Effect of Yeast *CTA1* Gene Expression on Response of Tobacco Plants to Tobacco Mosaic Virus Infection[1]

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The response of tobacco (*Nicotiana tabacum* L. cv Xanthi-nc) plants with elevated catalase activity was studied after infection by tobacco mosaic virus (TMV). These plants contain the yeast (*Saccharomyces cerevisiae*) peroxisomal catalase gene *CTA1* under the control of the cauliflower mosaic virus 35S promoter. The transgenic lines exhibited 2- to 4-fold higher total in vitro catalase activity than untransformed control plants under normal growth conditions. Cellular localization of the CTA1 protein was established using immunocytochemical analysis. Gold particles were detected mainly inside peroxisomes, whereas no significant labeling was detected in other cellular compartments or in the intercellular space. The physiological state of the transgenic plants was evaluated in respect to growth rate, general appearance, carbohydrate content, and dry weight. No significant differences were recorded in comparison with non-transgenic tobacco plants. The 3,3′-diaminobenzidine-stain method was applied to visualize hydrogen peroxide (H_2O_2) in the TMV infected tissue. Presence of H_2O_2 could be detected around necrotic lesions caused by TMV infection in non-transgenic plants but to a much lesser extent in the CTA1 transgenic plants. In addition, the size of necrotic lesions was significantly bigger in the infected leaves of the transgenic plants. Changes in the distribution of H_2O_2 and in lesion formation were not reflected by changes in salicylic acid production. In contrast to the local response, the systemic response in upper noninoculated leaves of both CTA1 transgenic and control plants was similar. This suggests that increased cellular catalase activity influences local but not systemic response to TMV infection.

Reactive oxygen species (ROS), such as $^{\cdot}O_2$, hydrogen peroxide (H_2O_2), and $^{\cdot}OH$, are associated with a number of physiological disorders in plants (Inzé and Van Montagu, 1995). Although ROS are produced as a product of normal cell metabolism, their levels are enhanced by exposure to biotic and abiotic stresses. It has been demonstrated that ROS, including H_2O_2, are a critical factor in the sequence of events taking place on the onset of infection, leading in many cases to hypersensitive response (HR) and the activation of the pathogenesis-related genes (PR), as well as in other processes associated with response to infection (for reviews, see Bolwell et al., 1995). The rapid generation of ROS as a result of pathogen attack is referred to as oxidative burst. A growing body of evidence suggests that this process is mediated by a membrane-bound NAD(P)H oxidase that resembles the phagocyte enzyme (Scheel, 2001). As the first step, the enzyme forms superoxide radicals, which are then converted to oxygen and hydrogen peroxide either spontaneously or by an extracellular superoxide dismutase. As an alternative, the contribution of other enzymes to the oxidative burst, like peroxidase, amine oxidase, or oxalate oxidase, is postulated (Bolwell et al., 1995; Allan and Fluhr, 1997; Zhou et al., 1998). It was recently shown that HR cell death was efficiently triggered when a balance between NO and H_2O_2 production (Delledonne et al., 2001) occurred. A number of antioxidant enzymes such as superoxide dismutases, ascorbate peroxidases, peroxidases, glutathione reductases, and catalases are involved in the specific detoxification of ROS.

There are many reports that indicate that catalases may play a critical role in plant defense mechanisms (Anderson et al., 1998; Dorey et al., 1998). In general, the importance of catalase could reside both in its direct antioxidant activity and its ability to affect signal transduction pathways that entail H_2O_2 as a signal. Changes in catalase activity after pathogen infection or treatment with salicylic acid (SA) suggest a role for catalase in the plant signal transduction cascade during plant-pathogen interactions (Chen et al., 1993). The in vitro inhibition of catalase (Chen et al., 1993; Sánchez-Casas and Klessig, 1994; Conrath et al., 1995) and ascorbate peroxidases by SA (Durner and Klessig, 1995) provided the first indications of the existence of a link between SA and the oxidative burst. Further study (Durner and Klessig, 1996) suggests that SA inhibits catalase by serving as an electron-donating substrate for peroxidative ac-

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tion of catalase, thereby trapping these enzymes in a partially inactive form. During this process, SA is expected to be converted into a one electron-oxidized SA free radical. Thus, interaction between SA and SA-binding catalase results in not only inhibition of catalase activity but also the generation of SA free radicals. Other reports, however, suggest that catalase inhibition may not be the main mechanism by which SA induces H_2O_2 accumulation (Rüffer et al., 1995; Ryals et al., 1995). A direct role for SA as a factor potentiating H_2O_2 production by plasma membrane NAD(P)H oxidase has been proposed (Kauss and Jeblick, 1995; Shirasu et al., 1997). Moreover, it was shown H_2O_2 may regulate SA accumulation (Bi et al., 1995; Léon et al., 1995; Neuenschwander et al., 1995).

Production of ROS, particularly H_2O_2, during response to abiotic stresses has also been proposed as a part of the signaling cascade leading to protection against these stresses (Doke et al., 1994). It was shown that catalase levels may in part determine cold, UV, or ozone sensitivity (Chamnongpol et al., 1996; Prasad, 1997).

Three classes of genes (*Cat1*, *Cat2*, and *Cat3*) coding for catalase activity were isolated and characterized in *Nicotiana plumbaginifolia* and other plant species (Willekens et al., 1994b; Yu et al., 1999). Cat1 is the most abundant catalase in leaves, whereas Cat3 is mainly found in seeds. Sequence and putative function of Cat1 and Cat3 suggest their peroxisomal localization. Several lines of evidence suggest that Cat1 is primarily involved in removing the H_2O_2 that is produced during photorespiration in leaf peroxisomes, whereas Cat3 scavenges the H_2O_2 that is formed in glyoxysomes during fatty acid degradation (Willekens et al., 1994a). *Cat2* mRNA levels rapidly increase after UV-B or ozone treatment, which suggests that Cat2 may play a role in stress protection (Willekens et al., 1994c). It has also been shown that levels of *Cat2* mRNA and protein and catalase activity increased around tobacco (*Nicotiana tabacum*) mosaic virus (TMV)-induced necrotic lesions in tobacco leaves (Dorey et al., 1998). In addition, the study of tobacco class 2 catalases (Yu et al., 1999) confirms the ability for direct interaction of Cat2 protein with SA. This suggests that SA-binding class 2 catalases may play an important role in development of disease resistance, possibly by serving as biological targets of SA.

To contribute to a better understanding of the role of H_2O_2 in plant response to viral infection, we have constructed an expression cassette containing the yeast (*Saccharomyces cerevisiae*) catalase A coding sequence, under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter and created tobacco plants that exhibited elevated levels of catalase. This study addresses the influence of peroxisomally expressed catalase on local and systemic responses to viral infection.

RESULTS

Yeast Catalases Are Poorly Inhibited by SA

Two yeast strains that carry loss-of-function mutations in either the *CTA1* or *CTT1* gene were used to measure a possible inhibitory effect of SA on catalase activity. In the $CTA1^{+}ctt^{-}$ strain, total observed catalase activity was attributed to catalase A (CTA1). Similarly, the $cta1^{-}CTT^{+}$ strain possessed only catalase T (CTT1) activity. Crude extracts were prepared from isogenic yeast strains grown in rich liquid medium collected at logarithmic phase of growth. Catalase activity of such extracts was measured in vitro in reaction mixtures containing various concentrations of SA. As shown in Figure 1, it was demonstrated

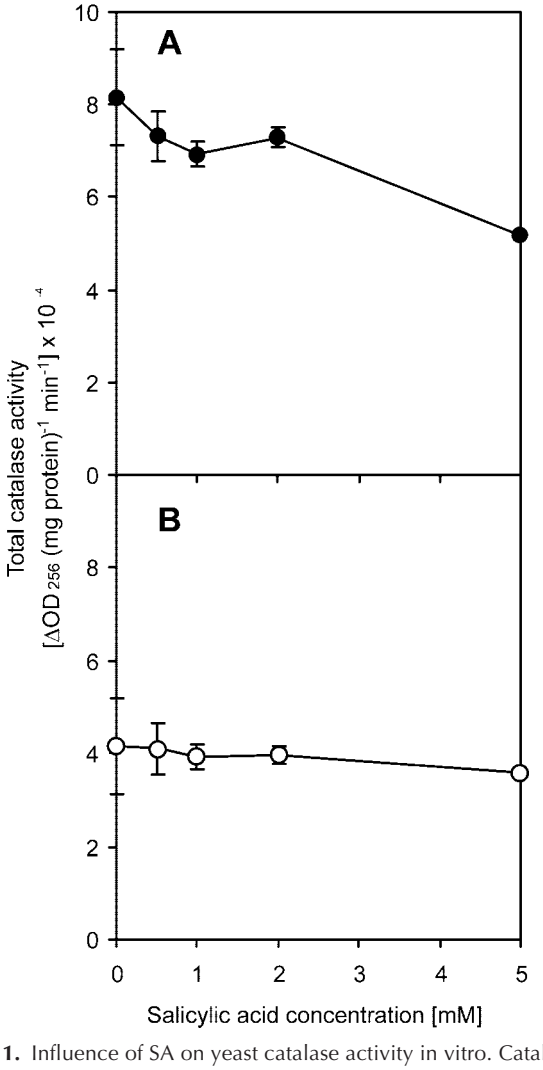

Figure 1. Influence of SA on yeast catalase activity in vitro. Catalase activity was measured in crude extracts isolated from isogenic yeast strains carrying mutations in either the *CTT1* (A, black symbols) or *CTA1* (B, white symbols) gene. Twenty microliters of extract (50–70 μg of protein) was added to a reaction mixture containing 0.05% (v/v) H_2O_2 and SA at appropriate concentration. Each point represents a mean of three assays performed with independently prepared extracts. Error bars represent the SD.

that the activities of the *CTA* and *CTT* gene products were relatively stable at all tested SA concentrations. Neither of the catalases were significantly inhibited by high concentrations (5 mM) of SA.

Transgenic Tobacco Plants Overexpressing Yeast Catalase Gene

Transgenic tobacco plants with increased catalase activity were created by expressing the yeast catalase gene *CTA1* under the control of the 35S CaMV promoter. The transgenic plants did not exhibit any visible morphological differences in comparison with healthy untransformed control plants under standard growth condition and under high-light intensity (16,000 lux). The presence of *CTA1* RNA in three selected lines was confirmed by northern blotting (Fig. 2A). The transgenic lines were then screened by western blotting for the presence of CTA1 protein (58.5 kD) in crude extracts isolated from fully developed leaves (Fig. 2B), using specific anti-CTA1 antibodies. The total catalase activity was also measured. All three lines (CTA1/2, CTA1/3, and CTA1/4) exhibited increased levels of catalase activity that was two to four times higher than the catalase activity of untransformed control plants (Fig. 2C).

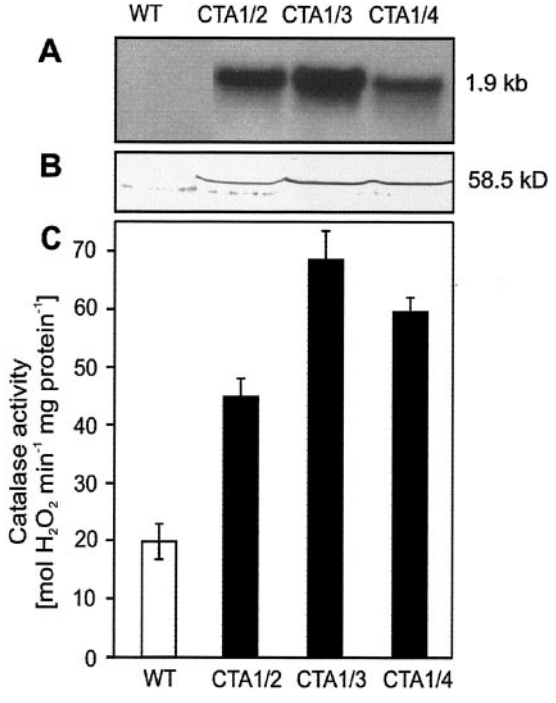

Figure 2. Expression of the yeast *CTA1* gene in three selected transgenic lines and in an untransformed line (WT). The presence of *CTA1* mRNA was detected by northern blotting (A, 10 μg of total RNA was loaded per lane), the presence of the CTA1 protein was detected by western blotting (B, 20 μg of protein was loaded per lane), and the catalase activity was measured in crude extracts from leaf tissue (C). Results shown in C are means of three measurements performed with independently prepared extracts. Error bars represent the SD.

A

Plant	Dry weight (mg/g fresh weight)
WT	0.0424 ± 0.0024
CTA1/4	0.0433 ± 0.0029

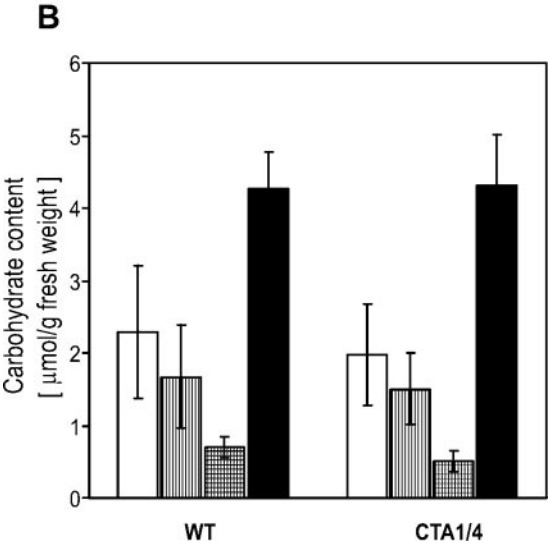

Figure 3. Assessment of the physiological state of the CTA1/4 line: dry weight (mg) per mg of fresh weight (A) and carbohydrate content (B). Leaf weight was measured for 10 leaves taken from a single plant. The ratio was calculated using obtained arithmetic means. Carbohydrate content was measured in three plants (□, Glc; ▥, Fru; ▦, Suc; ■, starch). The results are arithmetic means with error bars representing the SD.

Line CTA1/4 was chosen and used in all experiments described in the following sections of this paper. The physiological state of the CTA1/4 line was assessed using markers such as the fresh weight to dry weight ratio and the carbohydrate content (Glc, Fru, Suc, and starch) of leaf tissue. No differences were observed in the weight of leaf fragments of equal area (data not shown). The ratio of dry weight to fresh weight remained constant in both *CTA1*-transformed and control tobacco plants. Similarly, the carbohydrate content (Glc, Fru, Suc, and starch) was unchanged in the CTA1/4 plants compared with untransformed plants. These results are shown in Figure 3.

The Activity of Tobacco Catalases Is Inhibited by SA

It has been reported that catalase activities in various plant species are inhibited by SA (Sánchez-Casas and Klessig, 1994). To test the influence of SA on tobacco catalase activity, SA was included in the reaction mixture during catalase activity assay in vitro. In the absence of SA, the CTA1/4 line exhibited an over 2-fold increase in total catalase activity in crude extracts obtained from leaf tissue as compared

with untransformed control plants (Fig. 2). When SA was present in the reaction mixture during an in vitro assay, marked differences in total catalase activity were observed. As shown in Figure 4, in untransformed control plants exposed to SA concentrations of 1 mM, catalase activity decreased by 40% as compared with catalase activity in the absence of SA. No activity could be detected when the reaction mixture contained 5 mM SA. This result was in agreement with previous reports (Sánchez-Casas et al., 1994) demonstrating that other plant catalases were inhibited by SA. In contrast, total catalase activity in the CTA1/4 line was inhibited by only 35%, even in the presence of SA at concentrations as high as 5 mM. Interestingly, the difference in catalase activity between the CTA1 plants and untransformed plants was almost constant at all SA concentrations.

CTA1 Protein Is Located in Peroxisomes of Transgenic Plants

Peroxisome-localized catalase A of yeast has a well-defined targeting sequence (the so-called SKL motif) at its carboxyl terminus (Kragler et al., 1993). It has been shown that the SKL sequence is necessary and sufficient for targeting to plant peroxisomes. To test whether the CTA1 protein in the analyzed transgenic plants is indeed localized in peroxisomes, an immunogold localization approach was used to demonstrate its subcellular location. The CTA1 protein was detected using specific anti-CTA1 antibodies. Sections treated with anti-CTA1 antibodies are shown in Figure 5. In all analyzed sections, the majority of the signal was located in peroxisomes but not in the cytoplasm or intercellular space. Localization of the signal was not changed in TMV-infected tissue (data not shown).

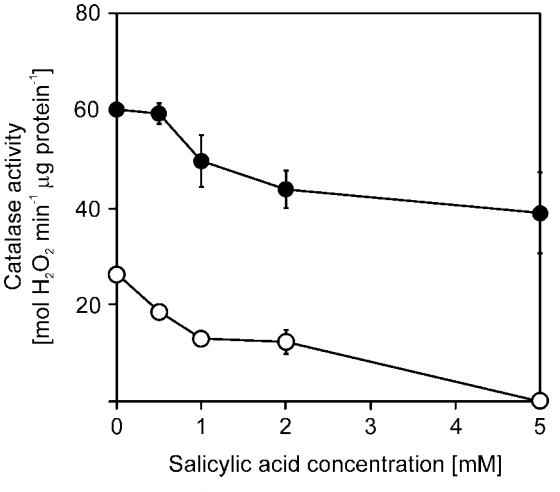

Figure 4. The influence of SA on in vitro catalase activity in untransformed plants (white symbols) and the CTA1/4 line (black symbols). Catalase activities were measured in crude extracts obtained from fully developed leaves in the presence of various SA concentrations. Each point is the mean of three independent experiments. Error bars represent the SD.

Defense Responses Are Impaired in CTA1 Plants

Tobacco infected with a necrotizing strain (U1) of TMV is able to mount an effective response and to limit pathogen presence to necrotic lesions that are formed in the course of the HR. The size of such necrotic lesions is generally used for assessment of the efficiency of the defense response (Van Loon, 1983).

CTA1/4 plants, similarly to untransformed control, responded to TMV infection with the formation of necrotic lesions. The first signs of tissue necrotization were visible on the 2nd d after infection, usually at 28 to 36 h postinoculation. Figure 6 shows typical TMV lesions on CTA1/4 plants and control plants 2, 4, and 7 d after infection with TMV. Necrotic lesions appeared on the CTA1 plants 3 to 6 h earlier, and after 48 h, a difference in diameter of lesion size could already be observed (Fig. 6). After 4 d, lesion size was significantly larger (45%) as compared with control plants. Such enlargement of lesion size was also present at 7 d postinfection, but no other macroscopically visible differences were detected between transgenic and control plants. A comparison of lesion size on leaves of untransformed tobacco plants and on the CTA1/4 plants is shown in Table I. A similar phenomenon was observed in other transgenic lines (CTA1/2, CTA1/3, and CTA1/4) expressing the *CTA1* gene (data not shown).

Direct Localization of H_2O_2 in Tobacco Leaves

The 3,3′-diaminobenzidine (DAB) staining method was employed to detect putative changes in H_2O_2 distribution in the transgenic CTA1 tobacco. In the experimental model used in this work, necrotic lesions begin to emerge between 26 and 32 h postinoculation. Leaves inoculated with TMV were DAB-stained to visualize areas of tissue with increased concentrations of hydrogen peroxide. To investigate early stages of lesion formation, tobacco leaves were collected 26 h postinoculation. At that time, lesions had just begun to emerge and appeared as barely visible needle marks. Leaves were incubated for 6 h in DAB solution. As shown in Figure 7, necrotic lesions on untransformed plants (Fig. 7A) were markedly stained by DAB, indicating the presence of H_2O_2 at the site of lesion formation. Such staining was almost completely absent on leaves of the CTA1/4 line (Fig. 7B).

Leaves were also collected at 30 h postinfection when necrotic lesions were already clearly visible and the tissue in the center had begun to collapse. They were treated with DAB as described above. Untransformed plants exhibited distinct brown rings around the lesions (Fig. 7C). This indicated the pres-

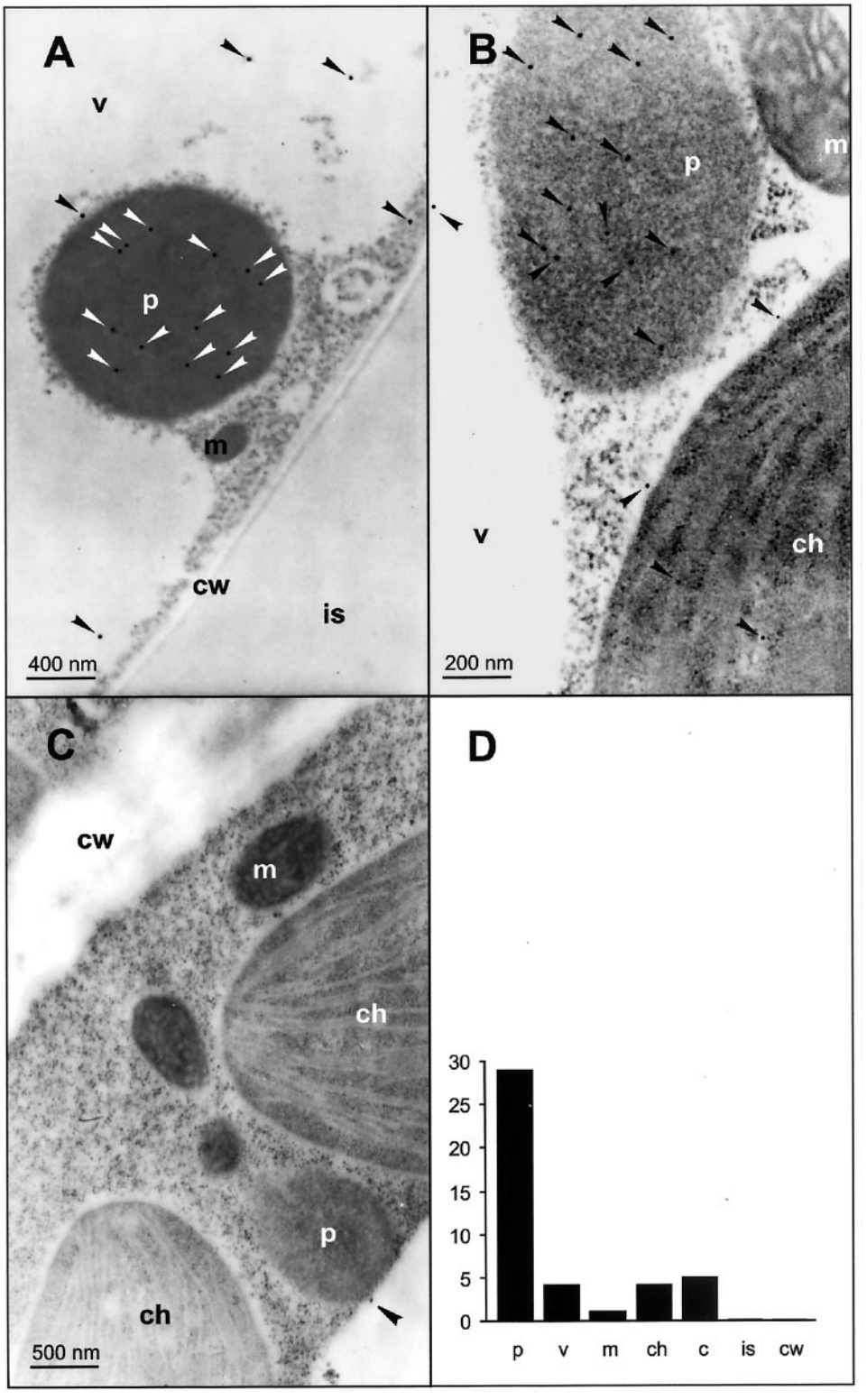

Figure 5. The subcellular localization of the CTA1 protein. A and B, CTA1/4 line; C, non-transgenic tobacco; and D, the number of gold particles in different cellular compartments. Positive immunolocalization (black dots) was counted on several independently obtained sections. Arrows indicate positions of gold labeling. Cellular compartments were abbreviated as follows: c, cytoplasm; ch, chloroplasts; cw, cell wall; is, intercellular space; m, mitochondria; p, peroxisomes; and v, vacuole.

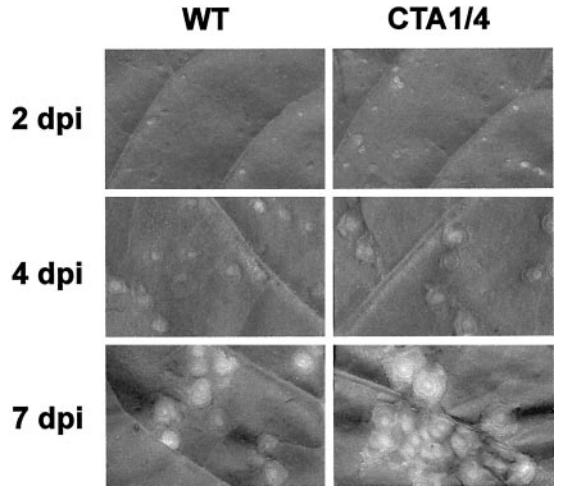

Figure 6. TMV lesion phenotype on CTA1 and control tobacco plants. CTA1-expressing (CTA1/4) and untransformed tobacco cv Xanthi-nc were inoculated with 1.5 μg of TMV strain U1, and sections of leaves were photographed 2, 4, and 7 d after inoculation.

ence of high concentrations of H_2O_2 in the tissue around the point of necrosis formation. Figure 7D shows clearly that the necrotization of the tissue of CTA1-transgenic plants had already occurred by this time point. The external rings surrounding necroses characteristic for untransformed tobacco cv Xanthi-nc plants are not present. It should be noted that Figure 7, A and B, shows lesions in magnification different from that on Figure 7, C and D. To account for the possibility of a time shift in lesion formation between untransformed and CTA1/4 plants, DAB detection was performed at other time points from 26 to 33 h postinoculation, but no similar ring patterns could be detected in either class of plants (data not shown).

Expression of Acidic PR Genes Is Affected in the CTA1 Plants

The hypersensitive reaction is associated with a coordinated set of metabolic changes and the synthesis of PR proteins. They are induced specifically in pathological situations and do not only accumulate in the infected leaves but are also induced systemically and are associated with the development of systemic acquired resistance (SAR). They are generally regarded as biochemical markers of the defense response.

Local expression of genes belonging to PR-1, PR-2, and PR-5 families in leaves infected with TMV was tested using northern analysis. Three fully developed leaves were inoculated with TMV and collected at 4 d postinoculation. Total RNA was isolated and analyzed with probes corresponding to the appropriate PR genes. As shown in Figure 8, the CTA1/4 line, similar to untransformed tobacco, was able to locally induce acidic isoforms of PR genes PR-1, PR-2, and PR-5, but the amount of accumulated mRNA was noticeably lower compared with untransformed control plants. The observed difference in expression was reproduced in several independent experiments. Remarkably, local expression of basic PR isoforms was unaffected in the transgenic plants, although these genes were also induced considerably as a result of TMV infection. Basic isoforms of PR genes were also slightly induced in mock-inoculated control plants, possibly as a response to some tissue damage that had occurred in the course of experimental procedures.

The SA Level Is Not Changed in the CTA1 Plants

Three TMV- or mock-inoculated leaves of the CTA1/4 plants and of untransformed control plants were collected. Levels of free SA and SA glucoside were measured in these leaves by HPLC. The SA level in mock-inoculated leaves was very low in untransformed plants, and no significant change was observed in the CTA1/4 line. In the infected tissue at 48 h postinoculation, the SA level increased approximately 10-fold (Fig. 9A). No significant differences were observed in the accumulation level of SA between the CTA1/4 plants and untransformed control plants. At 96 h postinoculation, SA concentration in the infected leaf tissue further increased 2.5-fold compared with 48 h postinfection (Fig. 9B). Again, the SA level in the CTA1/4 line was comparable with that of untransformed plants. Moreover, the proportion of free SA to SA-glucoside was unchanged in the CTA1/4 line compared with untransformed plants.

SAR in the CTA1 Plants

The observed differences in local defense response (increases in average necrotic lesion size and decreases in PR gene induction levels) between transgenic and wild-type plants raised the question as to whether similar changes would be observed in systemic leaves.

The effectiveness of the defense response in the systemic parts of the infected plant was assessed by measuring necrotic lesion size after secondary infection. Both CTA1/4 and untransformed control tobacco plants were inoculated with TMV. Seven days post primary inoculation, upper uninoculated leaves on the same plants were infected with TMV or were mock-inoculated. The size of necrotic lesions result-

Table I. *Size of necrotic lesions after TMV infection*

Plant	Lesion Size[a]	
	4 dpi	7 dpi
	mm	
Untransformed	1.65 ± 0.06[b]	2.75 ± 0.13[c]
Transgenic (CTA1/4)	2.40 ± 0.25[c]	3.95 ± 0.17[c]

[a] Lesion size is the mean of 40 lesions on four plants ± SD. [b] $P < 0.0005$. [c] $P < 0.001$.

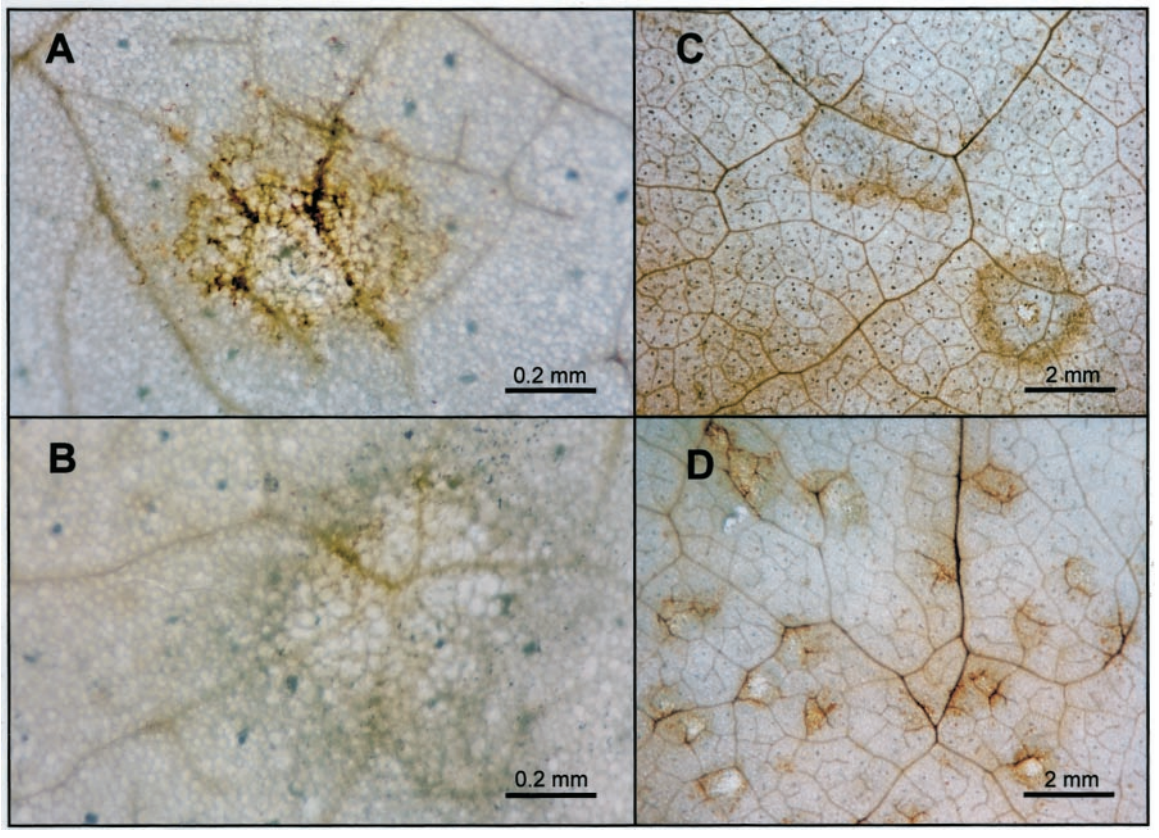

Figure 7. Visualization of H_2O_2 by DAB staining in leaf tissue after inoculation with TMV. A, Untransformed control plant 32 h postinfection (hpi); B, CTA1/4 32 hpi; C, untransformed control plant 36 hpi; and D, CTA1/4 36 hpi.

ing from primary or secondary infection was then measured at 7 d post secondary inoculation.

Necrotic lesions were markedly reduced in the secondary infection as compared with primary infection in both CTA1/4 and control plants (Table II). In primary and secondary infections, respective necrotic lesions remained larger on leaves of the CTA1/4 line as compared with leaves of untransformed plants. The ratio of size reduction remained constant.

Another experiment was designed to check PR gene activation in systemic leaves of the CTA1/4 plants. Seven days post primary inoculation with TMV, previously uninoculated leaves on the same plants were inoculated with TMV or were mock-inoculated. Leaves were collected at 7 d after secondary inoculation, and total RNA was isolated and hybridized with probes corresponding to the acidic isoforms of tobacco PR-1 and PR-2 genes. The induction level of acidic isoforms of the PR genes was unchanged in systemic leaves that had been mock-inoculated (Fig. 10A). In contrast, when systemic leaves on plants that had been previously challenged with pathogen were inoculated with TMV, untransformed control plants exhibited much higher accumulation of PR transcripts compared with the CTA1/4 plants (Fig. 10B). The additional bands in the PR-2 panel probably represent other PR-2 genes, which had been recognized by the probe in addition to the main transcript. The results of this experiment indicate that the efficiency of SAR induction is not affected in the CTA1/4 plants.

DISCUSSION

Rüffer et al. (1995) suggested that SA binding activity is a general property of iron-containing enzymes because SA can act as a siderophore, complexing metal ions. They analyzed the effect of SA on a variety of iron-containing enzymes and found that many of them could bind SA and that SA inhibited the activity of these enzymes. However, Durner and Klessig (1996) have shown that SA inhibits catalase by acting as an electron donating substrate, directing the enzyme activity into the slower peroxidative cycle. The biological significance of SA-dependent inhibition of catalase is unclear at the moment, but it has been suggested that it can be an important element of regulation of the HR, which promotes accumulation of high levels of H_2O_2 at the site of infection.

Sánchez-Casas and Klessig (1994) tested catalase activities from several plant species. They showed that catalases from tobacco, cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), Arabidopsis,

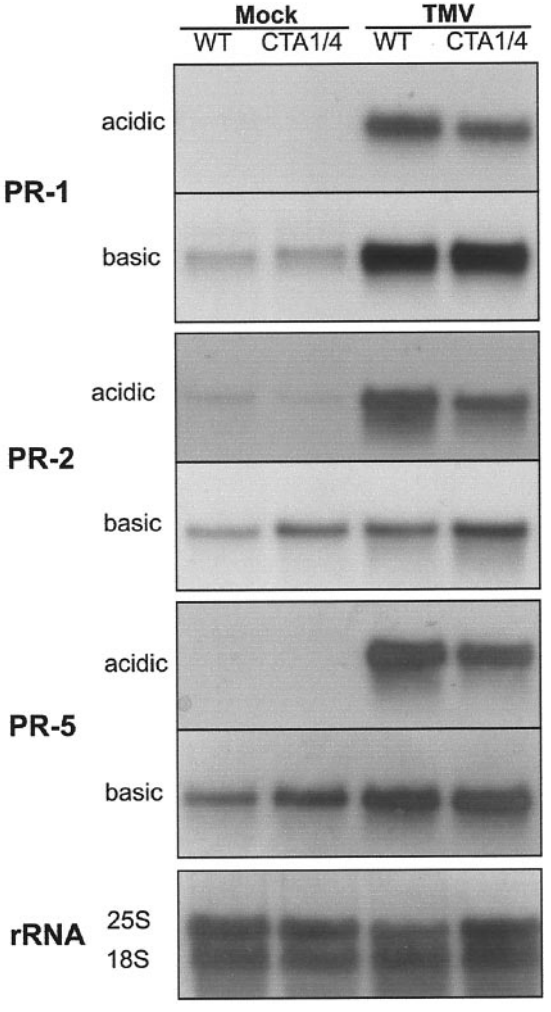

Figure 8. Local expression of genes coding for acidic and basic PR proteins. RNA was isolated at 4 d postinoculation from TMV- or mock-inoculated fully developed leaves. Each lane contained 10 μg of total RNA. After hybridization with a probe specific to an acidic PR isoform, the probe was removed and the same membrane was rehybridized with a probe for the corresponding basic isoform. The amount of RNA on the blot was visualized by rehybridizaton with a probe for rRNA. The experiment was done three times.

and soybean (*Glycine max*) are significantly inhibited by 1 mM SA (by 32%–66%, depending on the plant species), whereas such concentrations of SA did not have any influence on catalase activities in maize (*Zea mays*) and rice (*Oryza sativa*). Several other plant proteins are also known to bind SA, specifically ascorbate peroxidase (Durner and Klessig, 1995), SABP2 (Du and Klessig, 1997), and mitochondria-located SABP3 (D.F. Klessig, personal communication). However, no data has been published so far regarding the influence of SA on activity of yeast catalases. As shown in Figure 1, neither catalase A nor catalase T in yeast is significantly inhibited by SA in cellular extracts tested in vitro. One millimolar SA reduced the activity of peroxisomal catalase A by 12%, but taking into account considerable error in measurements without SA and in 1 mM SA, the difference could be even smaller if significant at all. Even SA concentrations as high as 5 mM inhibited catalase A activity by only 37%. No catalase T inhibition could be detected in the SA concentration range of 0 to 5 mM. Wild-type, *CTA1*-, or *CTT1*-deficient yeast strains were grown in media containing SA at various concentrations up to 5 mM and no differences in growth rate were recorded (data not shown).

The yeast gene *CTA1* coding for peroxisomal catalase A was chosen for plant transformation, because the product of this gene is not significantly inhibited by SA. Because it was known that SA levels rise dramatically in tobacco as a consequence of pathogen infection, it was important that the introduction of additional catalase activity was not influenced by possible interactions with SA. Another reason for choosing the yeast catalase gene is the fact that yeast catalase is not closely related to plant catalases in terms of sequence similarity. This fact minimized the risk of the transgene being silenced, which is a frequent phenomenon observed in the construction of transgenic plants.

One of the numerous selected transgenic lines that exhibited an approximately 3-fold increase in total catalase activity (CTA1/4) was chosen for detailed analysis and experiments to assess resistance against pathogen infection. Measurements of dry weight/fresh weight ratios and carbohydrate content are common markers indicating changes in plant metabolic condition. Neither of the assayed parameters was significantly different between CTA1/4 plants and untransformed tobacco cv Xanthi-nc plants (see Fig. 3). In addition, the overall appearance of the plants were unchanged.

The inhibitory effect of SA on catalase activity in CTA1/4 and control plants was different. For each concentration of SA tested, as shown in Figure 4, we observed a constantly higher level of catalase activity of approximately 30 to 40 mol H_2O_2 min^{-1} μg^{-1} protein in CTA1/4 lines as compared with nontransgenic plants. This value corresponded to the increase in total catalase activity observed in the CTA1/4 line (Fig. 2C). It is possible that this portion of the total catalase activity in the transgenic CTA1/4 line corresponded to the activity of the expressed yeast catalase that was not inhibited by SA as shown in Figure 1.

Two types of peroxisomal targeting sequences (PTS) have been identified. The more common Type 1 PTS is a tripeptide at the C terminus of the targeted protein, consisting of a small uncharged residue at position −3, a basic residue at position −2, and a non-polar residue at position −1 (iBo; de Hoop and Ab, 1992; Swinkels et al., 1992; Subramani, 1993). The Type 2 PTS is a nonapeptide (R-L/I-X_5-H/Q-L) located in the N-terminal signal sequence that is cleaved off after import into peroxisomes. In plants, several studies have shown that either the C-terminal

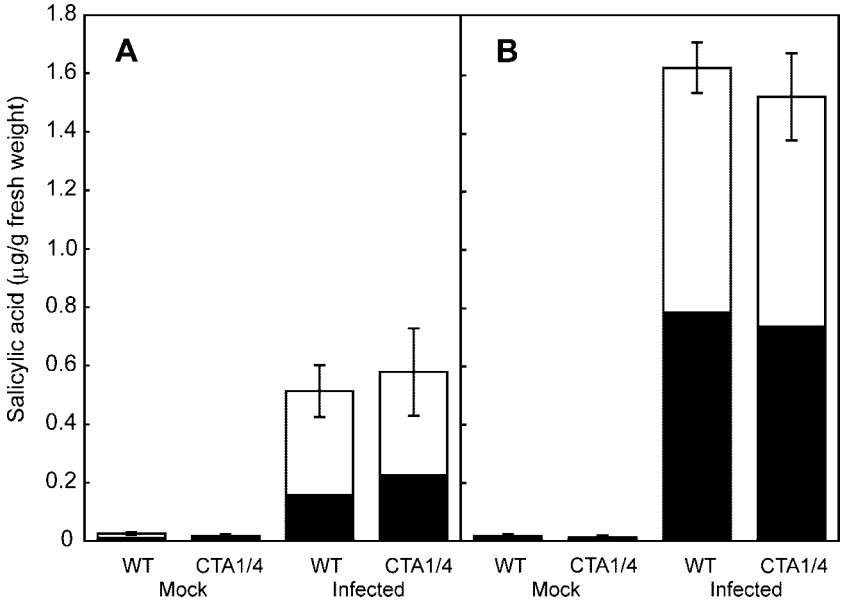

Figure 9. SA and SAG levels in the TMV- or mock-inoculated leaves 48 hpi (A) and 96 hpi (B). Results are the mean of two independent experiments. Inoculations were performed on three plants during each experiment. Error bars represent the SD. ■, SA; □, SAG.

or internal SKL (Ser-Lys-Leu) motif (or its variants) is essential for targeting to peroxisomes (Mullen et al., 1997). Several studies reported the presence of an internal SKL motif in the sequences of plant catalases (Gonzalez, 1991; Suzuki et al., 1994). For mammalian catalases, it was shown that a C-terminal -ANL sequence was both necessary and sufficient for import into peroxisomes. Although it does not fit well with the SKL motif, it was concluded that peroxisomal import takes place by using the same PTS1 mechanism (Trelease et al., 1996).

CTA1 catalase possesses an SKL-like PTS at its C terminus that was shown to be active in yeast (Kragler et al., 1993). This feature suggested that it may be sorted to peroxisomes in leaf tissue of tobacco plants, as suggested by Gould et al. (1990) who showed that peroxisomal protein import is conserved between yeast, plants, insects, and mammals. The immunogold localization experiments described in this study proved that the CTA1 protein, when expressed in transgenic tobacco, is indeed present almost exclusively in peroxisomes (Fig. 5).

Table II. *Formation of necrotic lesions after infection with TMV*

Upper uninfected leaves on plants challenged with TMV were inoculated with the same virus. Size of necrotic lesions was measured at 7 d post-secondary infection.

Plant	Lesion Size[a]	
	Local response (primary infection)	Systemic response (secondary infection)
	mm	
Untransformed	1.89 ± 0.06[b]	0.64 ± 0.10[c]
Transgenic (CTA1/4)	2.71 ± 0.15[c]	1.05 ± 0.08[b]

[a] Lesion size is the mean of 30 lesions on three plants ± SD. [b] $P < 0.0005$. [c] $P < 0.001$.

The CTA1/4 plants that exhibited elevated levels of catalase activity, responded less efficiently to infection with a necrotizing TMV strain. Data presented in Table I and in Figure 6 show that necrotic lesions were significantly larger in CTA1/4 plants as compared with untransformed control plants. This observation was confirmed in other transgenic lines expressing the *CTA1* gene. Levine et al. (1994) showed that H_2O_2 could play a dual role in the plant defense response depending on its concentration. At high concentrations, it triggers HR; whereas at low concentrations, it functions as a diffusible signal that induces cellular protectant genes that are involved in

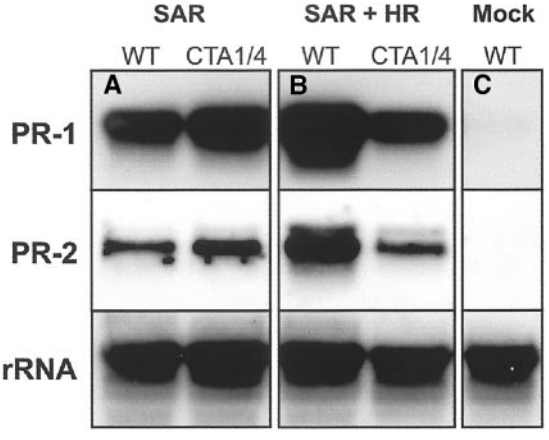

Figure 10. Expression of acidic PR-1 and PR-2 genes in the CTA1/4 and untransformed control plants during the systemic response to TMV infection. Seven days after the initial infection, upper uninoculated leaves were either mock inoculated (A) or inoculated with TMV (B). Ten micrograms of total RNA was loaded per lane. The blot was first probed with the PR-1 probe, then stripped, and reprobed with the PR-2 probe. Finally, the blot was probed with a probe for rRNA. The experiment was done three times.

blocking oxidant-mediated cell death. To explain the phenomenon of increased necroses observed in our transgenic lines one could assume that not only the concentration but also the time of action of the H_2O_2 could be important. To induce cell death, the signal may be brief, whereas activation of scavenger genes could require a more persistent signal. *N. plumbaginifolia* expressing the *Cat*2 gene in antisense orientation exhibited increased susceptibility to infection by *Pseudomonas syringae* pv. *syringae* (Chamnongpol et al., 1996). In contrast, Yu et al. (1999) tested Cat2 antisense *N. tabacum* transgenic plants and found that these Cat2-deficient plants exhibited a similar level of susceptibility to the same bacterial pathogen. In our case, after viral infection of *N. tabacum* expressing peroxisomal-targeted catalase increase of lesion size was observed (Table I; Fig. 6). These results indicate that catalase activity may contribute to the outcome of the plant-pathogen interaction. It should be noted that this effect depends on the nature of pathogen, conditions of growth, and the plant species.

Direct localization of H_2O_2 in the tissue demonstrated that in CTA1/4 plants, no H_2O_2 accumulation could be detected at early stages of lesion formation (Fig. 7B), whereas such accumulation was clearly visible within lesions of untransformed tobacco plants (Fig. 7A). At later stages, in untransformed tobacco plants (Fig. 7C), high concentrations of hydrogen peroxide were detected as a brown ring around developing necrotic lesions. In contrast, in the CTA1/4 plants, no such ring or similar structure was detected around necrotic lesions, and the overall H_2O_2 accumulation in the leaf tissue was greatly reduced (Fig. 7D). This observation clearly indicates that transgenic plants overexpressing catalase may have difficulties in attaining H_2O_2 levels as high as in untransformed plants during the oxidative burst. This suggests that, in addition to membrane- and cell wall-associated enzymes (Wojtaszek, 1997), peroxisomal enzymes can also play a role in ROS metabolism in the neighborhood of the infection site.

Impaired defense response to TMV infection in CTA1/4 plants coincided with a decrease in the levels of mRNA for PR genes coding for acidic isoforms of PR-1, PR-2, and PR-5 in the infected tissue (Fig. 8). These genes are known to be induced by SA and during pathogen infection (Brederode et al., 1991). Several studies correlated induction of various PR genes with elevated H_2O_2 levels in transgenic tobacco or potato (*Solanum tuberosum*) expressing fungal Glc oxidase (an H_2O_2-producing enzyme; Wu et al., 1997; Kazan et al., 1998). Elevated levels of PRs were also observed in transgenic tobacco plants expressing antisense catalase mRNA that exhibit a decrease in endogenous catalase activity (Takahashi et al., 1997). These reports described an increased SA concentration in tissues and also H_2O_2-dependent PR-1 gene induction. The H_2O_2-dependent PR-1 gene induction was strongly suppressed in NahG transgenic plants that are unable to accumulate SA (Bi et al., 1995; Neuenschwander et al., 1995). However, in CTA1/4 plants the SA level was similar to that in untransformed control plants (Fig. 9). Thus, it appears that the observed decrease in PR gene expression was unrelated to the SA concentration. It has been reported that, in addition to the best characterized SA-dependent induction of the PR genes, other SA-independent pathways also exist (Malamy et al., 1996; Pieterse and van Loon, 1999; Schaller et al., 2000). The observed change in the PR transcript levels may thus be attributed to perturbation of such a SA-independent pathway by the presence of increased catalase levels in the CTA1/4 line.

In contrast to the differences observed in the infected tissue, the expression of PR genes in the uninoculated tissue of infected plants was unchanged or even slightly increased in the CTA1/4 line (Fig. 10). Necrotic lesions formed after secondary TMV infection were larger on CTA1/4 plants, but based on the ratio of the size decrease between primary and secondary infection, the efficiency of SAR seems to be unchanged (Table II). Therefore, the increased catalase activity present in the CTA1/4 plants influences the dynamics and efficiency of local but not systemic responses. These results appear to contradict the hypothesis of Lamb and co-workers who suggested that ROS synthesized in tissues distal to the infection site ("micro-bursts") were an indispensable part of the pathway leading to SAR activation (Alvarez et al., 1998). However, in agreement with our results are recent experiments by Torres et al. (2002) using Arabidopsis *atrboh* knock-out mutants. These plants, which are devoid of an oxidative burst, were still able to establish SAR against virulent isolates of *Peronospora parasitica*.

Many biological processes are regulated by complex signaling networks of modular structure (Hartwell et al., 1999). Such organization implies the system is relatively robust i.e. insensitive to the precise values of biochemical parameters (Barkai and Leibler, 1997). Computational analyses carried out on the model of bacterial chemotaxis showed that single parameters (the rate constants) could be changed even by several orders of magnitude, and the output responses did not significantly vary from perfect adaptation (Barkai and Leibler, 1997). Similarly the model of segment polarity in fruitfly (*Drosophila melanogaster*), with given realistic initial conditions, performed in silico equivalently despite up to 100- or 1,000-fold variation in some of the parameters (von Dassow et al., 2000). In the plant kingdom, a modular pathway has been suggested to operate in abscisic acid signaling (Hetherington, 2001). Pathogen recognition by a plant cell also triggers a complex signaling network (for review, see Genoud et al., 2001) that possibly is similarly organized. An example of transducing signals via various routes in the plant defense response is PR gene induction resulting from either

SA-dependent or independent pathways (Malamy et al., 1996). In our experimental system, catalase activity has been 2- to 4-fold increased in comparison with untransformed tobacco plants leading to a reduced concentration of hydrogen peroxide. Although detailed analyses on virus multiplication, comprising measurement of the virus titer, have not been carried out, the lack of systemic symptoms strongly indicates the virus has not spread throughout the plants. It suggests resistance of the transgenic plants has not been lost but only impaired. This phenomenon could be discussed in terms of a modular signaling pathway.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants of tobacco (*Nicotiana tabacum* cv Xanthi-nc), resistant to TMV, were grown in growth chambers using a 16-h period of light (22°C) and 8 h of darkness (18°C). The light intensity was 5,000 to 6,000 lux, and the humidity was maintained at 65%. For all experiments, 6- to 9-week-old plants were used. For inoculation with TMV, carborundum-dusted leaves were rubbed with water or TMV strain U1 solution (1 μg ml^{-1}), and rinsed with water. Two to four leaves were inoculated on each plant and harvested together.

Construction of the 35S/CTA1 Transgenic Plants

The coding region of the yeast (*Saccharomyces cerevisiae*) catalase gene (*CTA1*) was excised from the YEp352 plasmid (Hill et al., 1986) with *Hin*dIII and *Sac*I. A fragment of 1,550 bp was cloned into the vector pFF19 (Timmermans et al., 1990) digested with *Sac*I/*Sma*I, and then enhanced CaMV 35S promoter/CTA1/3′NOS expression cassette was cloned as an *Eco*RI-*Hin*dIII fragment into the binary vector pGA482 (An, 1986) and transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Leaf discs from tobacco cv Xanthi-nc plants were transformed (Horsch et al., 1985) and kanamycin-resistant plants were regenerated on Murashige and Skoog medium (Murashige and Skoog, 1962) by standard methods. Presence of the transgene was checked by PCR with *CTA1*-specific primers (5′-ACTGTGGGTGGTGATAAAGGT-3′/5′-TCTCTGATAGCGGGATTGAAA-3′) and by using genomic Southern analysis with CTA1-specific probe. Primary transformants were allowed to self-fertilize, and R^0 seeds were collected and germinated on Murashige and Skoog medium with 300 μg mL^{-1} kanamycin. Selected Km^r seedlings R^1 generation were transferred into soil and grown for a few weeks before experimentation.

The Physiological State of the Transgenic Plants

Dry Weight/Fresh Weight Ratio

Ten fully developed leaves were taken from three 6-week-old tobacco plants at the same developmental stage, weighed, and lyophilized under vacuum for several hours. Leaves were then weighed again, and the ratio of dry weight to fresh weight was calculated.

Carbohydrate Content

For each preparation, 1 g of fresh leaf tissue was ground in liquid nitrogen, suspended in 80% (v/v) ethanol in 50 mM HEPES-KOH, pH 7.4, and incubated for 2 h at 37°C. The slurry was centrifuged at 14,000*g* for 15 min, and the supernatant was lyophilized. The dry supernatant was dissolved in 1.5 mL of 0.1 M imidazole-HCl, pH 6.9, centrifuged at 13,000*g* for 10 min to remove insoluble particles, and used for Glc, Fru, and Suc assays. The pellet was used for starch assay.

Glc and Fru

Spectrophotometric assays were performed in 1.5 mL of assay buffer (0.5 M imidazole-HCl, pH 6.9, 0.15 mM $MgCl_2$, 0.45 mM NADP, and 1 mM ATP) at 340 nm using 20 μL of each sample. Measurements and calculations were performed as described by Stitt et al. (1989).

Suc

Suc assay was performed as described by Stitt et al. (1989) for Glc and Fru assays, except that before assays, the samples were incubated for 15 min at 20°C to 25°C with 0.2 unit of invertase.

Starch

Starch content of each sample was measured using a commercially available reaction kit (Roche Molecular Biochemicals, Basel) following the manufacturer's protocol.

Gene Expression Analysis

RNA Analysis

Total RNA was isolated from leaves as described previously (Linthorst et al., 1993). For northern blots, 10 or 20 μg RNA was separated on a 1% (w/v) agarose gel in 15 mM sodium phosphate, pH 6.5, and transferred to Hybond N (Amersham, Buckinghamshire, UK) filters. Hybridization was performed at 65°C in 250 mM sodium phosphate, pH 7, 1 mM EDTA, 7% (w/v) SDS, and 1% (w/v) bovine serum albumin (BSA) with one of the following randomly labeled probes: (a) 1,550-bp *CTA1* cDNA (Cohen et al., 1988); (b) 900-bp acidic PR-1a cDNA (Cutt et al., 1988); (c) 790-bp basic *PR-1g* cDNA (Brederode et al., 1991 (d) 700-bp acidic PR-2d cDNA (Hennig et al., 1993); (e) 550-bp basic PR-2 cDNA (Brederode et al., 1991); (f) 800-bp acidic PR-5 (Brederode et al., 1991); (g) 700-bp basic PR-5 cDNA (Brederode et al., 1991); and (h) 1,200-bp 25S rDNA.

Protein Analysis

Proteins were extracted in buffer containing 50 mM Tris pH 8.0, 1 mM EDTA, 12 mM β-mercaptoethanol, and 10 μg mL^{-1} phenylmethylsulfonyl fluoride. Protein content was measured by the Bradford method using a commercially available reaction kit (Bio-Rad, Hercules, CA). Extracts were fractionated on a 12.5% (w/v) SDS-PAGE and subjected to immunoblot analysis using a specific goat anti-CTA1 polyclonal antibody (gift from Dr. A. Hartig) and alkaline phosphatase-conjugated anti-goat antibodies from Roche Molecular Biochemicals. Immunoblots were developed using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colorimetric detection kit from Roche Molecular Biochemicals.

Catalase Assays

Analysis of Yeast Catalase Properties

Yeast strains were grown in glycerol-containing medium at 30°C overnight with shaking. Cells were washed in homogenization buffer (20 mM sodium citrate, pH 6.5, 5 mM $MgSO_4$, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1% [w/v] polyvinylpyrrolidone), frozen in liquid nitrogen, thawed on ice, and disrupted by vortexing 3 × 30 s with glass beads (φ = 40 μm). Debris was removed by centrifuging at 13,000*g* for 5 min at 4°C, and the supernatants were used for catalase assays. Catalase activity was measured as was described by Aebi (1984) in assay buffer (20 mM sodium citrate, pH 6.5, 5 mM $MgSO_4$, 1 mM EDTA, and 0.05% [v/v] H_2O_2) by determining the absorbance change at 256 nm. Between 10 and 100 μL of extract were used for each measurement. Measurements were taken every 10 s for 1 min and each measurement was repeated three times. Results were standardized for the protein concentration of each extract.

Influence of SA on Yeast Catalase Activity

The inhibitory effect of SA was investigated by preparing modified assay buffer solutions containing SA at concentrations in the 0.5 to 5 mM range.

Catalase activity measurements were carried out as described above. The reactions were linear in these conditions for at least 3 min after addition of extracts.

Analysis of Tobacco Catalase Properties

Crude extracts were prepared by grinding 2 g of fresh leaf tissue in 10 mL of homogenization buffer. The following isolation steps were all carried out at 4°C. The resulting homogenates were filtered through four layers of cheesecloth and centrifuged at 40,000*g* for 30 min. Supernatants were transferred into new tubes, and ammonium sulfate was added to 45% saturation (0.32 g mL^{-1}). The samples were centrifuged at 20,000*g* for 20 min. Pellets were dissolved in 1 to 5 mL of dialysis buffer (40 mM sodium citrate pH 6.5, 10 mM $MgSO_4$, and 2 mM EDTA) and dialyzed overnight against 4 L of buffer. Finally, the samples were centrifuged at 3,500*g* for 15 min to remove undissolved proteins. The protein content was measured by the Bradford method. Catalase activity and the inhibitory effect of SA was measured as described before.

Direct Localization of H_2O_2 in Plant Tissue

Leaves from control and transgenic tobacco plants infected with TMV were taken 24 to 32 h postinfection, placed in 1 mg mL^{-1} DAB-HCl, pH 3.8 (as described by Thordal-Christensen et al., 1997), and incubated in the growth chamber for 6 h before sampling. Leaves were cleared in boiling ethanol (96%, v/v) for 10 min to remove chlorophyll and examined under a low-magnification light microscope. H_2O_2 was visible as a brown precipitate in the tissue.

Tissue Preparation and Immunogold Localization

Leaf pieces from noninfected plants transformed with *CTA1* gene or with the transformation vector pGA482 alone were fixed in 1.5% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 (Karnowsky, 1965), washed with 0.1 M cacodylate buffer, pH 7.2, dehydrated in an ethanol series, and embedded in LR White resin (medium grade; Sigma, St. Louis).

Sections were processed as follows for immunogold staining on uncoated nickel grids. The sections were immersed in 20 mM Tris-buffered saline (TBS), pH 7.5, containing 0.9% (w/v) NaCl, for 30 min; immersed in 1% (w/v) BSA diluted in TBS, pH 7.5, for 1 h; incubated in goat anti-CTA1 polyclonal antibodies diluted 1/50 in TBS, pH 7.5, for 1 h; washed in TBS, pH 7.5 (three changes for 10 min each); washed in TBS, pH 7.5, containing 1% (w/v) BSA (three changes for 10 min each); incubated with gold-conjugated rabbit anti-goat IgG serum (10 nM; Sigma) diluted 1/15 in TBS, pH 8.2, containing 1% (w/v) BSA, for 1 h; washed in TBS, pH 8.2, containing 1% (w/v) BSA (three changes for 10 min each); finally washed in TBS, pH 8.2 (two changes for 10 min each), and distilled water. The sections were post-stained with 2% (v/v) aqueous uranyl acetate, for 10 min. The control test, which gave no specific immunogold labeling, involved the omission of primary antibodies from the sequence. Sections were viewed in EM 100C (JEOL, Tokyo) at 80 kV.

Quantification and Characterization of SA and SAG

Free SA was extracted and quantified essentially as described by Raskin et al. (1989) with modifications described by Malamy et al. (1992). HPLC was performed as described previously (Malamy et al., 1990). The SAG was quantified as described by Malamy et al. (1992).

Statistical Analysis

Data are reported as the mean ± SD. The results were compared statistically by using a two-tailed Student's *t* test, and differences were considered significant if *P* values were <0.05.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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