where long-term observations have shown that neither sex disperses (*16*). However, our data further show that adult males never father offspring within their home pods (whether this is also true of killer whales must await parentage testing). By implication, pilot whales must mate when two or more pods meet or when adult males pay short visits to other pods. Both possibilities are supported by field observations. Large aggregations of whales have been reported, sometimes numbering well over 1000 individuals, and probably result from the temporary merging of several pods (*4*). On the other hand, there are also rare, seasonal sightings of small all-male groups (*4*). In either case, the transitory nature of these events is emphasized by the general failure of paternity testing to reveal fathers.

This behavior pattern is unusual for mammals. Normally, adult males living in social groups are expected to maximize their reproductive success by competing for access to females. This behavior may lead to harem polygyny, with one or a few dominant breeding males who either force subordinate males to disperse or prevent them from breeding (*2*). It appears that pilot whales neither show strong reproductive dominance nor disperse from their natal groups.

The ecology of the pilot whale provides a possible explanation for this behavior. If opportunities to mate with females in other pods are not limited, the optimal male strategy need not involve caring for his direct offspring. He might do better by helping the large number of known relatives in his natal pod (*17*). However, it is unclear what benefits a male could provide. Both defense and assistance in a communal feeding strategy are possibilities, but they lack observational support. Whatever the selective forces involved, it seems clear that the high degree of relatedness between pod members can explain to a large extent the extraordinary cohesion of pilot whale pods.

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8. In four cohorts ($n$ = 6, 3, 3, and 10), all paternal alleles were different. Pairs of alleles occurred in two cohorts of $n$ = 6 (one pair each) and one cohort of $n$ = 12 (two pairs). Of these four pairs, two were ambiguous because a mother-fetus pair shared both alleles. These data support only one possible instance of a male fathering four offspring. The estimated mean, assuming both ambiguities favored shared paternity, is 1.2 offspring per male per cohort.
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10. The empirical mean value of $V$ was 0.83.
11. Genotype frequency indices were calculated for an individual of genotype *ab* as the square root of $pf_a pf_b$, where $pf_a$ is the pod-specific frequency of allele *a*.
12. In 1000 runs, the mean $r$ value (± SD) was 0.0009 ± 0.112.
13. We reassigned alleles randomly to "mothers" (females of sufficient age) with up to nine offspring, using segregation ratios determined by sampling a binomial distribution. In the most extreme case (two-thirds of the pod in nine-offspring families) slight positive correlations were generated (mean ± SD = 0.082 ± 0.11).
14. A female reproduces first at age 5 and thereafter every 3 years with probability $P$. Founding and paternal alleles are selected, with full replacement, from $a$ alleles each at equal frequency. Simulations were stopped at target pod size $t$ or when the oldest female reached age 45. We simulated related founders by allowing the founder to reach age 60 and then deleting all individuals >45 years old. Mean $r$ values varied with $P$, $t$, and $a$ (default values 0.7, 100, and 100, respectively), but all lay in a range consistent with the empirical value.
15. Following Edwards's method of Support [A. W. F. Edwards, *Likelihood* (Cambridge Univ. Press, Cambridge, 1972)], Support = 13.3 (Leynar) and 9.7 (Midvágur), approximately $6 \times 10^5$ and $1.6 \times 10^4$ more likely.
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# A Laccase Associated with Lignification in Loblolly Pine Xylem


Wuli Bao,* David M. O'Malley, Ross Whetten, Ronald R. Sederoff



**Peroxidase has been thought to be the only enzyme that oxidizes monolignol precursors to initiate lignin formation in plants. A laccase was purified from cell walls of differentiating xylem of loblolly pine and shown to coincide in time and place with lignin formation and to oxidize monolignols to dehydrogenation products in vitro. These results suggest that laccase participates in lignin biosynthesis and therefore could be an important target for genetic engineering to modify wood properties or to improve the digestibility of forage crops.**


In 1933, Erdtman proposed that the final step in lignin biosynthesis was enzymatic oxidation of *p*-hydroxyphenylpropanoid compounds followed by a free radical coupling reaction (*1*). Peroxidase (E.C. 1.11.1.7) and laccase (E.C. 1.10.3.2) were postulated to carry out this oxidation because both enzymes produce dehydrogenation polymers (DHP), a lignin-like material, from monolignol precursors (*2*).

Laccase, in contrast to peroxidase, has rarely been studied in plants. A role for laccase in lignification was suggested by early studies with a fungal enzyme (*2*) but was later discounted because a purified plant laccase from the Japanese lacquer tree (*Rhus vernicifera* Stokes) was shown not to oxidize monolignols (*3*). Many researchers have associated peroxidases with lignification (*3–7*). In studies of green ash (*Fraxinus pennsylvanica* Marsh.) sapling stems, Harkin and Obst showed by histochemical staining with syringaldazine or furoguaiacin in the presence of $H_2O_2$ that the xylem tissue adjacent to the cambium contained large amounts of peroxidase activity (*4*). These researchers did not detect laccase activity when syringaldazine was used as the substrate without $H_2O_2$, and on this basis they concluded that peroxidase was the exclusive phenol oxidase responsible for the dehydrogenative polymerization of lignin precursors. Further evidence of peroxidase involvement in lignification was provided by the demonstration of peroxidase activity in differentiating poplar (*Populus x euramericana*) xylem (*7*) and in lignifying cell walls of differentiating tobacco (*Nicotiana tabacum* L.) xylem (*6*), although some phenol oxidase activity was detected in the absence of added $H_2O_2$ in tobacco xylem.

The recent characterization of a laccase purified from the cell culture medium of sycamore maple (*Acer pseudoplatanus* L.) has prompted a reevaluation of the role of this enzyme in lignification (*8–10*). We undertook a study of oxidative enzymes in differentiating xylem of loblolly pine (*Pi-*


Department of Forestry, P.O. Box 8008, North Carolina State University, Raleigh, NC 27695.

*To whom correspondence should be addressed.

*nus taeda* L.) to establish whether laccase participates in lignin polymerization and thereby test the hypothesis of exclusive peroxidase involvement.

Differentiating xylem in conifers is an excellent system for studies of lignin biosynthesis because of the high degree of specialization in wood formation. Crude cell wall preparations of loblolly pine xylem can oxidize phenolic compounds in the absence of added $H_2O_2$, which indicates that polyphenol oxidase activity is present. There are two types of polyphenol oxidases in plants, laccases and catechol oxidases, and they can be distinguished by substrate and inhibitor specificity, copper content, and molecular weight (*11*). We extracted the pine xylem cell walls with 1 M $CaCl_2$ (*12*), which releases most of the polyphenol oxidase activity as well as substantial amounts of peroxidase activity, and purified to homogeneity a glycoprotein (*13*) with characteristics of a laccase (*14*). Both SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1) and C4 reversed-phase high-performance liquid chromatography (HPLC) confirmed the purity of the protein.

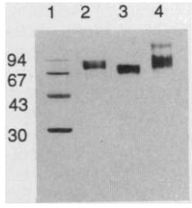


**Fig. 1.** Silver-stained SDS-polyacrylamide gel, showing the purified pine xylem laccase before (lane 2) and after (lane 3) deglycosylation (*15*). The top band of undenatured laccase (lane 4) coincides with enzyme activity, as deduced by staining with 0.68 mM DAF in 20 mM Bistris buffer, pH 5.9. The sizes of molecular markers (lane 1) are given in kilodaltons.

The apparent molecular weight of the glycosylated pine xylem laccase on SDS-PAGE was ~90,000 and that of the deglycosylated protein (*15*) ~70,000 (Fig. 1). Because the laccase activity was only partially denatured by SDS, the active enzyme could be detected on SDS gels. The isoelectric point of the laccase was ~9.0, and the optimum reaction pH was 5.9. Concentrated solutions of the laccase were blue with an absorbance peak at 610 nm, indicative of type I copper (*11*). Preliminary results from electron spin resonance (ESR) spectra also suggested the existence of a type I copper site in the laccase (*16*).

We determined the reaction rates of the pine xylem laccase with several phenolic substrates (Table 1) by measuring the rates of oxygen consumption. The fastest reaction rates were found for phenylhydrazine (an inhibitor of catechol oxidase) and pyrogallol. The laccase oxidized syringaldazine and catechol, which are commonly used substrates for peroxidase and catechol oxidase assays, but did not oxidize cresol, guaiacol, tyrosine, or ferulic acid. Moderate reaction rates were observed for the monolignols coniferyl alcohol and sinapyl alcohol, and a slow reaction rate was observed with *p*-coumaryl alcohol. EDTA and KCN, known inhibitors of laccase, reduced the rate of oxygen consumption. Diethyldithiocarbamate (1 mM) had no effect on oxygen consumption but reduced color formation in the spectrophotometric assay (*14*). The Michaelis constant ($K_m$) values (*17*) for coniferyl alcohol and sinapyl alcohol were 12 ± 1.3 mM and 25.4 ± 5.5 mM, respectively. The $K_m$ value could not be estimated for coumaryl alcohol because the amount of activity was small and coumaryl alcohol has low solubility. When 4-methylcatechol was used as substrate, the $K_m$ value for oxygen was 37 ± 3.5 μM.

**Table 1.** Substrate specificity of pine xylem laccase. Reaction rates were monitored with an oxygen electrode (YSI model 5300, Yellow Springs Instrument Co., Yellow Springs, Ohio, 1.7-ml chamber). Reaction mixtures contained 0.3 μg of laccase and 5 mM substrate in 20 mM Bistris buffer (pH 5.9). We determined the spontaneous oxidation rate by monitoring oxygen depletion in substrate solutions without enzyme. The oxygen content in air-saturated buffer at 25°C was assumed to be 240 μM (*8*). ND, not detected.

| Substrate | Activity (nkatal/mg laccase) |
|---|---|
| Coniferyl alcohol | 72.00 |
| Sinapyl alcohol | 46.67 |
| Coumaryl alcohol | 5.00 |
| Ascorbate | 9.33 |
| Catechol | 96.00 |
| Cresol | ND |
| Diaminofluorene | 480.00 |
| Ferulic acid | ND |
| Guaiacol | ND |
| Hydroquinone | 80.00 |
| 4-methylcatechol | 440.00 |
| Phenylenediamine | 640.00 |
| Phenylhydrazine | 1920.00 |
| Pyrogallol | 1360.00 |
| Syringaldazine | Active* |
| Tyrosine | ND |

*The solubility of syringaldazine was too low for detection of oxygen consumption, so laccase activity was monitored by the formation of a colored product.

**Table 2.** Composition of DHP made by pine xylem laccase. DHP was produced at 25°C in 100 μl of 20 mM Bistris buffer (pH 5.9) that contained 0.005% laccase. Monolignol substrate in methanol was added in 10-μg amounts at 10-min intervals for 2 hours. DHP was collected by centrifugation, dried under vacuum, then resuspended in dimethylformamide (DMF). DHP composition was analyzed by high-performance gel permeation chromatography (KD-802 column, Millipore, Bedford, Massachusetts) at 50°C, with DMF containing 50 mM LiCl and 50 mM NaOH as the mobile phase. Dichlorophenolindophenol, diaminobenzidine, and two degraded lignin samples (620 daltons and 2700 daltons) were used as standards.

| Monolignol substrate | Composition of DHP product (%) | | | | |
|---|---|---|---|---|---|
| | Dimer | Trimer | Tetramer | Pentamer | >Pentamer |
| Coniferyl alcohol | 6.7 | 44.5 | 24.2 | 19.8 | 8.4 |
| Sinapyl alcohol | 57.8 | 19.3 | 13.25 | 8.4 | 1.2 |

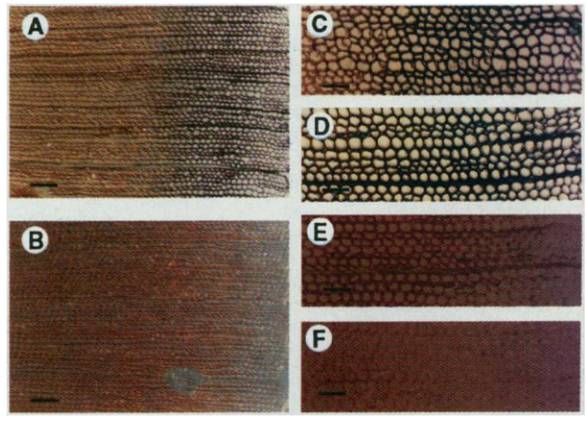


**Fig. 2.** Histochemistry of laccase and peroxidase in 30-μm cross sections of fresh pine stem xylem. The sections are oriented with the cambial side to the right and the mature xylem side to the left. (**A**) Sections were treated at 25°C with catalase (1 mg/ml; Sigma) in 20 mM tris HCl (pH 8) for 10 min then stained with 0.68 mM DAF in 20 mM Bistris (pH 5.9) for 30 min. Laccase activity was detected only in lignifying xylem near the cambium. (**B**) Section treated with 1% phloroglucinol (in a 25% HCl:75% ethanol solution), showing the cell walls containing lignin (red stain). (**C**) Higher magnification view of cell walls in the lignifying zone after staining for laccase. (**D**) Higher magnification view of cell walls stained for peroxidase, showing that cell walls both in the lignifying zone and in the mature xylem have peroxidase activity. (**E**) Higher magnification view of phloroglucinol staining, showing the weak staining of nonlignifying cell walls near the cambium. (**F**) Boiled control section showing no staining for laccase or peroxidase activity. Size bars equal 160 μm in (A) and (B) and 80 μm in (C) to (F).

The pine xylem laccase oxidized monolignols in vitro to form DHP (*18*). The reaction products were primarily trimers or tetramers of the monolignol (Table 2), although ~8% of the DHP from coniferyl alcohol was hexamers or larger molecules.

All of the detectable pine xylem laccase activity was bound to cell walls (Table 3) and could be attributed to a single protein. No other polyphenol oxidase activities were detected in the xylem soluble extract. Strong laccase activity was detected in differentiating xylem and in embryogenic callus, but no activity was detected in embryos, megagametophytes, or strobili. When crude $CaCl_2$-extracted protein samples were subjected to electrophoresis on SDS gels and stained for laccase activity, they produced one broad band that comigrated with the purified enzyme, which suggests that there is only one laccase isozyme in pine.

In sledge microtome sections cut from fresh blocks of pine stem, cells with laccase activity were detected by staining in 0.68 mM diaminofluorene (DAF) in 20 mM Bistris buffer (pH 5.9). Laccase activity was distinguished from peroxidase by its requirement for oxygen instead of exogenously added $H_2O_2$. Some sections were treated with catalase [1 mg/ml in 20 mM tris HCl (pH 8)] to remove endogenous $H_2O_2$, but no differences in staining were observed when sections were not treated with catalase. Only the walls of xylem cells in the zone of lignification had laccase activity (Fig. 2A). Phloroglucinol staining (Fig. 2, B and E) and ultraviolet fluorescent microscopy indicated the location of cells that were initiating lignification. Intense laccase activity staining was observed in the active lignifying zone but not in mature xylem (Fig. 2, A and C). Syringaldazine staining of sections in the absence of added $H_2O_2$ showed a pattern similar to that in the DAF-stained sections. Sections boiled in water showed no laccase activity (Fig. 2F). The addition of 50 mM EDTA to the stain solution inhibited laccase activity in the DAF-stained sections.

Peroxidase as well as laccase activity were detectable in lignifying tissues of loblolly pine by histochemical methods similar to those of Harkin and Obst (*4*). The peroxidase activity was present in both lignifying and nonlignifying tissues and was readily detected by soluble-enzyme assays in all of the crude tissue extracts studied (Table 3). Cross sections of woody stems stained more intensely with DAF and syringaldazine in the presence of added $H_2O_2$ (Fig. 2D). All tissues in the cross section, including nonliving mature xylem, showed peroxidase activity, in contrast with the more specific staining of laccase. No staining was obtained, with or without added $H_2O_2$, in sections that were boiled before staining.

Our analysis of loblolly pine xylem laccase challenges the hypothesis (*4*) that peroxidase is the only enzyme participating in lignification. Laccase is associated with lignification by four criteria. The enzyme is found in xylem, is associated with the cell wall, is present in lignifying cells, and can oxidize monolignols to DHP. The role of the laccase purified from sycamore maple cells in culture is less certain because the cultured cells do not make lignin (*9*, *10*), and the relation of other polyphenol oxidase activities in stems of sycamore maple (*9*) to the pine laccase is not clear. Savidge and Udagama-Randeniya (*19*) describe a coniferyl alcohol oxidase activity associated in time and place with lignification in the cell wall preparations of differentiating xylem of conifers. Similarly, in *Forsythia* sp., an insoluble cell wall fraction was shown to catalyze the formation of pinoresinol from E-coniferyl alcohol with no cofactors other than oxygen (*20*). The relation of these enzyme activities to the pine xylem laccase will become clearer once they have been purified.

**Table 3.** Laccase and peroxidase activities in different pine tissues and organs. Crude protein was extracted overnight at 4°C from 5 g of frozen, powdered plant material in 1 M $CaCl_2$ and 20 mM tris HCl (pH 8) and dialyzed. Xylem cell wall salt extract was obtained from the $CaCl_2$ extraction step in the laccase purification. Xylem soluble extract was the 10,000*g* supernatant from the first extraction of powdered frozen xylem. Laccase activity was determined by spectrophotometric assay (*14*). ND, not detected.

| Tissue | Activity (nkatal/mg protein) | |
|---|---|---|
| | Laccase | Peroxidase |
| Pollen strobili | ND | 1180.0 |
| Embryos and megagametophytes | ND | 20.0 |
| Shoot buds | ND | 2310.0 |
| Phloem and cambium | 0.019 | 820.0 |
| Differentiating xylem | 3.000 | 880.0 |
| Xylem soluble extract | ND | 820.0 |
| Xylem cell wall salt extract | 213.0 | 1070.0 |
| Embryogenic callus | 103.0 | 600.0 |

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