic acid carboxyl methyltransferase (SAMT). The methyl donor *S*-adenosyl-$_L$-methione (SAM) transfers a methyl group to the carboxylic acid of SA to form the methyl ester MeSA. SAMT was first identified in the *Clarkia breweri* and was classified into a new family of plant methyltransferases, the SABATH family (Ross *et al.*, 1999). SABATH stands for the substrates utilized by the enzymes—salicylic acid (SA), benzoic acid (BA), and theobromine

(TH) (D'Auria *et al.*, 2003). The SABATH family has been extensively studied with respect to floral scent, since volatile emission is closely correlated with pollinator attraction (Effmert *et al.*, 2005). MeSA and methylbenzoate (MeBA) are structurally related compounds synthesized by homologous enzymes. In some cases, both MeSA and MeBA can be synthesized by the same enzyme. Methyltransferases with dual substrate action are called benzoic acid/salicylic acid methyltransferases (BSMTs), while those acting only on benzoic acid are called benzoic acid methyltransferases (BAMTs). *BSMTs* have been identified in *Arabidopsis thaliana* (Chen *et al.*, 2003), *Petunia x hybrida* (Negre *et al.*, 2003; Underwood *et al.*, 2005), and *Oryza sativa* (Koo *et al.*, 2007). A BAMT has been identified in *Antirrhinum majus* (Murfitt *et al.*, 2000), while an SA-specific SAMT has been identified in *Hoya carnosa* (Effmert *et al.*, 2005). Other *Arabidopsis thaliana* family members can utilize substrates such as jasmonic acid (AtJMT) (Seo *et al.*, 2001), indole-acetic acid (AtIAMT) (Zubieta *et al.*, 2003; Qin *et al.*, 2005), gibberellins (AtGAMT) (Varbanova *et al.*, 2007), and a sesquiterpene, farnesoic acid (AtFAMT) (Yang *et al.*, 2006b). Interestingly, the majority of these substrates are plant hormones, and it has been suggested that methylation of these hormones may aid in their transportation across membranes (Yang *et al.*, 2006a). Based on studies of floral scent, methyltransferase activity is most likely involved in the synthesis of the flavor compound MeSA in tomato as well.

Consumers are often dissatisfied with the flavor of tomatoes (*Solanum lycopersicum*), since breeders have focused on traits such as fruit size, color, firmness, yield, and disease resistance, and flavor has generally been ignored. Tomato flavor is due to the interaction of the non-volatile components, sugars and acids, in addition to volatile components. Sugars and acids are responsible for the sweet and sour taste of tomatoes, while the volatile components contribute to the unique flavor of tomato. Over 400 volatile compounds have been identified as constituents

of tomato flavor, but only about 30 of these volatiles are believed to positively contribute to tomato flavor (Buttery and Ling, 1993). Based on their chemical structures, these compounds are believed to be derived from carotenoids, amino acids, and lipids (Buttery and Ling, 1993). It has been suggested that the volatile components of flavor serve as indicators of the nutritional composition of foods (Goff and Klee, 2006). In general, most of the important volatiles of tomato flavor are not synthesized until the fruits are ripe, most likely to facilitate seed dispersal (Tieman *et al.*, 2006). MeSA is an important volatile compound found in tomato (Buttery and Ling, 1993), and has been identified as a key compound differentiating the volatile profiles of tomato cultivars (Tikunov *et al.*, 2005). To date, MeSA synthesis has not been characterized in tomato or any other fruits. Since MeSA is an important component of tomato flavor, the purpose of this study was to identify and characterize the tomato *SAMT* gene (*LeSAMT1*) responsible for MeSA synthesis. In addition, the contribution of MeSA to tomato flavor was determined using transgenic lines that produced elevated levels of MeSA.

Results

Identification of *LeSAMT1*

Using BLAST analysis against the TIGR database, eight full-length tomato SAMT-related ESTs were identified by sequence similarity to the amino acid sequence of CbSAMT. The predicted amino acid sequence of LeSAMT1 was the closest homolog with 66% similarity and 54% identity to CbSAMT. The predicted protein is 362 amino acids with a molecular weight of 41.3 kDa. LeSAMT1 is even more closely related to solanaceous SAMTs as seen in the phylogenetic tree (Figure 3-1). Other SABATH family members are also represented on the tree, including AtIAMT (Zubieta *et al.*, 2003; Qin *et al.*, 2005) and AtGAMT (Varbanova *et al.*, 2007), which recognize the substrates indole-acetic acid and gibberellins, respectively. The amino acid sequence alignment (Figure 3-2) shows that LeSAMT1 contains all the previously

identified SA-binding and SAM-binding residues of the SABATH family of methyltransferases (Zubieta *et al.*, 2003). Especially noteworthy are the substrate binding residues that are identical to CbSAMT and PhBSMT1 at amino acid residues 153, 156, 232, 233, and 357 (Figure 3-2). The seven other tomato homologs were 41% to 60% similar to CbSAMT based on amino acid sequence (Table 3-1). These seven homologs were named *LeMT1-7* (*Solanum lycopersicum* methyltransferase) based on their amino acid sequence similarity to LeSAMT1 (Table 3-1). The amino acid sequence identity of these homologs is also shown (Table 3-2). The amino acid alignment of LeSAMT1 with the other LeMTS and two singleton sequences identified from the SGN database shows that these LeSAMT1 homologs potentially contain SA-binding residues (Figure 3-3). Both CbSAMT and PhBSMT1 have preferred activity on SA (Zubieta *et al.*, 2003; Negre *et al.*, 2003). However, when these residues were mutated by site-directed mutagenesis in a study by Zubieta *et al*. (2003), the resulting CbSAMT protein had a broader substrate specificity and could recognize substrates such as jasmonic acid and vanillic acid. Phylogenetic analysis of the SABATH family has shown that SAMTs specific for SA activity have a Met at position 153 of *C. breweri*, while BSMTs have a His residue at position 153 (Barkman *et al.*, 2007). This study went on to show that the wild-type SAMT of *Datura wrightii* with a Met-153 only produces MeSA, while a M153H substitution of a recombinant SAMT results in the production of MeSA and MeBA. Therefore, it appears that this active site Met is necessary for SA specificity and a His broadens the specificity to BA. LeSAMT1 contains a Met at this position, suggesting that it may preferentially have activity with SA.

Substrate Specificity and Kinetic Properties of LeSAMT1

To determine if LeSAMT1 could convert SA to MeSA *in vitro*, a recombinant GST-tagged LeSAMT1 was expressed in *Escherichia coli*. The N-terminal GST-LeSAMT1 was purified using the GST-affinity purification method (Figure 3-4) and assayed for activity with SA and

structurally related compounds. Compounds on which other known methyltransferases were active in this family were also tested, including jasmonic acid and indole-3-acetic acid. GST-LeSAMT1 had the highest activity with SA, which was normalized to 100% (Figure 3-5). LeSAMT1 had the next highest activity on BA, which was only four percent of the activity seen with SA. The kinetic properties of purified GST-LeSAMT1 were also determined. At 25^{o}C, SA had a K_m of 52 μM (Figure 3-6) and SAM had a K_m of 15 μM (Figure 3-7). V_{maxSA} was 138 pmol/mg/min and V_{maxSAM} was 85 pmol/mg/min. The $kcat_{SA}$ was 0.055 sec^{-1} and $kcat_{SAM}$ was 0.028 sec^{-1}. The K_m values for SA and SAM are within the range of reported values in other characterized methyltransferases, including CbSAMT, PhBSMT1, PhBSMT2, and SfSAMT (Effmert *et al.*, 2005). Since the *in vitro* data showed that LeSAMT1 specifically converted SA to MeSA, *LeSAMT1* was chosen for further analysis *in planta*.

Tissue-Specific Expression of *LeSAMT1*

After determining the substrate specificities of LeSAMT1, the expression of *LeSAMT1* was determined in various tissues. *LeSAMT1* transcript abundance and SA availability in ripening fruits were determined. Studies in *Petunia x hybrida* have shown that volatile emissions can be determined by both substrate availability and substrate preference of the methyltransferase (Negre *et. al.*, 2003; Underwood *et. al.*, 2005). These studies have shown that PhBSMT1 has a substrate preference for SA *in vitro*, but MeSA is not consistently detectable *in planta* because the substrate BA is more abundant. Therefore, PhBSMT1 catalyzes the synthesis of MeBA for floral scent instead of MeSA. *LeSAMT1* expression was first quantified by real-time pcr in flower buds, open flowers, young unexpanded leaves, and mature expanded leaves (Figure 3-8). For transcript abundance during fruit ripening, five stages of M82 fruits were examined—15 days after pollination (dap), mature green, breaker, turning, and ripe (Figure 3-9). *LeSAMT1* is

most highly expressed in immature and mature green fruits, followed by flower bud tissue, open flowers and young unexpanded leaves. *LeSAMT1* expression decreased as the fruits ripened, and expression levels in immature fruits were comparable to levels seen in flowers. The internal metabolite pools of MeSA (Figure 3-10) and SA (Figure 3-10) were also determined in ripening fruits. Since immature green fruits generally release low levels of volatiles from fresh tissue, the internal levels of MeSA were determined from frozen tissue of all fruit stages. Internal MeSA pools were highest in 15 dap fruits (Figure 3-10). No obvious changes in free SA occurred throughout fruit ripening in M82 (Figure 3-11). Internal pools of MeSA did not appear to correlate with either transcript abundance or SA availability, except at the 15 dap stage. However, MeSA is still present in ripe fruits and is considered important for tomato flavor.

Production of Transgenic Lines

To further elucidate the role of MeSA in tomato flavor and its other biological functions, overexpression and antisense transgenic lines in the M82 and Pearson backgrounds were made. The full-length cDNA of *LeSAMT1* was cloned under the control of the constitutive 35S figwort mosaic virus (FMV) promoter in the sense and antisense orientations. M82 is a commercial processing tomato, while Pearson is a variety with more locular compartments (Figure 3-12). Pearson has higher levels of endogenous MeSA than M82 in any season (Tables 3-4, 3-5; see y-axes in Figures 3-15 and 3-16). Transgenic fruits from plants grown in Live Oak, FL were analyzed in the Spring and Fall of 2006. One transgenic line in the Pearson background, pST1OE-6841-1, had over a 100-fold increase in MeSA emission with a 3000-fold increase in transgene abundance in the Spring of 2006 determined by quantitative real-time pcr (Tables 3-4 and 3-6). RNA was run on a gel as a loading control for each reaction (Figure 3-14). This transgenic line also had a 100-fold increase in MeSA emission in the Fall of 2006 (Table 3-4). The intensity of the MeSA peak was noticeably increased on the chromatogram from the

transgenic line (Figure 3-13). Another transgenic line in the M82 background, pST1OE-5220-2a, had an approximately 50-fold increase in MeSA emission in the Spring with over 3000-fold increase in *LeSAMT1* expression determined by quantitative real-time pcr (Tables 3-5 and 3-7). RNA was run on a gel as a loading control for each reaction (Figure 3-14). In the Fall this transgenic line had over 100 times the MeSA emission of M82 (Table 3-5). The absolute values of MeSA emissions varied between the Spring 2006 and Fall 2006 seasons, with the MeSA emissions being higher in the Fall. In addition, both cultivars varied in their endogenous levels of MeSA, indicated by the scales of the y-axes. MeSA emissions from Pearson were ten-fold higher than M82 in the Spring and six-fold higher than M82 in the Fall. Interestingly, *LeSAMT1* overexpression in both cultivars significantly increased MeSA emission in the transgenic lines over two seasons.

The antisense lines in Pearson (Figure 3-15) and M82 (Figure 3-16) showed approximately 50% reduction in MeSA emission that correlated with transcript abundance. Expression of *LeSAMT1* in the Pearson antisense line, pST1AS-6831-1, was reduced by about 50% in flower buds (Figure 3-17), while the M82 antisense lines showed a similar reduction (Figure 3-18). The flower bud tissue was chosen for expression analysis in the antisense transgenic lines because *LeSAMT1* in this tissue was more easily quantified than in the fruits. There is some variability in fruit MeSA emissions between the Fall and Spring 2006 seasons. For example, MeSA emissions from M82 were higher in the Fall than in the Spring. However, the antisense lines in the Fall had a 50% to 70% reduction in MeSA levels, while the Spring 2006 lines had a 40% to 50% reduction. The MeSA emission from the Pearson antisense line was reduced by 60% in the Spring and by 40% in the Fall.

Methylsalicylate Overproducers Taste Different and Are Preferred Equally to Controls

Ripe fruits from the *LeSAMT1* overexpressing line in the Pearson background, pST1OE-6841-1, were used in a triangle taste test to determine if panelists could taste the difference between the transgenic and control tomatoes. The pST1OE-6841-1 line was chosen for the taste panel because the Pearson variety has a more pleasant taste than the commercial processing tomato, M82. Even though pST1OE-6841-1 fruits produce over 100-fold higher MeSA than Pearson, these levels are well below the maximum daily MeSA intake of 740 μg/kg body weight determined by the Expert Panel of Flavor and Extract Manufacturing Association (FEMA) (Adams *et al.*, 2005). Therefore, these transgenic tomatoes have acceptable levels of MeSA for human consumption.

The triangle taste test was done using an untrained consumer panel. Seeds were removed from the control and transgenic tomatoes before giving the samples to the panelists. Tomatoes were sliced into small wedges and placed in plastic cups marked with a random three-digit code. Panelists were randomly given two transgenic pST1OE-6841-1 samples and one control, or two control samples and one transgenic, and asked to pick the odd sample. Results indicated that there was a significant difference between the control and the transgenic fruits ($p \leq 0.01$), with 50% of the panelists choosing the correct sample (Table 3-8).

Since the triangle test determined that there was a difference between the taste of the transgenic line and the control, a subsequent preference and likeability test was done on the same lines. MeSA emission was increased by approximately 80-fold in the transgenic fruits used for the preference test (Table 3-9). The likeability and preference tests were done at the Sensory Testing Facility in the Food Science and Human Nutrition Department at the University of Florida. The panel was an untrained consumer panel. Panelists were give two samples, Pearson

and pST1OE-6841-1, and asked to rate the aroma, sweetness, sourness, tomato flavor, and overall acceptability of the samples on a nine-point hedonic scale (Table 3-10). Panelists were then asked to pick which sample they preferred. Out of 67 panelists, 36 preferred the control and 31 preferred the transgenic (Table 3-11). The difference was not statistically significant. Therefore, the samples were preferred equally. Since this was a panel conducted on the University of Florida campus, the majority of the panelists were in 18 to 24 year age range (36 out of 67 panelists). Interestingly, a significant number of panelists in the 25 to 34 year old range preferred the transgenic sample (Table 3-12), but this was a small sample size (n = 11). The results from the likeability scores (Table 3-13) also showed that the samples were equally accepted and liked by the panelists, even though Pearson consistently scored higher than the transgenic line. The panelists that preferred the control tomato believed that it tasted more "tomato-like." These same panelists described the transgenic tomato as having a bland taste. In contrast, the panelists who preferred the transgenic tomato believed it had more flavor overall, so individual perception was a most likely a factor in choosing and describing the preferred sample.

Discussion

LeSAMT1 Is a Functional Salicylic Acid Carboxyl Methyltransferase

LeSAMT1, a tomato homolog to a known *SAMT* from *Clarkia breweri* (Ross *et al.*, 1999), was cloned, and recombinant GST-LeSAMT1 was shown to have specific *in vitro* activity to SA. This is expected from the amino acid sequence that shows a Met at position 156, which is present in SAMTs that specifically convert SA to MeSA (Barkman *et al.*, 2007). The transcript abundance and SA availability do not completely correlate with internal MeSA pools during fruit development. However, we do not currently have an antibody to LeSAMT1, so we do not know the protein levels during fruit ripening. When *LeSAMT1* was overexpressed, transgenic fruits had up to 100-fold higher MeSA emissions than the control fruits. Interestingly, overexpression of

LeSAMT1 significantly increased the MeSA from two cultivars of tomato with different endogenous levels of MeSA. MeSA production in the antisense lines from M82 and Pearson were reduced by about 50% over two seasons. This incomplete reduction in the antisense lines could be due to the presence of another methyltransferase present in fruits (Figure 3-3), since both cultivars showed a similar trend. So far seven full-length and two singleton SAMT-related methyltransferases have been identified in the tomato genome, so one of these may also be responsible for MeSA synthesis in fruits.

Flavor Panels

It was determined that transgenic tomatoes overproducing MeSA tasted different and were preferred equally to control tomatoes. It appears that the overproduction of MeSA in tomatoes is not necessarily an unfavorable trait, but it is not an overwhelmingly preferred trait, either. Personal preference and recognition of the trait may also influence which tomato is preferred. It was not known how often the panelists in the preference test consumed tomatoes or if they liked tomatoes. The transgenic and control tomatoes were preferred equally in a preference test, even though the controls tended to score higher in the likeability scores. Since the majority of panelists were under age 25, it is most likely that their familiarity with tomatoes is due to tomatoes consumed from the food service industry. These tomatoes are not picked at a fully ripe stage due to the transportation limitations of ripe fruits. Tomatoes picked before they are fully ripe have been reported to have a negative "off-flavor" affecting sweetness, sourness, and overall flavor (Kader *et al.*, 1977). If this panel was repeated, it would be beneficial to know the how frequently the panelists consume tomatoes and if they like tomatoes, which may produce different results.

Other studies have examined the effects of overexpression of flavor genes in tomato. The tomato alcohol dehydrogenase 2 gene (*ADH2*) was overexpressed in tomato and the resulting

transgenic fruits showed a disruption in the balance of lipid-derived 6-carbon volatiles (Speirs *et al.*, 1998), which are often described as "green" volatiles. The 6-carbon alcohols hexanol and *cis*-3-hexenol increased, which lowered the ratios of these alcohols to their precursor aldehydes. An increase in these alcohols correlated with an increase in ripe flavor according to a taste panel. Davidovich-Rikanati *et al*. (2007) found that overexpressing the geraniol synthase gene from lemon basil resulted in tomatoes that produced more monoterpenoid volatiles that were preferred by panelists in a preference test. The transgenic tomatoes were altered in a variety of monoterpenes not normally present in tomato and failed to develop a red color, but were still preferred by 60% of the panel and described as "tomato-like," "perfume," "rose," "lemongrass," and "geranium." Furthermore, it has been shown that different classes of tomato volatiles can decrease the effect of the taste of other classes of volatiles (Baldwin *et al.*, 2004). For example, increasing levels of "green" volatiles, such as the lipid-derived volatiles, can decrease the perception of "floral" volatiles. It has also been shown that varying the levels of MeSA can affect the desirable flavor of foods (Abraham *et al*., 1976). MeSA was added to black tea and the flavor was rated. MeSA levels up to 20 ppm imparted a desirable fragrant and flowery flavor, but above 25 ppm, the wintergreen flavor was more pronounced and the tea was described as bitter.

In the current study, the overproduction of MeSA was preferred equally, but those panelists that preferred the controls commented that the transgenic tomatoes tasted bland. Perhaps the overproduction of MeSA masked the other tomato volatiles which made the tomatoes "lose" their tomato-like taste. On the other hand, panelists that preferred the MeSA overproducing tomatoes believed that these tomatoes had more flavor. This particular volatile was perceived differently by the panelists, and some may be more sensitive to it than others. The

results of this study showed that increasing MeSA in tomato was neither highly desirable nor undesirable, and the flavor balance may have been perceived differently by panelists.

This is the first report of altering MeSA synthesis in fruits. Overexpression of *LeSAMT1* resulted in tomatoes with significantly higher levels of MeSA, which affected the taste of tomatoes. However, tomatoes with this "different" taste were preferred equally to control tomatoes by an untrained consumer panel, even though the controls scored consistently higher in likeability ratings. Since flavor is a highly complex trait involving primary and secondary metabolism, genetic engineering is a valuable tool for targeting specific biosynthetic genes involved in flavor. By altering the synthesis of single compounds in tomato fruits, the contribution of these volatiles to tomato flavor can be studied in a common background. The overexpression of *LeSAMT1* is an example of targeting a specific biosynthetic gene involved in the synthesis of MeSA and evaluating its contribution to tomato flavor.

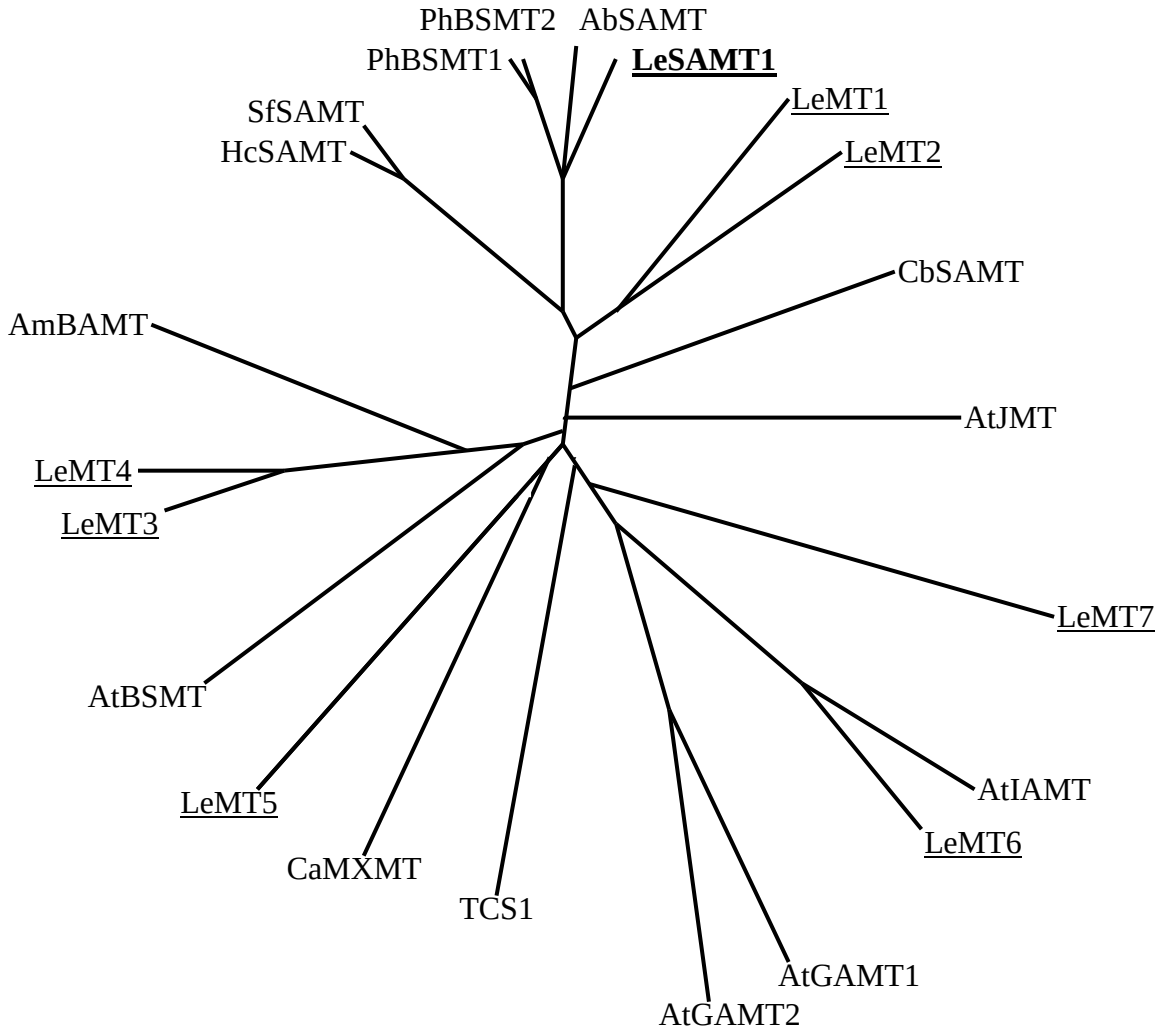


Figure 3-1. Phylogenetic tree of amino acid sequences of SABATH methyltransferases. LeSAMT1 is bolded. LeSAMT1 and the seven full-length tomato homologs related to LeSAMT1 are underlined. These tomato homologs are named LeMT1, LeMT2, LeMT3, LeMT4, LeMT5, LeMT6, and LeMT7. See Experimental Procedures for LeMT (tomato methyltransferase) sequence information. AbSAMT is *Atropa belladonna* (BAB39396), AmBAMT is *Antirrhinus majus* (BAB39396), AtGAMT1, AtIAMT, and AtJMT are *Arabidopsis thaliana* (NP_194372, NP_200336, AAG23343), CaMXMT is *Coffea arabica* 7-MXMT theobromine synthase (BAB39216), CbSAMT is *Clarkia breweri* (AAF00108), HcSAMT is *Hoya carnosa* (CAI05934), PhBSMT1 and PhBSMT2 are *Petunia x hybrida* (AAO45012 and AAO45013), SfSAMT is *Stephanotis floribunda* (CAC33768), and TCS1 is *Camellia sinensis* caffeine synthase (BAB12278). The unrooted dendogram was generated using the Phylip format.

Table 3-1. Percent similarities between amino acid sequences of LeSAMT1, LeMTs, and known methyltransferases.

	aa	LeSAMT1	CbSAMT1	PhBSMT1	PhBSMT2	LeMT1	LeMT2	LeMT3	LeMT4	LeMT5	LeMT6	LeMT7
LeSAMT1	362	100	66	85	85	65	63	58	52	50	43	40
CbSAMT1	359		100	67	67	60	56	57	51	52	41	42
PhBSMT1	357			100	100	68	66	57	50	51	44	41
PhBSMT2	357				100	68	66	58	50	51	44	41
LeMT1	347					100	71	58	49	52	40	40
LeMT2	357						100	54	47	49	40	40
LeMT3	353							100	78	49	42	39
LeMT4	390								100	44	41	44
LeMT5	322									100	42	44
LeMT6	369										100	38
LeMT7	305											100

Table 3-2. Percent identities between amino acid sequences of LeSAMT1, LeMTs, and known methyltransferases.

	aa	LeSAMT1	CbSAMT1	PhBSMT1	PhBSMT2	LeMT1	LeMT2	LeMT3	LeMT4	LeMT5	LeMT6	LeMT7
LeSAMT1	362	100	54	79	79	56	54	43	39	37	31	27
CbSAMT1	359		100	56	56	46	46	43	38	37	31	30
PhBSMT1	357			100	99	59	56	43	38	36	33	28
PhBSMT2	357				100	59	57	43	38	36	33	28
LeMT1	347					100	63	43	37	37	30	27
LeMT2	357						100	40	35	37	30	27
LeMT3	353							100	75	33	30	28
LeMT4	390								100	32	28	32
LeMT5	322									100	29	26
LeMT6	369										100	27
LeMT7	305											100

Figure 3-2. Alignment of the LeSAMT1 amino acid sequence with other known SABATH methyltransferases. Amino acid sequences were aligned using ClustalW. SAM/SAH binding residues are highlighted green, substrate-binding residues are blue, and other active site residues are yellow according to Zubieta *et al.* (2003). See Figure 3-1 for accession numbers and abbreviations.

```
LeSAMT1                 INNDGYNVSKCMRAVAEPLLLSQFDPKLIDLVFQKYEEIVS--KCMAKEDTEFINVTVSL 358
CbSAMT                  VEEEGYNVARCMRAVAEPLLLDHFGEAIIEDVFHRYKLLII--ERMSKEKTKFINVIVSL 354
PhBSMT1                 --NGGYNVSRCMRAVAEPLLVSHFDKELMDLVFHKYEEIVS--DCMSKENTEFINVIISL 353
AmBAMT                  KQRSGKFVADCVRAITEPMLASHFGSTIMDLLFGKYAKKIV--EHLSVENSSYFSIVVSL 361
AtJMT                   ALASGRRVSNTIRAVVEPMLEPTFGENVMDELFERYAKIVG--EYFYVSSPRYAIVILSL 385
AtBSMT                  -FEAGRNEANGIRAVSEPMLIAHFGEEIIDTLFDKYAYHVT--QHANCRNKTTVSLVVSL 376
AtIAMT                  ASEVGRAFASSCRSVAGVLVEAHIGEELSNKLFSRVESRATSHAKDVLVNLQFFHIVASL 383
AtGAMT1                 PDSYGRDRANYAQAGLKPIVQAYLGPDLTHKLFKRYAVRAA--ADKEILNNCFYHMIAVS 372
                            *   :   ::    ::   :. : . :* :             .    :

LeSAMT1                 TKKK- 362
CbSAMT                  IRKSD 359
PhBSMT1                 TKIN- 357
AmBAMT                  SRR-- 364
AtJMT                   VRTG- 389
AtBSMT                  TKK-- 379
AtIAMT                  SFT-- 386
AtGAMT1                 AVRV- 376
```

SAM/SAH Binding Residues

Substrate Binding Residues

Additional Active Site Residues

Figure 3-2. Continued

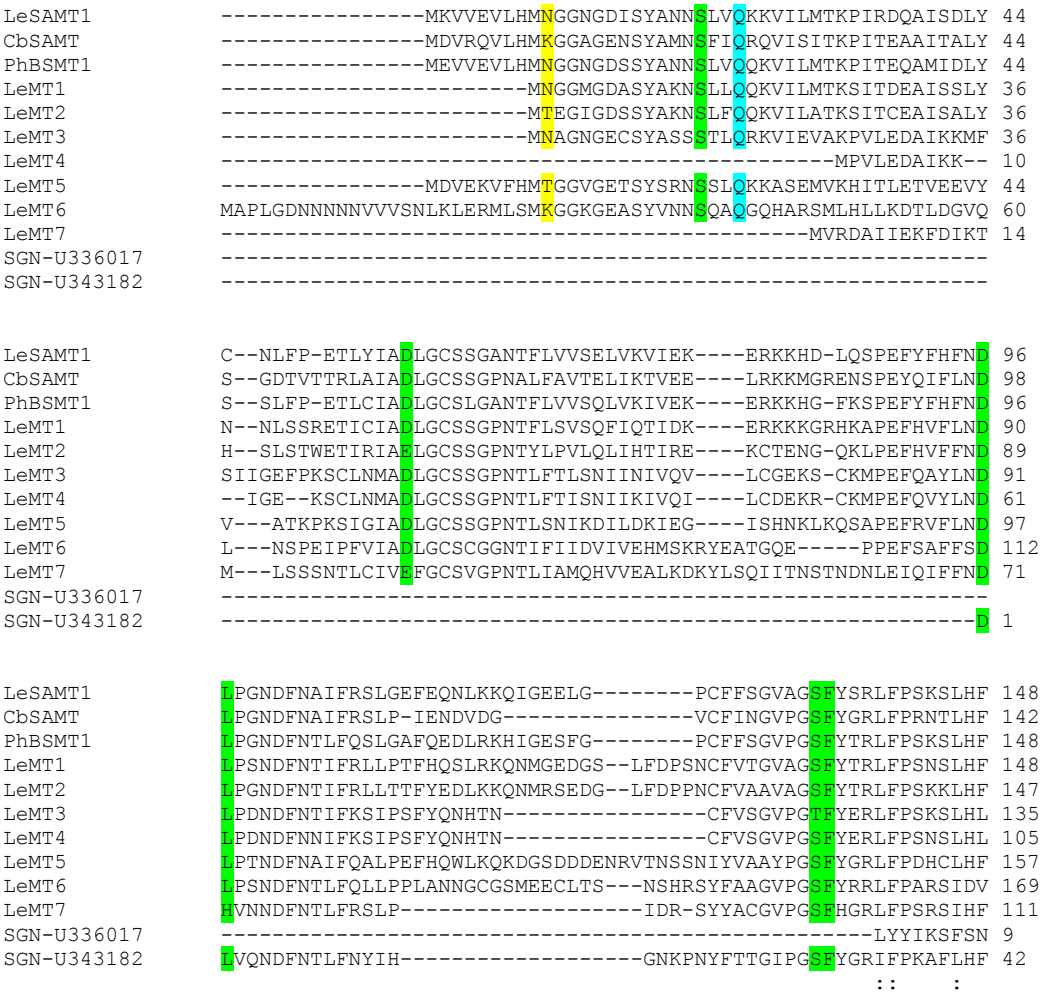


Figure 3-3. Alignment of LeSAMT1 and related tomato methyltransferase sequences. Full-length sequences of LeSAMT1-related homologs and two singleton sequences from the SGN database were aligned using ClustalW. See Experimental Procedures for LeMT (tomato methyltransferase) sequence information. The *Clarkia breweri* SAMT (AAF00108) and *Petunia x hybrida* BSMT1 (AAO45012) were also included in the alignment since they have known activity with SA (Ross *et al.*, 1999; Negre *et al.*, 2003). The two singleton sequences from the SGN database are SGN-U336017 (clone cTSB-1-J1, from a seed library) and SGN-U343182 (clone FA21BA02, from a mixed fruit library containing immature green, mature green, breaker, turning, and red ripe fruits). SAM/SAH binding residues are highlighted green, substrate-binding residues are blue, and other active site residues are yellow according to Zubieta *et al.* (2003).

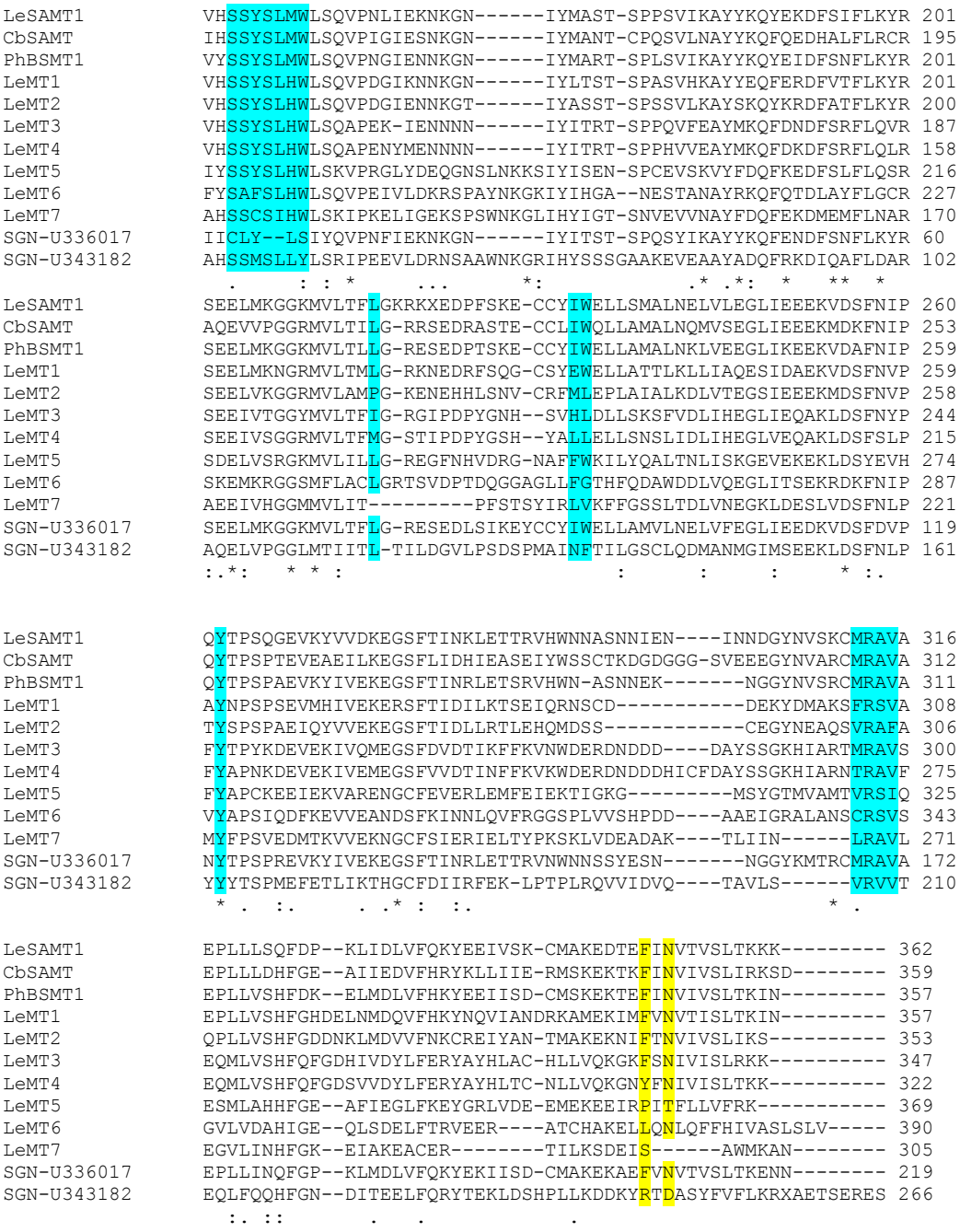


Figure 3-3. Continued

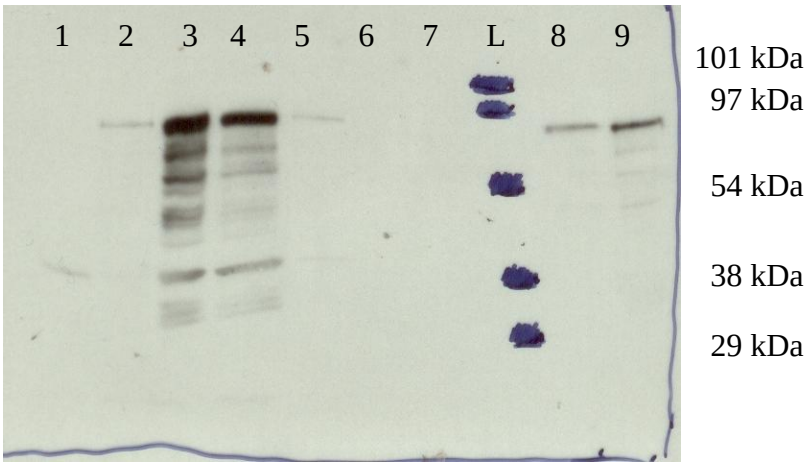


Figure 3-4. Purified GST-LeSAMT detected with α-GST antibodies. Lane 1, uninduced culture; Lane 2, induced culture; Lane 3, crude extract; Lane 4, flow through; Lanes 5,6,7, washes; L, ladder (prestained SDS-PAGE low range standards, BioRad); Lane 8, elution 1; Lane 9, elution 2. The GST-LeSAMT1 fusion protein is expected to be 67.6 kDa.

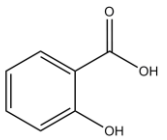
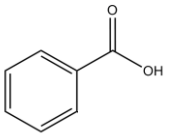
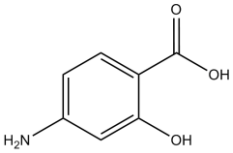
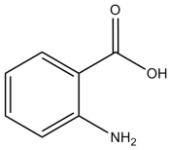
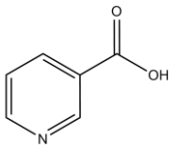
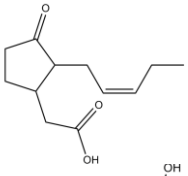
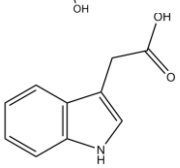
Structure	Substrate	Relative Activity
	Salicylic acid	100 ± 2.5
	Benzoic acid	4 ± 0.2
	p-Aminosalicylic acid	3 ± 0.3
	Anthranilic acid	2 ± 0.4
	Nicotinic acid	$\leq 1 \pm 0.2$
	Jasmonic acid	$\leq 1 \pm 0.7$
	Indole-3-acetic acid	$\leq 1 \pm 0.3$

Figure 3-5. Substrate specificities of LeSAMT1. Purified GST-LeSAMT1 was assayed with 1 mM of each substrate and 30 μM SAM. 2.85 μg protein was assayed at 25°C, pH 7.5 for one hour. Samples were performed in triplicate as well as a no enzyme control. 100% activity equaled 8.2 nmol/hr/mg protein.

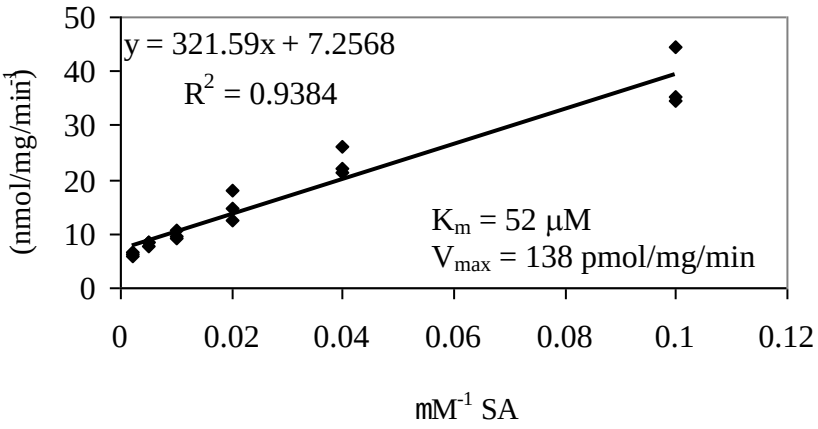


Figure 3-6. Lineweaver-Burke plot for the K_m of SA. ^{14}C-SAM was held constant at 75 μM. Assays were done at 25°C for two hours. Samples were performed in triplicate as well as a no enzyme control.

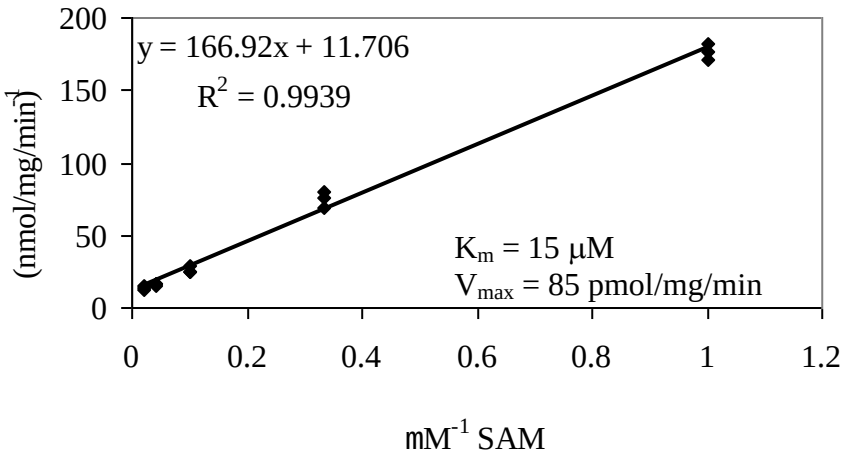
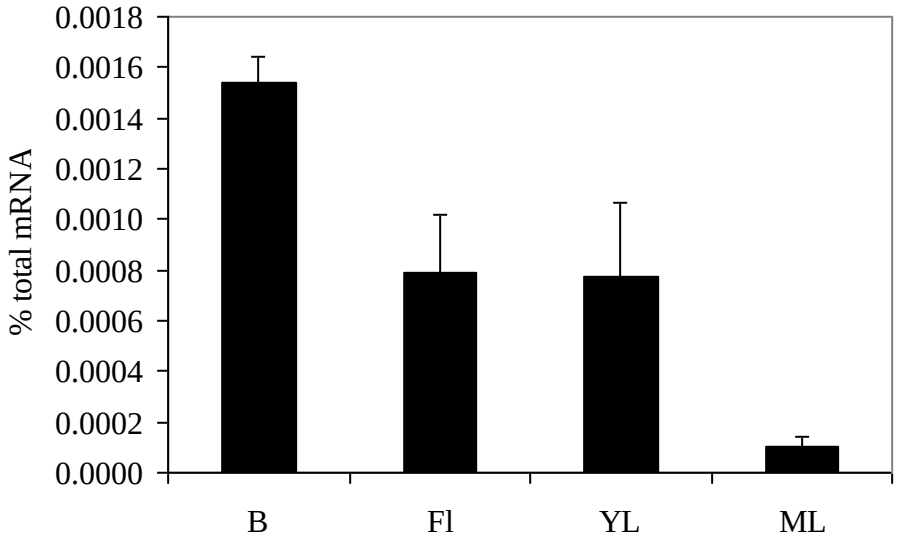


Figure 3-7. Lineweaver-Burke plot for the K_m of SAM. SA was held constant at 1 mM. Assays were done at 25°C for two hours. Samples were performed in triplicate as well as a no enzyme control.

A



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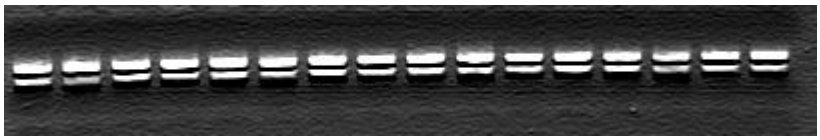
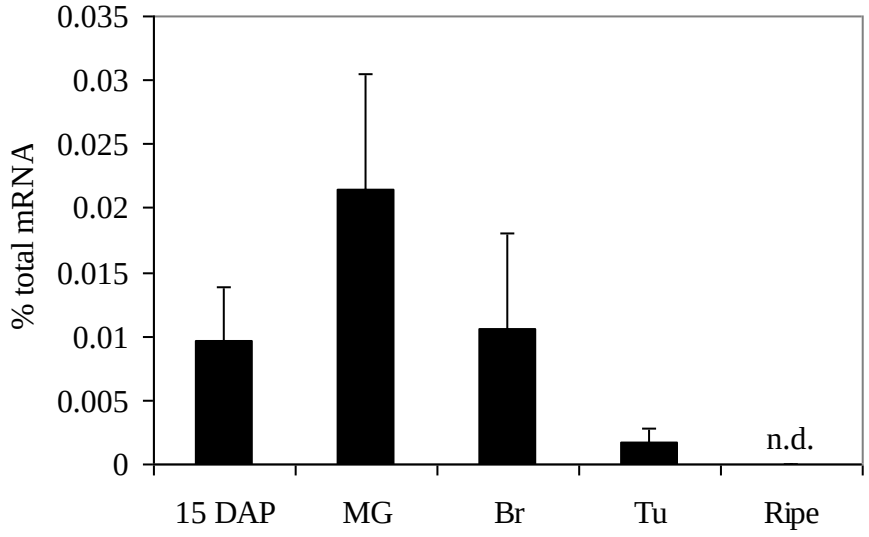


Figure 3-8. *LeSAMT1* tissue-specific expression in M82. A) B = bud, Fl = flower, YL = young leaf, ML = mature leaf. Error bars = SE. n = 4. B) 300 ng of each RNA sample was run on a 1% TBE gel and stained with ethidium bromide as a loading control for each reaction. Lanes 1-4, B; Lanes 5-8, Fl; Lanes 9-12, YL, Lanes 13-16, ML. RNA was collected from four biological replicates of vegetative and floral tissue from the field at Live Oak, FL and analyzed by quantitative real-time pcr (RT-PCR).

A



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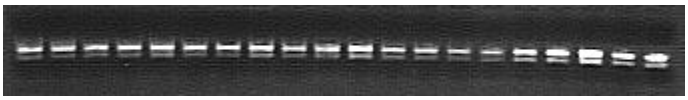


Figure 3-9. *LeSAMT1* expression during M82 fruit ripening. A) 15 DAP = 15 days after pollination, MG = mature green, Br = breaker, Tu = turning. Error bars = SE. n = 4. n.d. is not detectable. B) 200 ng of RNA from each sample was run on a 1% TBE gel and stained with ethidium bromide as a loading control for each reaction. Lanes 1–4, 15DAP; Lanes 5–8, MG; lanes 9–12, Br; Lanes 13–16, Tu; Lanes 17–20, Ripe. RNA was collected from 4 individual fruits from the greenhouse.

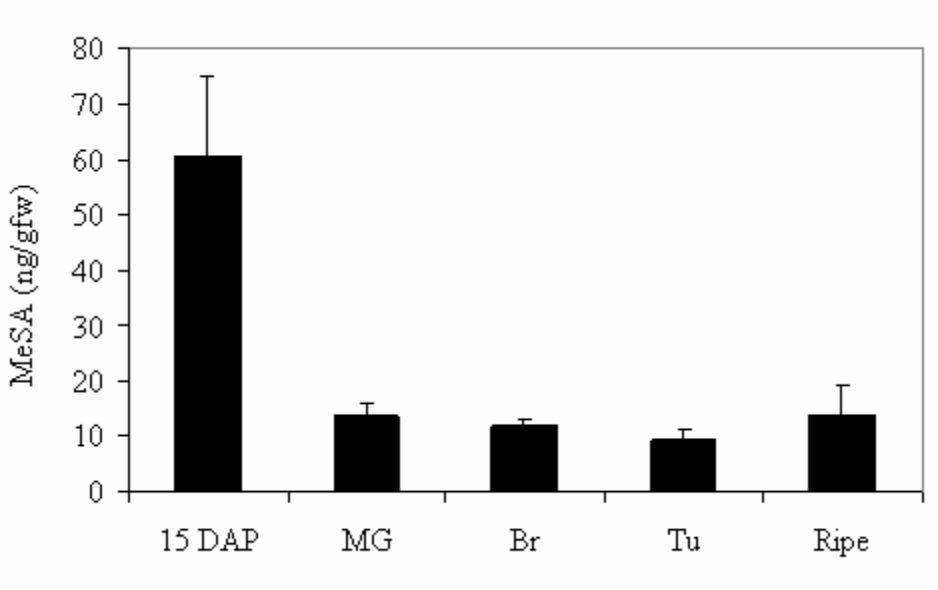


Figure 3-10. Internal pools of MeSA during M82 fruit ripening. Internal levels of MeSA from 4 individual greenhouse fruits of each stage. 15 DAP = 15 days after pollination, MG = mature green, Br = breaker, Tu = turning. Error bars = SE. n = 4.

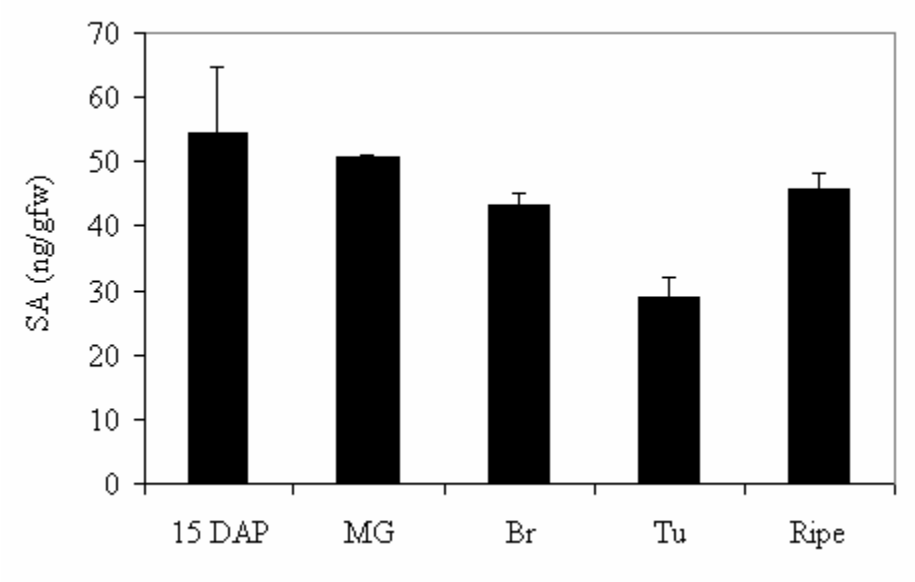


Figure 3-11. Internal pools of free SA during M82 fruit ripening. Internal levels of SA from 4 individual greenhouse fruits of each stage. 15 DAP = 15 days after pollination, MG = mature green, Br = breaker, Tu = turning. Error bars = SE. n = 4.

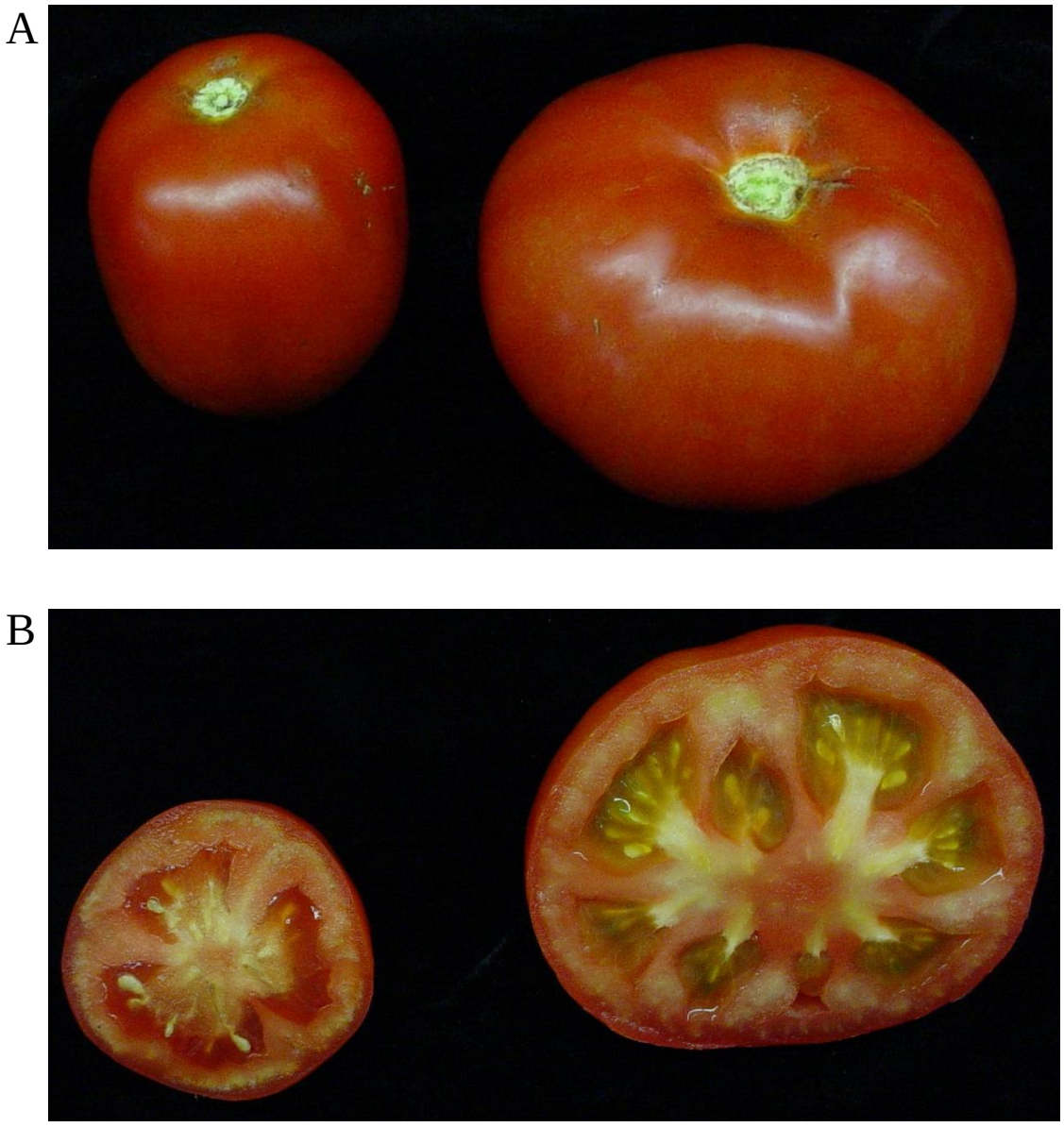


Figure 3-12. Comparison between cultivars M82 and Pearson. A) Whole fruit of M82 (left) and Pearson (right), B) Interior view of M82 (left) and Pearson (right).

Table 3-4. MeSA emission from pST1OE-6841-1 and Pearson ripe fruits.

Sample	Spring 2006	Fall 2006	
pST1OE-6841-1	48.97	78.08	MeSA ng/gfw/hr
	5.96	33.10	SE
Pearson	0.40	0.32	MeSA ng/gfw/hr
	0.08	0.10	SE

pST1OE-6841-1 is an *LeSAMT1* overexpressing line in the Pearson background. Plants were grown in Live Oak, FL. Error bars = SE. For Spring 2006, n = 17, 16. For Fall 2006, n = 4, 8.

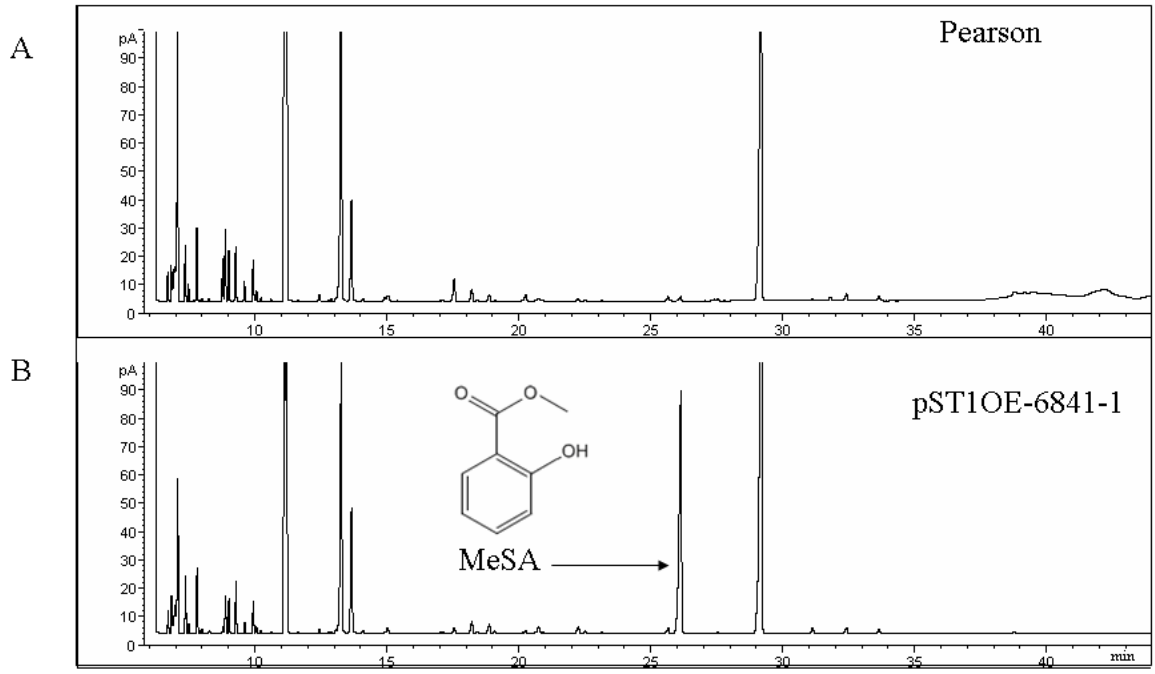


Figure 3-13. Chromatograms comparing volatile emissions from Pearson and pST1OE-6841-1 ripe fruits. A) Pearson chromatogram and B) pST1OE-6841-1 chromatogram. The MeSA peak ran at 26.13 min.

Table 3-5. MeSA emission from pST1OE-5220-2a and M82 ripe fruits.

	Spring 2006	Fall 2006	
pST1OE-5220-2a	1.77	12.69	MeSA ng/gfw/hr
	0.76	3.33	SE
M82	0.04	0.10	MeSA ng/gfw/hr
	0.01	0.02	SE

pST1OE-5220-2a is an *LeSAMT1* overexpressing line in the M82 background. Plants were grown in Live Oak, FL. Error bars = SE. For Spring 2006, n = 17, 13. For Fall 2006, n = 7, 8.

Table 3-6. *LeSAMT1* expression from pST1OE-6841-1 and Pearson ripe fruits.

	% total mRNA	STDEV
pST1OE-6841-1	8.14E-03	3.14E-03
Pearson	3.23E-06	3.84E-07

Several fruits were pooled in Spring 2006 and assayed by quantitative real-time polymerase chain reaction (RT-PCR) in duplicate. n = 2.

Table 3-7. *LeSAMT1* expression from pST1OE-5220-2a and M82 ripe fruits.

	% total mRNA	STDEV
pST1OE-5220-2a	1.12E-02	2.74E-03
M82	3.22E-06	3.72E-07

Several fruits were pooled in Spring 2006 and assayed by quantitative real-time polymerase chain reaction (RT-PCR) in duplicate. n = 2.

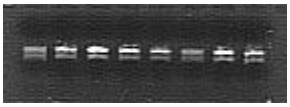
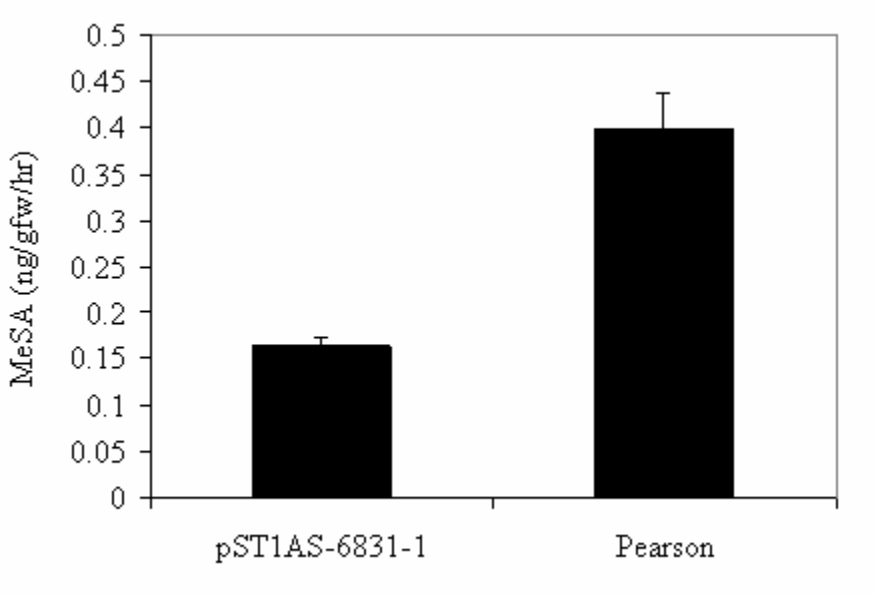


Figure 3-14. RNA gel of MeSA overproducing ripe fruits and control ripe fruits. Lanes 1–2, pST1OE-6841-1; Lanes 3–4, Pearson; Lanes 5–6, pST1OE-5220-2a; Lanes 7–8, M82. 200 ng of RNA was run on a 1% TBE gel and stained with ethidium bromide as a loading control for each reaction.

A



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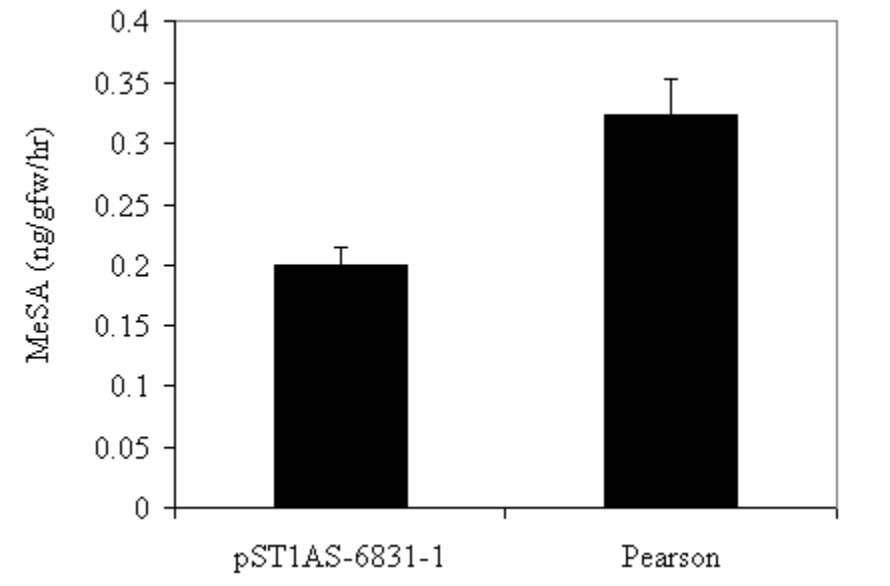
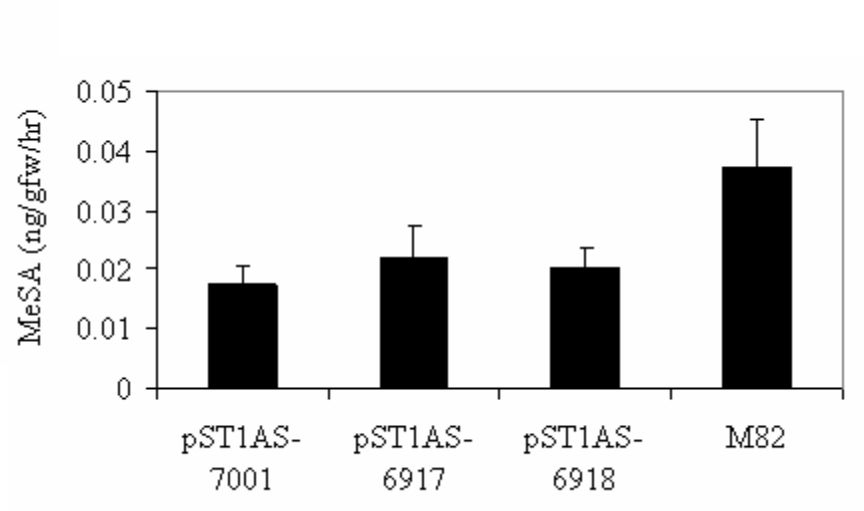


Figure 3-15. MeSA emission from pST1AS-6831-1 and Pearson ripe fruits. A) Spring 2006 and B) Fall 2006. pST1AS-6831-1 is an *LeSAMT1* antisense line in the Pearson background. Plants were grown in Live Oak, FL. Error bars = SE. For A), n = 3, 16. For B), n = 4, 8.

A



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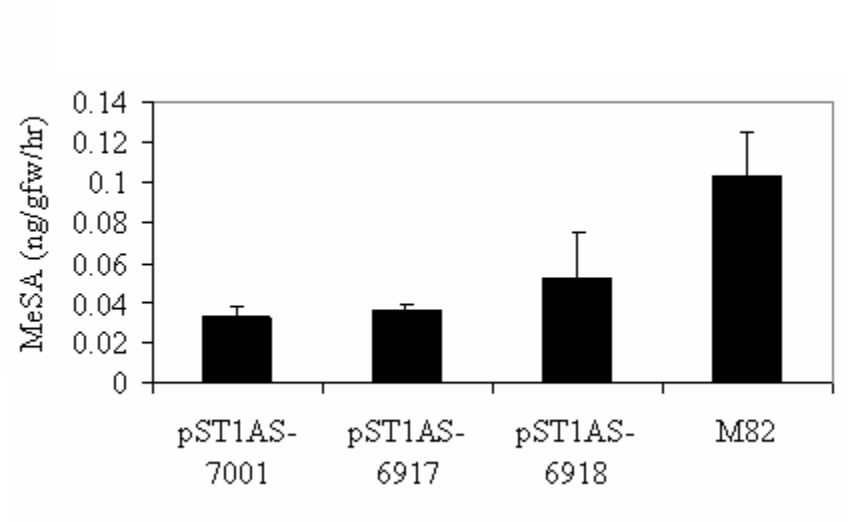


Figure 3-16. MeSA emission from *LeSAMT1* antisense fruits in the M82 background. A) Spring 2006 and B) Fall 2006. pST1AS-7001, 6917, and 6918 are *LeSAMT1* antisense lines in the M82 background. For A), n = 5, 3, 9, 13. For B), n = 6, 4, 5, 18.

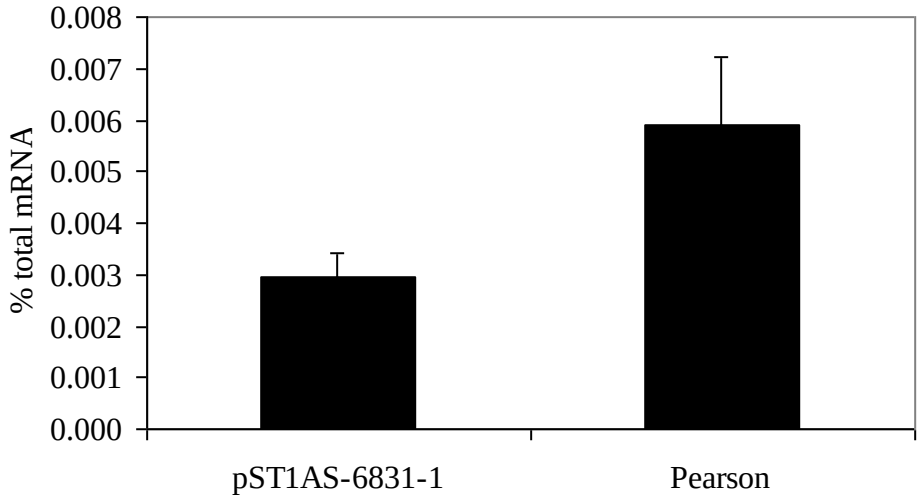


Figure 3-17. *LeSAMT1* expression from pST1AS-6831-1 and Pearson flower buds. pST1AS-6831-1 is an *LeSAMT1* antisense line in the Pearson background. Several flower buds were pooled and assayed by quantitative real-time polymerase chain reaction (RT-PCR) in duplicate from Spring 2006. Error bars = STDEV. n = 2. The quality of RNA was checked on a 1% TBE gel stained with ethidium bromide (not shown).

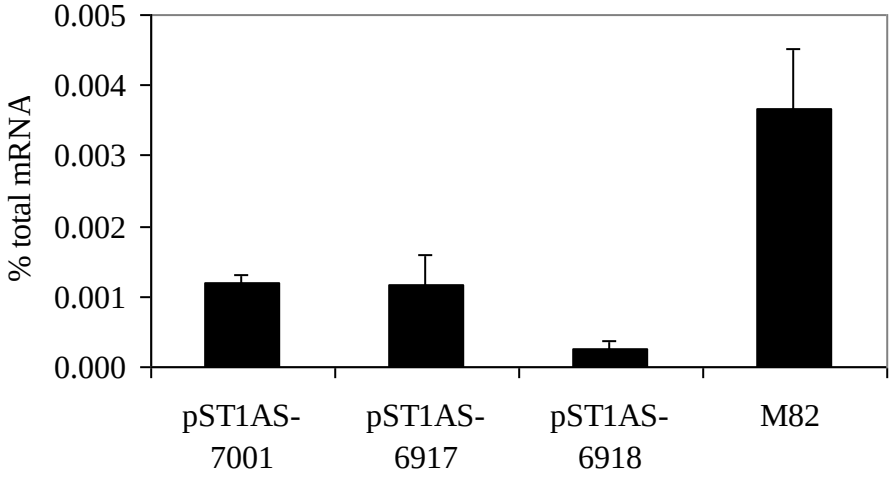


Figure 3-18. *LeSAMT1* expression from flower buds in M82 antisense lines. pST1AS-7001, 6917, and 6918 are *LeSAMT1* antisense lines in the M82 background. Several buds were pooled and assayed by quantitative RT-PCR from Spring 2006 in duplicate. Error bars = STDEV. n = 2. RNA was run on a 1% TBE gel stained with ethidium bromide as a loading control for each reaction (not shown).

Table 3-8. Triangle taste test results between Pearson and pST1OE-6841-1 fruits.

Total Number Correct	30
Number incorrect	30
Total	60

30 correct for p-value $\leq$ 0.01. A chi-square test was used to determine the significance of the number of correct responses. The probability of random guessing was assumed to be 33.33%.

Table 3-9. MeSA emission from fruits used in the preference and likeability tests.

	ng/gfw/hr	SE
pST1OE-6841-1	71.94	25.66
Pearson	0.87	0.13

pST1OE-6841-1 is a line overexpressing *LeSAMT1* in the Pearson background. Ripe fruits were collected in Spring 2007. Error bars = SE. n = 3.

Table 3-10. Hedonic scale parameters for the likeability taste test.

Value	Descriptor
1	dislike extremely
2	dislike very much
3	dislike moderately
4	dislike slightly
5	neither like nor dislike
6	like slightly
7	like moderately
8	like very much
9	like extremely

Table 3-11. Results for preference test between Pearson and pST1OE-6841-1 fruits.

Pearson preferred	36
pST1OE-6841-1 preferred	31
Total	67

The difference was not statistically significant according to a two-sided directional difference test. The samples were preferred equally.

Table 3-12. Preference test results for Pearson and pST1OE-6841-1 fruits by age range.

Pearson preferred	Age Range	pST1OE-6841-1 preferred	Age Range	Total in age range
22	under 18–24	15	under 18–24	37
1	25–34	*10	25–34	11
7	35–44	2	35–44	9
5	45–54	3	45–54	8
1	55–65	1	55–65	2
36	Total	31	Total	67

* indicates $p \leq 0.05$ according to a two-sided directional difference test.

Table 3-13. Cross-tabulated scores for likeability test comparing flavor attributes between Pearson and pST1OE-6841-1.

	Aroma	Sweetness	Sourness	Tomato Flavor	Overall Acceptability	
Pearson	429.00	414.00	387.00	435.00	435.00	Total Score
pST1OE-6841-1	427.00	399.00	374.00	426.00	429.00	
Pearson	7.00	6.00	5.00	7.00	7.00	Median
pST1OE-6841-1	7.00	6.00	5.00	6.00	7.00	
Pearson	6.40	6.18	5.78	6.49	6.49	Mean
pST1OE-6841-1	6.37	5.96	5.58	6.36	6.40	
Pearson	1.415	1.632	1.485	1.407	1.319	STDEV
pST1OE-6841-1	1.774	1.637	1.539	1.453	1.558	

Values for total scores were calculated by totaling the hedonic scores for each attribute. Means were calculated by dividing the total score by the total number of panelists (67). Differences were not statistically significant according to a one-way ANOVA.

CHAPTER 4
ROLE OF METHYLSALICYLATE IN RESPONSE TO BACTERIAL PATHOGEN INFECTION IN TOMATO

Introduction

Plants must respond to a variety of environmental stresses, including pathogen attack. Plant-pathogen interactions can be classified as compatible or incompatible. In a compatible interaction, the pathogen successfully infects the plant host and is able to multiply, therefore causing expansive disease symptoms. In this case the pathogen is virulent. On the other hand, an incompatible interaction occurs when the plant host is able to mount a resistance response to the pathogen, which results in limited growth of the pathogen. The plant host usually accomplishes this by localizing the cell death response to the area of infection, and the pathogen is considered to be avirulent. In this case, the defense response is localized at the site of infection, which is also called local resistance. In addition, plants can mount a systemic resistance to protect themselves from subsequent attack by a pathogen. This systemic acquired resistance response is designated SAR. In SAR, distal tissues not infected by a pathogen accumulate salicylic acid (SA) and upregulate pathogenesis-related genes (*PR* genes) that provide protection against subsequent infection. After the secondary infection, bacterial growth is restricted in the distal tissue.

SA has been implicated in both local resistance and SAR (Durrant and Dong, 2004). For example, the *sid2* (SA induction-deficient) mutant of *Arabidopsis thaliana* is more susceptible to local infection by *Pseudomonas syringae* and *Peronospora parasitica* and is impaired in SAR (Nawrtath and Metraux, 1999). The *sid2* mutant fails to accumulate SA in response to biotic and abiotic stress. The *sid2* mutant was later defined as ICS1 (isochorismate synthase), a step in the SA biosynthesis pathway, showing that normal levels of SA are required for local and systemic responses (Wildermuth *et al.*, 2001). Previous work in our lab has shown that action of the phytohormones jasmonic acid, ethylene, and SA are required for a successful infection by the

virulent pathogen *Xanthomonas campestris* pv. v*esicatoria (Xcv)* 93-1 in tomato (*Solanum lycopersicum*) (O'Donnell *et al.*, 2001; O'Donnell *et al.*, 2003). *Xcv* is the bacterial pathogen responsible for bacterial spot disease in tomato. Disease progression during *Xcv* infection can be divided into two phases. Primary symptoms include lesion formation on the abaxial surface of the leaf around 4 days post inoculation (dpi), while secondary symptom development begins around 8 dpi and includes the appearance of chlorotic patches on the blade of the leaf. Around 10 dpi, necrotic lesions appear within these chlorotic patches that eventually spread throughout the blade of the leaf by 16 dpi (O'Donnell *et al.*, 2001). Transgenic plants overexpressing a bacterial SA hydroxylase (*nahg*) are deficient in SA and have been used as a tool to study the role of SA in the response to *Xcv* (Gaffney *et al.*, 1993). Previous work in our lab has shown SA is required for symptom development during the course of *Xcv* infection in tomato (O'Donnell *et al*., 2001).

SA can also be converted to the volatile compound methylsalicylate (MeSA), which has also been implicated in defense in different plant-pathogen interactions (Schulaev *et al.*, 1997; Chen *et al.*, 2003; Koo *et al.*, 2007). MeSA is synthesized from SA via *S*-adenosyl-$_{L}$-methionine: salicylic acid carboxyl methyltransferase, or SAMT (Ross *et al.*, 1999). The Arabidopsis *S*-adenosyl-$_{L}$-methionine: benzoic acid/salicylic acid carboxyl methyltransferase (*AtBSMT*) was upregulated in response to the fungal elicitor alamethicin and thrip (*Plutella xylostella*) feeding (Chen *et al.*, 2003). It has been shown that MeSA can be converted to SA via an esterase from tobacco, salicylic acid-binding protein 2 (SABP2) (Kumar and Klessig, 2003; Forouhar *et al.*, 2005). This esterase is inhibited by its product, SA (Forouhar *et al.*, 2005). SABP2-silenced tobacco plants infected with tobacco mosaic virus (TMV) had larger lesions in the local infection and were impaired in SAR and their responsiveness to SA (Kumar and Klessig, 2003), suggesting a role for the MeSA pool in conversion back to SA. Here, we have examined the role

of MeSA in the tomato response to *Xcv*. We have used transgenic tomato lines overexpressing *LeSAMT1* that have significantly more MeSA to examine the effects upon disease symptom development.

Results

Mature Leaves of *LeSAMT1* Overexpressors Produce More Methylsalicylate

In order to establish a baseline for understanding the role of MeSA in pathogen infection of tomato plants, MeSA emissions from the control and MeSA overproducing line were examined. Volatile emissions were collected from mature leaves of M82 and the transgenic line pST1OE-5220-2a. Mature leaves from the transgenic line had a three-fold increase in MeSA emission over M82—a significant increase ($p \leq 0.01$) (Figure 4-1). Therefore, the constitutively expressed *LeSAMT1* is functional in mature leaf tissue, the stage used for bacterial inoculations.

LeSAMT1 Overexpressing Lines Show a Delayed Disease Response after *Xcv* 93-1 Inoculation

The purpose of this study was to examine the effect of MeSA on the local disease response to a virulent strain of *Xcv* (93-1). Leaves 3 and 4 of the M82 and pST1OE-5220-2a plants were inoculated with *Xcv* 93-1. The visible symptoms of disease progression in the M82 plants were similar to previously described symptom development in other cultivars of tomato (O'Donnell *et al.*, 2001). Briefly, pinpoint lesions appeared on the abaxial side of the inoculated leaves 5-6 days post inoculation (dpi). Mild chlorosis appeared on the tips of the leaflets 7–8 dpi, and by 9–10 dpi the chlorosis became even more severe and spread beyond the tip of the leaflet. The increasing severity of the chlorosis was accompanied by the appearance of necrotic spots and lesions. By 14 dpi, the necrotic lesions had grown larger and the tips of the leaflets became necrotic, also described as expansive necrosis (Figure 4-2). In an initial experiment, the bacterial growth was assayed in both the M82 and transgenic line during the course of disease. No

difference was observed between the two lines (Figure 4-3), indicating that overproduction of MeSA did not affect bacterial growth.

Interestingly, the MeSA overexpressing line was delayed in the development of necrotic lesions at 10 dpi. Ion leakage, a quantitative measure of cell death, showed that the transgenic line had a 30% reduction in cell death, which correlated with the later appearance of necrotic lesions (Figure 4-4). Eventually, the transgenic line succumbed to the disease and the infected leaves became necrotic. However, the onset of necrotic lesions was delayed by several days. Therefore, the symptom development was delayed in the transgenic line, prompting further investigation.

LeSAMT1 Overexpression Affects the Free Salicylic Acid and Methylsalicylate Pools during *Xcv* Infection

Since it is known that an increase in SA is essential for and precedes the appearance of necrotic lesions (O'Donnell *et al.*, 2001), internal levels of MeSA and SA were measured in the control and transgenic lines during the course of the disease. Internal metabolite levels are typically determined in frozen tissue and represent the pool of metabolites contained in the leaves, while the volatile emissions are collected from fresh tissue. Current methods for quantitating hormone levels in plant tissue rely on the derivatization of hormones to methyl esters so that the volatile derivatives can be collected by vapor phase extraction (Schmelz *et al.*, 2004). For example, SA is converted to its methyl ester, MeSA. However, the purpose of this experiment was to simultaneously measure SA and MeSA in the same tissue. Therefore, a new protocol was developed to extract endogenous SA and MeSA from the same sample. First, endogenous MeSA was extracted from leaves and the residual SA was derivatized to propyl-SA with a strong acid catalyst (described in Experimental Procedures). 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 seven-fold higher than M82 (Figure 4-5). In addition, the free SA pool was four-fold higher in the transgenic line at 10 dpi (Figure 4-5). Interestingly, an increase in MeSA correlated with an increase in free SA. At 12 dpi, the free SA levels in the wild-type reached a maximum, which correlated with the spread of necrosis. However, the MeSA levels were six-fold higher in the transgenic line and kept increasing at 14 dpi. Apparently the overexpression of *LeSAMT1* in the transgenic line resulted in an increase in MeSA as well as SA in response to the pathogen. Both MeSA and SA pools reached a maximum after 10 dpi, and the secondary chlorotic and necrotic symptoms developed until 14 dpi.

The rate of MeSA emissions from fresh leaves were also collected during the course of disease (Figure 4-6). By 12 dpi, the MeSA emitted from the leaves in the transgenic line reached a maximum. SA accumulation reached a maximum at 10 dpi in the transgenic line (Figure 4-5), so the timing of MeSA emission from the transgenic line lagged behind the substrate availability. At 14 dpi, the MeSA emission from the transgenic line remained higher than the control. During the course of *Xcv* 93-1 infection, the internal MeSA/SA pools and the MeSA emissions of the transgenic line were significantly increased.

LeSAMT1 Overexpression Affects the Conjugated Salicylic Acid and Methylsalicylate Pools during *Xcv* Infection

The conjugated pools of SA and MeSA were also examined. Glucoside conjugation is a general mechanism for hormone inactivation, and both SA and MeSA glucoside conjugates have been described (Dean *et al.*, 2005). The conjugated SA and conjugated MeSA pools started to increase only 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 4-7). Interestingly, this conjugation began at the same time as the delay in disease symptom

development occurred, 10 dpi. Therefore, overexpression of *LeSAMT1* caused the leaves of transgenic plants to accumulate a significantly higher level of all forms of SA during pathogen infection – free MeSA, conjugated MeSA, free SA, and conjugated SA.

Discussion

The purpose of this study was to examine the effect of *LeSAMT1* overexpression on the local plant-pathogen interaction between tomato and the virulent bacterial strain *Xcv* 93-1. The transgenic line overexpressing *LeSAMT1* showed a delay in the appearance of secondary chlorotic and necrotic symptoms, relative to the parental control, M82, but eventually succumbed to the pathogen. SA is required for the development of these secondary symptoms (O'Donnell *et al.*, 2001). Surprisingly, once SA accumulation was initiated in the transgenic line, the internal pools of free SA, free MeSA, conjugated SA, and conjugated MeSA increased significantly over those of M82.

Since the transgenic line is constitutively expressing *LeSAMT1*, data on SA and MeSA levels were consistent with the availability of SA to be the cause of the significant increase in MeSA accumulation and emission from the leaves after *Xcv* 93-1 inoculation. The results from this work show that in *LeSAMT1* overexpressing plants, SA levels are higher than the control after bacterial infection. Based on previous work in our lab (O' Donnell *et al*., 2001), the increase in SA would suggest that the leaves should have more severe secondary symptoms. However, this was not the case; the transgenic plants showed a delay in the onset of secondary symptoms. In TMV-infected tobacco, an increase in MeSA and SA was correlated with a decrease in lesion size (Schulaev *et al.*, 1997). It may be that MeSA provides some protection to the tissue to prevent the spread of lesions. Further work will be needed to determine the actual mechanism involved in the reduced rate of symptom development.

SA accumulation in the *LeSAMT1* overexpressing plants resulted in an increase in the entire SA/MeSA pool size. At 14 dpi the total SA pool of the transgenic line, including free and conjugated SA, was approximately three-fold higher than the controls (10 nmol and 3 nmol, respectively). The total MeSA pool of the transgenic line was approximately six-fold higher in the transgenic line relative to the control (12 nmol and 2 nmol, respectively). In addition, the pool of SA and all its derivatives in the transgenic line is approximately five-fold higher than the control (22 nmol and 5 nmol, respectively) at 14 dpi. Excess MeSA may be converted back to SA, or MeSA accumulation may be activating a feedback loop to SA synthesis. A MeSA esterase from tobacco, salicylic acid-binding protein 2 (SABP2), has been identified and silenced in transgenic tobacco plants (Kumar and Klessig, 2003; Forouhar *et al.*, 2005). This esterase converts MeSA to SA and is inhibited by its product, SA (Forouhar *et al.*, 2005). SABP2-silenced tobacco plants infected with TMV had larger lesions in the local infection and were impaired in SAR and their responsiveness to SA (Kumar and Klessig, 2003). Therefore, it is believed that SABP2 is involved in plant innate immunity in tobacco. The work in tobacco further suggests that MeSA may function as an important pool for conversion back to SA following pathogen infection. A more likely possibility for the increase in SA accumulation in the transgenic tomato plants is induction of SA biosynthesis. However, SA biosynthesis has not been fully characterized. Studies have shown that SA can be synthesized from phenylalanine via the phenylpropanoid pathway or isochorismate via the shikimate pathway (Yalpani *et al.*, 1993; Wildermuth *et al.*, 2001; Strawn *et al.*, 2007). The induction of SA biosynthesis genes was not examined in this study, but this system may be a useful tool to study the different branches of SA biosynthesis in the future.

MeSA emission has also been studied in other plant-pathogen and plant-herbivore interactions. A study by Huang *et al.* (2003) compared the volatile emissions, including MeSA, from tobacco plants infected with different strains of *Pseudomonas syringae*. They observed that MeSA was most highly induced by an avirulent strain, induced to a lesser extent by a virulent strain, and only induced in trace amounts by a nonpathogenic strain. In a dual fungal infection/insect feeding study, Cardoza *et al.* (2002) observed that peanut plants infected with the white mold *Sclerotium rolfsii* emitted MeSA in addition to other volatile compounds, but insect feeding alone did not induce MeSA emission. In a separate dual bacterial infection/insect-feeding study, Cardoza and Tumlinson (2006) observed that avirulent and virulent strains of *Xcv* induced different volatile emission profiles in pepper, and the different bacterial strains affected the timing of when insects preferred to feed on the plants. Notably, MeSA was induced during all treatments except insect feeding, but was the highest during the combined virulent *Xcv* infection and insect feeding treatment. In addition, the insect survival rate was increased by 25% on the infected plants, which indicates that biochemical changes in the plant during bacterial infection affect the plant's response to insect feeding. Therefore, MeSA emission is a common theme found during different plant-pathogen interactions, but its exact function is still unknown. The results of the current study suggest that MeSA may serve as a key metabolite regulating SA biosynthesis in response to SA accumulation after bacterial infection.

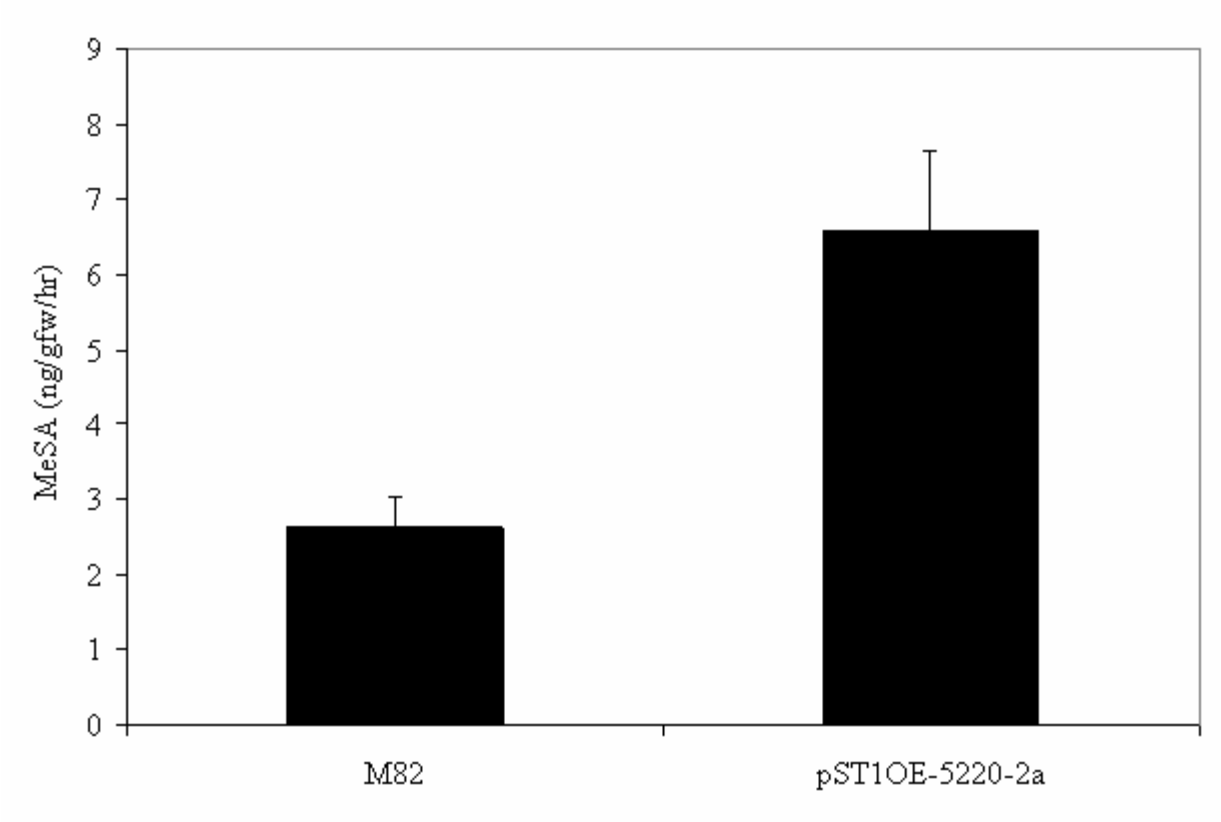


Figure 4-1. MeSA emission from mature leaves of M82 and pST1OE-5220-2a. Error bars = SE. n = 10. $p \leq 0.01$ by Student's t-test.

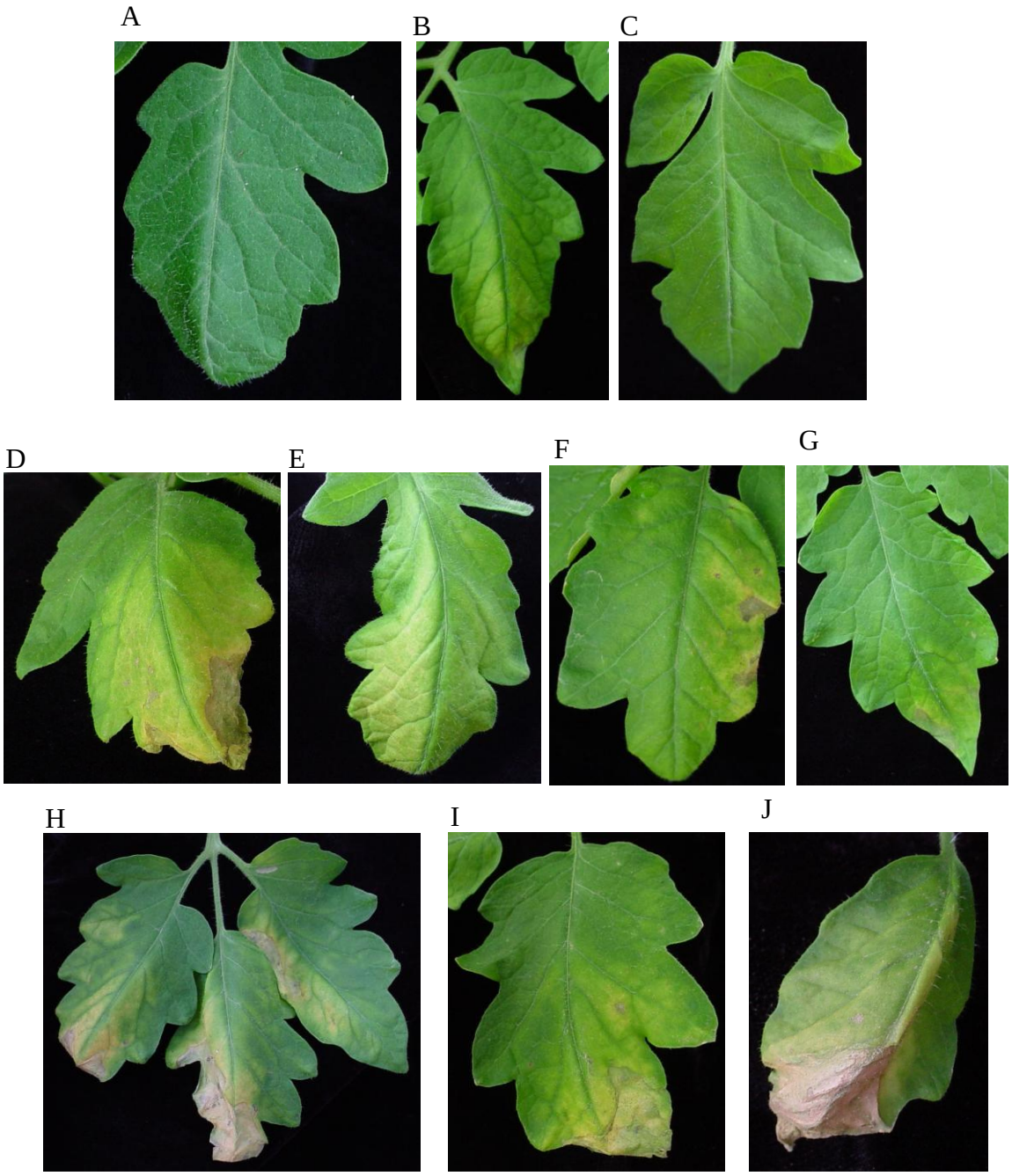


Figure 4-2. Secondary symptom development during *Xcv* 93-1 infection in tomato. A) Mock infected M82; B) M82 10 days post inoculation (dpi); C) pST1OE-5220-2a 10 dpi; D) and E), M82 12 dpi; F) and G), pST1OE-5220-2a 12 dpi; H) M82 14 dpi; I) and J), pST1OE-5220-2a 14 dpi.

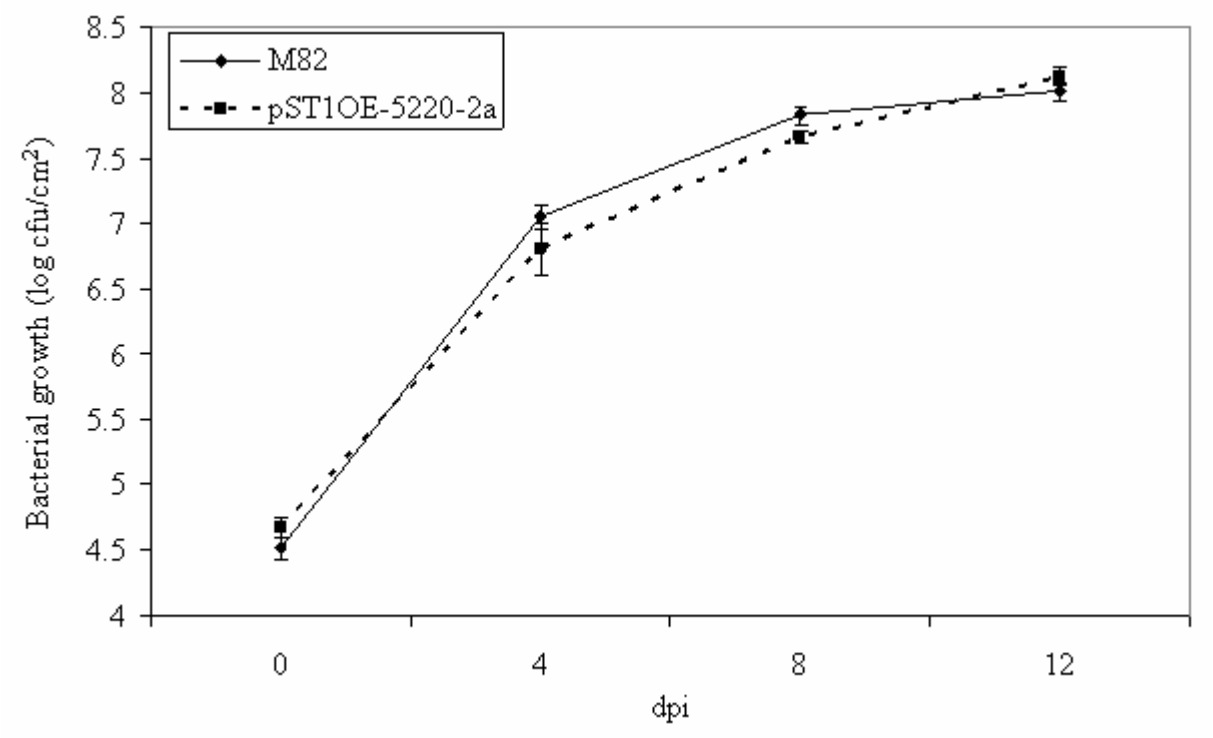


Figure 4-3. Bacterial growth during *Xcv* infection in tomato. dpi = days post inoculation. cfu = colony forming units. Error bars = SE. n = 6.

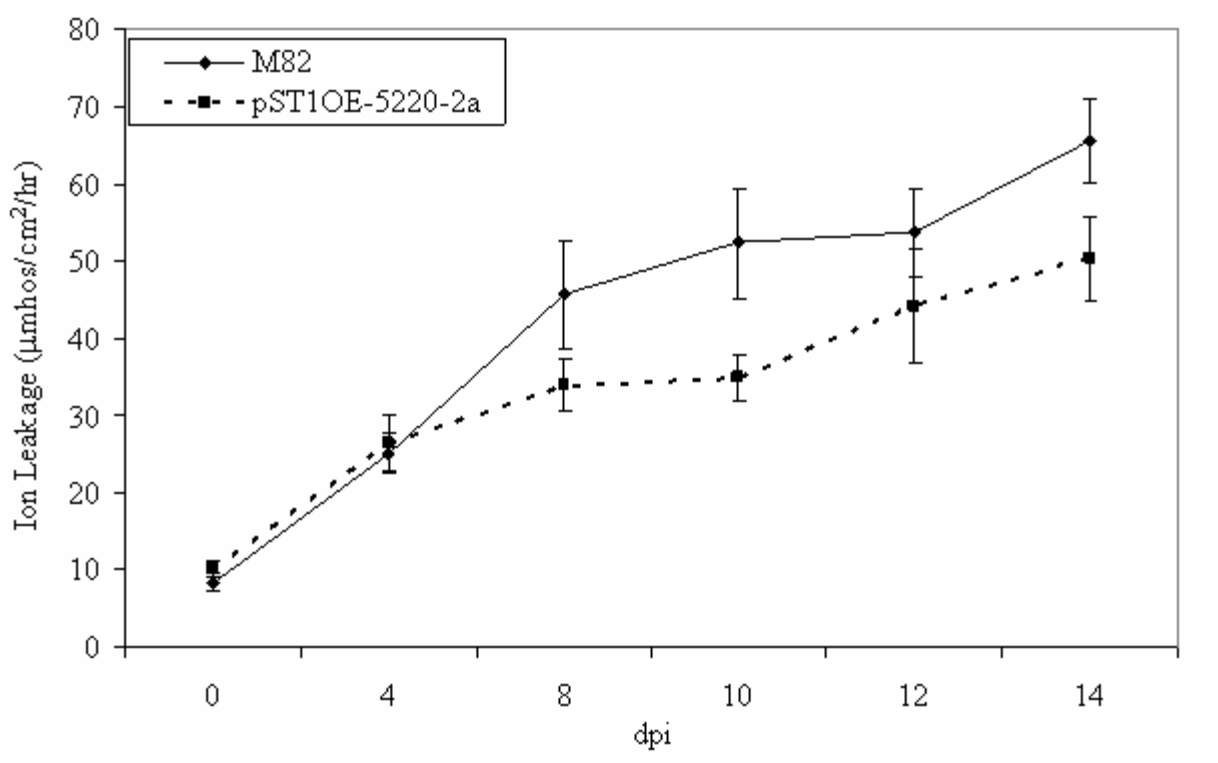
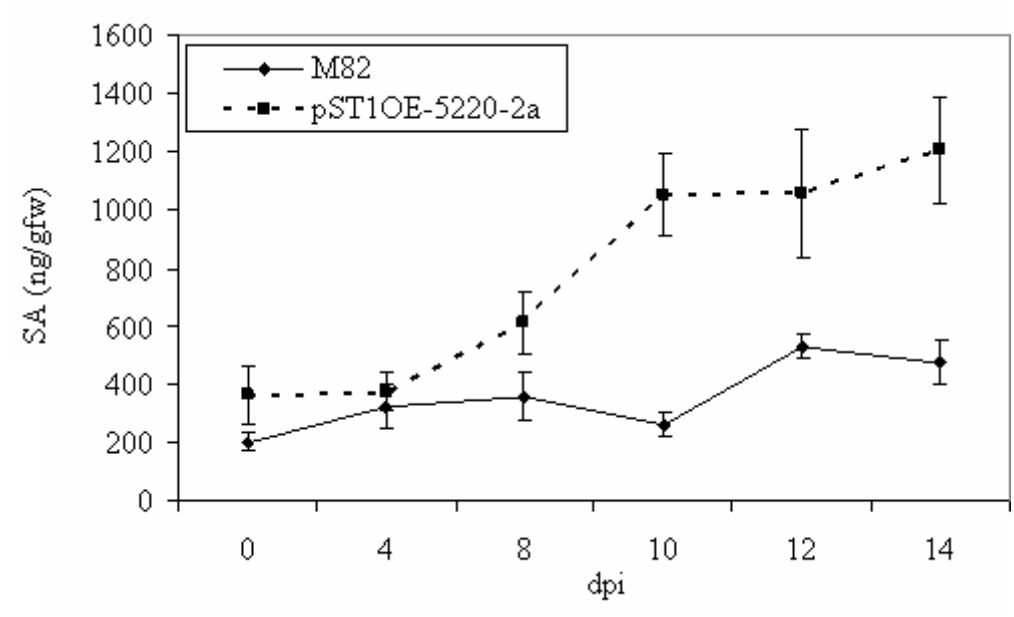


Figure 4-4. Ion leakage during *Xcv* infection in tomato. dpi = days post inoculation. Error bars = SE. n = 6.

A



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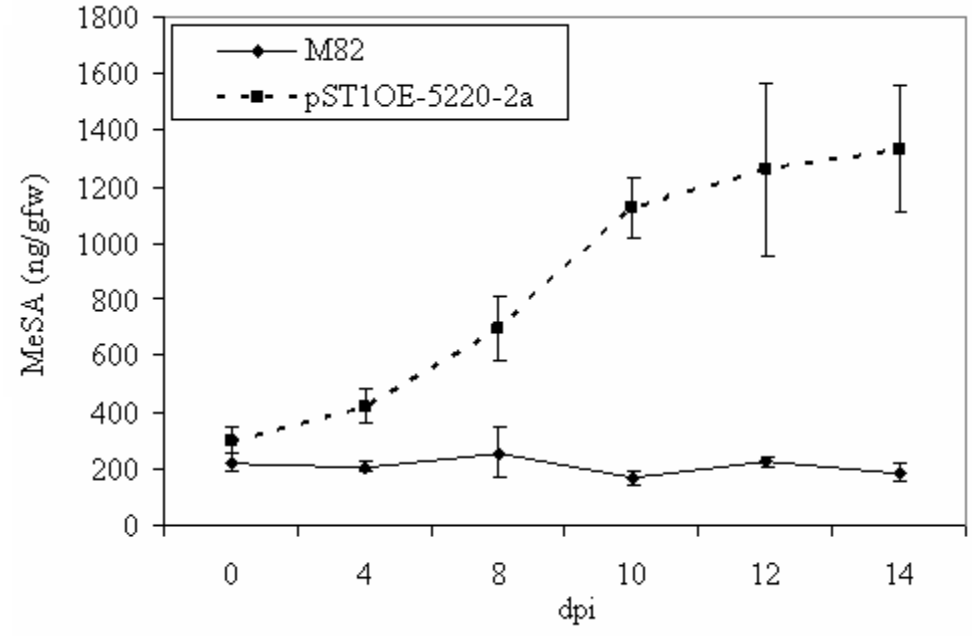


Figure 4-5. Internal pools of free SA and MeSA during *Xcv* infection in tomato. A) SA internal pool and B) MeSA internal pool. dpi = days post inoculation. Error bars = SE. n = 4.

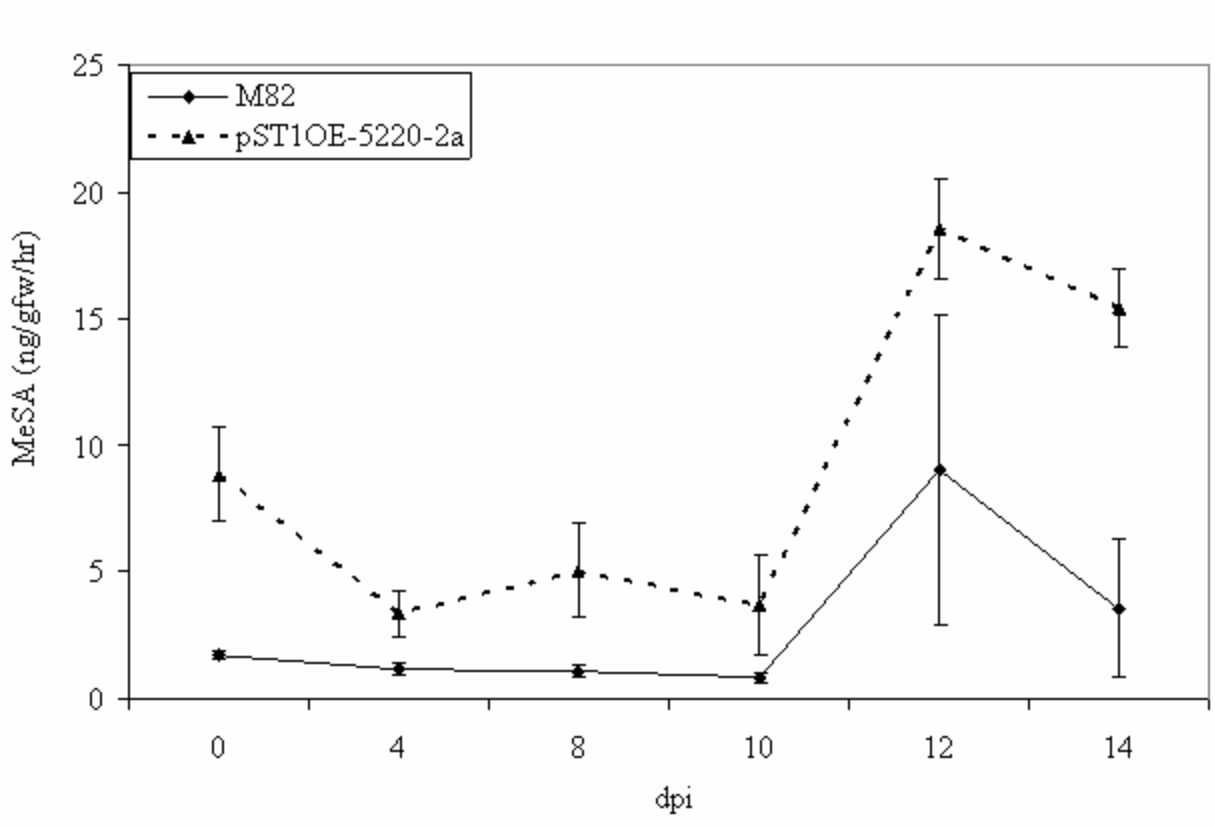
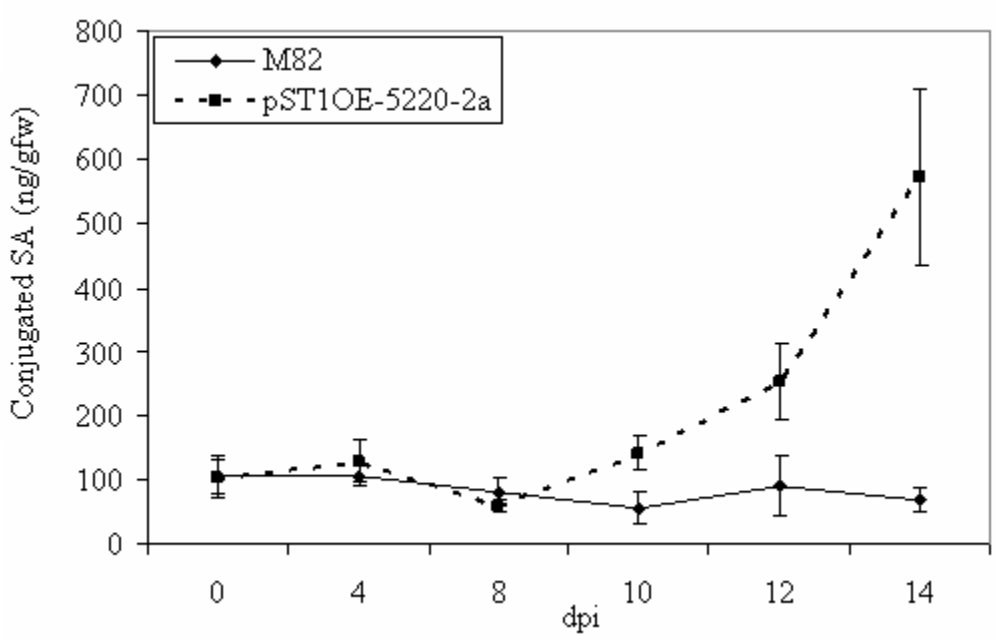


Figure 4-6. MeSA emissions during *Xcv* infection in tomato. dpi = days post inoculation. Error bars = SE. n = 3.

A



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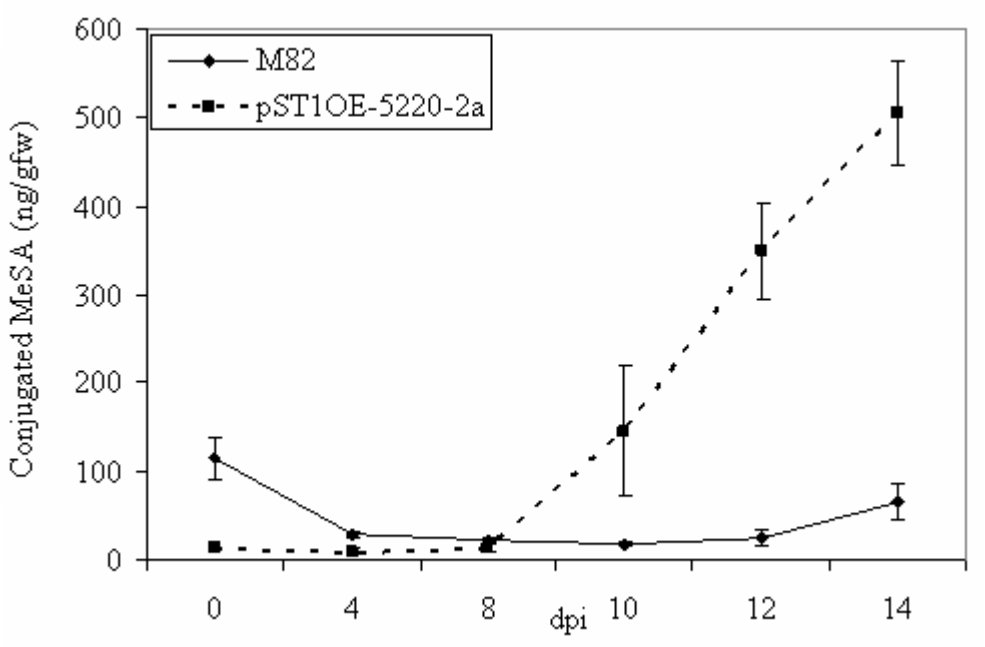

Figure 4-7. Internal pools of conjugated SA and MeSA during *Xcv* infection in tomato. A) Conjugated SA pool and B) Conjugated MeSA pool. dpi = days post inoculation. Error bars = SE. n = 4.

CHAPTER 5
GENERAL DISCUSSION

The purpose of this study was to characterize the contribution of methylsalicylate (MeSA) to tomato flavor and as well as any function in response to bacterial infection in tomato (*Solanum lycopersicum*). The substrate specificity as well as the enzyme kinetics of LeSAMT1 were determined *in vitro*. Plants constitutively expressing the gene responsible for MeSA synthesis in tomato, *S*-adenosyl-$_{L}$-methionine: salicylic acid carboxyl methyltransferase (*LeSAMT1*) were analyzed with respect to MeSA emission and flavor. In addition, one of these transgenic lines overexpressing *LeSAMT1* was used as a tool to examine potential roles of MeSA in bacterial pathogen responses.

LeSAMT1 was the closest tomato homolog to a known SAMT from *Clarkia breweri* (Ross *et al.*, 1999), and the *in vitro* enzyme activity was specific for converting the plant hormone salicylic acid (SA) to MeSA. Overexpression of *LeSAMT1* in transgenic tomatoes resulted in a significant increase of MeSA in fruits and leaves. An untrained consumer panel determined that MeSA overproducing fruits tasted significantly different than the control fruits. Subsequently, a preference taste test from an untrained consumer panel determined that the transgenic and control fruits were preferred equally. In addition, the transgenic and control fruits were rated according to their aroma, tomato-like flavor, sweetness, sourness, and overall acceptability. Even though the control fruits scored slightly higher in likeability and preference choice, the overall means did not indicate a significant preference over the transgenic fruits. Therefore, increasing the MeSA levels in the transgenic fruits did change the flavor of the tomatoes, but overall preference was determined by how the panelists perceived this change and both samples were liked by panelists.

Since the increased emission of MeSA was also seen in leaves, one of the transgenic lines was used to determine the role of MeSA during bacterial pathogen stress. After inoculation with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) 93-1, the transgenic line showed a delay in the onset of disease symptoms. Although delayed, the transgenic plants eventually reached the same endpoint with necrosis of the tips of the leaves. Bacterial growth was not affected in the transgenic line. However, the transgenic line accumulated significantly higher levels of SA, MeSA, conjugated SA, and conjugated MeSA following infection. Therefore, overexpression of *LeSAMT1* significantly altered the free and conjugated SA and MeSA pools in the transgenic line, which could be due to a feedback loop to SA biosynthesis. These MeSA overproducing lines may be useful tools for elucidating which branch of SA biosynthesis is affected in response to *Xcv* 93-1 infection. In the future, it would be useful to examine the free SA levels in the MeSA overproducing fruits to see if SA accumulates as it did in response to pathogen infection. In addition, numerous studies have shown that MeSA is a common volatile seen in the emissions of plants inflicted with herbivore damage. It would be interesting to see if increased levels of MeSA affect tomato-herbivore interactions with regard to attracting or repelling feeding insects.

Plants have evolved to use volatiles to attract pollinators, seed dispersing organisms, and to adapt to stress. These plant volatiles are components of floral scent and the flavors of foods, and may even possess medicinal properties. Humans have devised ways to take advantage of these volatile compounds for commercial use to improve upon fragrances, flavors, and pharmaceuticals. Understanding the molecular mechanisms of plant volatile production may aid in the development of improved crops by molecular breeding and may even help identify novel compounds useful for industrial purposes. However, it is difficult to target specific traits using traditional breeding practices. Genetic engineering has made it possible to study the effect of one

gene on specific biochemical pathways involved in a variety of plant processes. Using such a tool is advantageous when studying a complex trait such as flavor, so only one target compound will be altered. Using transgenic lines overexpressing *LeSAMT1*, the gene responsible for MeSA synthesis in tomato, this work demonstrated the effect of one volatile, MeSA, on tomato flavor and plant hormone pools in response to pathogen stress.

CHAPTER 6
EXPERIMENTAL PROCEDURES

Cloning of *LeSAMT1*

The full-length EST of *LeSAMT1* was identified in the TIGR database by homology to the amino acid sequence of *Clarkia breweri* CbSAMT (Ross *et al.*, 1999) and was amplified with primers (Fwd) CACCATGAAGGTTGTTGAAGTTCTTCACATGAATGGAGG and (Rev) TTATTTTTTCTTGGTCAAGGAGACAGTAACATTTATAAACTCAGTATCC from Flora-Dade (*Solanum lycopersicum*) bud cDNA. For *LeMT* (tomato methyltransferase) sequences, full-length clones from the TIGR database were ordered and sequenced. *LeMT*s were named according to their percent similarity to the predicted amino acid sequence of LeSAMT1 (Table 2-1). The following full-length clones from the TIGR database were assigned as *LeMT*s: *LeSAMT1*, cTOA4C17; *LeMT1*, cLEM7O9; *LeMT2*, cTOA14P1; *LeMT3*, cLEI13O14; *LeMT4*, cTOD6B16; *LeMT5*, cLEW1K6; *LeMT6*, cTOA28E18; *LeMT7*, cTOF25N7.

Production of Transgenic Plants

The full-length open reading frame of *LeSAMT1* was cloned into a vector containing the constitutive FMV 35S promoter (Richins *et al.*, 1987) in the sense or antisense orientation. *Solanum lycopersicum* (M82 and Pearson) were transformed by *Agrobacterium*-mediated transformation (McCormick *et al.*, 1986) with the kanamycin selectable marker. Plants were grown in the greenhouse for initial screening and planted in the field at the University of Florida North Florida Research and Education Center—Suwannee Valley in Live Oak, FL for additional analysis. In Spring 2006, the MeSA overproducing Pearson line pST1OE-6841-1 and the M82 antisense lines pST1AS-7001, 6917, and 6918 were a mixture of homozygous and heterozygous pcr-positive lines. The antisense Pearson line pST1AS-6831-1 and the M82 MeSA

overproducing line pST1OE-5220-2a were homozygous in Spring 2006. In Fall 2006, all lines were homozygous (pST1OE-6841-1-4, pST1AS-7001-1, pST1AS-6917-2, and pST1AS-6918-1).

Expression and Purification of GST-LeSAMT1

For protein expression and purification, *LeSAMT1* was cloned into the pENTR/D-TOPO Gateway vector (Invitrogen). The open reading frame was recombined into the N-terminal-GST-tag Gateway vector pDEST15 (Invitrogen) and transformed into *E. coli* strain BL21-AI (Invitrogen) for arabinose-inducible expression. Bacterial cultures were grown with 100 μg/mL carbenicillin to an OD_{600} of 0.4 and were induced with a 20% $_L$-arabinose solution for a final concentration of 0.2%. Cells were induced overnight (16 hrs) at 15^{o}C and harvested the next day. To harvest cells, cultures were centrifuged at 5000 g for 15 minutes and resuspended in lysis buffer—1X PBS, lysozyme, 10% v/v glycerol, and Bacterial Protease Inhibitor Cocktail (Sigma) at 4^{o}C. Cells were sonicated with a Fisher Sonic Desmembrator, Model 100 (Fisher) on level 1 for 10 cycles of 5 seconds on, 30 seconds off. Cells were centrifuged at 10000 g for 15 minutes and the GST-tagged protein was purified on a Glutathione Uniflow Resin (BD Biosciences Clontech) at 4^{o}C. Columns with 1.5 bed volumes of resin were equilibrated with 1XPBS. The extract was mixed with resin on a rotating wheel for 1 hour. The flow-through was collected and run through the column a second time. The column was washed with 16 bed volumes of 1X PBS and the GST-LeSAMT1 was eluted with Elution Buffer (10 mM glutathione, 50 mM Tris-HCl—pH 8.0, 20% v/v glycerol). Protein levels were quantified using Bradford Reagent (BioRad) and purification was checked with protein blotting using α-GST antibodies and visualized with ECL reagents (Amersham). The enzyme was stored on ice at 4^{o}C.

Kinetic Assays

Assay conditions for substrate specificity and K_m determination for GST-LeSAMT1 followed Zubieta *et al.* (2003) with some modifications. For substrate specificity assays, 2.85 μg of GST-LeSAMT1 was assayed in a 100 μL reaction containing 50 mM Tris-HCl—pH 7.5, 100 mM KCl, 2.8 mM BME, 1 mM substrate, and a 30 μM solution of 4:1 unlabeled SAM: ^{14}C-SAM, specific activity 11.04 mCi/mmol (Amersham). Substrates were diluted in EtOH with the exception of nicotinic acid, which was diluted in water. Assays were done in triplicate, including no enzyme controls. After one hour at 25^{o}C the reactions were stopped by adding an equal volume of hexanes. ^{14}C-MeSA was extracted from the organic layer by vortexing samples for 15 seconds and centrifuging at 13200 g for 2 minutes. Fifty μL of the hexane layer was counted for 5 min in 3 mL Ready Gel Scintillation Fluid (Beckman Coulter). 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 μg of purified GST-LeSAMT1 was used. ^{14}C-SAM was held constant at 75 μM. Two ^{14}C-SAM stock solutions were used with varying specific activity to minimize the use of radioactivity and to make sure the product counts were detectable. For the lower [SA] range, 200 μM of a 2:1 dilution (unlabeled SAM: ^{14}C-SAM) with a specific activity of 18.4 mCi/mmol was used. For the higher [SA] range, a 200 μM of a 4:1 dilution with a specific activity of 11.04 mCi/mmol of SAM was used. For the K_m of SAM, 3.42 μg of GST-LeSAMT1 was used and [SA] was held constant at 1mM. [SAM] was varied using two stock solutions with different specific activities. An undiluted specific activity of 55.2 mCi/mmol (10 μM) was used as the stock solution for the lower [SAM] range. A 200 μM stock of a 2:1 dilution with a specific activity of 18.4 mCi/mmol was used for the higher [SAM] range. The reactions for the K_m

determinations were stopped after two hours as described above. A preliminary experiment showed that the assay was linear after two hours.

Volatile Collection

Volatiles were collected from tomato fruits according to Tieman *et al.* (2006). For leaf volatile 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 one hour. Volatiles were eluted off the column with methylene chloride and run on a GC for analysis as described in Tieman *et al*. (2006).

LeSAMT1 Expression Quantification

Total RNA was extracted using the Qiagen RNeasy Plant Mini Kit and levels of *LeSAMT1* mRNA levels were quantified by real-time polymerase chain reaction (RT-PCR) using Taqman one-step RT-PCR reagents (Applied Biosystems). 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. *LeSAMT1* expression was determined using the following primer/probe set—Fwd: TCCCAGAAACATTATACATTGCTGAT, Rev: AATGACCTTAACAAGTTCTGATACCACTAA, Probe: (56-FAM) TGGGTTGTTCTTCTGGAGCGAACACTTT (3BHQ_1).

Samples were run on the BioRad iCylcer pcr detection system and quantified with the MyiQ software. The following pcr conditions were used: 48^{o}C, 30 min; 95^{o}C 10 min; 40 cycles of 95^{o}C, 15 sec; 60^{o}C 1 min. A sense strand was *in vitro* transcribed from plasmid DNA with ^{3}H-UTP (MAXIscript, Ambion) and was used to determine the absolute values of RNA in the sample.

Pathogen Inoculations

Xanthomonas campestris pv. *vesicatoria (Xcv)* 93-1 inoculations were done on M82 control plants and the MeSA overexpressing line pST1OE-5220-2a. Bacterial inoculations were performed on leaves 3 and 4 of 5 to 6 week old plants. Virulent *Xcv* 93-1 cultures were grown overnight in 100 mL of 0.7% Nutrient Broth (Difco Laboratories, Detroit, MI) at 28^{o}C. Cells were centrifuged at 5000 g for 10 minutes and resuspended in mock buffer (10 mM $MgCl_2$, 0.025% (v/v) Silwet L-77 in ultrapure H_2O). Cells were diluted to 1 x 10^6 cfu in mock buffer. For inoculations, leaves 3 and 4 were dipped in the bacterial suspension for 15 seconds. Leaves of mock-treated plants at 0 dpi were dipped in mock buffer only. Two to three plants were assayed per time point per measurement.

Ion Leakage

For ion leakage measurements, three plants were assayed, and each infected leaf was assayed separately (n = 6). Measurements in microohms^{-1} per cm^2 per hr (designated as µmho/cm^2/hr) are described in Lund *et al.* (1998).

Bacterial Growth Curves

Bacterial colony counts were performed on two leaves of three plants for each time point. Two ½ cm^2 discs were excised with a number 5 cork borer from representative leaflets for each time point. Discs were ground in 10 mM $MgCl_2$ and serial dilutions were plated on 0.7% Nutrient Broth, 1.5% Bacto-agar (Difco Laboratories, Detroit, MI). Plates were incubated at 30^{o}C for 2 days and colonies were counted for each time point.

Free Salicylic Acid and Methylsalicylate Extractions

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 needed to analyze both metabolites without interference. Briefly, 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, Mountainside, NJ, USA) and an internal standard mix containing 100 ng $^2H_6$-SA (CDN Isotopes, Pointe-Claire, Quebec, Canada) in EtOH and 100 ng of a lab-prepared $^2H_4$-MeSA standard in methylene chloride. The samples were extracted with 300 μL Extraction Buffer (2:1:0.005 1-propanol: H_2O: HCl) and shaken in a Fastprep FP 120 homogenizer (Qbiogene) for 30 sec. Then 1 mL methylene chloride was added and the samples were shaken an additional 10 seconds. Samples were centrifuged at 11300 g for one minute and the bottom methylene chloride layer was transferred to a 4 mL glass vial and sealed. The top aqueous layer was later used for glucoside extractions. First, free MeSA was collected from the methylene chloride phase. The glass vial was sealed with a cap containing a high-temperature septa and a column containing SuperQ resin was inserted into the septa, followed by a needle carrying a stream of N_2. The glass vial with the methylene chloride phase was placed on a 70^{o}C heating block and the vapor phase was collected just until the liquid evaporated. The column containing the MeSA was saved for recollection after the SA derivatization. The free SA remained in the dried vial and was derivatized to propyl-SA with 30 μL of a 2:1 mixture of 1-propanol: HCl. The samples were vortexed and placed in a 70^{o}C oven for 45 minutes. Samples were cooled to room temperature and 75 μL of a 1 M citric acid solution was added to stop the reaction. Samples were vortexed 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 minutes. Columns were rinsed with 200 μL ultrapure water and dried. Then the columns were eluted with 125 μL methylene chloride for CI-GC/MS. Free SA and MeSA levels were quantitated using the internal standard values as described in Schmelz *et al.* (2004). The propyl-SA derivatized from the endogenous SA ran at a retention time of 10.12 min and *m/z* of 181 and the $^{2}H_{6}$-SA-propylated standard ran at a retention time of 10.11 min and *m/z* of 185.

Conjugated Salicylic Acid and Methylsalicylate Extractions

The aqueous layer from the vapor phase extractions was transferred to a 4 mL glass vial and dried in a speed-vac overnight. After drying completely, the $^{2}H_{6}$-SA standard (100 ng) was added and dried with a stream of N_2. Then the $^{2}H_{4}$-MeSA standard was added and the vial was immediately sealed. The glucosides for MeSA and SA were acid hydrolyzed and derivatized in the same step by adding 30 μL of a 2:1 mixture of 1-propanol: HCl as described above. Samples were incubated for 45 minutes at 70^{o}C, followed by neutralization with 75 μL of 1 M citric acid. Hydrolyzed MeSA and SA were collected by vapor phase extraction in one step at 70^{o}C and kept on the heat block 2 minutes after drying. Columns were rinsed and eluted as described above. Samples were analyzed as described above.

Triangle Taste Test

Sixty untrained volunteer panelists participated in a triangle test to determine if the MeSA overproducing line pST1OE-6841-1 tasted different than the Pearson controls. Fruits were collected from the field and the seeds and locular gel were discarded. Panelists were given three samples of tomato slices: either two controls and one transgenic, or two transgenics and one control, in random order. Each sample was given a random three-digit code. Panelists were asked to smell the samples, taste the samples, and indicate which sample was different. Thirty panelists

were correct, and a p-value of ≤ 0.01 was assigned after a chi-square test assuming chance probability was 33.33% (Meilgaard *et al.,* 2007).

Likeability and Preference Tests

The likeability and preference tests were completed at the Sensory Testing Facility in the Food Science and Human Nutrition Department at the University of Florida according to Meilgaard *et al.* (2007). Seeds from the fruits of the MeSA-overproducing line and the Pearson control were removed and the fruits were cut into wedges. Each sample was given a random three-digit code and presented to the panelists in random order. Sixty-seven untrained panelists were asked to rate the aroma, sweetness, sourness, and overall acceptability of the two samples on a nine-point hedonic scale. Panelists were also asked to choose the tomato sample they preferred overall. The statistical significance of the preference test was analyzed by a two-sided directional paired-comparison test. The statistical significance of the likeability test was determined using a one-way ANOVA.

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BIOGRAPHICAL SKETCH

Michelle Lynn Zeigler was born July 16, 1980 and was raised in Clearwater, Florida. As an undergraduate student at the University of Florida, she participated in the University Scholars Program, where she worked under the direction of Dr. Joseph Ciardi in Dr. Harry Klee's lab and became interested in plant molecular biology. She graduated from the University of Florida in 2002 with a bachelor's degree in microbiology and was awarded an Alumni Fellowship in the Plant Molecular and Cellular Biology Graduate Program at the University of Florida. In Dr. Harry Klee's lab, she studied the role of methylsalicylate, or oil of wintergreen, in tomato flavor and its role in the response to the virulent bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria*.