Research Note

Role for Salicylic Acid in the Activation of Defense Responses in Catalase-Deficient Transgenic Tobacco

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Transgenic tobacco plants with severely reduced catalase activity were crossed with NahG plants that do not accumulate salicylic acid. Some of the progeny from the cross spontaneously developed necrosis when grown under strong light, similar to that observed on the parental severely catalase-deficient plants. However, in contrast to the parental catalase-deficient plants, these progeny plants did not constitutively express *PR-1* genes or develop enhanced resistance. Thus, salicylic acid appears to be required for the induction of these defense responses in catalase-deficient tobacco plants.

In tobacco (*Nicotiana tabacum*), as well as in many other plants, pathogen infection of resistant cultivars results in the hypersensitive response (HR), which is characterized by the formation of necrotic lesions at the site of infection and the restriction of pathogen growth and spread. In addition to this local resistance response, tissues distal to the infection site develop enhanced resistance to a secondary infection by the same or even unrelated pathogens. This enhanced disease resistance is generally termed systemic acquired resistance (SAR; for review, see Ryals et al. 1996; Wobbe and Klessig 1996). Associated with the HR and SAR is the expression of defense-related genes, such as those encoding the pathogenesis-related (PR) proteins. Synthesis of PR proteins has been widely used as a molecular indicator for the activation of plant defense mechanisms.

Salicylic acid (SA) has been implicated as one of the key components in the signal transduction pathway leading to plant resistance to various pathogens (Ryals et al. 1996; Wobbe and Klessig 1996). For instance, in tobacco, endogenous SA levels increase after pathogen attacks, which correlates with the expression of PR genes and the onset of SAR. Secondly, exogenous application of SA also induces PR gene expression and enhanced disease resistance. Thirdly, transgenic tobacco plants constitutively expressing the bacterial *nahG* gene (which encodes salicylate hydroxylase, an enzyme that destroys SA) show enhanced susceptibility to pathogen attacks and fail to develop SAR (Gaffney et al. 1993; Delaney et al. 1994).

In an attempt to elucidate the mechanisms of SA action in plant disease resistance, we have identified an SA-binding protein in tobacco leaves (Chen and Klessig 1991; Chen et al. 1993a, 1993b). It is catalase. The discovery that SA inhibited catalase's H₂O₂-degrading activity and that prooxidants such as H₂O₂ induced PR gene expression led to the proposal that one mechanism of SA's action is elevation of the level of H₂O₂ or H₂O₂-derived reactive oxygen species (ROS), which then serve as intermediates in the SA signal transduction pathway (Chen et al. 1993b). Consistent with this model, the following were observed: (i) 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH), two synthetic inducers of PR genes and enhanced resistance, also inhibit tobacco catalase (Conrath et al. 1995; Wendehenne et al., *in press*); (ii) SA, INA, and BTH inhibit the activity of tobacco ascorbate peroxidase, the other key H₂O₂-scavenging enzyme, *in vitro* (Durner and Klessig 1995; Wendehenne et al., *in press*); and (iii) antioxidants suppress SA-, INA-, or BTH-induced PR-I gene expression (Conrath et al. 1995; Wendehenne et al., *in press*).

The involvement of catalase inhibition by SA and elevated H₂O₂ levels in plant defense responses, however, has been called into question based on four observations: (i) tobacco plants infected with *Pseudomonas syringae* or leaf disks treated with SA did not show significant reduction in catalase activity (Bi et al. 1995); (ii) H₂O₂ levels did not increase during the establishment of SAR (Neuenschwander et al. 1995); (iii) PR-I gene activation by prooxidants such as H₂O₂ or 3-aminotriazole was suppressed in NahG transgenic tobacco (Bi et al. 1995; Neuenschwander et al. 1995); and (iv) very high levels of H₂O₂ induced the production of SA (León et al. 1995; Neuenschwander et al. 1995; Summermatter et al. 1995). These results argue that H₂O₂ acts upstream of SA in the signal transduction pathway rather than, or in addition to, acting downstream of SA.

To further investigate the involvement of catalase and ROS in the activation of defense responses, transgenic tobacco plants with severely depressed levels of catalase due to either sense co-suppression or antisense suppression were constructed (Chamnongpol et al. 1996; Takahashi et al. 1997). These plants spontaneously develop necrosis under high light, accumulate PR proteins, and show enhanced disease resistance. However, the antisense-suppressed, catalase-deficient plants (ASCAT1; Takahashi et al. 1997) also exhibited slightly

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to moderately elevated levels of SA and its glucoside. Thus, it is unclear whether the activation of defense responses was due to the reduction in catalase activity or the increase in SA levels.

To resolve this question, we have crossed two plants from the ASCAT1 #17 line (T2 generation; Takahashi et al. 1997) with homozygous NahG-10 plants (Gaffney et al. 1993). A total of 10 F1 progeny from two crosses (AN1 #1 to #5 and AN2 #1 to #5; Fig. 1A) were analyzed. Since the parental ASCAT1 #17 transgenic plant contained multiple copies of the antisense catalase insert (data not shown) and one or several of the insertions may be in a heterozygous state in the T2 plants, the catalase-deficient phenotype was still segregating. The two T2 plants of ASCAT1 #17 were also crossed with wild-type (wt) tobacco plants; progeny of these crosses (AW1 #1 to #5 and AW2 #1 to #5; Fig. 1A) served as controls to

which the AN1 and AN2 plants were compared. As the AN1 and AN2 plants were heterozygous for the *nahG* gene, progeny of a cross between the NahG plant and the wt plant served as another control (WN; Fig. 1A). All plants were grown at 20°C under a 14-h light cycle with strong light (20,000 to 28,000 lx).

Similar to the parental ASCAT1 plants, lesions spontaneously developed on the leaves of five AW and AN plants (Fig. 1B). In contrast, none of the six wt and WN plants developed lesions under these conditions. Due to the presence of multiple copies of the antisense catalase insert, AW and AN plants exhibited heterogeneity with respect to catalase protein levels and catalase activity (Figs. 2 and 3). Interestingly, the five plants (AW1 #2, AW1 #5, AN1 #2, AN1 #4, and AN2 #3) that had the most severely reduced catalase activity (Fig. 3) and the lowest levels of catalase protein (Fig. 2), also spontaneously developed necrosis (Fig. 2), while the others did not. This is consistent with the previous finding that development of necrosis correlated with greatly reduced catalase activity (>90%; Takahashi et al. 1997). Since these AN plants with severely depressed catalase levels developed necrosis just like

Fig. 1. A, Schematic representation of crosses performed between wild-type (wt), severely catalase-deficient tobacco (ASCAT1 #17 T2 generation), and NahG-10 plants. Abbreviations representing the progeny from each cross are listed at the bottom. **B**, Photographs of a wt leaf and the leaves of progeny from two crosses showing areas of spontaneously developing necrosis (indicated by arrows).

Fig. 2. Western blot (immunoblot) analysis of catalases and PR-1 proteins in leaf extracts. Leaf disks were taken either from leaves showing necrosis or from leaves at similar developmental stage on other phenotypically-normal plants. Soluble leaf proteins were extracted in buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM β -mercaptoethanol, and 10 μ g of phenylmethylsulfonyl fluoride per ml. Protein (10 μ g per lane) was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto a nitrocellulose membrane. Monoclonal antibodies (MAb3B6, MAb1F5, and MAb33G1) were used to detect catalase 1 (Cat1), catalase 2 (Cat2), and PR-1 proteins. Presence (+) or absence (-) of spontaneously developing necrosis is indicated at bottom of each lane. The number corresponds to individual progeny from the various crosses. See Figure 1A and text for description of the different crosses.

Fig. 3. Catalase activity present in the leaf extract. Catalase activity was measured with an O_2 evolution assay according to Takahashi et al. (1997). Catalase activity of the wild-type (wt) plants was defined as 100%. WN plants contain levels of catalase activity similar to those of the wt plants (data not shown). Catalase activity and standard deviation (shown as an error bar) were calculated for each plant with three independent extracts from the same leaf.

the severely catalase-deficient AW plants, elevated levels of SA do not appear to be required for the formation of necrosis. In contrast, SA seems to be required for the accumulation of PR-1 proteins in these catalase-deficient plants (Fig. 2). While the AW1 #2 and AW1 #5 plants accumulated substantial amounts of PR-1 proteins, AN1 #2, AN1 #4, and AN2 #3 plants produced little, if any, PR-1 proteins, even though they had very low levels of catalase and spontaneously developed necrosis. Thus, while stress resulting from severely reduced catalase activity likely induced necrosis, it appears to be insufficient for the activation of certain defense responses in the absence of elevated levels of SA.

SA was also necessary for the induction of enhanced resistance (i.e., SAR) to tobacco mosaic virus (TMV) in the catalase-deficient plants. As anticipated from our previous study (Takahashi et al. 1997), the severely catalase-deficient plants AW1 #2 and AW1 #5 not only developed necrosis and constitutively expressed *PR-1* genes, but also formed smaller lesions after TMV infection than the wt control or the other AW progeny with less depressed catalase levels (Fig. 4). In marked contrast, the two severely catalase-deficient AN progeny (AN1 #2 and AN1 #4) while developing necrosis, not only failed to express *PR-1* genes, but also formed TMV-induced lesions indistinguishable in size from those on the WN control and other AN plants with less depressed catalase levels.

In summary, our results indicate that SA is required for the activation of certain defense responses, such as *PR-1* gene expression and the development of enhanced resistance in the catalase-deficient progeny derived from crosses between AS-CAT1 #17 and the NahG plants. However, SA does not appear to be required for the development of necrosis, which is likely brought about by stress resulting from the severe reduction of catalase activity. This latter conclusion is consistent with the results from several other studies, which indicate that necrotic lesion formation can be uncoupled from disease resistance. For example, when NahG transgenic *Arabidopsis* plants were crossed to the lesion-forming *Arabidopsis* mutants *lsd2* and

Fig. 4. Lesion size 7 days post tobacco mosaic virus (TMV) infection. Leaves were inoculated with TMV according to Takahashi et al. (1997). Diameters of 20 randomly picked lesions from the same leaf were measured and the standard deviation (shown as an error bar) was calculated.

lsd4 (Dietrich et al. 1994), the progeny did not express *PR-1* genes or show enhanced disease resistance; lesion formation, however, was unaffected (Ryals et al. 1996). Another *Arabidopsis* mutant, *ndr1*, is susceptible to several pathogens that are avirulent on wt *Arabidopsis*. Interestingly, an HR similar to that occurring in wt plants also develops on *ndr1* mutant upon pathogen infection (Century et al. 1995). Finally, while NahG tobacco plants show enhanced susceptibility to several elicitor-producing *Phytophthora* spp. and fail to establish SAR after elicitor treatment, the necrosis response is unchanged with respect to that observed in wt tobacco plants (Keller et al. 1996). Hence, there seems to be an SA-independent pathway controlling the formation of necrosis, in addition to the SA-dependent pathway that leads to the induction of *PR* genes and enhanced resistance.

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