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MOLECULAR CHARACTERIZATION OF THE LIGNIN-FORMING PEROXIDASE: ROLE IN GROWTH, DEVELOPMENT AND RESPONSE TO STRESS

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PROGRESS SUMMARY REPORT

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SUMMARY

PUBLICATIONS ATTRIBUTABLE TO DOE FUNDING - 1992

Lagrimini, L. M. (1992). *Plant peroxidases: Under- and over-expression in transgenic plants, and physiological consequences* In PLANT PEROXIDASES 1980-1990: PROGRESS AND PROSPECTS IN BIOCHEMISTRY AND PHYSIOLOGY, Eds. Th. Gaspar and C. Penel, University of Geneva Press, In Press.

Diaz-De-Leon, F., Klotz, K. and Lagrimini, L. M. *Nucleotide sequence of the tobacco anionic peroxidase gene.* PLANT PHYSIOL. (In Press).

Sullivan, J. and Lagrimini, L. M. *Transformation of Sweetgum (Liquidambar styraciflua).* PLANT CELL REPORTS (In Press).

Lagrimini, L. M., Vaughn, J., Finer, J. M., Klotz, K., and Rubaihayo, P., *Expression of a tobacco peroxidase gene in transformed tomato plants.* J. AM. SOC. HORT. SCI. (In Press).

Chabbert, B., Monties, B., Liu, T.-T. Y., and Lagrimini, L. M. (1992). *Lignin content and composition in transgenic tobacco plants with altered peroxidase activity.* Proceedings of the Fifth International Conference for Biotechnology in the Pulp and Paper Industry, Kyoto, Japan.

Lagrimini, L. M., Liu, T-T and Joly, R. J. (1992). *Peroxidase overexpression in transgenic plants = wilting = decreased root growth.* Annual Meeting of the American Society of Plant Physiologists, Pittsburgh, PA, PLANT PHYSIOL. (SUPPLEMENT) **99**:35.

Sullivan, J. and Lagrimini, L. M. (1992). *Transformation of Liquidambar styraciflua.* Annual Meeting of the American Society of Plant Physiologists, Pittsburgh, PA, PLANT PHYSIOL. (SUPPLEMENT) **99**:46.

PROGRESS REPORT

This laboratory has continued its comprehensive study of the structure and function of plant peroxidases and their genes. Specifically, we are characterizing the anionic peroxidase of tobacco. During the past year we have (1) completed the nucleotide sequence of the tobacco anionic peroxidase gene, (2) joined the anionic peroxidase promoter to β-glucuronidase and demonstrated expression in transformed plants, (3) measured lignin, auxin, and ethylene levels in transgenic tobacco plants over-expressing the anionic peroxidase, (4) developed chimeric peroxidase genes to over- or under-express the anionic peroxidase in tissue specific manner in transgenic plants, and (5) over-expressed the tobacco anionic peroxidase in transgenic tomato and sweetgum plants.

(1) Complete Nucleotide Sequence of the Tobacco Anionic Peroxidase Gene.

Genomic DNA was isolated from *N. tabacum* leaf tissue and digested with *EcoR I* (* activity). These fragments were ligated into the lambda phage vector EMBL3 digested with *EcoR I*. A tobacco genomic DNA library consisting of over 100,000 genomic fragments was constructed. This library was screened with the TobAnPOD cDNA, and three genomic clones were isolated. Restriction analysis revealed that two of these clones were identical (λPOD1 and λPOD2). A second isolate, λPOD3 was unique. It was shown earlier that *N. tabacum* has four copies of the anionic peroxidase gene (Lagrimini *et al.*, 1987). By comparative blots of *N. tabacum*, *N. sylvestris*, and *N. tomentosiformis* DNA it could be determined that all clones were originally derived from *N. sylvestris*. Both λPOD1 and λPOD2 were determined to be pseudogenes by sequence analysis. The other unique genomic clone, λPOD3, was subcloned and subjected to DNA sequence analysis. The entire gene has been sequenced including more than 1000bp upstream from the start of transcription (Diaz-De-Leon *et al.*, in press). This lambda phage clone is 15kb in length, and contains 3.0 kb of 5'-flanking sequences. The transcription start site, TATA box, and GATA box directing expression to the shoot were identified.

(2) Chimeric Tobacco Anionic Peroxidase - β-Glucuronidase Gene.

Our objective is to characterize the tobacco anionic peroxidase promoter for *cis*-acting transcriptional elements through site-directed mutagenesis. Initially, the peroxidase promoter needed to be joined to the β-glucuronidase (GUS) gene to permit the detection of promoter activity. A single *Bgl II* site was inserted by site-directed mutagenesis just downstream from the transcriptional start site. A 1.5kb *EcoRI - BglII* promoter fragment was joined to the coding sequence of the GUS gene. This was followed by the CaMV 35S transcriptional terminator. This chimeric gene was tested for its efficacy by transforming into tobacco callus or leaf tissue, followed by a 48 hour incubation and localization of GUS activity with X-gluc. Transgenic tobacco plants harboring the chimeric peroxidase/GUS gene have been generated for further experimentation. The generation of promoter deletions is in progress.

(3) Analysis of Transgenic Plants Over-Expressing the Tobacco Anionic Peroxidase.

Transgenic plants which overproduce the anionic peroxidase by tenfold were phenotypically similar to wild-type plants until the plants flowered. Upon flowering the transformed plants began to wilt severely, respective of watering regime (Lagrimini *et al.*, 1990). We initially determined that wilting was not a result of water loss through the leaf surface, nor was it a result of abnormal vessel morphology in the stem and leaf veins. Since these results were published, we have measured leaf water potential, rate of water uptake, and root hydraulic conductivity. In all cases the values obtained were similar to control plants. Shoots from a peroxidase overproducer plant were grafted onto control root stock and *visa versa*. Shoots from a peroxidase overproducer plant did not wilt when grafted onto root stock of a control plant. This revealed that peroxidase overproduction exerts its effect on water relations through the roots.

Root growth rates were determined for control and overproducer plants. It was found that root mass increased at similar rates up until 40 days after germination. At this point, the roots from peroxidase overproducer plants stopped growing, although the shoot continued to grow at the same rate as the control plant. At 80 days post-germination the root mass of the peroxidase overproducer plant was 30% of the control plant for the equivalent shoot mass. Characterization of the root system at 80 days post-germination revealed that the control plant had >12 main root branches, while the overproducer plant had <4 main root branches. The length of the roots were similar in both plants; therefore, it was concluded that peroxidase exerted its effect at the level of root branching as opposed to elongation. Microscopic and chemical analysis of transformed roots revealed similar morphology to control plants; however, lignin levels were found to be 10-fold higher in coarse roots from plants overproducing peroxidase. It is currently not known if higher lignin levels are responsible for the marked decrease in root mass.

The decrease in branching of roots in transgenic plants over-expressing the tobacco anionic peroxidase is indicative of lower than normal auxin levels. Because peroxidase will oxidatively decarboxylate IAA *in vitro*, auxin levels were quantified in transgenic plants. Steady-state IAA levels in shoot tissues from peroxidase overproducer plants was similar to control plants at all stages of development. However, IAA levels in root tissue from transformed plants went from 500 ng/g fw before wilting to <100 ng/g fw after wilting. How this precipitous decline in IAA is related to peroxidase expression and decreased root growth will be the subject of further investigations. If increased peroxidase production results in accelerated IAA breakdown, root response to exogenously applied IAA should be suppressed in transformed root tissue. This hypothesis was tested by adding IAA or 2,4-D to growing roots, and measuring elongation rates and the extent of branching. As predicted, both auxins slowed root elongation and increased branching of control roots. Roots of peroxidase overproducer plants were effected equally with 2,4-D; however, root growth was insensitive to even the highest levels of IAA. This indicated that, for exogenous IAA, elevated peroxidase expression interferes with the physiological response to auxin. We are further characterizing the effect of peroxidase overproduction on endogenous IAA metabolism.

(4) Tissue Specific Over- and Under-Expression of the Tobacco Anionic Peroxidase.

Thus far, peroxidase expression has been altered in transgenic plants with utilized the CaMV 35S promoter. This promoter directs high levels of expression in most tissues with preference to phloem and epidermal tissue. This allowed us to gain information on potential anionic peroxidase functions *in planta*; however, more detailed information could be accessed only through more localized expression. We have already obtained promoters which are root specific, leaf specific, xylem specific, and inducible by heat shock or infection. The root promoter has been joined to both a sense and antisense anionic peroxidase coding sequence. These have been successfully transformed into tobacco, and will soon be tested for peroxidase activity in root tissue. The other constructs are currently being made.

Over-expression of the tobacco anionic peroxidase in transgenic plants resulted in increased lignification of leaf tissue (2-fold), stem tissue (2-fold), and root tissue (9-fold). Also, lignification occurred more rapidly in wounded tobacco pith tissue which over-expressed peroxidase. These observations suggest that the tobacco anionic peroxidase is required for lignification; however, we have not found lower lignin levels in transgenic plants which underproduce (antisense RNA) this enzyme by as much as 20-fold. This may indicate that this isoenzyme may not be the only isoenzyme capable of lignin synthesis, or the 35S promoter currently being used is not functional in xylem-forming tissue. We will attempt to obtain better suppression in lignin-forming tissue by expressing antisense RNA from the anionic peroxidase promoter. This promoter has previously been shown to be highly active in xylem-forming tissue.

(5) Expression of the Tobacco Anionic Peroxidase in Other Species.

Although not originally proposed, we have begun investigating the effects of the tobacco anionic peroxidase in other species. We have chosen two additional species for experimentation, *Lycopersicon esculentum* and *Liquidambar styraciflua*. We chose tomato because of the close relationship to tobacco and the relative ease of transformation to investigate properties such as disease and insect resistance. The wound-induced lignification and phenol deposition was similar in tomato fruit (Lagrimini *et al.*, in press) as was seen in tobacco. We screened for enhanced disease resistance in tomato seedlings expressing the tobacco anionic peroxidase. Young plants were challenged with *Fusarium* wilt, *Fusarium* rot, *Verticillium* wilt, and TMV (Lagrimini *et al.*, in press). No resistance was found to any of the pathogens as a consequence of peroxidase expression. Mature plants may be better protected by the tobacco enzyme; therefore, we are interested in challenging older plants and fruit with several pathogens.

Both transgenic tobacco and tomato plants were challenged with insect pests. We used corn earworm on tobacco basal stems and tomato fruit, dusky sap beetle on tomato stems, and aphids on tobacco leaves (Dowd and Lagrimini, manuscript in preparation). In all cases there was a significant increase in insect mortality and decrease in survivor weight in transgenic plants over-expressing the tobacco anionic peroxidase. We have speculated that peroxidase-induced insect resistance comes from either a perceivable change in cell wall texture or increased levels of anti-nutritive

phenolic compounds.

Liquidambar styraciflua (American sweetgum) was chosen because of its importance as a source of paper pulp, and the tobacco anionic peroxidase's role in lignification. A suitable transformation system for sweetgum was not available; therefore, development of such a system was essential for future genetic engineering experiments. An *Agrobacterium*-based transformation system was developed using shoot formation from cut leaves and petioles (Sullivan and Lagrimini, in press). Several plants have been generated which synthesize the tobacco anionic peroxidase. Sweetgum was also shown to possess a putative lignin-forming peroxidase with extensive homology to the tobacco isoenzyme.

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