Peroxidase Overproduction in Tomato: Wound-induced Polyphenol Deposition and Disease Resistance

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Abstract. Lignin composition in leaf, fruit, and fruit outer epidermis of transgenic tomato (*Lycopersicon esculentum* Mill.) plants that overproduce the enzyme tobacco anionic peroxidase (TobAnPOD) was analyzed. This enzyme may catalyze the polymerization of cinnamyl alcohols into lignin in tobacco (*Nicotiana tabacum* L.); therefore, we predicted that its presence in the transformed tissue would increase lignin levels in healthy and wounded tissue. Lignin levels in healthy plants increased by 20% in leaf, 49% in fruit, and 106% in fruit outer epidermal tissue. Mature-green fruit were aseptically wounded and incubated in darkness for up to 7 days. Soluble phenols in wounded transgenic fruit increased by more than 300% but changed little in control fruit. As with soluble phenols, lignin content in wounded transformed fruit increased by more than 20-fold but increased less than two-fold in control fruit. Transgenic seedlings overproducing TobAnPOD were screened for susceptibility to several pathogens, but resistance did not increase. Possible TobAnPOD roles in lignin biosynthesis, phenol metabolism, stress response, and disease resistance are discussed.

The plant cell wall is a primary defense against external threats, including pollutants, mechanical wounding, and intrusion by pathogenic microorganisms and insect pests. To defend the cell, the cell wall contains enzymes and substrates that can generate activated oxygen species and reactive phenolic radicals (Southerland, 1991) and erect barriers composed of cellulose, callose, lignin, and suberin (DeLeeuw, 1985). One cell wall enzyme that can catalyze many of these reactions is peroxidase. Peroxidases (EC 1.11.1.7, donor: hydrogen peroxide oxidoreductase) perform single electron oxidations on a wide variety of compounds in the presence of H₂O₂ or O₂ (Gaspar et al., 1982). There are various peroxidase isoenzymes in plants derived from a large gene family. Recent reviews describe many biochemical and physiological functions of peroxidase (Everse and Grisham, 1991; Lobarzewski et al., 1991). Some of the proposed functions include cross-linking cell wall polysaccharides (Fry, 1986), lignification (Grisebach, 1981), wound healing (Espelie et

al., 1986), phenol oxidation (Uddin et al., 1988), and pathogen defense (Hammerschmidt et al., 1982). Reactions catalyzed by peroxidases, while indications of the plant's response to stress, complicate the understanding of peroxidase function in plants.

Evidence collected so far suggests that tobacco anionic peroxidase (TobAnPOD) is needed to form lignin. The purified enzyme has a preference for cinnamyl alcohols and readily will catalyze their polymerization into lignin in vitro (Mäder, 1980; Mäder et al., 1977). Also, histological and biochemical evidence shows that TobAnPOD is localized in the cell walls of lignifying tissue (Lagrimini, 1991b). However, the abundance of peroxidase isoenzymes makes it difficult to prove that TobAnPOD is the primary enzyme involved in lignin synthesis. To understand better the possible functions of this enzyme, transgenic tobacco plants that greatly overproduce this enzyme were engineered (Lagrimini et al., 1990). Resulting phenotypes included chronic wilting, wound-induced browning, and greatly enhanced lignin synthesis in response to wounding (Lagrimini, 1991a). These phenotypes suggest that this enzyme has significant physiological functions in addition to lignification. More recently, tomato plants have been transformed with the chimeric TobAnPOD gene to overproduce this enzyme in a heterologous system (Lagrimini et al., 1992). As in tobacco, these tomato plants were severely wilted, and, consequently, fruit size was greatly reduced. This gene was introduced into tomato because phenylpropanoid metabolism and lignin biosynthesis in response to wounding and infection are important factors determining the overall postharvest quality of processing and fresh-market tomatoes (Robinson,

1991). The phenylpropanoid pathway, which is induced by wounding, affects tomato color, flavor, and texture (Bell, 1981). Also, mechanical harvesting and handling frequently damage the fruit and trigger several wound-specific processes.

Plant materials and wounding. Unless otherwise stated, all plant transformations were performed with the early season processing tomato cultivar OH 7814 (Berry and Gould, 1983), which is susceptible to all of the tomato pathogens used in this study. The hybrid tomato 'OH 1498' was used as a control because it is resistant to all pathogens tested. Producing transgenic tomato plants that express TobAnPOD was described previously (Lagrimini et al., 1992). Briefly, 'OH 7814' stem segments were co-cultivated with *Agrobacterium tumefaciens* harboring either the plasmid pCib 10 (control), which confers kanamycin resistance, or pML 507 (POD overproducer), which is composed of a chimeric TobAnPOD gene transcribed from the cauliflower mosaic virus 35S promoter inserted into pCib 10. Stem segments were placed on regeneration media containing kanamycin, shoots were rooted in potting soil, and plants homozygous for the foreign genes were selected through two generations of self-fertilizations. Tomato plants were propagated in the greenhouse with supplemental lighting (14-h photoperiod). One control plant (transformed with pCib 10) and two transgenic plants overproducing TobAnPOD (TobAnPOD 7-7 and TobAnPOD 7-B) were selected for further experiments.

Mature-green fruit were surface-sterilized briefly with 70% ethanol (EtOH) followed by 10% commercial bleach for 10 min, then wounded by aseptically cutting the fruit into two equal halves with a scalpel. The wounded sections were placed cut side up on moistened filter paper in petri dishes, sealed with parafilm, and incubated in darkness at 28°C. Tissues immediately frozen after excision were used as nonwounded controls.

Total soluble phenol and lignin determination. Total soluble phenol content was determined spectrophotometrically with the Folin-Denis reagent on methanol (MeOH) extracts of healthy and wounded tomato fruit (Swain and Hillis, 1959). A standard curve was generated with gallic acid. Lignin from leaf or fruit tissue was purified from an alcohol-insoluble cell wall fraction by preferentially solubilizing the lignin with thioglycolic acid using methods similar to those used by Bruce and West (1989) and Whitmore (1978). Tissue was homogenized (Polytron; Brinkman Industries, Westbury, N.Y.) in absolute MeOH. Cell walls were pelleted by centrifugation, washed once in MeOH, and pelleted again. The cell wall fraction was dried overnight at 60°C. Ten to 50 mg of alcohol-insoluble residue was suspended in 5 ml of 2 N HCl and 0.5 ml of thioglycolic acid (Aldrich Chemical Co., Milwaukee) in tightly sealed glass screw-cap tubes. The tubes were placed in a 100°C oil bath for 4 h and cooled to room temperature, and the lignin thioglycolate (LTGA) was pelleted by centrifugation at 30,000 × g for 10

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min at room temperature. The LTGA was washed once with H₂O and solubilized in 5 ml of 0.5 N NaOH for 18 h at 25°C. The extract was cleared by centrifugation (30,000× g, 10 min), transferred to glass conical centrifuge tubes, and the supernatant acidified with 1 ml of concentrated HCl. The LTGA was precipitated at 4°C for 4 h and the orange-brown precipitate pelleted by low-speed centrifugation. The LTGA was acid-base extracted once more to remove contaminants that absorb in the ultraviolet light spectrum. The final LTGA precipitate was dried and dissolved in 10 ml of 0.5 N NaOH, and absorbance was measured at 280 nm. A standard curve for lignin was generated with dehydroconiferyl alcohol polymerize synthesized from coniferyl alcohol and horseradish peroxidase using the method of Kirk et al. (1975).

Infection of plants and quantification of disease resistance. Three *Lycopersicon esculentum* genotypes were assessed for their level of resistance to the pathogens *Fusarium oxysporum* f.sp. *radicis-lycopersici* (crown and root rot), *F. oxysporum* f.sp. *lycopersici* race 2 (wilt), *Verticillium dahliae* (wilt), and tobacco mosaic virus strain 4 (TMV) as described by Erb and Rowe (1992). The procedure differed in that seedlings were inoculated with one pathogen only, and inoculations with *V. dahliae* and TMV were done 2 weeks after seed was sown. The genotypes tested included 'OH 7814', 'OH 1498', and TobAnPOD 7-7, which overproduces TobAnPOD. Seeds were sown in a flat containing 10 to 20 seedlings per genotype. The data are represented as an average of three replications. Seedlings were evaluated for *Fusarium* crown and root rot 18 days after inoculation, *Fusarium* wilt 11 days after inoculation, and *Verticillium* wilt and TMV 4 weeks after inoculation. An equal number of mock-infected plants was used in parallel for all treatments. The criteria for rating disease susceptibility are provided in Fig. 1. TMV infection was assessed by enzyme-linked immunosorbent assay with a kit purchased from EIGHTY (Elkart, Ind.).

Lignin composition of transgenic tomato plants. TobAnPOD also is referred to as the lignin-forming peroxidase (Lagrimini et al., 1987). This activity was inferred from its spacial and temporal localization relative to lignified tissue in the tobacco plant (Lagrimini, 1991 b; Lagrimini and Rothstein, 1987; Mäder, 1980). This assumption leads us to predict that TobAnPOD expression in transgenic tomato plants may increase lignification, given that the enzyme is limiting in nonlignified tissues. This hypothesis was confirmed when the lignin composition was determined in leaf, fruit, and fruit outer epidermal tissues from tomato plants expressing TobAnPOD. Lignin was extracted with thioglycolic acid, which was preferable to other methods used on soft tissues with low lignin levels (Bruce and West, 1989). We were confident in analyzing lignin at concentrations <1% of the total dry mass. The lignin content of leaf, fruit, and fruit outer epidermal tissues from peroxidase overproducer plants was higher than that in control plants (Table 1). This difference in lignifica-

tion could arise from substrate availability or differential expression of the CaMV 35S promoter. The observed increase in lignin in TobAnPOD-overproducing plants provides further evidence that the enzyme helps synthesize lignin.

Soluble phenol and lignin formation in wounded tomato fruit. Previous results with transgenic tobacco plants overproducing TobAnPOD indicated that the overproduction of this enzyme had a dramatic effect on wound-induced lignification and polyphenol metabolism (Lagrimini, 1991a). There were two reasons for examining the role of TobAnPOD in these secondary metabolic processes in tomato fruit: 1) fruit quality deteriorates from wounding, which can occur at several stages during harvest and postharvest handling, and 2) wounding leads to the production of many secondary metabolites that result in off-flavors, loss of firmness, and eventual decay (Haard,

1977; Robinson, 1991). For these reasons, mature-green tomato fruit producing TobAnPOD were wounded, and total soluble phenols was quantified at 0, 1, 2, 3, 5, and 7 days after wounding. Total soluble phenols in tomato fruit from control plants increased only slightly, even after 7 days (Fig. 2, top). However, soluble phenols rose rapidly—300% increase in 7 days—in fruit producing TobAnPOD at high levels (>1600-fold). Consequently, the high levels of TobAnPOD in the transformed tomato fruit significantly affected phenol metabolism during periods of stress, such as wounding. Just how an enzyme such as TobAnPOD affects soluble phenol concentration is unclear. Possibly, one or more substrates or products of a TobAnPOD-catalyzed reaction either directly or indirectly induces phenol synthesis in wounded tissue.

Wound-induced polymerization of phenol into lignin by TobAnPOD was also assessed.

Fig. 1. Disease susceptibility of transgenic tomato plants expressing TobAnPOD. Four tomato pathogens were used for quantifying disease resistance in three tomato cultivars: OH 1498 (resistant), OH 7814 (susceptible), and OH 7814TobAnPOD (producing tobacco anionic peroxidase). Three repetitions with 10 to 20 plants per repetition were used for each cultivar-pathogen pair. The rating system was defined as follows:

- (A) *Fusarium oxysporum* f.sp. *radicis-lycopersici*: 0 = failed to germinate; 1 = collapsed seedling; 2 = severe crown necrosis (shoot only slightly wilted or showing no symptoms); 3 = moderate crown necrosis (shoot showing no symptoms); 4 = slight crown necrosis (shoot showing no symptoms); and 5 = healthy.
 (B) *Fusarium oxysporum* f.sp. *lycopersici*: 1 = severely stunted and wilted; 2 = moderately stunted and wilted; 3 = slightly wilted and little or no stunting; and 4 = healthy.
 (C) *Verticillium dahliae*: 1 = severely stunted; 2 = moderate to slight stunting; and 3 = healthy.
 (D) Tobacco mosaic virus strain 4 was quantified by ELISA, and data presented are the mean absorbances (three replications) at 490 nm. The larger the number, the more virus in the tissue.

Table 1. Lignin composition in transgenic tomato plants producing tobacco anionic peroxidase. Percent change is with reference to the control plant 'OH 7814'. The mean $\pm$ SE for three samples is expressed;

Plant	Leaf		Fruit		Outer epidermis	
	Lignin (mean $\pm$ SE)	Change (%)	Lignin (mean $\pm$ SE)	Change (%)	Lignin (mean $\pm$ SE)	Change (%)
Control	0.393 $\pm$ 0.040	---	0.073 $\pm$ 0.005	---	0.255 $\pm$ 0.01	---
TobAnPOD 7-7	0.467 $\pm$ 0.032	19	0.098 $\pm$ 0.007	34	0.407 $\pm$ 0.02	80
TobAnPOD 7-B	0.474 $\pm$ 0.049	20	0.109 $\pm$ 0.009	49	0.525 $\pm$ 0.02	106

Fig. 2. (top) Wound-induced total soluble phenols (guaiacol equivalents) in mature-green tomato fruit wounded aseptically and then incubated in moist dark chambers at 28°C. Data collected from fruit of control plants, 'OH 7814' (□), and transformed plants, TobAnPOD 7-7 (◇) are shown. Each point is the mean of three replications; bar $\pm$ SE. (bottom) Wound-induced lignin deposition in mature-green tomato fruit isolated from control (□) and transformed tomato plants expressing TobAnPOD (◇) and aseptically wounded and incubated in darkness at 28°C. Each point is the mean of three replications; bar $\pm$ SE.

Tomato fruit producing TobAnPOD had a lignification rate that was 2.5-fold greater than in control fruit and that occurred 2 days postwounding as opposed to 3 days in control fruit (Fig. 2, bottom). Plants producing TobAnPOD also showed lignification 18-fold higher than in control fruit at 7 days postwounding (data not shown). These results indicated that the low TobAnPOD activity in control fruit may restrict lignification rate and extent.

Peroxidase expression and disease resistance. A correlation between peroxidase expression, lignification, and disease resistance has been proposed for various crops (Dean and Kuc, 1987; Graham and Graham, 1991a; Southerton and Deverall, 1990; Ye et al., 1990a), including tomato (De Leeuw, 1985; Reuveni and Ferreira, 1985). This role may involve producing activated oxygen species or reactive phenol radicals (Southerland, 1991), or forming physical barriers constructed of lignin and suberin (DeLeeuw, 1985). However, the precise role remains to be determined. Transgenic tobacco and tomato plants overproducing TobAnPOD showed higher soluble phenol and lignin concentrations in healthy and wounded tissue. Based on this observation, we tried to determine if transgenic

tomato plants were more resistant to infection. We chose four tomato pathogens: *F. oxysporum* f.sp. *radicis-lycopersici*, *F. oxysporum* f.sp. *lycopersici*, *V. dahliae*, and TMV. These pathogens penetrate the leaves, crown, and root. Of the three *L. esculentum* cultivars used for the infections, 'OH 7814' is susceptible to all four pathogens, TobAnPOD 7-7 (OH 7814) overproduces TobAnPOD, and OH 1498 is resistant to all four pathogens.

None of the plants producing TobAnPOD was more resistant to infection than the others. Thus, TobAnPOD expression in tomato plants failed to protect the young plants from infection by these pathogens (Fig. 1). There are several possible explanations for this result:

- This experiment was used on seedlings that were infected at a young age, and the amount of inoculum used was excessive. Mature field-grown tomato plants producing TobAnPOD may be field tolerant to the pathogens tested.
- Although the young seedlings overproduce TobAnPOD, there may not be the appropriate peroxidase substrates available in the infected tissues.
- Peroxidase-catalyzed reactions represent only one of several host responses to pathogenesis. Other responses include the pro-

duction of chitinase, glucanase, phytoalexins, and hydroxyproline-rich glycoproteins (Dixon and Lamb, 1990).

- The primary mechanism for pathogen resistance used in this work involves processes other than peroxidase-mediated reactions. In several instances, plants overproducing peroxidase were more susceptible to infection. This result may be a consequence of the observed water stress (Lagrimini et al., 1992).

The results presented here demonstrate the dramatic effect on phenol metabolism in transgenic tomato plants producing TobAnPOD. Overproducing this one isoenzyme results in soluble phenol accumulation in healthy and wounded fruit and enhancement of wound-induced phenol metabolism. Lignin concentration was significantly higher in tomato fruit and fruit outer epidermis, and wound-induced lignin formation in genetically engineered tomato fruit was dramatically elevated compared to that of the control. However, under our conditions of infection, the overproduction of TobAnPOD did not make the plants resistant to any of the four pathogens tested.

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