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**Towards a Detailed Understanding of the Structural Variability
in Lignins : A New Approach**

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by

N.G. Lewis and G.H.N. Towers

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Summary Statement:

This reporting period witnessed a number of exciting and important breakthroughs in the study of lignin synthesis in plants and resulted in a significant deviation from the work plan originally proposed. Taken together, our results reveal an emerging picture of a highly ordered assembly of the lignin polymer at the plasma membrane/cell-wall interface, in contrast to original considerations projecting a random (haphazard) series of coupling reactions. The main findings are as follows:

1. We have discovered that plants contain a *series* of tightly bound cell-wall oxidases (all presumed to be copper-containing) which catalyze a continuum of monolignol coupling reactions, each differing in stereospecificity and range of products formed. In this study, a laccase-like oxidase of narrow substrate specificity has been purified to apparent homogeneity in preparation for cloning and overexpression. We are proposing a general catalytic mechanism for these oxidases, which differ only by binding and orientation of the *two* substrate molecules prior to two, one electron oxidations leading to coupling. [This aspect is only briefly described in the Progress Report as it constitutes the main focus of the accompanying proposal.]

2. We have developed a model cell suspension culture system of loblolly pine (*Pinus taeda*) which can be induced to synchronously undergo cell-wall thickening and lignification. The cell wall lignin corresponds remarkably well with that found in secondary xylem tissue as evidenced by specific carbon-13 labeling. This system now permits us to meaningfully and systematically address issues of monomer transport into the cell-wall, regulation of lignin synthesis as a function of cell-wall development and its induction in response to various stresses (such as wounding, pathogen attack, etc.).

3. The *P. taeda* cultures, when grown in a sucrose medium, accumulated phenolic materials in the growth medium. Analysis of the constituents revealed that *p*-coumaryl alcohol and coniferyl alcohol had different metabolic fates at the plasma membrane/cell-wall interface, with the latter undergoing initial dimerization to yield a variety of lignans. On the other hand, *p*-coumaryl alcohol only underwent conversion via a peroxidase-catalyzed transformation in a process presumed to be dependent upon active H₂O₂ synthesis. This result provides some biochemical basis for lignin heterogeneity observed at the subcellular level, and may be the reason why middle lamella lignin is *p*-coumaryl alcohol rich.

4. The effect of wounding and pathogen attack on various plant species gave different results, dependent on the organism under investigation. Wounding of *Leucaena leucocephala* plants had no obvious deleterious effects on the structure of lignins formed *de novo*. By contrast, wounded potato tubers did not synthesize appreciable amounts of lignin, but instead formed a polyester comprised of hydroxycinnamate residues. Pathogen attack on barley seedlings by tobacco necrosis virus resulted in an increase in hydroxycinnamic acid formation. Similarly, abiotic elicitation of wheat cell cultures increased the amounts of hydroxycinnamic acid esters two-fold. These different results emphasize the need for careful structural identification of so-called "lignin-like" substances induced by external stresses.

5. Investigations of the phenylpropanoid constituents of various cereals/grasses revealed that both hydroxycinnamate oxidative coupling and photochemical dimerization reactions appear to be of general importance in cell-wall stiffening, with both perhaps being preludes to lignin deposition. Based on our exciting finding of a series of plasma membrane oxidases in woody plants, we are now in a unique position to extend these observations to cereals/grasses, etc., and define their precise role in phenylpropanoid polymer assembly.

6. Methodology was developed to detect minute amounts of amino acids in plants, and examine whether the conversion of argenolate into phenylalanine/tyrosine and subsequent deamination occurs via a closed recycling of nitrogen.

Progress Report:

The Progress Report is divided into two sections: the first section reflects the interlocking research completed to date in the laboratories of N.G. Lewis and G.H.N. Towers. In the broadest sense, the Lewis group has focused mainly on woody plants, whereas the Towers laboratory examined similar processes in cereals/grasses. The second section compiles published work, a summary of manuscripts in preparation, and work currently underway for year three of the existing grant.

1. Work Carried out in the N.G. Lewis and G.H.N. Towers Laboratories.

Lignins are considered to be heterogeneous polymers; in woody gymnosperms, they are mainly composed of *E*-coniferyl alcohol **2** together with smaller amounts of *p*-coumaryl alcohol **1**, whereas angiosperm lignins are mainly built up of a *ca* 1:1 mixture of both coniferyl **2** / sinapyl **3** alcohols together with small amounts of **1** (see Ref. 1). As an added complication, cereals/grasses contain hydroxycinnamic acids incorporated into the lignin polymer, as well as being associated with the arabinoxylans. In woody plants, *p*-coumaryl alcohol **1** is apparently preferentially deposited into the cell-corners and middle lamella during early stages of lignin deposition (2, 3), as well as into the compression wood of gymnosperms (4). But the reasons for this heterogeneity, in either a structural, biochemical or chemical context, are unknown. An additional complication is the question of monomer selection and transport from the cytoplasm into cell-wall, i.e., whether the monolignols **1-3**, their corresponding glucosides **4-6**, or both are involved. Indeed, even the subcellular location of monolignol synthesis (enzymes and intermediates) are also essentially unknown.

In the original work plan, we proposed a systematic study of how monolignol formation and transport, lignin heterogeneity at the subcellular level and induction of lignin synthesis by wounding/infection occurred. The results obtained to date are described below; a significant deviation for the original work plan occurred because of some exciting breakthroughs.

(i) **Monolignol formation and transport.** At the initiation of this study, the precise (sub)cellular location of most enzymes involved in monolignol **1-3** synthesis, and the mechanism of monomer selection and transport across the plasma membrane into the lignifying wall were unknown. As far as transport is concerned, monolignol glucosides **4-6** have often been implicated, although no consideration seems to have been given to the fate of the equimolar amounts of glucose which would be liberated by this process. An alternative viewpoint is that the phenolics are transported directly, and that glucosides serve simply in a storage capacity and are utilized only as needed.

The study of monomer transport into lignifying cell walls cannot readily be undertaken using intact plant tissues because the myriad of cells within a developing plant are at different developmental stages and the amounts of monolignols (and their glucosides) are generally very small. We reasoned that one way to potentially overcome such drawbacks was to develop a cell culture system, whereby monomer synthesis and transport across the plasma membrane were inducible. This was achieved using *P. taeda* suspension cell-cultures which upon transfer to an 8% sucrose solution both lignify and accumulate soluble phenolic compounds in the culture filtrate (Fig. 1a, Ref. 5; manuscript in finalization). At later stages these soluble compounds undergo polymerization to afford an insoluble lignin-like material [see section (ii)].

We investigated this process further to ascertain whether it had any bearing on monolignol transport. Thus, Fig. 1b shows the HPLC profile of soluble metabolites present in the culture filtrate 96 h post transfer, of which nine have been individually purified (HPLC, TLC), and their ¹H, ¹³C NMR, mass, FT-IR and UV spectra recorded. Compounds were identified by their spectral properties, comparison to authentic standards and published data as needed. Metabolites identified to date include the monolignols, *E*-*p*-coumaryl **1** and *E*-coniferyl **2** alcohols, and the lignans, guaiacylglycerol-8-*O*-coniferyl alcohol ether **7**, an unusual mixed dimer derived from *p*-coumaryl and coniferyl alcohols **8**, dehydroconiferyl alcohol **9** and its reduced analogue **10**, pinoresinol **11**, shonanin **12**, and todolactol **13** (Ref. 5, manuscript in finalization).

Several points are noteworthy: (a) the monolignol, *E*-*p*-coumaryl alcohol **1** is detectable in the culture filtrate medium at the very earliest stages of phenol accumulation and builds up in a linear fashion over the 96 h period. By contrast, *E*-coniferyl alcohol **2** is present only in minor amounts when compared to the accumulation of the corresponding *E*-coniferyl alcohol-derived dimers **7**, **9-13**. These findings, therefore, reveal a hitherto unexpected substrate specificity for initial monolignol coupling, and strongly suggest that *p*-coumaryl **1** and coniferyl **2** alcohols have very different metabolic fates during lignin assembly. Indeed, this observation may provide some clue(s) as to why middle lamella and compression wood lignins contain a higher proportion of *p*-coumaryl **1** units, in contrast to secondary wall lignins; (b) no monolignol glucosides, e.g., coniferin **5** and/or β -glucosidase activity were detected in the culture filtrate. Thus, if glucosides are involved, they must be hydrolyzed prior to their movement through the cell-wall and into the culture medium (perhaps via a cell-wall bound β -glucosidase). This question, as well as the fate of any glucose liberated is currently being addressed (see Section 2). (c) Several reports indicate that cell-wall lignins contain a dibenzylfuran substructure (albeit in relatively low amount) (e.g., see 5). It therefore seems significant that shonanin **12** accumulates in the medium, as it is an obvious candidate as a precursor of this substructure. But its formation cannot be rationalized simply on the basis of 8,8' coupling, as an additional reductive step (or steps) is obviously required; (d) the formation of the mixed monolignol dimer **8** (presumed to be derived from *p*-coumaryl **1** and coniferyl **2** alcohols) is also intriguing as it may represent the first covalent linkage (substructure) identified for *p*-coumaryl alcohol **1** in lignin; and (v) the 8-*O*-4' linked dimer **7** was surprisingly only present in minute amount, even though it is often considered to be the most prevalent substructure in native lignin [see (ii)].

We are also using these *P. taeda* cell cultures to investigate questions regarding the subcellular location of *E*-coniferyl alcohol **2**, and its glucoside *E*-coniferin **5**, (**6**) as well as the enzymes, UDPG-coniferyl alcohol glucosyltransferase, cinnamyl alcohol dehydrogenase (CAD), and phenylalanine ammonia lyase (PAL) (**7**). [Localizing the latter two enzymes was added to the original work plan to obtain a more comprehensive view of the subcellular localization of phenylpropanoid metabolism.] The progress to date on these aspects of monolignol formation and transport are detailed below:

(a) *Phenylalanine Ammonia-Lyase (PAL)*. This enzyme catalyzes the deamination of phenylalanine affording cinnamic acid, which is subsequently converted into hydroxycinnamic acids, flavonoids (i.e., their Phe-derived portion), lignans, lignins and the phenolic domain of suberins. (see Ref. 8). Polyclonal antibodies raised against PAL from loblolly pine (*P. taeda*) were provided by R.R. Sederoff (North Carolina State University) (**9**) (Fig. 2a). It is noteworthy that in contrast to other species (e.g., bean, rice), *P. taeda* PAL is apparently encoded by a single gene and only one isoform of the enzyme has been described (For a full discussion see Ref. 8). Ultrathin sections for immunogold staining were prepared from *P. taeda* cell suspension cultures (2,4-D line) and 21-day-old *P. taeda* seedlings. Following immunocytochemical staining and visualization by electron microscopy, PAL was observed to accumulate in the cytoplasm, with some labeling noted in the endoplasmic reticulum (Fig. 2b) (**7**). Interestingly, immunoresponses to PAL antibodies were also noted for other membrane-associated tissues (i.e., the plasma membrane and tonoplast) (Fig. 2b). No labeling occurred in the mitochondria or plastids. [Four sets of immunocytochemical controls were free of immunochemical responses.]

(b) *Cinnamyl Alcohol Dehydrogenase (CAD)*. This NAD(P)H-requiring enzyme catalyzes the formation of monolignols (e.g., coniferyl alcohol **2**) from the corresponding aldehydes (e.g., coniferaldehyde) and has been cloned from *P. taeda* (**10**) and tobacco (**11**). Monoclonal antibodies raised against *P. taeda* CAD were provided by R.R. Sederoff. Western blot analysis of protein extracted from *P. taeda* cambial tissue, visualized with CAD-monoclonal antibodies revealed a single band at 44 kD (Fig. 3). In an analogous manner to that described for PAL, immunogold staining of ultrathin sections was carried out, but apparently and unexpectedly revealed that CAD was mainly present in the chloroplasts of young seedlings (Fig. 4a) and plastids of 2,4-D-grown *P. taeda* cells (Fig. 4b). Immunoresponses (albeit small) were also observed for the endoplasmic reticulum and mitochondria/cytoplasm (Fig. 4b). [Similar findings have since been made with CAD from eucalyptus (A.M. Boudet, personal

communication).] But neither *Pinus* nor eucalyptus CAD N-terminal sequences contain any plastid-processing information. While this feature is not an absolute requirement for plastid processing, some additional lines of evidence are needed prior to making any firm conclusions regarding CAD subcellular location.

(c) *UDPG-Coniferyl Alcohol Glucosyltransferase*. We are also interested in determining the subcellular locations of *E*-coniferyl alcohol 2, its corresponding glucoside 5, and the enzyme, UDPG-coniferyl alcohol glucosyltransferase, (which catalyzes the formation of 5). This information is needed in order to be able to compare the relative locations of both CAD and UDPG-coniferyl alcohol glucosyltransferase (are they the same or different?), and to see whether the glucosides are storage products or not. To this end, polyclonal antibodies were raised against *E*-coniferyl alcohol 2 conjugated to bovine serum albumin (6). ELISA assays indicated that the antibodies reacted more strongly (by a factor of 2) with *E*-coniferyl alcohol 2 over its *Z*-analogue. But the antibodies also cross-reacted with lignans, hydroxycinnamic acids and monolignol glucosides (6) and hence are only of limited value. Consequently, our focus has shifted to the purification of *E*-coniferyl alcohol glucosyltransferase in order to obtain more definitive results regarding its subcellular localization; to this point, the enzyme has been purified to near homogeneity (>3350 fold purification) via a combination of ion exchange, gel filtration and affinity chromatography. Interestingly, this protein only displays a very slight substrate preference for *E*-coniferyl alcohol 2 over its *Z*-analogue, (*E*:*Z*, 2:1) in contrast to the marked preference for the *Z*-isomer with *Fagus grandifolia* (*E*:*Z*, 1:24) which accumulates the corresponding *Z*-isomer (12).

It should, therefore, be evident that these cell cultures offer an excellent means of studying a variety of questions involved in monolignol synthesis, storage and transport into the cell-wall.

(ii) **Lignin heterogeneity at the subcellular level.** This phase of the research was divided into two areas, namely lignification in woody plants and cereals/grasses, respectively. As far as woody plant lignification was concerned, we investigated whether the *P. taeda* cell suspension cultures could be used to study lignin deposition as a function of cell-wall development, and whether they would shed any light on cell-wall lignin heterogeneity.

Pinus taeda suspension cultures were initially maintained in growth medium containing 2,4-D, i.e., under conditions whereby phenylpropanoid metabolism is suppressed (13, 14). Under such circumstances, the cells contained only primary walls which were essentially unlignified, as determined by histochemical, ultrastructural, chemical and NMR spectroscopic analyses (15). However, they maintained a functional phenylpropanoid pathway as demonstrated by formation of the lignans, (-)-pinoresinol 11 and (-)-matairesinol 14. Administration of [1-¹³C]Phe to these cultures, followed by solid state carbon-13 NMR spectral analysis of their cell walls, demonstrated that phenylalanine was incorporated into the cell-wall matrix primarily as protein, rather than lignin. Successive transfer of the 2,4-D-grown cultures to NAA-containing medium induced cell-wall thickening (more or less corresponding to the deposition of a S₁ layer of the cell-wall) with concomitant lignification. The presence of lignin was confirmed by histochemical, ultrastructural, chemical, biochemical and NMR spectroscopic analyses. Interestingly, thioacidolysis did not reveal any notable differences in *p*-coumaryl 1 : coniferyl alcohol 2 derived ratios of the lignins from either cell line. But specific labeling of the lignin polymer *in situ* (NAA cell line) with [1-¹³C], [2-¹³C] and [3-¹³C]Phe and analysis of the cell-wall preparations by solid state carbon-13 NMR spectroscopy permitted the first direct determination of the *in situ* bonding patterns of a gymnosperm lignin. Several dominant interunit linkages were observed, including 8-O-4', furanofuran, phenylcoumarin and phenolic-linked monolignols, (Scheme 2) with the former predominating. Consequently, the lignin produced in culture corresponded remarkably well with that predicted for whole plants. Furthermore, the slow change from non-lignified to lignified cells also suggested that under these conditions the cultures may serve as a cell-wall developmental model system. It is also significant that soluble milled wood lignin derivatives prepared from these ¹³C-specifically enriched lignified tissues, gave ¹³C NMR spectra which clearly revealed the native lignin *in situ*. [This comprehensive study will appear later this year in the Journal of Biological Chemistry (15), galley proofs are appended].

As discussed earlier, when the non-lignified 2,4-D-grown cells were transferred to an 8% sucrose solution, they not only accumulated a large amount of soluble phenolics (including *p*-coumaryl **1** and coniferyl **2** alcohols and a series of dimers derived mainly from **2**, e.g., compounds **4-13**) (5) but they also lignified. Moreover, beyond 96 h post transfer, the phenolics in the *P. taeda* culture filtrate apparently underwent a H₂O₂-dependent peroxidase-catalyzed polymerization to afford a lignin-like polymer; this preparation however lacked any substantial amount of 8-O-4' linkages which predominate in native lignin. Importantly, it was only at the point of polymerization when *p*-coumaryl alcohol **1** (which built up steadily in the medium) underwent any significant conversion into the polymer, as did the coniferyl alcohol-derived dimers **7, 9-13**. This polymerization is presumed to be catalyzed by one or both of the two peroxidases present in the medium, and presumed to be activated only when H₂O₂ synthesis occurs. We do not yet know whether either isozyme displays any substrate preference for *p*-coumaryl **1** and coniferyl **2** alcohols, or the corresponding dimers **7-13**. The apparent conversion of *E*-coniferyl alcohol **2** into the dimeric lignans, such as **7, 9-13** prompted us to consider that the fate of monolignols **1** and **2** somehow differed at the plasma membrane/cell-wall interface. Indeed, we have now determined a biochemical basis for this difference, which began with the discovery that a tightly cell-wall bound O₂-requiring, laccase-like oxidase from *Forsythia* sp. catalyses the stereospecific coupling of two *E*-coniferyl alcohol **2** molecules to give optically pure (+)-pinoresinol **11**. [This is the first example of stereoselective coupling in phenylpropanoid metabolism (16, 17).] But the cell walls also contained a non-stereoselective coupling, copper-containing laccase-like oxidase (MW ~100,000) with a very narrow substrate preference for the *E*-coniferyl **2**, and *E*-sinapyl **3** alcohols; *E*-*p*-coumaryl alcohol **1** did not serve as a substrate. [This enzyme (now being cloned) and the other oxidases are discussed only briefly here since they constitute the bulk of the attached proposal.] Importantly, the *P. taeda* cell wall also contains an analogous tightly bound enzyme showing similar properties.

These discoveries have far reaching implications in the area of initial coupling reactions occurring at the cell-wall/plasma membrane interface. As discussed in the attached proposal, we predict that there exists a series of tightly wall-bound laccase-like enzymes capable of catalyzing monolignol coupling, but which differ in terms of stereospecificity and range of products formed. Indeed, we also propose that these reactions can only occur via 2 simultaneous 1 electron oxidations of two bound and highly oriented substrate molecules (see proposal). Thus, taken together, our results indicate that *E*-coniferyl alcohol **2** can undergo conversion at the plasma membrane/cell-wall interface to initially yield dimers whereas *p*-coumaryl alcohol **1** cannot. Consequently, a picture is beginning to emerge of a highly orchestrated series of reactions in lignification, which are both substrate specific and spatially and temporally distinct. Unraveling the mechanism of lignin assembly is the subject of the attached proposal.

Note also that with respect to lignin heterogeneity, we have begun to investigate how reaction wood, (i.e., a compression wood whose lignin has a high *p*-coumaryl alcohol **1** derived content) formation occurs in the gymnosperm, Douglas fir (*Pseudotsuga menziesii*). To this point, we have examined substrate specificities of various phenylpropanoid enzymes (e.g., O-methyltransferase, hydroxycinnamate CoA ligase, cinnamyl alcohol dehydrogenase) involved in monolignol synthesis but no trends favoring *p*-coumaryl alcohol **1** synthesis were evident.

Next, as far as lignins (and related phenolics) in cereals/grasses are concerned, these plant forms differ substantially from their woody counterparts in having hydroxycinnamates (e.g., *p*-coumaric/ferulic acid etc.) incorporated into their lignins. Prior to lignification, these acids are found ester-bound to various hemicelluloses (e.g., arabinoxylans) at specific sites. They are proposed to function in cell-wall stiffening reactions via oxidative coupling of adjacent hemicellulose chains by formation of diferulate bridges, and/or by photochemical coupling reactions to afford the corresponding cyclobutane dimers (i.e., truxillates; Scheme3). Additionally, these ester-bound phenols can also be envisaged to serve as a nucleation site for lignin synthesis to occur. It was, therefore, deemed important to first delineate the early steps of phenylpropanoid deposition leading to lignin synthesis in such tissues, prior to unravelling their biochemistry. In this regard, our initial work focused on the analysis of cell walls of bamboo, bamboo grass (18, 19), corn (*Zea mays*), sugar cane (*Saccharum officinalis*), barley (*Hordeum vulgare*), and kikuyu grass (*Pennisetum clandestinum*).

Diferulic acid was identified as a constituent of cell walls in all the plant materials examined so far. The discovery of various oxidizing enzymes, (purified in N.G. Lewis' laboratory), now permit us to address critical studies of these feruloyl ester-coupling reactions, since the regulation of the synthesis of diferulate bridges in the cell wall is a key element in the control of wall "stiffening". Identification of the enzyme(s) involved will be of importance to molecular biological approaches to wall modification, e.g., by appropriate antisense experimental strategies. Additionally, various truxillates **15-17** (cyclobutane dimers of hydroxycinnamic acids) were found in leaves and stems of bamboo and bamboo grass (Scheme 3), whereas in corn cell walls, six different cyclobutane dimers were detected. Interestingly, in this case, the amount of the photodimers were far less in stems than in leaves and may reflect either differences in the proximity and alignment of the double bonds of the side chains of the monomers *in situ* or the greater attenuation of light in stems; two properly aligned double bonds should be within ca 0.4 nm for photodimerization to be effectual. In the case of Kikuyu grass, we found that there are twelve types of cyclobutane dimers, formed under high light intensity ($400 \mu\text{mol s}^{-1}\text{m}^{-1}$) in leaves as young as 3-weeks old.

Lastly, UV-irradiation caused significant changes in the digestion of dark-grown barley coleoptiles cell walls by a partially purified xylanase from *Aspergillus niger* (20). Irradiation resulted in a reduced rate and extent of solubilization of pentoses and changes in their neutral sugar composition and molecular weight distribution. The inhibitory effects of wall-bound phenolics on digestion of forage fiber by ruminants are well established (21, 22).

(iii) Induction of lignification by wounding and infection. The processes that regulate cell-wall formation are poorly understood. Much of what is known, however, about the regulation of lignification of cell-wall phenylpropanoids (e.g., lignins, suberins, cell-wall bound phenolics) has been derived from studies in which changes to cell-wall phenolics have been induced either by wounding or infection. Rarely is the final wall-associated product analyzed beyond crude histochemical or (imprecise) chemical analyses, and the extent to which the stress-induced (poly)phenolics resemble "native" wall-bound phenolics is unclear. Consequently, knowledge gained with respect to changes in lignin structure, monomer composition and content and target enzyme activities or gene expression (and thereby clues about regulatory steps) are in doubt. We have addressed this question over the past several years by developing a system to precisely examine lignin structure *in situ*, i.e., [as discussed in (ii)] in which specifically labeled carbon-13 lignin precursors are incorporated into the cell-wall matrix (e.g., into lignin and/or wall-bound phenolics). We have now applied this approach to the question of wound-induced phenylpropane polymer structure. Two model systems have been examined; (a) *Leucaena leucocephala*, and (b) *Solanum tuberosum* (potato) tubers (23). Also, changes in the cell-wall phenolic composition of grasses in response to viral or abiotic elicitors have been investigated using (c) barley seedlings and (d) wheat cell cultures.

(a) *Leucaena leucocephala*. We had previously shown that hydroponically grown *L. leucocephala* plants rapidly and efficiently incorporate L-phenylalanine supplied through the growth medium into cell-wall lignin. ^{13}C NMR spectroscopic analysis of extractive-free stem tissue labeled with either $[1-^{13}\text{C}]$, $[2-^{13}\text{C}]$ or $[3-^{13}\text{C}]$ -phenylalanine established the structural features of native *Leucaena* lignin (24, 25). Next, two week old aseptic, hydroponically grown *L. leucocephala* seedlings were wounded (under aseptic conditions) with a razor cut along their stems and grown hydroponically in the presence of either $[1-^{13}\text{C}]$, $[2-^{13}\text{C}]$, $[3-^{13}\text{C}]$ or natural abundance phenylalanine for three weeks (with weekly medium exchange) during wound healing. A callus growth was evident in the region of the cut stem within one week of wound healing. Microscopic examination of cross-sections through the wound site revealed thickened cell walls in the xylem on the wound side of the stem. Isolation of the tissue surrounding the wound site (i.e., wound callus and underlying tissue) followed by extraction, yielded extractive-free cell wall preparations indistinguishable from those of non-wounded plants. In other words, the "wound-lignin" of *L. leucocephala* was not found to be different from native lignins (manuscript in preparation.)

(b) *Solanum tuberosum*. In a deviation from the original proposal, potato tubers were introduced as a second model system to investigate the nature of wound-induced "lignins". This

was done since controversy over the biopolyester suberin (comprising both aromatic and aliphatic domains) has recently come to light (26). The phenolic domain of suberin is often described as "lignin-like" based on nitrobenzene oxidation analysis. But, recently, the more precise analytical tool of thioacidolysis has revealed a much lower "lignin" content in the polymer than previously predicted (26). Moreover, ^1H and ^{13}C NMR spectral analysis of this polymer failed to yield any similarities with lignins (27, 28). After establishing both proper feeding conditions (with [^{14}C]-phenylalanine) and extractive procedures, [$1\text{-}^{13}\text{C}$], [$2\text{-}^{13}\text{C}$], [$3\text{-}^{13}\text{C}$] and natural abundance phenylalanines were separately administered to wound healing potato tuber slices (all under aseptic conditions). Surprisingly, the difference spectra obtained for carbon-13-enriched suberins revealed a polymer comprised largely of hydroxycinnamoyl moieties with only minute amounts of lignin (Fig. 5) (23).

These two examples emphasize the need for careful analysis of stress-induced changes in cell wall polymers prior to concluding that they are lignin, and demonstrate the power of *in situ* labeling in determining biopolymer structure to address such questions.

(c) *Barley Seedlings*. The effects on cell wall bound hydroxycinnamates, induced by infection with tobacco necrosis virus (TNV), is continuing. Preliminary results, reported last year, indicated significant increases, perhaps twofold, in ester bound cell wall hydroxycinnamates in response to viral infection. This is a totally new finding with respect to stress responses in a plant. The experiments are being repeated where we shall determine whether ether bound acids and lignin are also affected.

(d) *Wheat Cell Cultures*. Cell walls of wheat (*Triticum monococcum*) cell cultures contain esterified hydroxycinnamates, but cyclobutane dimers are absent. [It is possible that light intensities are inadequate in culture flasks.] Preliminary results indicated that elicitation with chitosan lead to a twofold increase in wall-bound hydroxycinnamates and the appearance of an unidentified elicited compound. In the literature there is much discussion about elicited increases in lignification with materials such as chitosan. What we have perceived so far, however, is that major differences in wall-bound phenolics may be the response of grasses to stresses. In other words, wall-bound esters may play a defensive role in grasses. Identification of the elicited compound, and characterization of the "lignins" in these materials is presently underway.

(iv) Phenylpropanoid biosynthesis. Phenylpropanoid compounds account for over 30% of the organic constituents of grasses. The sources of these compounds in grasses are L-phenylalanine and L-tyrosine which need to be continuously generated to provide cinnamic acids for lignins, flavonoids, etc. There is also a severe demand, by lignifying tissues, for amino nitrogen yet nitrogen does not appear to be a limiting factor in stepping up phenylpropanoid metabolism. This fact suggests that phenylpropanoid metabolism (including lignification) is linked to a specific and efficient recycling process for nitrogen, involving the aromatic amino acids and possibly arogenic acid as well. There is evidence that these aromatic amino acids are derived in plants from L-arogenic acid (which supplies both their carbon and nitrogen) (1, 29, 30). The nitrogen is subsequently lost in the first step of the general phenylpropanoid and lignin pathways through the mediation of the aromatic amino acid ammonia lyases. Arogenate thus appears to play a pivotal role in phenylpropanoid metabolism and would be expected to be amplified under stress conditions, e.g., pathological virus infections and wounding.

Arogenate has hitherto not been detected in plants and we have therefore devised a new analytical method for its detection at the pmole level using Pico-Tag technology developed by Waters (31, 32). This method allows us to analyze plant materials for amino acids including phenylalanine, tyrosine and arogenate. Standard arogenate was isolated and identified critically from a mutant of *Neurospora crassa*, ATCC 36373 (33) by ^{13}C NMR and mass spectrometry.

2. Published Work, Manuscripts in Preparation and Work Currently Underway.

To date, ten publications have appeared in print describing various aspects of the project. These include Eberhardt *et. al.* (J. Biol. Chem., 1993), Lewis (Plant Phenolics, 1993, In Antioxidants in Higher Plants, CRC Press), Lewis and Davin (Plant Polyphenols, 1992,

Plenum Press), Lewis (Methods in Lignin Chemistry, 1992, Springer-Verlag), Davin and Lewis (Recent Advances in Phytochemistry, 1992), Yamamoto *et al.* (Plant Physiol., 1990), Tachibana *et al.* (Phytochemistry, 1992 a, b) and McCallum *et al.* (Analyt. Biochem., 1991), McCallum *et al.* (J. Plant Physiol., 1992). Several of these papers reflect commitments by N.G. Lewis to various book chapters but which encompasses work carried out under DOE funding. Copies of these papers are attached.

(i) Manuscripts submitted / in preparation. Even though a significant redirection of the original work plan occurred, there are several papers which are in the final stages of preparation for publication and/or have been submitted. These are listed below; copies will be forwarded to DOE when galley proofs are received.

11. Lewis, N.G., Razal, R.A., Yamamoto, E., Towers, G.H.N. and Wooten, J.B. (1993) Determination of the Bonding Patterns of the Phenylpropanoid Side Chain of Lignin in *Leucaena leucocephala*. J. Amer. Chem. Soc. (submitted).
12. He, L., Davin, L.B., Sederoff, R.R. and Lewis, N.G. (1993) Intracellular Distribution of Phenylalanine Ammonia Lyase (PAL) in Loblolly Pine (*Pinus taeda* L.) (submitted).
13. Bernards, M.A., Lopez, M.L., Zajicek, J. and Lewis, N.G. (1993) The Phenolic Domain of Suberin (in preparation).
14. Bernards, M.A., Furlan, M. and Lewis, N.G. (1993) Phenylpropanoid Metabolites in *Pinus taeda* Cell Suspension Cultures (in preparation).
15. Bernards, M.A., Lopez, M. and Lewis, N.G. (1993) Wound Lignin in *Leucaena leucocephala* Stems (in preparation).
16. Davin, L.B., Bedgar, D.L., Paré, P.W., and Lewis, N.G. (1993) Purification and Partial Characterization of a Laccase-like Protein in the Cell Walls of *Forsythia intermedia* (in preparation).
17. Paré, P.W., Davin, L.B. and Lewis, N.G. (1993) A Stereoselective Coupling Enzyme in the Call Walls of *Forsythia intermedia* (in preparation).
18. Razal, R.A., Lewis, N.G. and Towers, G.H.N. (1993) A Picotag Determination of the Amino Acids, Phenylalanine, Tyrosine and Aroenic Acid (in preparation).

(ii) Work to be carried out in year 3 of existing grant. The goals for the third year have changed, and represent a deviation from the original work plan. They are outlined below:

(a) Monolignol synthesis and transport: Complete purification of UDPG-coniferyl alcohol glucosyl transferase and determine its subcellular location. Use the *P. taeda* cell suspension cultures to investigate how monolignol transfer occurs to the plasma membrane at the subcellular level (this is being done in collaboration with Andrew Staehelin) and determine the role of β -glucosidase and fate of the liberated glucose (during the putative hydrolysis of monolignol glucosides. Because of the changes in focus, we have not been able to - or will be able to - investigate the role of Z-monolignols in lignin synthesis in this project.

(b) Lignin heterogeneity at the subcellular level. On target, and completed as defined. Additionally, however, we are now cloning the laccase-like protein of narrow substrate specificity (as described in the proposal) and hope to have this completed by early 1994. We will also complete the identification of photodimers in the cell walls of grasses/cereals, and examine woody plant seedlings for the same.

(c) Induction of lignification by infection and wounding. This aspect of the project has been on target all this year, and we will now address the effect of simulated infection on the status of wall-associated (poly)phenolics. We shall also complete the study of the effects of viral infection on lignin and related phenylpropanoids.

(d) *Nitrogen recycling*: Determine whether *P. taeda* cultures are suitable for assaying nitrogen recycling in phenylpropanoid metabolism, and prepare [U- ^{14}C]- and [^{15}N]arogenate for the next phase of the study.

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E-Series

1: $R_1=R_2=R_3=H$

2: $R_1=R_3=H$; $R_2=OCH_3$

3: $R_1=H$; $R_2=R_3=OCH_3$

Z-Series

4: $R_1=Glc$; $R_2=R_3=H$

5: $R_1=Glc$; $R_2=OCH_3$; $R_3=H$

6: $R_1=Glc$; $R_2=R_3=OCH_3$

Scheme 1

Scheme 2

- 15: R₁=R₂=H; Dihydroxy truxillate
16: R₁=H, R₂=OCH₃; Dihydroxymethoxy truxillate
17: R₁=R₂=OCH₃; Dihydroxydimethoxy truxillate

Scheme 3

Figure 1. Analysis of sucrose-grown *Pinus taeda* cell culture filtrate. a) Time course of accumulation of soluble UV-absorbing compounds, b) Reversed-phase HPLC of soluble phenolic compounds. Numbered peaks refer to compounds shown in Scheme 1.

Figure 2. Immunological detection of PAL from *Pinus taeda* cambial tissue. A, Western blot of (1) soluble, (2) microsomal and (3) plasmamembrane associated proteins. Blots were probed with anti-PAL serum (1: 10,000 dilution). B, Immunogold labelling of *Pinus taeda* PAL. Thin section of 7-day old 2,4-D-grown *P. taeda* suspension cultures were probed with anti-PAL serum and subsequently stained with gold-labeled anti-rabbit antibodies. CW, cell wall; P, plastid; PM, plasma membrane; T, tonoplast; Ve, vesicle.

Figure 3. Western blot analysis of *P. taeda* CAD. Lane d, soluble proteins from cambial sap; MW, molecular weight standards.

Figure 4. Immunogold labelling of *Pinus taeda* CAD. A, *P. taeda* shoot cortical cell; B, 2,4-D-grown *P. taeda* cell cultures. Cp, chloroplast; CW, cell wall; G, golgi; M, mitochondrion; P, plastid; T, tonoplast.

Figure 5. Difference spectra of suberin-enriched preparations at various stages of extraction from wound healing potato (*Solanum tuberosum*) tubers previously administered L-[1-¹³C]-phenylalanine. Spectra were recorded after (a) DMSO extraction, (b) enzyme treatment and (c) alkaline hydrolysis. Difference spectra were generated by subtraction of natural abundance resonances obtained from an un-labelled control sample previously administered natural abundance phenylalanine. Only after alkaline hydrolysis has cleaved ester linkages were (presumed) monolignol resonances (e.g. 63.1 ppm) evident.

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