

Endogenous Methyl Salicylate in Pathogen-Inoculated Tobacco Plants¹

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The tobacco (*Nicotiana tabacum*) cultivar Xanthi-nc (genotype *NN*) produces high levels of salicylic acid (SA) after inoculation with the tobacco mosaic virus (TMV). Gaseous methyl salicylate (MeSA), a major volatile produced in TMV-inoculated tobacco plants, was recently shown to be an airborne defense signal. Using an assay developed to measure the MeSA present in tissue, we have shown that in TMV-inoculated tobacco plants the level of MeSA increases dramatically, paralleling increases in SA. MeSA accumulation was also observed in upper, noninoculated leaves. In TMV-inoculated tobacco shifted from 32 to 24°C, the MeSA concentration increased from nondetectable levels to 2318 ng/g fresh weight 12 h after the temperature shift, but subsequently decreased with the onset of the hypersensitive response. Similar results were observed in plants inoculated with *Pseudomonas syringae* pathovar *phaseolicola*, in which MeSA levels were highest just before the hypersensitive response-induced tissue desiccation. Transgenic NahG plants unable to accumulate SA also did not accumulate MeSA after TMV inoculation, and did not show increased resistance to TMV following MeSA treatment. Based on the spatial and temporal kinetics of its accumulation, we conclude that tissue MeSA may play a role similar to that of volatile MeSA in the pathogen-induced defense response.

Tobacco (*Nicotiana tabacum* L. cv Xanthi-nc) plants carrying the *N* gene produce high levels of SA after inoculation with TMV. An increase in the tissue content of SA follows the appearance of the virus-induced HR (Malamy et al., 1990). The correlation between SA accumulation and pathogen resistance has also been shown in cucumber (Metrax et al., 1990; Rasmussen et al., 1991), tomato (Hammond-Kosack et al., 1996), and Arabidopsis (Delaney et al., 1995). Following the primary infection, the inoculated plant becomes resistant to subsequent pathogen attack, both locally and systemically, in pathogen-free leaves. This phenomenon, called SAR, has attracted scientific interest for more than 60 years (Chester, 1933). The dependence of SAR on SA accumulation was shown in transgenic NahG tobacco plants expressing the salicylate hydroxylase, which accumulate little or no SA and do not exhibit SAR (Gaffney et al., 1993).

While investigating the movement and distribution of SA in TMV-inoculated tobacco, we discovered that the plants evolve large amounts of gaseous MeSA (Shulaev et al., 1997). In contrast, healthy or mechanically wounded plants did not evolve any detectable amounts of MeSA. Application of gaseous MeSA to healthy tobacco plants increased the expression of the *PR-1* gene and TMV resistance (measured as the size reduction of the TMV-induced lesions). The amount of MeSA produced by TMV-inoculated tobacco plants was sufficient to induce *PR-1* gene expression and resistance in healthy plants receiving air from the headspace of enclosed TMV-inoculated plants.

The accumulation of SA in tissue exposed to MeSA gas and labeling studies using [¹⁴C]SA and MeSA suggest that MeSA is synthesized from SA and acts by being converted back to SA in the target tissues. MeSA is not the only metabolite of SA found in plants. Large amounts of glucosyl SA accumulate in and around the HR lesions (Enyedi et al., 1992; Malamy et al., 1992). However, indirect evidence suggests that glucosyl SA is neither mobile nor biologically active and may function as a storage form of SA (Enyedi et al., 1992; Malamy et al., 1992). In contrast, data indicate that MeSA may serve as an airborne signal involved in inter- and intra-plant communication during the pathogen infection (Shulaev et al., 1997).

The fact that MeSA is naturally produced by a number of plants is well known (Wilson et al., 1987; Hamilton-Kemp et al., 1988; Hayden and Clough, 1990; Loughrin et al., 1993); however, the suggestion that gaseous MeSA serves as an inducible signal for resistance in tobacco prompted further investigation of this compound. MeSA, also known as oil of wintergreen, is a volatile liquid at room temperature (boiling point 220–224°C). Whereas previous work examined the effects of MeSA vapor (Shulaev et al., 1997), the work presented here investigates the production and role of nongaseous MeSA in tobacco plants using an analytical method developed for this purpose. In addition, this study provides further evidence that the conversion of MeSA to SA is required for the biological activity of this compound.

¹ This research was funded by grant no. 96-35304-3874 from the U.S. Department of Agriculture. Additional support was provided by the New Jersey Agricultural Experiment Station and the New Jersey Commission for Science and Technology.

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Abbreviations: HR, hypersensitive response; MeSA, methyl salicylate; PR protein, pathogenesis-related protein; SA, salicylic acid; SAR, systemic acquired resistance; TMV, tobacco mosaic virus.

MATERIALS AND METHODS

Plant Material and Inoculation with Pathogens

Wild-type and transgenic NahG (gift from J. Ryals, CIBA-Geigy, NC) tobacco (*Nicotiana tabacum* L. cv Xanthinn, NN genotype) seeds were germinated and grown as described previously (Yalpani et al., 1991). Fully expanded leaves of 6- to 8-week-old seedlings were inoculated with TMV strain U1 (5 μg per leaf) in 5 mM potassium phosphate buffer, pH 7.0, or mock inoculated with buffer by gently rubbing the leaves with carborundum, as described previously (Yalpani et al., 1991). Plants were kept under continuous illumination provided by incandescent and cool-white fluorescent lamps (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$). HR was induced by incubating TMV-inoculated plants at 32°C and then shifting them to 24°C after 4 d, as described previously (Malamy et al., 1992).

For bacterial inoculation, overnight cultures of *Pseudomonas syringae* pv *phaseolicola* (NPS3121) (Lindgren et al., 1986) (a gift from R. Mittler, Rutgers University, New Brunswick, NJ) were infiltrated into the abaxial surface of tobacco leaves using a sterile plastic syringe. Following the inoculation, 18-mm leaf discs immediately surrounding the infiltrated areas were sampled and analyzed.

For experiments with gaseous MeSA, 5-week-old wild-type and transgenic NahG tobacco plants were enclosed in 4-L gas-tight glass jars containing an upright cotton swab. The jars were opened daily and liquid MeSA was applied to the cotton swab to produce a concentration of 250 $\mu\text{g L}^{-1}$ in the headspace after evaporation. After 6 d of exposure to MeSA, leaves from one subset of plants were excised for SA and PR-1 determination. Another subset of plants was virus inoculated on all fully expanded leaves (see above), and the diameter of TMV-induced lesions was measured 72 h postinoculation with a low-power microscope.

Determination of SA

Total SA (the sum of free and glucosyl SA) was extracted and quantified as described previously (Enyedi et al., 1992). Leaf tissue samples (0.5 g fresh weight) were frozen in liquid nitrogen, ground to a fine powder, and sequentially extracted with 90 and 100% methanol. The combined methanolic extracts were vacuum dried and the pellets were resuspended in 5 mM sodium acetate buffer, pH 5.5, containing 80 units/g fresh weight β -glucosidase (Sigma). Following enzymatic hydrolysis (90 min at 37°C), the reaction was stopped with the addition of 10% TCA. The supernatant was partitioned with ethyl acetate:cyclopentane:isopropanol (100:99:1, v/v). SA was determined by fluorescence (excitation 301 nm, emission 412 nm) after separation on a C_{18} reverse-phase HPLC column (Waters). The column was maintained at 40°C and equilibrated in 0.5% glacial acetic acid:methanol (75:25, v/v) with a flow rate of 1.5 mL min^{-1} . Three minutes after injection, a methanol gradient (25–60%) was applied over 7 min, after which the methanol concentration was returned to 25%. All data were corrected for recovery using spiked samples.

Determination of MeSA

Leaf tissue samples (0.5 g fresh weight) were frozen and ground in liquid nitrogen. The resulting powder was resuspended in 2.5 mL of 100 mM phosphate buffer, pH 7.0, containing 0.125 $\mu\text{g mL}^{-1}$ vanillin as an internal standard. After 10 min of sonication and centrifugation at 3000g, the supernatant was loaded onto a 3-mL C_{18} column (Bakerbond SPE, J.T. Baker) preconditioned with 3 mL of 100% methanol and 3 mL of phosphate buffer added sequentially. MeSA was eluted with 2.5 mL of 80% methanol. The methanolic fraction was further diluted with water to 40% methanol (approximately 5 mL total volume), and loaded onto a second 3-mL C_{18} column preconditioned with 3 mL of 100% methanol and 3 mL of 40% methanol added sequentially. MeSA was eluted with 2.5 mL of 100% methanol. The volume of column eluent was reduced to 0.5 mL by partial drying under vacuum (SpeedVac, Savant Instruments, Holbrook, NY). The recovery of MeSA was 55 to 75%.

Vanillin and MeSA were detected and quantified by online UV absorption at 280 nm (model 486 tunable absorbance detector, Waters) and fluorescence detection at an excitation of 292 nm and an emission of 360 nm (model 474 scanning fluorescence detector, Waters), respectively. The samples (30- μL volume) were injected (model 717-Plus autosampler, Waters), and the compounds were separated on a C_8 reverse-phase HPLC column (Symmetry Shield RP_8 , Waters) maintained at 40°C. MeSA was separated during a 35-min acetonitrile (10–90%):1.2% acetic acid (90–10%) gradient (flow rate of 1 mL min^{-1}) maintained by HPLC pumps (model 510, Waters). Under these conditions the retention time for MeSA was approximately 22.04 min (Fig. 1, A and B). This procedure gave the detection limit of 200 ng/mL of MeSA (6 ng per injection). An absorption spectrum of an authentic MeSA standard, obtained using a photodiode array detector (model 996, Waters), was almost identical to that of a compound that coeluted with authentic MeSA. GC-MS analysis of TMV-inoculated tobacco leaf extracts demonstrated directly the presence of a compound with the retention time and fragmentation pattern of authentic MeSA (data not shown).

RNA Isolation and Analysis

Total RNA was isolated using the guanidine thiocyanate and phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA (10 μg) was separated by electrophoresis, transferred to nylon membrane (Zeta-Probe, Bio-Rad), and hybridized according to the manufacturer's protocols. PR-1 transcripts were detected with a corresponding ^{32}P -labeled tobacco cDNA probe (a gift from R. Smith, Rutgers University, New Brunswick, NJ) prepared by specific priming. As a control for loading and transfer efficiency, blots were stripped and rehybridized with an 18S gene probe.

RESULTS

MeSA Production in TMV-Inoculated Tobacco Leaves

Approximately 48 h after tobacco leaves were inoculated with TMV, HR lesions developed around the site of inoc-

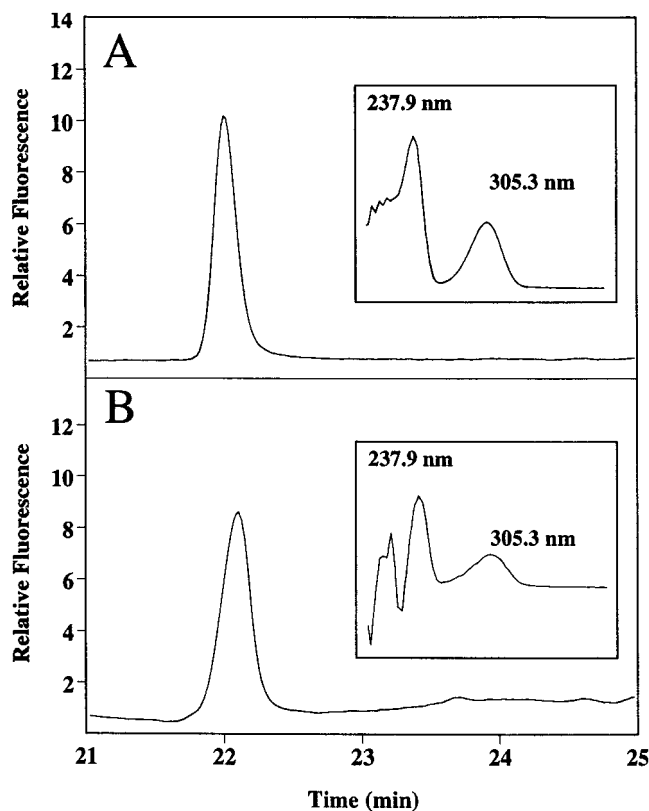


Figure 1. Identification of MeSA in leaf extracts of TMV-inoculated tobacco. HPLC-elution profile (fluorescence at 360 nm upon excitation at 292 nm) of authentic MeSA standard (A) and of leaf extract of TMV-inoculated tobacco obtained 12 h after temperature shift (B). Insets, UV-absorption spectra of the corresponding MeSA peaks from A and B.

ulation. At that time the SA concentration increased 8-fold and continued to accumulate in the inoculated leaf (Fig. 2A). MeSA accumulation was also first detected at the time of HR appearance and reached a maximum of 386 ng g⁻¹ fresh weight. MeSA levels remained relatively steady from 48 to 96 h postinoculation.

SA content increases systemically after TMV inoculation (Malamy et al., 1990), which at least partially explains its signaling role in SAR. This systemic SA increase is particularly pronounced in the leaf located directly above the inoculated leaf (Shulaev et al., 1995). To investigate the possible systemic mobility of MeSA, we measured the concentration of SA and MeSA in the leaf directly above the TMV-inoculated leaf. As expected, the SA concentration in the uninoculated leaf gradually increased, reaching a maximum of 318 ng g⁻¹ fresh weight at 144 h postinoculation (Fig. 2B). The uninoculated leaf also showed a significant but transient increase in nonvolatile MeSA content. Immediately following inoculation, MeSA concentration was below detection levels but reached 36 ng g⁻¹ fresh weight 96 h after the leaf below was inoculated with TMV. MeSA concentration returned to below detection levels at 144 h postinoculation. In most other experiments the MeSA concentration in the systemic leaf did not show such a rapid

decline and remained elevated for a longer period of time (data not shown).

Xanthi-nc tobacco plants inoculated with TMV do not develop HR lesions and do not accumulate SA when they are placed at 32°C following TMV inoculation (Kassanis, 1952; Malamy et al., 1992). Within 24 h of being returned to 24°C, inoculated plants respond with massive tissue necrosis and a dramatic accumulation of SA in the tissues to which the virus has spread. We used the temperature-shift experiment to amplify the biochemical changes associated with the HR response and MeSA production. MeSA concentration increased significantly as early as 8 h after temperature shift, and reached a maximum of 5858 ng g⁻¹ fresh weight after 12 h, just before the tissue started to collapse (Fig. 3). From 12 to 24 h the concentration of MeSA decreased in parallel with tissue desiccation during massive necrosis. However, tissue SA continued to accumulate throughout the 24-h period.

MeSA Production in *P. syringae*-Inoculated Tobacco Leaves

To establish whether the production of MeSA in tobacco is specific for TMV, we measured MeSA accumulation in tobacco leaves inoculated with the bacterial pathogen *P.*

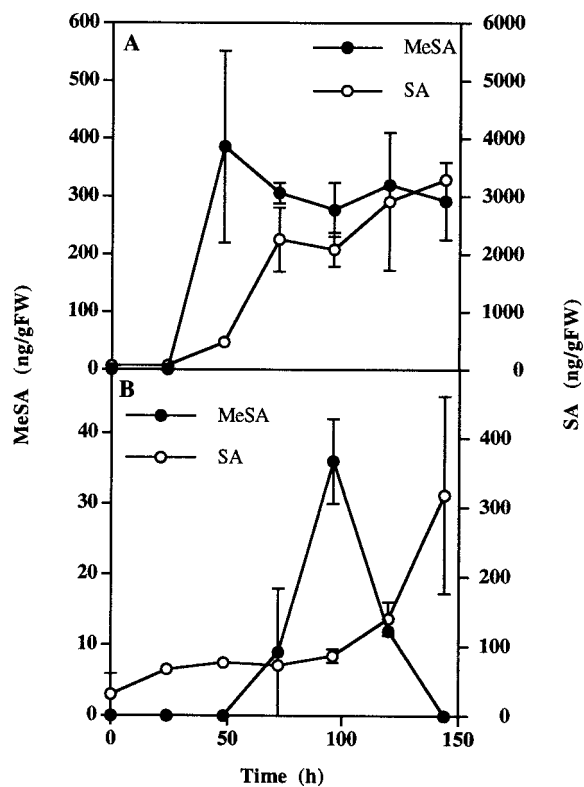


Figure 2. Accumulation of MeSA and SA in tobacco plants after TMV inoculation of a single leaf. Eight-week-old tobacco plants were inoculated on the third leaf from the bottom. MeSA and SA accumulation was analyzed in the inoculated leaf (A) and in the leaf located directly above it (leaf 8) (B). Each point is the mean \pm SE of three replicates. The experiment was repeated twice with similar results. FW, Fresh weight.

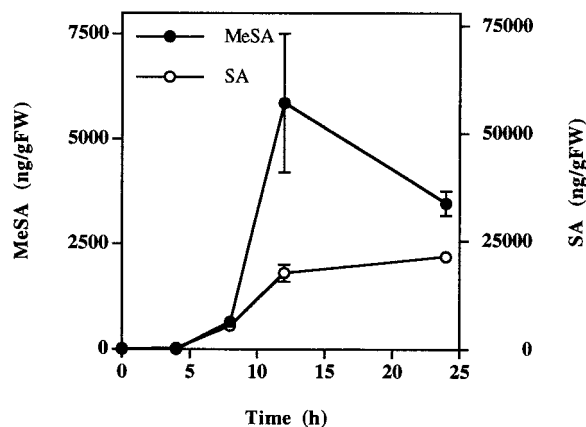


Figure 3. Accumulation of MeSA and SA in TMV-inoculated tobacco leaves after temperature shift. Tobacco plants were inoculated with TMV on a fully expanded leaf and kept at 32°C for 96 h. At time 0, the temperature was lowered to 24°C. Each point is the mean \pm SE of three replicates. The experiment was repeated twice with similar results. FW, Fresh weight.

syringae pv *phaseolicola*, which induces strong HR lesions around the site of infiltration 12 to 18 h postinoculation. Total SA concentration in the vicinity of the lesions reached 13,466 ng g⁻¹ fresh weight after 24 h (Fig. 4). Following the increase in tissue SA, a large increase in MeSA concentration of 1,126 ng g⁻¹ fresh weight was detectable 12 h postinoculation. However, by 18 h, when necrotic HR lesions had developed, the concentration of MeSA decreased to 504 ng g⁻¹ fresh weight. In contrast, tissue desiccation did not substantially reduce SA content.

Effect of MeSA on Transgenic NahG Tobacco Plants

In the previous experiments we observed that MeSA accumulation paralleled or followed an increase in tissue SA (Figs. 2, 3, and 4). To investigate whether SA accumu-

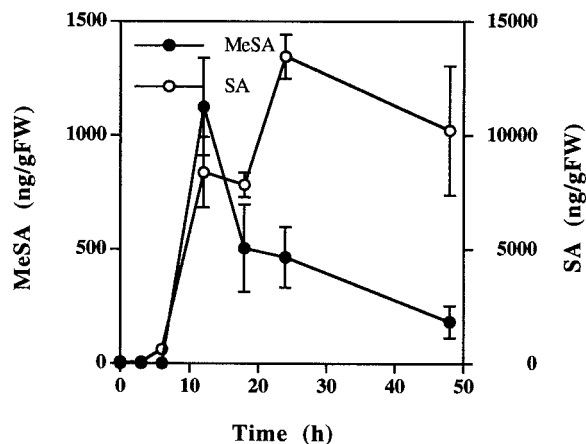


Figure 4. Accumulation of MeSA and SA in tobacco leaves inoculated with *P. syringae* pv. *phaseolicola*. One fully expanded leaf per plant was inoculated with a strain that induces the HR. The infiltrated tissue was harvested with a cork borer at the times indicated. Each point is the mean \pm SE of three replicates. The experiment was repeated with similar results. FW, Fresh weight.

lation is required for MeSA production, we used transgenic tobacco plants expressing the salicylate hydroxylase gene (*nahG*) from *Pseudomonas putida*, which converts SA to catechol (Gaffney et al., 1993). NahG tobacco plants, which are unable to accumulate SA, and wild-type plants were inoculated with TMV and kept at 32°C for 4 d. In contrast to the wild type, TMV inoculation of NahG tobacco plants did not cause SA or MeSA accumulation (Table I). Even though the leaf tissue of both wild-type and NahG plants became necrotic approximately 12 h after the temperature shift, the amounts of SA in NahG plants remained close to basal level, whereas MeSA was below the limit of detection.

Gaseous MeSA was shown to induce *PR-1* transcripts and to reduce the size of TMV-induced lesions in tobacco, presumably through conversion to SA (Shulaev et al., 1997). To further test this hypothesis, we investigated whether MeSA can induce *PR-1* transcripts and TMV resistance at low SA levels by exposing NahG plants for 6 d to 250 μ g L⁻¹ of gaseous MeSA. MeSA treatment of NahG plants resulted in only a 1.9-fold increase in leaf SA levels compared with a 193-fold increase in the wild type (Table II). In addition, the diameter of the TMV lesions on NahG leaves exposed to MeSA was 2.1 times greater than those on wild-type tobacco leaves, an indication of decreased TMV resistance. Moreover, MeSA treatment did not induce *PR-1* transcripts in NahG plants, in contrast to its dramatic effect in the wild-type plants (Fig. 5).

DISCUSSION

We previously demonstrated that gaseous MeSA produced in TMV-inoculated tobacco leaves acts as an airborne defense signal involved in the communication between infected and healthy plants (Shulaev et al., 1997). The amounts of gaseous MeSA produced after the infection were sufficient to induce expression of *PR-1* proteins and TMV resistance in nearby healthy plants.

SA glucoside, which is believed to be biologically inactive and immobile, was previously identified as a major metabolite of SA in TMV-infected tobacco tissue (Enyedí et al., 1992; Hennig et al., 1993; Edwards, 1994; Lee et al., 1995). Since MeSA is a volatile liquid at room temperature, earlier steps (such as drying) used in tissue extraction

Table I. SA and MeSA concentration in TMV-inoculated wild-type and NahG tobacco plants after temperature shift

Tobacco plants were inoculated with TMV and incubated at 32°C for 96 h. At time 0, the temperature was lowered to 24°C. Tissue was sampled 0 and 12 h after temperature shift. Each value is the mean of three replicates \pm SE. The experiment was repeated with similar results.

Plant	SA		MeSA	
	0 h	12 h	0 h	12 h
	<i>ng g⁻¹ fresh wt</i>			
Wild type	38 \pm 5	12,600 \pm 3,110	nd ^a	2318 \pm 110
NahG	59 \pm 25	63 \pm 2	nd	nd

^a nd, Not detected.

Table II. Effect of MeSA on SA concentration and TMV-lesion size in wild-type and NahG tobacco plants

Tobacco plants were enclosed in gas-tight glass chambers to which $250 \mu\text{g L}^{-1}$ MeSA was supplied daily. After 6 d of incubation the plants were sampled and analyzed for SA content. Each value is the mean \pm SE of three replicates. Plants were then inoculated with TMV, and the diameter of the TMV-induced lesions was measured 72 h postinoculation. Data represent the mean diameter \pm SE of at least 30 lesions per plant. The experiment was repeated with similar results. The diameters of TMV lesions in untreated wild-type and NahG plants were 1.328 ± 0.09 and 1.800 ± 0.11 mm, respectively.

Plant	SA		Lesion Diameter
	d 0	d 6	
	ng g^{-1} fresh wt		
Wild type	34 ± 1	6546 ± 683	0.852 ± 0.04
NahG	45 ± 4	84 ± 17	1.785 ± 0.08

prevented researchers from detecting MeSA as another important metabolite of SA. Measurements of MeSA in the air around virus-inoculated plants provided the first assessment, but underestimated the total MeSA production in the inoculated tissue. Thus, we have developed a method for measuring MeSA in plant tissues (Fig. 1). Our results demonstrate that large amounts of nonvolatile MeSA accumulate in the TMV-inoculated tissue around the time of its appearance in the gas phase (Shulaev et al., 1997).

The amounts of endogenous MeSA in pathogen-inoculated tobacco leaves represent about 10 to 20% of the total SA detected in nontemperature-shifted plants (Figs. 2 and 4), and approximately 30% in temperature-shifted tobacco. Since a substantial portion of MeSA escapes as a vapor, the total amounts of MeSA produced in inoculated leaves should represent an even larger fraction of total SA production. It is important to mention that the induction of MeSA production is not specific to TMV. *P. syringae* pv *phaseolicola*, a bean pathogen that is incompatible in tobacco (Lindgren et al., 1986), also caused dramatic MeSA accumulation, indicating that MeSA production is not pathogen specific.

It was shown earlier that MeSA is made from SA and acts by being converted back to SA (Shulaev et al., 1997). Transgenic tobacco plants expressing the salicylate hydroxylase gene *nahG* are unable to accumulate SA (Gaffney et al., 1993; Friedrich et al., 1995) and provided a useful tool to test the biochemical mechanisms of SA production and action. Since NahG plants inoculated with TMV were unable to accumulate detectable amounts of MeSA (Table I), it is likely that MeSA is synthesized from SA. In addition, the observation that MeSA could not induce TMV resistance (measured as smaller HR lesions) (Table II) and *PR-1* gene expression in NahG plants (Fig. 5) suggests that MeSA acts by being converted to SA. However, these results can also be explained by MeSA serving as a substrate for the salicylate hydroxylase. Another indication that SA is a precursor of MeSA comes from *Nicotiana glutinosa* \times *debneyi*, a tobacco hybrid that contains constitutively high levels of

SA (Yalpani et al., 1993). The MeSA concentration in healthy leaves ($n = 9$) of this hybrid is $480 \pm 248 \text{ ng g}^{-1}$ fresh weight, suggesting that the high levels of SA give rise to high levels of MeSA.

In inoculated leaves MeSA accumulation always paralleled the accumulation of endogenous SA (Figs. 2–4), consistent with the proposed synthesis of MeSA from SA. However, in the postinoculation period SA concentration increased continually and MeSA concentration decreased as HR lesions developed (Figs. 2–4). This observation agrees with our previously published observation that the highest production of gaseous MeSA is reached at the onset of tissue desiccation (Shulaev et al., 1997). Here we suggest that the differences in the kinetics of MeSA and SA accumulation can be at least partially explained by the increased volatilization of MeSA facilitated by tissue desiccation during HR-associated necrosis.

It has been previously suggested that SA may not be the only signal involved in SAR (Rasmussen et al., 1991; Ver-nooij et al., 1994; Shulaev et al., 1995), although it was shown to be required for the establishment of SAR (Gaffney et al., 1993). The observation that MeSA accumulation was detected in the healthy tobacco leaves located above the inoculated leaf (Fig. 2B) suggests that MeSA may function as a translocatable form of SA, into which it can be readily converted in the target tissue. Therefore, like the SA precursor benzoic acid (Shulaev et al., 1995), MeSA may play at least an accessory role in SAR signaling. We have detected MeSA in phloem exudates of TMV-inoculated leaves after a temperature shift, using a previously established exudate-collection technique (Yalpani et al., 1991; data not shown). Therefore, phloem translocation of non-gaseous MeSA may complement its aerial spread and provide an early warning defense system during SAR. However, relatively low levels of MeSA compared with SA argue for a supplemental rather than an exclusive role for MeSA in SAR signaling. On the other hand, MeSA vapor

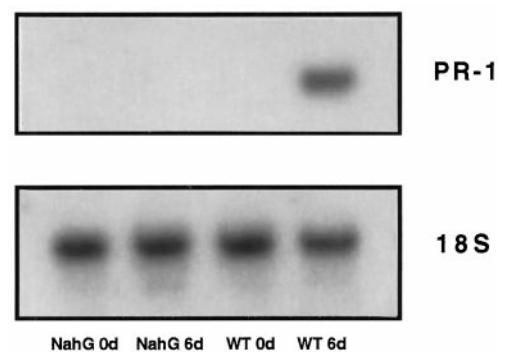


Figure 5. *PR-1* gene expression in wild-type (WT) and transgenic NahG tobacco plants exposed to gaseous MeSA. Five-week-old tobacco plants were enclosed in gas-tight glass chambers containing $250 \mu\text{g L}^{-1}$ MeSA. After the 6-d incubation period, fully expanded leaves were removed from plants and analyzed for *PR-1* gene transcription. Total RNA was isolated and the *PR-1* transcript was detected by hybridization with a cDNA probe (top). A probe for rRNA (18S) was used to demonstrate equal RNA loading (bottom).

may be the sole signal inducing defense responses in an adjacent plant. Further elucidation of MeSA biosynthesis and the cloning of MeSA biosynthetic enzymes may enable us to manipulate MeSA and SA levels in plants, thereby enhancing inter- and intra-plant resistance to pathogens.

Received July 30, 1997; accepted October 13, 1997.
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