Up-Regulation of Antioxidants in Tobacco by Low Concentrations of H₂O₂ Suppresses Necrotic Disease Symptoms

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ABSTRACT

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Pretreatment of tobacco leaves with low concentrations (5 to 10 mM) of H₂O₂ suppressed hypersensitive-type necrosis associated with resistance to *Tobacco mosaic virus* (TMV) or *Pseudomonas syringae* pv. *phaseolicola*. The same pretreatment resulted in suppression of normosensitive necrosis associated with susceptibility to *Botrytis cinerea*. This type of H₂O₂-mediated, induced disease symptom resistance correlated with enhanced host antioxidant capacity, i.e., elevated enzymatic activities of catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (POX) after viral and bacterial infections. Induction of genes

that encode the antioxidants superoxide dismutase (SOD), CAT, and APX was also enhanced early after TMV infection. Artificial application of SOD and CAT suppressed necroses caused by viral, bacterial, or fungal pathogens similarly as H₂O₂ pretreatment, implying that H₂O₂-mediated symptom resistance operates through enhancement of plant antioxidant capacity. Pathogen multiplication was not significantly affected in H₂O₂-pretreated plants. Salicylic acid (SA), a central component of plant defense, does not seem to function in this type of H₂O₂-mediated symptom resistance, indicated by unchanged levels of free and bound SA and a lack of early up-regulation of an SA glucosyltransferase gene in TMV-infected H₂O₂-pretreated tobacco. Taken together, H₂O₂-mediated, induced resistance to necrotic symptoms in tobacco seems to depend on enhanced antioxidant capacity.

During plant-pathogen interactions, the first line of plant defense consists primarily of the pathogen-associated molecular pattern (PAMP) recognition system that has been shown to confer basal resistance to a wide range of pathogens (34,68,69). If the PAMP system fails to recognize the pathogen as an invader, a second line of plant defense is induced. This is a cultivar/pathogen race-specific (“gene-for-gene”) resistance that can be regularly broken down by newly emerging pathogenic races (14,28). In “gene-for-gene” resistance plant cell and tissue death (necrosis) is usually associated with pathogen arrest at infection sites called the hypersensitive response (HR) (20,21). Interestingly, it has been shown that in several viral, bacterial and fungal infections plant resistance during an HR is independent of cell and tissue death (4,5,10,18,31,49,67, for a review see 30). Furthermore, an additional plant defense mechanism that activates antioxidants (agents that neutralize cell death-causing reactive oxygen species) has been described that is effective primarily against necrotic symptoms (3,15,32). Plant defense in this case means suppression of necrotic symptoms associated either with resistance (HR-type necrosis) or susceptibility (normosensitive necrosis) to pathogens and in some cases suppression of pathogen growth as a result of the activated antioxidant capacity. Interestingly, local and systemic acquired resistance responses elicited by salicylic acid (SA), a key mediator of plant defense responses, can also be associated with a similar activation of antioxidants (15,32).

Plant infections by pathogens are frequently associated with accumulation of reactive oxygen species (ROS) that induce oxidative stress in plants ultimately resulting in cell and tissue death (necrosis). Under natural conditions the up-regulation of antioxidant defense systems seems to be a general response to oxidative stress. Often this is the only evidence that oxidative stress has occurred in vivo (25). Interestingly, hydrogen peroxide (H₂O₂), one of the most important reactive oxygen species associated with oxidative stress, can up-regulate antioxidant systems even at very low concentrations (2,19,58). Application of low concentrations (5 to 20 mM) of H₂O₂ to tobacco leaves and pea seedlings stimulated the antioxidant capacity of plants, increasing thereby tolerance to abiotic stresses and enhancing seedling growth (2,19). It was supposed that the mechanism behind this phenomenon is one of the dual actions of H₂O₂. In low concentrations H₂O₂ is a diffusible signal for the induction of antioxidant and pathogenesis-related genes in adjacent plant tissues. However, high concentrations of H₂O₂ are related to the induction of death (necrosis) of plant as well as invading pathogen cells (6,38,64).

Here we show that low concentrations of H₂O₂ in plants suppress necrotic disease symptoms caused by viral, bacterial, and fungal infections by enhancing plant antioxidant capacity. The symptom resistance conferred by H₂O₂ is effective both against HR-type necrotic symptoms during host and non-host resistance to a viral and bacterial pathogen, respectively, and normosensitive necrosis associated with susceptibility to a fungal pathogen. In this type of induced resistance to disease symptoms, levels of the plant defense inducer SA are unchanged. Monitoring the in planta multiplication of viral and bacterial pathogens revealed that H₂O₂-mediated symptom resistance does not significantly influence pathogen levels. This type of induced resistance to disease symptoms may contribute to the reduction of pesticide inputs and human health and environmental hazards.

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MATERIALS AND METHODS

Plants and pathogens, pathogen inoculations. The U1 (common) strain of TMV (a gift of Milton Zaitlin, University of Arizona) was maintained on the Samsun (*nn*) cultivar of tobacco (*Nicotiana tabacum* L.). Systemically infected uppermost leaves of virus maintaining plants showing typical mosaic symptoms were used for mechanical virus inoculation. The sap of TMV-infected leaves homogenized in tap water (1 g of leaf in 10 ml of water) was rubbed onto healthy leaves of 60- to 70-day-old 'Xanthi' (*NN*) tobacco, a local lesion (HR) host of TMV. Both Samsun (*nn*) and Xanthi (*NN*) tobaccos were obtained from József Horváth, Pannon University, Hungary. Carborundum was used as an abrasive for both virus and mock inoculations. Inoculated plants were grown in the greenhouse (18°C, 160 $\mu\text{E m}^{-2} \text{ s}^{-1}$ supplemental light for 8 h/day, 75 to 80% relative humidity).

Pseudomonas syringae pv. *phaseolicola* isolate GSPB 1205 (from the bacterial culture collection 'Göttinger Sammlung phytopathogener Bakterien', a gift of Klaus Rudolph, Georg August University, Göttingen, Germany) was maintained on King's B medium. For bacterial inoculation, a 24-h-old liquid culture derived from a single bacterial colony was used with a final inoculum concentration of 10^8 cfu/ml adjusted with a spectrophotometer (13). The bacterial suspension was infiltrated into interveinal leaf segments of tobacco (Xanthi *NN*) that reacts with localized necrotic symptoms (non-host HR) to *P. syringae* pv. *phaseolicola*. Infiltration was done on the adaxial leaf side with a hypodermic syringe and needle. Inoculated plants were grown in the greenhouse as described above.

A tomato isolate (Bc-1) of *Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*) isolated in Tarcál (Tokaj region, Hungary) was maintained on potato dextrose agar (PDA). For inoculation, agar discs 5 mm in diameter were cut from 7-day-old cultures of the fungus and placed on the surface of tobacco leaves (Xanthi *NN*). Leaves were cut and put on wet filter paper in a Petri dish and held at 20°C in continuous light for at least 3 days.

Determination of virus levels and bacterial multiplication. To determine TMV concentrations, double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) from virus-infected whole tobacco leaves were performed as described in Király et al. (29), using a TMV-ELISA kit (Bioreba AG, Reinach, Switzerland). Briefly, 96-well polystyrene microplates were filled with TMV antibodies (IgG) diluted 1:1,000 in coating buffer (pH 9.6) and incubated at 37°C for 3 to 4 h. Following several washes by tap water and PBS-Tween (pH 7.4) wells were filled with leaf homogenate diluted 1:20 in PBS extraction buffer (PBS-Tween + 0.2% PVP + 0.2% BSA) and incubated at 4°C overnight. Following repeated washes, wells were filled with alkaline phosphatase-conjugated IgG (diluted 1:1,000 in PBS extraction buffer) and incubated at 37°C for 2 to 4 h. Finally, p-nitrophenyl phosphate at 1.0 mg/ml (substrate) was added to the washed wells and incubated at 37°C for 10 to 15 min. Absorbance values were read by a Multiskan ELISA plate reader (Thermo Scientific, Waltham, MA) at 405 nm.

Numbers of viable bacteria in tobacco leaves infected with *P. syringae* pv. *phaseolicola* were monitored by a modified serial dilution method as described by Ott et al. (46). In brief, eight leaf discs (1 cm^2 each) per sample were taken with a cork borer from infiltrated leaf areas, surface sterilized, and homogenized in 1 ml of K-phosphate buffer (10 mM, pH 7.0). Serial dilutions (10 $\times$, 100 $\times$, and 1,000 $\times$) were plated on King's B agar medium in Petri dishes (10 μl /dilution, at least six dilutions/Petri dish). Colony numbers per plated dilution were used to calculate the original numbers of bacterial cells present in 1 cm^2 of inoculated leaf tissue.

Treatment of tobacco with low concentrations of H_2O_2 and antioxidants. Tobacco plants were treated by spraying leaves with tap water or different concentrations of H_2O_2 (5, 7, 10, and

12.5 mM) until runoff 1 day before pathogen inoculation, or by infiltrating leaves with a mixture of horseradish superoxide dismutase and bovine liver catalase (Sigma-Aldrich Corp., St. Louis, MO) (SOD + CAT, 3,000 and 5,000 units/leaf, respectively) immediately after pathogen inoculation. Infiltration of SOD + CAT was done on the adaxial leaf side with a hypodermic syringe and needle until the leaf lamina was completely water-soaked.

To test the effect of H_2O_2 on growth of *B. cinerea*, PDA Petri dishes inoculated with the fungus were supplied daily with different concentrations of H_2O_2 (2.5, 5, 10, and 20 mM) by pipetting the solutions onto the inner edge of the Petri dishes. Distilled water was used as a negative control.

Activity assays of antioxidant enzymes. For the detection of antioxidant enzyme activities, 0.5 g of fresh leaf material/sample from virus-, bacteria-, or mock-inoculated whole tobacco leaves was homogenized on ice in 3 ml of 50 mM TRIS buffer (pH 7.8), containing 1 mM EDTA- Na_2 and 7.5% polyvinylpyrrolidone. In case of ascorbate peroxidase assays, the homogenizing buffer also contained 2 mM ascorbic acid. The homogenates were centrifuged (12,000 rpm, 20 min, 48°C), and the total soluble enzyme activities in the supernatant were measured spectrophotometrically. All assays were carried out at 25°C, using a model UV 160A spectrophotometer (Shimadzu, Kyoto, Japan). Enzymatic activity assays of CAT, guaiacol peroxidase (POX), ascorbate peroxidase (APX), glutathione S-transferase (GST), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) were carried out as described in our previous publications (15,29,32). For CAT assays, the decomposition of hydrogen peroxide was followed spectrophotometrically at 240 nm. Activity of POX was assayed by monitoring the formation of the conjugate product of guaiacol at 436 nm. APX activity was determined by following the disappearance of ascorbic acid at 290 nm. GST activities were monitored by assaying formation of the conjugate reaction product at 340 nm by using 1-chloro-2,4-dinitrobenzene as a substrate. GR and DHAR activities were measured by following the oxidation of NADPH (at 340 nm) and the reduction of dehydroascorbate (at 265 nm), respectively.

Total RNA extraction and gene expression analysis. At least 200 mg of fresh leaves/sample from TMV- or mock-inoculated whole tobacco leaves was homogenized in liquid nitrogen and used for total RNA isolation and subsequent reverse transcription (RT, i.e., first-strand cDNA synthesis) essentially as described in Höller et al. (26), except that only the oligo(dT) primer was used for RT reactions according to the instructions of the manufacturer (MBI Fermentas, Vilnius, Lithuania).

Quantitative real-time polymerase chain reaction (qPCR) to assay expression of tobacco antioxidant genes (*NtSOD*, *NtCAT*, and *NtAPX*) and an SA glucosyltransferase gene (*NtSGT*) was conducted in a DNA Engine Opticon 2 instrument (MJ Research, Waltham, MA) with the 2 $\times$ SYBR FAST Readymix Reagent (KAPA Biosystems, Woburn, MA) as recently described (26). Changes in gene expression were calculated by the $2^{-\Delta\Delta\text{CT}}$ method, using expression of a tobacco actin gene (*NtAct*, GenBank accession X69885) as an internal control. Gene expression in TMV-inoculated samples was also normalized to that in mock-inoculated samples.

Oligonucleotide primers used in RT-qPCR for amplifying tobacco (*N. tabacum*) sequences were the following: 5'-CGG AATCCACGAGACTACATAC-3' (5' primer [forward]) and 5'-GGGAAGCCAAGATAGAGC-3' (3' primer [reverse]) for a 230-bp tobacco actin (*NtAct*) cDNA fragment (GenBank accession X69885); 5'-GCCGTCCTTAGCAGCAGT-3' (5' primer) and 5'-ACAAGCAACCCTTCCACC-3' (3' primer) for a 420-bp *N. plumbaginifolia* and putative tobacco superoxide dismutase (*NtSOD*) cDNA fragment (GenBank accessions X55974 and EU123521); 5'-TCCGCTTGATGTGACTAAA-3' (5' primer) and 5'-TCCACCCACCGACGAATA-3' (3' primer) for a 501-bp *N.*

glutinosa and tobacco catalase (*NtCAT*) cDNA fragment (GenBank accessions AF006067 and U93244); 5'-CTGATGTTCCCTTCACCCTG-3' (5' primer) and 5'-CACTCCCAACTCTTCCTCCT-3' (3' primer) for a 451-bp tobacco ascorbate peroxidase (*NtAPX*) cDNA fragment (GenBank accessions U15933 and D85912); 5'-AAAGAAGTTGGCTCGGATA-3' (5' primer) and 5'-TTGGCTTGAAGACACTAAGG-3' (3' primer) for a 514-bp tobacco SA glucosyltransferase (*NtSGT*) cDNA fragment (GenBank accession AF190634). All oligonucleotide primers were designed with the aid of the Primer Premier 5 program (PREMIER Biosoft International, Palo Alto, CA).

The cDNA fragments amplified by RT-PCR were sequenced by an automated DNA sequencer (Eurofins MWG Operon, Ebersberg, Germany). In order to confirm identity of the obtained sequences, database searches were performed using the BLAST network service at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

Detection of the free and bound (glucosylated) forms of SA. The presence of free and bound (i.e., the conjugated, mostly glucosylated, acid hydrolyzable) forms of SA in TMV- and mock-inoculated whole tobacco leaves was detected by HPLC (high performance liquid chromatography) analysis. Samples for HPLC assays were prepared by separating organic and aqueous phases containing free and bound SA, respectively, and acid hydrolysis of aqueous phases before conducting HPLC analysis as described by Meuwly and Métraux (41) and Cole et al. (9).

Statistical analysis. Three independent biological experiments were conducted in each case with three to five replicates per treatment. Data represent the mean $\pm$ standard error. Significant differences between mean values were determined by the Student's *t* test ($P \leq 0.05$) or by one-way analysis of variance (ANOVA) ($P = 0.05$) with the aid of the computer program SPSS 15.0 (IBM Corporation, Armonk, NY).

RESULTS

Treatment of tobacco leaves with low concentrations of H₂O₂ or antioxidants results in suppression of necrotic symptoms caused by viral, bacterial, and fungal infections. In order to test whether low concentrations of H₂O₂ confer disease resistance to viral, bacterial, and fungal pathogens, tobacco ('Xanthi-nc') plants were pretreated with low concentrations of H₂O₂ 1 day before pathogen inoculation. Spraying of leaves with 5, 7, 10, and 12.5 mM H₂O₂ 1 day before inoculation suppressed HR-type necrotic lesions associated with host resistance to TMV compared with water-pretreated controls (Table 1; Fig. 1). Similarly, HR-type necrotic symptoms associated with non-host resistance to the bacterium *P. syringae* pv. *phaseolicola* were also

significantly suppressed by pretreatment with 5 mM H₂O₂. In addition, a significant reduction of normosensitive necrotic symptoms associated with susceptibility to the fungal pathogen *B. cinerea* was observed following the same H₂O₂ pretreatment (Fig. 1).

Abiotic stress tolerance of plants conferred by low concentrations of exogenous H₂O₂ correlates with the activation of several plant antioxidants (2,19). In order to gain insight into the role of antioxidants during H₂O₂-mediated plant resistance to pathogen-induced necrotic symptoms, the effect of simultaneous treatment with SOD and CAT was tested. In case of the previously mentioned viral, bacterial, and fungal infections, application of a mixture of SOD and CAT to tobacco leaves immediately after inoculation resulted in a suppression of tissue necrotization that was comparable to the effect of H₂O₂ pretreatment (Fig. 1).

Suppression of pathogen-induced necrotic symptoms in tobacco by H₂O₂ pretreatments is coupled to the induction of several antioxidant enzymes and genes. The facts that abiotic stress tolerance conferred by low concentrations of H₂O₂ correlates with enhanced plant antioxidant capacity (2,19) and treatment of tobacco with antioxidants (SOD + CAT) suppresses pathogen-induced necrotic symptoms (see above), led us to assay the activity and expression of several antioxidant enzymes/genes in infected tobacco leaves pretreated with low concentrations of H₂O₂.

In case of enzymatic activity assays, tobacco leaves were inoculated with TMV and *P. syringae* pv. *phaseolicola* 1 day after H₂O₂ pretreatment. Activities of antioxidant enzymes were determined 2, 3, and 4 days after inoculations. H₂O₂-mediated symptom resistance resulted in significantly enhanced enzymatic activities of CAT, POX, and APX both in mock- and pathogen-inoculated plants (Fig. 2). On the other hand, no significant changes were detected in enzymatic activities of GST, GR, and DHAR (data not shown).

Expression of three antioxidant genes (*NtSOD*, *NtCAT*, and *NtAPX*) in H₂O₂-pretreated, TMV-infected tobacco leaves was checked by quantitative real time RT-PCR. In plants pretreated by H₂O₂ a significant enhancement of gene expression occurred early, $\approx$ 3 to 6 h after TMV inoculation (Fig. 3). Identities of the cDNA fragments amplified by RT-PCR were checked by sequencing. As expected, the sequenced *NtSOD* fragment was highly identical to *SOD* genes from tobacco (*Nicotiana tabacum*) and *N. plumbaginifolia* (96 and 97% identity to GenBank accessions EU123521 and X55974, respectively). In case of *NtCAT*, the sequenced amplicon showed the highest identity to *CAT* genes from *N. tabacum* and three other *Nicotiana* species (95, 96, 97, and 98% identity to GenBank accessions U93244, EU998969, Z36975, and AF006067, respectively). The sequenced *NtAPX* fragment displayed the highest identity to two *APX* genes from tobacco (96 and 99% identity to GenBank accessions D85912 and U15933, respectively).

Suppression of pathogen-induced necrotic symptoms in tobacco by H₂O₂ pretreatments does not significantly alter pathogen multiplication. In order to detect any changes in pathogen multiplication that might occur in H₂O₂-pretreated plants ("immunized" to necrotic symptom development), we have monitored levels of TMV and *P. syringae* pv. *phaseolicola* in tobacco at 1, 2, and 3 days after inoculation in response to H₂O₂ pretreatments compared with water-treated controls. Titers of TMV 3 days after inoculation were essentially the same following all treatments. Interestingly, the previously mentioned antioxidant treatment (SOD + CAT) that suppressed tissue necrotization in a similar manner as H₂O₂ pretreatments also did not seem to influence TMV accumulation (Fig. 4). Similarly, multiplication of *P. syringae* pv. *phaseolicola* in tobacco leaves at 1, 2, and 3 days after inoculation was the same regardless of pretreatments with H₂O₂ or treatments with SOD + CAT, or water (Fig. 4). It is also likely that H₂O₂ does not significantly affect multiplication of *B.*

TABLE 1. Suppression of localized necrotic (HR-type) lesions in tobacco ('Xanthi-nc') leaves infected with *Tobacco mosaic virus* (TMV) 3 days after inoculation in response to treatments with H₂O₂ and superoxide dismutase (SOD) plus catalase (CAT)^a

Treatments	Number of lesions (cm ⁻²)	Lesion size (mm)
Control (water + TMV)	8.53 $\pm$ 1.85	1.57 $\pm$ 0.29
7 mM H ₂ O ₂ + TMV	5.80 $\pm$ 1.30**	0.70 $\pm$ 0.11*
10 mM H ₂ O ₂ + TMV	5.90 $\pm$ 1.25**	0.77 $\pm$ 0.20*
12.5 mM H ₂ O ₂ + TMV	6.07 $\pm$ 1.7**	0.84 $\pm$ 0.15*
SOD + CAT + TMV	5.90 $\pm$ 1.05**	0.79 $\pm$ 0.14*

^a Number and size of lesions were inspected visually on the third and fourth leaves of 8-week-old tobacco plants. Leaves were sprayed with low concentrations of H₂O₂ or tap water 1 day before TMV inoculation. A mixture of SOD and CAT (3,000 and 5,000 units/leaf, respectively) was injected into leaves 1 h after TMV inoculation. Values are means $\pm$ SD based on three independent biological experiments. Lesions were assayed in three plants for each treatment. * and ** indicate significant differences between control and treated plants at $P < 0.05$ and 0.01, respectively, as calculated by the Student's *t* test.

cinerea in tobacco leaves. The low concentration of H_2O_2 (5 mM) that suppressed normosensitive necroses caused by *B. cinerea* in tobacco leaves did not suppress growth of this fungus in an artificial medium for up to 6 days (data not shown).

Salicylic acid does not seem to play a role in H_2O_2 -mediated suppression of pathogen-induced necrotic symptoms in tobacco. We wanted to clarify the possible role of salicylic acid (SA), a central component of plant defense, in the H_2O_2 -mediated suppression of pathogen-induced necrotic symptoms in tobacco. It is known that most of the free SA produced in planta is converted to the bound, glucosylated form (SAG) by a pathogen-

inducible SA glucosyltransferase. SAG, as an inactive storage form, can be converted back to SA (Vlot et al. [60]). We found that expression of a tobacco SA glucosyltransferase gene (*NtSGT*) is induced early, ca. 3 to 6 h after TMV inoculation in water-pretreated control plants. On the other hand, a lack of early up-regulation of *NtSGT* was observed in H_2O_2 -pretreated (5 mM) and TMV-inoculated tobacco (Fig. 5A). Identity of the cDNA fragment(s) amplified by RT-PCR was checked by sequencing. As expected, the sequenced amplicon showed the highest identity to a tobacco *SGT* gene (99% identity to GenBank accession AF190634). Assays of the free and bound (glucosylated) forms of

Fig. 1. Treatment of tobacco (*Nicotiana tabacum* 'Xanthi') with low concentrations of H_2O_2 or antioxidants results in suppression of necrotic symptoms caused by viral, bacterial, and fungal infections. Plants were treated by spraying leaves with tap water or 5 mM H_2O_2 1 day before pathogen inoculation or infiltrating leaves with a mixture of superoxide dismutase and catalase (SOD + CAT, 3,000 and 5,000 units/leaf, respectively) immediately after pathogen inoculation. Leaves were inoculated with **A**, Tobacco mosaic virus, **B**, *Pseudomonas syringae* pv. *phaseolicola*, and **C**, *Botrytis cinerea*. Symptoms are shown at 3 days after inoculation. Representative results of three independent biological experiments are shown.

SA in TMV-infected tobacco indicated that H_2O_2 -mediated suppression of pathogen-induced necrotic symptoms is associated with unchanged levels of free and bound SA (Fig. 5B).

DISCUSSION

Here we show that pretreatment of Xanthi-nc tobacco with low concentrations (5 to 10 mM) of H_2O_2 1 day before pathogen inoculation suppressed HR-type necrotic symptoms associated with host and non-host resistance elicited by TMV or the bacterium *P. syringae* pv. *phaseolicola*, respectively. The same H_2O_2 pretreatment also resulted in a significant reduction of normosensitive necrotic symptoms associated with susceptibility to the fungal pathogen *B. cinerea*. We found that this type of H_2O_2 -mediated induced resistance to symptoms (i.e., "immunization" to necrotic disease symptoms) is associated with an enhanced antioxidant capacity of the tobacco host but not with a reduction in pathogen levels. In addition, higher than normal amounts of salicylic acid (SA) do not seem to play a role in this H_2O_2 -mediated resistance to symptom development.

It is important to point out that higher concentrations of reactive oxygen species (ROS) such as H_2O_2 may have a role in

the development of both the HR-type and normosensitive necrotic responses. Under pathogen attack, the rapid accumulation of ROS (i.e., the oxidative burst) in plants induces several host defense mechanisms, resulting in localized cell and tissue death at the penetration site and resistance to the pathogen (HR) (20,21). Thus, ROS accumulation is an effective process to combat biotrophic pathogens that depend on living host cells (23,27,48). However, it does not necessarily protect the host against necrotrophic pathogens like *B. cinerea*. In fact, the virulence of this latter pathogen correlates with the intensity of in planta development of ROS like H_2O_2 and the hydroxyl radical ($OH^\bullet$) (55,61). These oxidative processes could result in a massive perturbation of the plant redox status, contributing thereby to the development of normosensitive necrotic symptoms in the host.

The harmful effects of ROS (oxidative stress) can be counteracted by enzymatic and nonenzymatic antioxidants. Halliwell and Gutteridge (25) pointed out that often the only evidence that oxidative stress has occurred in vivo may be the up-regulation of antioxidant defense systems. In particular, H_2O_2 seems to play a dual role by eliciting localized death of plant and pathogen cells and as a diffusible signal for the induction of antioxidant and pathogenesis-related genes in adjacent plant tissues (6,38,64).

Fig. 2. Suppression of pathogen-induced local necrotic symptoms in tobacco ('Xanthi') by H_2O_2 pretreatments is coupled to the enhanced activity of several antioxidant enzymes. Plants were pretreated by spraying leaves with tap water or 5 mM H_2O_2 1 day before pathogen inoculation. Leaves were inoculated with **A, B, and C**, *Tobacco mosaic virus* (TMV) or **D, E, and F**, *Pseudomonas syringae* pv. *phaseolicola*. Mock = mock-inoculated leaves. DAI = days after inoculation. Means of three independent biological experiments $\pm$ SE are shown. Differences between time points (DAI) were nonsignificant, while the effect of H_2O_2 pretreatment was always significant as determined by analysis of variance ($P = 0.05$).

High, sustained levels of H_2O_2 in potato, tobacco, and cabbage achieved by transgenic approaches caused resistance to disease symptoms and pathogen multiplication at least in part by direct killing and/or inhibition of plant-pathogenic bacteria (6,37,65).

The present study is the first to show that pretreatment of tobacco plants with low concentrations (5 to 10 mM) of H_2O_2 ("immunization") induces resistance to symptom development by suppressing pathogen-induced host cell and tissue necroses but not pathogen multiplication, while enhancing activities of at least three antioxidant enzymes (catalase, guaiacol peroxidase and ascorbate peroxidase). Transcriptional changes in H_2O_2 -pretreated plants also support the role of antioxidants: expression of genes that encode SOD, CAT, and APX was significantly enhanced in H_2O_2 -pretreated plants early after TMV infection. Artificial appli-

cation of two antioxidant enzymes (SOD and CAT) suppressed the development of necroses caused by viral, bacterial, or fungal pathogens in a similar manner as H_2O_2 pretreatment implying that H_2O_2 -mediated "immunization" operates through the enhancement of plant antioxidant capacity. SOD is known to generate endogenous H_2O_2 through dismutation of superoxide (O_2^-), while H_2O_2 -degrading enzymes like catalase could be directly responsible for the suppression of necrotic symptoms (42). When inducing symptom resistance by antioxidant treatments we simultaneously applied SOD and CAT for two reasons. First, it should be emphasized that superoxide is a ROS that, similar to H_2O_2 , can be harmful at high concentrations to plants and animals as well as their pathogens (25,54). Second, although CAT plays a significant role in reducing high levels of H_2O_2 in planta, its affinity for H_2O_2 is rather low. Thus, CAT needs high H_2O_2 concentrations to work efficiently which can be ensured by the presence of active SOD (1,25).

Our results are in line with those of Gechev et al. (19) and Barba-Espin et al. (2) who demonstrated that pretreatment of tobacco plants or pea seedlings with 5 to 20 mM H_2O_2 (a mild oxidative stress) stimulates antioxidant enzymes like CAT, APX, and other peroxidases, confers tolerance to abiotic stresses and increases seed germination and growth. In addition, treatment of pepper fruits with 20 mM H_2O_2 significantly improves shelf life during postharvest storage (22). Furthermore, it was shown that this mild oxidative stress induces expression of a battery of genes

Fig. 3. Suppression of *Tobacco mosaic virus* (TMV)-induced local necrotic symptoms in tobacco ('Xanthi') by H_2O_2 pretreatments is coupled with the early induction of antioxidant genes (*NtSOD*, *NtCAT*, and *NtAPX*), as determined by real-time reverse transcription-quantitative polymerase chain reaction. Plants were pretreated by spraying leaves with tap water or 5 mM hydrogen peroxide (H_2O_2) 1 day before TMV inoculation. A relative gene expression of 100% represents transcript levels in leaves at the time of TMV inoculation. Means of three independent biological experiments $\pm$ SE are shown. Significant differences conferred by H_2O_2 pretreatments were calculated by the Student's *t* test, where *, **, and *** indicate significance at the 0.05, 0.01, and 0.001 levels of confidence, respectively. ns indicates non-significance.

Fig. 4. Suppression of pathogen-induced necrotic symptoms in tobacco ('Xanthi') by H_2O_2 pretreatments or antioxidants does not significantly alter pathogen multiplication. Plants were pretreated by spraying leaves with tap water or 5 mM H_2O_2 1 day before pathogen inoculation or infiltrating leaves with a mixture of superoxide dismutase and catalase (SOD + CAT, 3,000 and 5,000 units/leaf, respectively) immediately after pathogen inoculation. **A**, *Tobacco mosaic virus* (TMV) titers were assayed 3 days after inoculation (DAI) by enzyme-linked immunosorbent assay. **B**, Multiplication of *Pseudomonas syringae* pv. *phaseolicola* was monitored 1, 2, and 3 DAI. Means of three independent biological experiments $\pm$ SE are shown. Differences between time points (DAI) were significant, while the effects of H_2O_2 pretreatment and SOD + CAT treatment were always nonsignificant as determined by analysis of variance ($P = 0.05$).

and proteins associated with up-regulation of different signal transduction components and tolerance to abiotic stresses (2,58). Interestingly, high enzymatic activities of CAT and APX were also found in transgenic tobacco constitutively overexpressing a fungal glucose oxidase gene, producing elevated levels of H_2O_2 and displaying enhanced chilling tolerance (39). Similarly, high activities of antioxidant enzymes were observed in transgenic tobacco expressing a wheat oxalate oxidase (*OxO*) gene (62). These plants also produced higher than normal amounts of H_2O_2 and displayed increased tolerance to paraquat and high light stress. Inhibitors of *OxO* and scavengers of H_2O_2 blocked the stimulated tolerance to the abiotic stresses. It is worth to mention that H_2O_2 -mediated "immunization" via enhancement of antioxidant capacity is also effective in a plant pathogenic bacterium: exposing *Agrobacterium tumefaciens* cells to very low concentrations of H_2O_2 induced high catalase and peroxidase activities helping thereby bacteria to survive in the host plant (45). The finding that the survival of microbes in planta may depend on their antioxidant capacity suggests that unchanged pathogen multiplication in H_2O_2 -pretreated tobacco could be also a function of the ROS-scavenging capacity (H_2O_2 tolerance) of a given pathogen. It has been demonstrated that a low concentration of H_2O_2 inhibited fungal development in young (4-day-old) *Septoria tritici* cultures while a much higher concentration (50 mM H_2O_2)

was required to inhibit growth in older (16-day-old) cultures, reflecting the differing ability of a pathogen to tolerate H_2O_2 during different stages of its life cycle (51).

One characteristic that differentiates H_2O_2 -mediated symptom resistance from typical disease resistance responses is the suppression of pathogen-induced necrotic symptoms but not pathogen multiplication. One should emphasize that the fact that H_2O_2 pretreatment suppresses a part of the defense response (HR-type cell and tissue death) does not necessarily imply an enhancement of pathogen multiplication. Numerous studies had demonstrated that in several viral, bacterial, and fungal infections plant resistance during an HR is independent of cell and tissue death (4,5,10,18,31,49,67, for a review see 30). For example, in past years the generally accepted hypothesis regarding the suppression of spread and multiplication of plant viruses during HR was that the cause of virus resistance is the appearance of tissue necroses upon infection. However, this hypothesis was later criticized on the basis of new experiments (5,10) showing that in certain plant-virus interactions resistance associated with HR is independent of tissue necrosis. This idea is supported by our results, according to which in a local lesion host, pretreated with low concentrations of H_2O_2 or infiltrated with SOD and CAT, the lack of necrotization did not influence TMV levels compared with control plants which were only infected with the virus. Previously, we have also shown that at high temperature increased virus titers and suppression of TMV-induced HR-type necroses are independent phenomena in Xanthi-nc tobacco but are associated with up-regulation of an enzymatic antioxidant and down-regulation of superoxide (29). Similarly, bacterial multiplication did not change significantly in the H_2O_2 -pretreated or SOD plus CAT-treated leaves. This result is supported by previous studies showing that suppression of necrotic symptoms by albumin treatment or transgenic overproduction of cytokinin did not significantly alter bacterial numbers in resistant tobacco plants (4,33). Furthermore, bacterial resistance can be genetically uncoupled from tissue necrosis during an HR (11,18,67). As regards multiplication of the fungus *B. cinerea* in H_2O_2 -pretreated or SOD plus CAT-treated leaves, we did not assay fungal DNA or RNA levels in planta. However, the low concentration of H_2O_2 (5 mM) that suppressed normosensitive necroses caused by *B. cinerea* in tobacco leaves did not suppress growth of this fungus in an artificial medium for at least 3 days (53, this study). Therefore, it is reasonable to assume that application of 5 mM H_2O_2 does not significantly influence fungal multiplication in tobacco. This is supported by findings that *B. cinerea* indeed effectively tolerates H_2O_2 -induced oxidative stress in planta as well (53). In fact, even a *B. cinerea* mutant ($\Delta BAPI$) defective in a pivotal regulator of ROS detoxification and sensitive to 5 mM H_2O_2 in vitro can accumulate to similar levels as the wild type in infected bean leaves (53).

As regards the possible role of SA in this type of H_2O_2 -mediated, induced symptom resistance, one has to consider related earlier findings. The most direct evidence for the role of SA in plant resistance responses has come from experiments with transgenic plants unable to synthesize or accumulate high levels of SA. These tobacco and *Arabidopsis thaliana* plants displayed enhanced pathogen susceptibility and/or failed to develop systemic acquired resistance to a subsequent infection (12,17,63). Indeed, SA plays a key role in maintaining efficient resistance responses (i.e., a reduction in symptoms and pathogen levels) in higher plants infected with viruses, bacteria, or fungi (7,8,16, 24,35,40,43,44,47,57,60,66). Overproduction of SA by bacterial transgenes in plants also stimulates resistance to pathogens (59). However, overexpression of SA glucosyltransferase1 (*AtSGT1*) in transgenic *Arabidopsis* leads to reduced levels of both free and bound (glucosylated) SA and increased susceptibility to *P. syringae* pv. *maculicola* (52). While very high levels of SA glucosyltransferases may cause perturbations in disease resistance responses, it was also demonstrated that a rice SA glucosyl-

Fig. 5. Salicylic acid (SA) does not seem to play a role in H_2O_2 -mediated suppression of pathogen-induced necrotic symptoms in tobacco ('Xanthi'). Plants were pretreated by spraying leaves with tap water or 5 mM hydrogen peroxide (H_2O_2) 1 day before *Tobacco mosaic virus* (TMV) inoculation. **A**, Expression of a tobacco SA glucosyltransferase gene (*NtSGT*) in TMV-inoculated leaves, as determined by real-time reverse transcription-quantitative polymerase chain reaction. A relative gene expression of 100% represents transcript levels in leaves at the time of TMV inoculation. **B**, Levels of free and bound (glucosylated) SA in TMV- and mock-inoculated tobacco leaves 3 days after inoculation, as determined by high-performance liquid chromatography. Means of three independent biological experiments $\pm$ SE are shown. **A**, Significant differences conferred by H_2O_2 pretreatments were calculated by the Student's *t* test, where * and ** indicate significance at the 0.05 and 0.01 levels of confidence, respectively. ns indicates nonsignificance. **B**, The effect of H_2O_2 pretreatment was always nonsignificant as determined by analysis of variance ($P = 0.05$).

transferase (*OsSGT1*) is indeed a key mediator of development of chemically induced resistance to rice blast caused by *Magnaporthe oryzae* (56). Furthermore, expression of a tobacco SA glucosyltransferase gene (*NtSGT*) is induced early, only a few hours after inoculation with incompatible viral and bacterial pathogens that cause HR-type necrosis (36).

In our study a lack of early up-regulation of *NtSGT* in H₂O₂-pretreated and TMV-inoculated tobacco was associated with unchanged levels of free and bound (glucosylated) SA and enhanced resistance to HR-type necrosis compared with nontreated plants. It seems that higher than normal levels of SA do not play a role in H₂O₂-mediated resistance to symptom development which could differentiate this type of symptom resistance from the classical SA-mediated resistance responses (7,8,9,44,47,50,60). Unaltered SA levels could be one reason why the symptom resistance described by us is ineffective in reducing pathogen levels.

In summary, "immunization" to necrotic symptom development with a mild H₂O₂ stress seems to operate through an enhanced activity of host antioxidant genes and enzymes. Although pathogens are not inhibited, effective suppression of harmful disease symptoms during this H₂O₂-mediated, induced resistance might be envisaged as a complementary measure of future integrated pest management practices.

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