Tobacco Class I and II Catalases Are Differentially Expressed During Elicitor-Induced Hypersensitive Cell Death and Localized Acquired Resistance

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Expression of tobacco class I (CAT1) and class II (CAT2) catalases was analyzed in leaves reacting hypersensitively to tobacco mosaic virus (TMV) or to a fungal glycoprotein elicitor. In TMV-infected plants, *Cat1* transcript levels declined rapidly while *Cat2* transcripts accumulated strongly. The spatial and temporal changes in catalase transcripts, proteins, and activity during the hypersensitive reaction (HR) were further investigated in tobacco leaves infiltrated with a glycoprotein elicitor. Two functionally different zones were discriminated: the infiltrated tissue in which cells undergo the HR, called the HR-zone 1; and the surrounding tissue showing strong induced defense responses, called the LAR (Localized Acquired Resistance)-zone 2. Levels of *Cat1* and *Cat2* mRNA and proteins and catalase activity decreased in the HR-zone 1. In the LAR-zone 2, *Cat1* transcripts became rapidly undetectable, but levels of *Cat2* mRNA and protein and catalase activity increased. Catalase expression in elicitor-infiltrated leaves reflected that in TMV-infected leaves. A strong rise in hydrogen peroxide occurred in the HR-zone 1 and paralleled the CAT activity decline. No H_2O_2 increase was measured in the LAR-zone 2. There was no correlation between salicylic acid levels and catalase activity. Modulation of catalase activity in tobacco leaves undergoing the HR appeared predominantly supported by changes in catalase transcripts and proteins. We have shown that neither H_2O_2 nor salicylic acid can be the primary mobile signal diffusing from the HR-zone 1 to the LAR-zone 2 and inducing CAT2 expression. Furthermore, the signaling pathway responsible for decreased CAT2 expression in the HR-zone 1 does not involve reactive oxygen intermediates.

Additional keywords: gene expression, *Nicotiana tabacum*.

The hypersensitive reaction (HR) is an induced disease resistance mechanism by which plants cope with invading pathogens (Goodman and Novacky 1994). Phenotypically, the

HR results in plant cell death occurring at the site of pathogen penetration. Resistance is also induced both locally and systemically. Localized acquired resistance (LAR) occurs within a narrow zone around the HR lesion, and is characterized by a strong stimulation of defense responses (Dorey et al. 1997; Ross 1961). Systemic acquired resistance (SAR) develops in noninfected parts of the plant and only a subset of defense responses is triggered (Ryals et al. 1996). The biochemical responses observed in hypersensitively reacting plants are highly diverse, and are regulated by different signals (Hammond-Kosack and Jones 1996). Salicylic acid (SA) and hydrogen peroxide (H_2O_2) are two of those signals that play a crucial role in the establishment of local and systemic defense responses (Alvarez et al. 1998; Delaney et al. 1994; Levine et al. 1994).

Catalase (CAT) is one of the major enzymatic systems for the removal of H_2O_2 in plant cells (for a review see Willekens et al. 1995). A model was proposed in which SA binds and inactivates CAT enzymes, resulting in an increase in intracellular H_2O_2 that in turn acts as an inducer of defense genes like the PR-1 gene (Chen et al. 1993; Conrath et al. 1995; Durner and Klessig 1996). The model was first developed to explain the SA-mediated induction of SAR. There are reports, however, showing that in SAR tissues H_2O_2 may act rather upstream of SA (Bi et al. 1995; Neuenschwander et al. 1995). Furthermore, analysis of transgenic tobacco plants with severely reduced CAT activity revealed that CAT reduction per se was not a sufficient signal for PR-1 induction, and that rapid and strong expression of PR-1 required SA and occurred only when leaves developed necrotic symptoms (Chamnongpol et al. 1996, 1998; Takahashi et al. 1997). Whether CAT inactivation by SA plays a crucial role during the HR remains an open question.

Changes in CAT activity levels were observed in several but not all pathosystems. For instance, in bean leaves inoculated with an avirulent strain of *Pseudomonas syringae* pv. *phaseolicola* there was a decrease in CAT activity followed by a recovery and a further decrease when tissue collapsed; CAT activity first declined and then increased in the vicinity of the infiltration site (Adam et al. 1995). Using the same pathosystem, other authors observed a decrease in CAT activity in the infiltrated tissue, but not in the immediately surrounding tissue (Milosevic and Slusarenko 1996). In contrast, when examining CAT activity in the incompatible interaction between tobacco and *P. syringae* pv. *syringae*, Bi et al. (1995) did not

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measure significant changes. It should be pointed out that the pathogens themselves may express CAT proteins, as shown for phytopathogenic strains of *P. syringae* that contain multiple CAT activities in both periplasmic and cytoplasmic fluids (Klotz and Hutcheson 1992). Furthermore, these CAT activities were found to increase in response to treatment with H_2O_2 (Klotz and Hutcheson 1992). Such an observation may, in part, explain these apparently inconsistent results. Hence, in order to analyze the expression of plant CATs when local defense responses are induced, one should preferably use a system in which the pathogen does not express CAT proteins, i.e., a virus, or would be replaced by a molecule inducing all characteristics of a typical HR.

In the present study, we analyzed the expression of CAT genes in *Nicotiana tabacum* cv. Samsun NN leaves reacting hypersensitively to tobacco mosaic virus (TMV), the biological system for which the model of CAT inhibition by SA was proposed (Chen et al. 1993). *N. plumbaginifolia* *Cat1* (class I) and *Cat2* (class II) cDNA probes were used (Willekens et al. 1994a) since *Cat* genes from *N. tabacum* exhibit high identity (>97%) to *N. plumbaginifolia* *Cat1* and *Cat2* genes (Takahashi et al. 1997), and since CAT1 and CAT2 are the two major CATs expressed in mature tobacco leaves (Havir et al. 1996).

We further analyzed the spatial and temporal distribution of CAT mRNA, proteins and activity, and also the levels of H_2O_2 , which is the substrate of CATs, in tobacco leaves infiltrated with a fungal glycoprotein elicitor inducing the HR (Baillieul et al. 1995). This system clearly distinguishes spatially two zones of the overall HR response: (i) the infiltrated zone in which cells undergo events leading ultimately to cell death, called the HR-zone 1; and (ii) the surrounding tissue, about 5 mm in width, called the LAR-zone 2 since it showed strong induced defense responses (Dorey et al. 1997). These defense responses are obviously induced by a plant signal originating from the HR-zone 1 since the LAR-zone 2 was shown to remain free of elicitor (Dorey et al. 1997). We also investigated the signals involved in CAT2 expression and the question whether modulating the *in vivo* levels of SA would affect the levels of CAT activity.

RESULTS

Expression of *Cat1* and *Cat2* genes in tobacco leaves reacting hypersensitively to TMV.

Very striking differences in expression of *Cat1* and *Cat2* genes were found in tobacco leaves reacting hypersensitively to TMV (Fig. 1). A decrease in *Cat1* transcript levels occurred as early as 24 h after virus inoculation, i.e., before appearance of visible symptoms of necrotization. *Cat1* mRNA remained almost undetectable between days 2 and 7. In contrast, within the same period, *Cat2* gene expression was strongly stimulated. Accumulation of transcripts occurred as soon as 1 day post-inoculation and remained sustained during the course of the infection with a maximum at 7 days. These results indicated a striking differential expression of class I and II CAT genes during the typical HR of tobacco to TMV.

Levels of CAT transcripts, proteins, and activity in the HR-zone 1.

We then investigated the spatial and temporal expression of class I and II CATs in our experimental system, discriminating cells undergoing HR cell death and cells of the LAR zone.

Tobacco leaves were infiltrated either with 50 nM fungal glycoprotein elicitor or with water, and total mRNA and proteins were extracted from the infiltrated zone 1. We found a rapid decrease in both *Cat1* and *Cat2* mRNA levels (Fig. 2A, "Elicitor" lanes). The amount of *Cat1* and *Cat2* transcripts became almost undetectable after 4 h. A transient decrease in *Cat1* mRNA levels also occurred when tissue was infiltrated with water only as a control of treatment (Fig. 2A, "Water" lanes), while *Cat2* expression remained almost unaffected. Thus, expression of both *Cat1* and *Cat2* genes strongly decreased in cells undergoing the HR. The same mRNA samples had been probed against phenylalanine ammonia-lyase (PAL), *O*-methyltransferase (OMT), and hydroxymethylglutaryl CoA reductase (HMGR) cDNAs (see Figure 3 in Dorey et al. 1997), showing strong, increased steady-state levels of the messengers.

CAT enzyme is a tetramer composed of four similar or identical subunits. In *N. tabacum*, the molecular mass of CAT1 and CAT2 subunit polypeptides is 57 and 55 kDa, respectively (Takahashi et al. 1997). Immunoblot analysis revealed a strong drop in CAT1 and CAT2 levels (Fig. 2B, "E" lanes), and no CAT protein was detectable 18 h after elicitor-treatment. This observation correlated well with the analysis of the corresponding mRNA. The rapid decline in CAT transcripts in HR cells resulted in a parallel decrease in the amounts of the corresponding proteins.

Analysis of total CAT activity showed a decrease in the HR-zone 1 (Fig. 2C, solid circles) compared with tissue infiltrated with water (Fig. 2C, open circles). Similar kinetics were observed when total CAT activity was expressed on a fresh weight basis (data not shown). Hence, in the elicitor-treated zone, the decrease in CAT activity was well correlated with that in CAT protein levels. As an internal control of defense response induction, we also measured OMT and guaiacol peroxidase activities in the same samples. Five- and threefold increases in OMT-specific activity were found at 8 and 14 h, respectively (data not shown), which is in good agreement with our previous results from OMT gene expression analysis (Dorey et al. 1997). Specific guaiacol peroxidase activity also increased by two- and eightfold at 14 and 18 h, respectively (data not shown).

Levels of CAT transcripts, proteins, and activity in the LAR-zone 2.

Northern (RNA) blot analysis revealed a complex and differential pattern of *Cat1* and *Cat2* mRNA distribution in the

Fig. 1. Accumulation of *Cat1* and *Cat2* transcripts in tobacco leaves reacting hypersensitively to tobacco mosaic virus (TMV). Leaves were inoculated with TMV and total RNA was extracted at the different times of infection indicated (days), fractionated in agarose-formaldehyde denaturing gels, and hybridized with *Cat1* and *Cat2* probes.

LAR-zone 2. Levels of *Cat1* mRNA rapidly decreased as a result of elicitor and water infiltration and remained almost undetectable during the course of the experiment (Fig. 3A). In contrast, *Cat2* mRNA levels increased significantly above the control and remained high during the course of the experiment (Fig. 3A, "Elicitor" lanes). Water treatment alone had only a minor and transient effect on *Cat2* gene expression (Fig. 3A, "Water" lanes).

The pattern of CAT2 protein correlated with that of *Cat2* mRNA. Increased levels of CAT2 were detected in the LAR-zone 2 (Fig. 3B, "E" lanes) compared with the controls (Fig. 3B, "NT" and "W" lanes). Several assays with line 3B6 antibodies that are given as CAT1 and CAT2 specific (Takahashi

et al. 1997) revealed only a single band corresponding to CAT2 (data not shown). CAT activity appeared slightly stimulated (Fig. 3C). Similar kinetics were observed when total CAT activity was expressed on a fresh weight basis (data not shown). The results revealed that CAT activity was slightly stimulated in the LAR cells at the later times as an overall result of differential expression of CAT1 and CAT2. As for zone 1, we measured OMT and guaiacol peroxidase activities in zone 2 and we found, at 48 h, a five- and threefold increase over the control in OMT and peroxidase, respectively (data not shown).

Levels of H₂O₂ in the HR-zone 1 and LAR-zone 2.

H₂O₂ is the substrate of CATs and its accumulation is a characteristic feature of plant tissues undergoing the HR

Fig. 2. Analysis of catalase transcripts, proteins, and activity in the hypersensitive reaction (HR)-zone 1 of tobacco leaves infiltrated with 50 nM glycoprotein elicitor. **A**, RNA gel blot analysis. Total RNA was extracted at the different indicated times (hours) from nontreated ("NT" lane), water-infiltrated ("Water" lanes), and elicitor-infiltrated ("Elicitor" lanes) tissues. Blot was hybridized with *Cat1* and *Cat2* probes. **B**, Immunoblot showing CAT1 and CAT2 protein accumulation in nontreated ("NT" lane), water-infiltrated ("W" lanes), and elicitor-infiltrated ("E" lanes) tissues at indicated times after treatment (hours). Line 3B6 antibodies were used as probe. The same samples were blotted against line 1F5 antibodies, which are CAT2-specific, and only the 55-kDa protein band was revealed. **C**, Kinetics of catalase (CAT) activity. Total protein was extracted from nontreated (zero time point), water-infiltrated (open circles), and elicitor-infiltrated (solid circles) tissues, and CAT-specific activity was measured. Each time point represents mean and standard deviation from duplicates.

Fig. 3. Analysis of catalase transcripts, proteins, and activity in the LAR (Localized Acquired Resistance)-zone 2 of tobacco leaves infiltrated with 50 nM glycoprotein elicitor. **A**, RNA gel blot analysis. Total RNA was extracted at the different times indicated (days) from nontreated tissue ("NT" lane), and tissue surrounding water-infiltrated ("Water" lanes) and elicitor-infiltrated (LAR-zone 2, "Elicitor" lanes) tissues. Blot was hybridized with *Cat1* and *Cat2* probes. **B**, Immunoblot showing CAT2 protein accumulation in nontreated tissue ("NT" lane), and tissue surrounding water- ("W" lanes) and elicitor-infiltrated (LAR-zone 2, "E" lanes) tissues, at the times after treatment indicated (days). Line 1F5 antibodies were used as probe. **C**, Kinetics of catalase (CAT) activity. Total protein was extracted from nontreated (zero time point) tissue, and tissue surrounding water-infiltrated (open circles) and elicitor-infiltrated (LAR-zone 2, solid circles) tissues. Each time point represents mean and standard deviation from duplicates.

(Lamb and Dixon 1997). Thus, we further addressed the questions whether the glycoprotein treatment induced a burst of H_2O_2 and whether such a burst occurred in both HR-zone 1 and LAR-zone 2. The results are summarized in Figure 4. Water treatment (Fig. 4, open circles) did not cause significant changes in H_2O_2 levels in either the infiltrated (Fig. 4A) or the surrounding (Fig. 4B) tissues. However, H_2O_2 increased in the HR-zone 1 as soon as 10 h post-treatment and accumulated to a concentration of about 5 mM at 18 h (Fig. 4A, solid circles). It is noteworthy that in this zone the strong increase in H_2O_2 occurred at a time when CAT activity had already strongly decreased. In contrast, in LAR-zone 2, no significant increase in H_2O_2 was measured (Fig. 4B). Measurements performed in the LAR-zone 2 over a longer period of time, several days, did not show increased accumulation of H_2O_2 either (data not shown).

Relationships between CAT2 expression and ROIs.

Expression of CAT2 protein was further analyzed in relation to reactive oxygen intermediates (ROIs), particularly H_2O_2 , with both gain and loss of function experiments. Treatments of leaves with 5 mM H_2O_2 , or the H_2O_2 generator system, glucose oxidase/glucose, did not induce changes in CAT2 expression either at the infiltrated site (Fig. 5A) or in the surrounding zone 2 (Fig. 5B), compared with controls. This result suggested that (i) H_2O_2 was not the signal inducing the decrease in CAT2 in zone 1, and (ii) H_2O_2 was not the diffusing molecule signaling CAT2 stimulation in zone 2. This latter observation was further supported by the fact that co-infiltration of the elicitor with CAT enzyme or DPI, which was shown to block the oxidative burst in plant cells via the inhibition of NADPH oxidases (Levine et al. 1994), did not compromise the increased accumulation of CAT2 in the sur-

rounding zone 2 occurring when the elicitor was applied alone (Fig. 5B). The same treatments also did not compromise decreased CAT2 expression in zone 1, suggesting a signaling pathway independent from ROIs (Fig. 5A).

Is there a correlation between CAT activity and SA levels?

The above results revealed a complex expression of CATs in tissues undergoing HR and LAR, suggesting that, in addition to the hypothesized inhibition of CAT activity by SA, plant cells modulate CAT activity through modulation of CAT transcripts and protein levels. To address the question whether CAT inhibition by SA could have some biological relevance in our simplified system, we investigated the effects on CAT activity of modulation of SA amounts. Reduced SA levels were obtained by two means: (i) co-infiltration of the elicitor with α -aminooxy- β -phenylpropionate (AOPP), a known and efficient inhibitor of PAL activity (Dorey et al. 1997); or (ii) use of NahG tobacco plants that express a bacterial gene catabolizing SA into the inactive molecule catechol (Delaney et al. 1994). Levels of SA and CAT activity were measured in the HR-zone 1 at 14 h after treatment since SA accumulation was maximum at that time (Dorey et al. 1997), and since the remaining CAT activity was about 50 to 60% of the control (Fig. 2C). Results are shown in Figure 6A. A similar reduction in CAT activity, about 45% compared with the control, was obtained in Samsun NN plants treated with the elicitor alone or with both the elicitor and AOPP (Fig. 6, black bars). A 40% reduction in CAT activity was observed in NahG plants as a result of elicitor infiltration (Fig. 6, black bars).

Analysis of SA levels revealed very different relative changes. SA increased up to 200 ng per g of fresh weight (FW) in elicitor-infiltrated Samsun NN leaves, corresponding to an eightfold increase over the control (Fig. 6A, white bars). As expected (Dorey et al. 1997), co-infiltration of AOPP and the glycoprotein clearly reduced SA accumulation (about 50%). Amounts of SA in elicitor-treated NahG leaves remained low and similar to that in the control (Fig. 6A, white

Fig. 4. Kinetics of hydrogen peroxide levels in the hypersensitive reaction (HR)-zone 1 and the LAR (Localized Acquired Resistance)-zone 2 of tobacco leaves infiltrated with 50 nM glycoprotein elicitor. Amounts of H_2O_2 were measured in nontreated (zero time point), water-treated (open circles), and elicitor-treated (solid circles) leaves. Each time point represents mean and standard deviation from duplicates. **A**, Hydrogen peroxide in zone 1. **B**, Hydrogen peroxide in zone 2.

Fig. 5. CAT2 expression in tobacco leaves following treatments with H_2O_2 , H_2O_2 -generating compounds, elicitor and diphenylene-iodonium (DPI), and elicitor and catalase enzyme. Tobacco leaves were infiltrated with 50 nM glycoprotein (E), water (H_2O), 5 mM H_2O_2 , glucose oxidase (2.5 U/ml) + 2.5 mM glucose (GOG), 50 nM glycoprotein + 10 μ M DPI (E+DPI), 50 nM glycoprotein + bovine catalase enzyme, 100 U/ml (E+Cat), 10 μ M DPI (DPI), and bovine catalase enzyme, (100 U/ml) (Cat). Proteins were extracted, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted. Blots were probed against line 1F5 antibodies specific for CAT2. Analysis of proteins from **(A)** the infiltrated zone 1 at 14 h after treatment and **(B)** the surrounding zone 2 at 3 days after treatment.

bars), indicating that the salicylate hydroxylase was efficient in maintaining low levels of SA despite the elicitor treatment.

We also analyzed SA and CAT activity levels in the LAR-zone 2 of Samsun NN leaves 3 days after treatment (Fig. 6B). Both SA and CAT activity increased, compared with the control (fourfold increase for SA, and about 30% increase for CAT activity). Taken together, these results showed an absence of correlation between the levels of SA and those of CAT activity.

CAT2 stimulation in the LAR-zone 2 is SA-independent.

The above results strongly suggested that the stimulation of CAT2 in the LAR-zone 2 was not induced by ROI molecules that had diffused from zone 1 where they had accumulated. We further examined whether SA could be the signaling molecule stimulating CAT2 expression in the LAR-zone 2. Analysis of the latter zone in NahG leaves revealed the same CAT2 induction as in control wild-type plants (Fig. 7). These results suggested that SA was not the signal triggering CAT2 expression. As an internal control, we probed the same extracts against PR-1 antibodies. As expected, accumulation of

PR-1 proteins was suppressed in NahG plants, compared with wild-type plants (Fig. 7).

DISCUSSION

Our study on the expression of CAT1 and CAT2 during the HR of tobacco to TMV infection or to a fungal glycoprotein elicitor leads to five major findings: (i) CAT1 and CAT2 exhibited striking differential expression, the relative changes in transcript levels paralleled changes in the levels of CAT proteins and activity; (ii) in HR tissue both CAT1 and CAT2 strongly decreased with a concomitant burst of H_2O_2 ; (iii) on the other hand, CAT2 was induced in LAR tissue without any striking changes in H_2O_2 levels; (iv) in our experimental system based on the infiltration of an elicitor of the HR, the modulation of CAT activity through inhibition by SA apparently played only a minor role, if any; and (v) CAT2 expression in zones 1 and 2 is independent from ROI and SA signaling.

Here we demonstrate for the first time that a differential expression of class I and II CAT genes occurs during the typical HR of tobacco to TMV. A strong and sustained accumulation of *Cat2* transcripts occurred in hypersensitively reacting TMV-infected leaves, with a concomitant disappearance of *Cat1* transcripts. From data available in the literature on CAT regulation in plants upon compatible pathogen infection or chemical treatments it appears also that class II CATs are usually stimulated while class I CATs are rather repressed (Niebel et al. 1995; Willekens et al. 1994b; Williamson and Scandalios 1992). Hence, Willekens et al. (1995) have suggested that class II CATs are likely to play a role in defense against various environmental stresses. Our results disclose a similar role for class II CATs during the HR.

One major difficulty in the interpretation of overall differential gene expression during incompatible plant-pathogen interactions is that it does not necessarily reflect differential expression in the same cells but is likely to result from very distinct gene regulation in different zones of tissue, such as the HR and the LAR zones. One additional difficulty is that these zones are not fixed but extend radially in the course of an infection. Our results have shown that *Cat1* and *Cat2* gene expression in HR and LAR tissues of elicitor-infiltrated leaves reflected that found in leaves reacting hypersensitively to TMV. Levels of *Cat1* and *Cat2* mRNA dropped dramatically in the HR tissue, and CAT1 and CAT2 protein levels decreased as well. In the LAR-zone 2, expression of CAT2 was

Fig. 6. Levels of catalase (CAT) activity and of salicylic acid (SA) in tobacco plants co-infiltrated with both the elicitor and α -aminooxy- β -phenylpropionate (AOPP) or with the elicitor alone. **A**, Levels of CAT activity (black bars) and of SA (white bars) in the hypersensitive reaction (HR)-zone 1 of Samsun NN and transgenic NahG plants. W: tissue infiltrated with water. E: tissue infiltrated with the elicitor alone. E+AOPP: tissue co-infiltrated with the elicitor and 250 μ M AOPP. Effect of infiltration of AOPP alone was identical to infiltration of water. Values are means with standard deviation from duplicates. **B**, Levels of CAT activity and of SA in the LAR (Localized Acquired Resistance)-zone 2 of Samsun NN plants. Bar code as in **A**. Values are means with standard deviation from duplicates.

Fig. 7. CAT2 expression in the LAR (Localized Acquired Resistance)-zone 2 of elicitor-treated tobacco NahG leaves. Wild-type (WT) and NahG plants were infiltrated with 50 nM glycoprotein (E) or water (C). Proteins were extracted from the LAR-zone 2 at 3 days after treatment, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted. Blot was probed, as indicated, against line 1F5 antibodies specific for CAT2 and against antibodies specific for tobacco PR-1 proteins.

induced over a long period of time (several days) while CAT1 declined, and the corresponding proteins followed a similar pattern. The resulting CAT activity appeared significantly stimulated in the LAR zone. It should be noted that the CAT mRNA and protein levels found in control plants fluctuated slightly from one experiment to another, but these differences did not compromise the conclusions. Such differences reflected rather the fact that experiments had been performed with different batches of plants. The correlation between CAT mRNA and protein levels evidenced in our system is in good agreement with previous reports (Feierabend et al. 1992; Hertwig et al. 1992) showing that CAT proteins display a high turnover rate.

The drop of CAT mRNA in the HR tissue was not caused by cell death occurring several hours after elicitor application (Dorey et al. 1997) since at the same time a strong induction of PAL, OMT, and HMGR genes occurred in the same samples (see Figure 3 in Dorey et al. 1997). Similarly, cell death was also not the cause of the drop in CAT protein and activity levels because a stimulation of OMT and guaiacol peroxidase activities was measured in the same protein extract used to measure CAT proteins and activity.

For unknown reasons, CAT1 expression appeared particularly sensitive to the infiltration procedure itself, i.e., there was a rapid decline in CAT1 mRNA and protein levels in water-infiltrated leaves. In *N. plumbaginifolia*, CAT1 shows a circadian rhythm when plants are exposed to high light intensities (Willekens et al. 1994a). Such regulation for CAT1 in our experiments can be ruled out since plants were grown at low light intensities ($<100 \mu\text{E s}^{-1} \text{m}^{-2}$).

A hallmark of incompatible plant-pathogen interactions is the occurrence of an oxidative burst (Lamb and Dixon 1997). Treatment of tobacco leaves with the HR-inducing glycoprotein resulted after a few hours in a large increase in H_2O_2 that was localized to the HR tissue; this increase paralleled the decrease in CAT activity. Apparently, the plant strategy to elevate levels of H_2O_2 combines the stimulation of systems to produce it and the repression of other systems, like the CATs, whose function is to remove it. Milosevic and Slusarenko (1996) came to the same conclusion when analyzing the localization and changes in activity of CAT enzymes. Considering the method for quantification of H_2O_2 and the standard deviations, we cannot exclude the possibility that a very low increase in H_2O_2 also occurred in the LAR-zone 2 and escaped the analytical procedure. However, the same method was used by others to demonstrate the absence of elevated H_2O_2 levels in tobacco tissue developing SAR (Neuenschwander et al. 1995).

Based on the capacity of H_2O_2 to freely diffuse across dialysis membranes separating two populations of suspension cultured cells, a model was proposed in which H_2O_2 from the oxidative burst would diffuse to neighboring cells and induce antioxidant genes (Levine et al. 1994). Moreover, a recent report (Alvarez et al. 1998) described an ROI-mediated systemic induction of a glutathione S-transferase antioxidant (GST) gene in *Arabidopsis thaliana*. Gain and loss of function experiments performed on our system strongly suggested that H_2O_2 (or other ROIs) was not the signal diffusing from the HR-zone 1 to the LAR-zone 2 and inducing CAT2 expression. The discrepancy between GST and CAT2 distal expression suggests a differential regulation of these two antioxidant re-

sponses toward oxidative stress signaling or may reflect a different response in *A. thaliana* and tobacco. Experiments analyzing CAT2 and PR-1 expression in the LAR-zone 2 of elicitor-treated NahG plants further supported our previous results (Dorey et al. 1997) indicating that a molecule other than SA was the diffusing signal triggering expression of various defense genes. Although SA is a necessary intermediate for PR-1 expression (Yalpani et al. 1991; Delaney et al. 1994), it is not required for CAT2 expression in our system.

Application of H_2O_2 to leaf tissue via infiltration of a 5 mM solution or via an H_2O_2 generating system did not trigger CAT2 expression at the site of treatment, though the same treatments were shown to stimulate PR-1 accumulation (Chen et al. 1993). Moreover, a similar decrease in CAT2 expression was observed in tissue infiltrated with the elicitor alone or co-infiltrated with CAT or DPI, a molecule known to inhibit the oxidative burst (Levine et al. 1994). Hence, the decrease in CAT2 expression in the HR-zone 1 does not involve ROI signaling.

The attractive model of CAT inhibition by SA (Chen et al. 1993) has been much questioned. Though the in vitro inhibition activity has been clearly demonstrated (Conrath et al. 1995; Durner and Klessig 1996), its in vivo relevance is a matter of debate. SA amounts as high as $150 \mu\text{M}$ (Vernooij et al. 1994) were found in TMV-infected tobacco leaves. Because CAT can bind SA in vitro with a K_d of $14 \mu\text{M}$ (Conrath et al. 1995; Durner and Klessig 1996), it was suggested that CAT inhibition by SA may play an important role in tissues undergoing the HR (Chamnongpol et al. 1996; Vernooij et al. 1994). The highest levels of total SA, i.e., free and conjugated SA, that we found in elicitor-treated leaves were about 2 and $6 \mu\text{M}$ in the HR and the LAR zones, respectively. In our system, free SA represents about 10% of total SA (Baillieux et al. 1995). Consequently, CAT inhibition by SA is unlikely to occur in our simplified system, but we cannot exclude the occurrence of sites with locally high accumulation of SA.

Moreover, we could not find a correlation between the levels of CAT activity and of SA. Decreasing the in vivo SA levels by two different and independent approaches did not restore CAT activity in the HR-zone 1. Both CAT activity and SA levels increased in the LAR tissue, and this could be in agreement with a protective role of SA against CAT inactivation. Such a mechanism was proposed by Durner and Klessig (1996) in the case of high levels of ROIs resulting from the oxidative burst associated with the HR. However, no elevated H_2O_2 levels occurred in the LAR tissue.

In conclusion, our results suggest that the rapid decrease in CAT activity in the HR cells contributes to a mechanism of accumulating and maintaining high H_2O_2 levels. Indeed, all the cells of the HR zone are destined to death (Dorey et al. 1997). On the other hand, induction of CAT activity in the LAR zone could play a protective role against possible oxidative damage.

MATERIALS AND METHODS

Biological assays.

Nicotiana tabacum cv. Samsun NN and *N. tabacum* cv. Xanthi nc expressing the bacterial *NahG* gene (kindly provided by Ciba-Geigy, Research Triangle Park, NC) were grown in a glasshouse under controlled conditions and trans-

ferred into growth chambers 3 to 4 days before treatments. Light intensities in the growth chambers were between 70 and 90 $\mu\text{E s}^{-1} \text{m}^{-2}$. For TMV infection, three leaves of 3-month-old Samsun NN plants were inoculated with a suspension of purified virus in order to obtain about 300 lesions. The different solutions used were infiltrated with a syringe into the mesophyll of fully developed leaves of 3-month-old plants as described previously (Dorey et al. 1997). Zone 1 corresponds to the infiltrated tissue and is precisely delineated with a felt-tip marker. At different times after treatment, leaf disks were punched out with a cork borer from zone 1 and zone 2, which is just beyond the marker line. Tissue samples were frozen in liquid nitrogen and stored at -80°C , with the exception of samples used for H_2O_2 measurements, which were analyzed immediately after harvest. *Aspergillus niger* glucose oxidase was purchased from ICN (Orsay, France), H_2O_2 (stock solution, 30% wt/wt), DPI (diphenylene-iodonium), and bovine CAT from Sigma (St. Quentin Fallavier, France), and glucose from Roth (Lauterbourg, France).

Protein analysis and CAT activity measurements.

Tissue samples were collected as follows: 6 disks (13 mm in diameter) were punched out from six zone 1 areas of leaves of three different plants and 24 disks (6 mm in diameter) were punched out from the corresponding zone 2 areas. Under our conditions, the weight of a 1-cm² disk of nontreated leaf tissue was 30 mg. Tissue was ground in liquid nitrogen to a fine powder, which was then transferred to a pre-cooled (4°C) mortar and ground further with a pestle in 700 μl of 50 mM sodium phosphate buffer (pH 7.0) containing 0.1% (wt/vol) sodium metabisulfite. Polyclar AT was then added to the homogenate, which was centrifuged at 13,000 rpm for 10 min (202MK; Sigma, St. Quentin Fallavier, France). The supernatant was desalted over a HiTrap Desalting column (Pharmacia, Orsay, France) with 50 mM phosphate buffer (pH 7.0) as eluant. CAT activity was assayed in a volume of 1 ml containing 50 mM sodium phosphate buffer (pH 7.5), 10 mM H_2O_2 , and 40 μl of protein extract. The decrease in absorbance at 240 nm was monitored for the first 30 s; during this time the reaction showed first order kinetics. Results were expressed as CAT units per mg of protein, 1 unit corresponding to 1 μmol of H_2O_2 degraded per min. *O*-methyltransferase (OMT) and guaiacol peroxidase activities were measured according to Atanassova et al. (1995) and Ngo and Lenhoff (1980), respectively. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously (Baillieul et al. 1995). For some blots, detection was performed with the immun-star chemiluminescent kit of Bio-Rad (Ivry sur Seine, France). Monoclonal antibodies of lines 3B6 and 1F5 (Chen et al. 1993) were kindly supplied by D. Klessig (Rutgers University, Piscataway, NJ). Antibodies were used at a dilution of 1:5,000. Detection of PR-1 proteins was made with antibodies directed against PR-1b and diluted 500 times.

RNA analysis.

Procedures for RNA extraction and Northern hybridization have been described previously (Baillieul et al. 1995). The tobacco *Cat1* and *Cat2* cDNA fragments (Willekens et al. 1994a) were kindly supplied by D. Inzé and W. Van Camp (Gent University, Gent, Belgium). The amounts of RNA were

controlled by hybridization of the blots to a 25S rRNA probe from *Capsicum annuum* (gift of R. Schantz, IBMP, Strasbourg, France).

SA analysis.

Tissue samples for SA analysis were collected in the same way as those for protein analysis. The extraction procedure was according to Baillieul et al. (1995) with the following modifications. Tissue was ground in liquid nitrogen and then homogenized in 0.5 ml of methanol 90%. [^{14}C]Salicylic acid (1 nCi, 54 mCi mmol⁻¹) was then added as an internal standard. This amount of SA remained undetectable through high-pressure liquid chromatography (HPLC) analysis. After centrifugation, the residue was extracted again with 100% methanol (0.4 ml). The two methanol extracts were combined and then evaporated to dryness. The dried extract was resuspended in 0.5 ml of hot water (80°C). HCl (166 μl of 12 N) was then added and the samples were heated at 80°C for 45 min. Two ether partitions (1 ml) were performed to extract SA. Ether was evaporated under nitrogen and the residue was resuspended in 200 μl of 10% acetonitrile in 20 mM sodium acetate pH 5.0. SA was quantified by HPLC and the results corrected for loss of recovery by ^{14}C counting as described in Baillieul et al. (1995).

H_2O_2 analysis.

Tissue samples were collected as follows: 9 disks (13 mm in diameter) were punched out from nine zone 1 areas of leaves of three different plants and 36 disks (6 mm in diameter) were punched out from the corresponding zone 2 areas. H_2O_2 was extracted from leaf tissue according to Okuda et al. (1991). Tissue was ground in liquid nitrogen and 1 ml of 0.2 M HClO_4 . The slurry was centrifuged at 13,000 rpm for 10 min at 4°C (202MK; Sigma). The supernatant was neutralized with 4 M KOH and centrifuged at 1,000 rpm for 1 min (202MK; Sigma). Four hundred microliters of the supernatant was applied to a column filled with AG1-X8 resin (Bio-Rad). The column was washed with 800 μl of bi-distilled water. H_2O_2 was determined by a spectrophotometric assay. The reaction mixture contained 1 ml of the eluate, 400 μl of 12.5 mM 3-dimethylaminobenzoic acid in 0.375 M phosphate buffer (pH 6.5), 80 μl of 1.3 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) in water, and 20 μl of peroxidase (0.25 unit) in a total volume of 1.5 ml. The reaction was started by the addition of peroxidase at 25°C . The increase in absorbance at 590 nm was monitored.

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