Salicylic Acid Suppresses *Potato virus Y* Isolate N:O-Induced Symptoms in Tobacco Plants

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Accepted for publication 14 October 2005.

ABSTRACT

Nie, X. 2006. Salicylic acid suppresses *Potato virus Y* isolate N:O-induced symptoms in tobacco plants. *Phytopathology* 96:255-263.

The effects of salicylic acid (SA) and 1-aminocyclopropane-1-carboxylic acid (ACC) on the systemic development of symptoms induced by a severe isolate of *Potato virus Y* group N:O (PVY^{N:O}) in tobacco were investigated. Upon inoculation, the systemic development of symptoms in tobacco plants could be divided into three stages: virus incubation stage, rapid symptom-progress stage, and partial recovery and symptom-shifting stage. Treatment of seedlings with SA delayed the virus-induced necrosis in stems by 1 to 2 days. SA, not ACC, also significantly suppressed the symptom severity in stems. However, neither SA nor ACC treatment affected the partial recovery phenotype exhibited in the latterly emerged

upper parts of the plants. Further analysis indicated that the accumulation of PVY was retarded by SA at the early stage of infection, and the effects were more profound in stems than leaves. Peroxidase (POX) activity and pathogenesis-related (PR) genes *PR-1a* and *PR-1b* were enhanced by PVY infection. SA not only increased POX activity in stems and PR genes in stems and leaves of mock-inoculated plants, but also elevated the activity of POX in both leaves and stems and the expression of *PR-1a* in leaves of PVY-infected plants. Together, the results suggest that systemic acquired resistance plays a key role in suppressing PVY^{N:O}-induced symptom development through SA-mediated and ethylene-independent pathways. The symptom suppression was correlated with reduced replication/accumulation of virus at the early stage of infection. The results also suggest that neither SA nor ethylene plays a role in the recovery phenotype.

Plant-virus interaction is a complicated process. In a compatible interaction, systemic infection that involves virus multiplication, cell-to-cell movement and long-distance movement takes place, and the host plant exhibits a susceptible phenotype (13). In an incompatible reaction, systemic infection is disrupted, and the host plant demonstrates a resistant phenotype (13). Incompatible reactions are typically controlled by resistance (*R*) genes, and the resistance is virus species- and/or strain-specific (22). Besides the *R* gene (including the *N* gene) mediated resistance, systemic acquired resistance (SAR) which is inducible and pathogen-nonspecific has been recognized (10,37,43). SAR is generally coupled with elevated expression of pathogenesis-related (PR) genes (10, 43). In addition, reactive oxygen species (ROS) and associated enzymes also play a role in SAR (5,37).

Salicylic acid (SA) has been shown to be involved in responses to pathogen attack in various plant species. It appears that the induction of SA is essential in resistant responses against viruses, fungi, and bacteria (25,37). Depletion of SA by introducing SA hydroxylase (*nahG*) in tobacco plants results in the loss of *N* gene-mediated resistance against *Tobacco mosaic virus* (TMV) (6). Pretreatment of susceptible tobacco plants or plant tissues with SA or a SA derivative, aspirin, leads to a significant reduction in accumulation of TMV (1,47) and TMV RNA (7). The treatment also results in delays of systemic development of TMV-induced symptoms (32). Similarly, SA delays the *Potato virus X* (PVX) as well as the *Cucumber mosaic virus* (CMV)-induced symptoms in tobacco and *Arabidopsis* plants (23,27). In addition to SA, many other signal molecules including ethylene, NO, and

H₂O₂ are also involved in SAR in many different plant species (28,43,44).

Potato virus Y (PVY), genus *Potyvirus*, is a single-strand, positive RNA virus that induces severe symptoms in potato and tobacco plants (40). Multiple strains and substrains including common strain (PVY^O), tobacco vein necrosis strain (PVY^N), and potato tuber necrosis substrain (PVY^{NTN}) exist. Recently, a recombinant isolate group, PVY^{N:O}, whose genome comprises segments of PVY^O and PVY^N, has been identified (14,30,42). Like PVY^N and PVY^{NTN}, PVY^{N:O} induces vein clearing, vein necrosis, foliar deformation and stem necrosis in tobacco plants (30). Since its discovery in the mid-1980s (8), the isolate group has been widely reported in Europe and North America (14,34,42). The spread of PVY^{N:O} in North America is at least partially due to its indistinguishable serological property from PVY^O, and thus evading the serological indexing used in potato seed certification system (42).

Genomic analysis of PVY^{N:O} structure demonstrates that the genome portion (nucleotide 1 to approximately 2,400) derived from PVY^N might play a key role in its pathogenicity in tobacco plants (30). However, little is known about how tobacco plants respond to infections by the virus. Transgenic tobacco plants expressing cell wall invertase exhibit elevated *PR-Q* expression and lesions in leaves (16,17). These plants are PVY^N-resistant, and the resistance appears to be sugar mediated and salicylate independent (17). Meanwhile, resistance against PVY^N conferred by recessive *R* genes has been reported in tobacco, tomato, and pepper (3,22,33); however, the resistance can be overcome as the virus mutates (22).

In this study, the effects of SA and 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene biosynthesis, on PVY^{N:O}-induced systemic development of symptoms in tobacco plants were investigated. The results demonstrate that SA, not ACC, delayed symptom development and alleviated symptom severity in stems of tobacco plants challenged with PVY. The suppression of symptoms by SA was correlated with reduced replica-

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tion/accumulation of PVY^{N:O}, and was associated with elevated peroxidase (POX) activity and PR gene expression. This is, apparently, the first time that salicylate-mediated SAR against a potyvirus is demonstrated.

MATERIALS AND METHODS

Virus isolate and tobacco plants. PVY^{N:O} Mb58 was obtained from Manitoba, Canada (30), and was used to inoculate *Nicotiana tabacum* cv. Samsun plants.

Inoculation of *N. tabacum* cv. Samsun plants. Five fully expanded leaves (≈ 15 g) of PVY^{N:O} Mb58-infected tobacco plants were homogenized in 50 ml of inoculation buffer (10 mM sodium phosphate buffer, pH 7.5, with 32 mM Na₂SO₃) and filtered through double-layer cheese cloth. The extract was then used to inoculate 'Samsun' seedlings. Briefly, tobacco seedlings at five-leaf stage were dusted with Carborundum, and the top three leaves were rubbed gently with a pestle in the presence of 0.73 ml of virus extract or inoculation buffer (mock) per leaf. The plants were then maintained in the greenhouse with a light/dark cycle of 16/8 h at 18 to 24°C. The humidity in the greenhouse was 50 to 70%, and the sunlight was supplemented with artificial light. The symptom expression was recorded from day one up to 30 days postinoculation (dpi).

Treatments with SA and ACC. Tobacco seedlings were sprayed twice per day with various concentrations of SA (pH 7.0) or ACC solution containing 2 drops/liter Tween 20, started 3 days prior to inoculation and lasted as long as the plants were kept.

Enzyme activity measurements. Freshly sampled leaves and stem cortexes (including epidermises) were immediately frozen in liquid nitrogen and stored at -80°C. POX (EC 1.11.1.7) activity was measured based on the procedure described by Shetty et al. (38) with some modifications. Briefly, 0.3 g of frozen tissue was homogenized in 3 ml of ice-cold extraction buffer (50 mM potassium acetate, pH 5.2, 100 mM KCl, 1 M NaCl, 1 mM CaCl₂, and 1 mM ascorbic acid) using a Polytron (Kinematica GmbH, Kriens-Luzern, Switzerland) for 30 s. The solution was centrifuged at 12,000 $\times$ g for 12 min at 4°C. The supernatant was transferred to a 1.5-ml microtube and placed on ice. The enzyme activity was monitored spectrophotometrically at 20°C using 8453 UV-Visible Spectrophotometer (Agilent Technologies, Palo Alto, CA). To 1 ml of substrate solution containing 100 mM sodium acetate, pH 5.5, 360 μ M 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 0.63 mM H₂O₂, 10 μ l of enzyme extract was added and mixed. The absorbance at 405 nm (A_{405}) was recorded every 10 s for 4 min. The change of A_{405} versus time was plotted, and the linear portion was used to calculate the enzyme activity, which was expressed as A_{405} mg protein⁻¹.min⁻¹.

Catalase (CAT, EC 1.11.1.6) activity was measured according to the procedure described by Havir and McHale (15) with slight modifications. Crude enzyme was isolated by grinding 0.3 g of frozen tissue with 3 ml of extraction buffer (0.05 M potassium phosphate buffer, pH 7.5, 6.5 mM dithiothreitol [DTT]) on ice using a Polytron for 30 s. The solution was centrifuged at 13,500 $\times$ g for 15 min at 4°C. The supernatant was transferred to a 1.5-ml microtube and kept on ice. The activity of CAT was measured by monitoring the H₂O₂ change during the course of reaction. Briefly, to 1 ml of substrate buffer containing 30 mM H₂O₂ and 50 mM potassium phosphate buffer, pH 7.0, 50 μ l of enzyme was added. The absorbance at 240 nm (A_{240}) was monitored continuously for 4 min at 30°C, taking reading every 10 s using the kinetics mode of the 8453 UV-Visible Spectrophotometer (Agilent Technologies). The activity was expressed as A_{240} mg protein⁻¹.min⁻¹.

Protein assay. Protein was measured using the Bradford assay (2) and bovine serum albumin as the standard.

PVY level and gene expression analysis. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to assess the virus level and gene expression. Total RNA from leaf

or stem cortex was isolated as described previously (24,29). The RNA was then incubated with RNase-free DNase I (Roche Diagnostics, Indianapolis, IN) at 37°C for 10 min. Thereafter, the solution was extracted with water-saturated phenol once, phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) once, and chloroform/isoamyl alcohol (24:1, vol/vol) once. Total RNA was precipitated by adding 2 volumes of 95% ethanol at -20°C for 2 h, recovered by centrifugation at 12,000 $\times$ g for 20 min at 4°C, and quantified with a spectrophotometer (GeneQuant, Biochem, UK).

RT was carried out using random primers as described previously (29,31). Briefly, 1 μ g of total RNA in 2.5 μ l was mixed with 1 μ l (1 μ g) of hexanucleotides (Roche Diagnostics), incubated at 68°C for 8 min, and chilled on ice for 3 min. A total of 6.5 μ l of the RT mixture was added to provide a final concentration of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 2.5 mM MgCl₂, 1.0 mM of each dNTP (dATP, dTTP, dCTP, and dGTP), 5 units of RNasin Ribonuclease Inhibitor (Promega Corp., Madison, WI), and 100 units of Moloney murine leukemia virus reverse transcriptase (Promega). Samples were incubated at 42°C for 1 h and at 95°C for 2 min. Thereafter, 40 μ l of H₂O was added to the cDNA mix, and the resulting cDNA solution was used for semiquantitative PCR analysis of PVY level and gene expression.

Duplex PCR that included two pairs of primers, one targeting the cytochrome c oxidase subunit 1 (*COX1*), a control gene (29, 31), and the other targeting the gene of interest, was performed. The primers for *COX1* (accession no. BA000042), 5'-CTAATTCTGGGCTAGACATTGC3' and 5'-CTTCACATAGCTTTTCGTCTCC5', produced a fragment of 500 bp; the primers for *PR-1a* (accession no. X06930), 5'-CGACCTACGTCCATTTCACG3' and 5'-GCAAGTAGCTAGACCATCAAC3', produced a fragment of 266 bp; the primers for *PR-1b* (accession no. X66942), 5'-TAATAACGGTTTACGTGATCA3' and 5'-TGACTCATCACCTTGTTCACT3', produced a fragment of 244 bp; and the primers for PVY (41), 5'-ACGTCCAAAATGAGAATGCC3' and 5'-TGGTGTTCTGTGATGTGACCT3', produced a fragment of 480 bp. The amplification was conducted in a volume of 25 μ l containing 4- μ l aliquots of the cDNA mixture, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 μ M each of dNTPs (Promega), 0.1 μ g each of the sense and antisense primers, and 0.625 units of *Taq* polymerase (PE Applied Biosystems, Foster City, CA). Samples were amplified in 10, 15, and 20 cycles using a PTC-200 Thermocycler (MJ Research, Watertown, MA) with DNA denaturing at 92°C, primer annealing at 60 to 55°C (30), and primer extension at 72°C, each for 45 s. A final step of 10 min at 72°C was also included. A total of 10 μ l of the amplification product was fractionated by electrophoresis on 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide and photographed under UV illumination. A low mass DNA ladder (Gibco-BRL, Gaithersburg, MD) was used in the gel to determine the size of the amplified products. After the electrophoresis, the gel was blotted to Hybond-N+ (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions, and the resulting membrane was hybridized with probes corresponding to the gene of interest. Briefly, PCR amplification fragment of the gene of interest was extracted from agarose gel and used as template for labeling with the Gene Images AlkPhos Direct Labeling and Detection System (Amersham Biosciences) according to the manufacturer's instructions. After hybridization, the membrane was washed to remove the excess probe and incubated with the CDP-star chemiluminescent detection reagents (Amersham Biosciences). The chemiluminescent signals were detected by exposure of X-ray film (X-AR, Kodak) at room temperature.

RESULTS

Symptom development in *N. tabacum* cv. Samsun upon inoculation with PVY^{N:O}. Vein clearing in the newly emerged

leaves was the first visible response, which occurred approximately 7 dpi. At 7 to 8 dpi, necrosis appeared at the junctions of stem and petioles of leaves showing vein clearing; and at 8 to 9 dpi, isolated necrotic spots occurred in stem segment stretching from the bottom of the stem to three to four nodes above the inoculated leaves. At 10 to 14 dpi, rapid progress of necrosis took place in petioles of leaves showing vein netting and clearing and stem section ranging from bottom to three to four nodes above the inoculated leaves (Fig. 1A). Foliar distortion also occurred in these leaves. Concurrently, milder symptoms (mosaic, mottling, and mild leaf distortion) were observed in latterly emerged leaves, and no necrosis occurred in the petioles of these leaves and the stem sections where these leaves were located (Fig. 1A and B). The phenomenon resembled the recovery phenotype described in several virus–host systems (11,35,36). However, unlike the typical recovery in those systems, distinct mottling in the latterly emerged leaves became apparent as the leaves aged (Fig. 1A), indicating that the “recovery” was partial. Based on these observations, the interaction between PVY^{N:O} and tobacco plants could be divided into three stages, i.e., virus incubation stage (from inocu-

lation to ≈7 dpi), rapid symptom-progress stage (8 to 14 dpi), and partial recovery and symptom-shifting stage (≈15 dpi and later).

Effects of SA and ACC on PVY^{N:O}-induced symptoms in tobacco. Symptoms were recorded and scored based on the degree of stem necrosis and number of nodes/internodes that the necrosis stretched. A higher degree of symptom severity indicates more necrotic spots and each spot covers a larger area. As shown in Table 1, at 14 dpi, SA reduced PVY-induced symptoms by alleviating the degree of stem necrosis and the number of nodes/internodes that exhibited necrosis. The suppression became evident once the SA concentration increased to 1 mM. When SA concentration increased further to 5 mM, the degree of stem necrosis reduced by 50% and the symptom severity was approximately one-third of that of control (Table 1). However, plants treated with 5 mM SA exhibited a retarded growth rate and reduced leaf sizes in both PVY-inoculated and mock-inoculated plants (data not shown). To minimize these side effects, 1 mM SA was selected for further experiments.

SA delayed necrotic symptom initiation and development in stems by 1 to 2 days. At 10 dpi, light patchy necrosis (degree of

Fig. 1. Systemic development of symptoms induced by *Potato virus Y* isolate N:O (PVY^{N:O}) Mb58 in tobacco plants. Tobacco cv. Samsun seedlings were mechanically inoculated with PVY^{N:O} Mb58 and kept in the greenhouse for symptom development. **A**, Symptoms in plants at various days postinoculation (dpi). **B**, Enlarged picture of a plant at 14 dpi demonstrating the reduced symptoms in the upper part.

stem necrosis: 3 to 4) occurred on stems of water-treated plants, whereas one to two light necrotic spots (degree of stem necrosis: 1 to 2) were observed on stems of SA-treated plants (Fig. 2A and B). At 14 dpi, significant necrosis (degree of stem necrosis: 5 to

TABLE 1. Effects of various concentrations of salicylic acid (SA) and 1-aminocyclopropane-1-carboxylic acid (ACC) on symptom severity in tobacco plants inoculated with *Potato virus Y* (PVY^{N:O}) at 14 days postinoculation^a

Treatment	Stem necrosis		Score of symptom severity
	Degree of necrosis	Number of nodes	
SA (mM)			
0.0	7.00 ± 1.90	5.70 ± 0.75	41.10 ± 16.23
0.1	6.00 ± 1.97	4.90 ± 0.66	30.35 ± 14.01
1.0	6.00 ± 2.30	4.40 ± 1.02	24.30 ± 12.42
5.0	3.00 ± 1.70	3.60 ± 2.06	14.10 ± 9.58
ACC (μM)			
0.0	7.80 ± 1.12	5.20 ± 0.75	40.90 ± 9.03
10.0	5.75 ± 2.51	5.10 ± 0.73	39.25 ± 11.89
50.0	8.00 ± 0.87	6.10 ± 1.11	47.80 ± 13.79

^a The data shown are means ± standard deviations of four replicates from one of the three repeats of the experiment. The degree of necrosis was based on the area and color of the necrosis on stems (0, no necrosis; 1 to 2, one to two necrotic spots; 3 to 4, light patchy necrosis; 5 to 6, dark patchy necrosis; 7 to 8, heavy dark necrosis on one side of the stem or dark patchy necrosis around the stem; and 9 to 10, heavy dark necrosis around the stem). The score of symptom severity was obtained by multiplying degree of necrosis and number of nodes exhibiting necrosis.

8) was observed in water-treated plants, and light patchy necrosis (degree of stem necrosis: 3 to 4) occurred in SA-treated plants (Fig. 2B). The suppressive effects of SA on symptom development in stems became more evident at a later stage when the necrotic tissue, mainly the epidermis, darkened and became dead (Fig. 2). Unlike that in stems, symptoms in leaves did not appear to be significantly affected by SA treatment. In addition, SA treatment neither altered the overall symptom patterns nor affected the partial recovery and symptom-shifting in the plants.

Exogenous ACC did not affect PVY-induced symptom development and severity. No differences were observed among plants treated with 0 to 50 μM ACC in terms of degree of stem necrosis, number of nodes/internodes that exhibited necrosis, and the overall symptom severity (Table 1), suggesting that ethylene does not affect PVY^{N:O}-induced systemic development of symptoms in tobacco. Like SA, exogenous ACC did not affect the symptom patterns or the partial recovery in the plants.

Effects of SA on replication of PVY^{N:O} in tobacco plants. The relative amount of PVY was monitored in plants treated with various concentrations of SA at 6 dpi. As the concentration of SA increased, a relatively lower level of PVY was found in both leaves and stems, and this became more profound when SA concentration reached 1 mM (Fig. 3A). However, at 5 mM, SA not only lowered PVY levels but also interfered with the expression of *COX1*, a mitochondrial encoded gene, indicating nonspecific inhibitory effects of SA on plants at this concentration, coincided

Fig. 2. Effects of salicylic acid (SA) on *Potato virus Y* isolate N:O (PVY^{N:O}) Mb58 induced systemic development of symptoms in tobacco plants. Tobacco cv. Samsun seedlings were sprayed with 1 mM SA or H₂O 3 days prior to mechanical inoculation with the virus, and were sprayed continuously in the greenhouse until sampled. A, Whole plants at various days postinoculation (dpi). B, Stem segments from plants treated with water or 1 mM SA at various dpi.

with the visual observation of tobacco plants treated with 5 mM SA. The effect of 1 mM SA on PVY accumulation/replication/transportation was further examined during the virus incubation stage and rapid symptom-progress stage, i.e., 0 to 14 dpi. As shown in Figure 3B, PVY became detectable in the growing leaves at 4 dpi and in stems at 2 dpi in both SA-treated and untreated (H₂O-treated) plants. From 4 to 7 dpi, virus concentration increased dramatically in both stems and leaves, and thereafter, the concentration remained similar. In leaves, PVY accumulation was slightly retarded by SA treatment before 7 dpi; afterward, the virus reached the similar level in both SA-treated and untreated plants. In stems, lower levels of PVY were found in SA-treated plants than in untreated plants from 2 to 8 dpi, and thereafter, similar levels of PVY were observed in both treated and untreated plants (Fig. 3B), indicating that SA interferes with the replication/transportation of PVY in tobacco plants, especially in stems.

Effects of SA on activities of POX and CAT in PVY^{N:O}-challenged tobacco plants. To reveal the possible involvement of ROS-associated enzymes in PVY–tobacco interaction, activities of POX and CAT were monitored in both SA-treated and untreated plants after inoculation with PVY. At 14 dpi, a higher POX activity was observed in plants treated with SA compared with those in water control (0 mM) (Fig. 4). The increment of activity in the treated plants was associated with SA concentration: as SA concentration increased from 0 to 5 mM, POX activity increased approximately threefold (from ~98 to ~300) in leaves and ~35% (from ~750 to ~1,000) in stems. In leaves, ~20% increase of CAT activity was observed once the SA concentration reached 1 mM; and 5 mM SA did not further increase the activity of CAT. In

stems, no difference was observed between 0 and 0.1 mM SA on CAT activity, whereas 1 mM SA treatment resulted in a decreased enzyme activity level, and as SA concentration increased to 5 mM, a higher CAT activity was observed.

To further explore the dynamics of POX and CAT in plants during the course of PVY–tobacco interaction, the activities of these two enzymes were measured during the phase of rapid progress of symptoms, i.e., from 7 to 14 dpi. In mock-inoculated plants, a higher POX activity was observed in stems of SA-treated over untreated (H₂O-treated) plants, indicating the inductive effects of SA on POX in the tissue (Fig. 5). Infection of PVY resulted in elevated POX activity in both leaves and stems. As disease progressed, the activity of POX increased in both SA-treated and untreated plants, indicating the induction of POX by PVY. From 7 to 12 dpi, a significantly higher POX activity was recorded in SA-treated plants compared with untreated plants, and at 14 dpi, the difference of POX activity between the treated and untreated plants decreased. CAT activity exhibited a relatively small change in plants over the period of rapid symptom development (Fig. 5). Although CAT activity was slightly higher in SA-treated plants than in untreated mock-inoculated plants, the difference was not significant. In leaves, SA-treated plants demonstrated a higher CAT activity than untreated plants at 7 and 10 dpi, and the difference diminished once it reached 12 dpi. Thereafter, the activity in both treated and untreated plants decreased. In stem, slightly higher activity was recorded in SA-treated plants at 7 dpi in both mock- and PVY-inoculated plants. However, as disease progressed, CAT activity in untreated plants surpassed the treated plants and peaked at 10 to 12 dpi. Thereafter, the activity in both SA-treated and untreated plants decreased.

Fig. 3. Effect of salicylic acid (SA) on accumulation of *Potato virus Y* isolate N:O (PVY^{N:O}) Mb58 in tobacco plants. RNA from three leaves (position +1 to +3) emerging above the inoculated leaves (position 0) or stem cortex tissue from bottom to three nodes above the inoculated leaves was used to analyze PVY accumulation. Duplex semiquantitative reverse transcription-polymerase chain reactions (RT-PCR) including the primer pairs targeting the cytochrome c oxidase subunit I (*COX1*) and PVY were performed at 10, 15, and 20 cycles. Hybridization with the corresponding probes was performed after gel electrophoresis and blotting. **A**, Effects of various concentrations of SA on PVY accumulation in leaves and stem cortices at 6 days postinoculation (dpi). **B**, Effects of 1 mM SA on accumulation of PVY in leaves and stems at various days postinoculations.

Effects of SA on PR gene expression in PVY^{N:O}-challenged tobacco plants. The expression of PR genes, represented by *PR-1a* and *PR-1b*, were studied at the rapid symptom-progress stage. In mock-inoculated plants, the expression of both genes was higher in SA-treated than in untreated (H₂O-treated) plants (Fig. 6), indicating the inductive effects of SA on these genes. Infection of tobacco plants by PVY^{N:O} led to elevated expression of *PR-1a* and *PR-1b* in both SA-treated and untreated plants. And, except *PR-1b* in SA-treated plants, the expression of the genes increased as the disease progressed (Fig. 6), whereas PVY level did not change significantly during the time span (Figs. 3B and 6). SA augmented *PR-1a* expression in leaves of PVY-infected plants; however, an opposite trend was observed in stems. The compound did not appear to alter the expression of *PR-1b* in both leaf and stem cortex tissues of PVY-infected plants during the time course.

DISCUSSION

Examination of the systemic infection by PVY^{N:O} and disease development in tobacco cv. Samsun plants indicates that the infection/disease development can be divided into three stages: virus incubation stage, rapid symptom-progress stage, and partial recovery and symptom-shifting stage. The incubation stage was characterized by rapid accumulation of PVY without visible symptoms in the plants (Figs. 1 and 3). The second stage was symbolized by rapid progress of symptoms that included veinal, petiole, and stem necrosis, and leaf malformation (Fig. 1). The partial recovery and symptom-shifting stage was characterized by

milder symptoms in the latterly emerged leaves and stems (Fig. 1). It is noteworthy that severe necrotic symptoms were only limited to certain leaves and areas of stem during the course of PVY^{N:O}-tobacco interaction. Hypothesis regarding the recovery phenotype has been elucidated in various virus-host systems (21,35,36), and virus-induced gene silencing (VIGS) has been demonstrated to play a key role in the process (35,36). It is possible that initial infection of PVY^{N:O} on tobacco cv. Samsun plants might have triggered the VIGS-like responses in latterly emerging leaves and stems, and thus alleviated the symptom severity. However, it is unclear whether the VIGS-like process is the exclusive mechanism that might be involved in the partial recovery and symptom attenuation in the upper part of PVY^{N:O}-challenged plants. Nevertheless, the failure of exogenous SA and ACC to alter the overall symptoms patterns or to affect the recovery process during the course of PVY^{N:O}-tobacco interaction suggests that the signals lead to partial recovery and symptom-shifting is independent from SA and ethylene.

SAR is characterized by its inductive nature by a wide range of chemicals and stimuli including microbe invasions (9,12,37,39, 43). Unlike resistance mediated by *R* gene and/or RNA silencing, SAR is not pathogen specific (37,43). SA plays a key role in SAR in many plant species against various pathogens including fungi, bacteria, and viruses (37), and plant growth regulators such as jasmonic acid and ethylene have also been shown to be involved in the signaling pathways of SAR (9,39). When applied to tobacco cv. Samsun plants, SA at a concentration of 1 to 5 mM significantly reduced PVY^{N:O}-induced necrosis in stems, whereas the ethylene precursor ACC did not show any effects on the symptom

Fig. 4. Effects of salicylic acid (SA) on peroxidase and catalase activities in tobacco plants infected with *Potato virus Y* isolate N:O (PVY^{N:O}) Mb58. Tobacco cv. Samsun seedlings were sprayed with various concentrations of SA 3 days prior to mechanical inoculation with the virus, and were sprayed continuously in the greenhouse until sampled at 14 days postinoculation (dpi). Enzyme extracts from three leaves (position +1 to +3) emerging above the inoculated leaves (position 0) or stem cortices from bottom to three nodes above the inoculated leaves were used to analyze the enzyme activities. The x axis is the SA concentration and the y axis is the enzyme activity. The data shown are means $\pm$ standard deviations from four replicates. Experiments were repeated twice, and similar results were obtained.

severity (Table 1), suggesting that the suppression of PVY-induced symptoms is mediated by SA and is ethylene-independent. Similar observations and mechanisms have been reported in several pathogen–host systems (18,20,45). In *Arabidopsis*, activation of SA-mediated SAR results in efficient protection against *Turnip crinkle virus* (TCV), whereas ethylene-dependent induced systemic resistance (ISR) offers no protection against the virus

(45). It is possible that SA-dependent and ethylene-independent SAR is widely used in plants against viruses. SA retarded the symptom initiation in stems by 1 to 2 days. Similar to the SA-mediated resistance against TMV, PVX, and CMV in tobacco, in which delayed symptoms are accompanied by reduced level of virus (27,32), SA delayed PVY accumulation at the incubation stage (Fig. 3), suggesting that symptom develop-

Fig. 5. Dynamics of peroxidase and catalase activities during the rapid symptom-progress stage in tobacco plants infected with *Potato virus Y* isolate N:O (PVY^{N:O}) Mb58. Tobacco cv. Samsun seedlings were sprayed with H₂O or 1 mM salicylic acid (SA) 3 days prior to mechanical inoculation with the virus or mock, and were sprayed continuously in the greenhouse until sampled. Enzyme activities of peroxidase and catalase in leaf and stem cortex tissues were measured at 7 to 14 days postinoculation. Enzyme extracts from three leaves (position +1 to +3) emerging above the inoculated leaves (position 0) or stem cortices from bottom to three nodes above the inoculated leaves were used to analyze the enzyme activities. The data shown are means ± standard deviations from four replicates. Experiments were repeated twice, and similar results were obtained.

Fig. 6. Dynamics of pathogenesis-related (PR) gene expression during the rapid symptom-progress stage in tobacco plants infected with *Potato virus Y* isolate N:O (PVY^{N:O}) Mb58. Tobacco cv. Samsun seedlings were sprayed with H₂O or 1 mM salicylic acid (SA) 3 days prior to mechanical inoculation with the virus or mock (M), and were sprayed continuously in the greenhouse until sampled at 7, 10, 12, and 14 days postinoculation (dpi). RNA from three leaves (position +1 to +3) emerging above the inoculated leaves (position 0) or stem cortex tissue from bottom to three nodes above the inoculated leaves was used to analyze the PR gene expression. Duplex semiquantitative reverse transcription-polymerase chain reactions (RT-PCR) including the primer pairs targeting the cytochrome c oxidase subunit I (*COX1*) and the target gene were performed. Hybridization with the corresponding probes was performed after gel electrophoresis and blotting. Data shown were from 15 cycles of PCR with one representing *COX1*. The experiments were repeated twice, and similar results were obtained.

ment and PVY replication/accumulation are closely related. The reduced necrotic symptoms in the stems of SA-treated plants were at least partially attributed to the inhibitory effects of SA on PVY replication/transportation. Unlike the TMV- and CMV-tobacco systems where SA exhibits prolonged inhibitory effects on accumulation of the viruses (27,32), exogenous SA only demonstrated temporary inhibitory effects on PVY accumulation. These results suggest that PVY may have a mechanism to evade SA-mediated suppression of its replication and/or movement. The observation that there is no correlation between SA basal level and PVY^{NTN} resistance and, moreover, PVY^{NTN} infection results in elevated endogenous SA concentration in a susceptible potato cultivar (19) to some extent supports the evading hypothesis.

Elevated expression of PR genes and increased activity of ROS-associated enzymes are typical features of SAR (10,37,43). This study also demonstrated that PR genes and ROS-associated enzymes are correlated with SA-mediated suppression of PVY^{N:O}-induced systemic development of symptoms in tobacco plants. SA elevated POX activity and *PR-1a* and *PR-1b* expression in mock-inoculated plants and/or in PVY-infected plants during the course of virus-plant interaction at the rapid symptom-progress stage (Figs. 5 and 6), despite the fact that PR-1 proteins may not play a role in SAR against viruses (4). It is also interesting to note that, at the rapid symptom-progress stage, POX activity and *PR-1a* and *PR-1b* expression increased as the virus disease and systemic symptoms progressed. A set of PR genes, including *PR-1*, are elicited in susceptible *Arabidopsis* plants by several viruses including *Turnip mosaic virus*, a member of the genus *Potyvirus* (48). *Plum pox virus*, another potyvirus, also demonstrates its ability to enhance the abundance of *PR-1* transcript in leaves of susceptible peach plants (46). Given the fact that PVY^{NTN} can induce SA in susceptible potato plants (19), it is reasonable to speculate that the systemic infection of susceptible tobacco plants by PVY^{N:O} might have induced the endogenous SA, which in turn might have resulted in the induction of *PR-1* genes and POX. It does not, however, rule out the possibility that uncharacterized signaling pathways may be responsible for the regulation of PR genes in plants as suggested elsewhere (46,48).

In summary, this work demonstrates that SA-mediated and ethylene-independent SAR plays an important role in suppression of PVY^{N:O}-elicited necrotic symptoms in stems of tobacco plants. Given the complexity of PVY and PVY-host interactions as evidenced in this study as well as other studies (3,8,14,16,17,19,30,31), further research is needed to elucidate the mechanism(s) involved in SA-mediated resistance against the virus as well as the partial recovery and symptom-shifting phenomena in susceptible plants after systemic infection with the virus.

ACKNOWLEDGMENTS

I thank T. Molen for excellent technical assistance; K. Corey for initial greenhouse work and symptom observation; and H. Tai for critically reviewing the manuscript prior to submission.

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