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“Mechanisms of Lignin Biosynthesis During Xylogenesis in
Zinnia elegans”

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The original plan for this project was to use *Zinnia elegans* and its xylogenic cell differentiation system to dissect the role(s) played in lignin deposition by the three classes of laccases (*p*-diphenoloxidases) we had previously detected in zinnia stem extracts. The project was broadly divided into two general areas -- biochemistry/enzymology and molecular biology/genetic engineering -- each of which was pursued by one of two graduate students. As noted in the attached publication list, DOE funding for this project facilitated the publication of five (5) research papers, and two (2) more papers slated for peer-review publication are currently nearing completion. In addition, our research results were presented at nine (9) national and international scientific meetings over the past four years.

Some of the major findings reported in these papers and presentations are summarized below.

From *LaFayette et al. 1995 Plant Physiology* 107: 667-668:

- characterization of the first full-length laccase cDNA cloned from any plant (*Acer pseudoplatanus*).

From *Eriksson et al. 1995 Proc. 6th Intl. Conf. Biotech. Pulp and Paper Ind., Vienna, pp. 310-314Z*:

- demonstration that plant laccases are encoded by small gene families and that these families are sufficiently diverged as to prevent cross-hybridization of gene sequences, even under conditions of low-stringency;
- first demonstration of regenerable plant tissues transformed with laccase antisense gene constructs.

From *Dean et al. 1998 ACS Symp. Ser. 697:96-108*:

- reduction in stem lignin content in *Z. elegans* plants grown under copper-deficient conditions was shown to be correlated with a reduction in extractable laccase activity;
- demonstration that the relative abundances of high- and low-pI laccases in *Z. elegans* stem extracts varied with developmental stage of the plant;
- first demonstration of heterologous expression of a plant (*Acer*) laccase in transgenic plant cells;
- negative results for first attempt to reduce lignin content through down-regulation of laccase gene expression by transformation of yellow-poplar with an antisense laccase gene construct;
- demonstration that constitutive over-expression of *Acer* laccase in transgenic plants leads to a severely stunted phenotype.

From *LaFayette et al. 1999 Plant Mol. Biol.* 40:23-35:

- isolation and characterization of four different laccase cDNAs from a single plant species (*Liriodendron tulipifera*), as well as a laccase gene from rice (*Oryza sativa*);
- demonstration that although the gene products were strongly conserved, they were expressed to very different levels in lignifying xylem tissue;
- demonstration by heterologous expression in tobacco that all of these cDNAs encoded functional laccase enzymes.

From Liu *et al.* 1999 *Phytochemistry* (in press):

- demonstration in *Z. elegans* that a widely used histochemical method for detecting H_2O_2 in plant tissues is prone to misinterpretation due to wounding effects;
- demonstration that *Z. elegans* produces large amounts of H_2O_2 in peripheral collenchyma tissues where it may contribute to cross-linking and rigidification of non-lignified cell walls used for plant support.

The findings reported in these papers and in our presentations at meetings have garnered a great deal of interest, and several other laboratories have recently begun focusing increased attention on the potential role of laccases in lignin formation. Thus, DOE support for our work has led to findings that have stimulated a great deal of new research.

However, productivity on this project has lagged behind our expectations. Early in the project period, three unrelated problems arose making it difficult to achieve the project goals outlined in the original proposal. In early 1996, Dr. Dean left the Department of Biochemistry and Molecular Biology, but remained at the University of Georgia to establish a new laboratory in the Warnell School of Forest Resources. Both of the graduate students assigned to work on the project moved to the new laboratory, but research was disrupted for 6 to 7 months while the new laboratory was setup and reorganized. A 12-month no-cost extension of the grant period was requested and granted to cover time lost in the move.

A second problem arose in late 1997 and continued throughout 1998, when the senior graduate student responsible for molecular biology efforts on the project began a struggle with a neurological condition that limited her ability to work in the laboratory and eventually forced her to leave school in March 1999 without completing her Ph.D. We have had a difficult time trying to pick up the pieces of work she left unfinished, but we are making progress with the help of Dr. Bonnie McCaig who recently joined the lab to study laccase gene family phylogenies with the help of a NSF/Alfred P. Sloan Postdoctoral Fellowship in Molecular Evolution. (It is worth noting that it was the work on laccase genes accomplished with DOE support that enabled us to attract Dr. McCaig and her Fellowship to this laboratory.) We hope to be able to salvage enough material from the student's project to warrant publication with acknowledgment of DOE support. In particular, we are hoping to completely characterize the remaining gene sequences for one partial cDNA and two partial genomic DNAs that the student isolated from *Z. elegans*, but was unable to finish before her departure.

The third difficulty was principally a problem of biology which stemmed from the relatively low levels of laccase that can be recovered from *Z. elegans* stems. As noted in previous Progress Reports, maximal laccase activity can be extracted from *Zinnia* stems during a fairly short developmental window when the plant bolts to flower, and the enzyme yield from stems harvested at this time is highly dependent on the conditions under which the plant was grown. Thus, optimal yields are obtained if stems from plants grown in the greenhouse at midsummer (15 hr daylength) are extracted just before the apparent flower buds begin to open. However, the total amount of laccase enzyme that can be recovered from these prime tissues has been too low for us to complete our planned biochemical characterizations. However, our efforts in this regard are continuing, and we expect to have a research paper recounting our results submitted for peer-review within six months. (Completion of the paper has been made a prerequisite for graduation of the student working on this aspect of the project. The expected paper is noted on the Publication List as being *in*

preparation.)

This difficulty in recovering adequate amounts of active laccase from *Zinnia* stems was a major rationale for our efforts to clone laccase cDNAs from *Zinnia*. We had hoped to express the gene products heterologously as a way to easily generate sufficient enzyme for our biochemical characterizations and for the development of specific antibody probes. Because the student responsible for the molecular biology efforts was unable to isolate and express full-length clones in a reasonable amount of time, the student who had planned to do the biochemical characterizations for his dissertation work has focused most of his attention on characterizing laccase gene products, particularly the one from *A. pseudoplatanus*, heterologously expressed in transformed tobacco and yellow-poplar cells. He is currently working on a second draft of a manuscript describing his results from this work, and this is also noted on the Publication List as a manuscript *in preparation*. Of particular interest for our future work on this project have been his results using airlift bioreactors to maximize laccase production from transformed cell lines. His results have shown that once we recover a laccase cDNA, we can produce large quantities in liquid cultures of transformed plant cells, and this is significant because we were unable to do this using either bacteria or yeast.

DOE-SPONSORED PUBLICATIONS

Research Articles

LaFayette, P., Eriksson, K-E.L. and *Dean, J.F.D. (1995). Nucleotide sequence of a cDNA clone encoding an acidic laccase from sycamore maple (*Acer pseudoplatanus* L.). *Plant Physiol.* 107: 667-668.

(Note that the footnotes of this paper credit support to our previous DOE grant no. DE-FG02-92ER20082. This was an error that occurred because the paper was submitted for publication before we noticed that the project number had been changed for the renewal period. This manuscript was not listed in the Publication List include with our Final Report for that previous grant.)

Eriksson, K-E.L., LaFayette, P.R., Merkle, S.A. and Dean, J.F.D. (1995). Laccase as a target for decreasing lignin content in transgenic trees through antisense genetic engineering. *In: Proc. 6th International Conference on Biotechnology in the Pulp and Paper Industry*, E. Srebotnik and K. Messner (eds.), Facultas-Universitätsverlag, Vienna, pp. 310-314.

Dean, J.F.D., LaFayette, P.R., Rugh, C., Tristram, A.H., Hoopes, J.T., Merkle, S.A., and Eriksson, K-E.L. (1998). Laccases associated with lignifying tissues. *In: Lignin and Lignan Biosynthesis*, N.G. Lewis and S. Sarkanen (eds.), ACS Symp. Ser. 697: 96-108.

LaFayette, P., Merkle, S.A. Eriksson, K-E.L. and *Dean, J.F.D. (1999). Characterization and heterologous expression of laccase cDNAs from the lignifying xylem of yellow-poplar (*Liriodendron tulipifera*). *Plant Mol. Biol.* 40: 23-35.

Liu, L., Eriksson, K-E.L. and *Dean, J.F.D. (1999). Localization of hydrogen peroxide production in *Zinnia elegans* L. stems. *Phytochemistry (In press)*

Hoopes, J.T., Eriksson, K-E.L. & Dean, J.F.D. (2000). Characteristics of a sycamore maple laccase secreted by transformed tobacco cells in suspension culture. *Biochemistry (in preparation)*.

Hoopes, J.T., Eriksson, K-E.L. & Dean, J.F.D. (2000). Characteristics of a high-pI laccase isolated from stems of *Zinnia elegans* L. *Plant Physiol. (in preparation)*.

Meeting Abstracts

LaFayette, P., Merkle, S.A., Eriksson, K-E.L., and Dean, J.F.D. (1994). Molecular characterization of hardwood laccase genes and their application in lignin reduction via antisense technology. *Proc. 2nd Internatl. Symp. on Applications of Biotechnology to Tree Culture, Protection and Utilization*, Oct. 2-6, 1994, Minneapolis, MN. US Forest Service General Technical Report NC-175.

LaFayette, P.R., Rugh, C.L., Merkle, S.A., Eriksson, K-E.L. & Dean, J.F.D. (1995). Laccase as a target for decreasing lignin content. 23rd Southern Forest Tree Improvement Conference, June 20-22, 1995, Asheville, NC

LaFayette, P.R., Merkle, S.A., Eriksson, K-E.L. & Dean, J.F.D. (1995). Molecular characterization of hardwood laccase genes. *Plant Physiol.* 108S:180.

Hoopes, J.T., Tristram, A.H., LaFayette, P.R., Eriksson, K-E.L. & Dean, J.F.D. (1995). Isolation of laccases and their encoding genes from *Zinnia elegans*. *Plant Physiol.* 108S:325.

Dean, J.F.D., LaFayette, P.R., Tristram, A.H., Hoopes, J.T., Eriksson, K-E.L. (1995). Laccase involvement in lignification. Proc. 7th Cell Wall Meeting, Sept. 26-29, Santiago de Compostela, Spain.

Kim, M.K., LaFayette, P.R., Dean, J.F.D. & Merkle, S.A. (1997). Characterization of laccase genes from sweetgum (*Liquidambar styraciflua* L.). 24th Southern Forest Tree Improvement Conference, June 9-12, Orlando, FL-1068.

LaFayette, P.R. & Dean, J.F.D. (1997). Characterization of four laccase genes which are differentially expressed in cambium/lignifying tissue of yellow-poplar (*Liriodendron tulipifera*). *Plant Physiol.* 114S:132

LaFayette, P.R., Hoopes, J.T., and Dean, J.F.D. (1997). Members of a laccase gene family are differentially expressed during wood formation in yellow-poplar (*Liriodendron tulipifera* L.). 5th International Congress of Plant Molecular Biology, 21-27 September 1997, Singapore. Abstr. 1320

Dean, J.F.D., LaFayette, P.R., Hoopes, J.T., and McCaig, B.C. (1999) Characterization and expression of laccase genes from yellow-poplar xylem. IUFRO Forest Biotechnology '99, July 11-16, Oxford, England