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Down-regulation of Antioxidative Capacity in a Transgenic Tobacco which Fails to Develop Acquired Resistance to Necrotization Caused by TMV

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Antioxidant status was assayed in leaves of two local lesion hosts of tobacco mosaic virus (TMV), namely in wild-type Xanthi-nc tobacco and in NahG transgenic tobacco, the latter of which is not able to accumulate salicylic acid (SA) and therefore is unable to develop systemic acquired resistance (SAR). Activities of several enzymes related to antioxidative defense, and the levels of glutathione, chlorogenic acid and rutin were studied. The majority of antioxidant enzymes were less active in uninfected NahG tobacco than in Xanthi-nc. Furthermore, important enzymatic and non-enzymatic antioxidants were down-regulated in TMV-infected NahG plants, as compared to Xanthi-nc. Correspondingly, SA pretreatment primed the leaves for stronger induction of antioxidants in infected Xanthi-nc, but not in NahG tobaccos. The antioxidant status of NahG tobacco even decreased after an attempted induction of SAR, while the antioxidative level increased in Xanthi-nc leaves in which the SAR was successfully induced. After infection, a greater accumulation of superoxide and H_2O_2, and a more intensive necrotization was positively correlated with the reduced capability of NahG leaf tissue to detoxify reactive oxygen species.

Keywords: Antioxidant; Necrotic lesion; *Nicotiana tabacum* L; Reactive oxygen species; Tobacco mosaic virus

Abbreviations: AA, ascorbic acid; APX, ascorbate peroxidase; CAT, catalase; CGA, chlorogenic acid; DAB, 3,3-diaminobenzidine; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; DPI, diphenyleneiodonium chloride; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; HR, hypersensitive reaction; NBT, nitroblue tetrazolium (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3-3′-dimethoxy-4,4′-diphenylene(-ditetrazolium chloride); PAL, phenylalanine ammonia-lyase; POX, guaiacol peroxidase; PPO, polyphenol oxidase; QR, quinone reductase; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase; TMV, tobacco mosaic virus

INTRODUCTION

Systemic acquired resistance (SAR) in tobacco to necrotic lesions caused by tobacco mosaic virus (TMV) was first demonstrated by Ross.[1] Inoculation of one leaf of a local lesion host with the virus increased resistance of the remote leaves on the same plant to a lesion development after a subsequent TMV infection. SAR was expressed as a reduction in the number and size of virus-induced local lesions.[1] The most important progress in our understanding of biochemical events leading to SAR has been the discovery of salicylic acid (SA)-mediated signaling.[2] Following inoculation with the virus, SA accumulated at the site of infection, and later in distal parts of plants. Development of SAR has been shown to be associated with a coordinate expression of a set of genes.[3] Exogenous application of SA induced the same set of genes and spectrum of resistance specificities as found in biologically induced SAR.[3] Furthermore, transgenic *Arabidopsis* and tobacco plants were engineered to express a bacterial salicylate hydroxylase gene, whose product converts SA to catechol. This type of transgenic plants, which cannot accumulate SA, not only failed to develop SAR but also exhibited increased susceptibility to necrotization, as compared to the wild-type

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tobacco.[4] In the transgenic NahG tobacco, TMV-induced necrotization often spreads through the petiole to the stem and the adjacent leaves and this is associated with the presence of viral particles.[5] These results demonstrated that SA is crucial for both SAR and the primary resistance response to pathogen infection.

Infection of plants with necrotizing pathogens often results in rapid accumulation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) referred to as oxidative burst.[6] Likewise, TMV-induced cell death in tobacco has been proven to be associated with an oxidative burst.[7,8] Previous studies have suggested that SA may potentiate the oxidative burst and may increase cell death program in combination with ROS.[9,10] An early report showed that antioxidants were effective against TMV-induced tissue necrotization in tobacco.[11] Several observations have supported the potential function of plant antioxidants during resistant responses but many questions about their finer function and regulation remained unanswered.[12,13] To elucidate the role of ROS and antioxidants in SAR, SA-induced and TMV-initiated responses were assayed in the *N* (necrotic) gene-containing Xanthi-nc tobacco and its transgenic line expressing the *nahG* gene, which fails to develop SAR. Accumulation of $O_2^{\bullet -}$ and H_2O_2, levels of reduced and oxidized glutathione (GSH, GSSG), ascorbic acid, chlorogenic acid (CGA) and rutin, as well as activities of superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6), ascorbate peroxidase (APX, E.C. 1.11.1.11), glutathione reductase (GR, E.C. 1.6.4.2), glutathione *S*-transferase (GST, E.C. 2.5.1.18), dehydroascorbate reductase (E.C. 1.8.5.1), guaiacol peroxidase (POX, E.C. 1.11.1.7), quinone reductase (QR, E.C. 1.6.99.2) and polyphenol oxidase (PPO, E.C. 1.10.3.1) were assayed.

MATERIALS AND METHODS

Plant Materials and Treatments

Tobacco (*Nicotiana tabacum* L.) cultivar Xanthi-nc, which is a local lesion (HR) producing host of TMV and the genetically transformed Xanthi-nc carrying the *nahG* transgene were planted into soil and grown in the greenhouse (18–23°C, 160 mE m^{-1} s^{-1} for 8 h d^{-1} supplemental light, 75–80% RH). Seeds of transgenic line NahG-10 were kindly provided by NOVARTIS, Agricultural Biotechnology Research, Research Triangle Park, NC, USA.

The third and fourth true leaves of 8-week-old plants were inoculated with a suspension of the U_1 strain of TMV. Systemic responses were assayed in the fifth and sixth leaves 2 weeks after infection of the lower leaves. Visible symptoms and levels of ROS were also investigated after a second challenge TMV inoculation on the upper fifth and sixth leaves 2 weeks after the first infection of the lower leaves. Mock-inoculated plants were used as controls. TMV inoculation was carried out as described by Fodor *et al.*[14] except that abrasion was not used.

In separate experiments we tested the alterations of antioxidant enzymes and non-enzymatic antioxidants in TMV-infected leaves of wild-type Xanthi-nc, SA-deficient transgenic NahG, and SA-pretreated wild-type Xanthi-nc tobacco plants. Interveinal areas of the fifth and sixth leaves of Xanthi-nc plants were injected with 0.8 mM sodium salicylate (pH 7.0) by a hypodermic syringe until the leaf laminae were completely saturated. SA-injected leaves were inoculated by TMV 10 days after the treatment. Water-injected plants were used as controls.

To evaluate the inhibitory effect of diphenyleneiodonium chloride (DPI) on necrotic symptoms, discs ($d = 2$ cm) of fifth and sixth leaves of Xanthi-nc and NahG plants were floated on 5 μM DPI solution for 2 h 1 day post-inoculation. Control leaf discs were floated on water. After 2 h, leaf discs were placed on 0.5% agar plates and kept in the greenhouse until the visual inspection of tissue necrotization. Similarly, NaN_3 (2 mM) was used as inhibitor of POX. Unless stated otherwise, chemicals were purchased from Sigma–Aldrich Chemicals Inc. (St. Louis, MO, USA).

Determination of Antioxidants

To measure activities of SOD, CAT, GST, GR, APX, and DHAR and the levels of ascorbate and glutathione, we applied the methods detailed in our former experiments.[14] For the detection of antioxidant enzyme activities, 0.5 g leaf material was homogenized at 0–4°C in 3 ml of 50 mM TRIS buffer (pH 7.8), containing 1 mM EDTA-Na_2 and 7.5% polyvinylpyrrolidone. The homogenates were centrifuged (12,000 rpm, 20 min, 4°C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant. All measurements were carried out at 25°C, using a model UV 160A spectrophotometer (Shimadzu, Japan). Activity of POX was assayed by measuring the formation of the conjugate product of guaiacol at 436 nm. The reaction mixture (2.45 ml) contained 0.1 M sodium phosphate buffer (pH 6.0), 2 mM guaiacol, 1 mM H_2O_2, and 50 μl of leaf extract. The increase in A_{436} was measured as the conjugate was formed using an extinction coefficient of 26.6 mM^{-1} cm^{-1} for the conjugate. QR activity was measured in a reaction mixture (2.4 ml) contained 50 mM TRIS buffer (pH 7.5), 0.08% Triton X-100, 2.5 mM NADPH, 30 μM menadion, and 50 μl of leaf extract. QR activity was calculated using an extinction coefficient

of 6.2 $mM^{-1}\,cm^{-1}$ for NADPH. To measure PPO activity, 0.5 g leaf material was homogenized at 0–4°C in 2 ml 0.1 M phosphate buffer (pH 6.0) with 1 mM EDTA-Na_2. The homogenates were centrifuged (12,000 rpm, 20 min, 4°C), and the total soluble enzyme activities were measured spectrophotometrically in the supernatant at 25°C. PPO activity was assayed in a reaction mixture (2.2 ml) consisted of 0.1 M sodium phosphate buffer (pH 6.0), 1 mM EDTA-Na_2, 20 mM catechol, and 200 µl of leaf extract. The increase in A_{390} was measured as catechol was oxidized. PPO activity was calculated using an extinction coefficient of 0.95 $mM^{-1}\,cm^{-1}$ for the quinone product.

CGA and rutin were separated by thin layer chromatography. Leaf discs (1.0 g) were incubated in 3.0 ml of methanol on a rotary shaker (200 rpm, 2 days, 25°C). Then leaf extracts were separated on glass-backed silica plates (Kieselgel 60, Merck, Germany). Thin layer chromatography plates were activated by heating for 2 h at 80°C. Plates were spotted with 20 µl of sample and developed using ethyl acetate–acetic acid–formic acid–water (100:11:11:27, v/v). After chromatography, the plates were dried at 40°C in an air oven and sprayed with 1% methanolic solution of 2-aminoethyl diphenylborinate (Fluka). After drying, plates were sprayed with 5% ethanolic solution of PEG 4000 and dried again. Polyphenols were visualized under UV light and CGA and rutin were identified by R_F values (CGA: 0.45, rutin: 0.40) and specific staining (CGA: blue, rutin: orange), utilizing comigration with reference compounds. The spots were immediately analyzed with CS-930 densitometer (Shimadzu, Japan) at 340 nm. CGA and rutin contents were calculated on the basis of standard curves.

Histochemical Analysis of ROS

Histochemical staining for superoxide production in leaf tissue was based on the ability of $O_2^{\bullet -}$ to reduce NBT. Leaf discs were vacuum infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 w/v % NBT.[15] NBT-treated samples were incubated under daylight for 15 min and subsequently cleared in 0.15 w/v % trichloroacetic acid in ethylalcohol:chloroform 4:1 v/v for 1 day.[16] Catalase (2000 U ml^{-1}) and SOD (1600 U ml^{-1}) were put into the NBT staining solution to control the specificity of staining. Cleared samples were washed with water and placed in 50% glycerol in water prior to microscopic evaluation. $O_2^{\bullet -}$ was visualized as a purple coloration, and detected by MC 80 binocular stereomicroscope (Zeiss, Jena, Germany).

The solution for the histochemical detection of H_2O_2 contained 0.1% DAB (pH 3.8).[16] After vacuum infiltration, samples were incubated under daylight for 30 min and treated as described above.

Effects of DPI and NaN_3 on NBT and DAB staining were quantified photometrically using Shimadzu CS-930 scanner at 560 and 470 nm, respectively. Treatments with inhibitors were carried out as described above.

Statistics

At least three independent parallel experiments were carried out in each case. Two leaves from three different plants were individually tested in each experiment. Statistical analyses were performed by Student's *t*-test of the differences between two means. Differences were considered to be significant at $P = 0.05$ level.

RESULTS

Symptom Development in Wild-type and NahG Transgenic Tobaccos

Symptom development was monitored by visual inspection of TMV-infected leaves of Xanthi-nc and NahG plants. The first necrotic lesions became visible at around 40 h post-inoculation on the leaves of Xanthi-nc tobaccos. SA depletion in NahG tobacco delayed the timing of appearance of lesion development by 4–6 h, as compared to wild-type plants. However, the spreading of necrotic lesions was faster in NahG plants, therefore they produced significantly larger lesions upon TMV-infection, as compared to Xanthi-nc. Number of necrotic spots was also higher on NahG leaves (Table I).

TABLE I Effects of induction of SAR on number and size of necrotic lesions in TMV-infected tobacco plants

	Lesion size (mm)		Number of lesions (cm^{-2})	
Treatment	Xanthi-nc	NahG	Xanthi-nc	NahG
Control	1.49 ± 0.38	2.23 ± 0.47	7.68 ± 2.30	9.75 ± 2.15
TMV	0.72 ± 0.27*	2.18 ± 0.41	4.30 ± 1.74*	9.12 ± 1.94
SA	0.44 ± 0.15*	2.11 ± 0.36	2.36 ± 0.98*	8.89 ± 2.33

Number and size of TMV lesions were inspected visually on the fifth and sixth leaves of 10-week-old tobaccos. Induction of SAR was carried out by TMV infection on the third and fourth true leaves of 8-week-old plants. The fifth and sixth leaves were injected with SA 10 days before inoculation of the same leaves. TMV lesion size (mm) and lesion number were detected 3 days after inoculation. Values are means ± SD based on three independent assays. We measured 90 lesions from three plants in each experiment. *Significant difference between control and induced plants ($P < 0.01$).

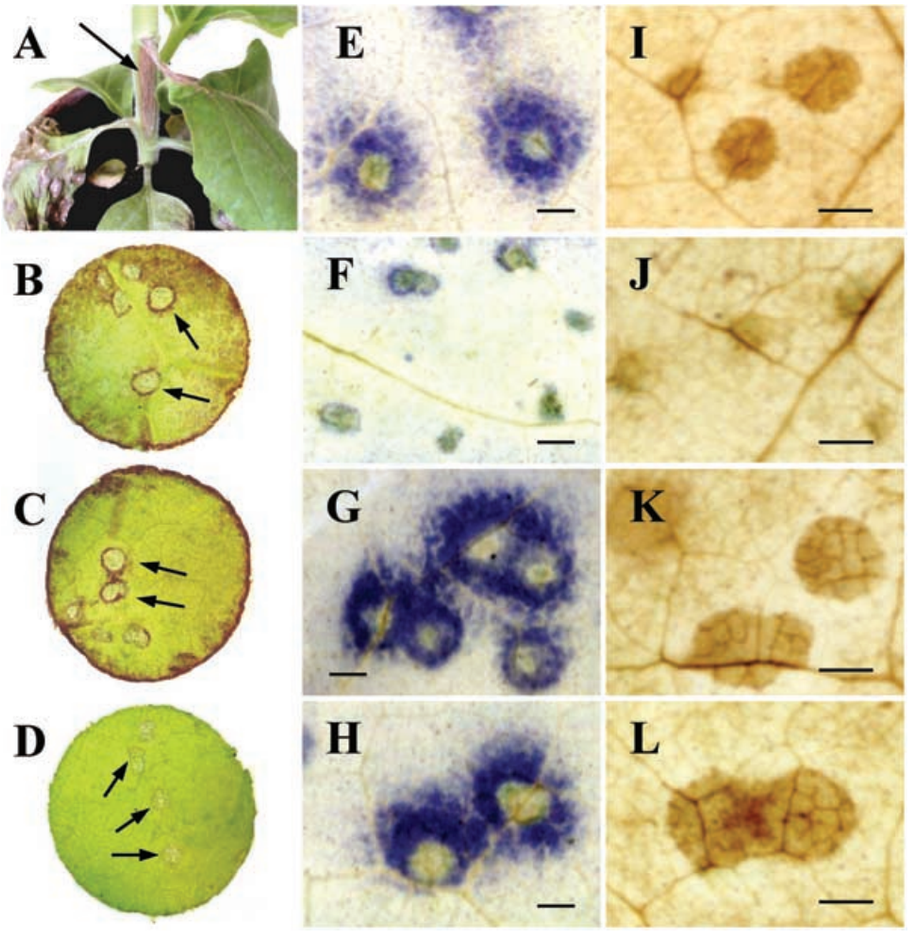

FIGURE 1 (A) TMV-induced necrosis spreads to the stem and to the uninfected upper leaf in a NahG plant. The arrow indicates necrosis on the stem. Superoxide-specific NBT staining in leaf discs ($d = 2$ cm) of Xanthi-nc leaves 2 days after TMV infection: (B) untreated; (C) treated with 2000 U ml^{-1} catalase; (D): treated with 1600 U ml^{-1} SOD. Arrows indicate the NBT-stained rings around necrotic spots. (E–H) Superoxide indicative blue formazan; and (I–L) H_2O_2-indicative reddish-brown DAB staining in tobacco leaves 2 days after TMV infection. Chlorophyll was removed after staining. Specific staining are seen at the sites of primary infection of wild-type Xanthi-nc (E and I) and NahG (G and K) tobaccos as well as the second challenge infection of wild-type Xanthi-nc (F and J) and NahG (H and L) plants. Challenge infection was carried out on the fifth and sixth upper leaves 2 weeks after the first inoculation on the lower leaves. Dyes were visualized using an MC 80 binocular stereomicroscope (Zeiss, Jena, Germany). Bar represents 1 mm.

Tissue necrotization in NahG plants has often extended from the inoculated leaves to the stem and the adjacent leaves (Fig. 1A).

Preinoculation of lower leaves with TMV induced SAR in remote leaves of Xanthi-nc tobacco, which resulted in decreased number and size of visible lesions (Table I). Pretreatment of leaves of Xanthi-nc with SA also induced resistance to a subsequent infection by TMV on the same leaf. SA-induced resistance was confined to the pretreated Xanthi-nc leaves. However, neither TMV infection nor SA treatment were able to induce SAR in NahG plants (Table I).

Both number and size of necrotic spots dropped in TMV-infected Xanthi-nc and NahG leaves following treatment with DPI (Table II). Considering both

TABLE II Inhibitory effect of DPI on number and size of necrotic lesions in TMV-infected tobacco leaves

	Lesion size (mm)		Number of lesions (cm^{-2})	
Treatment	Xanthi-nc	NahG	Xanthi-nc	NahG
H_2O	1.34 ± 0.32	2.06 ± 0.37	5.84 ± 2.12	6.21 ± 1.97
DPI	0.48 ± 0.11*	0.62 ± 0.16*	3.31 ± 1.35*	3.14 ± 1.11*

The fifth and sixth leaves of 10-week-old tobaccos were inoculated with TMV. One day after inoculation, eight leaf discs ($d = 2$ cm) were cut from each tobacco plant and incubated for 2 h with 5 μM DPI and then placed on agar plates. Control treatments used water alone. Number and size (mm) of viral lesions were calculated 3 days after inoculation. Values are means ± SD based on three independent assays. *Significant difference between control and DPI-treated plants ($P < 0.01$).

factors, necrotic lesion area decreased substantially, as compared to the water-treated control. Conversely, development of viral necrotic lesions was not affected significantly by treatment with sodium azide (data not shown).

Superoxide Burst

$O_2^{\bullet -}$ accumulated in the close vicinity of TMV lesions (Fig. 1B). Generation of $O_2^{\bullet -}$ was typically characteristic of living cells in the neighborhood of dead cells as it was detected by a double-staining procedure with NBT and Evans blue (data not shown). NBT reduction could be inhibited by SOD (1600 U ml^{-1}) in the staining mixture (Fig. 1D), however, CAT (2000 U ml^{-1}) did not affect the coloration (Fig. 1C).

NahG plants showed stronger NBT staining in TMV-infected leaves, whether or not they had been pre-inoculated with TMV on their lower leaves (Fig. 1G,H). Somewhat less $O_2^{\bullet -}$ accumulated around the viral lesions in Xanthi-nc leaves (Fig. 1E) than in the leaves of NahG plants that produced larger necroses. Importantly, induction of SAR in Xanthi-nc plants by a prior TMV infection of their lower leaves, led to reduced NBT staining in the TMV-infected upper leaves (Fig. 1F). Similar decrease in formazan deposition was found in those Xanthi-nc leaves, which were pretreated with SA before inoculation (data not shown). These results indicate a close correlation between the intensity of the $O_2^{\bullet -}$ production and the size of necrotic lesions caused by infection with TMV.

Pretreatment of TMV-infected Xanthi-nc leaves with DPI inhibited NBT staining by about 50% 2 days post-inoculation. This result is consistent with the effect of DPI on necrotic symptoms, since area of necrotic lesion was largely reduced in DPI-treated Xanthi-nc leaves. However, NBT staining was not affected by the peroxidase inhibitor NaN_3 in TMV-infected Xanthi-nc leaves.

H_2O_2 Accumulation

Inoculation of leaves with TMV resulted in H_2O_2 burst in both Xanthi-nc and NahG plants (Fig. 1I–L). The most intensive DAB staining was seen where cell death occurred. Expression of *nahG* gene led to extensive DAB polymerization as a result of larger necrotic lesions on NahG leaves, which are relatively more susceptible to necrotization (Fig. 1K,L). Accumulation of H_2O_2 in TMV-infected tobacco leaves suggests that appearance of visible lesions is accompanied by the evolution of H_2O_2.

Induction of SAR in the upper leaves of Xanthi-nc tobacco by a prior inoculation on their lower leaves decreased the intensity of DAB coloration caused by the second TMV challenge (Fig. 1J). The same was true for the action of SA-pretreatment on H_2O_2 burst (data not shown). The attempted induction of SAR in the NahG plants failed to decrease the burst of H_2O_2 in the upper leaves after the challenge inoculation (Fig. 1L).

The DAB-staining was also inhibited in DPI-treated leaves of TMV-infected Xanthi-nc tobacco by 50%. This result shows that accumulation of H_2O_2 mainly derives from dismutation of superoxide.

Antioxidants in Xanthi-nc and NahG Tobaccos

Activities of SOD, CAT, GST, GR antioxidant enzymes and the PPO were significantly lower in healthy, untreated and uninfected NahG tobaccos than in the wild-type Xanthi-nc, while APX, DHAR and QR activities were similar in NahG and Xanthi-nc plants (Fig. 2). None of the investigated enzymes were more active in plants expressing the *nahG* gene.

The level of ascorbate and GSH were somewhat lower in the untreated and uninfected leaves of NahG plants, as compared to wild-type Xanthi-nc tobacco (Fig. 2), but the ratio of GSH to GSSG and amounts of extractable phenolic compounds, the major ones being CGA and the flavonoid glycoside rutin,[17] were higher in NahG leaves than those in the wild-type Xanthi-nc plants (Figs. 2 and 3).

After TMV infection the ratio of GSH to GSSG increased in inoculated Xanthi-nc leaves, but it dropped in NahG plants 2 days after TMV infection, and remained significantly lower, as compared to Xanthi-nc tobacco (Fig. 3).

SA Pretreatment Primes the Induction of Antioxidants after TMV Infection

In the case of the TMV-infected leaves of SA-pretreated plants, pretreatment of Xanthi-nc tobaccos with SA strongly potentiated the induction of each antioxidant enzyme after TMV infection (Figs. 4–7). SA pretreatment not only enhanced the magnitude of stimulation of antioxidant enzymes, but it also prevented the transient decrease of antioxidant enzyme activities typical of infected wild-type Xanthi-nc tobacco. However, the pro-oxidant PPO was not affected by SA pretreatment (Fig. 7). This kind of predisposition of enzymes was not experienced in NahG plants: SA treatment did not exert significant effects on any enzyme activities in the NahG tobacco (data not shown).

Pretreatment of Xanthi-nc leaves with SA resulted in considerably higher contents of CGA, rutin and GSH at 1 and 2 days after inoculation with TMV (Figs. 5 and 7). In contrast to untreated Xanthi-nc and NahG, ascorbate content did not decrease in SA-pretreated Xanthi-nc leaves at later stages of infection (Fig. 6). SA treatment did not cause

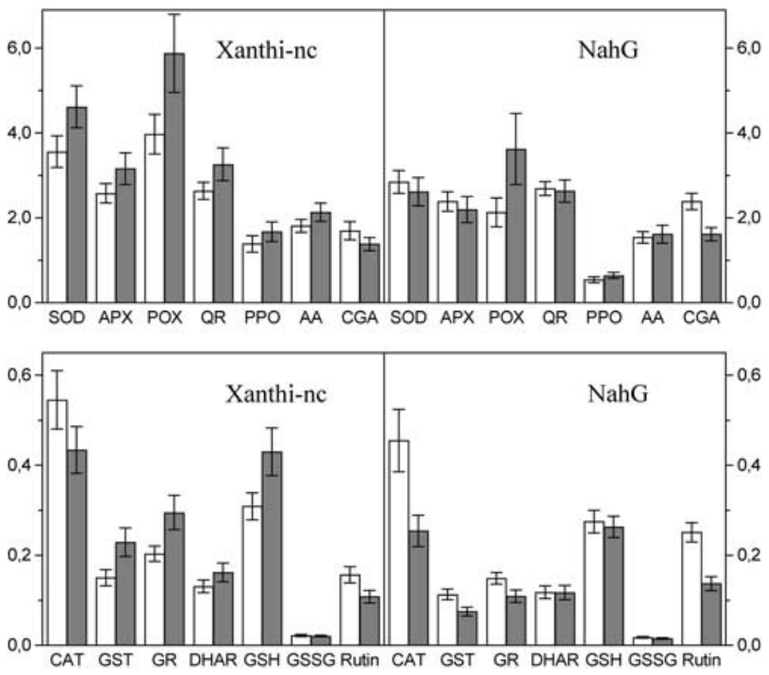

FIGURE 2 Changes in SOD (units/mg FW), APX (μM ascorbate/g FW min), POX (μM conjugate/g FW min), QR (μM NADPH/g FW min), PPO (μM catechol/g FW min), ascorbic acid (μM/g FW), CGA (mg/g FW), CAT (mM H_2O_2/g FW min), GST (μM conjugate/g FW min), GR (μM NADPH/g FW min), DHAR (μM dehydroascorbate/g FW min), GSH (μM/g FW), GSSG (μM/g FW), and rutin (mg/g FW) levels in the uninfected upper leaves of TMV-infected tobacco plants. The fifth and sixth true leaves of 10-week-old plants were tested 2 weeks after the inoculation of the third and fourth lower leaves. Mock-inoculated Xanthi-nc and NahG plants were used as controls. Means of three independent experiments are shown. At least three independently processed samples were analyzed in each experiment. Open bars are the data of control plants and gray bars represent the systemic effects of TMV infection of lower leaves ± SD ($n = 9$).

significant changes in the levels of the antioxidant substances measured in NahG leaves (data not shown).

Changes in Antioxidants in the Uninfected Upper Leaves of Tobacco Plants after TMV-infection on Their Lower Leaves

Activities of several antioxidant enzymes, such as SOD, POX, GST, GR, APX, DHAR and QR were stimulated systemically in the upper leaves of Xanthi-nc plants following TMV infection on the lower leaves (Fig. 2). Only activity of CAT decreased in upper leaves of Xanthi-nc, but much reduced levels of CAT were detected in upper NahG leaves that did not exhibit SAR. Activities of GST and GR also decreased and only POX became more active in upper leaves of NahG tobaccos (Fig. 2).

GSH and ascorbate levels increased in uninfected upper leaves of Xanthi-nc tobacco plants as a result of infection with TMV on their lower leaves. In contrast, they did not change systemically in TMV-infected NahG plants, in which the SAR did not develop (Fig. 2). Levels of GSSG did not exhibit significant changes in upper leaves of Xanthi-nc and NahG plants. Thus, ratio of GSH to GSSG showed a systemic increase in Xanthi-nc, but not in NahG leaves. As a result of TMV infection on the lower leaves, levels of CGA and rutin decreased in the upper leaves of Xanthi-nc

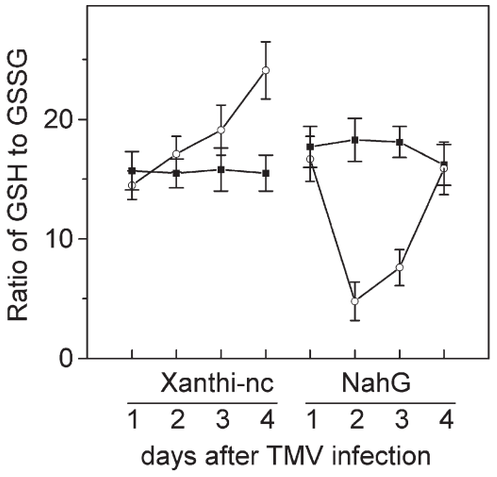

FIGURE 3 Ratio of reduced form of glutathione to oxidized glutathione (GSH:GSSG) in TMV-infected fifth and sixth true leaves of wild-type Xanthi-nc and transgenic NahG tobaccos. Means of three independent experiments are shown. At least three independently processed samples were analyzed in each experiment. Error bars represent ± SD ($n = 9$). (○) TMV-infected leaves; (■) mock-inoculated leaves.

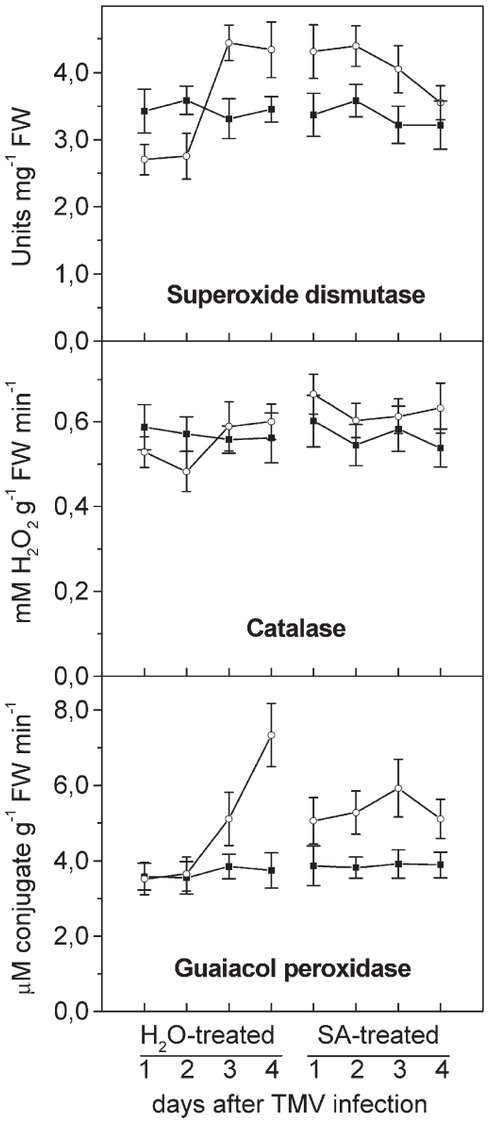

FIGURE 4 Activities of SOD, CAT, and POX in TMV-inoculated leaves of wild-type Xanthi-nc tobaccos with and without SA pretreatment. SA-pretreated and water-injected leaves of wild-type Xanthi-nc tobacco plants were tested. The fifth and sixth true leaves were inoculated with TMV 10 days after the injection of the same leaves with 0.8 mM SA. Means of three independent experiments are shown. At least three independently processed samples were analyzed in each experiment. Error bars represent ±SD ($n = 9$). (○) TMV-infected leaves; (■) mock-inoculated leaves.

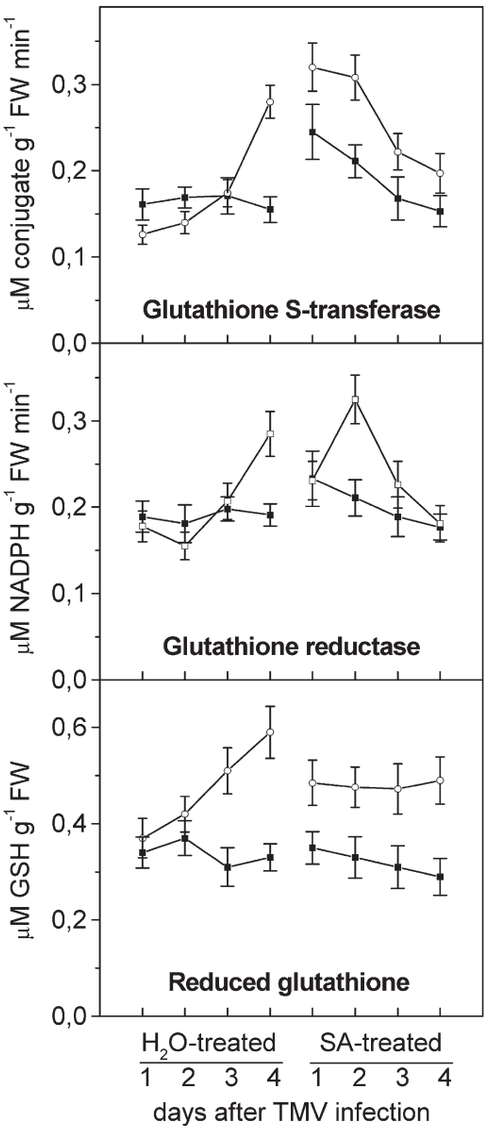

FIGURE 5 Activities of GST and GR and levels of GSH in SA-pretreated Xanthi-nc leaves upon TMV infection. See legend to Fig. 4 for details.

plants (Fig. 2). Interestingly, the decrease in CGA and rutin levels was somewhat stronger in NahG leaves.

DISCUSSION

According to Halliwell and Gutteridge,[18] the only evidence that oxidative stress has occurred *in vivo* may be the up-regulation of antioxidant defense systems, since antioxidants are capable of removing the excess of potentially damaging ROS. The assumption that ROS may have role in necrotic lesion development in plants and elevated antioxidant activities suppress the oxidative stress has prompted us to investigate the antioxidative defense responses of TMV-infected tobacco leaves.

Basic Levels of Antioxidants are Lower in NahG than in Xanthi-nc Tobacco

Activities of several enzymes such as SOD, CAT, POX, GST, GR, and PPO were lower in NahG, as compared to Xanthi-nc leaves (Fig. 2). Since NahG tobacco is deficient in SA accumulation, one can suppose that SA may stimulate activities of these

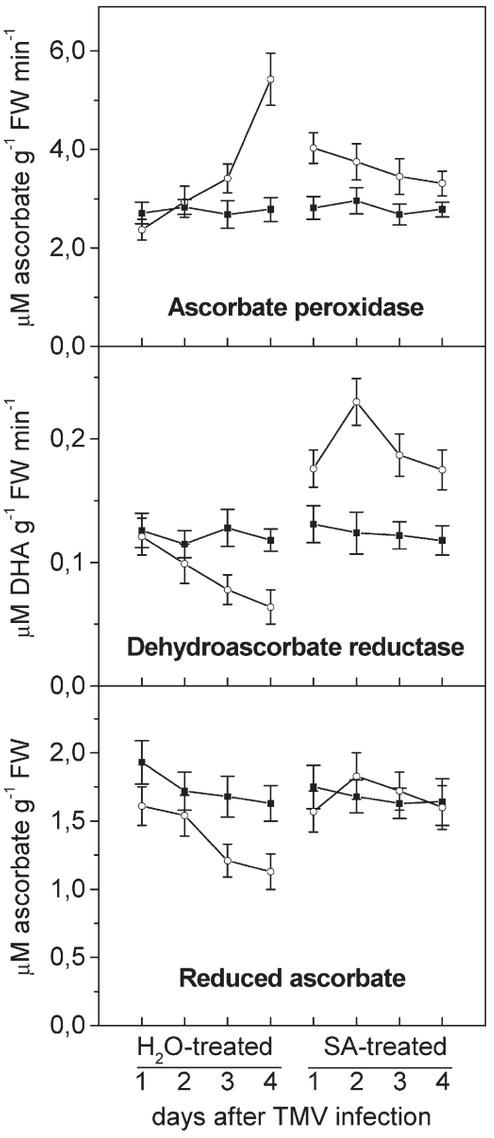

FIGURE 6 Activities of APX and DHAR and ascorbate levels in SA-pretreated Xanthi-nc leaves upon TMV infection. See legend to Fig. 4 for details.

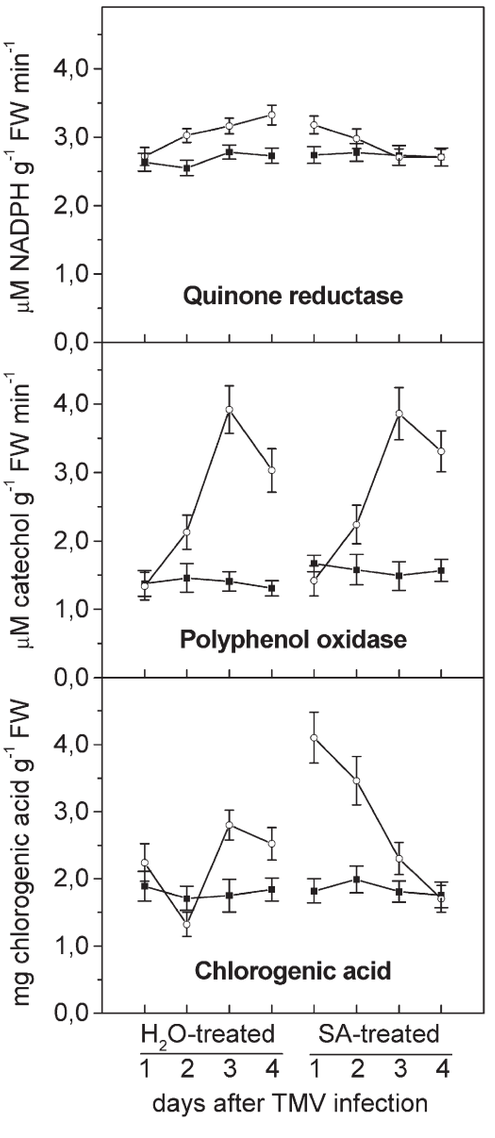

FIGURE 7 Activities of QR and PPO and levels of CGA in SA-pretreated Xanthi-nc leaves upon TMV infection. See legend to Fig. 4 for details.

enzymes. Indeed, exogenous application of SA induced activities of these enzymes.[19–23]

Ascorbate and glutathione contents were slightly lower in leaves of NahG as compared to Xanthi-nc. However, ratio of GSH to GSSG, which is an informative marker of cellular redox state, was somewhat higher in NahG leaves, probably because PPO and POX, which can oxidize polyphenols and thiols were down-regulated in NahG plants.

Antioxidative Defense is Incomplete in TMV-infected Lower Leaves of NahG Tobacco

Previous studies have shown that suppression of antioxidative defense during the interaction of plants with necrotizing pathogens can contribute to induction of cell death.[24–26] Inoculated NahG lower leaves showed a marked drop in the redox balance of glutathione, which could be critical for induction of cell death (Fig. 3). The hypothesis that SA accumulation is indispensable for keeping GSH:GSSG ratio under stress conditions has been confirmed by the observation that this ratio was largely reduced in NahG *Arabidopsis* plants after ozone fumigation, as compared to the wild-type plant.[27]

Recent reports have demonstrated that living cells around the pathogen-induced necroses exhibited significant induction of several antioxidative enzymes,[12,25,28] which may contribute to the restriction of necrotic lesions. In conclusion, the

down-regulation of antioxidant capacity in infected NahG leaves may contribute to the development of large necroses (and the lack of SAR after induction of resistance in this transgenic tobacco).

SA Potentiates Induction of Antioxidants

SA pretreatment potentiated induction of several defense genes after pathogenic attack.[29] Likewise, induction of each antioxidant enzyme assayed in our experiments with TMV-infected Xanthi-nc was enhanced by SA pretreatment (Figs. 4–7). The early and apparent induction of antioxidants in leaves possessing SAR may contribute to the lower rate of tissue necrotization after TMV infection by detoxifying the harmful ROS. However, exogenous SA was not able to increase activity of antioxidants and did not reduce the necrotization caused by TMV in the treated NahG tobaccos. Furthermore, the ratio of GSH to GSSG was higher and all of the investigated non-enzymatic antioxidants, such as GSH, ascorbate, CGA and rutin were also up-regulated in SA-pretreated and infected Xanthi-nc leaves (Fig. 2). Several natural phenolics, such as CGA are excellent antioxidants.[18,30] Previous results have shown that the absence of phenolics decreases the ability of cells to withstand ROS.[31] These results have suggested that both local and systemic resistance against necrotization may be related to the cellular levels of phenols. However, ascorbate and glutathione are important regulators that keep the redox state of phenolic compounds under control.[11,32]

Differences in Systemic Changes of Antioxidants Between Xanthi-nc and NahG Tobaccos

The level of GSH, activities of GSH-related enzymes, and, to a lesser extent, the level of ascorbate and activities of ascorbate-related enzymes and SOD increased in the uninfected upper leaves of Xanthi-nc concomitantly with the development of SAR.[14] Conversely, here we show that no increase in the levels of any of these antioxidants were observed systemically in the upper leaves of NahG tobacco, in which we tried to induce SAR by inoculating the lower leaves with TMV (Fig. 2).

It has been shown that SAR was expressed by a reduction in necrotic symptoms caused by viruses and bacteria rather than by a restriction of pathogenic growth.[33,34] This may be one factor in the manifestation of SAR. With regard to the concerted action of several antioxidants, we infer that small but coincident changes in the activities of antioxidants can contribute to resistance against oxidative cellular damage in Xanthi-nc tobacco after development of SAR. However, in NahG tobaccos, which lack the ability to develop SAR, we did not find stimulation of any antioxidants, except POX.

Oxidative Burst

ROS from the oxidative burst have been postulated to play a central role in triggering hypersensitive cell death and induction of defense genes in challenged cells.[12] Jabs *et al.*[35] demonstrated that $O_2^{\bullet -}$ was the key component in controlling cell death in a mutant *Arabidopsis thaliana* plant.

Accordingly, our data indicate that $O_2^{\bullet -}$ could be implicated in cell death induction, because the rate of TMV-induced tissue necrotization decreased in the presence of DPI, which inhibited accumulation of $O_2^{\bullet -}$ by 50%. DPI is an inhibitor of membrane bound NADPH oxidases that are proposed to be responsible for the synthesis of $O_2^{\bullet -}$ in plants.[12,35] Furthermore, accumulation of $O_2^{\bullet -}$ positively correlated with the rate of lesion growth in our studies with tobacco and TMV (Table II and Fig. 1E–H). Taken together, these results suggest that $O_2^{\bullet -}$ plays a pivotal role in the induction of cell death under these conditions. Our results with DPI and NaN_3 support the idea that NADPH oxidase, and not POX, is responsible, partly at least, for $O_2^{\bullet -}$ generation around viral necrotic lesions.

Accumulation of H_2O_2 was also characteristic of TMV-infected tobacco leaves, as was shown with DAB staining. We demonstrated that DAB staining was inhibited by DPI, suggesting that accumulation of H_2O_2 mainly derives from dismutation of $O_2^{\bullet -}$. Hydrogen peroxide may act in multiple defense pathways. It has been implicated in the cross-linking reactions leading to cell wall strengthening, in signaling that results in activation of peroxidase isoforms and other "defense genes", as well as in direct killing of invading pathogens.[6,12]

The $O_2^{\bullet -}$ burst was strong, activity of SOD was stimulated and activity of CAT was inhibited in TMV-infected NahG leaves, consequently the DAB staining that is specific for H_2O_2 was stronger at sites of virus infection in NahG, as compared to Xanthi-nc plants (Fig. 1K,L). As was expected, induction of SAR in the upper leaves of Xanthi-nc resulted in reduced accumulation of DAB polymer at the sites of restricted necroses (Fig. 1J). However, DAB staining did not change in the upper leaves of NahG plants, which could not exhibit SAR (Fig. 1L). It has been also demonstrated[27] that NahG *Arabidopsis* plants showed increased susceptibility to ozone-induced tissue necrotization, as compared to wild-type plants.

Mechanism of Action of SA in Local and Systemic Resistance

At present, the mode of action of SA in local and systemic resistance remains a disputed question because contradictory results have been published. It has been shown that SA may stimulate oxidative

burst and host cell death.[9,36] However, Mittler *et al.*[37] have suggested that SA may not be directly involved in the activation of ROS during the formation of necrotic lesions in tobacco to TMV. It would seem that SA has a dual role stimulating antioxidants in addition to the acceleration of oxidative burst, because several authors[14–18] have found that SA induced antioxidant activity. It is noteworthy that in *Arabidopsis* exposed to ozone in the presence of low levels of SA induced antioxidants and tolerance to ozone but high SA levels triggered oxidative burst and host cell necrosis.[27]

In Xanthi-nc tobacco, which exhibits local type of resistance (HR), both accumulation of SA and activation of oxidative burst are associated with the formation of tissue necrotization.[2,7] Induction of antioxidants regularly happens *in vivo* as a response to the oxidative stress[12,20] and may not have role in resistance to virus, however, it restricts the spread of necrosis. In this respect it seems worth to mention that recently several laboratories have shown that host necrosis and pathogen arrest are separate responses in resistant plants.[10,38,39] SA seems to have another action on virus resistance.[40] It was demonstrated that SA is an inducer of alternative oxidase[41] and alternative oxidation plays a role in defense against ROS.[42] This would be another type of action of SA that results in virus arrest.

However, in the case of SAR, which primarily means induced resistance to necrotization caused by TMV, accumulation of low levels of SA and up-regulation of antioxidants are induced in the resistant leaves of our wild-type Xanthi-nc plants before the challenge infection. This early antioxidant defense induced by SA, is able to suppress tissue necrotization caused by a subsequent challenge infection. In Xanthi-nc tobacco, in which SAR was already induced, up-regulation of the antioxidant capacity is induced well before the challenge infection, and therefore, it can suppress necrotization at the beginning of lesion development. In this latter case, SA mainly acts through stimulation of antioxidants.

CONCLUSIONS

In contrast to the enhancement of the antioxidant status in wild-type Xanthi-nc possessing SAR, levels of antioxidants even decreased in NahG leaves after an attempted induction of SAR. SA may play a primary role in maintaining active antioxidant defense to detoxify harmful ROS, causing thereby development of SAR. Although strong correlations do not necessarily imply causation, down-regulation of antioxidant defense in NahG tobacco appears to be an important factor in its inability to reduce tissue necrotization and to develop SAR.

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