An HR-Induced Tobacco Peroxidase Gene Is Responsive to Spermine, but Not to Salicylate, Methyl Jasmonate, and Ethephon

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In *Tobacco mosaic virus* (TMV)-infected tobacco plants carrying the *N* resistance gene, a hypersensitive reaction or response (HR) occurs to enclose the virus in the infected tissue. Although a contribution of peroxidases to the resistance has been proposed, no evidence has been presented that tobacco peroxidase genes respond to HR. Here, we describe the HR-induced expression of a tobacco peroxidase gene (*tpoxCI*) whose induction kinetics were slightly different from those of acidic and basic tobacco pathogenesis-related (PR) protein genes. Interestingly, *tpoxCI* was insensitive to the inducers of PR genes such as salicylic acid, methyl jasmonate, and ethephon. Spermine activated *tpoxCI* gene expression at a low level and both acidic and basic PR gene expression at a considerably higher level. These results indicate that the induced expression of *tpoxCI* is regulated differently from that of classical tobacco PR genes in the *N* gene-mediated self-defense system in tobacco plants.

Additional keyword: wounding.

In the interactions between *Tobacco mosaic virus* (TMV) and resistant tobacco cultivars carrying the *N* gene, infected tissues display a hypersensitive reaction or response (HR) that is associated with necrotic lesion formation to enclose the virus at the infected site. This phenomenon is characterized by diverse responses such as accumulations of salicylic acid (SA) (Malamy et al. 1990), jasmonic acid (JA) (S. Seo and Y. Ohashi, *unpublished*), ethylene (Ohtsubo et al. 1999), and spermine (Spm) (Yamakawa et al. 1998), callose deposition (Shimomura and Dijkstra 1975) and induction of pathogenesis-related (PR) proteins, which are the plant proteins induced in pathological or related situations (van Loon et al. 1994).

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PR proteins are proposed to be classified into 14 families on the basis of structural and functional properties (van Loon and van Strien 1999) and, in tobacco plants, expression profiles of five classical PR families (PR-1 to PR-5) have been studied in detail. They are composed of acidic and basic types, originally classified by their isoelectric points (Memelink et al. 1990; Brederode et al. 1991; Ohashi and Ohshima 1992). Genes for basic PR proteins are induced by wounding, and JA enhances (Brederode et al. 1991; Niki et al. 1998) while SA inhibits (Niki et al. 1998) their expression. In contrast, the expression of genes for acidic PR proteins is induced by SA but not by wounding (Brederode et al. 1991; Ward et al. 1991; Niki et al. 1998), and the effect of SA is inhibited in the presence of JA (Niki et al. 1998). Recently, Spm, which accumulates in the intercellular spaces of TMV-infected tobacco leaves developing necrotic lesions, was proposed to be an endogenous inducer of acidic PR-1, -2, -3, and -5 proteins and to confer resistance to TMV infection (Yamakawa et al. 1998). However, few studies have reported on the expression profiles of PR proteins other than the classical PR-1 to PR-5 and PR-6 families in tobacco.

Class III plant peroxidases (POXs; EC 1.11.1.7), which catalyze the oxidoreduction of hydrogen peroxide and various reductants, have been shown to be induced in pathological interactions involving plants and fungi (Thordal-Christensen et al. 1992; Harrison et al. 1995), bacteria (Reimers et al. 1992), viruses (van Loon and Geelen 1971; Lagrimini and Rothstein 1987), and a viroid (Vera et al. 1993). POX consists of multiple molecular forms (isoPOX) in single plant species, some of which do not respond to pathogen infection; therefore, only specific pathogen-inducible isoPOXs have been categorized in the PR-9 family (van Loon et al. 1994). Several studies have indicated an association of enhanced POX activity and induced resistance to TMV infection (Simons and Ross 1970) and *Pseudomonas syringae* pv. *tabaci* infection (Lovrekovich et al. 1968) in tobacco plants. In rice, an incompatible interaction with *Xanthomonas oryzae* pv. *oryzae* causes a more rapid and enhanced induction of a cationic POX than a compatible interaction (Reimers et al. 1992). In the defense system of plants against pathogen attack, POXs are pro-

posed to contribute essentially to the construction of strengthened physical barriers in cell walls (Vance et al. 1980; Fry 1986; Bowles 1990), formation of active oxygen species (Bolwell et al. 1995; Wojtaszek 1997), and production of antimicrobial substances (Kristensen et al. 1999). The induction of multiple isoPOXs in a single host-parasite interaction (Harrison et al. 1995; Chittoor et al. 1997) may reflect a contribution by each isoPOX to a specific process in the induced resistance.

Pathogen-inducible POX genes have been shown to be induced by defense-related signal compounds such as abscisic acid in tomato (Roberts and Kolattukudy 1989), jasmonates in rice (Schweizer et al. 1997) and *Stylosanthes humilis* (Curtis et al. 1997), SA in cucumber (Rasmussen et al. 1995) and parsley (Thulke and Conrath 1998), and ethephon, an ethylene-releasing agent, in tomato (Vera et al. 1993).

In tobacco, at least 12 isoPOXs were detected by activity staining after isoelectric focusing (Lagrimini and Rothstein 1987). Among them, two anionic isoPOXs (pI 3.5 and 3.75, respectively) were found to be the major isoPOXs in most organs and constitutively expressed in leaves (Lagrimini and Rothstein 1987). Analyses of transgenic plants over- or underproducing the anionic POX (Lagrimini 1991; Lagrimini et al. 1997a, 1997b) and the catalytic properties of purified enzyme (Gazaryan and Lagrimini 1996) suggested that this POX is associated with auxin degradation and the metabolism of phenolics. Upon TMV infection, total POX activity was increased, accompanied by the induction of two isoPOXs with a moderately anionic nature (pI 5.65 and 6.1, respectively) in tobacco leaves (van Loon and Geelen 1971; Lagrimini and Rothstein 1987). Although the anionic POXs (pI 3.5 and 3.75) were not induced by TMV infection at both activity and mRNA levels (Lagrimini and Rothstein 1987; Ward et al. 1991), Stintzi et al. (1993) reported that the two TMV-inducible isoPOXs with the molecular mass of 39 and 40 kDa have amino acid sequences identical to that of the anionic POX (pI 3.5). Since the 39- and 40-kDa POXs were only partially sequenced, it is not clear that they are really identical to the anionic POX (pI 3.5). Thus, there are no reports on the defined pathogen-inducible POX genes, to our knowledge.

To study pathogen-inducible isoPOXs in tobacco, we isolated novel cDNAs encoding putative POXs from a cDNA library prepared from TMV-infected tobacco leaves (Hiraga et al. 1999). Amino acid sequence alignment with known POXs revealed that one cDNA (*tpoxC1*) encodes a protein with the characteristic primary structure frequently found in pathogen-inducible POXs from other plant species (Chittoor et al. 1999). Here, we describe the induction of the *tpoxC1* gene by TMV-induced HR and its unique expression profile compared with classical tobacco acidic and basic PR genes, especially in response to defense-related signal compounds such as SA, methyl jasmonate (MeJA), ethephon, and Spm.

RESULTS

Genomic organization of the *tpoxC1* gene.

Genomic DNAs from *Nicotiana tabacum* cv. Samsun NN, the source of *tpoxC1* cDNA, and its parental species, *N. sylvestris* and *N. tomentosiformis*, were subjected to gel blot analysis to reveal the genomic organization of the *tpoxC1*

gene (Fig. 1). The one or two hybridization signals found in the genomic DNA from *N. tabacum* digested with *EcoRI*, *HindIII*, and *XbaI* suggest that *tpoxC1* exists as a single copy. The mobility of the most intense signal in the *N. tabacum* genome was almost the same as that from *N. sylvestris*. Thus, the *tpoxC1* gene is considered to have originated from the *N. sylvestris* genome. A less intense signal in *N. tomentosiformis* indicates the existence of a closely related gene in the *Nicotiana* species.

Organ-specific expression of the *tpoxC1* gene.

In healthy tobacco plants (50 cm in height), *tpoxC1* transcripts accumulated at a low level in roots, a trace level in flowers, and undetectable levels in upper leaves, lower leaves, stems, seeds, and shoot apices (Fig. 2). In contrast, the transcript for anionic POX, which was originally isolated by Lagrimini et al. (1987) and designated as tobacco PR-9 (van Loon et al. 1994), was abundant in almost all organs except for seeds, especially in stems and lower leaves (Fig. 2).

Fig. 1. Genomic DNA gel blot analysis of the *tpoxC1* gene. Genomic DNAs from *Nicotiana tabacum* (ta), *N. tomentosiformis* (to), and *N. sylvestris* (sy) were subjected to analysis as described in Materials and Methods. The image was visualized by PhosphorImager SI.

Fig. 2. Organ-specific expression of the *tpoxC1* gene. Total RNAs (20 µg per lane) were isolated from organs of tobacco plants (50 cm in height). Expression levels of *tpoxC1* and anionic POX (Lagrimini et al. 1987) genes were monitored by RNA gel blot analysis as described in Materials and Methods. The images were visualized by autoradiography.

HR-dependent induction of *tpoxC1* gene expression in TMV-infected leaves.

Transcript levels of *tpoxC1* and PR-1 genes were monitored with TMV- or mock-inoculated leaves incubated at 20°C, a permissive temperature for the necrotic lesion formation that occurs as a result of the *N* gene-mediated HR. Under these conditions, necrotic lesions appeared at about 32 h after TMV inoculation and increased in size with time. As shown in Figure 3, *tpoxC1* mRNA levels increased remarkably with the development of necrotic lesions in TMV-inoculated leaves but not in mock-inoculated leaves. The expression profile of an acidic PR-1 gene (*PR-1a*) was similar but accumulation of the transcript was a little faster, compared with that of *tpoxC1*. The transcript level of a basic PR-1 gene (*PRB-1b*) was significantly increased only by wounding (mock-inoculation) and enhanced by necrotic lesion formation. Although the expression level of all three genes studied here increased depending on the development of necrotic lesions, the induction kinetics seem to differ.

Then, we compared the expression profiles of these genes with the synchronous HR inducing system: TMV-inoculated leaves were incubated for 30 h at 30°C, under which conditions TMV multiplies without HR, and then at 20°C to induce the formation of synchronous necrotic lesions (Fig. 4A). Transcript of the *tpoxC1* gene was detected at 9 h after the temperature shift, coinciding with the appearance of necrotic lesions, peaked at 36 h, and decreased thereafter, while no signal was found in mock-inoculated leaves (Fig. 4B). When we used Samsun nn tobacco plants, which lack the *N* gene, no induced expression of the *tpoxC1* gene was observed even at 48 h after the temperature shift in TMV-inoculated leaves, nor in mock-inoculated leaves (data not shown). The expression of the anionic POX was suppressed slightly in both mock- and TMV-inoculated leaves after the temperature shift, as reported previously (Ward et al. 1991). Similarly to *tpoxC1*, acidic PR-1 was induced in TMV-infected Samsun NN leaves, but not in mock-inoculated leaves. On the wound-inducible basic PR-1

gene, the effect of the wounding by inoculation remained until soon after the temperature shift and the transcript level bottomed out at 12 h in mock-inoculated leaves. In TMV-inoculated leaves the transcript level of basic PR-1 gene was enhanced accompanying necrotic lesion formation after the temperature shift.

Unique response of the *tpoxC1* gene to *N* gene-mediated HR signals.

In TMV-inoculated Samsun NN tobacco leaves, the timing of *tpoxC1* induction and the appearance of necrotic lesions coincided (Fig. 4B), while no induction was observed in Samsun nn tobacco leaves, suggesting that the expression of *tpoxC1* depends on the *N* gene-mediated HR signals.

To analyze the response of the *tpoxC1* gene to HR, TMV-inoculated leaves were incubated for 40 h at 30°C, transiently shifted to 20°C, and then returned back to 30°C (Fig. 5A). Incubation at 20°C for more than 4 h induces signals sufficient for necrotic lesion formation depending on TMV infection and the *N* gene. In this system, 6 to 8 h of transient 20°C treatment resulted in more intense necrotic lesions 24 h after the temperature shift (16 to 18 h after the shift back to 30°C) than constant 20°C incubation for 24 h (Ohtsubo et al. 1999). Expression of *tpoxC1* was induced by 4 h of 20°C treatment and the level increased gradually on prolonged 20°C treatment, showing a similar expression pattern with the tobacco proteinase inhibitor (PI)-II gene (Fig. 5B). By contrast, expression levels of both acidic and basic PR-1 genes peaked at 6 h of treatment, and decreased after longer 20°C treatment (Fig. 5B), correlating with the intensity of necrotic lesions (Fig. 5A).

To study the response of *tpoxC1* to defense-related signal molecules, we treated tobacco leaf disks with SA, MeJA,

Fig. 3. Induction of *tpoxC1* and PR-1 genes in *Tobacco mosaic virus* (TMV)-infected tobacco leaves at 20°C. Total RNAs (20 µg per lane) were isolated from leaves harvested at the indicated time points. Expression levels of *tpoxC1* and PR-1 genes were monitored by RNA gel blot analysis as described in Materials and Methods. The images were visualized by PhosphorImager SI.

Fig. 4. Expression of tobacco POX and PR-1 genes during synchronous hypersensitive response. **A**, Time schedule for temperature treatment and sampling. *Tobacco mosaic virus* (TMV)- or mock-inoculated leaves were incubated for 30 h at 30°C and then at 20°C. **B**, RNA gel blot analysis of POX and PR-1 genes in Samsun NN tobacco plants (20 µg per lane). The images were visualized by autoradiography.

ethephon, and Spm, and analyzed the expression levels of *tpoxC1* and PR-1 genes (Fig. 6). Transcript for acidic PR-1, a representative acidic-type PR protein, was induced by SA and Spm in a dose-dependent manner, while MeJA and ethephon treatments had no apparent effect after 48 h. Transcript for basic PR-1, a representative basic-type PR protein, was induced 48 h after wounding, and the wound-induced expression was suppressed in the presence of 1 mM SA. In contrast, treatment with MeJA, ethephon, and Spm enhanced the wound-induced basic PR gene expression in a dose-dependent manner. Unlike the PR-1 genes, *tpoxC1* did not respond to SA, MeJA, or ethephon at all. Spm at 100 and 500 μ M induced *tpoxC1* gene expression at low levels.

Absence of systemic *tpoxC1* gene induction in uninoculated upper leaves.

When lower tobacco leaves were infected with necrotizing pathogens, the expression of both acidic and basic PR genes was systemically induced in upper uninoculated leaves (Ward

et al. 1991). POX activity has also been indicated to be enhanced systemically (Simons and Ross 1970; Lagrimini and Rothstein 1987). In the case of the *tpoxC1* gene, we could not detect the transcript at all in the upper uninoculated leaves 7 days after TMV inoculation of the lower leaves, while both acidic and basic PR-1 genes were apparently induced systemically in uninoculated leaves (Fig. 7).

DISCUSSION

We describe here a pathogen-inducible tobacco POX gene (*tpoxC1*) that does not respond to general defense-related signal compounds. Similar to that of tobacco PR genes, induction of *tpoxC1* depends on the *N*-gene mediated HR that occurs at a permissive temperature for the *N* resistance gene ($\leq 24^\circ\text{C}$) in TMV-infected tobacco leaves undergoing necrotic lesion formation.

We studied the response of *tpoxC1* to HR with three different HR inducing systems. The first is 20°C constant treatment,

Fig. 5. Response of *tpoxC1*, PI-II, and PR-1 genes to transient 20°C treatment in *Tobacco mosaic virus* (TMV)-inoculated leaves. **A**, Time schedule for the temperature treatment and sampling. TMV-inoculated leaves were incubated at 30°C for 40 h, then at 20°C for an appropriate period, and finally at 30°C again. All leaves were harvested 24 h after the temperature shift to 20°C . Intensity of the necrotic lesions was evaluated by the naked eye at 24 h after the temperature shift. **B**, RNA gel blot analysis of *tpoxC1*, PI-II, and PR-1 genes (20 μ g per lane). The images were visualized by autoradiography.

Fig. 6. Response of *tpoxC1* and PR-1 genes to hypersensitive response (HR)-related signal compounds. Leaf disks (20.5 mm in diameter) were floated on the solutions containing salicylic acid (SA), methyl jasmonate (MeJA), ethephon (ET), and spermine (Spm) at 20°C . Expression levels of *tpoxC1* and PR-1 genes at 48 h (for SA, MeJA, and ET) or 24 h (for Spm) were monitored by RNA gel blot analysis as described in Materials and Methods (20 μ g per lane). The images were visualized by autoradiography.

Fig. 7. Systemic expression of *tpoxC1* and PR-1 genes in *Tobacco mosaic virus* (TMV)-infected tobacco plants. Lower leaves (a leaf per plant) of tobacco plants (20 cm in height) were inoculated with TMV and upper uninoculated leaves were harvested at 0 or 7 days after inoculation. As a control, upper leaves were harvested at 7 days after mock-inoculation of lower leaves (a leaf per plant). Expression levels of *tpoxC1* and PR-1 genes were monitored by RNA gel blot analysis as described in materials and methods (20 μ g per lane). The images were visualized by autoradiography.

which induces continuous HR accompanied by normal necrotic lesion formation. The second is a temperature shift from 30 to 20°C, which induces synchronous HR at first and then continuous HR: necrotic lesions synchronously appear 9 h after the temperature shift and increase in size gradually at 20°C. The third is transient 20°C treatment (≥ 4 h), which induces synchronous HR within 9 h, but TMV escapes from necrotic lesions and spreads to neighboring uninfected cells on subsequent 30°C treatment.

Expression of *tpoxC1* was induced by all three systems. However, the response of *tpoxC1* to these HRs was not the same as those of PR-1 genes, which are frequently used as marker genes for HR. The difference was obvious with the third system, i.e., transient 20°C treatment. The levels of *tpoxC1* and PI-II transcripts were increased with the length of transient 20°C treatment. However, the levels of PR-1 transcripts peaked at 6 h of treatment, and decreased with longer treatment. Such response to transient 20°C incubation seems to correlate with the intensity of necrotic lesions. These results suggest that *tpoxC1* and PI-II expression is induced by continued *N* gene-mediated signaling, while the induction of PR-1 genes depends on the formation and intensity of necrotic lesions.

The profile of the response of *tpoxC1* to the signal compounds that accumulate in the leaves infected with necrotizing pathogens was unique, compared with that of other defense-related genes. Unlike acidic PR genes, *tpoxC1* did not respond to SA. MeJA and ethephon, which are effective inducers for basic PR and PI-II genes (Balandin et al. 1995; Niki et al. 1998), did not activate *tpoxC1* expression, as would be expected from the weak response of this gene to wounding. Spm was originally identified as an inducer for acidic PR-1, -2, -3, and -5 proteins (Yamakawa et al. 1998). In this paper, we originally indicated that Spm is an endogenous inducer for both *tpoxC1* and basic PR genes expression. The PR-1 to -5 proteins (Memelink et al. 1990; Niki et al. 1998), PR-11 (Melchers et al. 1994), and other defense-related proteins such as extensin (Memelink et al. 1993), have been reported to be induced by SA, jasmonates, or ethephon in tobacco plants. The insensitivity of *tpoxC1* to these compounds seems to be unique among tobacco defense-related genes. These results suggest the occurrence of unknown inducer(s) for *tpoxC1* in HR.

TMV-induced expression of *tpoxC1* was observed in NN but not in nn tobacco leaves, suggesting a possible association with the resistance. At a constant 22°C, TMV becomes restricted to the area in or around the necrotic lesions, while it easily escapes from necrotic lesions and spreads to noninfected, adjacent cells once the leaves are transferred to 32°C (Weststeijn 1981). Thus, the increase in *tpoxC1* transcript on prolonged, transient 20°C treatment may also support a possible defensive role for the gene product under temperatures at which the *N* gene is functional. The presence of N-terminal signal peptide in the *tpoxC1* gene product (Hiraga et al. 1999) suggests that *tpoxC1* is secreted outside of the cells and may function for pathogen resistance in cell walls or intercellular spaces. Combined studies on *tpoxC1* with transgenic, biochemical, and enzymological approaches would provide further information on the defensive function of the gene product.

It remains to be elucidated how many defense-related genes are activated coordinately and specifically in HR and how the

gene products function against the multiplication and spread of pathogens. A well-organized and orchestrated expression of PR genes or other defense-related genes, including *tpoxC1*, would be important for an effective self-defense of host plants against pathogen-attack. The unique responses of *tpoxC1* to HR-associated signals suggest some unknown mechanism behind the expression of defense-related genes.

MATERIALS AND METHODS

Plant material and pathogen inoculation.

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN and cv. Samsun nn) were grown in a temperature-controlled greenhouse at 20 to 30°C. Fully expanded upper leaves from 2-month-old tobacco plants were used for all experiments except for the examination of organ specific expression.

For virus infection, tobacco leaves were inoculated with a 10 μg of TMV per ml suspension and incubated at 20 or 30°C under continuous light at 100 $\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$.

Chemical treatments.

Leaf disks (20.5 mm in diameter) were floated on the chemical solutions and incubated at 20°C under continuous light at 100 $\mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$. SA solutions were adjusted to pH 7.0 with NaOH. Ethephon and Spm were dissolved in 10 mM MES (2-morpholinoethanesulfonic acid) at pH 5.5 and 7.5, respectively.

DNA gel blot analysis.

Tobacco genomic DNAs (10 μg) were digested with *EcoRI*, *HindIII*, and *XbaI*, fractionated in a 0.7% agarose gel and blotted onto a nylon membrane (Sambrook et al. 1989). Hybridization was performed with ^{32}P -labeled *tpoxC1* cDNA as described previously (Church and Gilbert 1984). Bovine serum albumin (BSA) was omitted from the hybridization buffer and the membrane was washed stringently at 65°C for 20 min in 0.2 $\times$ SSC (1 $\times$ SSC: 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% (wt/vol) SDS (sodium dodecyl sulfate). The image was visualized by PhosphorImager SI (Molecular Dynamics Japan, Tokyo).

RNA gel blot analysis.

Tobacco organs were frozen in liquid nitrogen immediately after sampling and stored at -80°C . Total RNA was isolated by the ATA method (Nagy et al. 1988). Denatured total RNAs (20 μg per lane) were separated in 1.2% agarose gels containing formaldehyde and blotted onto nylon membranes (Sambrook et al. 1989). Blots were hybridized with ^{32}P -labeled full length POX cDNAs and 3' untranslated regions of acidic (PR-1a; Matsuoka et al. 1987), basic (PRB-1b; Eyal et al. 1992) PR-1 and PI-II (Balandin et al. 1995) cDNAs at 42°C for 16 h in a solution containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 $\times$ Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.5% SDS, 3 $\times$ SSC, 50% deionized formamide, and 0.1 mg of denatured salmon sperm DNA per ml. The membrane was washed with 2 $\times$ SSC containing 0.1% SDS once for 5 min and twice for 10 min at room temperature and then with 1 $\times$ SSC containing 0.1% SDS 3 times for 15 min at 65°C. Images were visualized by autoradiography or PhosphorImager SI. Equal loading of RNAs was confirmed by monitoring the levels of ribosomal

RNA (rRNA) stained with methylene blue (Sambrook et al. 1989).

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