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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 - 1993

Dowd, P. F. and Lagrimini, L. M. ***Transgenic tobacco and tomato over-expressing tobacco anionic peroxidase are resistant to insects.*** ENTOMOLOGIA EXPERIMENTALIS ET APPLICATA (In press).

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Lagrimini, L. M., Vaughn, J., Erb, W. A. and Miller, S. A. (1993). ***Peroxidase overproduction in transgenic tomato plants: Analysis of wound-induced polyphenol deposition and disease resistance.*** HORTSCIENCE 28:218-221.

Diaz-De-Leon, F., Klotz, K. and Lagrimini, L. M. (1993). ***Nucleotide sequence of the tobacco anionic peroxidase gene.*** PLANT PHYSIOL. 101:1117-1118.

Sullivan, J. and Lagrimini, L. M. (1993). ***Transformation of Liquidambar styraciflua.*** PLANT CELL REPORTS 12:303-306.

Liu, T-T, Y. and Lagrimini, L. M. (1993). ***Lignin content and composition in tobacco plants with over and under expressed peroxidase.*** PLANT PHYSIOL (SUPPLEMENT) 102:579.

PROGRESS REPORT

Our group continues to focus on the characterization of the tobacco anionic peroxidase and its genes. Throughout this past year we have (1) generated transgenic plants expressing β -glucuronidase under control of the anionic peroxidase promoter, (2) characterized effectors of peroxidase gene expression in transformed protoplasts, (3) generated numerous transgenic plants which over- and under-express the anionic peroxidase in a tissue specific manner, (4) characterized the role of the anionic peroxidase in the metabolism of auxin, (5) introduced a marker (flag) into the anionic peroxidase primary protein sequence which will permit the identification of the recombinant protein in plant tissue, and (6) described the enhancement of insect resistance as a result of over-expression of the anionic peroxidase. Although our research program has continued along the lines of the original proposal, we have redirected a significant effort to the role which this enzyme plays in the metabolism of auxin, and conversely, the role which auxin plays in regulating the expression of the anionic peroxidase gene.

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

Our objective is to characterize the expression of the tobacco anionic peroxidase promoter with respect to its spacial and temporal patterns of expression, defining the effectors of gene expression, and the localization of *cis*-acting transcriptional elements through site-directed mutagenesis. The peroxidase promoter including 3kb of upstream sequences was joined to the β -glucuronidase (GUS) coding sequence. The resulting chimeric gene was introduced into tobacco using *Agrobacterium tumefaciens*. Fifteen plants for each *N. tabacum* and *N. sylvestris* were tested for expression by staining leaf samples with X-GLUC. As predicted, expression was localized to the vascular tissue and the epidermis. Five plants of each species were self-fertilized and progeny are currently being produced.

While transgenic plants were being grown out, the promoter-GUS gene was tested in a transient expression system using tobacco leaf protoplasts. A plasmid containing the chimeric gene was transformed into protoplasts with polyethylene glycol (PEG), incubated for 36 hours at 28°C in the dark, then a soluble extract was assayed for GUS activity fluorometrically (MUG). The results from 4 replicates were expressed as μ moles MU/min/mg protein.

(2) Characterized effectors of peroxidase gene expression in transformed protoplasts.

Protoplasts were incubated in the presence of possible effects such as IAA, BA, GA, ABA, ethylene, salicylate, methyl jasmonate, fungal glucan elicitor, and heat shock. The only effector which was found to alter promoter activity was IAA, which suppresses peroxidase activity in a concentration-dependent manner. Maximal suppression by IAA or NAA was 12-fold, and half-maximal suppression occurred at 30 μ M IAA or NAA. Similar results were found when peroxidase messenger RNA was quantified on RNA blots for leaf disks floated on medium containing IAA or NAA. The auxin inhibitor PCIB completely reversed the effect of IAA. Also, the inhibitor alone

increased the control level of promoter activity by as much as 50%. This result may indicate that endogenous IAA provides some degree of promoter suppression which is released by the competitive inhibitor.

These observations support our hypothesis that the anionic peroxidase plays a critical role in growth and development as opposed to many other peroxidases whose expression is regulated by external factors (stress and infection). It is also exciting to note that this is the first "constitutive" promoter characterized which is suppressed by IAA. There are many possibilities why the plant should wish to "turn off" this peroxidase with auxin. One attractive model proposes that the anionic peroxidase stops cell expansion through the introduction of covalent bonds between cell wall polymers. Those cells adjacent to the growing tip undergo expansion in part to the production of IAA in the meristem, and this same IAA keeps the anionic peroxidase in check at the transcriptional level, thus preventing any undesired cross-links in the wall. Another model involves the anionic peroxidase's role in IAA metabolism. This will be discussed later.

We are also interested in locating those upstream sequences which are important in directing tissue-specific expression and auxin down regulation. These sequences are being mapped by promoter deletions. Existing restriction sites have been used to create six 5' deletions in the peroxidase-GUS gene. The 3' deletions are being created with Exonuclease III. One feature in the peroxidase promoter which will be looked at is an AS-2 box (GATANGATA). This has been shown by others to be important in shoot-specific expression, which corresponds to the expression of the anionic peroxidase. An intact peroxidase-CAT gene will serve as a control. These deletions will be characterized in both protoplasts and transgenic plants.

(3) Generated numerous transgenic plants which over- and under-express the anionic peroxidase in a tissue specific manner.

Plants which either over- or under-express the tobacco anionic peroxidase have exhibited numerous phenotypes attributable to the altered expression of this enzyme. In many cases it has been difficult to trace the biochemical events which connect enzyme and phenotype. We have been hampered by the indiscriminate expression of the CaMV 35S promoter throughout the plant. We are proceeding to focus on the site of peroxidase action through the use of tissue-specific promoters to alter the expression of the anionic isoenzyme. Gene constructs which use a root-specific promoter, a leaf-specific promoter, and the anionic peroxidase's own promoter joined to either sense or antisense versions of the anionic peroxidase (cDNA) have been transformed into *N. tabacum* and *N. sylvestris*. Between 15-20 primary transformants for each construct in each species are beginning to flower in the greenhouse.

Tissue from these plants have been assayed for peroxidase activity to verify the gene construct and for actual transformation. Peroxidase activity has varied by as much as ten fold. Thus far, only one phenotype has been observed. A plant overexpressing peroxidase with a root-specific promoter was decapitated above the soil. The pith and vascular tissue through the stalk was stained black. Previously with the 35S promoter we observed some browning of the stem tissue, but we were quite impressed to see black, especially with a root-specific promoter. We will determine if

the peroxidase synthesized in the root is transported up the stem or if phenolic radicals formed by peroxidase in the roots are mobilized throughout the stem.

(4) Characterized the role of the anionic peroxidase in the metabolism of auxin.

We previously determined the purified anionic peroxidase will catalyze the oxidative decarboxylation of IAA with a K_m of $620\mu M$. Our objective is to see if this isoenzyme has a physiological role in the metabolism of IAA *in planta*. Previous measurements have shown both free and conjugated IAA concentrations were unchanged in transgenic plants. However, all morphological and physiological assessments of IAA action have shown a relationship between peroxidase levels and auxin-related growth effects. Leaf cells are smaller in transgenic plants with high peroxidase activity and larger in plants with low peroxidase activity. 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.

From the above observations it is clear that transformed plants exhibit increased (antisense) or decreased (sense) response to exogenous IAA. The following experiment was performed to examine endogenous auxin response. Shoot tips excised from control and transformed plants were placed on hormone-free media to permit rooting. The rate of root formation was used as an indicator of endogenous auxin activity. The data obtained were consistent with a role for peroxidase in the metabolism of IAA. Control cuttings produced on average 1 new root per day, plants over-expressing peroxidase produced 0.5 new roots per day, and plant under-expressing peroxidase produced 2 new roots per day. This is consistent with auxin's role in root formation, and peroxidase's role in removing IAA.

(5) Introduced a marker (flag) into the anionic peroxidase primary protein sequence which will permit the identification of the recombinant protein in plant tissue.

There are two reasons for introducing a tag into the primary sequence for the tobacco anionic peroxidase. 1) This allows one to perform site-directed mutagenesis on the protein coding sequence, express the engineered protein in transformed tobacco, then recover the altered enzyme with an affinity column. Biochemical properties of the engineered protein can then be determined *in vitro*. 2) This allows for localization of the anionic isoenzyme in tissue samples without interference from other isoenzymes. This is particularly important for peroxidase because of the homology between isoenzymes which limits the usefulness of antibodies.

We have synthesized an oligonucleotide which includes the code for 7 amino acids for the Kodak/IBI Flag peptide. Commercial monoclonal antibody can be purchased which recognizes this sequence. The Flag sequence was introduced by

site-directed mutagenesis at the C-terminus of the anionic peroxidase coding sequence. This site was selected by virtue of accessibility to antibody and minimal disruption of the protein. The synthetic peroxidase/Flag gene included the CaMV 35S promoter/terminator. This chimeric gene was transformed into tobacco with *Agrobacterium* and selected on kanamycin. A protein extract from leaf tissue of transformed plants was subjected to SDS-PAGE and blotted onto nitrocellulose. The Flag-M2 antibody was found to specifically recognize a single protein of 38kD. No binding was detected on protein from control plants. Also, the Flag antibody has been used to affinity purify the engineered protein, which has retained full peroxidase activity.

(6) Described the enhancement of insect resistance as a result of over-expression of the anionic peroxidase.

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.

CONTINUATION PROPOSAL

The characterization of the tobacco anionic peroxidase will continue essentially as outlined in the original proposal. Our objectives remain directed toward the understanding of peroxidase function in plants with the eventual goal of manipulating its expression to benefit plant health and productivity. I will briefly outline future directions in our research.

Peroxidase Gene Expression - Tissue Specific Expression

Transgenic plants harboring the anionic peroxidase/GUS reporter gene will be sectioned and stained with X-Gluc for localization of gene activity. By studying various tissues over the life of the plant we may better understand how this enzyme functions in growth and development. Transgenic plants with deleted regions of the peroxidase promoter will be examined for differences in expression. These patterns will be correlated with *cis*-acting elements within the promoter.

Peroxidase Gene Expression - Auxin Suppression

Now that we know that IAA will suppress the peroxidase promoter, we will use the deletion mutants to locate a *cis*-acting auxin suppressible element. We will also examine the effects of auxin application on transformed plant tissues to expand upon our protoplast results. Longitudinal sections of root and shoot meristems will be stained for GUS activity. If this enzyme is involved in cross-linking cell walls, we should see a correlation of GUS activity with the cessation of cell expansion.

Tissue Specific Over- and Under-Expression of the Tobacco Anionic Peroxidase

The transformed plants we have obtained will be thoroughly characterized for phenotypes including lignin synthesis, auxin metabolism, and phenolic metabolism.

Peroxidase-Mediated Auxin Catabolism

We will continue to perform physiological tests for auxin activity in transgenic plants over- or under-producing the anionic peroxidase. We will work with Dr. Michael Evans in the Plant Biology Department at OSU to examine root growth responses using real-time digitized video pictures. Auxin will be added to young transgenic seedlings to search for differences in auxin sensitivity relative to peroxidase expression. Also, in the absence of exogenous auxin, seedlings will be gravity stimulated to ascertain temporal differences based on peroxidase expression.

Site-Directed Mutagenesis of Peroxidase-FLAG

With the previous successes in hand we are currently performing site-directed mutagenesis on the POD-Flag protein. Three amino acids predicted to be critical in peroxidase function are being changed. His 42 is being changed to Gln, His 167 is

being changed to Ans, and Phe 140 is being changed to Ala. These altered proteins will be subjected to thorough kinetic analysis. Our primary objective is to determine those functional side groups critical to IAA oxidation, as opposed to phenolic peroxidation.

Peroxidase-Mediated Insect Resistance

Transgenic plants over-expressing the tobacco anionic peroxidase will be analyzed for phenolic composition to identify those compounds responsible for insect resistance.

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