Wound-Induced Expression of a Tobacco Peroxidase Is Not Enhanced by Ethephon and Suppressed by Methyl Jasmonate and Coronatine

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In tobacco plants, wounding induces production of a set of defense-related proteins such as basic pathogenesis-related (PR) proteins and proteinase inhibitors (PIs) via the jasmonate/ethylene pathway. Although class III plant peroxidase (POX), inducing ethylene and ethopon in contrast to other wound-inducible genes such as PR-I and PI-II genes. And treatment with MeJA and coronatine, biological analogs of jasmonate, rather suppressed the POX expression. Salicylic acid, an antagonist of jasmonate-induced POX expression, did not suppress the wound-induced expression of POX. Only spermine, which is reported as an endogenous inducer for acidic PR genes in tobacco mosaic virus-infected tobacco leaves, could induce POX gene expression. These results suggest that wound-induced expression of the POX gene is regulated differently from that of the basic PR and PI-II genes.

Key words: Ethylene — Jasmonic acid — Peroxidase — Spermine — Tobacco (Nicotiana tabacum) — Wounding.

Wounding is one of the severest environmental stresses that inhibits normal growth and reproduction of plants, and enables pathogens to penetrate easily into plant tissues. Wounded plant tissues display diverse responses for restoration of damaged tissues and resistance to pathogen infection such as suberization (Dean and Kolattukudy 1976), crosslinking of cell wall proteins (Bradley et al. 1992) and production of antimicrobial substances (Dixon and Paiva 1995) accompanying both local and systemic induction of a set of defense-related proteins including pathogenesis-related (PR) proteins, proteinase inhibitors (PIs) and enzymes involved in phenylpropanoid metabolism.

Jasmonates (Farmer and Ryan 1990), ethylene (O’Donnell et al. 1996) and abscisic acid (ABA) (Peña-Cortés et al. 1989) have been shown to be chemical mediators for wound-induced expression of the POX gene in tomato and potato plants. Systemin (Pearce et al. 1991), ABA (Peña-Cortés et al. 1989) and electric current (Wildon et al. 1992) have been postulated as signal mediators for systemic expression of POX. In tobacco plants, jasmonates (Seo et al. 1995) and ethylene (Niki et al. 1998) accumulate in wounded mature leaves, and exogenous application of these compounds also induces the wound-responsive tobacco POX and basic PR genes (Brederode et al. 1991, Niki et al. 1998, Ohtsubo et al. 1999). Thus, jasmonates and ethylene are considered the major signal compounds for wound-induced gene expression in plants.

Class III plant peroxidase (EC 1.11.1.7, POX), which catalyzes the oxoreduction of hydrogen peroxide and various peroxidents, is induced in response to wounding in several plant species (Roberts et al. 1988, Ishige et al. 1993, Ito et al. 1994). A highly anionic POX of potato is one of the most extensively studied wound-inducible POXs and was shown to be associated with suberization (Espelie et al. 1986). Wound-inducible POX genes are activated by exogenous application of ABA in potato and tomato (Roberts and Kolattukudy 1989), ethopon, a ethylene releasing agent in azuki bean and rice (Ishige et al. 1993, Ito et al. 1994), and methyl jasmonate (MeJA) in Stylosanthes humilis (Curtis et al. 1997). In tobacco leaves, wounding was shown to increase total POX activity and induce the expression of a cationic POX in leaves (Lagrimini and Rothstein 1987). Kawaoka et al. (1994b) identified a cis-acting element and a transcription factor for a wound-inducible horseradish POX gene using transgenic tobacco plants. However, the regulatory mechanism for wound-induced expression of POX genes is poorly understood. To study the physiological function of POX in self defense systems in plants, we isolated two novel POX cDNAs from tobacco mosaic virus (TMV)-infected tobacco leaves.

Abbreviations: ABA, abscisic acid; COR, coronatine; MeJA, methyl jasmonate; P1, proteinase inhibitor; POX, class III plant peroxidase; PR, pathogenesis-related; SA, salicylic acid; TMV, tobacco mosaic virus.

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Materials and Methods

Plant materials, pathogen inoculation and wound treatment—Fully expanded upper leaves of 2-month-old tobacco plants (Nicotiana tabacum cv. Samsun NN), grown in a greenhouse were used for all experiments except for the examinations of organ specific or systemic gene expression. Detached leaves were inoculated with TMV (10 μg ml⁻¹) and incubated at 20°C under continuous illumination (100 μE m⁻² s⁻¹). For wound treatment, detached leaves were cut into pieces with a razor blade and incubated at 20°C under the continuous illumination.

Treatment with chemicals—Leaf disks (20.5 mm in diameter) were floated on 10 mM MES-KOH buffer (pH 5.5) containing chemicals and incubated at 20°C under continuous illumination (100 μE m⁻² s⁻¹).

DNA gel blot analysis—Tobacco genomic DNA (10 μg) was digested with EcoRI, HindIII and XbaI, separated on a 0.7% agarose gel and blotted onto a Hybond-N nylon membrane (Amersham, Buckinghamshire, U.K.). Hybridization was performed with 32P-labeled tpoxNI cDNA as described previously (Church and Gilbert 1984). Bovine serum albumin was omitted from the hybridization buffer and the membrane was washed three times for 5 min and once for 15 min in 20 mM Na2HPO4 and 1% SDS (pH 7.2) with H3PO4 at 65°C. The image of the signals was visualized by autoradiography.

RNA gel blot analysis—Total RNA (20 μg lane⁻¹) isolated by the GATA method (Nagy et al. 1988) was separated on 1.2% agarose gels containing formaldehyde and blotted onto Hybond-N nylon membranes. Blots were hybridized with 32P-labeled full length tpoxNI cDNA and 3' untranslated regions of basic PR-1 (PRB-1b; Eyal et al. 1992) 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 × Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 0.5% SDS, 1 × SSC (1 × SSC: 15 mM sodium citrate and 150 mM NaCl), 50% deionized formamide and 0.1 mg ml⁻¹ denatured salmon sperm DNA. The membranes were washed with 2 × SSC containing 0.1% SDS once for 5 min and twice for 10 min at room temperature and then with 1 × SSC containing 0.1% SDS 3 times for 15 min at 65°C. Images were visualized by autoradiography or PhosphorImager SI (Molecular Dynamics Japan, Tokyo). Equal loading of RNA was confirmed by monitoring the levels of ribosomal RNA (rRNA) stained with methylene blue (Sambrook et al. 1989).

Results

Genomic organization of tpoxNI—Genomic DNA from Nicotiana tabacum cv. Samsun NN, the source of tpoxNI cDNA (Hiraga et al. 1999), and its parental species, N. sylvestris and N. tomentosiformis, was subjected to gel blot analysis to reveal the genomic organization of tpoxNI (Fig. 1). tpoxNI cDNA hybridized to one to four fragments in the genomic DNA from N. tabacum digested with XbaI, HindIII and EcoRI. The signal from EcoRI-digested DNA was apparently more intense than that from XbaI or HindIII-digested DNA, indicating the presence of two or more genomic fragments of similar size in EcoRI-digested genomic DNA. The amino acid sequence deduced from tpoxNI cDNA is highly homologous to two cationic POXs purified from medium of tobacco cell suspension culture (de Marco et al. 1999). Two separate partial polypeptides of both cationic POXs, which comprise 146 amino acid residues in total, were sequenced and displayed 97% or 95% identity with corresponding regions deduced from the tpoxNI nucleotide sequence. Hence, tpoxNI comprises a gene family in the N. tabacum genome. The signals from the N. tabacum genome migrated to almost the same positions as those from the N. sylvestris genome, indicating that tpoxNI originated from the N. sylvestris genome. Signals in N. tomentosiformis indicate the existence of a closely related gene in the Nicotiana species.

Organ specific expression of tpoxNI—RNA gel blot
analysis showed that tpoxN1 mRNA accumulated at a high level in roots, at a trace level in lower leaves and not at all in upper leaves, stems, flowers, seeds and shoot apices of mature tobacco plants (50 cm in height) (Fig. 2) indicating that expression of tpoxN1 is spatially regulated at the mRNA level. A predominant accumulation of POX transcripts in roots is to be expected given that all detectable tobacco POX isoenzymes were found in roots (Lagrimini and Rothstein 1987).

Rapid induction of tpoxN1 by wounding—Transcript for tpoxN1 accumulated rapidly after wounding: it was detectable at 30 min, increased gradually, reached a maximum level at 4 h and had decreased slightly at 8 h (Fig. 3). In contrast, transcript for tobacco basic PR-1 (PRB-1b; Eyal et al. 1992) gene, which is a marker gene for wound response, appeared 8 h after cutting and that for tobacco PI-II (Balandin et al. 1995) gene, which is also a marker gene, was not detected. Methylene blue staining indicated equal loading of RNAs in each lane. Thus, tpoxN1 was induced more rapidly by wounding than basic PR-1 and PI-II genes.

Induction of tpoxN1 by TMV infection—tpoxN1 cDNA was isolated from a cDNA library from TMV-infected and necrotic lesion-forming leaves of Samsun NN tobacco plants (Hiraga et al. 1999). Then, we examined whether the expression of tpoxN1 is induced by necrotic lesion formation in TMV-infected tobacco leaves incubated at 20°C (Fig. 4). In these conditions, necrotic lesions appeared about 36 h after inoculation. The level of tpoxN1 transcript transiently increased 1 and 2 d after mock-inoculation. In TMV-infected leaves, it increased similarly at 1 and 2 d after inoculation, and it was maintained at high levels at 4 d and then decreased at 6 d. Expression of both basic PR-1 and PI-II genes was induced by mock inoculation and further enhanced by necrotic lesion formation in TMV-infected leaves. However, the timing of maximal induction was delayed and the expression was kept at high levels for longer time periods than tpoxN1.

Systemic induction of tpoxN1 in wounded tobacco plants—When lower leaves of young tobacco plants (20 cm in height) were mock-inoculated, transcript of tpoxN1 was accumulated in upper unwounded leaves 7 d after the treatment (Fig. 5). The expression level was further enhanced by TMV infection of lower leaves in which necrotic lesions were formed. These results suggest that the signal(s) for tpoxN1 expression is systemically transmitted in plants from wounded lower leaves to the upper unwounded leaves, and such signal(s) is increased by necrotic lesion formation. In contrast to tpoxN1, basic PR-1 and PI-II genes

![Fig. 3](image)

Fig. 3 Accumulation of tpoxN1, basic PR-1 and PI-II transcripts on cutting. Leaf pieces were harvested at indicated time points after cutting.

![Fig. 4](image)

Fig. 4 Expression of tpoxN1, basic PR-1 and PI-II genes during necrotic lesion formation in TMV-infected tobacco leaves. Mock- or TMV-inoculated leaves were incubated at 20°C and harvested at indicated time points after inoculation.

![Fig. 5](image)

Fig. 5 Systemic expression of tpoxN1, basic PR-1 and PI-II genes in tobacco plants on wounding or necrotic lesion formation in lower leaves. Lower leaves (one leaf per plant) of young tobacco plants (20 cm in height) were inoculated with TMV or sterile water and incubated at 20°C. Total RNAs were isolated from upper untreated leaves after 7 d.
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![Diagram]

Fig. 6 Responses of *tpoxNl*, basic PR-1 and PI-II genes to defense-related signal compounds. Total RNAs were isolated from the leaf disks after 24 or 48 h of treatment with indicated concentrations of salicylic acid (SA), methyl jasmonate (MeJA), coronaotide (COR), ethephon (ET) and spermine (Spm) or immediately after disking (0 h).

transcripts accumulated systemically at low levels in plants with necrotic lesions but not in mock-inoculated plants.

Unique response of *tpoxNl* to defense-related signal compounds—To study the characteristics of *tpoxNl*, the response to defense-related signal compounds was studied (Fig. 6). To confirm the effect of chemicals, the expression of basic PR-1 and PI-II genes as wound-inducible control genes was also examined. All genes used were induced 24 or 48 h after wounding. Salicylic acid (SA) did not affect wound-induced expression of *tpoxNl* but suppressed that of basic PR-1 and PI-II at 1 mM consistent with previous results (Niki et al. 1998). Interestingly, expression of *tpoxNl* was suppressed by MeJA in contrast to basic PR-1 and PI-II genes whose expression was enhanced by MeJA in a dose dependent manner. Coronatine (COR), which is a structurally related compound to jasmonic acid and has similar biological activity (Fey et al. 1994), induced the expression of basic PR-1 and PI-II genes at 0.5 to 5 μM. This is consistent with a previous observation that COR has jasmonate-like activity at a lower concentration than jasmonates (Fey et al. 1994). Wound-induced expression of *tpoxNl*, in contrast, was suppressed by COR even at 0.5 μM and the inhibition was increased at higher concentrations. Decreased levels of transcripts for all three genes following 20 μM COR treatment would be a consequence of its toxic effect. Etechphon treatment clearly enhanced the wound-induced expression of basic PR-1 and PI-II genes whereas expression of *tpoxNl* was not affected. Spermine (Spm) was recently identified as an endogenous inducer for acidic PR-1, 2, 3 and 5 proteins conferring resistance to TMV infection (Yamakawa et al. 1998). It also induced *tpoxNl* as well as basic PR-1 and PI-II genes in a dose dependent manner at 10 to 100 μM. The decrease in transcript levels at 500 μM may be a result of the toxic effect of Spm.

Discussion

Here, we showed the wound-induced rapid and systemic induction of a tobacco Pox gene (*tpoxNl*). This gene exhibits quite different responses from those of known wound-inducible genes of tobacco to defense-related signal compounds such as jasmonates, ethephon and SA. Our results suggest that *tpoxNl* is a novel type of wound-inducible Pox gene and its expression is regulated through unknown signaling pathway(s) different from the wound-inducible basic PR-1 and PI-II genes in tobacco plants.

Induction of Pox gene expression or activity by wounding has been reported in many plant species including tobacco (Lagrimini and Rothstein 1987), tomato (Roberts et al. 1988), potato (Roberts et al. 1988), cucumber (Svalheim and Robertsen 1990), azuki bean (Ishige et al. 1993), rice (Chittoor et al. 1997, Ito et al. 1994), horseradish (Kawaoka et al. 1994a), *Stylosanthes humilis* (Harrison et al. 1995) and sweet potato (Kanazawa et al. 1965, Huh et al. 1997). In these studies, the time course of wound-induced expression was monitored at intervals of hours or days, and the expression at early time points was not closely examined. Induction of *tpoxNl* within 30 min after cutting would be the most rapid response among known Pox genes suggesting that the gene product is required for the early process of wound response.

In cucumber and tobacco plants, POXs were reported to be systemically induced by pathogen infection (Lagrimini and Rothstein 1987, Rasmussen et al. 1995), however it was not clear whether they are systemically induced by wounding. In the present study, we demonstrated systemic induction of a Pox gene by wounding for the first time. Similar to the case in leaves infected locally, expression of *tpoxNl* was strongly induced systemically in the uninoculated leaves when necrotic lesions were formed in the TMV-inoculated lower leaves of the same plants, suggesting that the signal for wound-induced expression of *tpoxNl* is enhanced both locally and systemically by TMV.
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infection.

Although all of *tpoxNI*, basic PR-1 and PI-II genes were induced by wounding, we found some differences in the expression profile between *tpoxNI* and the other two genes. At first, accumulation of *tpoxNI* transcript by wounding is hastened compared to that of basic PR-1 and PI-II genes: the time points of maximum accumulation for *tpoxNI*, basic PR-1 and PI-II genes were 4 h, 1 to 4 d and 3 to 4 d, respectively (Fig. 3, 4). Next, *tpoxNI* is systemically induced by simple wounding while basic PR-1 and PI-II transcripts were not detected in the same conditions (Fig. 5). Thirdly, the response of *tpoxNI* to several defense-related signal compounds was different from that of basic PR-1 and PI-II genes: wound-induced expression of basic PR-1 and PI-II genes was enhanced by MeJA, COR and ethephon and suppressed by SA while that of *tpoxNI* was not affected by ethephon and SA, and rather suppressed by MeJA and COR (Fig. 6). In tobacco plants, jasmonates and ethylene are considered endogenous positive mediators for wound-responsive genes such as basic PR-1, 2 and 5, and PI-II (Seo et al. 1995, Niki et al. 1998, Ohitsu et al. 1999, Seo et al. 1999), and so the negative response of *tpoxNI* to these signal compounds is characteristic. Although *tpoxNI* as well as basic PR-1 and PI-II was activated by Spm, it is not clear whether Spm is an endogenous inducer for these genes in wounded tobacco leaves because Spm accumulates in the intercellular spaces of TMV-infected and necrotic lesion-forming leaves, but not in wounded tobacco leaves (Yamakawa et al. 1998). Finally, *tpoxNI* was activated by TMV infection like basic PR-1 and PI-II genes, however its transcript level decreased more transiently than that of basic PR-1 and PI-II genes. Altogether, *tpoxNI* expression would be regulated differently to basic PR-1 and PI-II expression and the wound-induced expression of *tpoxNI* is possibly mediated by some endogenous signal compound(s) other than jasmonates and ethylene in tobacco plants. A similar phenomenon was reported in Arabidopsis: wound-induced local and systemic expression of Aco, Ck and Wr3 genes did not depend on jasmonates (Titsarenko et al. 1997).

Like *tpoxNI*, several wound-inducible POX genes are also induced by pathogen infection or elicitor treatment (Mohan and Kolattukudy 1990, Harrison et al. 1995, Chittoor et al. 1997, Kristensen et al. 1999). In contrast, a rice POX gene (*POX5.1*) was indicated to be induced preferentially by wounding rather than pathogen infection (Chittoor et al. 1997) whereas another rice POX gene (*POX22.3*) was reported to be induced during incompatible interaction with *Xanthomonas oryzae pv. oryzae* but not by wounding (Chittoor et al. 1997). Thus, each POX gene responds differently to wounding and pathogen infection. It is interesting to study how the expression of these distinct types of POX genes are regulated in response to wounding or pathogen infection and what kinds of signal compounds are involved in the regulation.

Rapid wound response is an indispensable system for the growth and reproduction of plants. Rapid induction of *tpoxNI* by wounding suggests a possible unique role in the quick establishment of a self-defense response to wounding. The response of *tpoxNI* to known defense-related signal compounds was quite different from that of basic PR-1 and PI-II genes implying the presence of unknown regulatory mechanism(s) for *tpoxNI* gene expression. Regulated wound-inducible expression of the *tpoxNI* gene should be further studied for a better understanding of the self-defense system against wounding in plants.

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