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Wound-Induced Deposition of Polyphenols in Transgenic Plants Overexpressing Peroxidase[1]

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ABSTRACT

Tobacco (*Nicotiana tabacum*) plants transformed with a chimeric tobacco anionic peroxidase gene have previously been shown to synthesize high levels of peroxidase in all tissues throughout the plant. One of several distinguishable phenotypes of transformed plants is the rapid browning of pith tissue upon wounding. Pith tissue from plants expressing high levels of peroxidase browned within 24 hours of wounding, while tissue from control plants did not brown as late as 7 days after wounding. A correlation between peroxidase activity and wound-induced browning was observed, whereas no relationship between polyphenol oxidase activity and browning was found. The purified tobacco anionic peroxidase was subjected to kinetic analysis with substrates which resemble the precursors of lignin or polyphenolic acid. The purified enzyme was found to readily polymerize phenolic acids in the presence of H_2O_2 via a modified ping-pong mechanism. The percentage of lignin and lignin-related polymers in cell walls was nearly twofold greater in pith tissue isolated from peroxidase-overproducer plants compared to control plants. Lignin deposition in wounded pith tissue from control plants closely followed the induction of peroxidase activity. However, wound-induced lignification occurred 24 to 48 hours sooner in plants overexpressing the anionic peroxidase. This suggests that the availability of peroxidase rather than substrate may delay polyphenol deposition in wounded tissue.

Peroxidases (EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase) have been implicated in several primary and secondary metabolic functions including the regulation of cell elongation (10), cross-linking of cell wall polysaccharides (9), lignification (13), wound-healing (7), pathogen defense (15), and phenol oxidation (31). Several reviews have been written on the numerous biochemical and physiological functions of peroxidase (6, 11, 12, 18). Although peroxidases are ubiquitous to vascular plants and are crucial to growth and the response to environmental stimuli, many of the proposed functions for peroxidases are based on *in vitro* data. The many potential substrates and multitude of isoenzyme forms have made it difficult to determine the actual *in vivo* role for the various peroxidase isoenzymes using standard biochemical techniques. The tools of recombinant DNA technology have been applied to generate plants with modified peroxidase activity, resulting in unique phenotypes, which can be analyzed for potential *in vivo* peroxidase functions.

The anionic peroxidase isoenzyme of tobacco is considered crucial to the formation of lignin; however, it is not known what other functions it may impart to the plant (23, 26). A wound-induced browning reaction that occurs in pith tissue excised from tobacco plants which overproduce the tobacco anionic peroxidase isoenzyme is currently described (24). The browning reaction as seen in damaged plant tissue refers to the deposition of polyphenolic acids which can cross-link to proteins and other cell wall constituents. Precipitation of proteins by polyphenols leads to an astringent flavor and a decrease in palatability (28), and polyphenols contribute to decreased digestibility through the denaturation of protein and cross-links to carbohydrates (*e.g.* lignocellulose) (20). Excessive browning is characteristic of many fruits and vegetables that have been handled harshly or stored for long periods. Much of the wound-induced browning in plants has previously been attributed to diphenol oxidases (diphenol oxygen oxidoreductase, EC 1.10.3.2) generally referred to as polyphenol oxidases (27).

In addition to browning, wounding results in the polymerization of cinnamyl alcohols into lignin and suberin (7). Wound-induced deposition of polyphenols is characteristic of infection and is widely considered to play a role in disease resistance (1, 4). For these reasons, it is necessary to gain a better understanding of the enzymes which are responsible for the deposition of polyphenols. Pith tissue from transgenic plants which overproduce the tobacco anionic peroxidase isoenzyme was characterized for color formation, peroxidase and polyphenol oxidase activities, soluble phenols, and lignin composition before and after wounding. In addition, the purified anionic peroxidase was analyzed for the kinetics of polymerization of lignin and polyphenolic acid precursors. The regulation of peroxidase expression, the role which peroxidase plays in plant polyphenol metabolism, and how these factors may affect disease resistance and post-harvest quality are discussed.

MATERIALS AND METHODS

Plant Material

Transgenic *Nicotiana tabacum* cv Coker 176 plants harboring either a chimeric peroxidase gene or a vector-only control were propagated from seed of second generation transgenic plants in the greenhouse (24). Plants, upon reaching sexual maturity, were harvested for analysis. Stems were sterilized with 70% ethanol and pith tissue was aseptically excised with

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a cork borer. Longitudinal explants were cut into equivalent 5 mm discs and placed onto sterile filter paper saturated with Murashige-Skoog medium without hormones and kept in Petri dishes (22). The tissue was stored for various lengths of time at 28°C in the dark.

Tissue Color

Tissue color was quantified with a Minolta Chroma Meter II according to the L^*,a^*,b^* color scale (5). Pith sections were measured for light reflectance at various times after wounding. Four tissue sections were averaged for each reading, with little variation seen between replicates.

Tissue Blots

Stems from either control or peroxidase overproducer plants were carefully cut into 5 mm cross-sections and blotted onto prewetted nitrocellulose filters (3). Firm pressure was applied for 15 s before the tissue was removed. The filters were rinsed briefly in PBS (10 mm sodium phosphate [pH 6.0], 150 mm sodium chloride), then placed in PBS containing 0.6 mg/mL 4-chloro-1-naphthol for 1 min. Hydrogen peroxide was then added to a final concentration of 0.09% (v/v). A blue color reaction localizing peroxidase activity occurred almost immediately and was stopped by washing the filters with PBS.

Tissue Extraction and Enzyme Assays

Pith tissue was homogenized in three volumes (w/v) of 100 mM sodium phosphate-citrate buffer (pH 5.5) with a Polytron blender (Brinkman Industries). This material was centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was used immediately for enzyme assays. Total protein content was determined by the Bradford reagent method (Bio-Rad Corp). Peroxidase activity was assayed spectrophotometrically at 470 nm in 0.28% (v/v) guaiacol, 0.3% (v/v) hydrogen peroxide. Polyphenol oxidase activity was determined by monitoring oxygen consumption with an oxygen electrode (Rank Brothers, Cambridge, England) with caffeic acid or chlorogenic acid as substrate.

Lignin and Total Phenol Determination

Lignin and lignin-related polymers were quantified by dissolving the alcohol insoluble cell wall residue in acetyl bromide followed by measurement of lignin absorbance at 280 nm (19, 33). Briefly, 0.5 g of pith tissue was homogenized in methanol, centrifuged, and the residue was washed sequentially with methanol, ethyl acetate, and water (17). The final residue (≈10 mg) was taken to dryness under vacuum overnight, weighed, and solubilized in 25% (v/v) acetyl bromide in acetic acid at 70°C for 30 min. Samples were diluted to equal volumes with acetic acid and the absorbance at 280 nm was measured immediately. A standard curve for lignin was generated with dehydroconiferyl alcohol polymerizate synthesized from coniferyl alcohol and horseradish peroxidase by the method of Kirk *et al.* (21). Total soluble phenolic content was determined spectrophotometrically with the Folin-Denis reagent on methanol extracts of pith tissue (32), with guaiacol used to generate a standard curve.

Enzyme Kinetics

The tobacco anionic peroxidase was purified to homogeneity as described previously (23). All assays, excluding pH activity profile, were performed at 25°C in 100 mM sodium phosphate-citrate buffer (pH 5.5). When indicated, hydrogen peroxide was added to the reaction to a final concentration of 59 mM, and purified anionic peroxidase was added at 1.34 μg/mL. Reaction of syringaldehyde was measured at 307 nm, caffeic acid was assayed at 450 nm, and chlorogenic acid was measured at 330 nm in a Shimadzu dual beam UV/VIS recording spectrophotometer (27). Where appropriate, a Lineweaver-Burk plot of the data was made and a Michaelis constant determined.

RESULTS AND DISCUSSION

Wound-Induced Browning of Pith Tissue

Pith tissue was extracted from second generation transgenic tobacco plants harboring either a chimeric anionic peroxidase gene under control of the cauliflower mosaic virus 35S promoter (507) or control plants transformed with a vector-only construct, *pCib10* (30). Plants with the chimeric peroxidase gene were previously shown to have 10-fold higher leaf peroxidase activity than that of control plants (24). Figure 1 shows peroxidase activity through cross-sections of stem from either control plants (wild-type peroxidase activity) or those transformed with the chimeric peroxidase gene (507). Peroxidase activity in the stem was localized to the xylem and epidermal tissue of control plants. However, 507 plants synthesized the anionic peroxidase isoenzyme in pith and throughout all tissues of the stem.

Pith tissue was aseptically removed from sexually mature plants and sectioned into equivalent 5 mm discs. These discs were placed onto filter paper which was saturated with medium which included salts, vitamins, and sucrose (no hormones). The tissue was incubated at 28°C in the dark for 0, 1, 2, 3, 5, or 7 d. After 24 h, a distinctive browning response was observed in pith tissue from the peroxidase overproducer plant (Fig. 2). The browning intensified for 5 d after wounding then leveled off. To quantify the extent of browning, a colorimeter was employed to measure color and light intensity by the L^*,a^*,b^* color system. A graph which displays green-red (a^*) reflectances over the time course of the experiment is shown in Figure 3. Red reflectance best represents browning. Although pith tissue from both plants looked identical at time zero, the reflectance meter was able to detect a red character in the 507 pith. Control pith never exhibited measurable red character (browning). The browning response was a result of peroxidase overproduction. Several other transformed plants of *N. tabacum* and *N. sylvestris* overexpressing the anionic peroxidase demonstrated the same phenotype (data not shown). These results suggest that enhanced anionic peroxidase activity is correlated with wound-induced browning of tobacco pith tissue.

Peroxidase and Polyphenol Oxidase Activity

Polyphenol oxidase (diphenol oxygen reductase, 1.10.3.2) catalyzes the oxidative polymerization of phenolic acids (*e.g.*

Figure 1. Tissue blot for peroxidase activity of control and overproducer plants. Tobacco stem tissue was blotted onto nitrocellulose filters and stained for peroxidase activity with 4-chloro-1-naphthol and H_2O_2. Top, control plant; bottom, transformed plant overproducing peroxidase. Lighter regions in the center of the pith are variable and due to uneven contact with the nitrocellulose filter.

caffeic acid, ferulic acid, or chlorogenic acid) into brown-colored polyphenolics (27). The effect of wounding and infection on peroxidase and polyphenol oxidase enzymatic activity has been demonstrated in several organisms (22). Total peroxidase and polyphenol oxidase activity of pith tissue at various times after wounding were assayed (Fig. 4). The initial peroxidase activity in pith tissue from the 507 plant was more than twofold greater than from the control plant. Elevated peroxidase activity was predicted on the basis of previous results with leaf tissue (24). There was an overall increase in peroxidase activity over time after wounding. Depressions in peroxidase activity at d 1 and 3 are likely due to the enzyme becoming bound to cell wall components (22). Peroxidase activity in 507 plants mimics the same pattern of induction as in control plants, albeit at an elevated level.

Because polyphenol oxidase activity results in browning, it was necessary to measure polyphenol oxidase activity on the chance that overproduction of peroxidase leads to browning indirectly through the induction of polyphenol oxidase. Initial polylphenol oxidase activity and the induction of polyphenol oxidase activity by wounding were similar in both control and 507 plants (Fig. 4). It is not believed that the differences seen between control and 507 plants are significant. This observation diminishes the possibility that polyphenol oxidase is responsible for the browning seen in peroxidase overproducer plants. Browning does not result from elevated levels of wound-induced peroxidase activity in control plants after 48 h. However, browning is associated with elevated levels of the anionic peroxidase isoenzyme in 507 plants during the initial 24 h after wounding. The browning seen as a result of the anionic peroxidase may be due to the presence of this isoenzyme in the cell wall and/or the ability of this enzyme to utilize phenolic acids as substrate. This is in contrast to the wound-induced peroxidase isoenzymes which are localized to the vacuole (26).

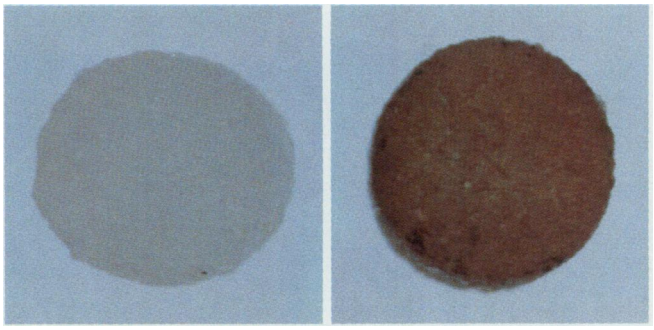

Figure 2. Pith tissue from control and peroxidase overproducer tobacco plants 48 h after wounding. Control (left) and peroxidase overproducer (right) pith tissue 48 h after excision from the plant.

Anionic Peroxidase Kinetics

If the tobacco anionic peroxidase contributes to browning *in vivo* as seen in 507 plants, the purified enzyme should be able to polymerize phenolic acids *in vitro*. The anionic peroxidase isoenzyme was purified to homogeneity from tobacco leaf tissue as previously described (23). Kinetic measurements were taken with syringaldehyde, which closely resembles a

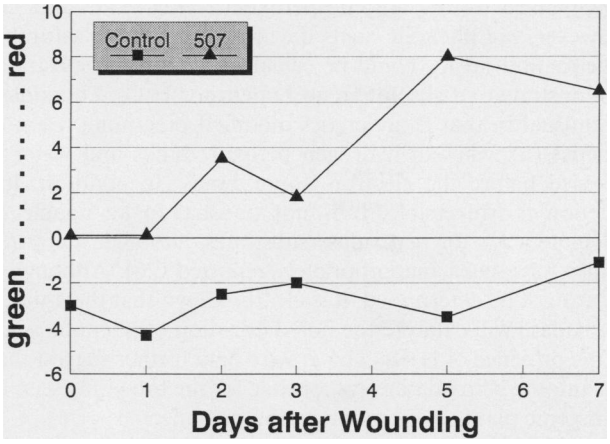

Figure 3. Wound-induced browning of tissue over time as represented by (a^*) green-red color reflectance measurements of wounded pith tissue. Control (■) and peroxidase overproducer (507, ▲) pith tissue was excised and light reflectance measurements were taken 0, 1, 2, 3, 5, and 7 d after wounding.

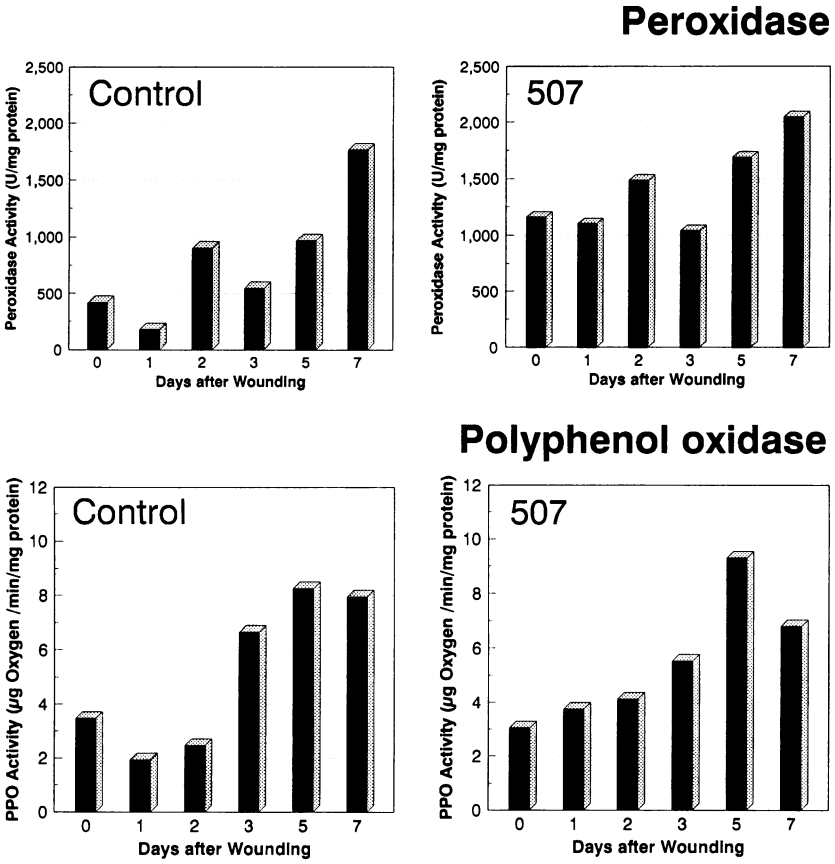

Figure 4. Peroxidase and polyphenol oxidase activity in control and 507 pith tissue at various times after wounding. Peroxidase activity was determined spectrophotometrically with guaiacol and H_2O_2. Polyphenol oxidase activity was measured by O_2 consumption with caffeic acid as donor. H_2O_2 was not required for polylphenol oxidase activity as determined by the addition of 10 μg catalase/mL. All assays were performed in pH 5.5 buffer at 25°C.

lignin precursor, and caffeic or chlorogenic acid, which are abundant phenolic acids in tobacco. A pH activity profile was made with all three substrates with maximal activity at pH 5.5 ± 0.5 (data not shown). Syringaldehyde displayed classical saturation kinetics with the anionic peroxidase, and a K_m of 270 μM was determined (Fig. 5, A and B). Also, in the presence of H_2O_2, both caffeic acid and chlorogenic acid were readily polymerized by the anionic peroxidase (Fig. 5, C and D). However, the phenolic acids did not demonstrate saturable kinetics and no K_m could be calculated. All three substrates demonstrated an absolute requirement for H_2O_2. Peroxidase is unusual in that it undergoes modified ping-pong reaction kinetics (6), wherein hydrogen peroxide binds and water is released before the electron donor binds. In addition, the reaction is irreversible. It is not unusual to be unable to calculate a K_m for peroxidase substrates, although many K_m values have been inappropriately reported (26). Although a K_m cannot be determined, it is clearly shown that the anionic peroxidase will catalyze the polymerization of phenolic acids in the presence of H_2O_2. The *in vitro* data further suggest that the anionic peroxidase is responsible for the browning seen in transgenic plants overproducing this enzyme.

Soluble Phenols and Lignin Synthesis

The ability of the anionic peroxidase to polymerize phenolic acids *in vivo* would, in part, rely upon the availability of substrate in the peroxidase overproducer plant. Total alcohol extractable phenols in wounded pith tissue from control and 507 plants were assayed. The initial concentration of soluble phenols was nearly twofold greater in 507 pith than control tissue. However, after wounding, the levels of soluble phenols in 507 tissue were equivalent to those seen in control tissues (Fig. 6). A sufficient concentration of soluble phenols was present initially in the peroxidase overproducer to provide substrate for peroxidase-catalyzed browning. Wounding presumably resulted in the release of phenolic acids stored in the vacuole, which were rapidly polymerized by the cell wall-associated anionic peroxidase in the 507 plants. The reason that 507 plants had higher initial levels of soluble phenols compared to control plants is unknown. However, the decrease in soluble phenols at 1 d after wounding in both control and 507 tissue may represent their polymerization by peroxidase.

The anionic peroxidase isoenzyme is considered to be responsible for lignin biosynthesis based on its affinity for cinnamyl alcohols, its location in the cell wall, and its expression in lignified tissue (23, 26). Wound-induced lignification and suberization, which are catalyzed by peroxidase, have important implications in wound healing and disease resistance (4, 7). Therefore, we sought to determine if the lignin composition of healthy pith tissue was higher in peroxidase overproducer plants and if the rate and timing of wound-induced lignification varied in peroxidase overproducer plants. As can be seen in Figure 6, lignin-type polymers made up 0.3% of the pith cell wall alcohol insoluble residue in

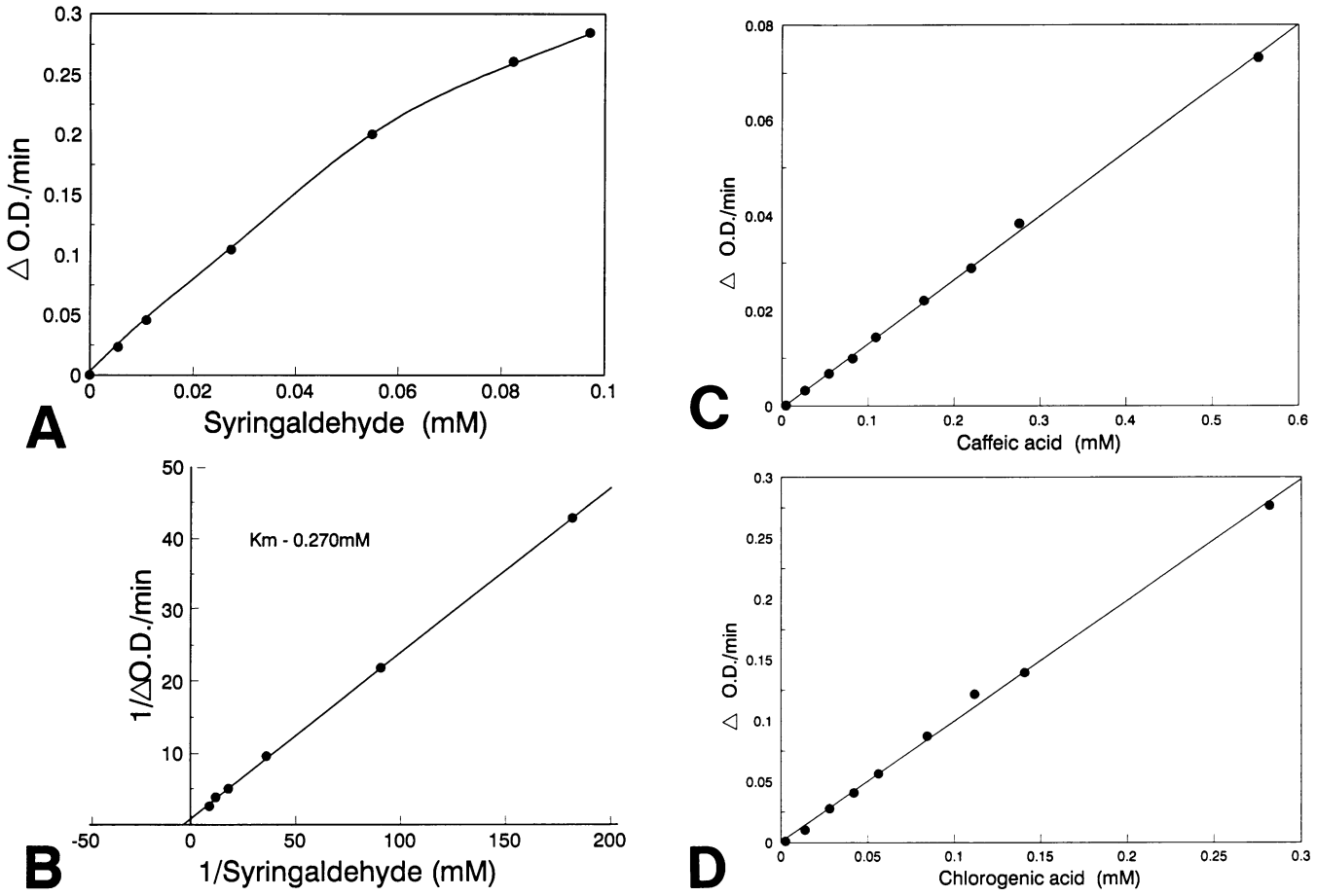

Figure 5. Reaction kinetics of purified tobacco anionic peroxidase. Purified anionic peroxidase isoenzyme was reacted with varying concentrations of syringaldehyde, caffeic acid, or chlorogenic acid. Primary (A) and secondary (B) plots for syringaldehyde (307 nm) and primary plots for (C) caffeic acid (450 nm) and (D) chlorogenic acid (330 nm) are shown. All reactions were carried out at pH 5.5 in 59 mM H_2O_2 at 25°C .

control tobacco plants. Comparably, leaf cell walls of control plants were approximately 1.0% lignin. Alcohol insoluble residue from all tissue samples was off-white in color, thus showing little visible evidence of highly polymerized phenolic acids in cell walls. Cell walls from unwounded pith tissue of peroxidase overproducer plants were composed of 0.6% lignin, still relatively low, but twofold higher than in control tissue. Because pith tissue normally synthesizes little peroxidase compared to lignified tissue (xylem, etc. [23], Fig. 1), the twofold higher lignin content in 507 plants implies there are adequate levels of lignin precursors (cinnamyl alcohols) available in pith tissue and peroxidase may be limiting with respect to the extent of lignification seen.

Wounded pith tissue from control tobacco plants shows little change in lignin composition for 2 d, followed by a gradual rise which peaks at 1.3% lignin between d 5 and 7. No differentiation was made between lignin, suberin, and related phenolic polymers. Therefore, the observed absorbance is likely a reflection of the combined contribution of these related polymers. The time-course of lignification observed with control tissue closely resembles that which has been seen in infected cucumber (4). However, wounded pith tissue from 507 plants shows an increase in the percentage of lignin between d 1 and 2 and subsequently levels off at 1.1% lignin. Although the final content of lignin is the same in both wounded control and 507 tissue, lignification in the 507 tissue occurred 1 to 2 d sooner than that observed in control plants. The delayed induction of peroxidase activity seen in control plants (Fig. 4) resulted in the delay of wound-induced lignification (Fig. 6). This delay is in contrast to the rapid induction of other phenylpropanoid pathway enzymes (less than 24 h) by wounding or infection (2, 25).

It is not known whether the relatively slow induction of peroxidase activity in response to wounding in control plants is an effect of transcription rate, mRNA stability, translational control, or related to heme availability. Roberts *et al.* (29) have reported the steady-state mRNA levels of a suberin-associated peroxidase in wounded potato and tomato. They showed peroxidase mRNA levels peaking 4 d after wounding, suggesting either transcriptional control or regulation of mRNA stability. Peroxidase differs from other phenylpropanoid enzymes in that its activity is dependent upon the coordinate induction of heme synthesis, and heme synthesis is dependent on the availability of soluble iron (18). This complicates the regulation of peroxidase synthesis, and the availability of heme may slow down the wound-dependent

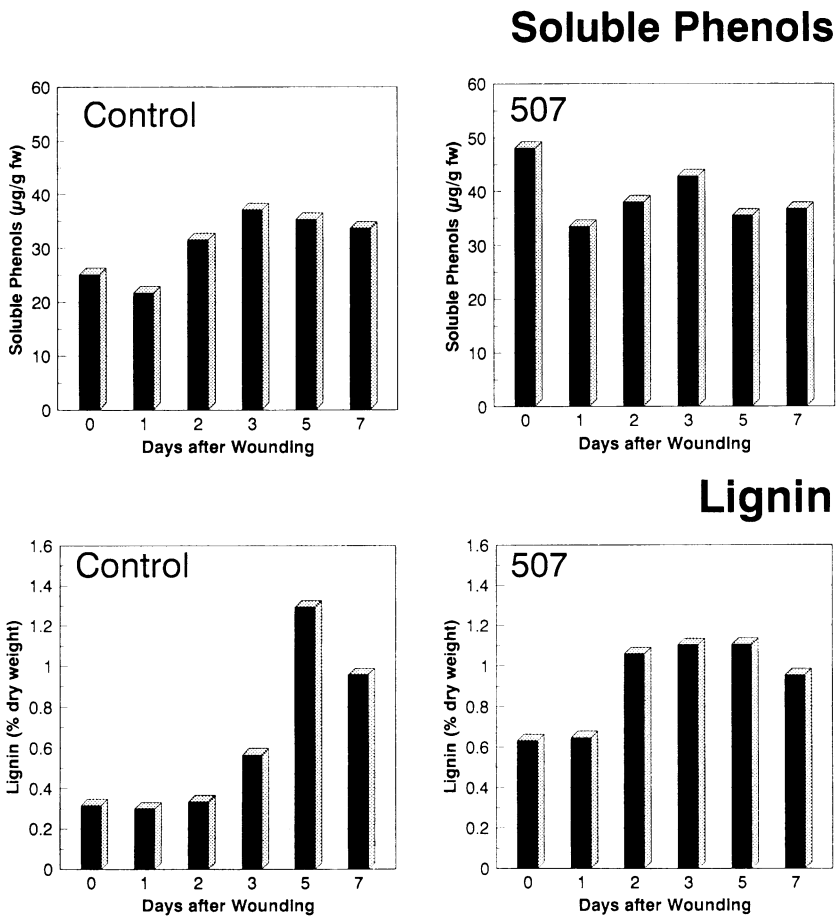

Figure 6. Soluble phenols and lignin-type polymer levels at various times after the wounding of pith tissue. Phenols in alcohol extractable material were quantified with the Folin-Denis reagent and are represented as μg/g fresh weight. Lignin in alcohol insoluble residue was determined by first solubilizing with acetyl bromide and recording the absorbance at 280 nm.

induction of peroxidase activity. The deposition of phenolic monomers into cell walls is a plant response to wounding, infection, or other forms of stress (14). Peroxidase has a crucial role in the final step in this process and the understanding of peroxidase expression will open possibilities in crop improvement through the development of stress-tolerant plants.

CONCLUSIONS

These results strongly suggest that the enhanced synthesis of the tobacco anionic peroxidase leads to elevated levels of lignin in unwounded pith tissue and excessive polymerization of phenolic acids in wounded tissues. Transformed plants were shown to express the anionic peroxidase in all tissues, most noticeably pith tissue which normally has little peroxidase activity (Figs. 1, 2). Pith tissue from the peroxidase overproducer plant browns within 24 h of wounding, while control tissue does not brown even after 7 d (Fig. 3). The maximal level of peroxidase activity induced by wounding is the same in control and peroxidase overproducer tissue (Fig. 4), thus suggesting a regulatory limitation on the total amount of active peroxidase. This limitation may be imposed by the availability of heme. Initially, the presence of enhanced peroxidase activity in 507 pith tissue and the absence of significant differences in polyphenol oxidase activity when compared to control tissue suggest that peroxidase is catalyzing the browning reaction (Fig. 4).

In vitro data with the purified anionic peroxidase and phenolic acids as substrates verify that this isoenzyme will polymerize phenolic acids in the presence of H_2O_2 (Fig. 5). The presence of elevated levels of soluble phenols initially in 507 pith (Fig. 6) may be important to the browning response observed in peroxidase overproducer plants. Browning in wounded plant tissue is complex with a multitude of enzymes (oxidases and peroxidases) and substrates (chlorogenic acid, caffeic acid, ferulic acid, etc.) which all may contribute to a reddish-brown end product. It has been confirmed that, if phenolic acids and H_2O_2 are present in the cell wall space, the anionic peroxidase is fully capable of polymerizing these compounds. The anionic peroxidase that is present in the cell wall may, under normal circumstances, utilize phenolic acids to form covalent cross-links between lignin (via —OH groups), carbohydrate (via —COOH groups), and proteins (via tyrosines—OH) (8, 9, 16). These results show the potential for altering polyphenol metabolism (and thus cell wall structure, disease resistance, browning, etc.) through the molecular transformation of peroxidase gene expression.

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