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Temperature-Dependent Induction of Salicylic Acid and Its Conjugates during the Resistance Response to Tobacco Mosaic Virus Infection

Jocelyn Malamy, Jacek Hennig, and Daniel F. Klessig[1]

Waksman Institute, Rutgers, The State University of New Jersey, P.O. Box 759, Piscataway, New Jersey 08855

Increases in endogenous salicylic acid (SA) levels and induction of several families of pathogenesis-related genes (PR-1 through PR-5) occur during the resistance response of tobacco to tobacco mosaic virus infection. We found that at temperatures that prevent the induction of PR genes and resistance, the increases in SA levels were eliminated. The addition of exogenous SA to infected plants at these temperatures was sufficient to induce the PR genes but not the hypersensitive response. However, when the resistance response was restored by shifting infected plants to permissive temperatures, SA levels increased dramatically and preceded PR-1 gene expression and necrotic lesion formation associated with resistance. SA was also found in a conjugated form whose levels increased in parallel with the free SA levels. The majority of the conjugates appeared to be SA glucosides. The same glucoside was formed when plants were supplied with exogenous SA. These results provide further evidence that endogenous SA signals the induction of certain defense responses and suggests additional complexity in the modulation of this signal.

INTRODUCTION

Plants respond to pathogens in a variety of ways. Some plants are able to restrict an infecting pathogen to a small region near the site of infection. The tissue collapses in this region, giving rise to a necrotic lesion. Concurrently, pathogen multiplication ceases and pathogen spread is arrested. The localization of a pathogen accompanied by the occurrence of necrotic lesions is referred to as a hypersensitive response (HR), and plants displaying this response are said to be resistant to the restricted pathogen. In contrast, susceptible plants do not exhibit the HR; the pathogen is not localized, but replicates and spreads throughout the plant.

The HR occurs in many plants in response to a variety of different pathogens and is accompanied by multiple molecular and biochemical changes. We are studying the response of tobacco to tobacco mosaic virus (TMV) as a model for plant–pathogen interactions. The tobacco cultivar Xanthi-nc is resistant to TMV and responds hypersensitively to TMV infection. Furthermore, once an infected Xanthi-nc plant has undergone the HR, it is better able to resist a second TMV infection, restricting the virus to a significantly smaller region than during the initial HR. This increased resistance, known as "acquired resistance," is seen in response to challenge infections of both the inoculated leaf and the distal, uninoculated leaves, suggesting that a signal has passed from the initial infection site to the rest of the plant establishing systemic acquired resistance (SAR) (for review, see Matthews, 1981).

Genetic analyses have shown that the presence of the single dominant N locus in Xanthi-nc controls the HR, but neither the function of this locus nor the mechanism of resistance is understood. However, a large number of proteins accumulate following TMV infection of plants carrying the N locus. Two major lines of evidence suggest that these proteins, termed pathogenesis-related (PR) proteins, play a role in the resistance response and in the establishment of SAR. First, they are not produced after infection of a nearly isogenic, susceptible cultivar (Xanthi, genotype nn) (Matthews, 1981). Second, shortly after the appearance of the PR proteins at infection sites, they also begin to accumulate in the uninoculated leaves of plants inoculated with TMV. This systemic occurrence of PR proteins correlates with the systemic nature of acquired resistance. Although no activity has been assigned to the extensively studied PR-1 protein family, several of the other PR protein families have enzymatic activities consistent with roles in defense against bacterial and fungal pathogens (Kauffman et al., 1987; Legrand et al., 1987), which also induce the production of PR proteins. None of the PR proteins has been shown to play a role in defense against viral attack (Cutt et al., 1989; Linthorst et al., 1989). However, because of the strong correlation between PR gene induction and the HR and SAR, we have studied PR genes as markers for induction of defense responses (Ward et al., 1991; for reviews, see Carr and Klessig, 1989; Cutt and Klessig, 1992).

Recently, we have shown that the endogenous level of salicylic acid (SA), a potent chemical inducer of the PR proteins,

[1] To whom correspondence should be addressed.

dramatically increases following TMV infection (Malamy et al., 1990). The observation that this increase is seen only in resistant cultivars, precedes PR-1 gene induction, and occurs systemically led us to propose that SA is a natural signal in the induction of the PR genes and other defense responses. A complementary study by Métraux et al. (1990) in cucumber showed that increases in SA levels also correlated with the induction of SAR.

To further analyze the relationships among SA, PR gene induction, and resistance, we took advantage of the temperature sensitivity of the resistance response of tobacco to TMV. When Xanthi-nc (genotype NN) plants are inoculated with TMV and incubated at temperatures of >28°C, the replication and spread of the TMV are not restricted, necrotic lesions are not formed, and the PR genes are not induced. However, when the infected plants are moved to lower temperatures (22°C), PR proteins accumulate and the HR is rapidly activated (Kassanis, 1952; Gianinazzi, 1970). Here we report that increases in SA levels were blocked at elevated temperatures, but rose rapidly and dramatically after we shifted plants to normal temperatures. This rise preceded the appearance of necrotic lesions and PR gene induction. Furthermore, we found that SA was produced de novo in both a free form and as an SA glucoside following infection.

RESULTS

Increases in SA Levels Correlate with HR and PR-1 Gene Induction in Plants Subjected to Temperature Shifts

If SA is in the pathway leading to the induction of defense responses, temperatures that prevent the resistance response might also block increases in endogenous SA levels. This result would indicate that the temperature-sensitive step precedes the induction of SA. Indeed, Figure 1A shows that when Xanthi-nc plants were inoculated at elevated temperatures (32°C), SA levels did not increase, whereas SA levels in control plants inoculated and maintained at 22°C rose 10- to 20-fold. In addition, there was no PR-1 gene induction or necrotic lesion formation at the high temperatures (data not shown). The absence of SA increases at 32°C is particularly compelling in light of the demonstration by Van Loon (1983) and our own observation (data not shown) that plants express PR proteins in response to exogenous SA at this temperature. This is consistent with the model that the lack of induction of the PR-1 gene is due to the absence of a rise in SA levels.

When plants inoculated with TMV are shifted from 32 to 22°C, resistance is established, necrotic lesions form (Weststeijn, 1981, 1984), and PR-1 genes are induced, as shown in Figure 1B. Therefore, the block to the HR and PR-1 gene induction imposed at elevated temperatures is reversible. If SA functions as an essential signal in the induction of resistance and PR-1 genes, endogenous SA levels should rapidly increase when

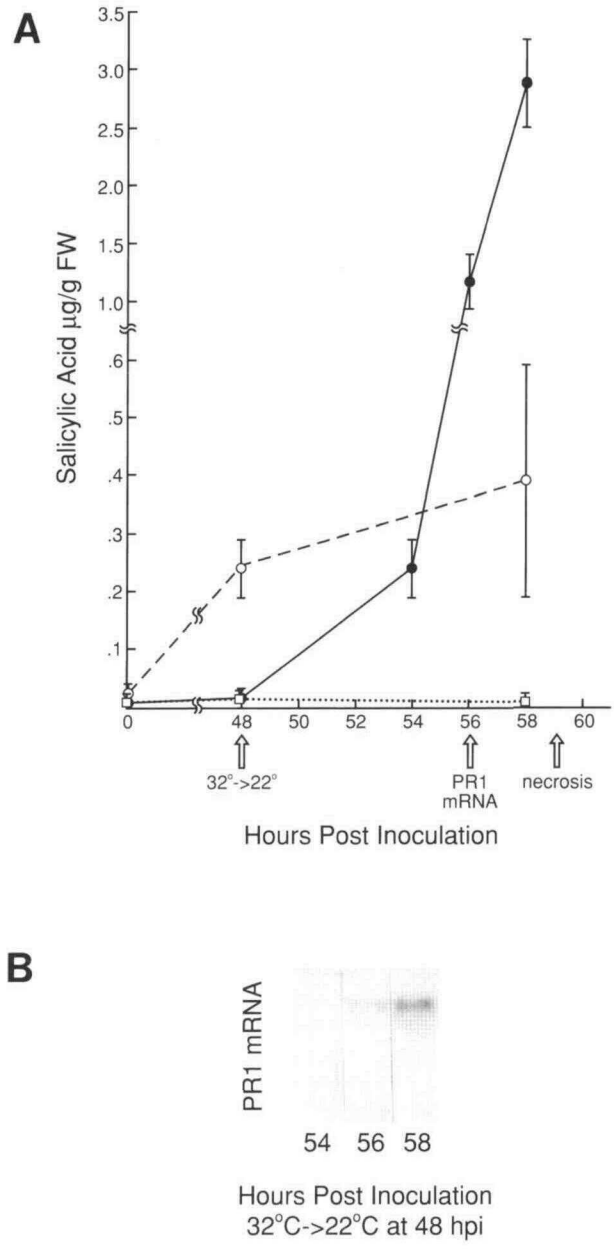

Figure 1. SA and PR-1 mRNA Levels after Inoculation with TMV at Elevated Temperatures.

(A) SA levels determined at various times after TMV inoculation. Plants were infected and maintained at 22°C (– – –), 32°C (····), or transferred from 32 to 22°C 48 hpi (——). Graph points represent an average of SA values determined for three plants. Error bars reflect standard deviations. Necrotic lesions, indicative of the HR, appeared at 59 hpi or 11 hr after the shift from 32 to 22°C. In plants infected and maintained at 22°C, lesions appeared at 42 to 43 hpi under our conditions. The experiment was repeated three times. FW, fresh weight.

(B) RNA gel blot analysis. PR-1 mRNA levels were determined 6, 8, and 10 hr after plants were shifted from 32 to 22°C (at 48 hpi). mRNA was extracted from opposite halves of leaves analyzed for SA content in **(A)**. Only one of three similar samples is shown for each time point.

plants inoculated with TMV are shifted to permissive temperatures. Indeed, when plants inoculated with TMV were shifted from 32 to 22°C 48 hr postinfection (hpi), SA levels rose 10-fold over basal levels by 6 hr postshift (hps) (Figure 1A). By 10 hps, SA levels increased 100- to 200-fold above basal levels, 10 times higher than the increase found in plants grown and infected at 22°C (Figure 1A). PR-1 mRNA was first detectable 8 hps (Figure 1B), and tissue collapse associated with HR was evident at 9 to 11 hps. Thus, the initial rise in SA levels preceded the induction of PR-1 genes by at least 2 hr and appearance of necrotic lesions by 3 to 5 hr. These findings provide a further correlation between increases in SA levels and induction of the HR and PR-1 genes. In addition, they demonstrate that the temperature-sensitive step in the resistance response precedes the production of endogenous SA.

Addition of SA Is Not Sufficient for Restoration of the HR at High Temperature or after Temperature Shift

If induction of the HR were solely dependent on the increases in SA levels that follow TMV infection, application of exogenous SA to plants inoculated and maintained at 32°C should result in formation of lesions. This would be consistent with the demonstration that treatment of uninfected plants with exogenous SA induced PR protein accumulation at 32°C as well as 22°C, as discussed above. However, no lesions appeared on TMV-inoculated leaves of plants maintained at 32°C that were injected with 1 mM SA 48 hpi (data not shown). In similar experiments, infected plants maintained at 32°C for 48 hpi were injected with 1 mM SA and then immediately shifted to 22°C. In this case, although SA was supplied 6 hr before endogenous levels were expected to increase, lesion formation was not accelerated. In fact, when SA was injected into one half-leaf of a plant that had been inoculated and maintained at 32°C and water injected into the other half-leaf, the SA-injected half-leaf developed symptoms ~2 hr later than the water-injected half-leaf after shifting to 22°C. One such leaf, at 11 hr after the temperature shift, is pictured in Figure 2. Thus, the addition of SA is not sufficient for induction of the HR in plants infected with TMV at either 32 or 22°C.

Identification of Conjugated Forms of SA in TMV-Inoculated Leaves of Tobacco

In previous studies, we analyzed the level of SA in leaves of plants inoculated with TMV by extracting SA from an aqueous extraction buffer into an organic solvent and analyzing the extracted material by HPLC. Back extraction of the aqueous phase confirmed that SA was quantitatively removed by the organic extraction. However, when the aqueous phase after organic extraction was subjected to acid hydrolysis (100°C, pH 1.0 to 1.5, 30 min) and then extracted with the same organic

Figure 2. Effect of SA Treatment on Necrotic Lesion Development following Temperature Shift.

Plants were inoculated and maintained at 32°C until 48 hpi. Then, one-half of each inoculated leaf was injected with 1 mM SA (pH 6.5) until the entire half-leaf appeared soaked, and the other half was injected with water. Plants were then shifted to 22°C and photographed 11 hps. Pictured is one of the 12 leaves treated, all of which showed the identical delay in lesion development in the SA-treated half.

solvent, additional SA could be extracted into the organic phase (see Methods). This indicates that in TMV-infected tissue, SA can be found both as a free acid and conjugated to another compound(s) through an acid-hydrolyzable linkage. Analysis of SA released by acid hydrolysis thus allows indirect determination of the levels of SA conjugates.

Using the sequential extraction procedure described above, the levels of conjugated SA were determined at various times following TMV infection, as represented in Figure 3. SA conjugates were present at only very low levels (0.02 to 0.05 μg/g fresh weight of tissue) in uninfected or mock-infected plants. The levels of SA conjugates following TMV infection increased dramatically between 24 and 48 hpi, paralleling the initial rise in free SA. By 48 hpi, levels of SA conjugates rose 15- to 20-fold. The amount of conjugated SA at 48 hpi was approximately two times greater than the amount of SA existing as a free acid (Figure 3).

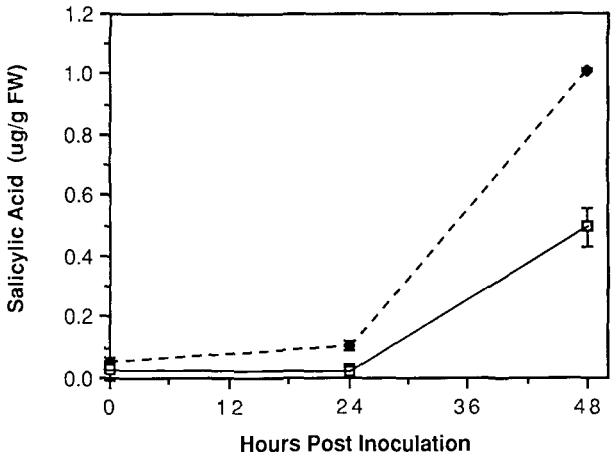

Figure 3. Free SA and Acid-Hydrolyzable SA Conjugates following TMV Inoculation.

Graph points are averages of SA values from two plants. At each time point, free (——) and conjugated (— — —) SA levels were determined from the same leaf material. Error bars reflect standard deviations. The experiment was repeated three times. FW, fresh weight.

SA conjugates were present at very low levels (<0.01 μg/g fresh weight of tissue) in plants maintained at 32°C, and the levels did not increase following TMV inoculation at 32°C, as shown in Figure 4. When plants infected with TMV were shifted from 32 to 22°C 48 hpi, levels of SA conjugates rose 100-fold over basal levels by 10 hps, similar to the increase observed for free SA (Figure 4). These experiments demonstrated that SA conjugates, like free SA, are produced de novo after TMV infection and their induction is reversibly blocked by elevated temperatures.

Characterization of SA Conjugates

Many phenolic acids in plants exist as sugar conjugates. Glucosides are particularly common (Harborne, 1964, 1980). In addition, the alcohol of SA, o-hydroxybenzyl alcohol, has been identified in glucosylated forms in several plant systems (Harborne, 1964, 1980). Therefore, it seemed likely that the SA conjugates detected after TMV infection might be SA–glucose conjugates. To test this, the conjugates were subjected to β-glucosidase digestion. β-Glucosidase specifically releases β-linked terminal D-glucose residues. Hence, release of free SA from conjugates treated with β-glucosidase would strongly imply that the conjugates were SA glucosides. SA–glucose esters should not be digested by this enzyme.

Extracts of infected tissues at various times after infection were divided in half; β-glucosidase was added to one-half, whereas the other half served as an undigested control (see Methods). Free SA was then extracted from each half. Less than 0.1 μg/g fresh weight SA was released by β-glucosidase in samples from tissue harvested immediately after inoculation. At all subsequent times, twofold to fourfold more free SA was present in sample halves incubated with β-glucosidase than in undigested sample halves, as shown in Figure 5A. SA glucoside levels, calculated by subtracting SA levels in control samples from SA levels in digested samples, rose to ~7.0 μg/g fresh weight of tissue by 8 days postinfection. Further addition of enzyme did not release additional SA (data not shown), indicating that the β-glucosidase digestion had gone to completion. Thus, SA was produced as a glucoside after TMV infection at levels onefold to fourfold the levels of free SA (Figure 5A).

To determine whether there are additional forms of conjugates, samples were digested with β-glucosidase, all free and released SA was removed, and any remaining conjugates were then acid hydrolyzed. Little if any additional SA was released by acid hydrolysis of enzyme-treated samples obtained from plants 2 and 4 days after inoculation, as shown in Figure 5B. Additional SA was released from enzyme-digested samples obtained from plants 8 days after inoculation upon acid hydrolysis (Figure 5B). The nature of this minor fraction of enzyme-resistant, acid-labile conjugates that were observed at late time points is unknown. However, the vast majority of SA conjugates appeared to be SA glucosides. This is further supported by the finding that the ratio of free SA to SA glucosides (Figure 5A) was similar to ratios for free SA versus acid hydrolyzable conjugates (Figure 3).

Analysis of the Fate of Exogenously Supplied SA

Because exogenously supplied SA has been shown to induce PR genes and resistance in tobacco (White, 1979), the fate

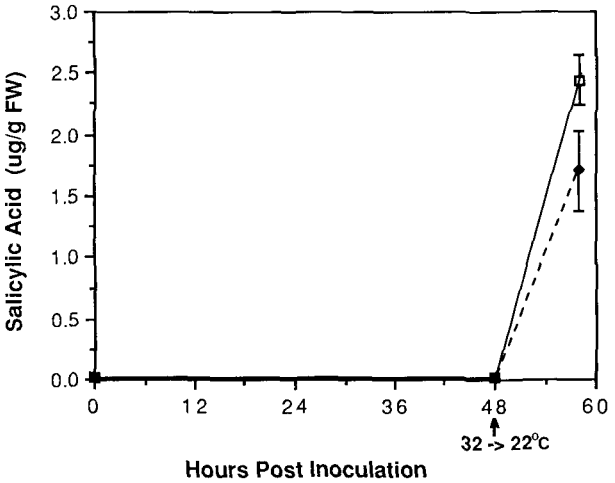

Figure 4. Free SA and Acid-Hydrolyzable SA Conjugates after Temperature Shift.

Plants were inoculated and maintained at 32°C for 48 hpi and then shifted to 22°C. Free (——) and conjugated (— — —) SA levels were determined at various times after the temperature shift. Graph points are average values from three plants. Error bars reflect standard deviations. The experiment was repeated three times. FW, fresh weight.

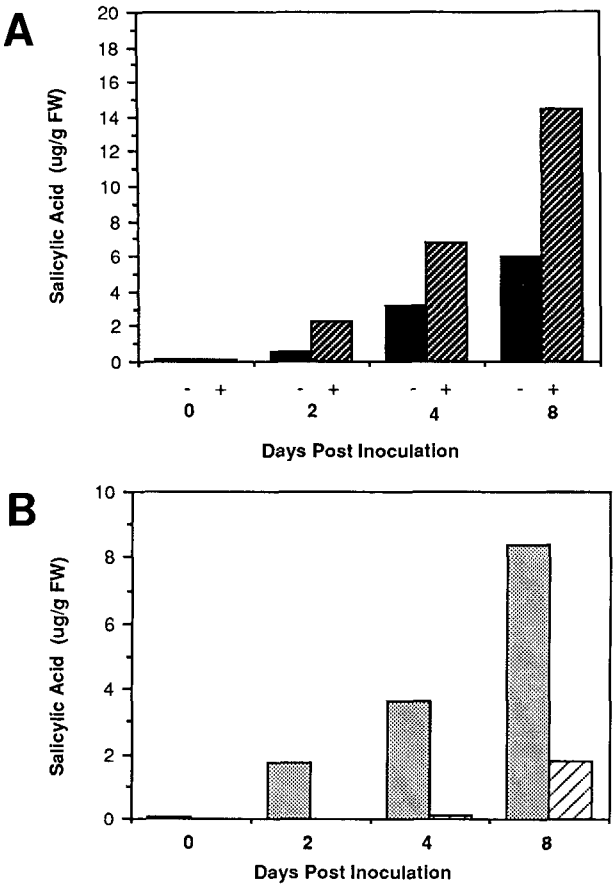

Figure 5. Multiple Forms of SA Produced after TMV Inoculation.

(A) SA released by β-glucosidase treatment. Leaf samples from various times after TMV inoculation were incubated in the presence (+) or absence (−) of β-glucosidase. Solid black bars represent averages of values of free SA in the absence of enzyme. Hatched bars represent averages of combined values of free SA and SA released by β-glucosidase. Two plants were analyzed at each time point.

(B) Enzyme-resistant SA conjugates. Following β-glucosidase digestion and extraction of free SA, samples were subjected to acid hydrolysis to release any remaining conjugates. Bars for SA glucosides (dots) represent averages of values obtained by subtracting the amount of free SA present in control samples from the amount present after enzyme digestion. Bars for nonglucosidic conjugates (diagonals) represent averages of values of SA released by acid hydrolysis after enzyme digestion of each sample. Two plants were analyzed at each time point. The experiment was repeated twice.

of this SA, specifically whether or not it is converted to glucosides, becomes biologically relevant. To address this question, ^{14}C-labeled SA was introduced in uninfected excised tobacco leaves (see Methods). After 2 days, labeled tissue was analyzed for free SA and SA glucoside content. Results from one representative experiment are presented in Table 1. Less than 2% of the labeled material recovered by methanol extraction could be partitioned into organic solvent, indicating that the majority of the label was no longer in the form of free SA. However, after β-glucosidase digestion, ~80% of the label could be extracted into the organic solvent. This is in agreement with data from other systems (Towers, 1964; Ben-Tal and Cleland, 1982) in which nearly all exogenously supplied SA was shown to be converted to a conjugated form. The release of the label by β-glucosidase strongly suggests that the majority of exogenously supplied SA is conjugated to glucose to form the same conjugate observed in infected tissue.

DISCUSSION

The experiments described above provide further evidence that SA plays a role in defense responses, including the induction of the PR-1 genes. The increases in SA levels characteristic of the resistance response to TMV infection were completely abolished when plants were infected and maintained at 32°C. At this temperature, the HR and the induction of the PR-1 genes were also blocked. While these studies were in progress, Yalpani et al. (1991) independently showed that the SA rise was inhibited at high temperatures. In addition, we found that when plants were shifted from 32 to 22°C, SA levels remained at basal levels for the first 4 hr (data not shown), but by 6 hps had risen 10- to 20-fold. Necrotic lesions appeared 9 to 11 hr after the temperature shift. Therefore, the rapid induction of SA is consistent with the observation that the entire process of localizing virus is completed by 12 hps, in contrast to the 36- to 48-hr period required when a plant is infected at 22°C. The rapid and precisely timed development of PR-1 gene induction and necrosis after the temperature shift allowed us to demonstrate that SA increases preceded the induction of the PR-1 genes by at least 2 hr and the appearance of necrotic lesions by 3 to 5 hr.

Interestingly, Weststeijn (1981) showed that when tobacco plants infected at 32°C were transferred to 22°C for short periods of time and then returned to the elevated temperature,

Table 1. Release of Exogenously Supplied ^{14}C-labeled SA by β-Glucosidase

Sample No.[a]	Total[b] (%)	Free SA (%)	β-Glucosidase-Labile Conjugates (%)	Remainder[c] (%)
1	100	1.40	80.0[d]	8.6
2	100	1.38	74.6[e]	10.5

[a] Leaves from two plants were independently labeled.
[b] Label recovered by methanol extraction.
[c] Label remaining in aqueous phase after extraction of free and β-glucosidase-released SA.
[d] Incubation of tissue from sample 1 in the absence of β-glucosidase resulted in release of 6.5% of total label.
[e] Incubation of tissue from sample 2 in the absence of β-glucosidase resulted in release of 1.4% of total label.

necrosis ensued only when the 22°C incubation periods exceeded 6 hr. Increases in SA levels were first observed at this time. This suggests that the increase in SA levels may be the signal that commits the infected leaf to lesion formation. However, our observation that the addition of exogenous SA to infected plants at 32°C did not result in lesion formation suggests that SA, although perhaps essential, is not sufficient. Multiple pathways may be inhibited at the high temperature, and many factors may be involved in establishment of HR after temperature shift. For example, the burst of ethylene synthesis associated with TMV infection is also blocked at 32°C and restored at 22°C (DeLaat and Von Loon, 1983). Alternatively, SA may be the signal responsible for induction of the PR genes and/or SAR, but may not be involved in the development of necrosis and the HR. In this model, the high-temperature block may interfere with the ability of the infected leaf to initiate multiple pathways involved in defense responses. The absence of increases in SA levels would be one consequence of this block, and the lack of viral localization and necrosis an unrelated consequence.

Unexpectedly, SA levels increased 100- to 200-fold when plants were infected at 32°C and transferred to 22°C 48 hpi. This increase is 10-fold higher than the increase in SA levels after a 22°C infection of the same duration. Because SA production is blocked at elevated temperatures, this "hyperinduction" may be due to the accumulation of some precursor or signal essential for endogenous SA production. Release of the block could then result in a burst of SA. Alternatively, if infection of a cell triggers the production of SA, the hyperinduction of SA could be explained by the relatively large number of infected cells in the temperature-shifted plant. TMV replicates and spreads at 32°C, and therefore many more cells are infected during the 48-hr incubation period at 32°C than in the plant maintained at 22°C, where the spread of virus is rapidly restricted to a small zone around the infection site.

We have demonstrated that endogenously produced SA exists in a conjugated form in addition to the free form reported previously (Malamy et al., 1990). This finding suggested the possibility that the increase in free SA after infection might be due to the release of SA from preexisting conjugated forms. However, only very low levels of SA conjugates were detected in uninfected or mock-infected leaves, indicating that both free SA and SA conjugates are produced de novo following infection. The ability of β-glucosidase to hydrolyze the vast majority of SA conjugates in TMV-infected leaves strongly suggests that SA glucosides are the major form of SA conjugates in this system. It is likely that the appearance of SA conjugates reflects a normal metabolic process for handling the large quantities of free phenolic acids produced after TMV infection. In fact, free phenolic acids are rare in plants, perhaps due to their toxicity. Most exist as sugar conjugates (Harborne, 1980). Several naturally occurring conjugates of SA and its derivatives, including SA glucosides, have been identified in various plant systems (Harborne, 1964). However, if only free SA or only certain conjugates are biologically active, the partitioning of free SA among these forms may significantly affect the signaling of defense responses.

Labeling studies have shown that when plants are supplied with ^{14}C-labeled SA, only 1 to 2% of the label can be recovered in the form of free SA 2 days after application. It has been demonstrated that when the related compounds benzoic acid and *o*-hydroxybenzyl alcohol are exogenously supplied to plants in the presence of UDP-glucose, they will be rapidly converted to their glucosides and glucose esters (Towers, 1964). In addition, Yalpani et al. (1990) reported the existence of an SA–UDP–glucosyl transferase activity in oat roots that apparently is responsible for detoxifying high levels of exogenously supplied SA in that system. However, after TMV infection, 20 to 40% of the total endogenous SA exists in the free form (Figure 3). Therefore, it is possible that a specific mechanism exists to prevent conjugation of the entire endogenous free SA pool after TMV infection, resulting in the high free SA levels observed. Alternatively, these high levels of free SA may reflect the continuous biosynthesis of SA. In either case, the rapid and efficient conjugation of exogenously supplied SA may have important consequences for the interpretation of any experiments involving SA uptake, especially if SA conjugates are biologically inactive. For example, the concentration of SA that must be added exogenously to leaves to induce the PR-1 genes is 10 to 100 times greater than the approximated concentration of endogenous SA following TMV infection (White et al., 1986; Malamy et al., 1990). This discrepancy may be due, in part, to the efficient conversion of the added SA to inactive conjugates. Further studies are clearly required to evaluate the role of SA glucosides.

The temperature shift data presented here provide additional evidence for the involvement of endogenous SA in the signaling of defense responses. The recent identification and partial characterization of a soluble SA binding protein in tobacco leaves by Chen and Klessig (1991) are also consistent with the role of SA as a signaling molecule. The demonstration that SA glucosides are induced in addition to free SA after TMV infection suggests that there may be greater complexity in the regulation of SA signaling than previously suspected.

METHODS

Plant Material and Growth Conditions

Tobacco plants (*Nicotiana tabacum* cv Xanthi-nc) were grown at 22°C in growth chambers programmed for a 14-hr light and 10-hr dark cycle. For high-temperature experiments, plants were transferred to 32°C Conviron chambers 2 to 3 days before inoculation. Tobacco mosaic virus (TMV) strain U1 was used at a concentration of 1 μg/mL in 50 mM phosphate buffer at pH 7.5 in all experiments. Two to three leaves were inoculated on each plant and harvested together.

RNA Preparation and RNA Gel Blots

Total RNA was extracted, and 10 to 20 μg of total RNA was run per lane on formaldehyde gels and transferred to nitrocellulose membranes as described by Berry et al. (1985). RNA gel blots were probed with a pathogenesis-related 1b (PR-1b) cDNA clone (Cutt et al., 1988).

Quantitation of Free SA

Free salicylic acid (SA) was extracted and quantitated essentially as described by Raskin et al. (1989). One gram of frozen tissue was ground in 3 mL of 90% methanol and centrifuged at ~6000*g* for 15 min. The pellet was back extracted with 3 mL of 100% methanol and centrifuged. Methanol extracts were combined, centrifuged at 1500 to 2000*g* for 10 min, and dried at 40°C under vacuum. Extracts were then resuspended in either 2.5 mL of 5% trichloroacetic acid (temperature shift experiments) or in 2.5 to 5 mL of water at 80°C (SA conjugate experiments). When water was used as the solvent, an equal volume of 0.2 M sodium acetate buffer (pH 4.5) was added, and the pH was adjusted to 1 to 1.5 with HCl before extraction. Free SA was extracted into 2 volumes of cyclopentane/ethyl acetate/isopropanol 50:50:1. Back extractions, when performed, used 2 volumes of the same solvent. The organic extract was dried under nitrogen and analyzed by HPLC as described by Raskin et al. (1989).

Quantitation and Characterization of SA Conjugates

SA conjugates were indirectly quantitated by acid hydrolyzing the compounds that remained in sodium acetate buffer after organic extraction and analyzing the released SA by HPLC. Acid hydrolysis was performed by incubating the samples in a boiling water bath at pH 1 to 1.5 for 30 min and then extracting free SA as above. β-Glucosidase digestion was performed as described by Southerton and Deverall (1990). For each sample, the dried methanol extract was resuspended in 5 mL of water at 80°C, and the solution was divided into two equal portions. To one portion, an equal volume of 0.2 M acetate buffer (pH 4.5) containing 0.1 mg/mL β-glucosidase (22 units/mg; Sigma) was added, while buffer alone was added to the other portion. Both portions were incubated at 37°C overnight. After digestion, samples were acidified to pH 1 to 1.5 with HCl. SA was extracted and back extracted from each half for quantitation by HPLC. Samples were then adjusted to pH 4.5 with NaOH for additional enzyme digestion or acid hydrolyzed at 100°C for 30 min, as described above, before extraction of additional released SA.

Labeling Experiments

Excised leaves were labeled with 7-^{14}C-SA (specific activity 56 mCi/mmol) by applying a mixture of 25 μL of 7-^{14}C-SA and 25 μL of SA (1.1 mM; Sigma) to the petioles of excised leaves in 10-μL aliquots. Leaves were allowed to imbibe drops for ~10 min before subsequent drops were applied. Labeled leaves were placed in covered beakers with water for 2 days in a growth chamber under normal growth conditions.

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