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THE MOLECULAR CHARACTERIZATION
OF THE
LIGNIN-FORMING PEROXIDASE

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PROGRESS SUMMARY REPORT
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SUMMARY

My research program focuses entirely on the study of the lignin-forming peroxidase of tobacco. Ever since our cloning and sequencing of the first plant peroxidase cDNA, we have pioneered in the introduction of the tools of molecular biology to the study of plant peroxidases. A significant part of our effort has been focused on the construction and analysis of transgenic plants which either over- or under-express the tobacco anionic peroxidase. This research has not only supported the role for this enzyme in lignification, but has opened the door to our understanding of additional metabolic functions including auxin metabolism and insect defense. As you will learn, this enzyme's role in auxin catabolism has led to numerous phenotypes in transgenic plants.

More recently, our attention has been directed towards the analysis of peroxidase gene expression. From this work we have learned that the anionic peroxidase gene is expressed at high levels in the xylem-forming cells, epidermis, and trichomes. This expression pattern supports its role in lignification and host defenses. We have also learned that this gene is down-regulated by auxin which indicates a strong relationship between auxin and the anionic peroxidase.

PUBLICATIONS ATTRIBUTABLE TO DOE FUNDING

1. Lagrimini LM, Joly RJ, Liu T-T Y (1994) **Peroxidase overproduction in transgenic plants inhibits root growth.** PLANT PHYSIOL (Manuscript submitted for publication).
2. Dowd PF, Lagrimini LM **Examination of transgenic tobacco and tomato expressing high levels of tobacco anionic peroxidase for resistance to insects.** ENVIRONMENTAL ENTOMOLOGY (In press).
3. Lagrimini LM (1993). **Analysis of peroxidase function in transgenic plants.** IN PLANT PEROXIDASES: BIOCHEMISTRY AND PHYSIOLOGY, Eds. K.G. Welinder, S.K. Rasmussen, C. Penel, H. Greppin, University of Geneva, pp. 301-306.
4. Lagrimini LM, Vaughn J, Erb WA, Miller SA (1993). **Peroxidase overproduction in transgenic tomato plants: Analysis of wound-induced polyphenol deposition and disease resistance.** HORTSCIENCE 28:218-221.
5. Diaz-De-Leon F, Klotz K, Lagrimini LM (1993). **Nucleotide sequence of the tobacco anionic peroxidase gene.** PLANT PHYSIOL. 101:1117-1118.
6. Sullivan J, Lagrimini LM (1993). **Transformation of *Liquidambar styraciflua*.** PLANT CELL REPORTS 12:303-306.
7. Lagrimini LM, Rubihayo P, Klotz K, Vaughn J, Berry S, Finer JM (1992). **Expression of a tobacco peroxidase gene in transformed tomato plants.** J. AM. SOC. HORT. SCI. 117:1012-1016.

PROGRESS REPORT

The objectives set forth upon the initiation of this project were *i)* to determine how lignin synthesis is regulated through the control of peroxidase gene expression and the availability of substrate, *ii)* to establish the role of the anionic peroxidase in auxin metabolism, with special attention to auxin transport and tissue responsiveness, *iii)* to elucidate all possible *in planta* reactions catalyzed by the anionic peroxidase through the use of transformed plants with elevated or reduced levels of this enzyme in specialized tissues, and *iv)* to characterize the anionic peroxidase gene, its patterns of expression, and potential *cis* and *trans*-acting regulatory elements. A summary of progress thus far which includes both published and unpublished work will be presented in 5 sections: (1) the effect of the TobAnPOD on root growth and development, (2) the role of the TobAnPOD in auxin metabolism, (3) the role of the TobAnPOD in lignification and insect resistance, (4) spacial and temporal localization of TobAnPOD gene expression, and (5) the effectors of TobAnPOD gene expression.

(1) Phenotypes of transgenic plants over- or under-expressing the TobAnPOD.

Phenotype of plants underexpressing the TobAnPOD. Transgenic tobacco plants which underexpress the TobAnPOD by > 20-fold (via antisense RNA) were observed for morphological and physiological differences. We observed that greenhouse-grown plants had thickened leaves and often had misshapen leaves with uneven edges and protruding midveins (Lagnini, 1992). Frequently, there are additional pairs of leaf lamina emerging from the midvein and secondary veins (Fig. 1, insert). The lamina always emerge as pairs with discernable abaxial and adaxial surfaces. This unusual phenotype was frequently seen in greenhouse-grown transgenic plants during the Summer months, however, was never observed when plants were grown in the Winter with supplemental lighting or in growth chambers (16 h photoperiod). We are currently trying to determine if this phenotype is initiated by temperature or light intensity. Transgenic plants with < 5% of the peroxidase activity of control plants were observed to grow considerably slower than control plants in

Figure 8. Growth progress for control and transformed tobacco plants underexpressing the TobAnPOD.

environmental chambers, therefore, we followed-up with an examination of growth rates. Control and transformed tobacco plants with 20-fold reduced peroxidase activity were grown in environmental controlled chambers at 28°C and 16h photoperiod. Three replicates were sacrificed at 30, 37, 44, 51, and 58 days post germination for shoot and root fresh and dry weight determinations. The total mean dry weights for control and experimental plants are plotted in Fig. 8. The plants with reduced TobAnPOD activity accumulate dry mass significantly slower than control plants, and are only slightly greater than 50% the mass of control plants at

flowering. This difference was reflected in both shoot and root mass. The shoot/root ratio was approx. 2.5 for all plants throughout the experiment. These experiments indicate the importance of the TobAnPOD in maintaining normal plant growth. Follow-up experiments will help determine if this phenotype is an effect of the enzyme's role in cell wall biogenesis or auxin metabolism. **Progress during granting period:** At the beginning of this granting period we had constructed transgenic plants which underproduce the TobAnPOD through the introduction of antisense RNA, however, we had no confirmed phenotypes. Was the TobAnPOD necessary for plant growth and development? We now know the TobAnPOD is required for normal plant growth, and a possible role for the enzyme in auxin metabolism is suggested.

Effect of the TobAnPOD on root growth and development. It has been known for some time that the ten-fold overexpression of the TobAnPOD in transformed plants using the CaMV 35S promoter results in chronic wilting. In our most recent publication (Lagrimini *et al.*, 1994), we describe this wilting phenotype in detail. Briefly, we discovered that the plants wilted because root growth decreased sharply as the plants matured until the root system was no longer able to supply sufficient water to the growing shoot. At the time of flowering the root mass was 30% that of control plants. Upon examination of the root system in transformed plants, we observed significantly less branching than control plants. We concluded that wilting resulted from insufficient root mass which was a consequence of suppressed root branching. There are two probable ways which peroxidase overexpression could result in less root branching. TobAnPOD overproduction which results in 9-fold higher lignin content in roots could restrict the outgrowth of lateral root primordia through the pericycle, or the enzyme's overproduction increases the rate of IAA turnover in roots which inhibits lateral branching. The latter model is supported by the loss of communication between the roots and the shoot. Why did the shoot continue to grow in the absence of root growth? A physical restriction of root growth should result in a suppression of shoot growth. **Progress during granting period:** At the beginning of this granting period we had a wilting phenotype in TobAnPOD overproducer plants with no understanding of the mechanism. Now we understand that the TobAnPOD inhibits root growth (root branching) in these plants, and a connection between TobAnPOD expression and auxin metabolism has been established.

Figure 9a. Root elongation rates for control and transformed tobacco roots in the absence of exogenous auxin.

Figure 9b. Root elongation rates for control and transformed tobacco roots in 50µM IAA.

(2) The role of the TobAnPOD in auxin metabolism.

As indicated above, there is an apparent correlation between TobAnPOD activity and auxin metabolism. It has been known for some time that peroxidase will oxidatively decarboxylate IAA *in vitro*, however, there has been considerable controversy over the role of peroxidase in the catabolism of IAA *in vivo*. Many of the phenotypes seen in transgenic plants with altered peroxidase activity reflect what may be an auxin effect. We have conducted several experiments to assess the effect of applied IAA on the growth and development of these transformed plants. In one experiment, TobAnPOD overproducing or control seedlings were placed onto MS medium containing no hormones or 50 μ M IAA. At seven day intervals the length of selected roots was measured. On hormone-free medium, control roots elongated somewhat faster than those roots which overexpressed the TobAnPOD (Fig. 9a). On medium containing 50 μ M IAA the elongation rate of control roots decreased sharply, however, those roots overproducing the TobAnPOD elongated at the same rate (Fig. 9b). This clearly indicates that the TobAnPOD is capable of interfering with auxin action, likely through the metabolism of IAA. In another experiment, tobacco plants overexpressing the TobAnPOD were hybridized with plants which were transformed with a chimeric gene containing an early auxin-induced promoter (IAA 4/5) joined to GUS (courtesy of S. Theologis). This allowed us to test if applied IAA was capable of inducing gene expression. The results shown in Fig. 10 indicate that the overexpression of the TobAnPOD blocked the induction of the IAA 4/5 promoter by auxin. **Progress during granting period:** Initially, we had phenotypes which resembled plants with altered auxin metabolism, however, we had no evidence that the TobAnPOD could oxidize IAA *in vivo*. We now know that transformed plants overexpressing the TobAnPOD are unresponsive to exogenous IAA at the level of gene expression.

Figure 10. Incubation of roots from control and transformed tobacco plants with varying concentrations of IAA for 24h.

(3) The role of the TobAnPOD in lignification and insect resistance.

Lignification in plants with suppressed TobAnPOD activity. There is considerable evidence that the TobAnPOD participates in the process of lignification. 1) The purified enzyme will catalyze the free radical polymerization of monolignols *in vitro*. 2) The enzyme is expressed at high levels in the cell walls of lignifying tissues. 3) The 10-fold overexpression of the TobAnPOD results in a 2-10 increase in lignin content. We obtained a 2-fold increase in lignin content in leaf tissue, a 4-fold increase in lignin content in stem tissue, and a 10-fold increase in lignin content in root tissue. This is strong evidence that the TobAnPOD can carry

Figure 11a. Lignin content in leaf samples from control (WT) and 4 plants underexpressing the TobAnPOD.

Figure 11b. Lignin content in coarse root samples from control (WT) and 4 plants underexpressing the TobAnPOD.

as determined by thioglycolic acid solubilization is shown for stem, leaf, and root tissues in Fig. 11. Although we found some variability between transformed plants, we often found >10% reduction of lignin content in leaf and root samples. It is interesting to note that we detected approx. a 10% increase in lignin content in woody stem tissue. This result could indicate that other enzymes may function in lignification or substitute for the TobAnPOD in its absence. It could also be indicative of the dual function for the TobAnPOD i.e., auxin metabolism. If peroxidase under-producer plants actually have higher auxin levels due to

out the free radical polymerization of monolignols *in vivo*. However, this does not prove that the TobAnPOD is the sole enzyme involved in lignification in the unaltered plant. To test if the TobAnPOD is the primary enzyme responsible for lignification, transgenic plants were constructed using antisense RNA driven by the CaMV 35S promoter to suppress peroxidase activity by as much as 97%. Several homozygous lines have been obtained in *N. sylvestris* and *N. tabacum* with >20-fold suppression of peroxidase activity. Lignin content

Figure 11c. Lignin content in woody stem samples from control (WT) and 4 plants underexpressing the TobAnPOD.

slower degradation, then auxin will promote vascularization i.e., lignification which could possibly mask the reduction of TobAnPOD activity. **Progress during granting period:** We knew at the beginning of the period that overexpression of the TobAnPOD resulted in higher lignin levels. Now we know that reduced TobAnPOD activity can lower lignin content in some tissues. We also have indications that this may not be the only enzyme involved in lignification, and the possibility that this enzyme may metabolize IAA could complicate the interpretation of the data.

Overexpression of the TobAnPOD results in enhanced insect resistance. Both transgenic tobacco and tomato plants overexpressing the TobAnPOD have been tested for insect resistance (Lagrimini et al., 1992, Dowd and Lagrimini, 1994). This analysis was performed because of published results indicating peroxidase and phenoloxidase may contribute to insect defense through the generation of reactive quinones. Briefly, transformed tobacco and tomato plants showed a significant increase in insect mortality in feeding studies with corn earworm and dusky sap beetle. Significantly fewer aphids were also counted on the leaves of transformed plants. **Progress during granting period:** An exciting new role for the TobAnPOD has been uncovered with the correlation of insect resistance with TobAnPOD expression. Future research will explore the usefulness of this enzyme in engineering "natural" insect resistance in crop plants.

(4) Spatial and temporal localization of TobAnPOD gene expression.

Another objective of this work was to characterize the TobAnPOD gene and identify its patterns of expression. We have completed the sequence of the TobAnPOD gene including 1.4kb of the promoter (Diaz et al., 1993). The transcription start site has been mapped by SI analysis, and examination of the promoter has revealed a TATA box and an AS-2 box. The AS-2 box has been implicated in shoot-specific expression. We have joined 3kb of promoter sequence to the GUS gene, and stably transformed this chimera into *N. tabacum* and *N. sylvestris*. Plant tissues at various stages of development have been developed for GUS activity with X-gluc to identify TobAnPOD expression patterns. We observed that the TobAnPOD gene is expressed in the trichomes throughout the plant including the flower petal (Fig. 2, insert). The gene is also expressed in the epidermis of the shoot and in xylem-forming cells (Fig. 3, insert). As the plant matures, expression is also detected in the secondary cambium and in the endodermis surrounding the vascular bundles in the shoot. In roots, expression is limited to older roots near the base (cortex) and at lateral root junctions (Fig. 4, insert). Gene expression is observed in cortical cells at the first indication of lateral root primordia formation (Fig. 5, insert), and continues in the cortical cells at the lateral root junction (Fig. 6, insert). Expression of the gene in the vascular tissue relates to its role in lignification. The gene's expression in the epidermis could be involved in growth and auxin metabolism. Its expression in the trichomes and the lateral root junctions may be related to a role in defense. We have already indicated that the overexpression of the TobAnPOD in transformed plants results in enhanced insect resistance (Dowd and Lagrimini, 1994).

Progress during granting period: It has been difficult localizing TobAnPOD expression through conventional staining methods because of numerous related peroxidases. The GUS chimera has permitted us to identify expression in tissues such as trichomes and lateral root junctions which may have gone unnoticed. Localizing gene expression is most valuable in defining possible functions for the TobAnPOD.

(5) The effectors of TobAnPOD gene expression.

In addition to spacial and temporal information concerning TobAnPOD gene expression, it would be very helpful in determining those signals which affect TobAnPOD gene expression. Tobacco protoplasts were transfected with the TobAnPOD promoter-GUS fusion gene and incubated with various phytohormones and activators of gene expression (Fig. 12). We found that benzyladenine and ethylene had no significant effect on expression levels. Gene expression was reduced 30% with 100 μ M abscisic acid and increased by 30% with 1 μ M gibberellic acid. TobAnPOD gene expression was significantly inhibited by IAA, with half-maximal suppression at 35 μ M IAA. The auxin inhibitor parachlorophenylisobutyric acid increased expression by 40% at 100 μ M, and negated the suppressive effect of IAA. When transformed roots (TobAnPOD/GUS) were incubated for 48h in 50 μ M IAA there was a 2-fold decrease in GUS activity. Incubation in the presence of the auxin inhibitor PCIB (100 μ M) resulted in a 2-fold induction of GUS activity. However, there was one region immediately behind the root cap where GUS activity increased in response to auxin (Fig. 7). **Progress during granting period:** Initially, we were unaware of which metabolic messengers would regulate TobAnPOD gene expression. We now know that auxin is a good inhibitor of TobAnPOD transcription. These results suggest that the expression of the TobAnPOD gene is under close regulatory control by auxin. Because the TobAnPOD also degrades auxin, an interesting feedback regulatory scheme is developing.

Figure 12. Relative GUS activity for transformed tobacco protoplasts incubated for 36h with various phytohormones.

Figure 1. Phenotype of transgenic plants underexpressing the tobacco anionic peroxidase.

Figure 2. Expression of the tobacco anionic peroxidase in the trichomes of flower petals.

Figure 3. Expression of the tobacco anionic peroxidase in tobacco stem sections.

Figure 4. Expression of the tobacco anionic peroxidase in lateral root junctions.

Figure 5. Expression of the tobacco anionic peroxidase in lateral root primordia.

Figure 6. Expression of the tobacco anionic peroxidase in lateral root junctions.

Figure 7. Expression of the tobacco anionic peroxidase in the auxin-treated root growing tip.