The Plant Cell, Vol. 7, 1691–1701, October 1995 © 1995 American Society of Plant Physiologists

Is Salicylic Acid a Translocated Signal of Systemic Acquired Resistance in Tobacco?

Vladimir Shulaev, José León, and Ilya Raskin[1]

AgBiotech Center, Cook College, Rutgers University, P.O. Box 231, New Brunswick, New Jersey 08903-0231

Salicylic acid (SA) is a likely endogenous signal in the development of systemic acquired resistance (SAR) in some dicotyledonous plants. In tobacco mosaic virus (TMV)-resistant Xanthi-nc tobacco, SA levels increase systemically following the inoculation of a single leaf with TMV. To determine the extent to which systemic increases in SA result from SA export from the inoculated leaf, SA produced in TMV-inoculated or healthy leaves was noninvasively labeled with $^{18}O_2$. Spatial and temporal distribution of ^{18}O-SA indicated that most of the SA detected in the healthy tissues was synthesized in the inoculated leaf. No significant increase in the activity of benzoic acid 2-hydroxylase, the last enzyme involved in SA biosynthesis, was detected in upper uninoculated leaves, although the basal level of enzyme activity was relatively high. No increases in SA level, pathogenesis-related PR-1 gene expression, or TMV resistance in the upper uninoculated leaf were observed if the TMV-inoculated leaf was detached up to 60 hr after inoculation. Apart from the inoculated tissues, the highest increase in SA was observed in the leaf located directly above the inoculated leaf. The systemic SA increase observed during SAR may be explained by phloem transport of SA from the inoculation sites.

INTRODUCTION

Acquired or induced resistance, which develops after inoculation with necrotizing pathogens, has been recognized by plant pathologists for >60 years (Chester, 1933; Gilpatrick and Weintraub, 1952). Both localized acquired resistance and systemic acquired resistance (SAR) were extensively studied by Ross (1961a, 1961b), who was the first to introduce definitions of these phenomena. However, the intercellular signal transduction mechanisms leading to the development of localized acquired resistance and SAR have yet to be determined.

The phenomenon of SAR suggests that there is a signal that originates at the site of infection and moves throughout the plant. Dean and Kuc (1986a, 1986b) have shown that such a signal is produced by an infected leaf and that detachment of this leaf before the development of the hypersensitive response (HR) blocks the induction of SAR. Grafting and stem girdling experiments with cucumber and tobacco suggest that the SAR signal moves in the phloem (Jenns and Kuc, 1979; Guedes et al., 1980; Tuzun and Kuc, 1985).

Recent evidence indicates that salicylic acid (SA) plays an important role in plant defense against pathogen attack and is essential for the development of SAR (for review, see Yalpani and Raskin, 1993; Ryals et al., 1994). Interest in the role of SA in plant defense started from the observation that exogenously applied aspirin or SA induces resistance to tobacco mosaic virus (TMV) in tobacco (White, 1979). This increase in resistance was correlated with the accumulation of pathogenesis-related (PR) proteins, generally assumed to be markers of the defense response (Ward et al., 1991). Later, it was shown that development of the HR and SAR is accompanied by a dramatic increase in the level of endogenous SA in the inoculated leaves and in the systemically protected tissue (Malamy et al., 1990). SA levels increase systemically following TMV inoculation of Xanthi-nc tobacco that carries the *N* resistance gene to TMV but not in a susceptible cultivar (Malamy et al., 1990). Similarly, inoculation of cucumber plants with tobacco necrosis virus, or the fungus *Colletotrichum lagenarium*, leads to a dramatic rise in SA levels in the phloem, which is preceded by the development of SAR (Métraux et al., 1990). Levels of SA in pathogen-free tissues of inoculated plants have been shown to be sufficient to induce PR proteins and greater resistance to subsequent inoculation (Yalpani et al., 1991; Enyedi et al., 1992). Transgenic tobacco plants carrying a bacterial salicylate hydroxylase gene cannot accumulate SA and therefore do not develop SAR (Gaffney et al., 1993), whereas Arabidopsis plants carrying the same genes show much higher susceptibility to various pathogens (Delaney et al., 1994).

The pathway of SA biosynthesis has been recently elucidated in tobacco, in which SA is synthesized from cinnamic acid via benzoic acid (Yalpani et al., 1993). The latter reaction is catalyzed by a benzoic acid–inducible benzoic acid 2-hydroxylase (BA2H), which functions as a cytochrome P450 monooxygenase (León et al., 1993). β-*O*-D-Glucosylsalicylic acid is the major metabolite of exogenous and endogenous SA in tobacco (Enyedi et al., 1992; Malamy et al., 1992), although production of the glucose ester has also been reported (Edwards, 1994). Large quantities of glucosylsalicylic acid (79 μg/g fresh

[1] To whom correspondence should be addressed.

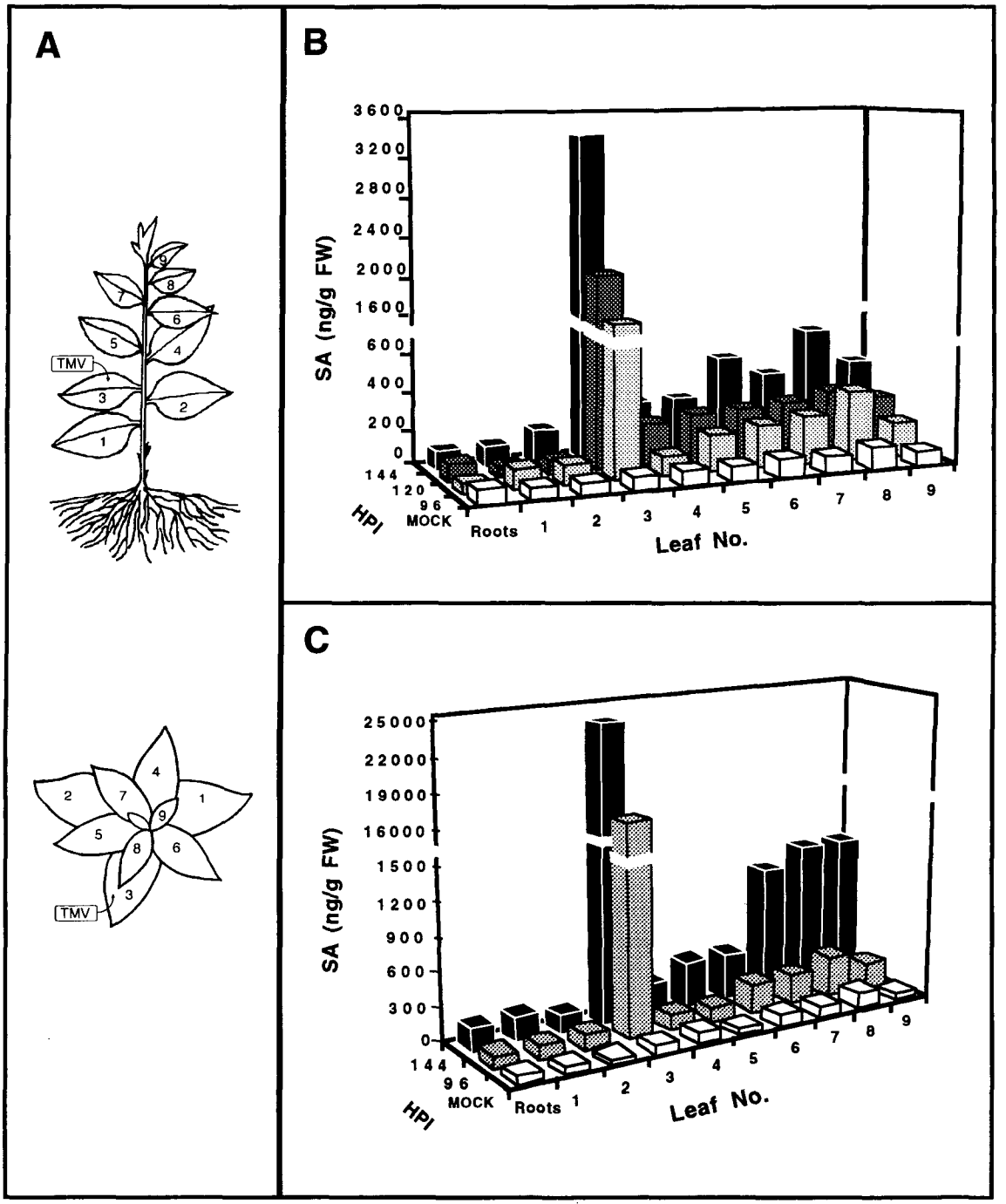

Figure 1. Kinetics of SA Accumulation throughout a Tobacco Plant after TMV Inoculation of a Single Leaf in the Middle of the Plant.

SA levels were determined at 24-hr intervals in roots and in the upper and lower leaves. The experiment was repeated twice with similar results.
(A) Arrangement of the leaves in a 6-week-old tobacco plant (side and top view).
(B) Temporal and spatial distribution of free SA in a 6-week-old tobacco plant after inoculation of leaf 3 with TMV. Each bar represents the mean of four replicates ± SE. The experiment was repeated with similar results.
(C) Temporal and spatial distribution of total SA (the sum of free and glucosylsalicylic acid) in a 6-week-old tobacco plant after inoculation of leaf 3 with TMV. Each bar represents the mean of four replicates ± SE. The experiment was repeated with similar results.
FW, fresh weight; HPI, hours postinoculation; mock, mock inoculated.

weight) accumulate in and around the TMV-induced HR lesions in tobacco (Enyedi et al., 1992). Little glucosylsalicylic acid has been detected in phloem and in systemically protected leaves (Enyedi et al., 1992).

Although much experimental data accumulated to date support the role of SA as a primary long-distance signal in SAR, other results suggest that SA may not be the translocated signal responsible for the induction of SAR (Rasmussen et al., 1991; Vernooij et al., 1994). Data presented in this study directly demonstrate that a large amount of SA synthesized in the TMV-inoculated leaf is translocated to upper uninoculated leaves. The magnitude and timing of this transport indicate that SA may be a major translocated SAR-inducing signal in tobacco.

RESULTS

Spatial and Temporal Distribution of SA Accumulation after Inoculation with TMV

SA levels increase systemically following TMV inoculation of tobacco containing the *N* gene (Malamy et al., 1990; Enyedi et al., 1992). We studied the temporal and spatial patterns of SA accumulation in 6-week-old Xanthi-nc tobacco plants following the inoculation of a single leaf with TMV (Figure 1). At the time of inoculation, leaf 3 was fully expanded (Figure 1A). Free and total (the sum of free SA and glucosylsalicylic acid) SA levels were measured at 24-hr intervals in roots and in leaves above and below the inoculated leaf (Figures 1B and 1C).

Increases in free SA in the upper uninoculated leaves were detected 96 hr after inoculation (Figure 1B). The largest increase in SA, other than in the inoculated leaf, occurred in leaf 8, which was located directly above the inoculated leaf and has the strongest vascular connection with the inoculated source leaf. SA levels continued to increase for 144 hr postinoculation. Increases in total SA paralleled temporally and spatially the increases in free SA levels (Figure 1C). Little or no increase in total or free SA occurred in the roots or in leaves located below the inoculated leaf. The fact that the highest SA increase occurred in the strongest sink leaf to the inoculated leaf further supports the hypothesis that the SAR-inducing signal is translocated in the phloem. Based on this information, all additional experiments were conducted by inoculating leaf 3 of 6-week-old tobacco plants and following the SA levels in leaf 8, which showed the largest systemic increase in SA. In all these experiments, we report the total tissue amounts of SA (the sum of free and glucosylsalicylic acid), which is a more biologically relevant measure of tissue SA.

SA Labeling with $^{18}O_2$

BA2H was hypothesized to be a cytochrome P450 monooxygenase (León et al., 1993). Monooxygenase activity incorporates an oxygen atom from O_2 into a product. Thus, if the reaction occurs in an atmosphere containing $^{18}O_2$, the products will be labeled with oxygen-18 and can subsequently be identified and quantified by mass spectroscopy. Labeling with oxygen-18 has been used to study the biosynthesis of abscisic acid (Creelman and Zeevart, 1984), caffeic acid (Fritz et al., 1974), and *p*-coumaric acid (Fritz and Andresen, 1978). The technique is particularly valuable for the study of the role of SA in SAR for two reasons: it tests the proposed mechanism of SA formation from benzoic acid, and it allows noninvasive in situ labeling of endogenously synthesized SA and subsequent monitoring of its movement throughout the plant.

To perform oxygen-18 labeling, we enclosed a TMV-inoculated leaf of a tobacco plant in a transparent, temperature-controlled plastic chamber initially containing 20% $^{18}O_2$, 80% N_2, and 0.1% CO_2. The rest of the plant remained exposed to the ambient air containing $^{16}O_2$. The experiment was terminated 5 days after inoculation, and levels of SA were determined in all leaves. SA purified from the β-glucosidase–hydrolyzed extracts of the inoculated leaf (leaf 3) and from leaf 8 was derivatized with ethereal diazomethane and subjected to gas chromatography–mass spectrometry analysis.

A TMV-inoculated leaf kept in the $^{18}O_2$ atmosphere showed a typical HR. SA distribution throughout the plant was not affected by the labeling procedure (data not shown). As expected, the TMV-inoculated leaf showed a dramatic increase in SA (Table 1). SA levels also increased systemically, with the highest level of 1.1 μg of SA per gram of fresh weight detected in leaf 8. The mass spectrum of methylated SA isolated from the upper

Table 1. Distribution of ^{18}O-Labeled SA in TMV-Inoculated Xanthi-nc Tobacco (Inoculated Leaf Labeling)[a]

	SA (μg/g FW)[b]						
Leaf Position	TMV-Inoculated Plant	Control Plant	SA Increase (μg/g FW)	% ^{18}O Labeled	^{18}O-Labeled SA (μg/g FW)	Theoretical Limit of ^{18}O-SA (μg/g FW)	% SA Transported
Inoculated leaf	15.71	0.12	15.59	45.60	7.16	NA[c]	NA
Upper leaf	1.10	0.17	0.93	26.32	0.29	0.42	69.05

[a] The experiment was repeated twice with similar results.
[b] FW, fresh weight.
[c] NA, not applicable.

uninoculated leaf 4 days after the lower leaf was exposed to $^{18}O_2$ contained a new molecular ion with an *m/z* of 154 as well as the molecular ion with an *m/z* of 152 of the unlabeled methyl salicylate (MeSA) (compare Figures 2A and 2B). This suggests that only one atom of oxygen-18 is incorporated into the SA molecule. The position of the oxygen-18 atom in the molecule was determined from the fragmentation pattern of methylated SA. Loss of the methoxyl group from the molecular ion of ^{18}O-MeSA produces an ion with an *m/z* of 122 that is still shifted by two mass units compared with the ion with an *m/z* of 120 from a MeSA standard. This ion fragments further by elimination of two CO groups. The elimination of the first CO produces an ion with an *m/z* of 92 that is not shifted in the

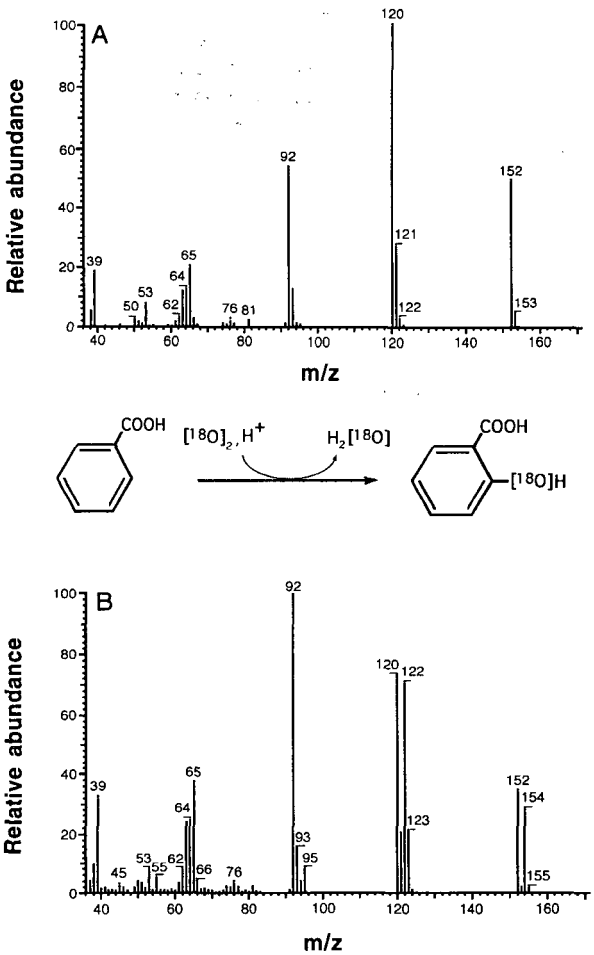

Figure 2. Electron Impact Mass Spectral Analysis of Derivatized SA.

(A) The mass spectrum of the authentic 2-hydroxybenzoic acid methyl ester (SA–methyl ester).

(B) The mass spectrum of the fraction from the $^{18}O_2$-labeled leaf coeluting with authentic SA–methyl ester. Diazomethane-treated HPLC-purified SA from the TMV-inoculated leaf was injected onto a gas chromatograph interfaced to a mass spectrometer.

spectrum of labeled MeSA. Using ^{14}C-labeled SA, it was shown that the first loss of CO involves a ring carbon (Occolowitz, 1968). The loss of oxygen-18 at this step suggests that a heavy oxygen atom is incorporated into the 2-hydroxyl group of SA.

The presence of peaks at both *m/z* 152 (^{16}O-MeSA) and 154 (^{18}O-MeSA) in the mass spectrum indicates that both unlabeled ("light") and labeled ("heavy") SA are present in the plant extracts. By monitoring the peaks at *m/z* 154 and *m/z* 152, we determined the ratio of heavy to light SA. These measurements show that 45.6% of total SA isolated from the TMV-inoculated leaf contained heavy oxygen (Table 1). This was very close to the molecular fraction of $^{18}O_2$ to $^{16}O_2$ in the leaf chamber, which averaged 50% for the duration of labeling (see Methods). The similarity between these two numbers indicates almost stoichiometric incorporation of heavy oxygen into the hydroxyl group of SA.

The appearance of labeled SA in the upper leaf suggests that SA made in the inoculated leaf was translocated into the upper uninoculated leaf. Twenty-six percent of total SA isolated from the upper uninoculated leaf was labeled with oxygen-18 (Table 1; calculated as the ratio of the molecular ions *m/z* 154 and *m/z* 152). If all the SA in the upper leaf came from the lower leaf, the ratio of heavy to light SA in the upper leaf would be the same as that in the bottom leaf, that is, 45.6%. However, upper leaves contained background levels of unlabeled SA (0.17 μg/g fresh weight), which diluted labeled SA coming from the inoculated leaf. From the ratio of heavy to light SA in extracts from the lower and upper leaves, corrected for the background SA level, the amount of SA transported to the upper leaf was estimated as follows (Table 1): the amount of heavy SA in the upper leaf was 26.32% of 1.10 μg/g fresh weight or 0.29 μg/g fresh weight. The theoretical maximum level of heavy SA in the upper leaf of the inoculated plant (0.42 μg/g fresh weight) was calculated as 45.60% of 0.93 μg/g fresh weight (the ratio of labeled SA in the source leaf multiplied by the increase in SA in the upper leaf compared with the control). The amount of labeled SA detected in the upper leaf of the inoculated plant was 69% of the amount that would be expected if all the increase was due to import of SA from the source leaf. This means that at least 69% of the total SA increase in the upper leaf can be explained by the import of SA.

To obtain additional confirmation of the above result, we repeated the heavy oxygen labeling experiment, leaving the inoculated leaf in the ambient air and enclosing the upper uninoculated leaf inside the chamber containing 20% $^{18}O_2$, 80% N_2, and 0.1% CO_2. Total SA extracted from the upper leaf after 4 days of labeling contained oxygen-18. However, the proportion of labeled SA was 21% (Table 2), significantly lower than in the experiment in which the label was applied to the lower leaf (Table 1). This result would be expected if SA synthesized in the upper leaf was diluted by unlabeled SA imported from the inoculated leaf.

Because the increase in SA in the upper leaf was not as large as that in the inoculated leaf, newly synthesized SA would also be diluted somewhat by the preexisting basal level of SA,

Table 2. Distribution of ^{18}O-Labeled SA in TMV-Inoculated Xanthi-nc Tobacco (Upper Leaf Labeling)[a]

Leaf Position	SA (μg/g FW) TMV-Inoculated Plant	SA (μg/g FW) Control Plant	^{16}O-SA Remaining after Labeling (μg/g FW)[b]	SA Increase (μg/g FW)	^{18}O-Labeled SA (μg/g FW)	Theoretical Limit of ^{18}O-SA (μg/g FW)	% SA Transported
Inoculated leaf	15.70	0.12	NA[c]	NA	NA	NA	NA
Upper leaf	0.84	0.32	0.13	0.71	0.17	0.40	57.54

[a] The experiment was repeated twice with similar results.
[b] Assuming an SA turnover rate of 25.5% per day. FW, fresh weight.
[c] NA, not applicable.

if this pool does not turn over rapidly. To estimate the turnover rate for SA in the upper leaf, leaf 3 of a healthy tobacco plant was infiltrated with a 0.5 mM solution of ^{14}C-labeled SA (56 mCi/mmol). Twenty-four hours after infiltration, the bottom leaf was detached. The specific activity of SA extracted from the upper leaf (leaf 8) was determined at 0 and 24 hr after detachment of the infiltrated leaf. At time 0, SA isolated from leaf 8 had a specific activity of 36.3 mCi/mmol. Within 24 hr, the specific activity of SA isolated from leaf 8 decreased to 27.1 mCi/mmol. This suggests that the SA turnover rate in leaf 8 is 25.5% per day. An autoradiograph of the upper leaf (leaf 8) of the plant 24 hr after infiltration of leaf 3 with a 0.5 mM solution of ^{14}C-labeled SA (56 mCi/mmol) or benzoic acid (56 mCi/mmol) shows the presence of the label in the vascular tissue and throughout the leaf blade (Figures 3A and 3C). More label moved in the upper leaves of plants infiltrated with SA than in plants infiltrated with the same amount of benzoic acid. An autoradiograph of the upper leaf of the control plant that was not infiltrated with labeled SA or benzoic acid had no detectable image at this exposure time.

Based on the average ratio of oxygen-18 and oxygen-16 in the incubation atmosphere and the ratio of heavy to light SA in extracts from the upper leaf, corrected for the background SA level and the SA turnover rate, we calculated the amount of SA transported to the upper uninoculated leaf. Because the basal level of SA in the upper leaf was 0.32 μg/g fresh weight, 0.13 μg/g fresh weight of ^{16}O-SA should remain in the upper leaf after 72 hr of labeling (assuming a turnover rate of 25.5% per day). Therefore, the total SA that appeared in the leaf during labeling is 0.84 − 0.13 = 0.71 μg/g fresh weight. The average proportion of oxygen-18 in the leaf chamber was 56.7% of the total oxygen. If all of the accumulated SA was made in the upper leaf, the amount of heavy SA would be expected to be 56.7% of 0.71, or 0.40 μg/g fresh weight. However, the total amount of ^{18}O-SA in the upper leaf was 0.17 μg/g fresh weight—only 42.5% of the expected. This suggests that 58% of that SA accumulated in the upper leaf may be accounted for by SA import. This result complements the experiment in which the TMV-inoculated leaf was exposed to $^{18}O_2$ (Table 1).

BA2H Activity Levels and SA Content in Upper Uninoculated Leaves of TMV-Inoculated Tobacco Plants

In Xanthi-nc tobacco, the level of free benzoic acid in the inoculated leaf increases following inoculation with TMV (León et al., 1993). This increase can be correlated with the induction of BA2H, an enzyme that is highly induced by TMV inoculation. To determine whether induction of SA biosynthesis in the upper uninoculated leaf could explain the SA increase, we measured BA2H activity and benzoic acid levels in leaf 8 following inoculation of leaf 3 with TMV. Daily measurements of BA2H activity in leaf 8 did not show significant changes in the enzyme activity up to 5 days postinoculation. BA2H activity in leaf 8 averaged 2.40 nmol/hr per g fresh weight ± 0.24 SE (n = 4), indicating relatively high basal levels of enzyme activity. The levels of free benzoic acid in this leaf averaged 12.49 ng/g fresh weight ± 1.2 SE (n = 4) and did not change significantly when measured each day for 5 days following TMV inoculation of leaf 3.

Effect of Detachment of the Inoculated Leaf on Systemic SA Increases, TMV Resistance, and PR-1 Gene Expression

Fourteen groups of tobacco plants (three plants per group) were inoculated with TMV (5 μg per leaf) at time 0. Inoculated leaves were detached at the indicated time intervals for a total period of 96 hr after inoculation and assayed for free and total SA content (Figure 4A). Leaf 8 was kept on the plants for 96 hr and subsequently analyzed for free and total SA (Figure 4B). Statistically significant increases in free and total SA in the inoculated leaf were first observed 48 and 36 hr after inoculation, respectively. No significant increases in free or total SA in the upper leaf could be measured when the TMV-inoculated leaf was detached 72 and 60 hr after inoculation, respectively. Therefore, SA accumulation in the upper leaves could be prevented by removing the inoculated leaf before it accumulated substantial amounts of SA.

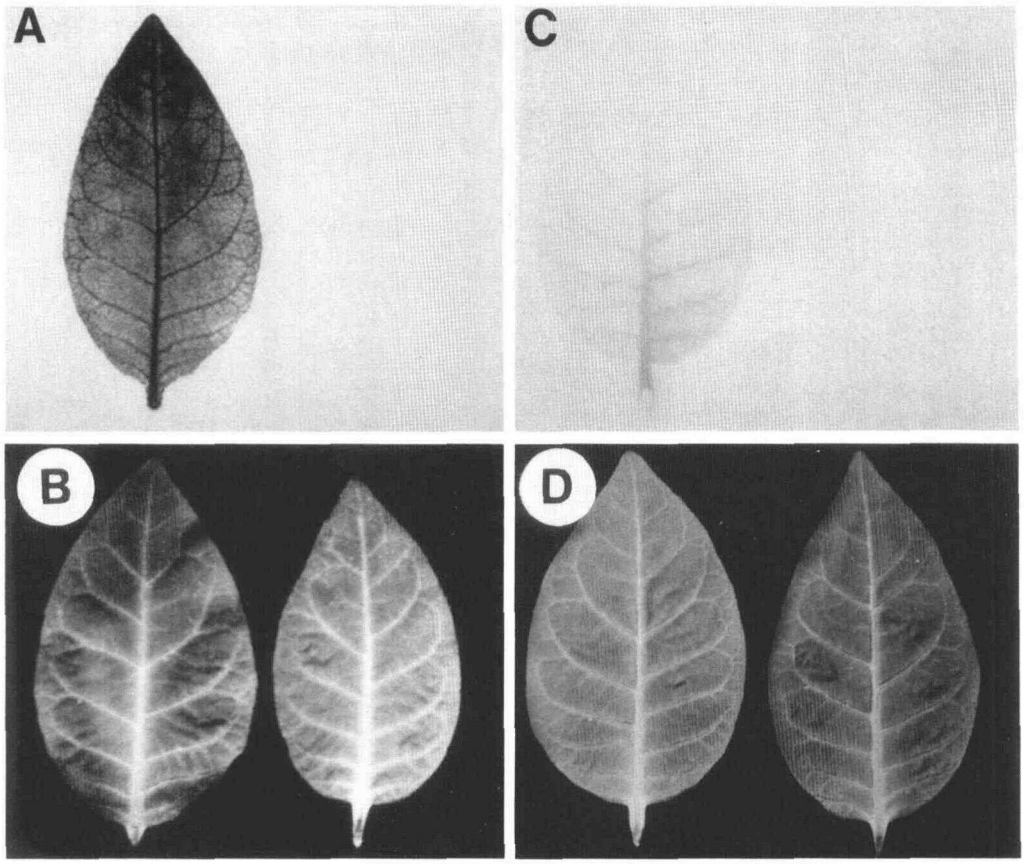

Figure 3. Autoradiogram of Upper Tobacco Leaf after Infiltration of the Lower Leaf with ^{14}C-SA.

This experiment was repeated three times with similar results.
(A) Autoradiograph of leaf 8 excised from a tobacco plant 24 hr after infiltration of leaf 3 with 56 mCi/mmol of ^{14}C-SA solution (left) or with buffer (right, image not visible).
(B) Corresponding light photograph of **(A)**.
(C) Autoradiograph of leaf 8 excised from a tobacco plant 24 hr after infiltration of leaf 3 with 56.5 mCi/mmol ^{14}C-benzoic acid solution (left) or with buffer (right, image not visible).
(D) Corresponding light photograph of **(C)**.

Interestingly, significant reduction in lesion size was obtained in parallel experiments in which TMV resistance in leaf 8 was determined as a function of leaf 3 detachment time (Figure 4C). Increased resistance, quantified as a reduction in lesion diameter, closely coincided with the increases in SA in leaf 8 and was not observed when leaf 3 was detached 60 hr after inoculation. However, keeping the inoculated leaf on the plant for 72 hr resulted in a statistically significant 27% reduction in lesion diameter when compared with detachment at time 0. SAR in leaf 8 was further strengthened when the inoculated leaf was kept on the plant for over 72 hr. In addition, no significant PR-1 gene expression in leaf 8 was observed unless the inoculated leaf 3 was kept on the plant for 120 hr (Figure 5). Therefore, PR-1 gene expression in leaf 8 coincided with increases in SA and TMV resistance in this leaf. None of these three phenomena occurred in leaf 8 unless leaf 3 was kept on the plant long enough to accumulate substantial amounts of SA.

DISCUSSION

To be a translocated signal responsible for SAR, SA must be exported from the inoculated leaf in amounts sufficient to explain its accumulation in the upper leaves. Export of SA from leaves inoculated with various necrotizing pathogens and its appearance in the phloem have been confirmed in tobacco and cucumber (Métraux et al., 1990; Rasmussen et al., 1991, Yalpani et al., 1991). The physicochemical properties of SA make it well suited for long-distance phloem transport (Yalpani et al., 1991). However, it has never been demonstrated that

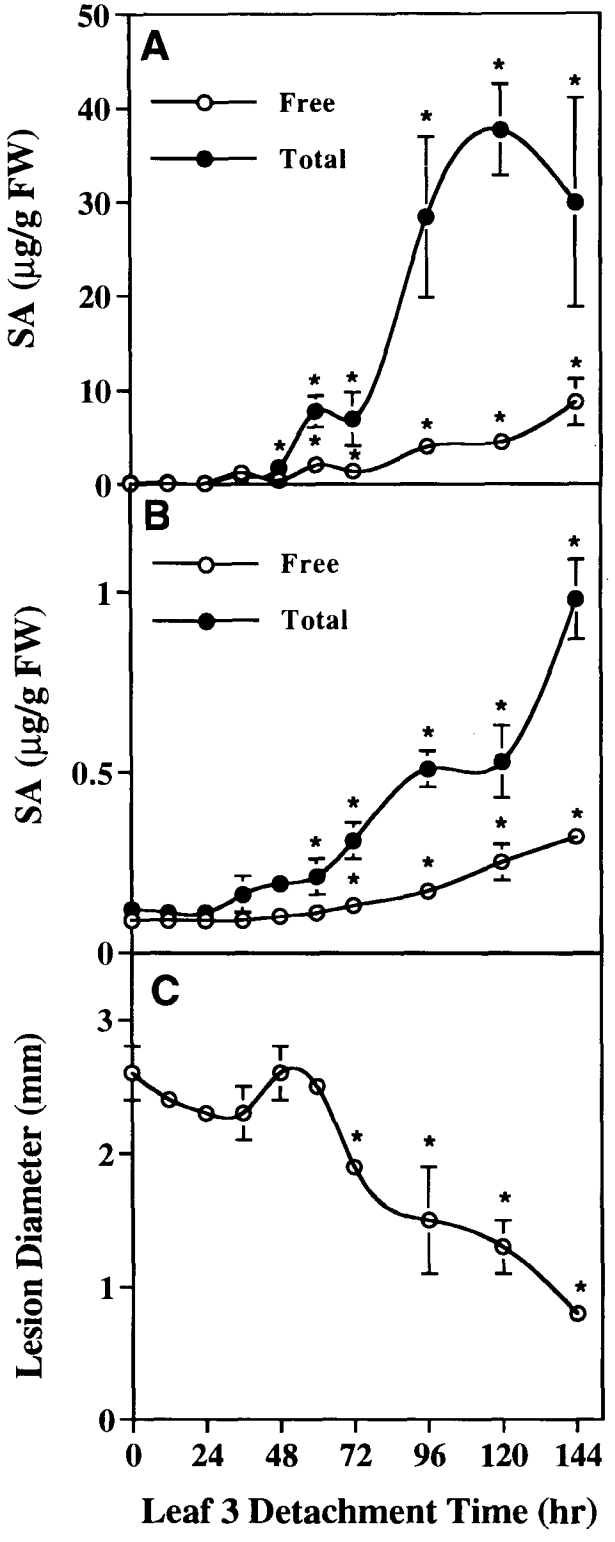

Figure 4. Effect of Detaching a TMV-Inoculated Leaf on SA Levels and TMV Resistance in Leaf 8.

Tobacco plants (six plants per each leaf detachment time; 60 plants total) were inoculated on leaf 3 with 5 μg of TMV. The inoculated leaf was detached at the indicated intervals and assayed for free and total (free plus conjugated) SA. Six days after inoculation of leaf 3, levels of free and total SA were determined for half of the plants (three plants per each detachment time). The remaining plants were inoculated on leaf 8 with 2.5 μg of TMV per leaf. Lesion diameter was measured 7 days later. This experiment was repeated twice with similar results.
(A) Levels of free and total (free plus conjugated) SA in leaf 3 at the time of leaf detachment. Data represent the mean of three replicates ± SE.
(B) Levels of free and total SA in leaf 8 at 6 days after inoculation of leaf 3. Data represent the mean of three replicates ± SE.
(C) Diameter of TMV-induced lesions in leaf 8 at 7 days after inoculation. Data represent the mean diameter ± SE of 50 lesions per leaf. Values are the mean of triplicates ± SE.
FW, fresh weight; *, statistically significant change compared with the corresponding time 0 (Student's *t* test at P = 0.05).

the SA accumulating in the systemically protected tissue comes from the inoculated leaves.

Noninvasive, in vivo labeling of SA with oxygen-18 provides direct proof that a major part of the systemic SA increase can be explained by SA import from the inoculated leaf. Another advantage of this technique is that it does not change the pool sizes of SA precursors. Oxygen-18 labeling takes advantage of the final reaction of SA biosynthesis catalyzed by BA2H. BA2H incorporates one oxygen atom into the hydroxyl group of SA, using benzoic acid and molecular O_2 as substrates (León et al., 1993). The specificity of oxygen-18 incorporation into the hydroxyl group and a high labeling efficiency (Table 1) rule out the possibility of indirect labeling of SA via SA-independent label movement to the upper leaf. Thus, the possibility that heavy oxygen incorporation into SA is an indirect effect caused by the reduction of $^{18}O_2$ to H_2O during respiration followed by subsequent incorporation of transported ^{18}O-labeled H_2O into SA in the upper uninoculated leaf is not consistent with the high specific activity obtained with the BA2H reaction mechanism. Heavy oxygen–containing water would be highly diluted with the large amount of nonlabeled water present in the plant tissue. This precludes the possibility of a high labeling efficiency, even if some ^{18}O-labeled H_2O is photosynthetically converted back to $^{18}O_2$ in the upper leaf. It was shown previously that heavy oxygen enrichment of water recovered from both shoot and root axes and cotyledons of maize seedlings grown in an atmosphere containing 10.6% $^{18}O_2$ is <0.2% (Fritz and Andresen, 1978). Exchange of the oxygen atom from the hydroxyl group of SA with oxygen present in water is also improbable, because this reaction requires a high temperature (over 100°C) and catalysts (Gragerov and Ponomarchuk, 1959). The systemic movement of SA in the tobacco plant was also confirmed by the detection of ^{14}C-labeled SA in the upper leaf (leaf 8) when ^{14}C-labeled SA was infiltrated into leaf 3 (Figures 3A and 3B). Although less quantitative and more invasive, this experiment independently supports the $^{18}O_2$-labeling experiment.

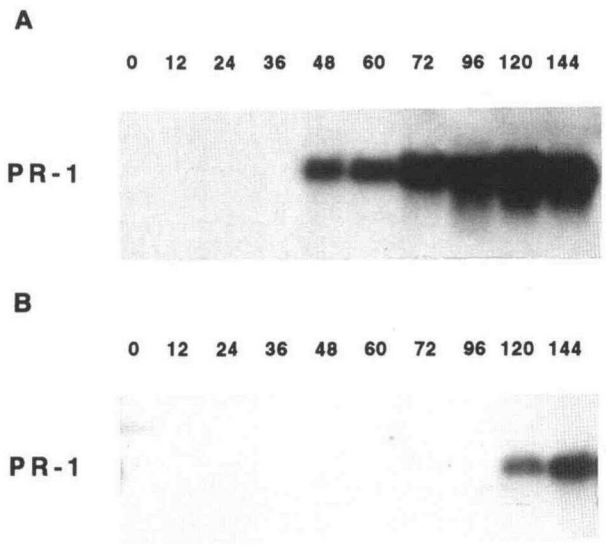

Figure 5. PR-1 Gene Expression in Upper Uninoculated Leaf 8 Is Affected by Detaching TMV-Inoculated Leaf 3.

Tobacco plants were inoculated on leaf 3 with 5 μg of TMV. Inoculated leaves were detached at different intervals after inoculation. RNA was isolated from TMV-inoculated leaf 3 at the time of detachment and from upper uninoculated leaf 8 at 6 days after inoculation of leaf 3. Thirty micrograms of RNA was loaded in each lane, and PR-1 transcripts were detected with tobacco PR-1 cDNA. The numbers above the lanes indicate the time (in hours) of detachment of leaf 3. RNAs from **(A)** and **(B)** were run on the same gel and exposed for the same time. The experiment was repeated twice with similar results.

(A) Total RNA from TMV-inoculated leaf 3 isolated at the time it was detached.

(B) Total RNA from upper uninoculated leaf 8 isolated at 6 days after leaf 3 was inoculated with TMV.

Our data indicate that a substantial part (60 to 70%) of SA accumulated in the upper leaves arrives from the inoculated leaf. To account for the rest of the SA increase, we hypothesize that benzoic acid, which shares similar physicochemical properties with SA and serves as its immediate precursor, is also exported into the upper leaves. In fact, our results are consistent with the movement of ^{14}C-labeled benzoic acid from leaf 3 to leaf 8 (Figures 3C and 3D). It is known that levels of free and conjugated benzoic acid increase dramatically in TMV-inoculated tobacco tissue, parallel with SA increases (Yalpani et al., 1993). Thus, benzoic acid, along with SA, may be transported in phloem to the upper leaves, where it is rapidly converted to SA by a constitutive basal level of BA2H activity. This explanation is consistent with all experimental results.

Our observation that the highest systemic increase in SA could be observed in the youngest leaves located directly above the inoculated leaf further supports the hypothesis that SA is translocated in the phloem (Figure 1). When $^{14}CO_2$ is assimilated by single fully expanded leaves of tobacco (Shiroya et al., 1961; Jones et al., 1962) or sugar beet (Joy, 1963), the distribution of radioactivity throughout the plant follows a pattern determined by the vascular connections between leaves. In tobacco, radioactive assimilates preferentially appear in the leaves that are directly above the labeled source leaf (Jones et al., 1962). Moreover, the labeled assimilates transported from the source leaves are found predominantly in the youngest leaves.

The data on SA distribution in tobacco plants following inoculation with TMV can explain some of the discrepancies in the published measurements of SA levels in upper uninoculated leaves. Often, only small SA increases are observed in the leaves of the nodes immediately above the inoculated leaf (Enyedi and Raskin, 1993; Vernooij et al., 1994). These leaves are located approximately opposite to the inoculated leaf and have relatively little direct vascular connection with it. Even a 59% increase in tissue SA can cause a detectable induction of PR proteins in tobacco extracellular fluid (Yalpani et al., 1991). Thus, even small systemic increases in SA, such as those detected by Vernooij et al. (1994), may be sufficient for the induction of PR proteins and induced resistance. Vernooij et al. (1994) have also demonstrated that inoculated transgenic tobacco rootstocks expressing a bacterial salicylate hydroxylase gene, which significantly reduces SA accumulation, nevertheless induces SAR and some SA accumulation in nontransgenic scions. Based on this observation, the authors concluded that SA is not the primary translocated signal responsible for the induction of systemic resistance in tobacco. However, expression of salicylate hydroxylase does not completely block SA accumulation in transgenic tobacco. The transgenic plants exhibit at least a twofold increase in SA levels in inoculated leaves (Gaffney et al., 1993); however, the increases in and around the lesions may be much larger. In fact, Vernooij et al. (1994) observed induction of SA-inducible PR proteins in TMV-inoculated plants expressing salicylate hydroxylase. Therefore, it is possible that enough SA is produced and exported from the HR tissue to account for systemic SA increases.

Another argument against the systemic signaling role of SA comes from the study of cucumber plants inoculated with the bacterial pathogen *Pseudomonas syringae* pv *syringae* (Rasmussen et al., 1991). In these plants, the systemic accumulation of SA was observed even when the inoculated leaf was cut off before SA could be detected in its phloem exudates.

Similar experiments were performed with TMV-inoculated tobacco using direct and highly sensitive analysis of tissue SA corrected for the recovery (Figure 4). The data indicate that detaching the inoculated leaf before it starts to accumulate SA completely blocks systemic SA increases in the upper leaves. Even more importantly, PR-1 gene expression and SAR were not detected in the upper leaf unless lower leaves were allowed to accumulate SA before its detachment from the plant (Figure 4). This observation provides additional evidence in favor of SA as a primary translocated signal. It is possible, however, that the SAR signaling mechanism in tobacco inoculated

with TMV is different from that in cucumber inoculated with *P. s. syringae*.

This paper describes definitive measurements of SA movement in plants undergoing SAR. The results suggest that, at least in the upper leaves of TMV-inoculated Xanthi-nc tobacco, SA is an important translocated signal involved in SAR and systemic induction of PR proteins. Therefore, there is no reason to invoke the existence of another, yet unidentified signal that induces secondary SA accumulation in the pathogen-free leaves. However, caution should be used in extrapolating these results to other plant–pathogen interactions because it is entirely possible that other plants and pathogens may use different mechanisms of long-distance signaling.

METHODS

Plant Material

Tobacco (*Nicotiana tabacum* cv Xanthi-nc) seeds (gift from D.F. Klessig, Rutgers University) were germinated in a synthetic soil medium (Pro-Mix BX; W.R. Grace, Fogelsville, PA) and grown as previously described (Yalpani et al., 1991). Plants were maintained at 24°C under continuous illumination provided by cool-white fluorescent lamps (200 μmol m^{-2} sec^{-1}). For studies on the levels of salicylic acid (SA), greenhouse-grown 6-week-old Xanthi-nc plants were used. Inoculations with the U1 strain of tobacco mosaic virus (TMV) were as described by Enyedi et al. (1992). All experiments were repeated at least once with similar results.

SA Labeling with $^{18}O_2$

$^{18}O_2$ (99 atomic %) was purchased from the Isotech Company (Miamiburg, OH). For labeling with $^{18}O_2$, Xanthi-nc tobacco plants were either inoculated with 5 μg of TMV per leaf or mock inoculated with buffer. Twenty-four hours after inoculation, the TMV-inoculated leaf was enclosed in a transparent gas-tight chamber. The atmosphere within the chamber (total volume 2.5 L) was replaced with a mixture containing 80% N_2, 20% $^{18}O_2$, and 0.1% CO_2. Controlled circulation of 500 mL/min of gases through the leaf chamber was provided by an air supply unit (MF; Analytical Development Co., Hodderson, England). Excess moisture was removed by passing the gases through a Drierite column (W.A. Hammond Drierite Co., Xenia, OH).

Five days after inoculation, SA was extracted from inoculated leaf 3 and upper uninoculated leaf 8. SA was purified using HPLC (see below), derivatized with ethereal diazomethane, and subjected to gas chromatography–mass spectrometry analysis. The number of oxygen atoms incorporated into the SA molecule, as well as their position, was determined from the fragmentation pattern of the methyl salicylate (MeSA) molecular ion derived from the high resolution mass spectrum. The ratio of "heavy" and "light" SA in plant extracts was determined by monitoring the peaks at *m/z* 154 (^{18}O-MeSA) and *m/z* 152 (^{16}O-MeSA).

Throughout the labeling experiment, the concentration of heavy oxygen in the leaf chamber was monitored. During the total period of incubation, the ratio of heavy oxygen in the chamber gradually declined from 100 to 10%. From these data, we estimated that the average molecular fraction of $^{18}O_2$ to $^{16}O_2$ in the leaf chamber was 50% for the experiment in Table 1 and 57% for the experiment in Table 2. We verified that the decrease of $^{18}O_2$ and the increase in $^{16}O_2$ was due to photosynthetic and respiratory activity of the leaf and not to gas leaks in the chamber. Experiments in which the upper uninoculated leaf was exposed to heavy oxygen were performed in exactly the same way, except that the upper leaf was exposed to $^{18}O_2$ 24 hr after the inoculation of the lower leaf. Experiments were terminated 5 days after inoculation.

Transport of ^{14}C-SA and ^{14}C-Benzoic Acid

^{14}C-SA with a specific activity of 56 mCi/mmol and ^{14}C–benzoic acid with a specific activity of 56.5 mCi/mmol were purchased from Du Pont–New England Nuclear (Boston, MA). Leaf 3 of a 6-week-old healthy tobacco plant was infiltrated with 1 mL of a 0.5 mM solution of ^{14}C-SA (56 mCi/mmol) or 1 mL of a 0.5 mM solution of ^{14}C–benzoic acid (56.5 mCi/mmol). Twenty-four hours later, leaf 8 was detached and autoradiographed in a Molecular Dynamics (Sunnyvale, CA) PhosphorImager screen. Autoradiography was performed under normal laboratory conditions. After a 16-hr exposure time, screens were scanned using a Molecular Dynamics PhosphorImager. Data were analyzed with ImageQuant software (Molecular Dynamics).

Extraction and Quantitation of SA

SA was extracted from leaf samples (0.5 g) and quantified by spectrofluorescence using HPLC as previously described (Enyedi et al., 1992). Plant extracts were injected onto a Dynamax 60A 8-μm guard column (4.6 mm × 1.5 cm; Rainin Instrument Co., Emeryville, CA) linked to a Dynamax 60A 8-μm C-18 column (4.6 mm × 25 cm) and maintained at 40°C. The sample was fractionated isocratically with 23% (v/v) methanol in 20 mM sodium acetate buffer, pH 5.0, at a flow rate of 1.5 mL/min. Hydrolysis of leaf extracts with almond β-glucosidase (Sigma) was performed as previously described to extract SA and its glucose conjugate (Enyedi et al., 1992). All data were corrected for SA recovery, which ranged from 46 to 80%.

Gas Chromatography–Mass Spectrometry

The HPLC fraction that co-chromatographed with authentic SA was collected from multiple injections, pooled, and dried under vacuum. The residue was resuspended in ethyl acetate, and insoluble buffer salts were removed by centrifugation at 5000*g* for 15 min. The supernatant was dried under vacuum and dissolved in a small volume of 100% methanol, and an excess of diazomethane in ether was added. After 30 min at room temperature, the sample was dried under N_2. The residue was dissolved in dichloromethane, and the sample was analyzed by gas chromatography–mass spectrometry.

Gas chromatography was performed on a DB-5MS capillary column (30 m × 0.32 mm, 0.25 mm film thickness; J and W Instruments, New Brighton, MN) with a column temperature program (50°C for 3 min, 50 to 300°C at 10°C/min) using Varian 3400 gas chromatography. The end of the gas chromatography capillary column was inserted directly into the ion source of a high-resolution, double-focusing magnetic sector mass spectrometer (MAT 8230; Finnigan Corp., San José, CA) via a heated transfer line maintained at 280°C. The mass spectrum of the fraction coeluting with SA–methyl ester was determined in the electron ionization mode, scanning masses 35 to 350 atomic mass units

at a rate of 1.0 sec per decade. A Finnigan MAT SS 300 data system was used for acquisition and data processing.

Oxygen Measurements

Samples were analyzed using a high-resolution mass spectrometer (ZAB-T; VG Analytical, Manchester, England). The mass spectrometer was operated in the electron ionization mode, scanning masses 1 to 100 atomic mass units at a rate of 1.0 sec per decade. The mass spectrometric data were acquired and processed using a VG Opus data system. Samples were injected into the mass spectrometer via the septum inlet reservoir with a 1-mL gas-tight syringe. From the peak areas, the amounts of oxygen-18 and oxygen-16 were computed and the oxygen-18 abundance (atomic %) calculated as follows:

$$^{18}O \text{ abundance} = \frac{^{18}O}{^{18}O + {}^{16}O} \times 100$$

Extraction and Analysis of Benzoic Acid 2-Hydroxylase Activity

Benzoic acid 2-hydroxylase (BA2H) activity was assayed as previously described (León et al., 1993). Samples of 0.3 g of tobacco leaf tissue were frozen in liquid nitrogen and ground in a chilled mortar, and the resulting fine powder was resuspended in 1 mL of extraction buffer (20 mM Hepes, pH 7.0, containing 12.5 mM 2-mercaptoethanol, 10 mM sorbitol, 1% polyvinylpyrrolidone, and 1 mM phenylmethylsulfonyl fluoride). The suspension was vortexed, sonicated for 2 min, and vortexed again before being centrifuged for 10 min at 10,000*g*. The supernatant was used for enzyme assays. All extraction procedures were performed at 4°C. The reaction mixture contained, in a final volume of 0.5 mL, 10 μmol of Hepes buffer, pH 7.0, 1 μmol of benzoic acid, 1 μmol of reduced β-nicotinamide adenine dinucleotide phosphate and up to 200 μL of enzyme extract. The reaction mixture was incubated for 30 min at 30°C, and thereafter 250 μL of 15% (w/v) trichloroacetic acid was added to stop the reaction. After vortexing and centrifuging for 5 min at 10,000*g*, the supernatant was partitioned twice with 500 μL of ethyl acetate/cyclopentane/isopropanol (100:99:1). The upper organic phase was dried by vacuum centrifugation in a sample concentrator (SVC 200 SpeedVac; Savant Instrument Co., Farmingdale, NY), and the pellet was resuspended in 150 μL of 55% (v/v) methanol. The methanolic solution was filtered through 0.2-μm nylon filters. SA was quantified by HPLC as described previously (Yalpani et al., 1993). BA2H activity was expressed as nanomoles of SA formed per hour per gram fresh weight of tissue.

RNA Isolation and Blot Hybridization Analysis

Total RNA was extracted at various times after TMV inoculation, using the single-step guanidinium method (Chomczynski and Sacchi, 1987). RNA was fractionated (30 μg per lane) on formaldehyde agarose gels (Lehrach et al., 1977). After electrophoresis, RNA was transferred onto a probe membrane (Zeta; Bio-Rad). Blot hybridization and membrane washing were performed as suggested by the manufacturer. Tobacco PR-1 mRNA was detected with a radioactive probe prepared from the PR-1 cDNA (gift of E. Ward, Ciba-Geigy Corp., Research Triangle Park, NC) by random priming.

Measurements of Resistance to TMV

The diameter of necrotic lesions, a measure of resistance (Roberts, 1984), was determined by using a stereo microscope. Results for each treatment are presented as a mean of at least 50 randomly selected lesions from four different plants.

ACKNOWLEDGMENTS

We thank Dr. Ilan Chet, Dr. Peter Day, and Dr. Michael Moynihan for reading the manuscript and for their useful suggestions; Dr. Gerald Berkowitz for use of equipment; and Dr. Thomas Hartman and Dr. Joseph Lech for assistance with mass spectrometry. This research was supported by grants from the U.S. Department of Agriculture (Competitive Research Grants Office) and the U.S. Department of Energy, Division of Energy Biosciences. Additional support was given by the New Jersey Agricultural Experiment Station and the New Jersey Commission for Science and Technology.

Received June 23, 1995; accepted August 21, 1995.

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