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TOWARDS THE SPECIFICATION OF CONSECUTIVE STEPS IN MACROMOLECULAR LIGNIN ASSEMBLY

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Key Word Index—*Pinus taeda*; Pinaceae; lignins; lignans; H_2O_2; regulation; peroxidase; laccase; potassium iodide.

Abstract—When *Pinus taeda* cell suspension cultures are exposed to 8% sucrose solution, the cells undergo significant intracellular disruption, irregular wall thickening/lignification with concomitant formation of an 'extracellular lignin' precipitate. However, addition of potassium iodide (KI), an H_2O_2 scavenger, inhibits this lignification response, while the ability to synthesize the monolignols, *p*-coumaryl and coniferyl alcohols, is retained. Lignin synthesis (i.e. polymerization) is thus temporarily correlated with H_2O_2 generation, strongly implying a regulatory role for the latter. Time course analyses of extracellular metabolites leading up to polymer formation reveal that coniferyl alcohol, but not *p*-coumaryl alcohol, undergoes substantial coupling reactions to give various lignans. Of these, the metabolites, dihydrodehydrodiconiferyl alcohol, shonanin (divanillyl tetrahydrofuran) and its apparent aryl tetralin derivative, cannot be explained simply on the basis of phenolic coupling. It is proposed that these moieties are the precursors of so-called reduced substructures in the lignin macromolecule. This adds a new perspective to the lignin assembly mechanism.

INTRODUCTION

Evolution of the lignin pathway was selected for the successful adaption of vascular plants to a dry-land habitat [1, 2], a process which also required substantial alterations in both biopolymeric composition and the modes of cell wall assembly. As a consequence, when lignified tracheary elements form, specific cells of apical meristematic roots and shoots change their nuclear and cytoplasmic activities to ultimately afford such specialized walls [3]. The lignins are heterogenous with respect to tissue type, e.g. lignins in fibres of white birch (*Betula papyrifera* Marsh) are mainly (~ 90%) sinapyl alcohol (**3**) derived, whereas vessel and middle lamella lignins predominate in moieties ($\gtrsim$90%) originating from coniferyl alcohol **2** [4] (Scheme 1). Heterogeneity in gymnosperm lignins has also been observed, e.g. compression wood [5] and middle lamella lignins [6] of black spruce (*Picea mariana*) have higher *p*-coumaryl alcohol (**1**) contents than that of secondary wall lignin which is mainly coniferyl alcohol (**2**) derived. Surprisingly, little consideration has been given to the physiological advantages conferred by such programmed changes in lignin biopolymer composition, i.e. in terms of specific or distinctive cell-wall matrix properties conferred to particular tissue types.

In spite of the careful orchestration of lignin monomeric deposition processes in different tissues/cell wall types, the polymers themselves are often viewed as being formed via a random, dehydrogenative polymerization of monolignols **1**–**3**, i.e. in reactions considered to be catalysed via H_2O_2-dependent peroxidase(s) [7], O_2-requiring laccases [8] or both [9]. Because of the intractable nature of the resulting wall-bound polymers, much of our knowledge of lignin structure comes from the dissolution and characterization of lignin-derived monomeric, dimeric and trimeric fragments, released, for example, during 'acidolysis' treatment [reviewed in refs 10 and 11]. While such experimental protocols undoubtedly yield valuable information, they suffer from two major drawbacks. The first is that the lignins are 'hydrolysed'

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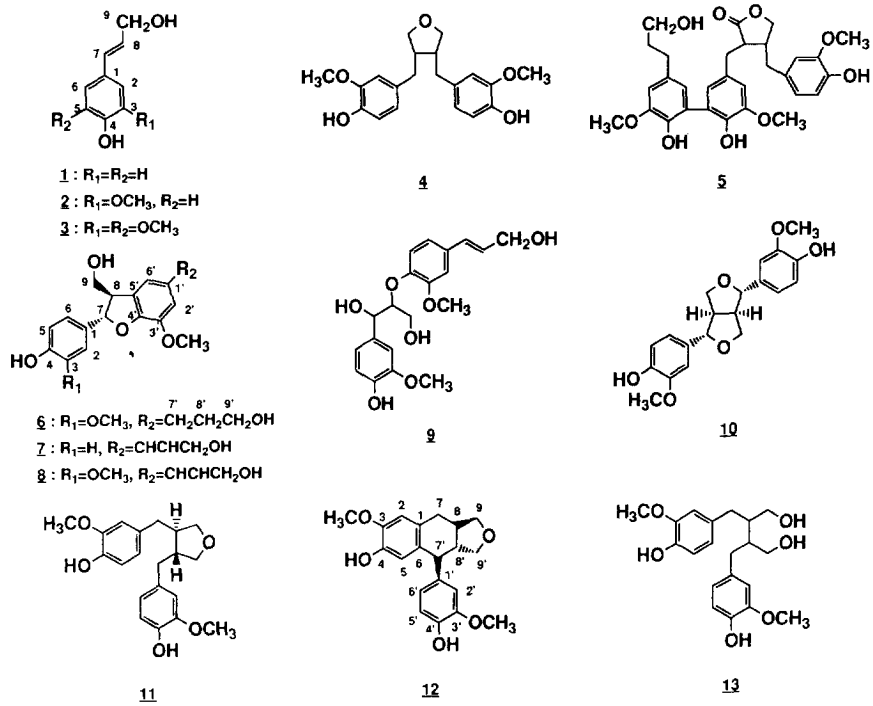

Scheme 1. Various monolignols and lignans isolated from gymnosperm plants and cell cultures.

from tissues/subcellular compartments harbouring more than one lignin type [reviewed in ref. 12]. The second drawback is that fragment yields are generally low and artefact formation cannot be arbitrarily disregarded. [An important example of such limitations was observed recently in *Picea abies* cell culture lignin, where the so-called β-1 substructure (Fig. 1, substructure E) was thought to be present as *ca* 7–9% of the native macromolecule [10], but apparently absent when examined using 2D-COSY, *J*-resolved and other 2D NMR techniques [13, 14]. Consequently, the putative β-1 substructure may only be an artefact of isolation.]

In any event, various approximations of lignin structure (bond type and frequency) have been made, one such example being summarized in Fig. 1 for a gymnosperm lignin (adapted from Adler [10]). But other putative lignin fragments have also been characterized, which surprisingly seem to be derived via oxidoreductive processes, e.g. divanillyl tetrahydrofuran **4**, the trimer **5** and dihydrodehydrodiconiferyl alcohol **6** (Scheme 1). These substructures cannot simply be formed on the basis of monolignol **1**–**3** polymerization. Furthermore, although putatively only present as a small component of the lignin polymers [11], their presence raises fascinating questions regarding the precise mode of lignin assembly and heterogeneity, the influence of such substructures on cell-wall matrix properties and differences in tissue specificity.

In principle, cell suspension cultures engendering specific developmental processes (e.g. tracheid, ray cell formation), or defence responses, can be systematically studied to probe the mechanism of lignin assembly and how modulation of its synthesis affects wall matrix properties.

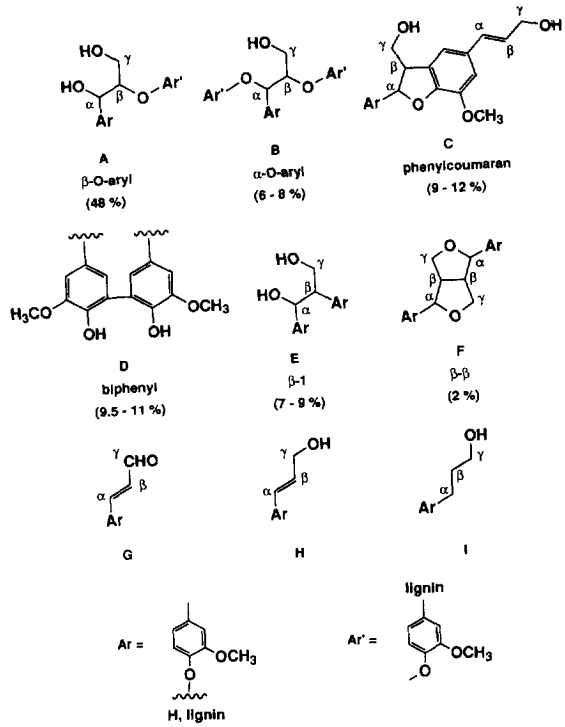

Fig. 1. Proposed main substructures in gymnosperm lignins (adapted from Adler [10]).

Important progress has been made in such areas. For example, *Zinnia elegans* mesophyll cells can be induced to undergo tracheary element formation in culture. But in the presence of limiting amounts of AOPP (α-aminooxy-

β-phenylpropionic acid, a phenylalanine ammonia-lyase inhibitor), substantial lignification is prevented from occurring even though the tracheary element skeleton is still formed [15], apparently demonstrating the independence of both the lignin and cellulose biosynthetic processes. In related studies, cell suspension cultures of the gymnosperm, *Pinus taeda*, have been employed to establish lignin bonding environments *in situ* combining judicious carbon-13 specific-labelling and solid-state ^{13}C NMR spectroscopy [16]; importantly, this revealed convincing evidence for the dominance of the so-called β-*O*-aryl bonding environment *in situ* (substructure A in Fig. 1). There have also been numerous reports of extracellular lignins being formed in cell suspension cultures, particularly when exposed to differing concentrations of sucrose [13, 17–19]. For example, with sycamore (*Acer pseudoplatanus*) suspension cells, exposure to sucrose (up to 6%) resulted in lignins apparently being deposited in the protoplasts, in plates between cells as well as in the 'medium' [17]. This was accompanied by the corresponding cells undergoing progressive cellular disorganization and irregular cell wall thickening ultimately resulting in cell death.

In this paper, we have investigated the formation of extracellular lignin synthesis in *P. taeda* cell suspension cultures exposed to sucrose solution [19]. This was in order to establish the following: (i) whether oxidoreductive steps are involved in the synthesis of the various lignin substructures, and whether this occurs prior to, as part of, or following phenolic coupling; (ii) the consecutive process of lignin assembly, and whether O_2–laccase, H_2O_2-dependent peroxidases, or both, are involved; and (iii) whether by judicious choice of inhibitor, lignin synthesis (i.e. monolignol coupling) can be inhibited without interfering with the biosynthesis of monolignols.

RESULTS AND DISCUSSION

Transfer of unlignified *Pinus taeda* cells (2,4-D line) [16, 19] to an 8% sucrose solution induced formation of an extracellular lignin-like precipitate within four to six days. As previously noted for sycamore [17], the *P. taeda* cells went through a series of marked cytoplasmic changes within four to seven days of transfer, as determined by electron microscopy. First, the cell walls underwent irregular expansion to *ca* 0.483 μm ($n = 16$), while cellular contents displayed considerable disorganization (data not shown). Interestingly, this cellular disruption was also accompanied by the formation of electron opaque structures which were apparently either fused or associated with the plasma membrane, as well as accumulating in the vacuole; these may be involved in transport of lignin precursors. Whichever case holds, these observations point to a series of essentially uncharacterized biochemical events in the programmed conscription of cellular components during wall assembly, ultimately leading to cell death.

As these metabolic 'perturbations' continued within the *P. taeda* cells, the culture medium turned cloudy and within four to six days post-transfer resulted in the formation of an 'extracellular precipitate'. This precipitate was collected by centrifugation, washed, and studied by ^{1}H and ^{13}C NMR spectroscopic techniques (TOCSY, DEPT, HETCOR, HMQC and HMBC). By analysing the TOCSY spectrum, crosspeaks were assigned (Table 1) based on expected chemical shifts of the H_α, H_β and H_γ protons of lignin structural units A–C and F–I (Fig. 1). Further support for the presence of all of these units, except substructure B, was obtained by HETCOR and HMQC (substructures A, C and F–I) and HMBC (substructures G–I) analyses, respectively (Table 1 and Fig. 1).

Table 1. ^{1}H and ^{13}C chemical shifts of observed crosspeaks in TOCSY, HETCOR, HMQC and HMBC NMR spectra for protons and carbons of proposed substructural units (Fig. 1) in lignin

NMR Experiment	Crosspeak type	Substructural type A	B	C	F	G	H	I
TOCSY	H_α–H_β	4.77–4.30	5.28–4.78	5.49–3.45	4.66–3.04	6.78–7.63	6.48–6.23	2.54–1.70
	H_β–H_γ	4.30–3.62	4.78–3.43	3.45–3.65	3.04–4.16	7.63–9.62	6.23–4.10	1.70–3.46
	H_α–H_γ	4.77–3.62	5.28–2.43	5.49–3.65	—	—	—	2.54–3.46
HETCOR and/or HMQC	C_α–H_α	71.2–4.77	—	87.0–5.49	83.1–4.66	121.5–6.78	129.5–6.48	31.5–2.54
	C_β–H_β	84.5–4.30	82.2–4.78	53.5–3.45	53.7–3.04	153.5–7.63	129.0–6.23	34.7–1.70
	C_γ–H_γ	60.2–3.62	60.2–3.43	60.2–3.65	61.7–4.16	194.0–9.62	61.7–4.10	60.2–3.46
HMBC	H_α–C_β	—	—	—	—	—	6.48–129.0	2.56–34.7
	H_α–C_γ	—	—	—	—	—	6.48–61.7	2.56–60.2
	H_β–C_α	—	—	—	—	7.63–126.2	6.23–129.5	1.70–31.5
	H_β–C_γ	—	—	—	—	7.63–194.0	—	1.70–60.2
	H_γ–C_α	—	—	—	—	9.62–126.2	—	—
	H_γ–C_β	—	—	—	—	—	4.10–129.0	—

—, Crosspeak was not observed.

No clear evidence for the presence of the C_α-H_α crosspeak for substructure B could be observed, indicating that, at best, it only occurs as a very minor moiety within the polymer formed in the culture filtrate. In an analogous manner, no crosspeaks corresponding to protons and carbons of interunit linkage E (Fig. 1) were observed in the 2D NMR spectra (*vide supra*), in agreement with previous findings [13, 14]. To establish that the lignin was being formed *de novo* [2-^{13}C]Phe (99 atom% ^{13}C) was next administered to the *P. taeda* cell suspension cultures upon transfer to 8% sucrose. Following uptake and metabolism by the cells, the [2-^{13}C]Phe-labelled extracellular lignin was analysed spectroscopically as before. The ^{13}C $\{^1H\}$ NMR difference spectrum of this precipitate (natural abundance spectrum subtracted from the corresponding ^{13}C enriched spectrum) showed four regions of enhancement at δ34.7 (substructure I), 52.5–53.6 (C, F), 83.5–85.5 (A), 126.2–129.0 (H) as well as a very small resonance at 194 ppm (Fig. 2). These structural units were also identified in the cell suspension culture lignin from *Picea abies* [13, 14]. In an analogous manner, natural abundance solid-state ^{13}C NMR spectroscopy revealed that the cell walls of these sucrose-treated *P. taeda* cultures were also lignified (to be published). Thus, lignin synthesis had occurred in both the cell walls and the culture filtrate.

Having established that the cell cultures of *P. taeda* formed an 'extracellular lignin' *de novo* in response to sucrose solution treatment, attention was next given to the time course of formation of soluble aromatic components present in the sucrose solution leading up to precipitate formation. In this respect, a rapid increase in the amount of soluble UV-absorbing material (λ = 280 nm) was observed over the four to six days following transfer of the unlignified *P. taeda* cells to an 8% sucrose solution. One such example is shown in Fig. 3A. Significantly, the 'extracellular lignin' precipitate was only visible four to seven days post transfer, coincident with a dramatic decrease in this UV-absorbance. Subsequent analyses of the culture medium by reversed-phase HPLC also revealed the progressive accumulation of a complex mixture of phenolics as early as 24 hr post-transfer. This accumulation reached a maximum within four to six days, and then abruptly declined coincident with formation of the 'extracellular-lignin' precipitate. Figure 3B shows a typical chromatogram of the phenolics accumulating within 48 hr prior to extracellular lignin formation.

Of these soluble phenolics, nine metabolites have been isolated and identified to date, including *p*-coumaryl (**1**) and coniferyl (**2**) alcohols, dihydrodehydrodiconiferyl alcohol (**6**), 3-demethoxydehydrodiconiferyl alcohol (**7**), dehydrodiconiferyl alcohol (**8**), guaiacylglycerol-β-*O*-coniferyl alcohol ether (**9**), pinoresinol (**10**), shonanin (**11**) and isoshonanin (**12**) (Scheme 1). Several points are noteworthy: although *p*-coumaryl alcohol (**1**) was formed rapidly (i.e. within 24 hr), it did not appear to undergo substantial further metabolism until the point of extracellular precipitate formation, following which it was essentially no longer detectable. [The only evidence for metabolism of **1**, prior to extracellular lignin synthesis, was formation of small amounts of the dimer **7**, presumably derived from **1** and **2**]. To our knowledge, this is only the second report of a mixed dimer built up from *p*-coumaryl (**1**) and coniferyl (**2**) alcohols [20]. In contrast, *E*-coniferyl alcohol (**2**) was only present in a very small amount, and was presumably rapidly converted into the lignans **6**–**12**. This strongly suggests that monolignols **1** and **2** have quite distinct metabolic fates in *P. taeda*. This is an important finding since it may help explain previous observations (discussed in the Introduction) which suggest that *p*-coumaryl (**1**) and coniferyl (**2**) alcohols are differentially transported during the formation of specific wall layers in 'secondary' wall assembly.

Additionally, the accumulation of dihydrodehydrodiconiferyl alcohol (**6**), shonanin (**11**) and isoshonanin (**12**), prior to polymer formation may also provide an explana-

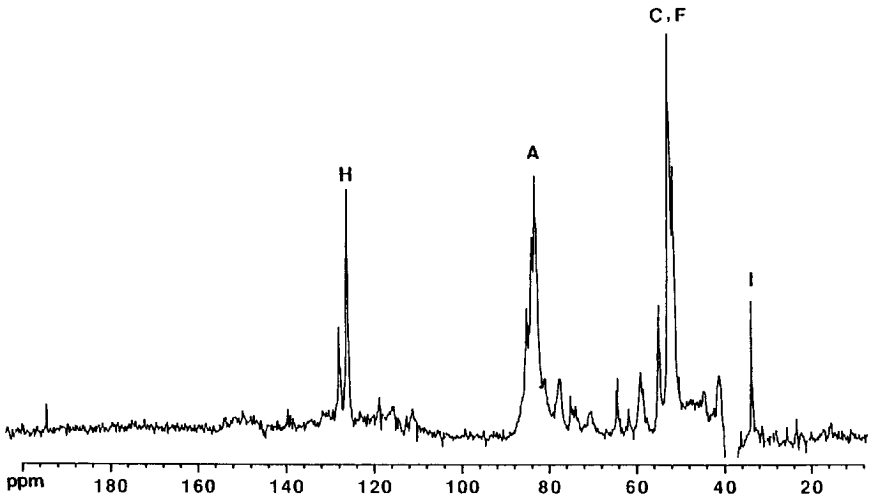

Fig. 2. ^{13}C$\{^1H\}$ NMR difference spectrum of *P. taeda* extracellular lignin derived from [2-^{13}C] phenylalanine; natural abundance resonances have been subtracted from the labelled spectrum. For explanation of substructures see Fig. 1.

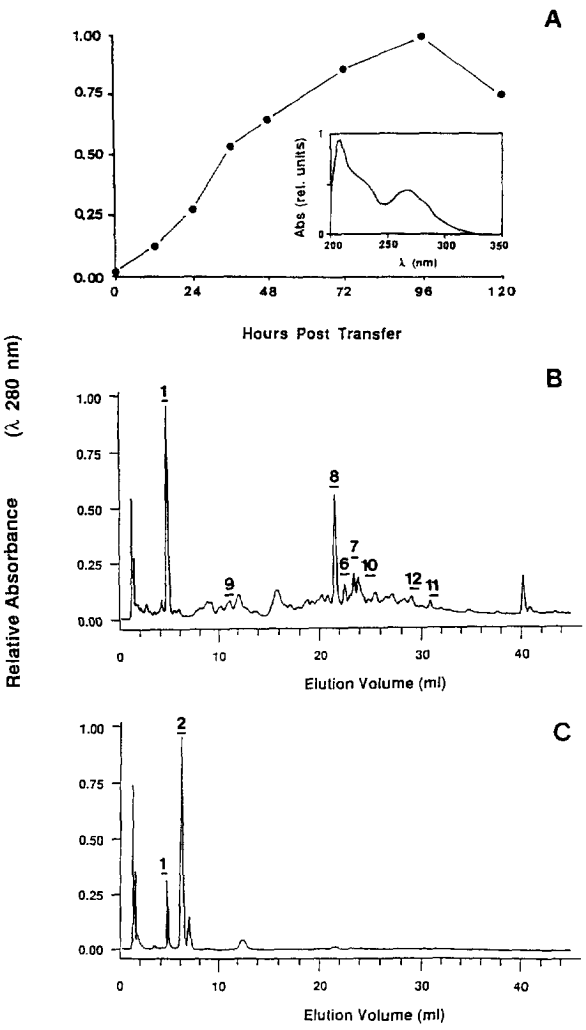

Fig. 3. Spectroscopic analyses of *P. taeda* cell culture filtrates. (A). Changes in UV-absorbance as a function of time ●, UV absorbance measurements. (B/C). HPLC chromatographic separation of soluble phenolics accumulated within 48 hr. Cells treated with 8% sucrose (B); and 20 mM KI/8% sucrose (C). For explanation of structural assignments to peaks, see Scheme 1.

tion for previous observations [10, 11] that some lignin-derived fragments possess (a) reduced side-chains, (b) divanillyl tetrahydrofuran (**4**) (cf. shonanin **11**) and (c) aryltetralin substructures, respectively. As a working hypothesis, it can be proposed that **6** is formed directly via reduction of dehydrodiconiferyl alcohol **8**. On the other hand, shonanin (**11**) may originate from pinoresinol (**10**) via appropriate oxidoreductive transformations, with isoshonanin (**12**) then presumably resulting from cyclization of **11**. [In this context, it is important to note that lignan biosynthetic studies in *Forsythia* species revealed that pinoresinol (**10**) undergoes two sequential benzylic ether reductive steps to give (+)-lariciresinol and (−)-secoisolariciresinol (**13**), with dehydrogenation of the latter affording (−)-matairesinol (summarized in ref. 21).] (Elucidation of the biochemical pathways to **6**, **11** and **12** will be the subject of a future report.)

Mode of extracellular lignin synthesis

Given the apparently different metabolic fates of each monolignol, the next question of interest was the mechanism of formation of the extracellular lignin precipitate, derived from *p*-coumaryl alcohol (**1**) and the soluble lignans **6**–**12**. As described earlier, three biochemical processes have been proposed for lignin synthesis, which involve either H_2O_2-dependent peroxidase(s) [7], O_2-requiring laccase(s) [8], or a combination of both [9]. Thus, aliquots of culture medium (collected 96 hr post-transfer) were analysed for soluble oxidase (i.e. laccase-like) [22] and peroxidase [23] activities. Three separate assay systems were employed: conventional spectrophotometric assays using ABTS and guaiacol–H_2O_2 as substrates, respectively, as well as one utilizing the monolignols **1** and **2** ($\pm H_2O_2$). No evidence for soluble oxidase activity was detected under any of the assay conditions employed. On the other hand, peroxidase activity was consistently present and steadily increased until precipitate began to appear (data not shown). A more intriguing observation, however, was that of intermittent H_2O_2 formation (measured indirectly via a guaiacol-peroxidase assay) [24]. Although not observed in every case, H_2O_2 was only detectable (12–83 nmol per ml culture filtrate) just prior to, or during, extracellular lignin synthesis. This indicated, that, at least for 'extracellular lignin' formation, H_2O_2 generation was critically important and, indeed, could be a regulatory step in lignification. These findings are consistent with previous histochemical observations using *Z. elegans* that suggested an important temporal and spatial correlation between H_2O_2 synthesis and lignification [25]. Surprisingly, however, there is little rigorously known about the mode of H_2O_2 formation, and its regulation, in cell walls.

With the knowledge that H_2O_2 generation apparently played a critical role in 'extracellular lignin' formation, it was timely to next attempt to determine the effect of an H_2O_2 scavenger on monolignol, lignan or lignin accumulation. Thus, *P. taeda* cells were cultured (in the presence of 8% sucrose as before) for periods of one–seven days, but now with different concentrations of the known H_2O_2 scavenger, KI (0, 0.2, 2, 5, 10, 15, and 20 mM) [26, 27]. It was found that, at concentrations of > 5 mM KI, no extracellular lignin synthesis occurred. HPLC analysis of the resulting culture filtrate at different time intervals revealed only the presence of the monolignols, *p*-coumaryl (**1**) and E. coniferyl (**2**) alcohols, i.e. all of the coupling reactions which normally led to lignan and lignin formation were apparently inhibited (Fig. 3C). Further proof that these moieties only corresponded to monolignols **1** and **2** was obtained from their UV, mass and ^{1}H NMR spectral data. (In an analogous manner, inspection of the solid-state ^{13}C NMR spectrum of cell wall preparations following 20 mM KI treatment revealed that they were unlignified, in contrast to treatment with 8% sucrose alone, i.e. lignification in both the medium and cell walls had been disrupted.) This is the first example of a plant system where lignin/lignan synthesis is inhibited, whilst maintaining the ability to form the monolignols **1** and **2**.

It was next instructive to establish that monolignols **1** and **2** were being formed *de novo*. Consequently, [U-^{14}C]Phe (378 kBq, 13.43 GBq/mmol) was administered to the *P. taeda* cultures in the presence of 20 mM KI and 8% sucrose as before. Following two days, the culture medium was extracted, and monolignols **1** and **2** recovered by preparative HPLC and converted into their corresponding 3,5-dinitrobenzoyl derivatives. The absolute incorporations of [U-^{14}C]Phe into [U-^{14}C]*p*-coumaryl (**1**) and [U-^{14}C]coniferyl (**2**) alcohols were determined to be 2.3 and 7.4%, respectively.

Taken together, these results demonstrated that the addition of > 5 mM KI to the *P. taeda* cell cultures prevented both lignan and lignin synthesis from occurring, but not the *de novo* formation of monolignols **1** and **2**. Importantly, comparable results were obtained when sodium iodide was used instead, indicating that this effect was due to iodide alone and not the effect of K^+. That potassium iodide effectively scavenged H_2O_2 was also established as follows: 100 nmol/ml H_2O_2 was incubated with 20 mM KI, in the presence and absence of 22.5 nkat/ml peroxidase. As can be seen from Fig. 4, the H_2O_2 was decomposed in both instances, albeit more rapidly in the presence of peroxidase [27]. We next examined whether potassium iodide exhibited any inhibitory effect on the catalytic properties of a purified *Forsythia* laccase *in vitro*; however, even at 40 mM concentrations of iodide, no inhibitory activity was detected (to be published).

It can, therefore, be concluded that, following treatment of *Pinus taeda* cell suspension cultures with 8% sucrose, the initial formation of dimeric lignans and the resulting lignins (both intra- and extracellular) is a consequence of H_2O_2-dependent peroxidase activities. No evidence for involvement of any oxidases was obtained under the conditions employed. Our results, therefore, strongly suggest that the availability of H_2O_2 controls the overall sequence of events in this culture system. If this hypothesis is correct, it also suggests that, initially, the soluble peroxidases present in the culture medium display a marked substrate preference for coniferyl (**2**) over *p*-coumaryl (**1**) alcohol in lignan synthesis.

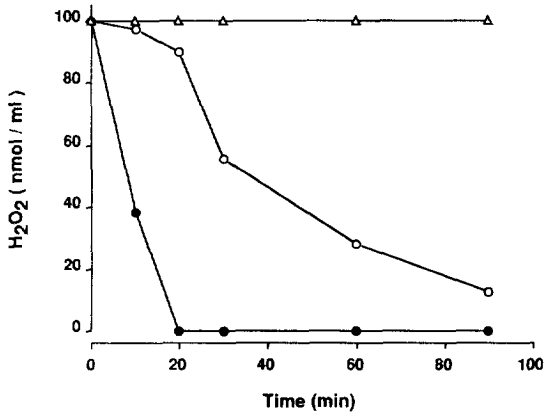

Fig. 4. Decomposition of hydrogen peroxide in solutions containing 20 mM KI/8% sucrose (○) and 20 mM KI/8% sucrose/horseradish peroxidase (●). Control experiments (△) were conducted using 8% sucrose solution alone.

Obviously, these findings do not, at present, allow us to distinguish whether the mode of coupling observed is developmental, tissue-specific or a stress/defence response. Nevertheless, it is confidently expected that intensive investigations under way using this particular model system will furnish an unprecedented opportunity to elucidate and resolve these matters.

CONCLUSIONS

The effect of transferring *Pinus taeda* cell suspension cultures to an 8% sucrose solution resulted in significant alterations in cell wall and cellular metabolism. Rapid dissolution of the familiar cytoplasmic organelles occurred with concomitant irregular cell wall thickening and lignification. At the same time, the culture filtrate accumulated various phenolic constituents; their identification revealed that both *p*-coumaryl (**1**) and coniferyl (**2**) alcohols had quite distinct metabolic fates with the latter apparently undergoing rapid dimer formation. Isolation and spectroscopic analysis of the metabolites resulted in the identification of nine components, three of which (**6**, **11** and **12**) had undergone additional reductive transformations prior to polymer formation. Their formation may account for the previously inexplicable occurrence of such substructures in lignin [10, 11]. These metabolites accumulated in the culture filtrate until *ca* four to six days post-transfer, following which a lignin-like extracellular precipitate was formed. Its formation was apparently temporarily associated with H_2O_2 synthesis, suggesting a previously unrecognized pivotal regulatory role for H_2O_2-generation in lignin formation. By contrast, only monolignol synthesis occurred when a known H_2O_2 scavenger, KI, was used, suggesting an exclusive role for H_2O_2-dependent peroxidase-catalysed transformations under the conditions employed.

It now appears that there are a number of highly orchestrated and defined biochemical events involving monolignol transport into the plasma membrane/cell wall interface, cooperative oxidative coupling reactions, and post-coupling biochemical modifications (e.g. to shonanin-like structures) ultimately leading to lignin synthesis.

Moreover, these findings now invoke another series of unanswered questions: do laccases and laccase-like oxidases or H_2O_2-dependent peroxidases have different physiological roles in lignin synthesis? Is one type involved in defence responses and the other development, or are there subtle tissue specificities that need to be defined? These issues will constitute the basis of future work.

EXPERIMENTAL

Chromatography, reagents and spectral analyses. Solvents for extraction, TLC, and CC were of reagent grade or better. HPLC grade solvents were used for the HPLC systems described below. HPLC, TLC and CC were

performed as described [16]. Solution state ^1H and ^{13}C NMR spectra were recorded, using a Bruker AMX 300 instrument, in $CDCl_3$ with TMS as an internal standard, whereas solid-state ^{13}C cross-polarization magic-angle spinning (CPMAS) spectra were obtained at 100.6 MHz on a Chemagnetics CMX-400 spectrometer. FTIR spectra were obtained from samples dispersed as thin films. MS were recorded in either the EI-MS (70 eV) or FAB-MS (with glycerol as matrix) mode. UV spectra were obtained from MeOH solutions.

Cell cultures. Cell cultures of *P. taeda* were maintained on a modified Brown and Lawrence medium containing 3% sucrose and 2,4-D (11.3 μm) as auxin [16]. Lignification was induced by transferring 7-day-old cells (1 ml PCV per 10 ml medium) to a sterile solution of 8% sucrose [19]. KI treatment was carried out by transferring 7-day-old cells (1 ml PCV per 10 ml medium) to a sterile solution of 8% sucrose containing various concentrations (0.2, 2, 5, 10, 15, and 20 mM) of KI. Cultures were maintained on a Lab-Line (Melrose Park, IL) model 3590 orbital shaker (80 rpm) under constant illumination (25–45 $\mu mol\, s^{-1}\, m^{-2}$) provided by a mixture of two fluorescent (Philips, Cool White) and incandescent (Philips) lights at 24 $\pm$ 1°. Cell growth measurements and other chemical analyses were carried out on freeze-dried tissue: briefly, cells were collected by filtration (Miracloth, Calbiochem) under gentle suction, rinsed with H_2O, frozen by liquid N_2, freeze-dried and stored at − 80°.

Analysis of culture filtrate. (i) *UV measurements.* Portions of culture filtrate (100 μl) were removed under aseptic conditions at different time intervals, centrifuged (12 000 *g*), with the supernatant diluted with methanol (1 : 10), and measured directly at $\lambda = 280$ nm (Fig. 3A).
(ii) *HPLC analyses.* Aliquots of culture filtrate (100 μl) were collected under aseptic conditions, filtered (0.45 μm), and 50 μl analysed directly on a Novapak reversed-phase C-18 column (5 μm, 150 × 3.9 mm i.d., Waters). Compounds were eluted either with MeCN–3% HOAc (1 : 19) for monolignols, or a gradient of MeCN in 3% HOAc for lignans at 1.0 ml min^{-1} as follows: initial conditions 10% MeCN for 5 min, followed by sequential gradients to 40% (30 min), and then to 95% MeCN (5 min). The eluent was monitored simultaneously at 262 and 280 nm.

Analysis of extracellular precipitate. Four-day-old cells from 500 ml of culture were filtered through glass wool and washed with H_2O (500 ml). The extracellular ppt. was collected from the filtrate by centrifugation (12 000 *g*, 15 min), and washed with H_2O (500 ml × 3) centrifuging as above after each wash. The final pellet was suspended in a small vol. of H_2O (total 30 ml), transferred to a 50 ml conical bottom tube and freeze-dried to afford a pale yellow powder (59 mg). The residue was redissolved in DMSO-d_6 and subjected to TOCSY, HETCOR, HMQC and HMBC NMR analyses (see Table 1).

Isolation of proteins from culture filtrate. Before and after centrifugation (to collect the extracellular precipitate described above), proteins were isolated from the culture filtrate as follows: the filtrate was concentrated approximately 10-fold (to 70 ml) using an Amicon ultrafiltration apparatus (YM 10 membrane), loaded onto a Sephadex G-25M column (2.5 × 60 cm) pre-equilibrated with phosphate buffer (100 mM, pH 7.0) and eluted with the same (1.4 ml min^{-1}). The protein fraction (monitored at 280 nm) was collected (*ca* 90 ml), divided into 3 ml aliquots and made to 10% sucrose (w/v) by the addition of solid sucrose. Fractions were stored at − 80° until assayed.

Enzyme assays. Peroxidase and oxidase assays were carried out on culture filtrate protein concentrates after stored samples were thawed and desalted, using PD 10 gel filtration columns (Pharmacia), into an appropriate buffer. For peroxidase assays, samples were desalted into phosphate buffer (100 mM, pH 6.25), whereas for oxidase assays, proteins were desalted into citrate buffer (50 mM, pH 5,0).

Peroxidase assays. Peroxidase activity was measured using three different assay conditions. The first [23] utilized a conventional guaiacol–H_2O_2 assay (9 mM guaiacol, 9 mM H_2O_2, monitored spectrophotometrically at 470 nm for 5 min at 30°). A second spectrophotometric assay was developed using monolignols. The assay consisted of 1 μM monolignol (*p*-coumaryl, coniferyl or sinapyl alcohol), 200 μl desalted protein concentrate and phosphate buffer in a final volume of 980 μl. The reaction was initiated by the addition of 20 μl 12.5 mM H_2O_2 and monitored for up to 10 min at either 262 nm (*p*-coumaryl and coniferyl alcohol) or 273 nm (sinapyl alcohol) at 30°. The third assay condition consisted of 250 μl desalted protein concentrate, 20 μl 5 mM monolignol and 20 μl 10 mM H_2O_2. Multiple assays were incubated at 30° for up to 60 min. At various times, samples were removed and the reaction stopped by the addition of 70 μl 100 mM Na ascorbate and 20 μl glacial acetic acid. After mixing and centrifugation (10 min, 10 000 *g*), 100 μl aliquots were analysed by HPLC as described above.

Oxidase assays. Oxidase activity was assayed in one of three ways. The first [22] was a spectrophotometric assay using 2,2′-azino-bis-(3-ethylbenzenethiazoline-6-sulphonic acid) (ABTS) as substrate at 0.03% (w/v) final concentration in a 1.0 ml cuvette, with up to 300 μl protein concentrate, monitoring at 420 nm for 5 min at 30°. The other two assay systems utilized monolignols as substrate, and were as described above except that H_2O_2 was omitted.

Metabolite isolation from P. taeda *cell culture filtrate.* Aliquots of sucrose-grown *P. taeda* cell filtrate (100 ml) collected 4 days post transfer were mixed with saturated NaCl solution (30 ml) and partitioned with EtOAc (150 ml × 4). The pooled organic solubles from 500 ml cell culture filtrate were evaporated to dryness (101 mg), redissolved in a minimum volume of CH_2Cl_2 and applied to a silica gel column (2 × 20 cm). Sequential elution with CH_2Cl_2–MeOH (49 : 1, 500 ml), CH_2Cl_2–MeOH (19 : 1, 500 ml), CH_2Cl_2–MeOH (9 : 1, 500 ml) and CH_2Cl_2–MeOH (1 : 1, 500 ml) gave 98 fractions (20 ml).

Frs 13–15 were combined and concd *in vacuo* to yield 3.37 mg. The residue was dissolved in a minimum volume of $CHCl_3$, applied to a prep. silica gel (Merck) TLC plate (500 μm), and eluted (× 2) with $CHCl_3$–MeOH (49 : 1). Four bands (detected by UV) were collected, eluted with

EtOAc, filtered and evapd to dryness to give **2** (0.50 mg), **10**, (0.85 mg), **11** (0.45 mg) and **12** (0.33 mg).

Frs 21–24 were combined and concd *in vacuo* to yield 8.04 mg, then redissolved in a minimum volume of $CHCl_3$, applied to a prep. silica gel TLC plate (500 μm) and eluted ($\times 2$) with hexane–Me_2CO (7:3). A single UV absorbing band was collected, eluted with EtOAc, filtered and evapd to dryness to afford pure **1** (6.06 mg).

Frs 30–32 were combined and concd *in vacuo* to yield 7.03 mg. This was dissolved in a minimum volume of $CHCl_3$, applied to a prep. TLC plate (500 μm), and eluted ($\times 2$) with hexane–Me_2CO (1:1). Three UV absorbing bands were collected, eluted with EtOAc, filtered and evapd to dryness to afford pure **6** (0.87 mg), **7** (0.59 mg) and **8** (4.30 mg).

Frs 33–34 were combined and concd *in vacuo* to yield 3.40 mg, then redissolved in a minimum vol. of $CHCl_3$, applied to a prep. silica gel TLC plate (500 μm), and eluted ($\times 2$) with hexane–Me_2CO–MeOH (11:8:1). A single UV absorbing band was collected, eluted with EtOAc, filtered and evapd to dryness to afford pure **9** (1.02 mg).

Compounds **1**, **2**, **6**–**11** were identified based on their ^{1}H NMR, MS and IR spectral properties. Spectral data for **1** and **2** [28], **6** and **8** [29], **9** [30], **10** [31] and **11** [32] have been reported elsewhere.

3-Demethoxydehydrodiconiferyl alcohol, (7S,8S)- $\Delta^{7'(E)}$ 7-0-4',8-5'-*lignan* (**7**). FT-IR cm^{-1}: 3339, 1603, 1519, 1489, 1266, 1240; ^{1}H NMR (300 MHz, $CDCl_3$): δ3.93 (3H, *s*, OMe), 3.95 (2H, *m*, H-9), 4.28 (2H, *d*, $J_{9'-8'} = 5.9$ Hz, H-9'), 5.53 (1H, *d*, $J_{7-8} = 6.7$ Hz, H-7), 5.65 (1H, *s*, OH), 6.20 (1H, *dt* $J_{8'-9'} = 5.9$ Hz, $J_{8'-7'} = 16.0$ Hz, H-8'), 6.56 (1H, *d*, $J_{7'-8'} = 16.0$ Hz, H-7'), 6.80 (2H, *d*, $J_{3-2} = J_{5-6} = 8.2$ Hz, H-3 and H-5), 6.87 (2H, *br s*, H-2' and H-6'), 7.26 (2H, *d*, $J_{2-3} = J_{6-5} = 8.2$ Hz, H-2 and H-6); FAB-MS: 329 [12, MH]$^+$, 311 [28, M $-$ H_2O]$^+$.

Isoshonanin (**12**). FT-IR cm^{-1}: 3418, 1603, 1514, 1465, 1372, 1271, 1218, 1153; ^{1}H NMR (300 MHz, $CDCl_3$): δ2.24 (*m*, $\Sigma J \sim 25.8$ Hz, 2H, H-8, H-8'), 2.70 (*dd*, 1H, $J_{7a,7b} = 15$ Hz, $J_{7a,8} = 9.7$ Hz, 7a), 2.96 (*dd*, 1H, $J_{7b,7a} = 15$ Hz, $J_{7b,8} = 4.2$ Hz, 7b), 3.50 (1H, CH_2-9'a), 3.54 (1H, CH_2-9'a), 3.70 (*d*, 1H, $J = 9.8$ Hz, H-7'), 3.80 (*s*, 3H, OMe), 3.82 (1H, CH_2-9'b), 3.85 (*s*, 3H, OMe), 4.2 (1H, CH_2-9b), 5.35 (*br s*, 1H, OH), 5.50 (*br s*, 1H, OH), 6.37 (1H, $j_{5-2} = 0.8$ Hz, H-5), 6.55 (*d*, 1H, $J_{2',6} = 1.8$ Hz, H-2'), 6.60 (*br s*, 1H, H-2), 6.63 (*dd*, 1H, $J_{6'2'} = 1.8$ Hz, $J_{6'5'} = 8.0$ Hz, H-6'), 6.82 (*d*, 1H, $J_{5',6'} = 8.0$ Hz, H-5'); ^{13}C NMR (75.47 MHz, $CDCl_3$): δ32.5 (C-7), 42.4 (C-8'), 49.6 (C-7'), 50.6 (C-8), 55.8 and 55.9 (2 $\times$ OMe), 72.4 (C-9'), 73.2 (C-9), 110.4 (C-2), 111.0 (C-2), 114.3 (C-5'), 115.2 (C-5), 121.5 (C-6'), 127.6 (C-1), 133.0 (C-6), 136.4 (C-1'), 143.7 (C-4), 144.4 (C-4), 145.0 (C-3), 146.7 (C-3); MS *m/z* (rel. int.): 342 [100, M$^+$], 311 [10], 241 [18], 137 [28], 111 [10], 97 [18] and 83 [25].

Chemical synthesis. Dihydrodehydrodiconiferyl alcohol (**6**): to a stirred solution of dehydrodiconiferyl alcohol **8** (1.75 mg, 4.9 nmol) in EtOAc (2 ml) was added Pd-C (10%, 1.75 mg) under hydrogen at room temperature. After 1 hr the reaction mixture was filtered on Celite and silica H, and the solvent evapd *in vacuo* to give an oil. Compound **6** (1.45 mg, 83.3%) was purified by HPLC (Novapak C-18, 3.9 $\times$ 150 mm, Waters), with isocratic elution [MeCN–H_2O containing 3% HOAc, 1:4] at 1 ml min^{-1}.

Shonanin **11**: synthesized from secoisolariciresinol (**13**) (10.8 mg, 0.030 mmol) as adapted from ref. [33]. Purification by prep. TLC (hexane–EtOAc, 7:3) yielded **11** (6.43 mg, 63.3%).

Isoshonanin **12**: synthesized from **11** (16.2 mg, 0.047 mmol) as adapted from ref. [34]. Purification by prep. TLC ($CHCl_3$–MeOH, 49:1) yielded **12** (5.2 mg, 32.3%).

Incorporation of [*U*-^{14}C] *Phe into* [*U*-^{14}C] p-*coumaryl* (**1**) *and coniferyl* (**2**) *alcohols.* L-[U-^{14}C] Phe (378 kBq, 13.43 GBq mmol^{-1}) was administered to 7-day-old 2,4-D grown cells (2.5 ml PCV) in 25 ml of a sterile solution of 8% sucrose, with and without 20 mM KI, respectively. [Aliquots of culture medium (100 μl) were collected under aseptic conditions with 50 μl subjected to HPLC analyses as described above.] For the KI treatments, *p*-coumaryl (**1**) (5 mg) and coniferyl (**2**) (5 mg) alcohols were added as radiochemical carriers to 50 ml aliquots of 20 mM KI–8% sucrose-grown *P. taeda* cell culture filtrate collected 2 days post-transfer. These were then mixed with satd NaCl solution (10 ml) and partitioned with EtOAc (50 ml $\times$ 3). The organic layers were combined, dried, evapd to dryness (22.5 mg), and the whole reconstituted in MeOH (0.5 ml). The monolignols were separated on a μ-Bondapak C_{18} reversed-phase HPLC column (10 μm, 300 $\times$ 7.9 mm i.d., Waters) eluted with MeCN–3% HOAc (1:19) at 3.0 ml min^{-1}. [Eight injections in total were made (8 $\times$ 50 μl), with the corresponding monolignols collected separately and lyophilized to give **1** (4.2 mg) and **2** (3.5 mg), respectively.] Each monolignol was separately dissolved in pyridine (1.5 ml) under N_2. To these were added 3,5-dinitrobenzoyl chloride (32 mg, 138 μmol), and the mixtures stirred at 35° for 60 min. The reaction mixtures were diluted with $CHCl_3$ (10 ml), extracted with HCl (1 M, 3 $\times$ 5 ml), $NaHCO_3$ (1 M, 3 $\times$ 5 ml), and washed with H_2O (2 $\times$ 5 ml). The $CHCl_3$ layers were individually dried (Na_2SO_4), and the solvent removed *in vacuo*. Each crude product was reconstituted in a minimum vol. of EtOAc and purified using prep. silica gel TLC plates with toluene–EtOAc (9:1). Each derivative was collected and recovered, then reconstituted in EtOAc (300 μl) and further purified using a Waters μ-Porasil normal phase column (10 μm, 300 $\times$ 7.9 mm i.d.), eluted with hexane–EtOAc (21:4) at a flow rate of 3 ml min^{-1} to give the 4,9-*O*,*O*-[3',5'-dinitrobenzoyl] derivatives of **1** (4.9 mg, 4.5 kBq, 26.8% recovery, 2.3% absolute incorporation) and **2** (6.2 mg, 18.7 kBq, 35% recovery, 7.4% absolute incorporation).

Administration of natural abundance and [2-^{13}C] *Phe.* Natural abundance and L-[2-^{13}C] Phe (99 atom % ^{13}C), 10 mM final concentration, were individually administered to 7-day-old 2,4-D treated *P. taeda* cells (2.5 ml CV) in 25 ml of a sterile solution of 8% sucrose. Cells were harvested at weekly intervals from 1 to 4 weeks, with cell wall preparations and extracellular precipitate isolated as follows.

(i) *Cell wall preparations.* These were prepared as previously described [10]. Typical yields of extractive-free cell wall preparations, obtained from *ca* 385 mg of freeze-dried [2-^{13}C]Phe administered and natural abundance Phe administered cell walls, were 153 and 155 mg, respectively. The resulting extractive-free cell walls were subsequently analysed by solid-state ^{13}C NMR spectroscopy.

(ii) *Extracellular precipitate.* This was isolated as previously described in 'Analysis of Extracellular Precipitate'. Typical yields for [2-^{13}C]Phe and natural abundance Phe administered cells were 59 and 55 mg, respectively. The residues were individually dissolved in DMSO-d_6 and analysed by solution-state ^{13}C NMR spectroscopy (see Table 1).

Effect of KI *and* KI–*peroxidase on* H_2O_2 *stability.* The procedure used was modified from ref. [24] and involved the measurement of the rate of peroxidative oxidation of guaiacol by H_2O_2. The reaction mixture consisted of 20 mM KI/8% sucrose solution (1.96 ml), 10 mM H_2O_2 (20 μl), in the presence or absence of peroxidase (20 μl, 0.1 mg ml^{-1}, Horseradish Type II, 200 u mg^{-1} Sigma). Each was subjected to the same conditions as the cell cultures. Appropriate controls using H_2O_2 without KI were carried out. Aliquots (100 μl) of each reaction mixture were removed at specific time intervals and added to the assay solution consisting of potassium phosphate buffer (100 μl, 100 mM, pH 6.25), guaiacol (10 μl, 25 mM in H_2O), peroxidase (10 μl, 1 mg ml^{-1} in 100 mM phosphate buffer) and H_2O (780 μl). The assay mixture was incubated for 2 min at room temperature with the absorbance at 470 nm directly measured and converted to H_2O_2 equivalents from a calibration curve.

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