Activity, Isozyme Pattern, and Cellular Localization of Peroxidase as Related to Systemic Resistance of Tobacco to Blue Mold (*Peronospora tabacina*) and to Tobacco Mosaic Virus

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ABSTRACT

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Stem injection of tobacco cultivar Ky 14 with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus induced systemic resistance to both pathogens. The treatment also elicited a systemic increase in peroxidase activity which was positively correlated with induced resistance. Increases were evident in cytosol, intercellular fluid, and cell wall fractions. Upon challenge with *P. tabacina*, peroxidase activity further increased in the induced plants and remained higher after challenge as compared to the control plants. The isozyme patterns of peroxidases on isoelectric focusing gels showed an increase of two anionic peroxidases. Both peroxidases were positively correlated with induced resistance.

Peroxidases play an integral part in the biosynthesis of plant cell wall components, including lignin, suberin, and cross-linked extensin (7,8,14). Lignification and wall thickening are well-known plant defense responses to pathogens, particularly to fungi (7,8, 28,29,30). Peroxidases have, therefore, been studied extensively in many plant-pathogen interactions. However, a role for peroxidase in resistance has not been unequivocally established in any plant-pathogen interaction.

Increased activity of peroxidase was associated with induced systemic resistance in cucumber to a variety of pathogens and in tobacco to tobacco mosaic virus (TMV)(11,13,25,33). Lignification and wall apposition are mechanisms of induced resistance in cucumber (4,9,27,34). In induced tobacco, peroxidase activity was higher before challenge in uninfected leaves, and it increased more rapidly after challenge with TMV than in controls (25). Peroxidase, therefore, may have a role in induced resistance of cucumber and tobacco to TMV. Increased activity of peroxidase in tobacco later was reported to have no role in induced resistance to TMV and *Pseudomonas solanacearum* (20,31). The authors suggested that the increase in peroxidase activity was a reflection of physiological changes associated with, but not responsible for, induced resistance.

There are at least 12 isozymes of peroxidase in tobacco (13,16), and their biological functions are not yet completely understood (7). Cationic peroxidases are located in the central vacuole (15) and catalyze the synthesis of H_2O_2 from NADH and H_2O (15,17,18). The moderately anionic peroxidases are highly expressed in response to wounding (1,5,13). Highly anionic peroxidases are associated with cell walls and polymerize cinnamyl alcohols in vitro (13,14,16,17). They are suggested to be involved in lignification and cross-linking of extensin monomers and feruloylated polysaccharides (7,8,14).

Leaf inoculation with TMV of tobacco plants containing the *N*-gene for resistance to TMV (hypersensitive reaction) and stem inoculation with *Peronopora tabacina* of tobacco with or without the *N*-gene induce systemic protection against blue mold (35,36). Induction with both pathogens also elicits systemic accumulation of pathogenesis-related proteins and increased activities of β-1,3-glucanase and chitinase (21,35,36). Pathogenesis-related proteins and activities of β-1,3-glucanase and chitinase are closely associated with induced resistance to blue mold but not to TMV (21,35,36). These proteins and enzymes are primarily located in intercellular spaces where they may contact and inhibit fungal pathogens (2,22,32,35). The purpose of this study was to reexamine the role of peroxidases, particularly the anionic peroxidases, in induced systemic resistance of tobacco to blue mold.

MATERIALS AND METHODS

Plants, pathogens, and inoculation. Tobacco (*Nicotiana tabacum* L.) cultivar Ky 14 plants were produced as described previously (35). Inocula of *P. tabacina* and TMV for induction and challenge were prepared, and plants were inoculated as described previously (35). Plants were inoculated (induced) with TMV or *P. tabacina*. Induced plants were challenged with *P. tabacina* or TMV at 12 or 21 days after induction, respectively (35). Resistance to blue mold and TMV was determined 6 days after challenge by measuring lesion diameter (35).

Extraction, fractionation, and assay of peroxidases. Frozen (−20 C) leaf tissues were homogenized in 10 mM sodium phosphate buffer, pH 6.0, (3 ml/g tissue), in a prechilled mortar at 4 C. The homogenates were filtered through four layers of cheesecloth, and the filtrates were centrifuged at 12,000 *g* for 30 min. The supernatant was used for determination of soluble peroxidase activity.

Freshly collected leaf tissues were vacuum-infiltrated with water and blotted dry with paper towels as described by Rathmell and Sequeira (23). Intercellular fluids were collected by centrifugation of infiltrated leaves at 1,500 *g* for 10 min. After centrifugation, the infiltrated tissues were homogenized, filtered, and centrifuged as described above. Peroxidases residing in this fraction were considered to be present in the cytosol. The supernatants of these homogenates were used as preparations of peroxidase present in cytosol. The sediments of cell walls were washed six× by centrifugation with 10 mM sodium phosphate buffer, pH 6.0, once with water, once with 0.5% Nonidet P-40 containing 2 mM $Na_2S_2O_5$, and four× with the same phosphate buffer. Cell wall peroxidase was then extracted with 0.2 M $CaCl_2$ in 4 mM $Na_2S_2O_5$ for 12 hr with constant stirring at 4 C. After centrifugation at 12,000 *g* for 30 min, the supernatant was collected and used for the determination of ionically bound cell wall peroxidase.

Peroxidase activity was assayed with guaiacol as the hydrogen donor as described by Hammerschmidt et al (11). The reaction mixture consisted of 0.25%, v/v, guaiacol and 0.1 M H_2O_2 in 10 mM sodium phosphate buffer, pH 6.0. A series of dilutions of the crude enzyme preparations (0.1 ml) were added to 3 ml of the reaction mixture. Changes in absorbance at 470 nm were recorded, and the activity of peroxidase was expressed as change in absorbance at 470 nm per g fresh weight or per mg protein

per min. Protein was assayed by the method of Bradford (3) with γ-globulin as standard.

Isoelectric focusing gel electrophoresis and in vitro detection of peroxidase. The method of Lagrimini and Rothstein (13) for flat bed isoelectric focusing gel electrophoresis of peroxidases and in vitro detection was used. Ampholines (Pharmacia LKB, Sweden) with a pH range of 3–10 were used as electrolyte carriers. The gels (1.5 mm in thickness) were prefocused at 15 W for 1 hr at 10 C. The samples (50 μg protein) then were applied to the catanodic side of the gel and electrophoresed for an additional 2 hr at 15 W. After focusing, the gels were soaked in 500 ml of 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl for 30 min with gentle shaking to remove ampholines and equalize the pH. Peroxidase isozymes were detected by dipping gels for 10 min in 250 ml of the phosphate buffer containing 0.6 mg of 4 chloro-1-naphthol per ml and 0.1 M H_2O_2 on a shaker (70 rpm). Quantitation of peroxidase isozymes was performed with a LKB ultroscan densitometer.

RESULTS

Time course of peroxidase activity in leaf homogenate and intercellular fluids. Peroxidase activity increased significantly in uninfected leaves of induced plants 6 days after inoculation of lower leaves with TMV (Fig. 1A). Peroxidase activity increased rapidly 6–12 days after inoculation, while activity in the water-treated controls remained essentially at the same level during the same time period. In plants stem-injected with *P. tabacina*, leaf peroxidase activity increased 9 days after injection and continued to rise slowly up to 17 days and very rapidly between 17 and 21 days (Fig. 1B). Upon challenge with *P. tabacina*, peroxidase activity was further increased in induced and control plants and remained higher in the induced plants (Fig. 1).

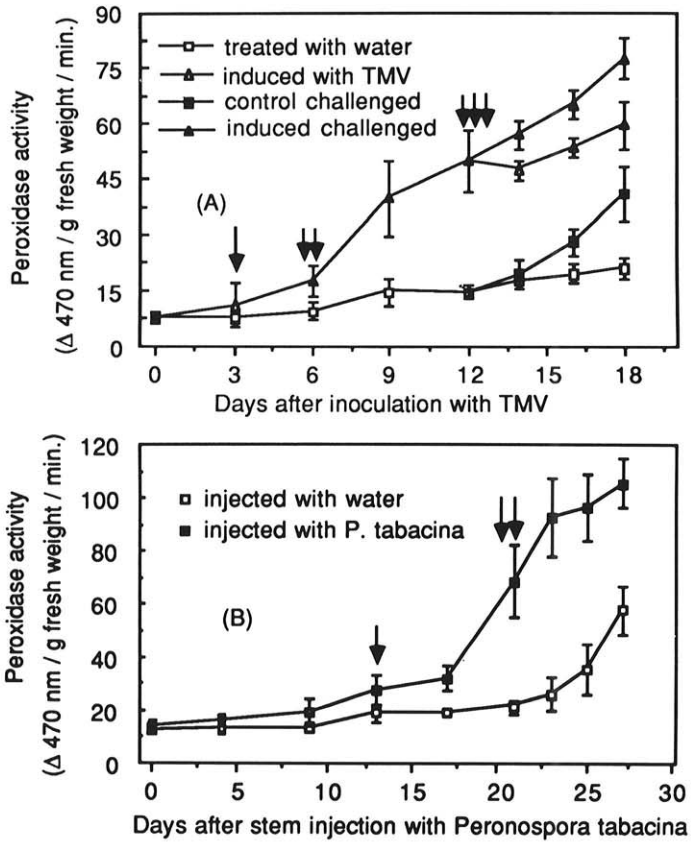

Fig. 1. A, Effect of inoculating the three lower leaves of tobacco plants with tobacco mosaic virus (TMV) on total peroxidase activity in younger leaves above before and after challenge with *Peronospora tabacina*. Data are the means (±SD) of three separate experiments with 8–10 plants per treatment per experiment. Single, double, and triple arrows indicate induced resistance just evident, clearly evident, or at maximum and the time of challenge, respectively. **B,** Effect of inoculating tobacco stems with *P. tabacina* on total peroxidase activity in the leaves before and after challenge with *P. tabacina*. Data are the means of two separate experiments with 10 plants per treatment per experiment. Single arrow indicates when induced resistance was first evident and double arrows indicate the time of challenge and when induced resistance was at maximum.

Five isozymes of peroxidase, designated by isoelectric points, were present in extracts of leaf homogenates from noninduced plants (Table 1). Isozymes P35 and P37 increased markedly in leaves from plants induced either with TMV or *P. tabacina* (Table 1). These two isozymes also increased more rapidly in the induced than in the control plants 2–4 days after challenge with *P. tabacina* and remained higher in the induced as compared to the control plants during the period of experiment (Fig. 2). P68 appeared 6 days after challenge with the blue mold pathogen. P54 and P62 were barely apparent 4 days after challenge and increased markedly 6 days after challenge with *P. tabacina* (Fig. 2). Control plants usually had more of these three isozymes than the induced plants 6 days after challenge (Fig. 2). By this time, the control plants had severe blue mold symptoms.

Peroxidase activity in the intercellular fluids obtained from systemically protected leaves of the induced plants was much higher than that from the noninduced plants 12 days after inoculation with TMV (Table 2) and 21 days after inoculation with *P. tabacina*. A time course on the TMV-induced plants showed that increased peroxidase activity was evident 3 days after inoculation with TMV (Fig. 3). Isozymes P35 and P37 increased markedly in intercellular fluids obtained from the induced as compared to those from control plants (Fig. 4).

Effect of inoculation and leaf removal on peroxidase activity. Removal of the TMV-inoculated leaves at various time intervals after inoculation with TMV and measurement of peroxidase 12 days after inoculation revealed that increased peroxidase activity was induced between 3 and 6 days after inoculation with TMV. Peroxidase activity increased progressively thereafter (Fig. 5). Peroxidase activity in the intercellular fluids and in leaf homogenates increased as the number of TMV-inoculated leaves increased, as did the level of protection against blue mold and TMV (Table 3).

Cellular localization of increased peroxidase activity. The specific activity of peroxidase in intercellular fluids and in the salt-

TABLE 1. Peroxidase isozymes in leaves of control plants and uninfected leaves of plants induced with tobacco mosaic virus (TMV) or with *Peronospora tabacina*

		P. tabacina[a]		TMV					
		S-fraction		S-fraction		ICF		Cell Wall	
Isozymes	pI	C	I	C	I	C	I	C	I
P93	9.3							+[b]	+
P88	8.8							+	++
P83	8.3							+	+
P81	8.1							+	+
P68	6.8							+	++
P62	6.2	+	+	+	+	+	+	+	+
P57	5.7	+	+	+	+	+	+	+	+
P54	5.4					+	+	+	+
P49	4.9	+	+	+	+	+	+	+	+
P46	4.6					+	+	+	+
P37	3.7	+	+++	+	+++	+	+++	+	+++
P35	3.5	+	+++	+	+++	+	+++	+	+++

[a] Leaves from stem-injected plants were sampled 21 days after stem injection with *P. tabacina*. Uninfected leaves of control or induced plants were harvested 12 days after induction with TMV. Enzyme preparations were obtained from leaf homogenates, intercellular fluids, or cell walls as described in Materials and Methods. S-fraction = supernatant from leaf homogenate before removal of ICF; ICF = intercellular fluids; cell wall = salt extracts of cell wall preparations; C = control plants treated with H_2O; I = plants induced with TMV or *P. tabacina*.

[b] A + indicates the presence of the peroxidase isozyme with the indicated pI value. A ++ indicates an increase in activity of one- to twofold and a +++ a greater than twofold increase of activity of the peroxidase isozyme in induced plants as compared to the controls, as determined by densitometry.

soluble fraction of cell walls was much higher than in the soluble fraction (Table 2). Increased activity of peroxidase was found in all the fractions of extracts from induced as compared to control plants. Twelve distinct isozymes were detected in salt extracts of cell wall preparations (Table 1). Isozymes P35 and P37 increased markedly, and P68 and P88 slightly, in cell wall extracts of the induced as compared to the control plants (Table 1).

DISCUSSION

Stem inoculation with *P. tabacina* or leaf inoculation with TMV elicited a systemic increase in peroxidase activity. Activities of peroxidase in homogenates of leaves and in extracts from intercellular spaces increased more rapidly in plants induced with TMV than in plants induced with *P. tabacina*. The peroxidase increase is closely associated with the development of induced resistance to *P. tabacina* and TMV in the systemically protected leaves (36), and the similarity of biochemical changes induced by TMV or *P. tabacina* suggests that they may elicit similar multicomponent mechanisms for resistance (12).

Increasing the number of leaves inoculated with TMV increases the level of protection against *P. tabacina* and TMV. The close association of increased peroxidase activity with protection, however, does not prove that peroxidase has a role in induced resistance (11,20,31,36). Peroxidase does not have an important role in induced resistance to TMV in tobacco (36). Plants induced by inoculation with TMV and held at 23 C were well protected systemically against TMV and *P. tabacina*, and peroxidase levels were elevated in induced as compared to control plants before

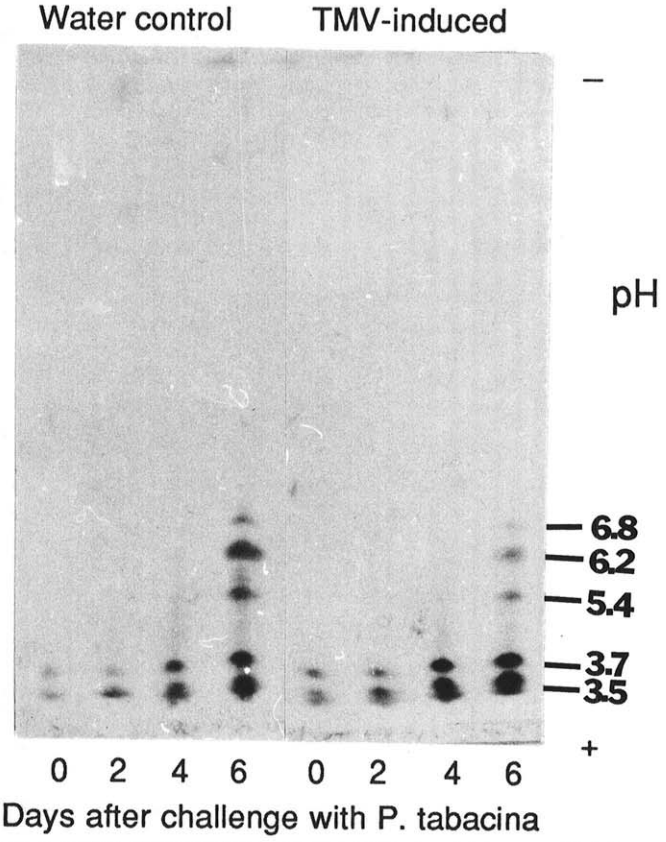

Fig. 2. Effect of inoculating three lower leaves of tobacco with tobacco mosaic virus (TMV) and subsequent challenge of uninfected protected leaves with *Peronospora tabacina* on leaf peroxidase isozymes. Plants were challenged with *P. tabacina* 12 days after inoculation with TMV. Isoelectric points are indicated on the right.

TABLE 2. Localization of enhanced peroxidase activity in tobacco plants induced with tobacco mosaic virus (TMV)

	Peroxidase activity ±SD (Δ_{470nm}/mg protein/min)			
			Ionically cell wall bound	
			Days after challenge[c]	
Treatment[a]	S-ICF[b]	Intercellular fluid	(0)	(6)
Water control	0.7 ± 0.2	20.0 ± 1.1	25.6 ± 8.8	46.4 ± 5.2
TMV-induced	5.2 ± 2.3	96.5 ± 12.4	102.4 ± 25.1	145.6 ± 18.6

[a] Tobacco plants were inoculated on three lower leaves with TMV or water. Systemic uninfected leaves were harvested 12 days after inoculation for peroxidase assays.
[b] Supernatant from leaf homogenate after removal of intercellular fluids.
[c] Plants were challenged with *Peronospora tabacina* 12 days after inoculation with TMV.

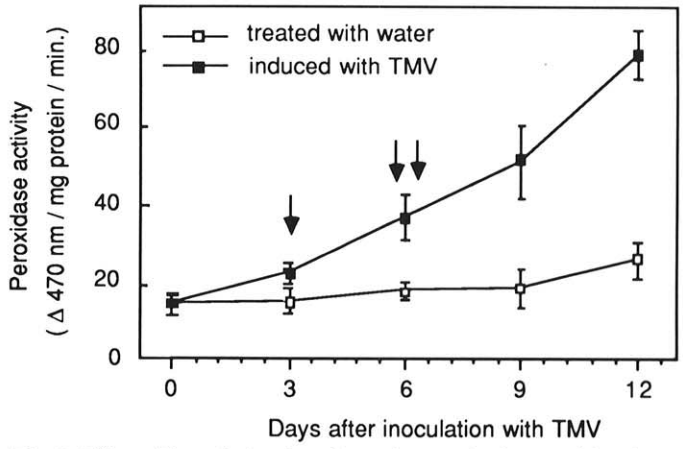

Fig. 3. Effect of inoculating three lower leaves of tobacco with tobacco mosaic virus (TMV) on peroxidase activity in intercellular fluids extracted from upper uninfected leaves at various times after inoculation with TMV. Data are the means(±SD) of two separate experiments with 10 plants per treatment per experiment. Single arrow indicates when induced resistance was first evident and double arrows when induced resistance was clearly evident.

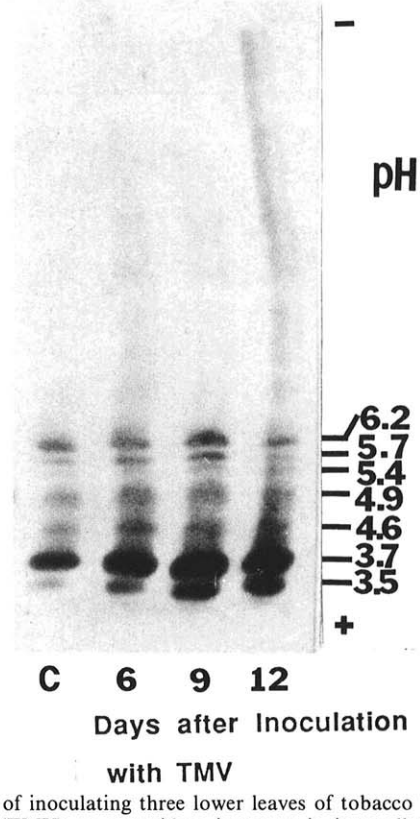

Fig. 4. Effect of inoculating three lower leaves of tobacco with tobacco mosaic virus (TMV) on peroxidase isozymes in intercellular spaces of leaves distant from those inoculated with TMV. Isoelectric points of peroxidases are marked on the right. C = H_2O control 12 days after inoculation with TMV.

and after challenge (35,36). When plants were held at 28 C after challenge, systemic protection against *P. tabacina* but not TMV was evident, though peroxidase activity was enhanced in the induced as compared to control plants before and after challenge with the pathogens (36). Van Loon (31) also reported evidence against a role for peroxidase in induced systemic resistance of tobacco to TMV.

Tobacco has at least 12 isoperoxidases (13,16). Peroxidases have not been shown to have direct antifungal properties, unlike other systemic inducible enzymes such as chitinases and β-1,3-glucanases (2,24). Peroxidase may function in disease resistance indirectly by affecting biochemical processes which in turn influence disease resistance. Increased activities of peroxidase were found in all the cellular fractions from induced plants in the present study. High specific activities of peroxidase in extracts from intercellular spaces and from cell walls suggest that peroxidase is secreted outside of tobacco leaf cells, either bound to cell walls or present in the intercellular spaces. Although biological functions of all the peroxidases are not completely understood, they are known to have a role in secondary wall biosynthesis (7,8,14). Peroxidases also participate in superoxide generation and lignification (7,8,14, 18,28,29,30). A systemic increase in peroxidase activity is associated with induced systemic resistance in cucumber (11). Increased lignification, hydroxyproline-rich glycoprotein accumulation, cell wall apposition, and chitinase activity are all positively correlated with induced resistance in cucumber (4,9,10,19,27,34).

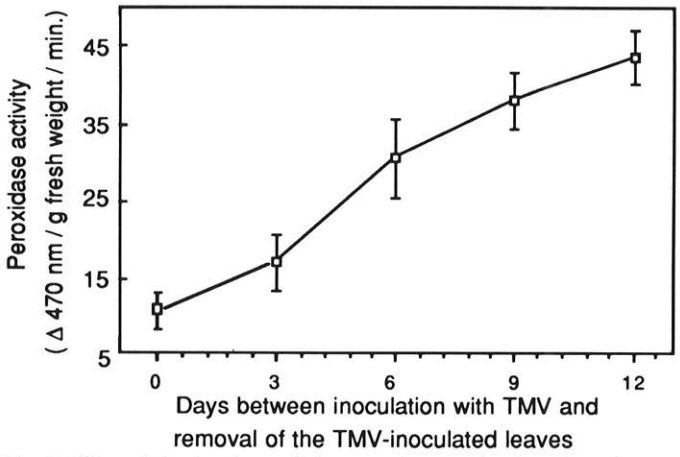

Fig. 5. Effect of the time intervals between inoculating three lower leaves of tobacco with tobacco mosaic virus (TMV) and removal of the TMV-inoculated leaves on systemic peroxidase activity in the uninfected leaves. Peroxidase activity was determined 12 days after inoculation with TMV. Data are the means (±SD) of three separate experiments with four plants per treatment per experiment.

TABLE 3. Effect of the number of leaves inoculated with tobacco mosaic virus (TMV) on peroxidase activity and induced resistance against *Peronospora tabacina* and TMV in leaves distant from those inoculated with TMV

Leaves inoculated (No.)	Peroxidase activity in[a]		Average lesion diameter[b](mm)	
	S-fraction	ICF	*P. tabacina*	TMV
0	5.5±0.2	19.1±2.5	17.9±2.4	3.61±0.54
0.5	19.6±6.6	37.0±10.6	6.4±2.8	2.26±0.72
1	24.6±2.6	52.5±22.8	3.4±2.0	1.02±0.60
2	45.4±4.1	59.5±10.2	1.7±1.4	0.55±0.21
3	54.5±8.7	86.7±8.3	0.9±0.5	0.30±0.18

[a] Expressed as changes in absorbance at 470 nm/g fresh wt per min for leaf homogenates (S-fraction) and as changes in absorbance at 470 nm/mg protein per min for intercellular fluids (ICF). Four plants were used for each treatment, and the experiment was repeated twice.

[b] Plants were challenged with *P. tabacina* or TMV 12 days after induction with TMV. Lesion diameter (±SD) was measured 6 days after challenge on leaves distant from those inoculated with TMV.

Other associated changes, such as accumulation of pathogenesis-related proteins, increased activities of chitinase and β-1,3-glucanase, must also be considered when considering peroxidase activity. It also is important to study individual isozymes, because different isozymes may have different biological functions (7,15, 17,18). The lack of in-depth studies in the past may explain the uncertainty of the role of peroxidase in plant host-pathogen interactions.

Two highly anionic peroxidases, which are associated with secondary cell walls (8,13), increased as a function of induced resistance. They are not detected in callus of tobacco (13). Lignin precursors such as coniferyl alcohol have been shown to be preferred substrates of highly anionic peroxidases of tobacco in vitro (18). They, therefore, are thought to play a critical role in lignification (7,8,14). Extensin and lignin of systemically protected leaves of the induced plants are greatly increased as compared to control plants (Ye and Kuc, *unpublished data*). Lignin contents also are increased in and around necrotic lesions in TMV-infected leaves of tobacco (26). Important enzymes in the metabolic pathways leading to lignification are greatly stimulated soon after inoculation with TMV in resistant tobacco (6). Therefore, lignification and secondary cell wall thickening are thought to inhibit cell to cell spread of virus in localized infections (26). However, TMV becomes systemic from localized infection sites when plants are transferred from 23 C to above 28 C by 12 days after inoculation (36). These cell wall changes, on the other hand, would affect blue mold development. After penetration through the epidermis, hyphae of the blue mold fungus grow in intercellular spaces. Pathogenesis-related proteins, chitinases, β-1,3-glucanases, and lignification of the middle lamellae of cell walls would be the early chemical and physical barriers encountered by the fungus. The fungus then penetrates cell walls and produces an intracellular nutrient-absorbing organ, the haustorium, to establish a successful infection. Highly anionic peroxidases may have a role in induced resistance to fungal diseases by enhancing phenolic oxidation and cell wall lignification.

Moderately anionic peroxidases were also associated with cell walls (Table 1) (1,16). They were at a very low level in intercellular spaces or leaf homogenates of healthy leaf tissue. This is in agreement with a previous report (13). Their activity was greatly increased after infection with TMV or *P. tabacina*, and when symptoms appeared on leaves. These isozymes are greatly increased upon wounding (1,13), and isozyme patterns in TMV-infected and wounded leaves are very similar (1,13). The isozymes also are suggested to have a role in suberization of healing tissues (5).

Peroxidase activity was progressively higher in induced plants as the time period between inoculation and removal of TMV-inoculated leaves increased. Plants with inducer leaves removed 6, 9, and 12 days after inoculation were progressively better protected than plants with inducer leaves removed 3 days after inoculation (35). The more leaves that were inoculated with TMV, the higher the systemic peroxidase activity and the higher the level of induced systemic resistance. Thus, there is a positive correlation between peroxidase activity and the level of induced resistance to *P. tabacina*.

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