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Local and Systemic Responses of Antioxidants to Tobacco Mosaic Virus Infection and to Salicylic Acid in Tobacco[1]

Role in Systemic Acquired Resistance

József Fodor, Gábor Gullner, Attila L. Ádám, Balázs Barna, Tamás Kömives, and Zoltán Király*

Plant Protection Institute, Hungarian Academy of Sciences, P.O. Box 102, Hungary H-1525 Budapest

Changes in ascorbate and glutathione levels and in activities of ascorbate peroxidase, catalase, dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione *S*-transferase (GST), and superoxide dismutase (SOD) were investigated in tobacco mosaic virus (TMV)-inoculated lower leaves and in non-inoculated upper leaves of *Nicotiana tabacum* L. cv Xanthi-nc. In separate experiments the effects of exogenous salicylic acid (SA) were also studied. Symptom appearance after TMV inoculation was preceded by a slight, transient decline of ascorbate peroxidase, GR, GST, and SOD activities in the inoculated lower leaves, but after the onset of necrosis these activities and the glutathione level substantially increased. Ascorbic acid level and DHAR activity declined and dehydroascorbate accumulated in the inoculated leaves. In upper leaves, the glutathione level and the activities of GR, GST, and SOD increased 10 to 14 d after TMV inoculation of the lower leaves, concomitantly with the development of systemic acquired resistance. From the six distinct SOD isoenzymes found in tobacco leaves, only the activities of Cu,Zn-SOD isoenzymes were affected by TMV. SA injection induced DHAR, GR, GST, and SOD activities. Catalase activities were not modified by TMV infection or SA treatment. It is supposed that stimulated antioxidative processes contribute to the suppression of necrotic symptom development in leaves with systemic acquired resistance.

Infection of plants by necrogenic pathogens often results in enhanced protection against a challenge (second) infection, not only in the inoculated leaves but also systemically, in the healthy leaves located above the inoculated site. The biochemical mechanisms of these phenomena, referred to as local acquired resistance and SAR, are poorly understood (Kuc, 1982; Ryals et al., 1996). A substantial increase in endogenous SA content was observed in infected lower leaves and, to a lesser extent, in the uninfected upper leaves of plants following viral and fungal inoculations (Malamy et al., 1990, Métraux et al., 1990). The onset of SAR was accompanied by the systemic expression of a number of genes (SAR genes) encoding different stress proteins (pathogenesis-related proteins) (Malamy et al., 1990; Ward et al., 1991). The corresponding set of mRNAs was also induced by virus infection and by exogenously applied SA (Ward et al., 1991).

In transgenic tobacco plants expressing a bacterial *NahG* gene encoding salicylate hydroxylase (which transforms SA to catechol), little or no SA was detected after TMV infection, and these plants were unable to exhibit a SAR response (Gaffney et al., 1993). This defect in SA accumulation led to increased susceptibility to various pathogens, proving that SA is essential for disease resistance (Delaney et al., 1994). However, the role of SA as a systemic signal of SAR remained a debated question (Rasmussen et al., 1991; Vernooij et al., 1994; Mölders et al., 1996).

Some years ago a cDNA encoding a SA-binding protein was isolated and found to encode a CAT (EC 1.11.1.6) isoenzyme (Chen et al., 1993). SA was shown to inhibit CAT activity in vitro and to induce an increase in H_2O_2 concentration in plant tissues. The accumulation of H_2O_2 was supposed to mediate SA action by activating SAR genes (Chen et al., 1995). However, recent observations showed that H_2O_2 treatment can induce SA accumulation in plants and suggested that H_2O_2 affects the induction of SAR gene expression upstream of SA in signal transduction (Bi et al., 1995; León et al., 1995; Neuenschwander et al., 1995). SA pretreatment was recently found to increase dramatically the competence of plant tissues to trigger a burst of H_2O_2 in response to subsequent elicitor treatment. The increase in H_2O_2 level was not due to a decrease in the rate of degradation but, rather, to an increase in H_2O_2 synthesis (Fauth et al., 1996).

An increased production of reactive oxygen derivatives has been observed in various pathogen-infected tissues (Baker and Orlandi, 1995). H_2O_2 seems to play a central role in this oxidative burst, acting as a signal for localized death of challenged cells (hypersensitive reaction) and as a diffusible signal for the induction of cellular protectant genes in adjacent cells (Levine et al., 1994). Recently, the importance of superoxide was also pointed out (Jabs et al., 1996). However, little information is available about the

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* Corresponding author; e-mail zkir@planta.nki.hu; fax 36–1–1563–698.

Abbreviations: AP, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate; DHAR, DHA reductase; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GR, glutathione reductase; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; SA, salicylic acid (2-hydroxybenzoic acid); SAR, systemic acquired resistance; SOD, superoxide dismutase; TMV, tobacco mosaic virus.

role of protective antioxidative systems in plant response to pathogen attacks, particularly in the induction of SAR. Decreased GSH and increased ascorbate levels and SOD (EC 1.15.1.1) activities were found in oat (*Avena sativa*) after infection with virulent *Drechslera* spp. (Gönner and Schlösser, 1993). In compatible barley powdery mildew interactions the ascorbate-glutathione cycle and other antioxidative enzymes were activated, which may have diminished the damaging effects of oxidative stress (El-Zahaby et al., 1995). Recently, GST (EC 2.5.1.18) activity has also been shown to be induced by fungal infection or by a fungal elicitor (Mauch and Dudler, 1993; Hahn and Strittmatter, 1994; Levine et al., 1994). Viral infections also alter GST activities (Gullner et al., 1995a, 1995b). GST, by its glutathione-peroxidase-like activity, is able to catalyze the breakdown of lipid hydroperoxides that derive from lipid peroxidation processes (Bartling et al., 1993).

To investigate the possible role of antioxidants in the tobacco-TMV interaction, and particularly in the development of SAR, we examined the changes of ascorbate and GSH levels and activities of AP (EC 1.11.1.11), CAT, DHAR (EC 1.8.5.1), GR (EC 1.6.4.2), GST, and SOD in both the upper and lower leaves of *Nicotiana tabacum* L. cv Xanthi-nc plants after TMV inoculation of the lower leaves. Since SA has been shown to be necessary in the local signal transduction for resistance, the effect of exogenous SA treatments on these antioxidative systems was also investigated in separate experiments in the lower leaves of tobacco plants.

MATERIALS AND METHODS

Plant, Pathogen Inoculation, and SA Treatment

Seeds of tobacco (*Nicotiana tabacum* L. cv Xanthi-nc) were planted in soil and grown under normal greenhouse conditions (18–23°C, 160 mE m^{-2} s^{-1} for 8 h d^{-1} supplemental light, 75–80% RH). For each experiment 55- to 60-d-old plants were used.

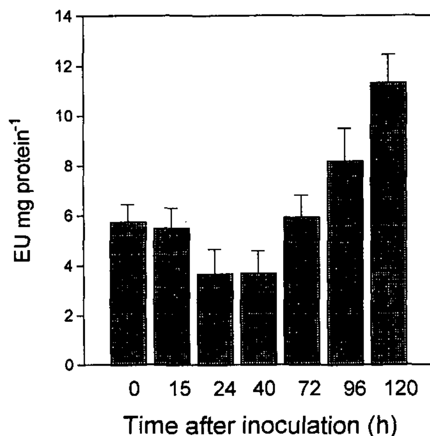

Figure 1. Changes in the specific activity of soluble SOD in TMV-infected tobacco leaves. One enzyme unit (EU) is defined as the enzyme activity that inhibits the reduction of *p*-nitroblue tetrazolium by 50% in the activity assay mixture.

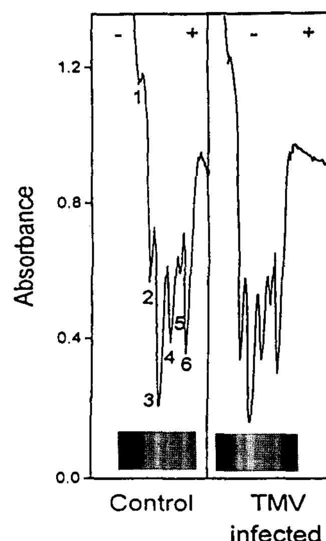

Figure 2. SOD isoenzymes from the upper leaves of tobacco as detected after anodic gradient PAGE and specific staining by densitometric scanning of gels. The traces show isoenzymes in control plants and in plants in which the lower leaves had been infected by TMV 14 d before isoenzyme analysis. SOD isoenzyme bands are numbered in the order of increasing electrophoretic mobility. The insets show the corresponding gel patterns after activity staining.

The third and fourth true leaves (third and fourth leaf position above hypocotyl) of plants were inoculated with a suspension of the U_1 strain of TMV. The virus was maintained in *N. tabacum* L. cv Samsun plants. Leaves of maintaining plants showing typical disease symptoms of TMV were ground (1 g in 10 mL of 10 mM sodium phosphate buffer, pH 7.0) in a mortar and the homogenate was used for inoculation, with Celite (Sigma) as an abrasive. The changes in antioxidative systems were investigated in both the TMV-inoculated lower leaves and in the uninfected upper leaves (fifth and sixth leaf positions above the hypocotyl) of tobacco after different periods. Mock-inoculated plants were used as controls. For testing the appearance of SAR on the upper leaves, a second (challenge) TMV inoculation of the upper leaves was carried out 10 to 14 d after the first inoculation of the lower leaves. After the challenge inoculation only the visible symptoms were investigated.

In separate experiments an aqueous solution (0.8 mM) of sodium salicylate was injected into the interveinal areas of lower leaves of tobacco plants with a hypodermic syringe until the leaf lamina was completely water-soaked. The effect of SA treatments on the antioxidative systems was investigated only in the treated lower leaves after different times, but the GST activities were determined in untreated upper leaves as well. Water-injected plants were used as controls. Chemicals were purchased from Sigma.

Total Soluble SOD Activity and Separation of SOD Isoenzymes

For enzyme assays cell-free homogenates were prepared at 0 to 4°C. Treated and control leaf tissue (0.5 g) was

Table I. *SOD activity in extracts of tobacco upper leaves 14 d after inoculation of the lower leaves with TMV*

Isoenzyme activities were determined by densitometric analysis after gradient PAGE and specific staining of the gels and are expressed as a percentage of the total unit peak area of the control. SOD isoenzyme bands are numbered in order of increasing electrophoretic mobility. Data are the means ± SD of three independent experiments.

Band No.	SOD Type	Control Leaves	Leaves having SAR
		% of total	
SOD1	Mn	7.2 ± 2.9	8.8 ± 4.5
SOD2	Cu,Zn	16.4 ± 1.9	24.5 ± 3.4[a]
SOD3	Fe	24.5 ± 2.6	29.2 ± 1.8
SOD4	Cu,Zn	20.1 ± 2.8	21.2 ± 3.0
SOD5	Cu,Zn	16.7 ± 3.9	21.8 ± 2.1
SOD6	Cu,Zn	15.1 ± 1.5	20.6 ± 3.0[a]
Total		100.0	126.1

[a] Significant differences at P = 0.05 level.

frozen with liquid N_2 in a mortar and homogenized with a pestle with 2 mL of 0.1 M sodium phosphate buffer (pH 7.8) containing 4% soluble PVP and 5 mM 2-mercaptoethanol. The homogenates were centrifuged (12,000*g*, 20 min, 4°C), and the total soluble SOD activity was determined in the supernatant by the method of Dhindsa et al. (1981). The protein content of the samples was measured by the method of Bradford (1976). Aliquots of supernatants containing 40 μg of protein were run on native anodic gradient (4–20%) PAGE to separate SOD isoenzymes. Following electrophoresis, negative SOD activity staining was carried out with riboflavin and *p*-nitroblue tetrazolium chloride, as described by Beauchamp and Fridovich (1971). To identify Cu,Zn-SOD, Fe-SOD, and Mn-SOD isoenzymes, gels were incubated in the staining solution supplemented with 1 and 2 mM KCN or 2 and 5 mM H_2O_2 (Barna et al., 1993; Ádám et al., 1995). Activity of SOD isoenzymes was quantified following scanning with a densitometer (model CS-930, Shimadzu, Kyoto, Japan) at 560 nm. The SOD activities were calculated as total integrated intensity in arbitrary units for each isoenzyme resolved (Ádám et al., 1989, 1995).

Measurement of Other Antioxidative Enzyme Activities

Cell-free leaf extracts were prepared as described by Uotila et al. (1995). Spectrophotometric methods were used to determine the various total enzyme activities. AP activity was determined according to the method of Nakano and Asada (1981), except that the concentrations of H_2O_2 and ascorbic acid were 0.5 and 0.25 mM in the assay mixture, respectively. The oxidation of ascorbic acid was followed at 290 nm. CAT activities were determined as described by Aebi (1984). DHAR and GR activities were measured by following the reduction of DHA (at 265 nm) and the oxidation of NADPH (at 340 nm), respectively (Klapheck et al., 1990). GST activities were determined by measuring the formation of the conjugate reaction product at 340 nm using 1-chloro-2,4-dinitrobenzene as the substrate (Mauch and Dudler, 1993).

Ascorbate and Glutathione Assays

Ascorbate and DHA levels were determined by enzymatic methods using the ascorbate oxidase enzyme as described by Wise and Naylor (1987) and Foyer et al. (1983), respectively.

Acid-soluble nonprotein thiol levels were measured spectrophotometrically with DTNB (De Kok and Graham, 1989). GSH and GSSG levels were measured by an enzymatic recycling method using the GR enzyme, as described previously (Smith, 1985).

The GSH content of tobacco leaves was determined by the reverse-phase HPLC method after derivatization of thiols with DTNB. Thiol derivatization was carried out by mixing 1.5 mL of 0.1 M sodium phosphate buffer (pH 7.8) with 0.5 mL of plant extract and with 0.2 mL of 10 mM DTNB reagent dissolved in buffer. The samples were centrifuged for 15 min at 12,000*g* (4°C). Control samples were prepared by replacing the DTNB solution with buffer. HPLC separations were carried out on a ChromLab column (250 × 4 mm; Central Research Institute for Chemistry, Budapest, Hungary) packed with reverse-phase Nucleosil C-18 (5 mm; Central Research Institute for Chemistry, Budapest, Hungary). DTNB derivatives were separated by isocratic elution with a mixture of 0.023 M ammonium acetate solution (pH 5) and methanol (92:8), as described by Uotila et al. (1995). The mixed-disulfide derivatives were monitored spectrophotometrically at 340 nm in the effluent.

Table II. *Local effect of 0.8 mM SA treatment of lower tobacco leaves on SOD enzyme activity at different times after treatment*

For details of data presentation, see Table I.

Band No.	Control Leaves	SA-Treated Leaves		
		24 h	48 h	72 h
		% of total		
SOD1	16.7 ± 2.7	22.2 ± 4.9	17.9 ± 2.4	22.6 ± 4.2
SOD2	19.1 ± 1.7	19.4 ± 1.6	23.4 ± 7.2	21.3 ± 2.0
SOD3	27.1 ± 3.6	39.9 ± 4.4[a]	34.7 ± 1.7[a]	41.0 ± 5.0[a]
SOD4	14.1 ± 1.9	17.9 ± 1.0	15.7 ± 1.0	16.7 ± 2.1
SOD5	10.2 ± 0.4	16.3 ± 2.7[a]	13.4 ± 2.3	13.8 ± 2.9
SOD6	12.8 ± 2.5	16.5 ± 1.2	15.9 ± 4.1	16.3 ± 1.4
Total	100.0	132.2	121.0	131.7

[a] Significant differences at P = 0.05 level.

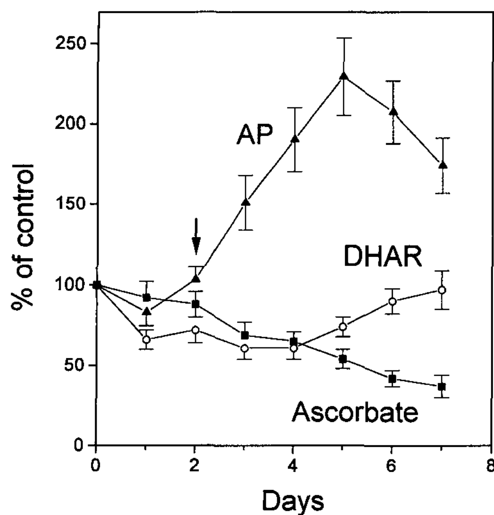

Figure 3. Alterations of ascorbate levels, AP, and DHAR activities in TMV-inoculated lower leaves of tobacco. Means ± SDs of three replicate experiments are shown. Ascorbate levels, AP, and DHAR activities in control plants were 1.51 ± 0.13, 2.2 ± 0.17, and 0.11 ± 0.03 μmol g^{-1} fresh weight min^{-1}, respectively (n = 4). ▲, AP; ○, DHAR; ■, ascorbate. The arrow indicates the appearance of necrotic symptoms.

Statistics

At least three independent, parallel experiments were carried out in each case. The significant difference between mean values was evaluated by Student's t test. Differences were considered to be significant at P = 0.05.

RESULTS

SOD Activity and Isoenzyme Pattern

Visible necrotic lesions appeared on inoculated leaves approximately 2 d after inoculation by TMV. The inoculation significantly altered SOD activities both locally (in the infected lower leaves) and systemically (in the uninfected upper leaves). The spectrophotometric determination of SOD activities showed that the manifestation of symptoms was preceded by the decrease in soluble SOD-specific activity in the infected lower leaves (Fig. 1). The subsequent considerable increase in SOD activity (up to 198% of control) started only after the appearance of necrotic symptoms.

Typically, after gradient PAGE of crude leaf extracts, six distinct SOD isoenzymes (designated SOD1–6, in order of increasing migration) could be detected (Fig. 2). SOD2, SOD4, SOD5, and SOD6 were cyanide-sensitive (Cu,Zn-SOD), SOD3 was H_2O_2-sensitive (Fe-SOD), and SOD1 was not sensitive to the above inhibitors (Mn-SOD) (data not shown). The molecular mass was between 35 and 95 kD.

Two days after TMV inoculation the activity of the SOD2 isoenzyme (Cu,Zn-SOD) showed the most marked decrease (data not shown). Later, however, the SOD activity (especially Cu,Zn-SOD bands) started to increase in infected leaves, as observed previously by Montalbini and Buonaurio (1986).

The SOD activity of upper leaves was significantly higher than in uninfected plants (by 26%) 14 d after inoculation (Table I), when SAR was already fully developed. The activities of SOD2 and SOD6 isoenzymes were induced (both of them Cu,Zn-SOD) (Fig. 2). Treatment of lower leaves with 0.8 mM SA also augmented SOD activities by 21 to 32% in the treated leaves (Table II). This concentration of SA did not result in phytotoxic symptoms, but 1.0 to 1.2 mM SA levels led to wilting and drying of the leaf edges. The activity of isoenzyme SOD3 (Fe-SOD) was elevated most considerably. The total SOD activity in SA-treated tissue was significantly higher than in control plants, even 5 to 7 d after SA treatment (data not shown).

Other Antioxidative Enzymes

CAT activity was investigated in both the upper and lower leaves of tobacco plants up to 14 d after TMV infection or SA treatment of lower leaves. The activities in the upper and lower leaves were 950 ± 161 and 652 ± 97 μmol H_2O_2 g^{-1} fresh weight min^{-1}, respectively, in intact plants (n = 4). No significant changes in CAT activity were detectable in TMV-infected or SA-treated leaves. These data are in agreement with the results of Summermatter et al. (1995) in plants after a bacterial infection. On the contrary, the activities of enzymes of the H_2O_2-detoxifying ascorbate-glutathione cycle (AP, DHAR, and GR) were substantially altered by TMV infections. In the infected lower leaves the AP activity gradually increased (after a transient decrease) up to 230% of control 5 d after inoculation and then declined (Fig. 3). In contrast, the DHAR activities decreased up to 4 d postinoculation, to 61% of control, and then returned to the normal level (Fig. 3). The

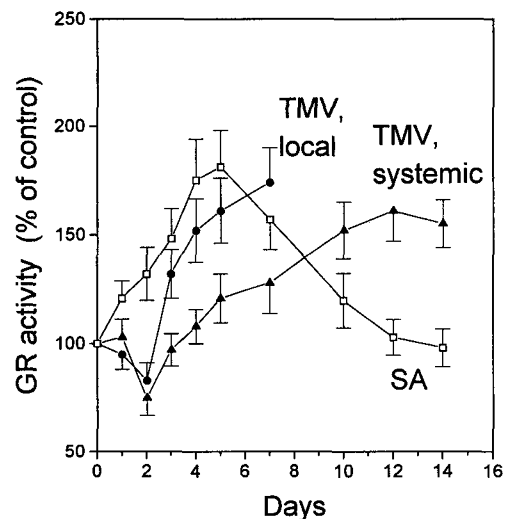

Figure 4. Changes of GR enzyme activity in the lower and upper leaves of tobacco after inoculation of the lower leaves with TMV. The local effect of 0.8 mM SA injection is also presented. Means ± SDs of three replicate experiments are shown. GR activities in control plants were 0.13 ± 0.01 and 0.17 ± 0.02 μmol NAD(P)H g^{-1} fresh weight min^{-1} in the lower and upper leaves, respectively (n = 4). ●, Infected lower leaves after TMV infection; ▲, uninfected upper leaves after TMV infection; □, SA-treated lower leaves.

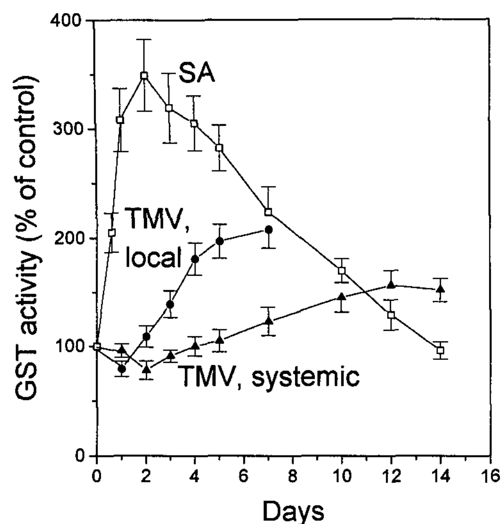

Figure 5. Alterations of GST activity in the lower and upper leaves of tobacco after inoculation of the lower leaves with TMV. The local effect of 0.8 mM SA is also presented. Means ± SDs of three replicate experiments are shown. GST activities in control plants were 0.08 ± 0.009 and 0.12 ± 0.01 μmol g^{-1} fresh weight min^{-1} in the lower and upper leaves, respectively (n = 4). Symbols are as for Figure 4.

AP and DHAR activities were not modified in the uninfected upper leaves (in which SAR was developed) up to 14 d postinoculation. SA injection (0.8 mM) did not alter AP activities significantly in the treated lower leaves within the above period. DHAR activities increased up to 160% of control 1 d after SA injection and then declined and returned to the control level 4 d following SA injection (data not shown).

A significant decrease in GR activity was found in the inoculated lower leaves 48 h after TMV inoculation (Fig. 4). However, after 3 d the GR activity had already increased, reaching 175% of control 7 d postinoculation. In contrast to AP and DHAR, TMV infection of the lower leaves led to a noticeable induction of GR activity in the upper leaves 12 d postinoculation (160% of control) (Fig. 4). In separate experiments exogenous SA treatment (0.8 mM) considerably induced GR activity (up to 181% of control) in the injected lower leaves (Fig. 4).

Markedly induced GST activities were also observed in tobacco after TMV inoculation. In the inoculated lower leaves the GST activities increased up to 7 d (preceded by a rapid, transient decline), reaching 210% of control (Fig. 5). In the upper leaves GST activity started to increase later, approximately 10 d after inoculation of the lower leaves, and 12 d after inoculation it reached 156% of control (Fig. 5). Single, exogenous SA treatment (0.8 mM) led to a dramatic and rapid GST induction 16 h after injection (Fig. 5), which is in accordance with results from previous studies (Gullner et al., 1995a). The GST activity increased to 350% of control 2 d after SA injection and then declined to the control level. GST activity was the antioxidant most significantly affected by SA treatment. To determine whether the SA treatment has any systemic effect, we also measured GST activities in the upper leaves of tobacco plants after SA treatment of the lower leaves. A single SA injection did not cause any significant alteration in GST activity in the untreated upper leaves up to 7 d postinoculation.

Ascorbic Acid and Glutathione Levels

Gradually decreasing ascorbic acid contents were observed in TMV-inoculated lower leaves (Fig. 3), down to 37% of control 7 d postinoculation. Concomitantly with the decline in ascorbate level, the DHA content of infected leaves markedly increased (Table III). These changes led to a considerable increase in the ratio of DHA to the total ascorbate pool (ascorbate plus DHA), which was probably a consequence of oxidative stress (Table III). In contrast to inoculated lower leaves, no significant modification of ascorbic acid content could be found in the uninfected upper leaves. SA treatments did not alter ascorbic acid levels significantly in the injected leaves.

GSH and nonprotein thiol levels were determined in parallel experiments in the same acidic leaf extracts. In the untreated leaves GSH accounted for 92 ± 5% (n = 4) of the nonprotein thiol level. The GSH-specific HPLC measurements showed considerably increased GSH levels in both the TMV-inoculated lower leaves and the untreated upper leaves (concomitantly with the development of SAR) (Table IV). SA treatment (0.8 mM) also led to elevated GSH levels in the injected leaves (Table IV). No accumulation of

Table III. *The effects of TMV inoculation on the reduced ascorbate and DHA contents of inoculated lower tobacco leaves*

Means ± SD of three replicate experiments are shown.

Treatment	Reduced Ascorbate Content	DHA Content	Percentage of DHA of the Total Ascorbate Pool
	μmol g^{-1} fresh wt		
Control leaves	1.38 ± 0.15	0.11 ± 0.02	7.4
TMV-inoculated leaves			
After 2 d	1.26 ± 0.11	0.12 ± 0.01	8.7
After 4 d	0.93 ± 0.08[a]	0.15 ± 0.01[a]	13.9
After 6 d	0.71 ± 0.06[b]	0.21 ± 0.02[b]	22.8

[a,b] Significant differences between control and inoculated leaves at P = 0.05 and 0.01, respectively.

Table IV. *Alterations of GSH levels determined by HPLC in both the upper and lower leaves of tobacco after TMV inoculation of the lower leaves and the local effect of 0.8 mM SA*

For details of data presentation, see Table III.

Days after Treatment	Lower Leaves			Upper Leaves (Systemic Effect)	
	Control	TMV-inoculated	SA-treated	Control	TMV-inoculated
			μmol g⁻¹ fresh wt		
1	0.31 ± 0.03	0.32 ± 0.03	0.35 ± 0.04	0.38 ± 0.03	0.37 ± 0.03
3	0.30 ± 0.02	0.48 ± 0.05[a]	0.49 ± 0.06[a]	0.39 ± 0.03	0.39 ± 0.04
7	0.33 ± 0.03	0.58 ± 0.07[b]	0.39 ± 0.05	0.38 ± 0.02	0.42 ± 0.05
12	0.29 ± 0.02	n.d.[c]	0.31 ± 0.03	0.35 ± 0.02	0.46 ± 0.06[a]
14	0.31 ± 0.02	n.d.	0.28 ± 0.03	0.36 ± 0.05	0.57 ± 0.07

[a,b] Significant differences between control and inoculated leaves at P = 0.05 and 0.01, respectively. [c] n.d., Not determined because of leaf tissue collapse.

any other thiol compound in these tissues could be detected. For this reason, the simpler spectrophotometric assay utilizing DTNB was used instead of HPLC for a more detailed investigation of thiols. In accordance with the measurement of GSH content, TMV infection led to a substantial elevation of nonprotein thiol levels in both the inoculated lower leaves and the uninfected upper leaves having SAR. In the inoculated leaves the thiol content gradually increased up to 5 d (reaching 190% of control) (Fig. 6). The thiol content increased much later and to a lesser extent in the uninfected upper leaves than in the infected ones, reaching 143% of control 2 weeks postinoculation (Fig. 6). A single SA injection (0.8 mM) also caused a rapid elevation of thiol levels at the site of application (Fig. 6).

For a better characterization of cellular redox processes the changes in the ratio of GSSG level to the total glutathione pool (GSH plus GSSG) were also investigated in separate experiments. The GSSG content decreased slightly in TMV-inoculated lower leaves 4 d postinoculation. This decrease in GSSG levels coincided with the considerable increase in GSH level (Table V). These changes resulted in the marked decline of GSSG/total glutathione ratio in the infected leaves 4 and 6 d after inoculation. The GSSG content did not change significantly in the uninfected upper leaves (Table V). SA injection, however, led to a marked accumulation of GSSG in the injected tobacco leaves 2 and 4 d after treatment (Table V).

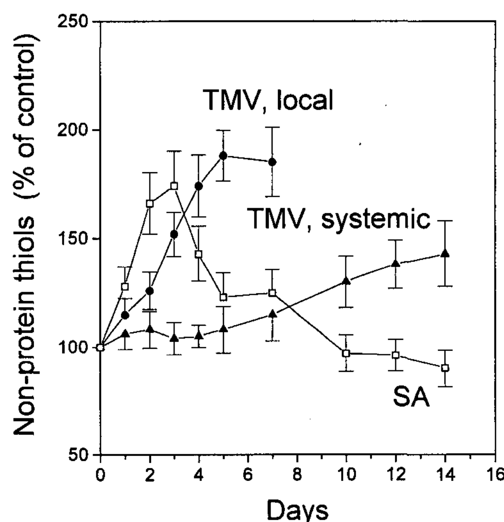

Figure 6. Changes of nonprotein thiol levels in lower and upper leaves of tobacco after inoculation of the lower leaves with TMV. The local effect of 0.8 mM SA treatment is also presented. Means ± SDs of three replicate experiments are shown. Thiol levels in control plants were 0.34 ± 0.03 and 0.41 ± 0.03 μmol g^{-1} fresh weight in the lower and upper leaves, respectively (n = 4). Symbols are as for Figure 4.

DISCUSSION

TMV inoculation led to an oxidative burst (the increased production of the superoxide anion) in the hypersensitively reacting tobacco cv Samsun NN (Doke and Ohashi, 1988). Similar changes have also been observed in our experimental system in cv Xanthi-nc tobacco (J. Fodor and Z. Király, unpublished results). Oxidative burst has been shown to induce the expression of a variety of defense genes (Levine et al., 1994; Baker and Orlandi, 1995). Our results showed that TMV infection also led to substantial changes in the antioxidative systems of tobacco. Several antioxidative enzyme (AP, GR, GST, and SOD) activities decreased transiently in the infected leaves prior to the appearance of visible symptoms. Ascorbate levels and DHAR activities decreased even after symptom expression. The diminished antioxidative capacity in the infected leaves probably facilitates the accumulation and action of oxygen free radicals and lipid peroxidation processes, which may finally lead to cell necrosis.

The sustained decrease in the ascorbic acid level can be explained by decreasing DHAR activities and the strong induction of AP activity after the appearance of necrotic symptoms. However, most of the antioxidative enzyme (AP, GR, GST, and SOD) activities were strongly induced following a transient decline, concomitantly or following symptom expression in the infected leaves, together with a noticeable increase in GSH levels. It is noteworthy that the accumulation of H_2O_2 as a consequence of chilling stress led to increased activities of the antioxidative CAT and guaiacol peroxidase enzymes in maize (Prasad et al., 1994). These findings suggest that genes encoding antioxidative enzymes can be activated by the increased production of

Table V. *The effect of TMV inoculation and SA injection on GSH and GSSG contents of infected (or SA-treated) lower and uninfected upper leaves of tobacco*

For details of data presentation, see Table III.

Treatment	GSH	GSSG	Percentage of GSSG of Total Glutathione Pool
	μmol g⁻¹ fresh wt		
Lower leaves			
Control	0.28 ± 0.02	0.020 ± 0.002	6.6
TMV inoculation			
After 2 d	0.33 ± 0.02[a]	0.018 ± 0.002	5.1
After 4 d	0.40 ± 0.03[b]	0.016 ± 0.001[a]	3.8
After 6 d	0.53 ± 0.04[b]	0.017 ± 0.002	3.1
SA injection			
After 2 d	0.45 ± 0.04[b]	0.033 ± 0.003[b]	6.8
After 4 d	0.36 ± 0.03[a]	0.034 ± 0.003[b]	8.6
After 6 d	0.31 ± 0.02	0.021 ± 0.002	6.3
Upper leaves			
Control	0.34 ± 0.03	0.022 ± 0.002	6.1
TMV inoculation (systemic effect)			
After 12 d	0.44 ± 0.03[a]	0.025 ± 0.003	5.4
After 14 d	0.47 ± 0.04[a]	0.024 ± 0.002	4.9

[a,b] Significant differences between control and inoculated leaves at P = 0.05 and 0.01, respectively.

active oxygen derivatives in TMV-infected plants. It is possible that these antioxidative processes protect plant tissues from lipid peroxidation processes around the infection sites and thereby protect them from the spreading of necrotic lesions.

The increased DHA content and DHA/total ascorbate redox ratio in the infected leaves probably reflect the oxidative effects of TMV infection. The decline of DHAR activity can also contribute to the accumulation of DHA. The GSSG content and GSSG/total glutathione redox ratio decreased in the infected leaves, which is in contrast to the ascorbate results. This effect was supposedly due to the strongly induced GR activity in the infected tissues.

Increased GSH levels (De Kok and Stulen, 1993) and elevated AP (Kubo et al., 1995), GR (Edwards et al., 1994), GST (Kömives and Dutka, 1989; Uotila et al., 1995), and SOD (Madamanchi et al., 1994) activities have been detected in plants exposed to various abiotic stress effects. Similarly, elevated ascorbic acid (Gönner and Schlösser, 1993) and GSH levels (May et al., 1996a) and increased AP (El-Zahaby et al., 1995), GST (Mauch and Dudler, 1993; Gullner et al., 1995a), and SOD (Montalbini and Buonaurio, 1986; Ádám et al., 1995) activities were reported in plants after pathogen attack or elicitor treatment. Obviously, the activation of antioxidative defense systems in plants by abiotic and biotic stresses is a general phenomenon and probably contributes to increased resistance against a subsequent stress.

Exogenously applied antioxidants often lead to increased stress resistance. An early report showed an antioxidative, protective effect of ascorbate and GSH against necrotic viral infection (Farkas et al., 1960). Modulation of plant cell GSH levels altered the transcription of various defense genes and the biosynthesis of phytoalexins (Wingate et al., 1988; Guo et al., 1993). The enhanced resistance of muskmelon (*Cucumis melo*) and tomato (*Lycopersicon esculentum*) roots against *Fusarium oxysporum* brought about by herbicides coincided with the significant increase in GSH levels (Bolter et al., 1993). However, the depletion of GSH in Arabidopsis did not alter its responses to various pathogens (May et al., 1996b). Stress resistance can also be increased by genetic manipulation of antioxidative enzymes (Bowler et al., 1992; Strohm et al., 1995) or by selecting plants with high antioxidative capacity (Barna et al., 1993).

SA treatments activated both nonenzymatic and enzymatic scavengers: it readily elevated GSH levels and induced DHAR, GR, GST, and SOD activities in the treated leaves. In particular, GST activities were dramatically elevated, which is in accordance with earlier data (Gullner et al., 1995a). It is noteworthy that some proteins encoded by SA-inducible mRNAs showed limited but significant homologies to plant GST isoenzymes (Droog et al., 1993). In our experiments a single SA injection was ineffective in the induction of GST activity in the uninfected upper leaves, supporting earlier suggestions that SA is not an endogenous signal but rather is necessary for local resistance (Vernooij et al., 1994). SA was found to inhibit CAT (Chen et al., 1995) and AP (Durner and Klessig, 1995) activities in vitro. On the other hand, no changes in CAT activity could be detected in Arabidopsis, in which the SA level was artificially elevated (Summermatter et al., 1995). This finding is in accordance with our results. The lack of inhibition may be due to the difference between extractable CAT activity from SA-injected tissue (measured in this paper) and the in vitro inhibition of the enzyme by SA, which is readily reversible (Chen et al., 1995).

In our experiments GSH levels and GR, GST, and SOD activities significantly increased in the upper leaves after TMV infection of the lower leaves, and these changes were concomitant with the development of SAR. It was shown by Strobel and Kuc (1995) that pathogens and chemicals that triggered SAR against necrotic symptoms also enhanced the resistance systemically against the damage of pro-oxidant chemicals such as paraquat and $CuCl_2$.

One can suppose that the systemic induction of SOD and the GSH-related antioxidative systems contribute to the appearance of SAR by the increased capacity of scavenging active oxygen species during a second (challenge) infection. It is important to note that SAR generally means resistance against necrosis (symptom expression) and not necessarily against pathogens; smaller or fewer necrotic spots appear on leaves having SAR than on uninduced leaves without the restriction of pathogen multiplication (Sziráki et al., 1980; Doss and Hevesi, 1981). However, in other host-pathogen relationships SAR may cause resistance to bacterial and fungal pathogens (Uknes et al., 1992).

In conclusion, antioxidative processes were substantially activated in both the lower and upper leaves of tobacco following TMV inoculation of the lower leaves. In the uninfected upper leaves the induction of GSH level, GSH-related enzyme activities, and, to a lesser extent, SOD activities occurred concomitantly with the appearance of SAR. These antioxidants were also induced by the injection of SA. Further studies comparing incompatible and compatible host-virus interactions are necessary for a better understanding of the role of antioxidative processes in viral infections. Furthermore, investigation of transgenic tobacco having the *NahG* gene, which does not accumulate SA and does not exhibit SAR, will provide deeper insights into the role of antioxidative processes.

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