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Cyanide Restores *N* Gene–Mediated Resistance to Tobacco Mosaic Virus in Transgenic Tobacco Expressing Salicylic Acid Hydroxylase

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Salicylhydroxamic acid (SHAM), an inhibitor of alternative oxidase (AOX), blocks salicylic acid–induced resistance to tobacco mosaic virus (TMV) but does not inhibit pathogenesis-related PR-1 protein synthesis or resistance to fungal and bacterial pathogens. We found that the synthetic resistance-inducing chemical 2,6-dichloroisonicotinic acid also induced *Aox* transcript accumulation and SHAM-sensitive resistance to TMV. The respiratory inhibitors antimycin A and KCN also induced *Aox* transcript accumulation and resistance to TMV but did not induce PR-1 accumulation. Tobacco plants of the TMV-resistant cultivar Samsun NN transformed with the salicylic acid hydroxylase (*nahG*) gene could no longer restrict virus to the inoculation site, resulting in spreading necrosis instead of discrete necrotic lesions. Treatment with KCN restored TMV localization and normal lesion morphology. SHAM antagonized this effect, allowing virus escape and spreading necrosis to resume. The results demonstrate the importance of the SHAM-sensitive (potentially AOX-dependent) signal transduction pathway in mediating virus localization early in the hypersensitive response.

INTRODUCTION

Salicylic acid (SA) plays a pivotal role in plant disease resistance (reviewed in Raskin, 1992; Ryals et al., 1996; Durner et al., 1997). When plants are challenged with an avirulent pathogen, the interaction of the products of the pathogen's avirulence and the host's resistance genes triggers resistance via a signal transduction system that includes SA as a key component (Delaney et al., 1994; Staskawicz et al., 1995; Baker et al., 1997). In the case of tobacco cultivars possessing the *N* resistance gene, inoculation with tobacco mosaic virus (TMV) induces the hypersensitive response (HR)—rapid programmed host cell death at the infection site. The spread of TMV is restricted to a small zone adjacent to the necrotic lesions, and the triggering of the HR is accompanied by an increase in SA levels (Malamy et al., 1990). In addition, parts of the plant distal to the primary infection site nonetheless acquire an enhanced degree of resistance to secondary infection, and this systemic acquired resistance (SAR) is preceded by a rise in the concentration of SA (Malamy et al., 1990).

SAR is effective against TMV and a broad spectrum of other pathogens (Delaney et al., 1994). In tobacco plants containing the *N* gene, SAR against TMV is characterized by the production of fewer and smaller necrotic lesions in response to secondary infection with the virus (Ross, 1961a, 1961b). The rise in SA levels and the induction of SAR are accompanied by the activation of a range of genes, including those that encode pathogenesis-related (PR) proteins (Bowles, 1990). The occurrence of PR proteins has been used in the past as a molecular marker for SAR induction (Ward et al., 1991; Kessmann et al., 1994). However, although many PR proteins are antibacterial or antifungal, none has yet been shown to act against viruses. Furthermore, the induction of PR protein accumulation and the induction of resistance to TMV appear to be activated by independent branches of the defensive signal transduction pathway (Chivasa et al., 1997).

Several observations indicate that the accumulation of SA, and not host cell death, is largely responsible for inducing TMV localization. For example, inhibition of cell death by depletion of oxygen does not interfere with virus localization (Mittler et al., 1996). Furthermore, *N* gene–containing transgenic plants expressing the bacterial *nahG* gene, encoding the SA-degrading enzyme SA hydroxylase, cannot restrict the spread of virus or of virus-induced necrosis (Bi et al., 1995; Ryals et al., 1995; Mur et al., 1997). Finally, exogenous SA confers resistance to TMV on susceptible tobacco cultivars that do not respond hypersensitively to TMV (White et al., 1983; Chivasa et al., 1997). Thus, accumulation of SA is required for the establishment of both local and systemic acquired resistance to TMV.

Other compounds, for example, acetylsalicylic acid (aspirin; White, 1979), 2,6-dihydroxybenzoic acid (Van Loon, 1983), 2,6-dichloroisonicotinic acid (INA; Ward et al., 1991),

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and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Friedrich et al., 1996), also induce PR protein accumulation and resistance to TMV. BTH and INA can induce SAR in transgenic tobacco expressing SA hydroxylase and can induce SAR in nontransgenic tobacco without causing SA accumulation. This indicates that both compounds induce SAR independently of SA accumulation, leading to the conclusion that they both act either at the same position as SA or at positions downstream of SA in the defensive signal transduction pathway (Vernooij et al., 1995; Friedrich et al., 1996).

Recently, we showed that in tobacco, the signal transduction pathway culminating in SAR branches downstream of SA. One branch is insensitive to salicylhydroxamic acid (SHAM), leads to PR protein induction, and confers resistance to fungal and bacterial pathogens (Chivasa et al., 1997). The other branch is sensitive to inhibition by SHAM and is required for the induction of SAR to TMV but not for PR protein gene induction (Chivasa et al., 1997). SHAM is an inhibitor of alternative oxidase (AOX), the terminal oxidase of the alternative respiratory pathway (Laties, 1982; Raskin, 1992; McIntosh, 1994; Day et al., 1995). Thus, our results suggest that the AOX may play a role in the signal transduction pathway leading to SAR against viruses (Chivasa et al., 1997). Independently, work by another group has also implicated AOX in the induction of resistance (Lennon et al., 1997). Here, we report further studies on the SHAM-sensitive signal transduction pathway that leads to resistance to viruses. In addition to providing further evidence that AOX is a component of this pathway, we have also demonstrated that the SHAM-sensitive signaling pathway is essential for *N* gene–mediated virus localization early in the HR to TMV.

RESULTS

Antimycin A and KCN Induce Resistance to TMV but Do Not Induce PR-1 Accumulation

In earlier work, it was shown that an inhibitor of AOX enzyme activity, SHAM, blocked SA-induced resistance to TMV but did not block induction of synthesis of the PR-1 protein. This seemed to suggest that AOX participates in the induction of SAR against TMV but that it does not participate in the induction of PR protein gene expression (Chivasa et al., 1997). If this is true, it would be expected that induction of *Aox* gene expression should enhance resistance to TMV without the induction of PR-1. The metabolic inhibitors antimycin A (AA) and KCN inhibit electron transfer in the cytochrome pathway at complexes III and IV, respectively (Stryer, 1981). This inhibition of normal cytochrome pathway activity triggers *Aox* gene induction (Vanlerberghe and McIntosh, 1992). Therefore, we were curious to determine whether AA and KCN stimulated resistance to TMV without inducing PR-1 protein gene expression.

We found that AA, SA, and INA induced at least a twofold increase in accumulation of the *Aox* gene transcript in Xanthi nn (TMV-susceptible genotype) tobacco leaf discs treated with solutions of these compounds for 3 days (Figure 1). Furthermore, AA-treated leaf discs displayed levels of resistance to TMV that were comparable with the resistance induced by SA (Figure 2A). We reported previously that SA inhibits TMV replication overall and disproportionately inhibits the accumulation of TMV coat protein (CP) mRNA more than the accumulation of full-length TMV RNA (Chivasa et al., 1997). Interestingly, AA also repressed TMV CP mRNA more than it did full-length RNA (Figure 2A).

We also probed protein gel blots with a polyclonal antibody raised against PR-1 to determine whether AA induces PR-1 protein gene expression. AA failed to induce PR-1 accumulation (Figure 2B), despite inducing resistance to TMV. Likewise, KCN inhibited TMV RNA accumulation in a fashion similar to AA and induced *Aox* gene transcription but failed to induce PR-1 accumulation (data not shown). These results indicate that AA and KCN can activate the defensive signal transduction pathway downstream of the point at which the virus-specific (SHAM-sensitive) and PR protein–specific (SHAM-insensitive) signaling pathways diverge.

We wanted to make sure that the decrease in TMV RNA and protein induced by AA and KCN was due not merely to poisoning of the plant tissue. Therefore, buffer- and AA- or KCN-treated Xanthi nn and Xanthi NN leaf discs were fed with a mixture of ^{35}S-labeled cysteine and methionine. It was found that neither of these metabolic inhibitors caused a decrease in the incorporation of radiolabel into trichloroacetic acid–insoluble material. Similarly, analysis of the radiolabeled protein by SDS-PAGE and autoradiography showed that neither AA nor KCN (both at 50 μM) caused a major overall decrease in protein synthesis, although AA did appear to cause a slight increase in the relative amounts of a

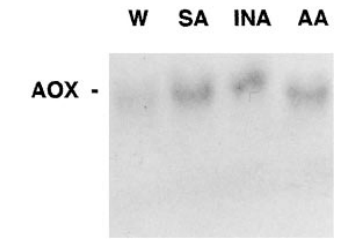

Figure 1. Induction of *Aox* Transcript Accumulation.

For RNA gel blot analysis, equal amounts of RNA extracted from Xanthi nn leaf discs were treated with water (W), 1 mM SA, 1 mM INA, and 50 μM AA for 3 days. The blot was hybridized with a ^{32}P-labeled *Aox*-specific DNA probe. Densitometric measurements revealed a minimum of twofold increase in accumulation of the transcript in chemically treated tissue above the water control (data not shown). The position of the *Aox* gene transcript (AOX) is indicated.

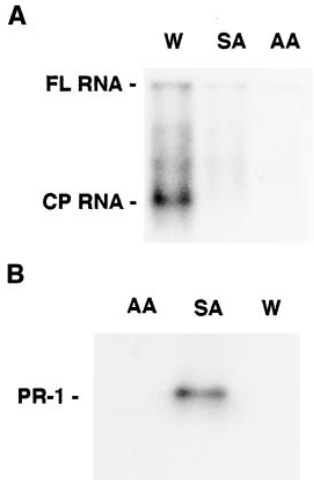

Figure 2. Effect of AA on the Accumulation of TMV-Specific RNAs and PR-1 Protein in Xanthi nn Tobacco.

(A) RNA gel blot analysis of plus-sense single-stranded TMV RNAs in TMV-inoculated leaf discs. Xanthi nn tobacco leaf discs were pretreated with water (W; negative control), 1 mM SA (positive control), and 50 μM AA for 3 days before inoculation with TMV (10 μg/mL). RNA was extracted 2 days after inoculation. Equal amounts of total RNA were probed with a ^{32}P-labeled riboprobe specific for plus-sense TMV RNA. The positions of the full-length (FL) genomic TMV RNA and the subgenomic CP mRNA (CP RNA) are indicated.
(B) Protein gel blot analysis of PR-1 in Xanthi nn tobacco leaf discs. Total soluble protein from leaf discs treated as given in **(A)** for 5 days was fractionated using SDS-PAGE. Proteins were electroblotted to a nitrocellulose filter and probed with a polyclonal rabbit anti–PR-1 serum. The position of the PR-1 protein is indicated.

few polypeptide bands (Figure 3). Therefore, at least at these concentrations, AA and KCN were inducing a specific resistance mechanism, not an incidental shutdown of virus replication or gene expression caused by a generalized inhibition of host metabolism.

KCN Restores *N* Gene–Mediated Resistance to TMV in Transgenic Plants Expressing SA Hydroxylase

We found that resistance to TMV could also be induced by KCN in transgenic tobacco plants that are not able to accumulate SA. SH-L transgenic tobacco plants express the bacterial SA hydroxylase (*nahG)* gene, and as a result, exogenous SA fails to induce SAR and PR protein accumulation (Bi et al., 1995). However, INA induces both SAR and PR proteins in SH-L plants (Bi et al., 1995). Despite possessing the *N* gene for TMV localization, TMV-inoculated SH-L tobacco plants fail to localize virus around the necrotic lesion. This results in virus escape by cell-to-cell movement into surrounding tissues, giving rise to spreading necrosis manifested as “spidery” (irregularly shaped) lesions (Figure 3E; see Mur et al., 1997). SH-L plants treated with KCN before TMV inoculation developed fewer and smaller visible necrotic lesions relative to buffer-treated controls (Table 1 and Figures 4A to 4C). Whereas there was a mixture of circular and spidery lesions on buffer-treated controls, KCN treatment prevented the development of spidery lesions, indicating successful containment of virus around lesions (Figures 4D and 4E). SHAM antagonized the effect of KCN, restoring the spidery lesion morphology and increasing the size and number of lesions (Table 1 and Figure 4A).

It has been observed that under certain conditions, such as anoxia, hypersensitive cell death can be uncoupled from resistance (Mittler et al., 1996). Therefore, the resistance status of the leaf tissue was analyzed by immunoblotting, and consistent with the visual symptoms, KCN treatment reduced TMV CP accumulation in the treated leaf tissue, with this effect being reversed by SHAM (Figure 5). Thus, the reduction in visible necrotic lesions observed on KCN-treated leaves was a result of increased resistance to TMV and not an inhibition of lesion formation only. We could not test the effect of AA on lesion development in whole plants because it was not taken up efficiently through the roots. Overall, these results demonstrate that the resistance to TMV induced by KCN does not require accumulation of SA and support the view that KCN feeds into the virus-specific defensive signal transduction pathway downstream of both the position of SA and the point at which the virus- and bacteria/fungi–specific signaling pathways diverge.

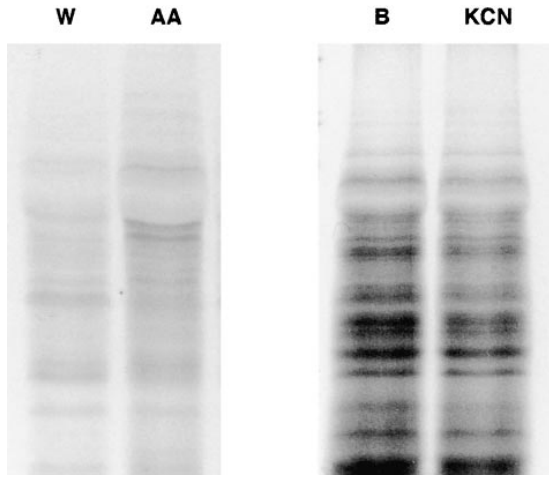

Figure 3. Protein Synthesis in Plant Tissue Treated with AA or KCN.

Xanthi NN tobacco leaf discs were floated on water (W) or 50 μM AA (left) or on 20 mM potassium-phosphate buffer (B) or 50 μM KCN (right) for 3 days. Each pretreated disc was fed for 2 hr on 10 μL of a 7:3 (v/v) mixture of L-^{35}S-methionine and L-^{35}S-cysteine. Equal amounts of trichloroacetic acid–insoluble radioactivity were analyzed by SDS-PAGE and autoradiographed.

Table 1. Effect of Chemical Pretreatment on the Size of TMV-Induced Lesions on SH-L Transgenic Tobacco

	Lesion Size (mm)[b]	
Treatment[a]	Circular Lesions	Spidery Lesions
Buffer	5.7 ± 0.8	10.54 ± 2.0
KCN	2.6 ± 0.4	ND[c]
KCN + SHAM	4.39 ± 0.7	10.24 ± 1.1
	$LSD_{0.05}$ = 0.59	$LSD_{0.05}$ = 1.49

[a] SH-L transgenic tobacco plants were watered with buffer, KCN, or a combination of KCN and SHAM for 3 days and then inoculated with 0.1 μg/mL TMV.

[b] The diameter of circular lesions and the length of the longest axis of spidery lesions were measured 5 days after inoculation, and the mean ±SD of 10 lesions from each treatment is given.

[c] ND, none detected, but a mean of 0 mm was used for calculating least significant difference.

Effects of SHAM and INA on PR-1 Accumulation in Transgenic Plants Expressing SA Hydroxylase

Previous findings showed that SHAM preferentially abolished SA-induced SAR to TMV but not SAR to bacterial and fungal pathogens in tobacco. However, SHAM was also a good inducer of synthesis of the antifungal PR-1 protein (Chivasa et al., 1997). Given the similar chemical structures of SA and SHAM, we suspected that this paradoxical result was due to the breakdown of a small amount of SHAM to SA in the plant or to the presence of traces of SA in the SHAM solutions that were applied to the plants. To test this idea, we investigated PR-1 accumulation in SH-L tobacco leaves treated with SA, INA, and SHAM. It was found that SHAM did not induce PR-1 in the SH-L transgenic tobacco leaves, despite being a strong inducer of PR-1 accumulation in nontransgenic plant tissue (Figure 6). Treatment of SH-L plant tissue with INA induced PR-1 accumulation, showing that there was no problem with uptake of chemicals into these tissues (Figure 6). The results confirm our hypothesis that traces of SA (from breakdown or contamination of SHAM) were responsible for the PR-1 accumulation induced by SHAM in nontransgenic tobacco.

Having determined that SHAM-induced PR-1 gene induction was an artifact, we were able to determine whether INA-induced PR-1 synthesis is SHAM sensitive. PR-1 accumulation was induced in SH-L transgenic tobacco by INA in both the presence and absence of SHAM (Figure 7C), showing that the SHAM-sensitive signal transduction pathway is not required for INA-induced PR-1 accumulation.

INA-Induced Resistance to TMV Is SHAM Sensitive

INA feeds into the SAR signaling pathway at either the same position or downstream of SA (Bi et al., 1995; Vernooij et al., 1995). Induction of acquired resistance to TMV by SA is sensitive to the AOX inhibitor SHAM, suggesting the involvement of alternative respiration in this pathway (Chivasa et al., 1997). We performed experiments to establish whether INA-induced resistance to TMV is also SHAM sensitive. Xanthi nn tobacco leaf discs were pretreated by floating them on water, 1 mM INA, or a combination of 1 mM INA and 1 mM SHAM. After 3 days, the discs were inoculated with TMV; 2 days later, total soluble protein was extracted and analyzed by immunoblotting. SHAM antagonized the INA-induced reduction in the accumulation of TMV RNA (Figure 7A) and CP (Figure 7B). This shows that INA-induced resistance to TMV does act via the SHAM-sensitive branch of the defensive signal transduction pathway and, like SA-induced resistance, appears to be dependent on AOX activity.

Aox Transcript Accumulation Correlates with Lesion Numbers in Tobacco That Responds Hypersensitively to TMV

We were curious to investigate the changes in *Aox* gene expression in resistant (NN genotype) and susceptible (nn genotype) cultivars of tobacco infected with TMV. As shown in Figure 8A, Xanthi NN leaf tissue infected with TMV had significantly higher levels of the *Aox* transcript than did mock-inoculated tissue. Densitometric measurements revealed a progressive increase in the amount of the transcript with increasing virus concentration and number of necrotic lesions, namely, two- and threefold increases for 0.1 and 0.5 μg/mL TMV, respectively (data not shown). This indicates that *Aox* transcript accumulation is responsive to the level of infection and suggests that synthesis of the transcript is higher around individual lesions. There was no difference in the accumulation of the *Aox* transcript in mock- and TMV-inoculated leaf tissue of the susceptible cultivar Xanthi nn (Figure 8B).

DISCUSSION

This work confirms that the signal transduction pathway responsible for the induction of SAR diverges downstream of SA into a SHAM-sensitive branch, which activates resistance to viruses, and a SHAM-resistant branch, which activates PR protein synthesis together with expression of resistance to bacteria and fungi. The work has also lent further support to the hypothesis that AOX is involved in the induction of resistance (Lennon et al., 1997), specifically in the induction of resistance to viruses (Chivasa et al., 1997). We have found that a synthetic SAR-inducing chemical, INA, and two metabolic poisons, AA and KCN, also induce resistance to viruses via the SHAM-sensitive signaling pathway. Like SA, these compounds induce the same mechanism of

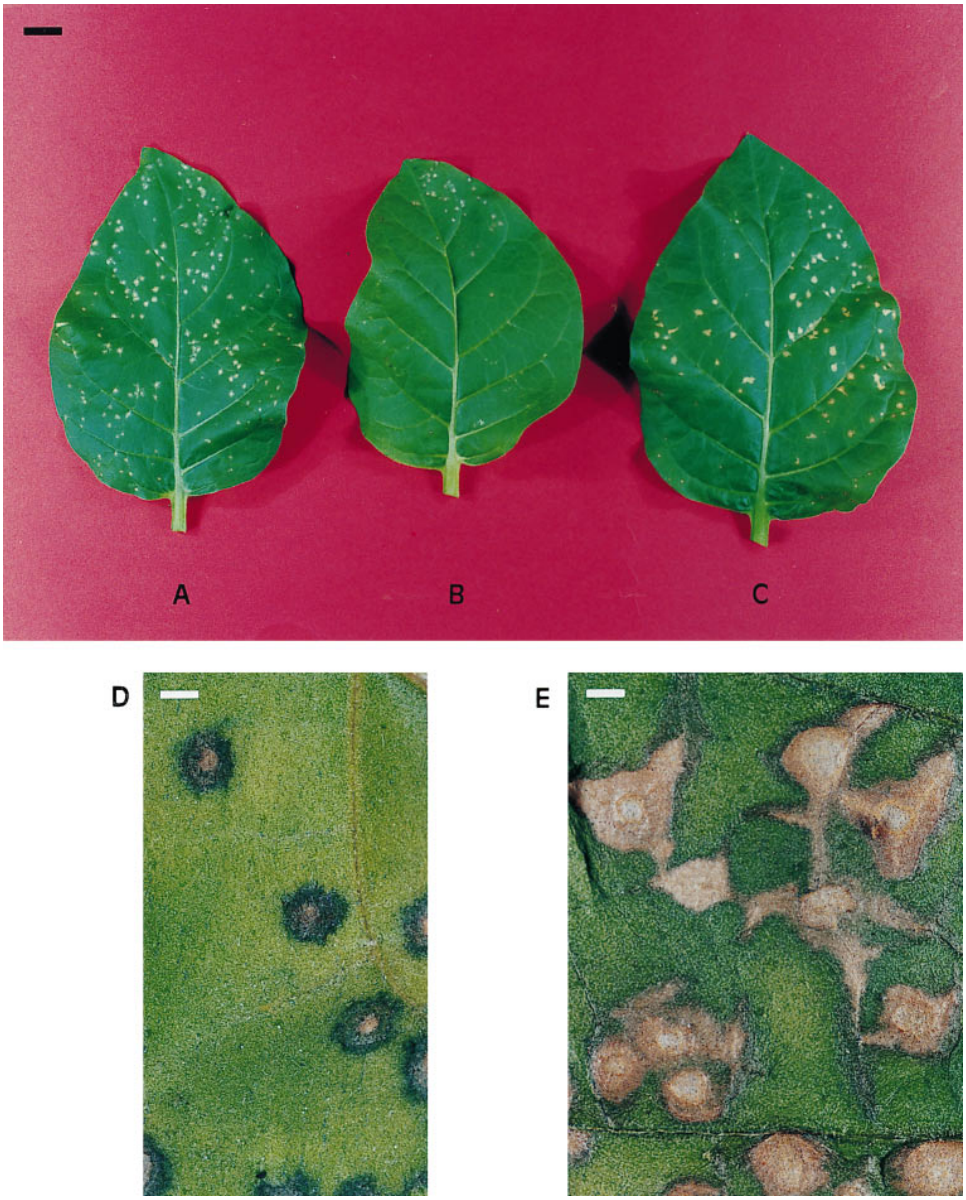

Figure 4. Restoration of the *N* Gene Resistance Phenotype in SH-L Tobacco by KCN.

(A) to **(C)** SH-L plants were watered for 3 days with a combination of 0.5 mM KCN plus 1 mM SHAM **(A)**, 0.5 mM KCN only **(B)**, and 20 mM potassium-phosphate buffer, pH 6.8 **(C)**, before inoculation with 0.1 μg/mL TMV. Leaves were photographed 5 days after inoculation. The detached leaves were kept alive by feeding with water for an additional 2 days and rephotographed in close-up.

(D) and **(E)** By 7 days after inoculation, it was apparent that the few necrotic lesions present on KCN-treated leaves had remained small and roughly circular in morphology **(D)**, whereas the control leaves had a mixture of circular and spidery (irregularly shaped) necrotic lesions that were beginning to merge **(E)**, as did the leaves treated with KCN plus SHAM (data not shown).

The bar in **(A)** to **(C)** = 23 mm; bars in **(D)** and **(E)** = 2 mm.

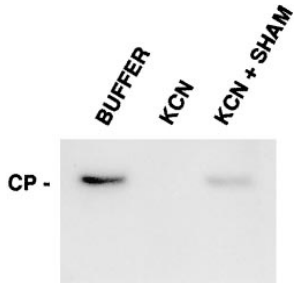

Figure 5. Effect of KCN on the Accumulation of TMV CP in SH-L Tobacco.

For gel blot analysis, proteins were extracted from leaves of SH-L plants watered with 20 mM potassium-phosphate buffer, 0.5 mM KCN, or a combination of 0.5 mM KCN and 1 mM SHAM for 3 days before inoculation with 0.1 μg/mL TMV. Protein was extracted from the leaves 5 days after inoculation, and equal amounts from each sample were analyzed by immunoblotting using a polyclonal rabbit anti–TMV CP antiserum. The position of the CP is indicated.

resistance to TMV. Specifically, they induce interference with viral replicase function, leading to an overall decrease in viral RNA accumulation and a change in the relative accumulation of the full-length genomic and subgenomic TMV RNAs.

Our results support the contention of Mur et al. (1997) that SA is required for virus localization early on in the HR. These workers attributed the spidery morphology of TMV-induced lesions on SH-L tobacco to unhindered movement of virus resulting from the absence of SA (Mur et al., 1997). We believe that this "early" role for SA in virus localization must require the participation of the SHAM-sensitive signal transduction pathway for two reasons: (1) because KCN treatment of SH-L tobacco before inoculation significantly reduced lesion size, restored normal lesion morphology, and decreased TMV CP accumulation; and (2) because SHAM antagonized the effect of KCN. These results, together with our earlier work showing that virus-specific SAR was abolished by SHAM (Chivasa et al., 1997), led us to conclude that the SHAM-sensitive signaling pathway is essential for *N* gene–mediated TMV localization as well as for the subsequent establishment of acquired resistance to the virus.

We have found that the metabolic inhibitors AA and KCN can activate resistance to TMV, most likely by induction of AOX and subsequent activation of the SHAM-sensitive signaling pathway. However, an early pioneering study shows that NaCN suppressed TMV multiplication in tobacco (Woods, 1940). Our study showed, using radiolabeling proteins, that the cyanide was having a selective effect on virus replication. This type of experiment would not have been possible at the time the earlier study was conducted. Nonetheless, in retrospect, it appears that Woods (1940) was probably the first to demonstrate a link between respiratory metabolism and resistance to viruses in plants.

Three new observations support a role for AOX in the induction of resistance to viruses. First, inducing *Aox* gene expression independently of SA with the respiratory inhibitors AA and KCN induced resistance to TMV but did not induce PR-1 protein synthesis. Second, the inhibitor of AOX enzyme activity, SHAM, abolished INA- and KCN-induced resistance to TMV in the susceptible cultivar Xanthi nn and abolished cyanide-induced restoration of the normal *N* gene resistance phenotype in SH-L tobacco. Unfortunately, we could not test the effect of SHAM on AA-induced resistance to TMV because coinjection of both solutions resulted in severe phytotoxicity. Third, consistent with a role for AOX in virus resistance, we have shown that *Aox* gene induction is correlated positively with TMV resistance in tobacco tissue. *Aox* transcript levels were increased by TMV infection of Xanthi NN tobacco in proportion to virus inoculum concentration and lesion numbers. In contrast, *Aox* transcript levels were not increased in either mock-inoculated plants or TMV-inoculated plants of the susceptible cultivar Xanthi nn. Furthermore, when exogenous SA is fed to Xanthi nn tobacco, there is increased resistance to TMV (White et al., 1983), and we have shown that this resistance is accompanied by *Aox* gene induction (Figure 1; Chivasa et al., 1997).

Our findings are consistent with the results of Lennon et al. (1997), who showed that AOX protein accumulation was increased in directly inoculated and noninoculated leaves of Xanthi NN tobacco infected with TMV but was not increased in the corresponding leaves of TMV-infected SH-L transgenic tobacco plants. Our results, together with those of Lennon et al. (1997), provide mounting evidence in support of a role for AOX in resistance to viruses. However, the only way to definitively test the role of AOX in resistance to viruses is to use transgenic plants containing modified levels of AOX (Vanlerberghe et al., 1994) when they are made generally available.

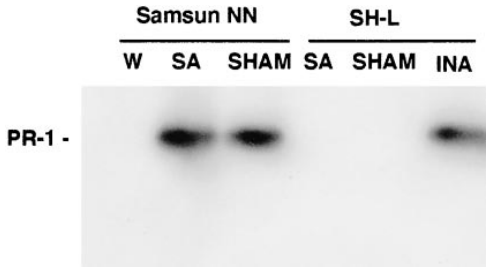

Figure 6. PR-1 Accumulation in Chemically Treated Samsun NN and SH-L Transgenic Tobacco Leaf Tissue.

Leaves detached from Samsun NN and SH-L transgenic tobacco plants were fed via the petiole with water (W) and 1 mM SA, 1 mM SHAM, or 1 mM INA for 5 days. Total soluble protein was analyzed by immunoblotting using a polyclonal rabbit anti–PR-1 antiserum. The position of the PR-1 protein is indicated.

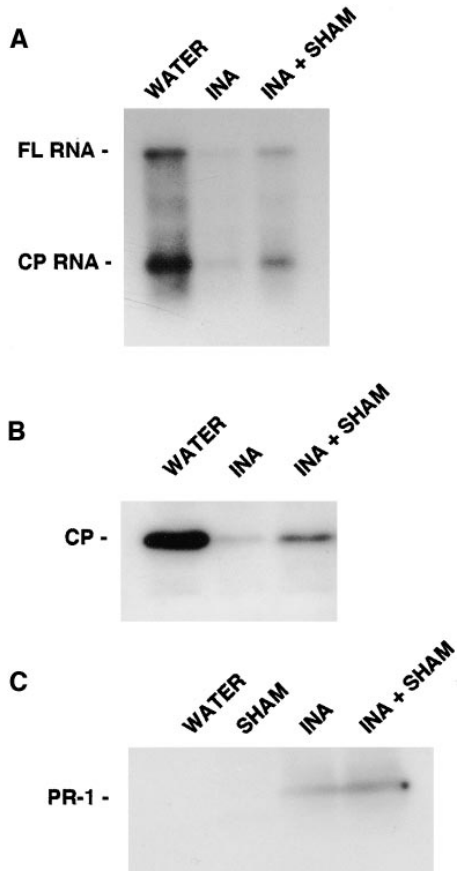

Figure 7. Effects of SHAM on INA-Induced Resistance to TMV and PR-1 Accumulation.

Xanthi nn leaf discs were floated for 3 days on water, 1 mM INA, or 1 mM INA combined with 1 mM SHAM. After inoculation with TMV (10 μg/mL), the discs were incubated for an additional 2 days on the same solutions before extraction of soluble protein and total RNA.

(A) RNA gel blot analysis of the total RNA samples using a ^{32}P-labeled riboprobe specific for plus-sense TMV RNA. The positions of the full-length (FL) genomic TMV RNA and the subgenomic CP mRNA (CP RNA) are indicated.

(B) Immunoblot analysis of protein samples analyzed using a rabbit polyclonal anti–TMV CP antiserum.

(C) Immunoblot analysis of protein samples extracted from SH-L leaf tissue treated for 5 days with water, SHAM, INA, and INA mixed with SHAM. The blot was probed with a rabbit polyclonal anti–PR-1 antiserum.

Curiously, Lennon et al. (1997) found that although AOX protein levels increased throughout hypersensitively responding and SA-treated plants (in line with our *Aox* transcript accumulation data), there was no overall increase in AOX enzyme activity. One possible explanation for this paradoxical result might be that although increased amounts of the AOX protein accumulate in all cells of the plant, the enzyme may be activated only in cells infected with the virus or in cells close to the hypersensitive lesion. This idea is consistent with our finding that *Aox* gene expression correlates positively with lesion numbers, suggesting that cells close to lesions express higher levels of AOX. This idea is also supported by evidence for a transitory burst in total respiration in virus-infected plant tissue (Yamaguchi and Hirai, 1959; Weintraub et al., 1960; Takahashi and Hirai, 1964; Baur et al., 1967; Dwurazna and Weintraub, 1969). It is conceivable that this respiratory burst provides the increased amounts of pyruvate and NADPH needed to activate the AOX enzyme (Millar et al., 1993; Vanlerberghe et al., 1995). Therefore, further investigations of the role of AOX activity during the HR may need to concentrate on tissue immediately adjacent to the hypersensitive lesions.

In conclusion, we have strengthened the evidence for the existence of a new virus-specific branch of the defensive signal transduction pathway. This branch plays a key role both in the induction of SAR to viruses and in the induction of virus localization during the primary HR. The branch diverges from the signaling pathway leading to PR protein gene induction at a point downstream of SA. This was illustrated by the fact that SHAM did not block INA-induced PR-1 accumulation but did block INA-induced resistance to TMV. In addition, the *Aox* gene–inducing metabolic inhibitors

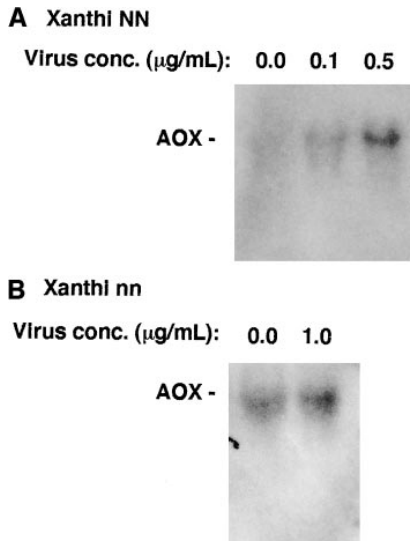

Figure 8. Effect of TMV Infection on *Aox* Transcript Accumulation in Tobacco Plants.

(A) Xanthi NN plants were inoculated on one lower leaf with 0, 0.1, or 0.5 μg/mL TMV.

(B) Xanthi nn plants were inoculated similar to the plants in **(A)**, with 0 or 1.0 μg/mL TMV.

RNA gel blot analysis was conducted using a ^{32}P-labeled *Aox*-specific DNA probe. The position of the *Aox* transcript is indicated at left in **(A)** and **(B)**. The autoradiograph in **(B)** was overexposed to reveal basal *Aox* transcript levels. conc., concentration.

KCN and AA induced resistance to TMV in nontransgenic Xanthi NN and Xanthi nn tobacco as well as in transgenic tobacco expressing SA hydroxylase. However, they did not induce PR-1 accumulation in any of these plants. This confirmed that KCN and AA activate the virus-specific signal transduction pathway at a position that is downstream of both SA and the point at which the virus-specific and PR protein–specific signaling pathways diverge (Figure 9). The ability of metabolic poisons to induce resistance to TMV further implicates AOX in the induction of resistance to viruses in plants.

METHODS

Chemicals

Ten millimolar stock solutions of salicylic acid (SA; Sigma), salicylhydroxamic acid (SHAM; Sigma), and 2,6-dichloroisonicotinic acid (INA; Maybridge Chemical, Cornwall, UK) were prepared fresh weekly in deionized water. Antimycin A (AA; Sigma) was dissolved in isopropanol to make a 30 mM stock. For treatment of plant tissue, we prepared a solution of 50 μM AA by diluting the stock in deionized water and infiltrated it through the lower epidermis into the intercellular space by using a syringe. Control treatments contained an equivalent dilution of isopropanol. Before application to plant tissue, SA, INA, and SHAM solutions were diluted to 1 mM. AA, SA, INA, and SHAM solutions were adjusted to pH 6.5 with KOH. KCN (BDH Chemicals, Leicester, UK) was used at a concentration of 50 μM for treatment of leaf discs and 0.5 mM for treatment of whole plants. KCN solutions were prepared in 20 mM potassium phosphate buffer, pH 6.8, and control treatments used buffer alone.

Plant Growth Conditions

Tobacco (*Nicotiana tabacum*) plants were raised in a greenhouse maintained at 25°C with supplementary lighting in winter to give a 16-hr photoperiod. The tobacco cultivars used were Xanthi nn, Xanthi NN, Samsun NN, and Samsun NN transformed with a bacterial SA hydroxylase enzyme (*nahG*) gene under the control of the cauliflower mosaic virus 35S promoter (SH-L plants; Bi et al., 1995). The plants used in these experiments were 6 to 8 weeks old. Inoculation of leaf tissue with tobacco mosaic virus (TMV; strain U1) was performed as described previously (Chivasa et al., 1997).

Radiolabeling of Proteins

To evaluate the effect of inhibition of the cytochrome pathway on tobacco protein synthesis, leaf discs (five per treatment) of Xanthi NN and Xanthi nn tobacco floated on water and AA or buffer and KCN for 3 days were incubated for 2 hr separately on 10 μL of a 7:3 (v/v) mixture of L-^{35}S-methionine and L-^{35}S-cysteine (Promix, Amersham International, Buckinghamshire, UK) at a concentration of 2.86 μCi/mL. Total soluble proteins were extracted, and trichloroacetic acid–insoluble radioactivity was quantified using a liquid scintillation analyzer (Tri-Carb 2000; United Technologies, Chicago, IL).

Protein and RNA Extractions

Soluble proteins were extracted and subjected to protein gel blot analysis by using polyclonal rabbit antisera raised against the TMV coat protein (CP) and the tobacco PR-1 protein, using previously described methods (Carr et al., 1987; Carr and Zaitlin, 1991). Purification of total single-stranded RNA was performed using the method of Berry et al. (1985), and RNA gel blot analysis of TMV-specific RNAs

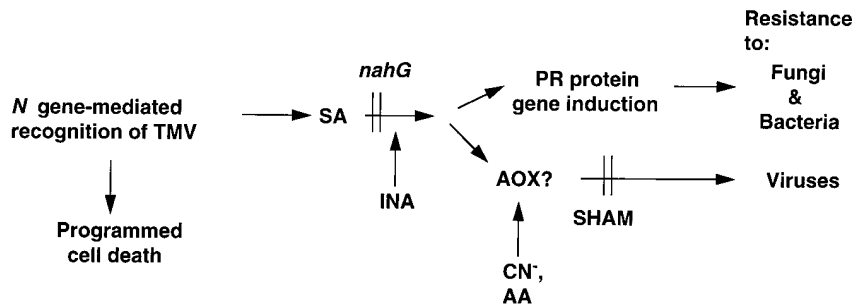

Figure 9. Model for Induction of Resistance to Viruses via the SHAM-Sensitive Signaling Pathway.

N gene–mediated recognition of TMV results from the interaction of the *N* gene product (Whitham et al., 1994) with sequences of the 126-kD replicase protein of TMV (Padgett et al., 1997). This results in the initiation of programmed cell death close to the point of inoculation and the accumulation of SA. Subsequent signaling steps are blocked in transgenic plants expressing the bacterial *nahG* gene for SA hydroxylase. The synthetic resistance inducer INA feeds in at the same point or subsequent to SA in the pathway (Bi et al., 1995; Vernooij et al., 1995). Downstream of this point, the pathway splits in two. The SHAM-insensitive signaling pathway leads to the induction of PR protein synthesis and subsequently to resistance to bacteria and fungi. The SHAM-sensitive pathway leads to the induction of resistance to viruses. The involvement of AOX activity is suggested because the pathway can be inactivated by SHAM, an inhibitor of AOX enzyme activity, and activated by inducers of *Aox* gene expression (CN^-, AA). The pathways work together to provide resistance to a broad range of pathogens. The scheme is simplified and does not include the many intermediate steps in defensive signal transduction revealed by analysis of Arabidopsis mutants. For a full discussion of these, see Durner et al. (1997).

using strand-specific ^{32}P-labeled riboprobes was performed using previously published methods (Carr and Zaitlin, 1991). The *Aox* transcript was detected on RNA gel blots by hybridization with ^{32}P-labeled *Aox* DNA fragment as described previously (Chivasa et al., 1997). Densitometric measurements were performed using autoradiographs of RNA and protein gel blots using the Molecular Dynamics ImageQuant v2.0 densitometer (Molecular Dynamics, Chesham, UK).

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