Plant Physiol. (1987) 84, 438–442
0032-0889/87/84/0438/05/$02.00/0

Tissue Specificity of Tobacco Peroxidase Isozymes and Their Induction by Wounding and Tobacco Mosaic Virus Infection

Received for publication June 6, 1986 and in revised form February 6, 1987

L. MARK LAGRIMINI* AND STEVEN ROTHSTEIN
CIBA-GEIGY Biotechnology Unit, P. O. Box 12257, Research Triangle Park, North Carolina 27709

ABSTRACT

Peroxidases (EC 1.11.1.7) have been implicated in the responses of plants to physical stress and to pathogens, as well as in a variety of cellular processes including cell wall biosynthesis. Tissue samples from leaf, root, pith, and callus of *Nicotiana tabacum* were assayed for specific peroxidase isozymes by analytical isoelectric focusing. Each tissue type was found to exhibit a unique isozyme fingerprint. Root tissue expressed all of the detectable peroxidase isozymes in the tobacco plant, whereas each of the other tissues examined expressed a different subset of these isozymes. In an effort to determine which peroxidase isozymes from *Nicotiana tabacum* are involved in cell wall biosynthesis or other normal cellular functions and which respond to stress, plants were subjected to either wounding or infection with tobacco mosaic virus. Wounding the plant triggered the expression of several cationic isozymes in the leaf and both cationic and anionic isozymes in pith tissue. Maximum enzyme activity was detected at 72 hours after wounding, and cycloheximide treatment prevented this induction. Infection of tobacco with tobacco mosaic virus induced two moderately anionic isozymes in the leaves in which virus was applied and also systemically induced in leaves which were not inoculated with virus.

Induction of new cell wall biosynthesis (4, 14) has been proposed to be an important defense mechanism in response to pathogen stress. Peroxidases (EC 1.11.1.7) are induced by wounding and are presumably involved in the repair of damaged cell walls (2). Peroxidases have been shown to catalyze the polymerization of phenolic compounds into lignin and form cross-links between extensin, lignin, and feruloylated polysaccharides (for a review, see Griesbach [5]). The levels of peroxidase expression and its isozyme patterns have been shown in several plant systems to be altered by stress, chemicals, and infection (4). It is currently unclear which isozymes are responsible for the various functions of peroxidase. Initial studies on peroxidase isozymes from tobacco showed that the anionic isozymes have a high affinity for phenolic substrates and that the cationic isozymes are capable of H_2O_2 formation (10, 19). It has previously been difficult to characterize the different peroxidase isozymes in part because of the absence of a reproducible, quantitative method of resolving them. In an effort to define a role for the many isozymes detected on gels, we describe the separation of tobacco peroxidase isozymes by isoelectric focusing, a method which is highly reproducible and easily quantified. We have examined the tissue-specific expression of the isozymes and the effects of wounding and infection. This work sets a framework for future analysis of the different peroxidase isozymes and the genes which code for them.

MATERIALS AND METHODS

Plant Materials and Tissue Culture. *Nicotiana tabacum* L. cv Xanthi plants were grown from seed in the greenhouse with 14-h daily light periods. Tobacco callus cultures were derived from sections of pith from *N. tabacum* L. cv Xanthi. Callus was grown on agar plates and subcultured every 14 d. The medium contained Murashige-Skoog salts, B5 vitamins, 2% sucrose, 0.4 mg/L 2,4-D, 0.05 mg/L kinetin, and 0.8% agar, and callus was grown in fluorescent light at 28°C (6).

Wounding and TMV[1] Infection. Tobacco leaves were wounded by crushing with a hemostat at a 45° angle to the midvein. Pith was wounded by cutting out 5-mm wedges from the stem at 5-cm intervals or by aseptically removing the pith with a cork borer and slicing into 3-mm cross-sections. These sections were incubated for various times in a moist dark chamber either in water alone or water containing 5 μg/ml cycloheximide. In all cases tissue samples were combined from three or more mature healthy plants. All experiments were repeated at least three times with similar results.

Eight-week-old Xanthi plants were infected with TMV by gently rubbing the leaves with carborundum and then applying an aqueous virus suspension with a cotton applicator. Approximately 50 necrotic lesions were detected 2 d after infection. Excessive wounding of the leaves was avoided.

Extraction Procedure, Protein Assay, and Enzyme Assay. Tissue samples were homogenized on ice with a Polytron homogenizer (Brinkman Industries) at setting 8 for 20 s in 10 mM sodium phosphate buffer (pH 6.0). The ratios of buffer volume to tissue mass were as follows: 3 to 1, for leaf tissue; 3 to 1 for root tissue; 2 to 1 for pith tissue; and 1 to 1 for callus tissue. The homogenate was centrifuged for 20 min at 15,000*g*, and the cleared supernate was saved for analysis. Protein content was determined by the Bradford reagent method (BioRad). Peroxidase activity was assayed in 0.28% guaiacol, 0.05 M sodium phosphate buffer (pH 6.0), and 0.3% H_2O_2. The increase in absorbance was monitored at 470 nm in a Gilford spectrophotometer. Samples prepared for isoelectric focusing included 1% PVP to decrease binding by phenolic compounds which may affect the number of isozymes (21).

Flat Bed Isoelectric Focusing and *in Situ* Peroxidase Staining. Tissue extracts were subjected to analytical flat bed isoelectric focusing on polyacrylamide gels containing ampholines in the pH range 3.5 to 9.5 (LKB, Bromma, Sweden). The samples were subjected to electrophoresis for 1.5 h at 0.125 W/cm² at 10°C. After focusing, the gels were soaked for 30 min in 500 ml of PBS (10 mM sodium phosphate buffer [pH 6.0], 150 mM NaCl) to remove the ampholines and equalize the pH throughout the gel. The isozymes at the extremes of the pH gradient would otherwise appear to have low enzymatic activity. Several different chromogenic substrates were tested and gave similar results. Of the

[1] Abbreviation: TMV, tobacco mosaic virus.

substrates guaiacol, dianisidine, and 4-chloro-1-naphthol, the latter gave the best results for long-term gel storage and scanning densitometry. The peroxidase isozymes were visualized *in situ* by soaking the gel for 10 min in 100 ml of PBS containing 0.6 mg/ml 4-chloro-1-naphthol, and 0.16% H_2O_2 (J Conroy, personal communication).

Scanning Densitometry. Quantitation of peroxidase isozymes *in situ* was performed with a Zeineh SLR-504 scanning densitometer (Biomed Instruments, Fullerton, CA).

RESULTS

Tissue-Specific Expression of Tobacco Peroxidase Isozymes. Soluble extracts were prepared from the leaves, pith, and roots of 8-week-old tobacco plants and also from callus tissue derived from excised tobacco pith. Extract prepared from 15 mg fresh weight tissue was applied to flat-bed polyacrylamide isoelectric focusing gels (pH range 3.5–9.5). As shown in Figure 1, each of the four plant parts tested showed a unique isozyme pattern. Only the root was found to express the full complement of 12 isozymes found in the tobacco plant. Leaf, pith, and callus each expressed a different subset of the 12 isozymes found in roots. These results are summarized in Table I. Each of the peroxidase isozymes is named by its isoelectric point.

Wounding and Its Effect on Total Peroxidase Activity. The effect of stress on peroxidase activity was tested by wounding either leaf or pith tissue and then assaying for total soluble peroxidase activity. Tobacco leaves were wounded by crushing. Soluble extracts were prepared and assayed at several time points after wounding. As shown in Fig. 2*A*, peroxidase activity increased linearly beginning 8 h after wounding and reached maximal induction after 72 h. There was a 5- to 6-fold increase in total peroxidase activity (Table II), while the concentration of soluble protein remained constant relative to fresh weight of tissue (data not shown). Unwounded leaves from a plant which was wounded did not show increased peroxidase levels (data not shown). Although we chose to base our measurements on tissue weight, we found no significant change in soluble protein concentrations during the time course of these experiments.

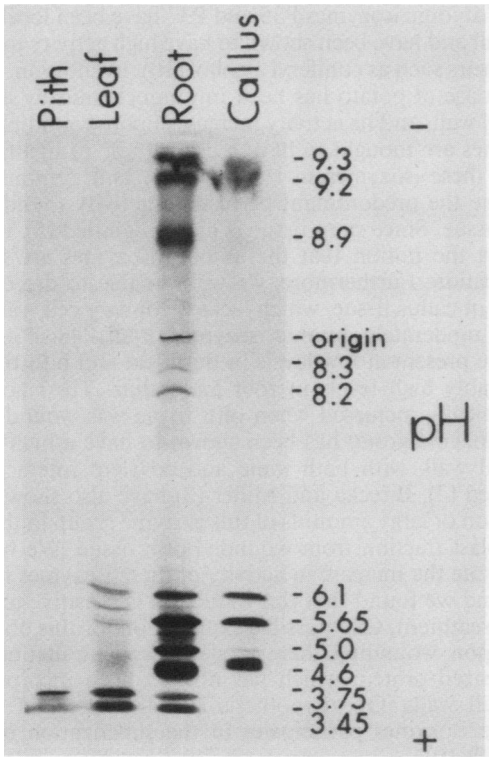

FIG. 1. Tissue specificity of tobacco peroxidase isozymes. The supernatant from 15 mg fresh weight of four different tobacco tissues was electrophoresed on isoelectric focusing gels and stained for peroxidase activity with 4-chloro-1-naphthol. The isoelectric points are marked on the right side of the figure.

Table I. *Patterns of Peroxidase Expression in Tobacco*

The isozymes of peroxidase found in tobacco are summarized as to the tissue in which they are expressed, and whether or not their expression is induced by wounding or TMV infection.

Isozyme	pI	Tissue Location of Peroxidase Isozymes						
		Tissue expressed[a]				Wound inducible		TMV inducible
P93	9.3	R			C	L	P	
P92	9.2	R			C	L	P	
P89	8.9	R			C	L	P	
P83	8.3	R						
P82	8.2	R						
P61	6.1	R	L		C		P	L
P56	5.65	R	L	P	C		P	L
P50	5.0	R	L		C		P	
P48	4.85	R		P	C		P	
P46	4.6	R			C		P	
P37	3.75	R	L	P				
P35	3.5	R	L	P				

[a] R, root; P, pith; L, leaf; C, callus.

The effect of wounding was also tested on tobacco pith. The total peroxidase activity exhibited a linear increase of 15-fold during the period between 8 to 48 h postwounding (Fig. 2B; Table II). Later time points were not analyzed because of tissue decay. Similar results were also obtained by notching the stem of a live plant and later extracting the pith.

In a parallel experiment, tobacco pith sections were incubated in 5 μg/ml cycloheximide, a protein synthesis inhibitor. In this case, no induction of peroxidase activity was detected. Therefore, the increase in peroxidase activity appears to require *de novo* synthesis of protein rather than enzyme activation.

Effects of Wounding on the Expression of Tobacco Peroxidase Isozymes. To determine which peroxidase isozymes were affected by wounding, samples from wounded leaf tissue were analyzed by isoelectric focusing. The leaves from 8-week-old tobacco plants were wounded by crushing with a hemostat, and tissue samples were taken at 0, 8, 24, 48, and 72 h postwounding. Equivalent weight samples were applied to isoelectric focusing gels and were stained for peroxidase activity. As shown in Figure 3, the 5- to 6-fold increase in total peroxidase activity is a result of the increased activity of cationic isozymes, primarily the P89 isozyme (Table II) which is not detectable in normal leaf tissue. The activity of anionic peroxidase isozymes remained constant after wounding.

In a similar manner, tobacco pith tissue was wounded and samples were applied to isoelectric focusing gels. Extracts from freshly excised pith were taken after incubation for 0, 8, 24, and 48 h in a moist dark chamber. In a parallel experiment, pith sections were incubated with 5 μg/ml cycloheximide for 24 or 48 h. As shown in Figure 4, the 15-fold increase in peroxidase activity 48 h after wounding can be attributed to both the cationic and moderately anionic peroxidase isozymes (Table II). Wounded pith tissue expressed most of the isozymes found in root tissue. The presence of cycloheximide effectively inhibited the induction of all new isozymes, suggesting that they accumulate as the result of new protein synthesis. The induction of the cationic isozymes in wounded pith parallels the induction seen in leaf tissue.

Induction of Peroxidase Isozymes by Infection with TMV. An

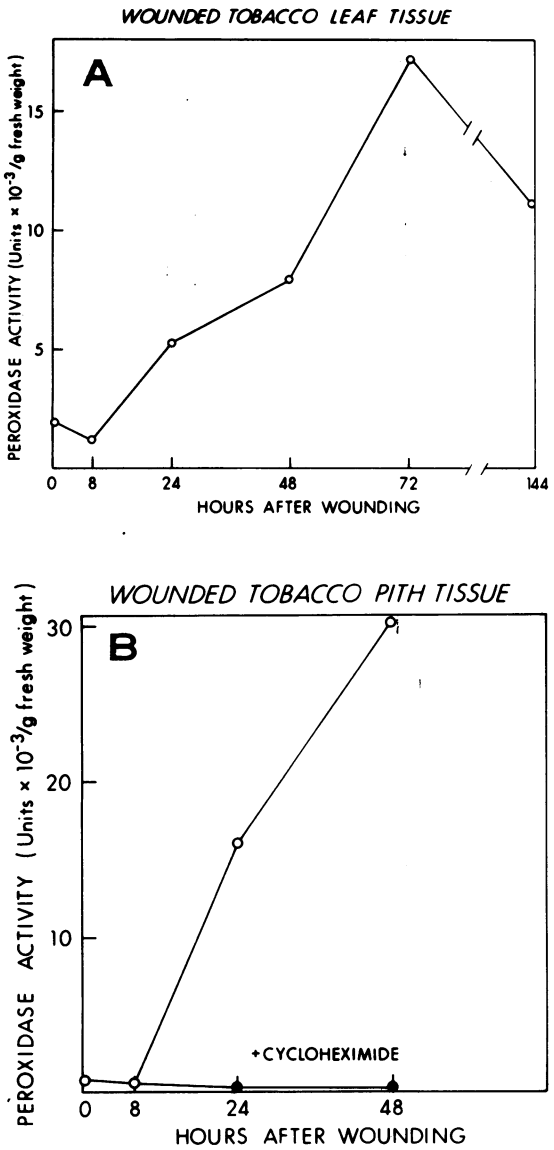

FIG. 2. Effect of wounding on total peroxidase activity. Total peroxidase activity of extracts from wounded tobacco leaf or pith tissue were assayed with guaiacol and H_2O_2.

increase in total peroxidase activity has been reported to occur in leaves infected with TMV (15, 21, 22). We wished to determine the effect of TMV infection on the induction of peroxidase isozymes, both in the leaves in which virus was applied and in those leaves which were not infected. TMV was applied to the lower leaves of 10-week-old *Nicotiana tabacum* cv Xanthi plants, and approximately 50 lesions per leaf were detected 2 d after infection. In a parallel experiment, similar plants were mock inoculated by rubbing the leaves with carborundum alone. Tissue samples were taken from both inoculated and uninoculated leaves 10 d after infection. Extracts were prepared and applied to isoelectric focusing gels, and the gels were stained to identify the peroxidase isozymes.

Mock inoculated plants showed a slight increase in the activities of the cationic peroxidase isozymes (Fig. 5). These isozymes were also induced by crushing the leaves, and their increase can be attributed to wounding. However, in TMV infected leaves, two moderately anionic peroxidase isozymes, P56 and P61, were specifically induced (Fig. 5). These isozymes were not induced by wounding alone (Fig. 3; Table II). Analysis of tissue from an uninoculated leaf of a plant which had been infected with TMV revealed that the P61 and to a lesser extent the p56 isozyme were induced. The activity of P56 in the upper uninfected leaves was often so low it could not be detected (Fig. 5).

This 'systemic' induction of peroxidase is presumably caused by the translocation of some type of signal from the site of infection and correlates with the systemic resistance phenomenon (15, 18, 19).

DISCUSSION

The use of isozymes as genetic and biochemical tools in the study of plant physiology is well established. Analyses of peroxidase isozymes have been used to study tissue specificity (1), developmental regulation (17), and the effects of tissue culture (1). In addition, peroxidases have been implicated in wound healing (2, 13) and disease resistance (12). Before one can assign particular functions to different peroxidase isozymes, isolate the purified proteins, and ultimately obtain the genes for the various isozymes, a reproducible, quantitative method for resolving the various isozymes must be developed. Previously, the tobacco peroxidase isozymes were analyzed by several different methods, including starch gels (1), native polyacrylamide gels, and isoelectric focusing (12). Flat bed isoelectric focusing provides a rapid, reproducible, and quantitative means of characterizing the peroxidase isozymes and provides the isoelectric point of the protein, which is invaluable in the purification of the isozymes (12).

Tobacco root, leaf, pith, and callus tissue display distinctive sets of peroxidase isozymes. We found that root tissue possesses all 12 isozymes found in the tobacco plant. This is similar to the number of tobacco peroxidase isozymes determined by native polyacrylamide gel electrophoresis (8). Each of the other tissues displays a different subset of these isozymes in the healthy plant; pith has 4, leaf has 5, and callus has 8 isozymes.

The anionic isozymes P35 and P37 have been localized on the cell wall and have been shown to have high activity toward lignin precursors such as coniferyl alcohol (10). In addition, the anionic peroxidase of potato has been immunochemically localized on the cell wall, and its activity increases upon wounding (3). These isozymes are thought to have a critical role in lignification. We found these isozymes in leaf and root and demonstrated that they are the predominant peroxidase activity found in healthy stem tissue. Since stem tissue is highly lignified (5), these results support the notion that the anionic isozymes are involved in lignification. Furthermore, we were unable to detect these isozymes in callus tissue, which lacks secondary cell walls.

The moderately anionic isozymes, P46, P48, P50, P56, and P61 are present at low levels in fresh leaf and pith tissue and in reasonably high levels in root and callus. Their activity most dramatically increased when pith tissue was wounded (Fig. 4). This isozyme group has been shown to have a high affinity for the cell wall, with both ionic and covalent interactions being observed (2). Birecka and Miller (2) have also shown the accumulation of large amounts of this isozyme group in the unbound protoplast fraction from wounded pith tissue. We were able to quantitate the increase in activity of these isozymes in wounded pith, and we found that this induction is sensitive to cycloheximide treatment. One possible explanation for this observation is that upon wounding there is a rapid accumulation of newly synthesized protein which has not yet been incorporated into new cell walls. One hypothesis is that this set of moderately anionic isozymes participates in the suberization of wounded tissue (3, 10).

Some investigators have found increases in peroxidase activity as well as qualitative changes in the isozyme pattern after infection of a hypersensitive plant with TMV (15, 21, 22). They have proposed that the changes in peroxidase activity aid the plant in deterring any additional pathogen challenge. However, a clear

Table II. *Distribution of Peroxidase Activity between the Isozymes of Normal, Wounded, and Infected Tobacco Tissues*

These values were obtained from densitometric scanning of isoelectric focusing gels which were stained for peroxidase activity. The distant leaf in the TMV infected plant refers to an uninoculated leaf from an infected plant.

Esozyme	Induction by Wounding and TMV Infection						
	Wounded leaf		Wounded pith		Infected plant		
	Normal	Wounded	Normal	Wounded	Uninfected	Infected	Distant
P93	0	1.46	0	5.25	0	0.032	0
P92	0	0.67	0	1.09	0	0.042	0
P89	0	8.06	0	8.94	0	0.014	0
P83	0	0	0	0	0	0	0
P82	0	0	0	0	0	0	0
P61	0.31	0.7	0	5.27	<0.1	0.45	0.45
P56	0	0	<0.1	3.55	0	0.49	0
P50	0.34	0.78	0	1.01	<0.1	<0.1	<0.1
P48	0	0	<0.1	0.95	0	0	0
P46	0	0	0	<0.1	0	0	0
P37	0.24	0.25	1.13	2.29	0.91	0.91	0.91
P35	2.1	5.06	0.87	1.63	2.09	2.08	2.08
Total units	3	17	2	30	3	4.03	3.44

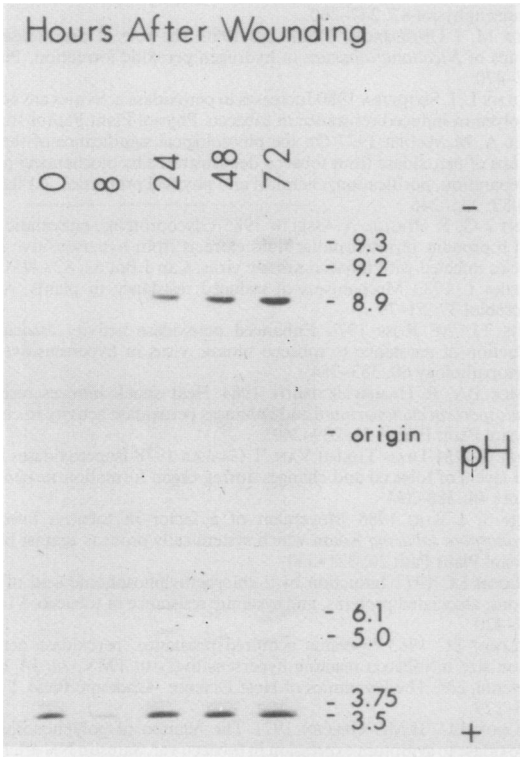

FIG. 3. Effect of wounding on tobacco leaf isozymes. The leaves of 8-week-old tobacco plants were wounded by crushing with a hemostat. Tissue samples were taken at 0, 8, 24, 48, and 72 h postwounding. Extract from 15 mg of fresh weight tissue was electrophoresed on acrylamide isoelectric focusing gels pH 3.5 to 9.5 and stained *in situ* for peroxidase activity.

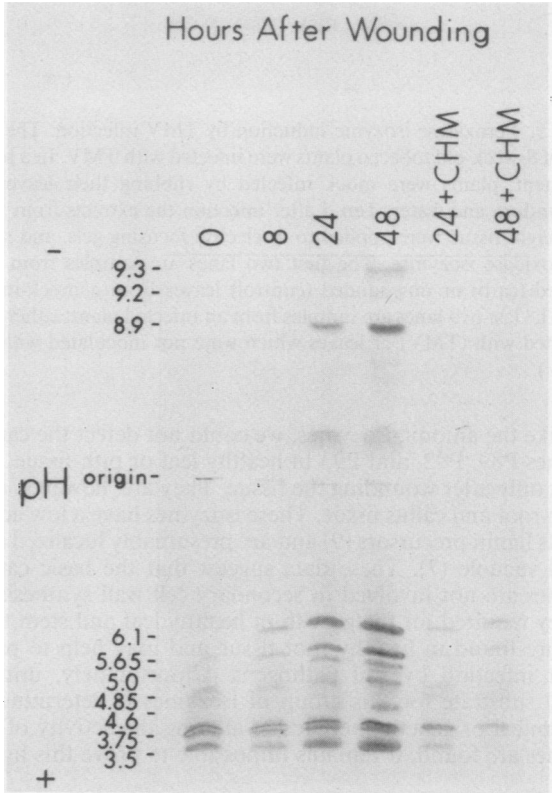

FIG. 4. Effect of wounding on tobacco pith isozymes. Pith was extracted from 8-week-old tobacco plants, diced into 3 mm sections, and incubated in a moist dark chamber. Parallel samples were incubated in 5 μg/ml cycloheximide. Tissue extracts equivalent to 15 mg fresh weight tissue were isolated at 0, 8, 24, and 48 h postwounding, applied to isoelectric focusing gels, and stained for peroxidase activity.

cause and effect relationship has not been established (11, 22). We have found that isozymes within the moderately anionic group have increased activity in leaves from plants infected with TMV (Fig. 5). The isozymes P56 and P61 specifically increase in response to TMV infection in both the inoculated and uninfected leaves. This type of response is not found by wounding leaves. Peroxidases are certainly important in the reinforcement of the cell wall, and it is not unreasonable to assume that they have a role in the containment of a pathogen. These two enzymes have provided us with a reliable marker for TMV infection. However, further characterization is necessary before their actual function, if any, in disease resistance is resolved.

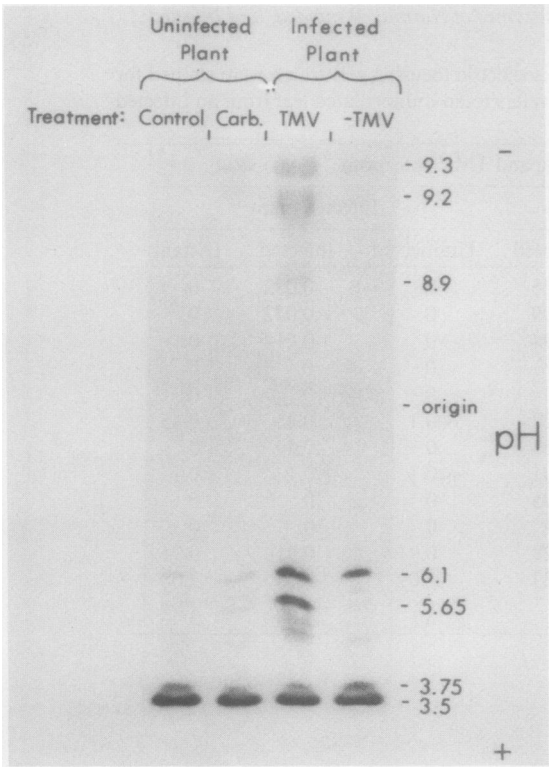

FIG. 5. Peroxidase isozyme induction by TMV infection. The lower leaves of 8-week-old tobacco plants were infected with TMV. In a parallel experiment, plants were mock infected by rubbing their leaves with carborundum and water. Ten d after infection the extracts from 15 mg fresh weight tissue were applied to isoelectric focusing gels, and stained for peroxidase isozymes. The first two lanes are samples from either wounded (carb) or unwounded (control) leaves from a mock-infected plant. The last two lanes are samples from an infected plant: either leaves inoculated with (TMV) or leaves which were not inoculated with virus (−TMV).

Unlike the anionic isozymes, we could not detect the cationic isozymes P89, P92, and P93 in healthy leaf or pith tissue. They appear only after wounding the tissue. They are, however, abundant in root and callus tissue. These isozymes have a low activity towards lignin precursors (9) and are presumably localized in the central vacuole (7). These data suggest that the basic cationic isozymes are not involved in secondary cell wall synthesis, nor are they required for the growth of healthy leaf and stem tissue. They are found in healthy root tissue and may help to protect against infection by soil pathogens. Unfortunately, until the natural substrate for this group of isozymes is determined, or biochemical or genetic methods of altering the activity of these isozymes are found, it remains impossible to prove this hypothesis.

The experiments presented in this paper describe the expression of individual peroxidase isozymes of tobacco. These data should facilitate the determination of the molecular basis of tissue specific expression, the response to wounding and infection, and the eventual identification of a function for each of the different isozymes.

LITERATURE CITED

1. BASSIRI A, PS CARLSON 1979 Isozyme patterns in tobacco plant parts and their derived calli. Crop Sci 19: 909–914
2. BIRECKA H, A MILLER 1974 Cell wall and protoplast isoperoxidases in relation to injury indoleacetic acid and ethylene effects. Plant Physiol 53: 569–574
3. ESPELIE KE, VR FRANCESCHI, PE KOLATTUKUDY 1986 Immunochemical localization and time course of apperance of and anionic peroxidase associated with suberization in wound-healing potato tuber tissue. Plant Physiol 81: 487–492
4. GASPER T, C PENEL, T THORPE, H GREPPIN 1982 Peroxidases. A survey of their biochemical and physiological roles in higher plants. University of Geneva Press, Geneva, Switzerland
5. GRISEBACH H 1981 Lignins. *In* EE Conn, ed, The Biochemistry of Plants, Vol 7. Academic Press New York, pp 451–478
6. HARMS CT, JJ OERTLI, JM WIDHOLM 1982 Characterization of amino acid analogue resistant somatic hybrid cell lines of *Daucus carota* L. Z Pflanzenphysiol 106: 239–249
7. MÄDER M 1986 Cell compartmentation and specific roles of isoenzymes. *In:* H Greppin, C Penel, Th Gaspar, eds, Molecular and Physiological Aspects of Plant Peroxidases. University of Geneva, Switzerland, pp 247–260
8. MÄDER M, Y MEYER, M BOPP 1975 Localization of peroxidase isozymes in protoplasts and cell walls of *Nicotiana tabacum* L. Planta 122: 259–268
9. MÄDER M, A NESSEL, M BOPP 1977 On the physiological significance of the isozyme groups of peroxidase from tobacco demonstrated by biochemical properties. II, pH optima, Michaelis-constants, maximal oxidation rates. Z Pflanzenphysiol 82: 247–260
10. MÄDER M, J UNGEMACH, P SCHLOSS 1980 The role of peroxidase isozyme groups of *Nicotiana tabacum* in hydrogen peroxide formation. Planta 147: 467–470
11. NADOLNY L, L SEQUEIRA 1980 Increases in peroxidase activities are not directly involved in induced resistance in tobacco. Physiol Plant Pathol 16: 1–8
12. NESSEL A, M MÄDER 1977 On the physiological significance of the isozyme groups of peroxidase from tobacco demonstrated by biochemical properties. I. Separation, purification, chemical and physical properties. Z Pflanzenphysiol 82: 235–246
13. PARENT J-G, R HOGUE, A ASSELIN 1985 Glycoproteins, enzymatic activities and b proteins in intercellular fluid extracts from hypersensitive *Nicotiana* species infected with tobacco mosaic virus. Can J Bot 63: 928–931
14. SEQUEIRA L 1983 Mechanisms of induced resistance in plants. Annu Rev Microbiol 37: 51–79
15. SIMONS TJ, AF ROSS 1970 Enhanced peroxidase activity associated with induction of resistance to tobacco mosaic virus in hypersensitive tobacco. Phytopathology 60: 383–384
16. STERMER BA, R HAMMERSCHMIDT 1984 Heat shock induces resistance to *Cladosporium cucumerinum* and enhances peroxidase activity in cucumbers. Physiol Plant Pathol 25: 239–249
17. THORPE TA, M TRAN THANH VAN, T GASPAR 1978 Isoperoxidases in epidermal layers of tobacco and changes during organ formation *in vitro*. Physiol Planta 44: 388–394
18. TUZUN S, J KUC 1985 Movement of a factor in tobacco infected with *Peronospora tabacina* Adam which systemically protects against blue mold. Physiol Plant Path 26: 321–330
19. VAN LOON LC 1977 Induction by 2-chloroethylphosphonic acid of viral-like lesions, associated proteins, and systemic resistance in tobacco. Virology 80: 417–420
20. VAN LOON LC 1983 Systemic acquired resistance, peroxidase activity and lesion size in tobacco reacting hypersensitively to TMV. *In:* JA Bailey, BJ Deverall, eds, The Dynamics of Host Defense. Academic Press, Sydney, pp 123–129
21. VAN LOON LC, JLMC GEELEN 1971 The relation of polyphenoloxidse and peroxidase to symptom expression in tobacco var. Samsun NN after infection with tobacco mosaic virus. Acad Sci Hung 6: 9–20
22. WESTSTEIJN EA 1976 Peroxidase activity in leaves of *Nicotiana tabacum* var. Xanthi nc. before and after infection with tobacco mosaic virus. Physiol Plant Pathol 8: 63–71