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BIOCHEMICAL, MOLECULAR, AND PHYSIOLOGICAL ASPECTS OF PLANT PEROXIDASES

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ALTERED PHENOTYPES IN PLANTS TRANSFORMED WITH CHIMERIC TOBACCO PEROXIDASE GENES

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INTRODUCTION

Peroxidases have been implicated in a variety of secondary metabolic reactions including lignification (5), cross-linking of cell wall polysaccharides (2), oxidation of indole-3-acetic acid (4,7), regulation of cell elongation (3), wound-healing (1), phenol oxidation (16), and pathogen defense (6). However, due to the many different isoenzymes and even more potential substrates, it has proven difficult to verify actual physiological roles for peroxidase. We are studying the molecular biology of the tobacco peroxidase genes, and have utilized genetic engineering techniques to produce transgenic plants which differ only in their expression of an individual peroxidase isoenzyme. Many of the *in planta* functions for any individual isoenzyme may be predicted through the morphological and physiological analysis of transformed plants.

Previous studies have shown that the anionic peroxidase of tobacco is loosely associated with the cell wall and is the predominant form in leaf and stem tissue (Fig. 1)(11). The anionic peroxidase of *N. tabacum* is a heme containing glycoprotein with relative molecular weights of 36,000 and 37,000 daltons and isoelectric points of 3.5 and 3.75 respectively (10). It was determined that both isoenzymes are encoded by a single gene and differ by post-translational modifications (8). This peroxidase isoenzyme is predominantly expressed in epidermal and xylogenic tissue (Fig. 2). The anionic isoenzyme is loosely associated with the cell wall, and readily polymerizes cinnamyl alcohols into lignin *in vitro* (12,13). It is not certain what other possible functions this isoenzyme performs. Described here are the construction of transgenic tobacco plants with either elevated or reduced peroxidase activity.

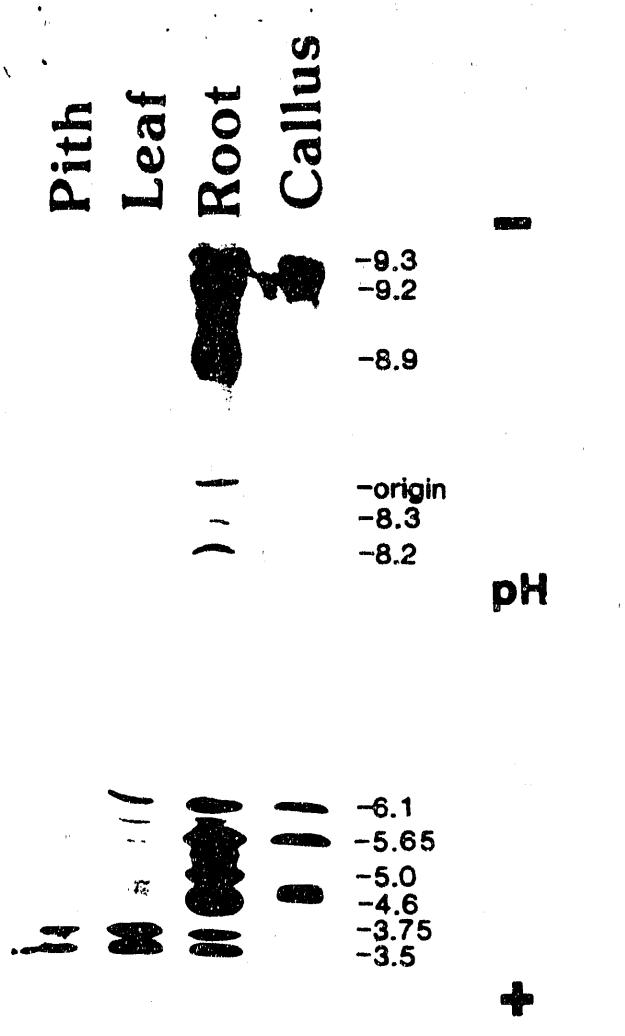

Figure 1. *Peroxidase isoenzymes of tobacco. N. tabacum tissue extracts were applied to isoelectric focusing gels then stained for peroxidase activity with 4-chloro-1-naphthol. Isoelectric points are indicated on the right.*

RESULTS and DISCUSSION

Gene isolation and plants transformations

A cDNA clone for the highly anionic peroxidase isoenzyme of tobacco has recently been obtained (10). The complete DNA sequence was determined, from which the predicted protein sequence was found to be ≈ 44% homologous to both horseradish and turnip cationic peroxidases (14,17). The messenger RNA for this gene was detected in large amounts in stem tissue and to a much lesser extent in leaf and root tissue. The tobacco anionic peroxidase cDNA (1252 bp Eco RI fragment) was inserted between the cauliflower mosaic virus (CaMV) 35S promoter and terminator such that the anionic isoenzyme would be produced at high levels in most tissues when transformed into tobacco plants (8). Suppression of the synthesis of the anionic isoenzyme was achieved through the use of antisense RNA (15). The 5' half of the peroxidase cDNA was inserted between the CaMV promoter and terminator in an inverted fashion such that antisense RNA would be produced (9). The antisense RNA will specifically inhibit the synthesis of the anionic peroxidase in transformed plants.

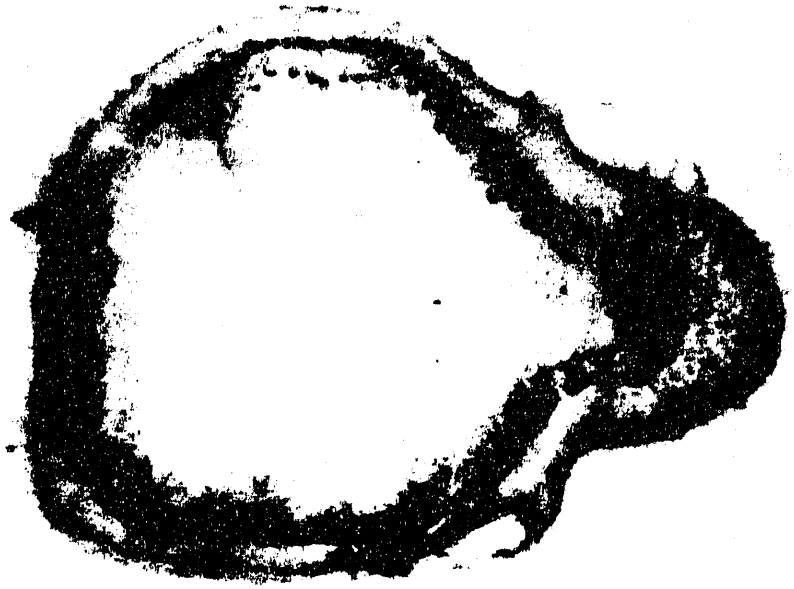

Figure 2. *Peroxidase tissue blot of tobacco stem. Fresh cut tobacco stem was pressed briefly onto wet nitrocellulose filter paper. Peroxidase activity was revealed by staining the filter in 4-chloro-1-naphthol. Xylem and epidermis stains heavily for peroxidase activity.*

Tobacco plants were transformed with *Agrobacterium tumefaciens* harboring recombinant plasmids with the altered peroxidase genes and kanamycin resistance genes, and were regenerated expressing either a chimeric antisense RNA gene for the anionic peroxidase or overproduce this same isoenzyme. Total leaf peroxidase activity in transformed *Nicotiana tabacum* and *Nicotiana sylvestris* plants was determined. Total peroxidase activity was increased by more than 10-fold in overproducer plants and was reduced by as much as 20-fold in antisense plants (Fig. 3). Peroxidase xymograms showed that the anionic peroxidase isoenzyme is selectively elevated or suppressed by the antisense RNA. The initial transformants *N. sylvestris* and *N. tabacum* were self-fertilized. The seed representing the R1 generation were sown, plants were grown to maturity, and also self-fertilized. Plants from the R2 generation which were kan ^{R}/kan^{R} and presumably homozygous for the antisense peroxidase gene were thus chosen on the basis of 100% germination of seed on agar plates containing kanamycin.

Analysis of transformed plants

The transformed plants displayed a number of distinctive phenotypes which could be directly correlated to altered peroxidase expression.

The most dramatic phenotype involves the chronic severe wilting in plants which overproduce the anionic peroxidase (8). These plants appear normal until approximately the time which they reach sexual maturity at which they begin wilting (Fig. 4). Plants do not respond to watering, however, improve upon exposure to low light.

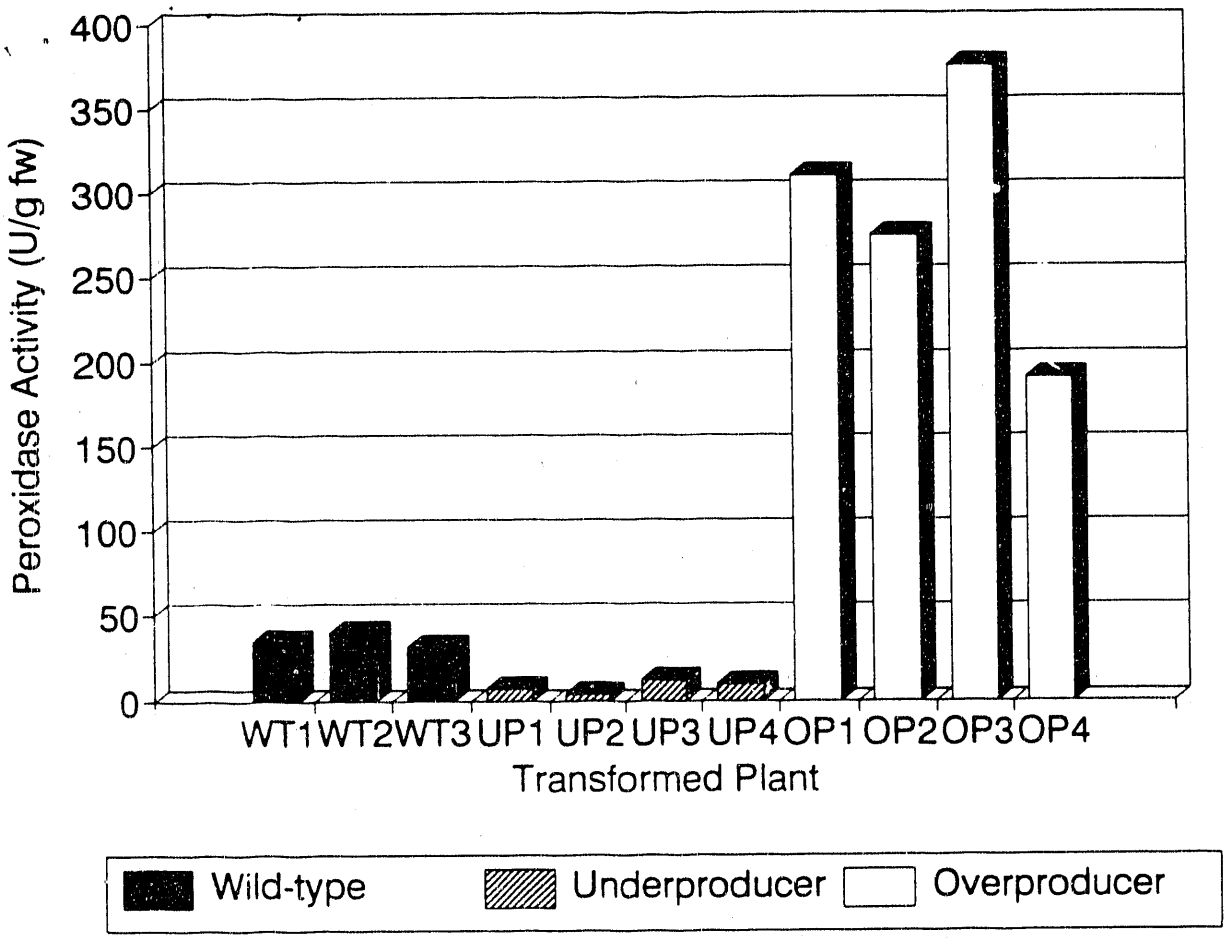

Figure 3. *Histogram of total leaf peroxidase activity in control and transformed tobacco plants.*

We have determined that the stomata are responsive and are tightly closed while leaves are wilting. Steady-state leaf porometer measurements agree with this result showing sub-normal transpiration through the leaf surface. Microscopic examination of leaf and stem tissue also reveals no visible defect in water transport. Pressure bomb and xylem conductivity measurements show that the vasculature in the shoot is fully capable of conducting water at wild-type levels (data not shown). Grafting experiments have recently shown that immature shoots (scions) from overproducer plants will not wilt when grafted onto wild-type root stock. We are further examining water absorption by the roots.

A relationship was found between leaf thickness and peroxidase expression. Leaf sections were prepared from equivalent, fully-expanded leaves from wild-type, twenty-fold peroxidase underproducer plants, and seven-fold peroxidase overproducer plants, then examined for cell size, number, and morphology (9,15). Higher peroxidase activity resulted in thinner leaves and smaller densely packed cells, while reduced peroxidase activity correlated with larger, loosely-packed leaf cells. Cell number between the upper and lower epidermis was unchanged in all the plants tested. Peroxidase overproduction resulted in leaves which averaged 30% thinner than

wild-type. Antisense RNA suppression of peroxidase activity resulted in leaves which averaged 17% thicker than wild-type. The differences in leaf cell size can be attributed to either a fluxuation in cell turgor pressure or extensibility of the leaf cell walls. A cell wall-associated peroxidase such as the one studied here could have an effect on either component of cell expansion.

Figure 4. *Wilting phenotype of transformed N. sylvestris. Mature wild-type (left) and peroxidase overproducer (right) plants.*

One of the predicted functions of peroxidase is in the catabolism of auxin as an indole-3-acetic acid oxidase (4,7). The tobacco anionic isoenzyme will efficiently decarboxylate IAA *in vitro*, and exogenously applied auxin is rapidly decarboxylated by tobacco plants (data not shown). These results along with the effect of peroxidase activity on leaf cell size led us to believe that peroxidase may control endogenous auxin levels. Analysis of IAA levels in wild-type, underproducer, and overproducer plants revealed no change in auxin levels (9). We can conclude that a cell wall-associated peroxidase is not involved in auxin catabolism, and that free IAA is not likely transported through the cell wall/apoplast. Presumably, either free IAA is transported through the symplast (plasmodesmata) or IAA is transported through the cell wall in a protected form (amino acid conjugates). These results do not exclude the possibility that an intracellular peroxidase may be involved in the catabolism of endogenous auxin.

Phenotypes were examined which relate to lignin synthesis and cell wall structure. Whole leaves were bleached with lactic acid: phenol: water then stained for lignin with phloroglucinol. A comparison of leaves from wild-type, overproducer, and under-producer plants are shown in figure 5. The extent of lignification of vascular tissue

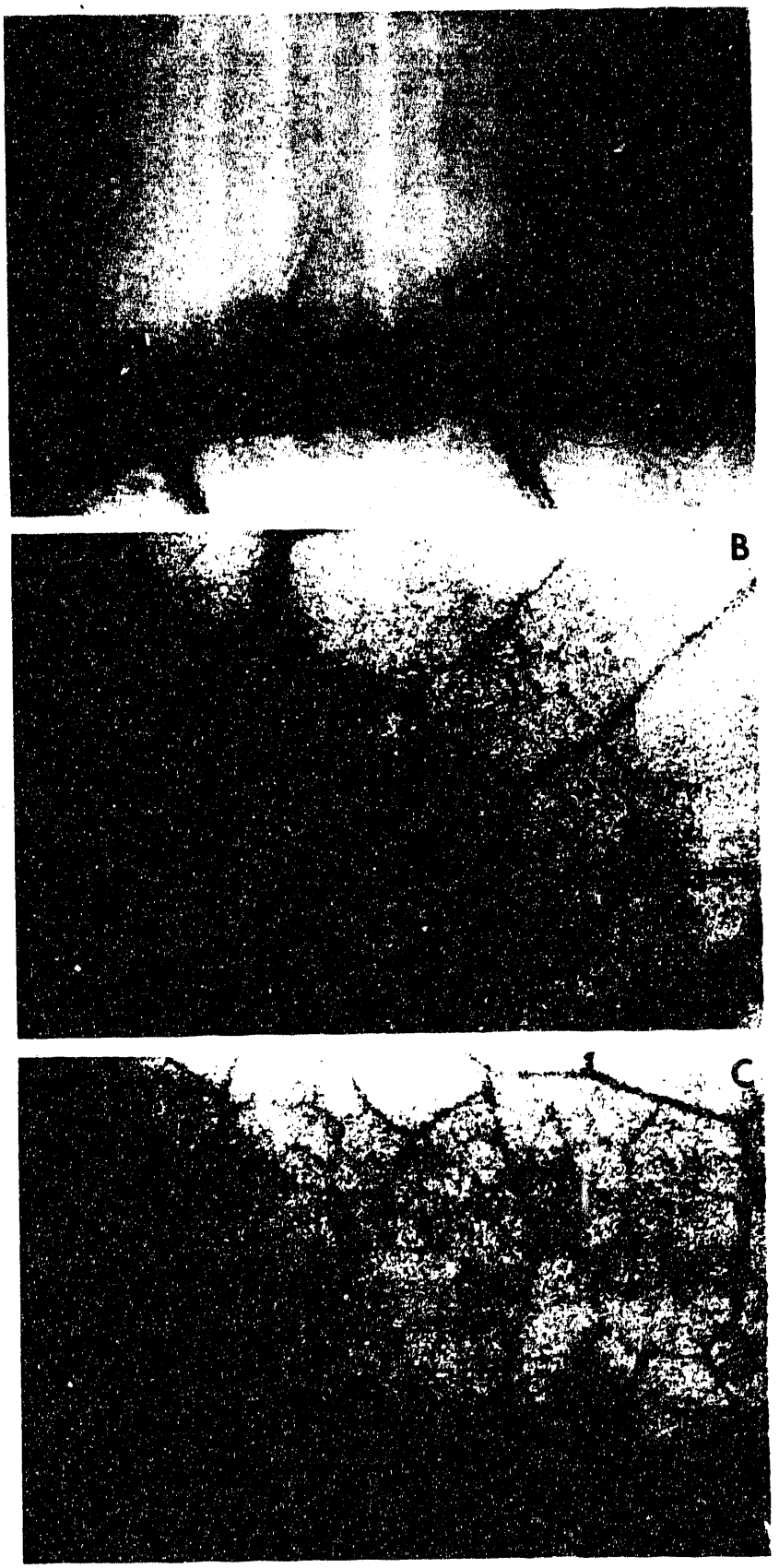

Figure 5. ***Lignin stain*** *in whole leaves from transformed plants. Equivalent* ***expanded leaves from peroxidase*** *underproducer (A), wild-type (B), and overproducer (C)* ***plants were bleached with*** *lactic acid : phenol : water and stained for lignin with phloroglucinol.*

and cell walls correlate well with peroxidase expression. Less peroxidase - less lignin. More peroxidase - more lignin. Another experiment was performed in which pith tissue from wild-type and underproducer plants was exposed to air for various periods of time. We found that wild-type tissue would dry out with minimal change in size,however, underproducer tissue would collapse to a fraction of its original size. (Fig. 6). We have not yet determined whether this relates to water loss or cell wall structure. Other phenotypes include: severe browning of wounded tissue in over-producer plants, defective seed germination in overproducer plants, and female infertility in peroxidase underproducer plants. It is hoped that each of these phenotypes will give us some insight into the *in planta* functions of this remarkable enzyme.

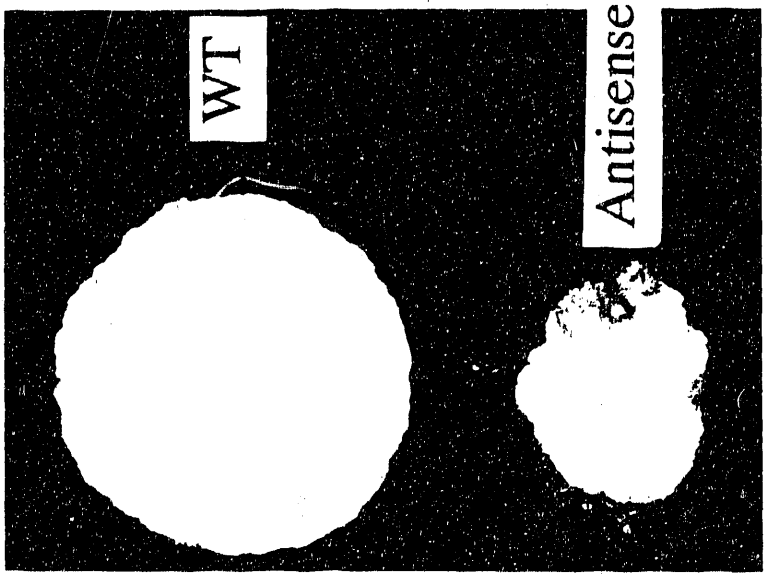

Figure 6. *Pith tissue from wild-type and peroxidase underproducer plants which has been exposed to air for 24 hours. Excised pith tissue was exposed to the air for 24 hours then photographed.*

Recently the chimeric CaMV 35S/tobacco anionic peroxidase gene has been transferred into tomato plants. These plants express the tobacco peroxidase at high levels and exhibit a similar phenotype of chronic severe wilting. The tomato fruit appears normal, however, browns rapidly upon wounding. The phenotypes described here appear to be general and not species specific. We are currently measuring lignin levels in tomato fruit, and their response to stress. Other tobacco peroxidase genes are being studied in the lab, and we are developing transgenic plants in which the spacial and temporal expression of peroxidase genes can be more closely regulated. This will prove useful in the determination of where and when peroxidase exerts its physiological effect. The results presented here illustrate the usefulness of molecular biology and genetic engineering as another tool in the study of a complex enzyme family such as peroxidase, yet highlights the need for a multi-disciplinary approach.

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