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Expression of a Chimeric Tobacco Peroxidase Gene in Transgenic Tomato Plants

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Abstract. **Tomato plants *(Lycopersicon esculentum* cv. OH 7814) were transformed via *Agrobacterium tumefaciens* with a chimeric tobacco anionic peroxidase (EC 1.11.1.7) gene joined to the cauliflower mosaic virus (CaMV) 35S promoter. Transgenic plants obtained by selection on kanamycin were found to have more than five times the total leaf peroxidase activity of control plants. Transformed tomato plants chronically wilted upon reaching sexual maturity. Two independently selected transformants were self-fertilized, and progeny were obtained that were homozygous for the foreign gene. Isoelectric focusing gels stained for peroxidase activity revealed a new tomato leaf peroxidase isoenzyme with a pI of 3.75, which is similar to that seen in *Nicotiana sylvestris* L. Mature tomato fruit were found to have up to 1600-fold higher peroxidase activity in transformants expressing the tobacco anionic peroxidase (TobAnPOD) than control plants. Tissue blots showed the tobacco enzyme evenly distributed throughout the tomato fruit tissue. Progeny plants possessing the tobacco peroxidase gene (now homozygous) showed stunting, and fruit size was reduced by >80%. However, fruit set was normal and the rate of ripening was not altered from control plants. Fruit from transformed plants were found to have normal pigmentation, but the soluble solids concentration was 400% higher than in control tomato fruit. This result was predicted from the peroxidase-induced water stress. Possible roles for the tobacco anionic peroxidase in growth, development, and stress resistance are discussed.**

Plant peroxidases (donor : hydrogen-peroxide oxidoreductase) carry out single-electron oxidations of a wide variety of compounds in the presence of H_2O_2 or O_2 (Gaspar et al., 1982). Cinnamyl alcohols, phenolic acids, aromatic amines, and indoles are some of the natural compounds that serve as electron donors in peroxidase-catalyzed reactions. In addition to the large variety of potential substrates found in plants, there are also a multitude of peroxidase isoenzymes. Tobacco and tomato each have > 12 peroxidase isoenzymes (Lagrimini and Rothstein, 1987; Marangoni et al., 1989). This makes it difficult to assign specific functions to individual peroxidases. Some of the physiological processes in which peroxidases have been implicated include the regulation of cell elongation (Goldberg et al., 1986), cross-linking of cell wall polysaccharides (Fry, 1986), lignification (Grisebach, 1981), wound-healing (Espelie et al., 1986), pathogen defense (Hammerschmidt et al., 1982), and phenol oxidation (Strivastava and Huystee, 1977). Several reviews have been written on the numerous biochemical and physiological functions of peroxidase (Everse and Grisham, 1991; Greppin et al., 1986).

Although peroxidases are ubiquitous to vascular plants and are certainly crucial to growth and development, many of the proposed functions for peroxidases are based solely on in vitro data. It has been difficult to determine the actual in vivo role for individual peroxidase isoenzymes using standard biochemical techniques, due in part to the many substrates and isoenzyme forms. Based on prior biochemical and cytological data, the TobAnPOD is considered to participate in the formation of lignin (Lagrimini, 1991; Mäder et al., 1977). Recently, transgenic tobacco plants have been generated with altered peroxidase activity (Lagrimini et al., 1990). These transgenic tobacco plants had a chimeric TobAnPOD gene under control of the CaMV 35S promoter introduced to direct the overexpression of the TobAnPOD. The plants were characterized by chronic wilting, which began at the time of flowering (Lagrimini et al., 1990). This result suggested a connection between peroxidase activity, root growth, and water relations. Also, a wound-induced browning reaction occurred in pith tissue excised from tobacco plants that overproduced the TobAnPOD isoenzyme (Lagrimini, 1991). This browning reaction in damaged plant tissue was caused by deposition of polyphenolic acids in cell walls.

Tomato plants may have as many as 12 peroxidase isoenzymes, however, tomato fruit expresses only one isoenzyme with an isoelectric point of 3.5 (Evans, 1970). The tomato enzyme has an approximate molecular weight of 40-42 kD. Unlike the TobAnPOD, this enzyme has a strict requirement of Ca for activity (Marangoni et al., 1989), and the tomato fruit enzyme has been implicated in the oxidation of indole3-acetic acid (Brooks, 1986). These characteristics differ from those of TobAnPOD (Lagrimini and Rothstein, 1987), and these enzymes may perform different functions in their respective tissues and organisms.

In this study, tomato plants were transformed with this same chimeric TobAnPOD gene to determine if the dramatic phenotypes seen in tobacco could also be obtained in tomato. The effect of TobAnPOD on the tomato fruit with respect to food quality was also investigated, since peroxidase has been implicated in excessive browning and fiber formation in harvested fruits and vegetables (Haard, 1977). Also, the precipitation of proteins by polyphenols can lead to an astringent flavor and a decrease in palatability (Ozawa et al., 1987). Polyphenols can also contribute to decreased digestibility through the denaturation of protein and cross-links to carbohydrates (e.g., lignocellulose) (Jung and Fahey, 1983). For these reasons, we were

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Abbreviations: CaMV, cauliflower mosaic virus; POD, peroxidase; TobAnPOD, tobacco anionic peroxidase.

interested in studying the consequences of expressing the TobAnPOD gene in transgenic tomato plants, with the expectation of altering some of these characteristics.

Materials and Methods

Plant growth and transformations. The construction of a chimeric tobacco peroxidase gene under control of the CaMV 35S promoter was described previously (Lagrimini et al., 1990). This gene is contained within the binary plant transformation vector pCib10 that provides selection of plant material on the antibiotic kanamycin (Rothstein et al., 1987). The resulting plasmid, pML507, was transformed into *A. tumefaciens* strain A136 harboring the Ti helper plasmid pCib542, which was used in all plant transformations (Rothstein et al., 1987). *Agrobacterium* was grown as described previously (Lagrimini et al., 1990).

Seeds of an early season processing tomato OH 7814 (Berry and Gould, 1983) were surface sterilized for 15 min in a 15% commercial bleach solution containing two drops Tween-20 per milliliter. Seeds were then rinsed five times with sterile water and plated on OMS medium that contained Murashige and Skoog (MS) salts (Murashige and Skoog, 1963), Gamborg's B5 vitamins (Gamborg et al., 1968), 2% sucrose and 0.8% agar (pH 5.8). All cultures were maintained at 28C and illuminated under a 16:8 (light : dark) photoperiod at 30 $\mu Em^{-2}s^{-1}$. Two weeks after planting, stem segments (1 cm in length) were excised and dipped into a 2-day-old culture of *tumefaciens.* The stem segments were then blotted using dry sterile filter paper and transferred to OMS medium supplemented with 2 mg kinetin and 0.02 mg indole-3-acetic acid (IAA)/liter. Following a 3 day cocultivation period, the stem segments were transferred to the same medium but containing 500 mg carbenicillin and 100 mg kanamycin. Controls for transformation included tomato stem segments with no *Agrobacterium*, no antibiotic, or plants transformed with the vector plasmid pCib10.

Stem pieces with developing calli were transferred every 3 to 4 weeks to fresh medium containing carbenicillin and kanamycin. After 3 months, kanamycin-resistant shoots were excised from the callus and transferred to the OMS medium containing 500 mg carbenicillin and 50 mg kanamycin/liter for rooting. Roots were formed on this growth regulator-free medium after 4 weeks. The rooted shoots were then transferred to soil and kept in a mist chamber for 4 days before their transfer to the greenhouse.

Peroxidase assays and isoelectric focusing. Leaf, root, and fruit tissue (0.5 g) from greenhouse-grown control or TobAnPOD transformed plants were analyzed for total peroxidase activity. The tissue was homogenized with a Polytron blender (Brinkman Industries, Westbury, N.Y.) in three volumes of cold grinding buffer (10 mM sodium phosphate pH 6.0, 0.5 M NaC1, 5 mM sodium metabisulfite). A cleared extract was obtained by centrifugation at 10,000× *g* for 20 min. Total peroxidase activity was determined at 25C by the increase in absorbance at 470 nm in 0.28% (v/v) guaiacol, 0.05 M sodium phosphate buffer pH 6.0, and 0.3% (v/v) H_2O_2 (Lagrimini and Rothstein, 1987).

Individual peroxidase isoenzymes were identified by isoelectric focusing. Equivalent aliquots of tissue were applied to precast flat-bed polyacrylamide isoelectric focusing gels (LKB Pharmacia, Piscataway, N.J., pH 3.5-9.5). After electrophoresis for 1.5 h at 30 W, the gels were removed and equilibrated in PBS [10 mM sodium phosphate (pH 6.0) 150 mM sodium chloride] for 20 min before being developed for peroxidase activity with 4-chloro-1-naphthol (0.6 $mg \cdot ml^{-1}$ 4-chloro-1-naphthol, PBS pH 6.0).

Tissue blots. Mature green tomato fruit from either control or TobAnPOD transformed plants were carefully cut into 5 mm cross-sections and blotted onto prewetted nitrocellulose filters (Miesle et al., 1991; Spruce et al., 1987). Firm pressure was applied for 15 sec before the tissue was removed. The filters were rinsed briefly in PBS, then placed for 1 min in PBS containing 0.6 mg·ml-1 4-chloro-1-naphthol. 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.

Soluble solids concentration, color, and firmness determinations. Soluble solid concentration was determined with a refractometer and expressed in °BRIX. Tomatoes were disrupted with a polytron tissue homogenizer and centrifuged for 10 min at 10,000× g to obtain a cleared supenatant for refraction measurements. Tissue color was quantified with a Minolta Chroma Meter II (Osaka, Japan) according to the *L*a*b** color scale (DeMan, 1976). Mature-green and red-ripe tomato fruit were placed on their sides and measured for light reflectance. Tomato fruit firmness was determined with an AccuForce III Model 100 digital force gauge (Ametek, Largo, Fla.) joined to a lift motor automated operation. Tomatoes were placed on their sides for peak force measurements recorded in newtons.

Results and Discussion

Transformations and selection of transgenic plants. A chimeric tobacco anionic peroxidase gene constructed previously (Lagrimini et al., 1990) was transformed into the commercial tomato OH 7814 tomato with *A. tumefaciens.* This chimeric gene under control of the CaMV 35S promoter directs the synthesis of the TobAnPOD throughout the transformed tomato plant. Tomato stem segments were co-cultivated with *A. tumefaciens* strain A136 harboring the Ti helper plasmid pCib542 and either the binary vector pCib10 or the vector plasmid containing the chimeric TobAnPOD gene pML507. Outgrowth of kanamycin resistant tissue from stem segments was very slow, taking 3 months for the formation of shoots. Only 6 weeks were required for shoot formation from nontransformed cotyledon tissue of the same cultivar (Uddin et al., 1988). Although transgenic tissue was resistant to kanamycin, the time required for shoot production was increased on the antibiotic containing medium. This delay in shoot formation from the transgenic tissue may be related to incomplete resistance to kanamycin, the use of different explants, or the lack of contribution of kanamycin-sensitive tissues to the selected transformed tissues. Once shoot formation was initiated, proliferation and recovery of shoots and plants were similar to that obtained with nontransformed shoots placed on regeneration media.

Table 1. Peroxidase activity in tomato leaf tissue from two control and three individual R_0 transformed plants. Total leaf peroxidase activity as determined spectrophotometrically with guaiacol and H_2O_2 as substrates were indicated for control and TobAnPOD transformed plants.

Plant	Peroxidase activity (units/g fresh wt)
Control A	178
Control B	182
TobAnPOD5A	267
TobAnPOD77	1135
TobAnPOD7B	1056

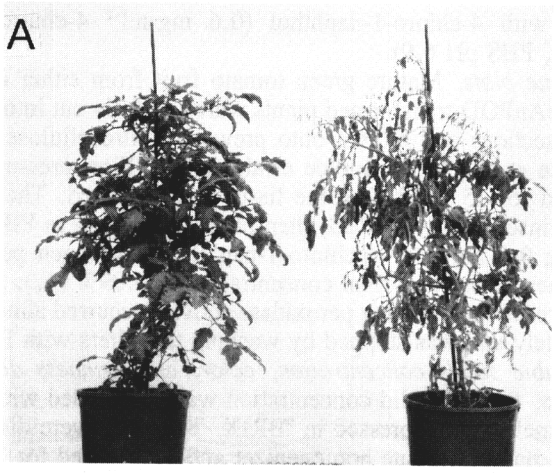

Fig. 1. Transgenic tomato plants expressing the chimeric tobacco anionic peroxidase gene. **(A)** Control (left) and TobAnPOD transformed (right) tomato plants immediately following the onset of chronic wilting. **(B)** and **(C)** Transformed (left) and control (right) tomato plants during the latter stages of fruiting. These photographs demonstrate the chronic wilting and the diminished fruit size that result from the expression of the TobAnPOD.

Five individual transformants (two controls and three TobAnPOD) were rooted on hormone-free media, and plantlets were transferred to the greenhouse. Leaf tissue was subsequently assayed for peroxidase activity. Total leaf peroxidase activity was found to be as much as 5-fold higher in plants transformed with the chimeric TobAnPOD gene as compared to control plants transformed with only the pCib10 plasmid conferring kanamycin resistance (Table 1). Initial transformed plants (R_0) were allowed to reach maturity in the greenhouse and were self-fertilized. Progeny that germinated on kanamycin were then grown to maturity. These R_1 plants were self-fertilized as before, and progeny was analyzed for segregation of the chimeric peroxidase gene and kanamycin resistance by enzymatic activity and growth on kanamycin, respectively. Those R_1 plants homozygous for the foreign gene, as determined by 100% segregation of kanamycin resistance, were selected for further studies (TobAnPOD5A, TobAnPOD77, and TobAnPOD7B). The chimeric tobacco peroxidase gene was found to be stable and segregated as a single dominant gene in the transgenic tomato plants.

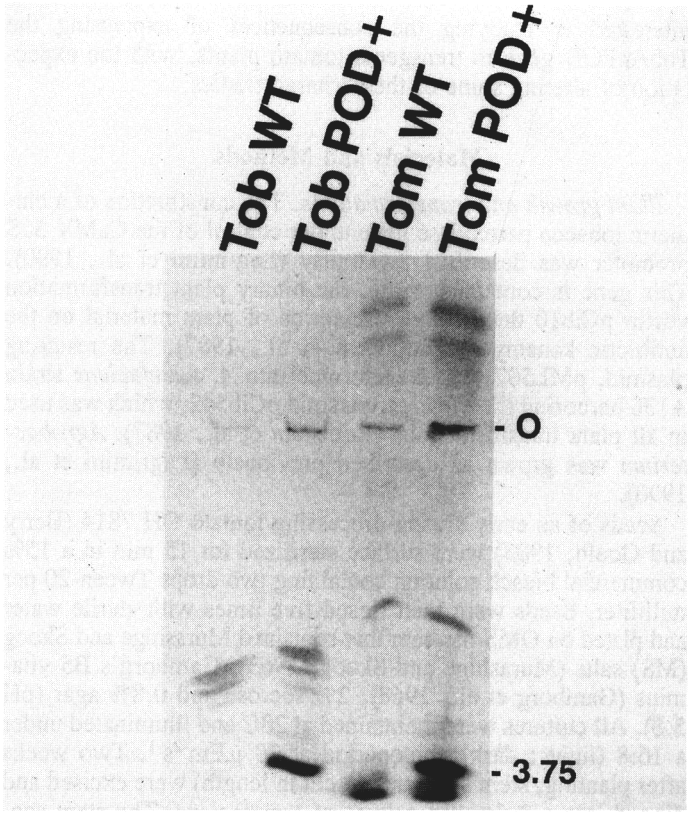

Fig. 2. Isoelectric focusing gel developed for peroxidase isoenzymes in control and transformed plants. The first two lanes show leaf peroxidase isoenzymes of *N. sylvestris* wild-type (Tob WT) and plants overexpressing the TobAnPOD (Tob POD +). The next pair of lanes are leaf peroxidase isoenzymes from wild-type (TomWT) tomato and plants transformed with the chimeric TobAnPOD gene (Tom POD +).

Phenotype of transformed tomato plants. Although it was possible to obtain seed from transgenic tomato plants expressing the TobAnPOD, the mature plants did not appear normal. The seedlings propagated in the greenhouse were indistinguishable from control OH7814 plants and remained normal in appearance until the plants began to flower. At this time, the transformed tomato plants began to chronically wilt (Fig. 1A). As in tobacco (Lagrimini et al., 1990), this wilting was continuous regardless of watering regimen, resulting in a significant reduction of fruit size (Fig. 1 B and C). Fruit size in plants expressing the TobAnPOD was reduced >5-fold as compared to control plants. Chronic wilting seen in trangenic tobacco plants was not caused by increased water loss through the leaves (Lagrimini et al., 1990), but there was a significant reduction in root growth rate and root mass. A mature control tomato plant (at flowering) had a shoot : root ratio of 6:1. A transformed plant overproducing peroxidase had a shoot : root ratio of 14:1, the difference being accounted for by a decrease in root mass. The presence of the

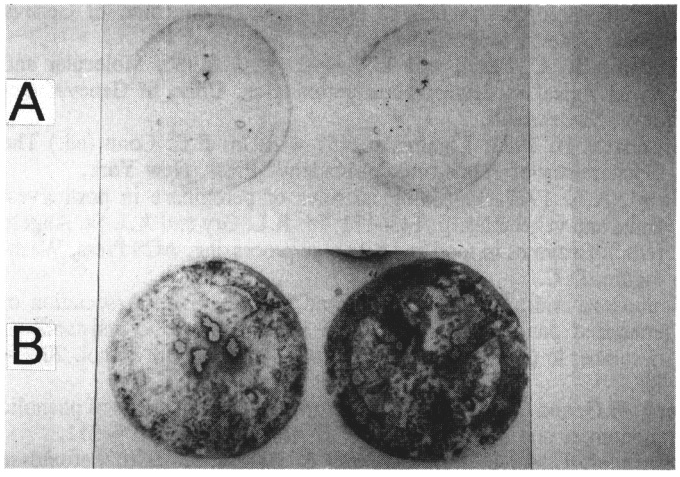

Fig. 3. Tissue blot to localize peroxidase activity in control and transformed tomato fruit expressing the TobAnPOD. Mature-green tomato fruit from control **(A)** and TobAnPOD **(B)** transformed plants were cut into 5-mm tranverse sections and blotted onto moist nitrocellulose filters. The filters were immediately stained for peroxidase activity with 4-chloro-1-naphthol and photographed. Blots are pictured at equal sizes for comparative purposes, although transformed fruit were considerably smaller.

TobAnPOD in root tissue appears to interfere with root growth in tobacco and tomato. Because of this, it is difficult at this time to determine if the reduction in fruit size is primarily a consequence of the expression of the TobAnPOD in the tomato fruit or a secondary effect of the chronic wilting, although the latter is more probable.

Analysis of peroxidase activity. Because the leaf total peroxidase activity was increased by as much as 5-fold in transformed plants expressing the TobAnPOD (Table 1), a soluble extract from tomato leaves was subjected to isoelectric focusing and subsequently stained for peroxidase activity to characterize the foreign tobacco protein in more detail. Transformed tomato plants showed a new band with an apparent pI of 3.75 (Fig. 2). This same chimeric TobAnPOD gene also resulted in the overproduction of a single peroxidase isoenzyme in *Nicotiana sylvestris* (Fig. 2). This event was presumed to indicate differences in posttranlation processing of the TobAnPOD gene product. The striking similarity between the isoelectric point of the TobAnPOD in *N. sylvestris* and *L. esculentum* suggests a similar pattern of posttranslation glycosylation.

Peroxidase activity in tomato fruit tissue of 07814 was extremely low, and the enzyme required extraction with high salt. While less than one unit of peroxidase activity per gram fresh weight was obtained from control plants, transgenic plants had 1600-fold higher peroxidase activity (Table 2). This enormous difference likely reflects the expression of the CaMV 35S promoter in fruit tissue and the solubility of the TobAnPOD.

Localization of TobAnPOD activity. The spacial pattern of peroxidase expression in tomato fruit was examined by using tissue blots stained for peroxidase activity (Spruce et al., 1987). Control tomato fruit blotted onto nitrocellulose resulted in minimal peroxidase activity (Fig. 3A) as predicted from the spectrophotometric data (Table 2), and this peroxidase activity was localized predominantly to the epidermal region of the fruit pericarp. Blots of fruit from plants expressing high levels of the TobAnPOD are shown in Fig. 3B. The TobAnPOD activity was seen in all tissues throughout the tomato fruit.

Assessment of fruit quality We were interested in determining the effect of the tobacco anionic peroxidase on fruit quality for future genetic improvements. Visual inspection of fruit revealed that, although fruit size was greatly reduced, the fruit set, rate of ripening, and overall appearance reflected that of healthy normal fruit. Red-ripe control and transformed fruit were similar in color quality (data not shown). Light reflectances from three selected fruit from control and transformed tomato plants were recorded with a Minolta Chroma Meter II. *L*a*b** values were insignificantly different between the plants. An excessive amount of the TobAnPOD in the tomato had no effect on color development in the fresh fruit.

The outer pericarp from the fruit expressing the tobacco anionic peroxidase was noticeably thicker than control fruit (data not shown). We wished to determine if this apparent thickness resulted in improved fruit firmness. A comparison between control and transformed fruit at mature green and red ripe stages showed no significant differences in peak force readings (data not shown). These results indicated that the increased thickness in transformed fruit did not translate into increased resistance to skin puncture. Therefore, the components of the outer pericarp that contribute to the peak resistance to puncture are unchanged in plants that express the tobacco peroxidase.

The final criteria for tomato quality was the effect of TobAnPOD expression on the amount of solutes in the fruit. Soluble solids, which represent sugars and organic acids in the fruit, are a concern with processed tomatoes because of the energy required to remove excess water. Water stress would be expected to affect the solute solid concentration through decreased water content and decreased weight. On average, a 4-fold increase in solute concentration was observed in fruit obtained from plants expressing the TobAnPOD (Table 3). This value corresponds closely with the 30% decrease in fruit size. The increase in concentration is likely a consequence of the water stress induced by the expression of the tobacco anionic peroxidase.

Conclusions. The introduction of a chimeric TobAnPOD gene into tomato plants resulted in the synthesis of a new peroxidase isoenzyme with a pI of 3.75 in leaf and fruit tissue. Total peroxidase activity was increased by up to 1600-fold in fruit tissue with no apparent effect on ripening or color quality. However,

Table 2. POD activity from individual mature-green and red-ripe tomato fruit from transformed plants expressing the TobAnPOD. Total peroxidase activity in tomato fruit expressed in guaiacol units per gram fresh weight are presented for control OH 7814 and plants transformed with chimeric TobAnPOD gene.

	Mature green		Red ripe	
Plant	POD activity (units/g fresh wt)	Fold overproduction	POD activity (units/g fresh wt)	Fold overproduction
OH 7814	0.656	---	0.752	---
TobAnPOD77	221	336x	222	293x
TobAnPOD7B	1080	1646x	980	1303x

Table 3. Soluble solids concentration in red-ripe tomato fruit harvested from control (OH7814) and transformed tomato plants. High speed supernatant obtained from freshly harvested red-ripe tomato fruit was determined for refractive index and °BRIX (n = 3, SE < 1°BRIX).

Fruit type	Refractive index	°BRIX
OH7814	1.337	2.8
TobAnPOD77	1.349	10.8
TobAnPOD7B	1.353	13.1

these experiments with trangenic tomato plants showed that the TobAnPOD can have dramatic effects on root growth and water relations, which may likely have resulted in the observed 80% decrease in fruit size and 400% increase in fruit solute concentration. The presence of high levels of the TobAnPOD in both tobacco and tomato root tissue resulted in diminished root growth and chronic wilting upon reaching maturity. Although the mechanism of the inhibition of root growth remains unknown, this observation identifies the TobAnPOD or similar peroxidases from other species, it may be possible to genetically modify root growth and obtain considerable variation in plant water relations, perhaps affecting drought tolerance.

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