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# Basic peroxidases: The gateway for lignin evolution?

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**Abstract**

Lignins are cell wall phenolic heteropolymers which result from the oxidative coupling of three monolignols, *p*-coumaryl, coniferyl and sinapyl alcohol, in a reaction mediated by peroxidases. The most distinctive variation in the monomer composition of lignins in vascular plants is that found between the two main groups of seed plants. Thus, while gymnosperms lignins are typically composed of G units, with a minor proportion of H units, angiosperms lignins are largely composed of similar levels of G and S units. The presence of S units in angiosperm lignins raises certain concerns in relation with the step of lignin assembly due to the inability of most peroxidases to oxidize syringyl moieties. *Zinnia elegans* is currently used as a model for lignification studies: – first because of the simplicity and duality of the lignification pattern shown by hypocotyls and stems, in which hypocotyl lignins are typical of angiosperms, while young stem lignins partially resemble those occurring in gymnosperms. Secondly, because of the nature of the peroxidase isoenzyme complement, which is almost completely restricted to the presence of a basic peroxidase isoenzyme, which is capable of oxidizing both coniferyl and sinapyl alcohol, as well as both coniferyl and sinapyl aldehyde. In fact, the versatility of this enzyme is such that the substrate preference covers the three *p*-hydroxybenzaldehydes and the three *p*-hydroxycinnamic acids. The basic pI nature of this peroxidase is not an exceptional frame point in this system since basic peroxidases are differentially expressed during lignification in other model systems, show unusual and unique biochemical properties as regards the oxidation of syringyl moieties, and their down-regulation in transgenic plants leads to a reduction in lignin (G+S) levels. Basic peroxidase isoenzymes capable of oxidizing syringyl moieties are already present in basal gymnosperms, an observation that supports the idea that these enzymes were probably present in an ancestral plant species, pre-dating the early radiation of seed plants. It also suggests that the evolutionary gain of the monolignol branch which leads to the biosynthesis of sinapyl alcohol, and of course to syringyl lignins, was not only possible but also favored because the enzymes responsible for its polymerization had evolved previously. In this scenario, it is not surprising that these enzymes responsible for lignin construction appeared early in the evolution of land plants, and have been largely conserved during plant evolution.

*Abreviations:* 4CL – *p*-hydroxycinnamate CoA ligase; C3H – *p*-coumarate-3-hydroxylase; C4H – cinnamate-4-hydroxylase; *p*-CA – *p*-coumaric acid; CAD – coniferyl alcohol dehydrogenase; CAld5H – coniferylaldehyde-5-hydroxylase; CCR – *p*-hydroxycinnamoyl-CoA reductase; CoI – compound I; CoII – compound II; G – guaiacyl unit; H – *p*-hydroxyphenyl unit; PAL – phenylalanine ammonia-lyase; S – syringyl unit.

## Lignins

The xylem constitutes the longest pathway for water transport in vascular plants. It is a simple pathway of low resistance, which enables water to be transported in large quantities with great efficacy, especially from the roots to the leaves (Kozela and Regan, 2003). Most terminally differentiated cells fulfill specialized functions until they die but, in the case of the xylem, its function does not really begin until after cell death

(Roberts and McCann, 2000). Acquisition of function by xylem elements involves cell death and, therefore, functional water-conducting cells have no membranes or organelles, and what remains are the thick, lignified cell walls, which form hollow tubes through which water can flow with relatively little resistance (Kozela and Regan, 2003). Terminal xylem elements (water-conducting cells) are thus internally coated with lignins, which confer resistance against the tensile forces of the water columns they contain, and also impart water impermeability. This process of sealing plant cell walls through lignin deposition is known as lignification, and provides mechanical strength to the stems, protecting cellulose fibers from chemical and biological degradation (Grabber et al., 1998). In this context, plant cell wall lignification is one of the main restrictive factors in the use and recycling of plant biomass (Anterola and Lewis, 2002).

Lignins represent the second most abundant organic compound on the earth's surface after cellulose, and account for about 25% of plant biomass (Higuchi, 1990). They are found specifically in vascular plants (tracheophyta) and occur selectively in greatest quantity in the secondary cell walls of particular cells which form parts of woody tissues, such as fibers, xylem vessels, tracheids, and sclereids. Lignins have been identified in pteridophytes (ferns, lycophytes and horsetails), widely considered to be the first vascular plants, and are likely to have played a key role in the colonization of the terrestrial landscape by plants during the Ordovician to Silurian transition, 400 to 450 million years ago (Niklas, 1997). However, although they have putatively been identified in club mosses, they are absent from mosses which have no tracheids and, of course, from algae (Lewis and Yamamoto, 1990). Thus, and from the botanical standpoint, the phenomenon of lignification is essentially associated with the acquisition of the vascular structure by plants.

Lignins are three-dimensional, amorphous, heteropolymers which result from oxidative coupling of the three *p*-hydroxycinnamyl alcohols (monolignols, Figure 1A), *p*-coumaryl (I), coniferyl (II) and sinapyl (III) alcohols, in a reaction mediated by peroxidases (Ros Barceló, 1997). The cross-coupling reaction produces an optically inactive hydrophobic heteropolymer (Ralph et al., 1999) composed by H (*p*-hydroxyphenyl), G (guaiacyl), and S (syringyl) units, respectively. The fact that the three monomer constituents of lignins differ as to the extent of their methoxylation (Figure 1A) and that for each one of the three building blocks the radical has at least five mesomeric forms (Figure 1B), suggests that a plethora of substructures may be formed during dimerization, i.e, $C_{m,n} = m\ (m + 1)\ /\ n\ (n - 1) = 120$, where $m$ is the number of mesomeric forms to be combined (i.e. $m = 3 \times 5 = 15$) and $n$ the polymerization degree (i.e., $n = 2$).

Phenylpropanoid units are interconnected in lignins by means of a series of ether and carbon-carbon linkages (see Ralph et al., 2004b, for review), which lead to the main sub-structures: guaiacylglycerol-$\beta$-aryl ether, phenylcoumaran, diarylpropane, resinol, biphenyl, and diphenyl ether, as well as other substructures of lesser importance. The most frequent $\beta$-*O*-4 bonds are present in guaiacylglycerol-$\beta$-aryl ether substructures, which are the targets of most lignin depolymerization processes, including thioacidolysis. In contrast, other inter-units bonds, such as $\beta$-5 (in phenylcoumaran), $\beta$-1 (in diarylpropane), $\beta$-$\beta$ (in resinol), 5-5 (in biphenyl) and 5-*O*-4 (in diphenyl ether), are very resistant to degradation. At the chemical level, lignins are therefore ill-defined polymers whose monomeric composition varies greatly, as does the nature of their inter-unit linkages. This means that the expression 'lignins' is preferable to the use of 'lignin', since a great diversity of chemical structures probably exists within natural lignins (Boudet, 1998).

However, the most distinctive variation in lignin monomer composition in vascular plants is that found between the two main groups of seed plants. Thus, in gymnosperms, lignins are typically composed of G units, with a minor proportion of H units, while in angiosperms lignins are mainly composed of similar levels of G and S units (Higuchi, 1990). Caution should be exercised, however, when attempting to define lignin composition as a function of taxonomy, since there are some gymnosperms in which S moieties predominate, and some angiosperms in which lignins are principally of the G type (Lewis and Yamamoto, 1990). In grasses (see Ralph et al., 2004a, for review), lignins are more complex, since they also contain significant amounts of ester bound *p*-coumaric acid (Ralph et al., 1994). In such scenario, it may be concluded that the chemical complexity of lignins has increased during the course of plant evolution from ancient pteridophytes and gymnosperms to the most evolved grasses.

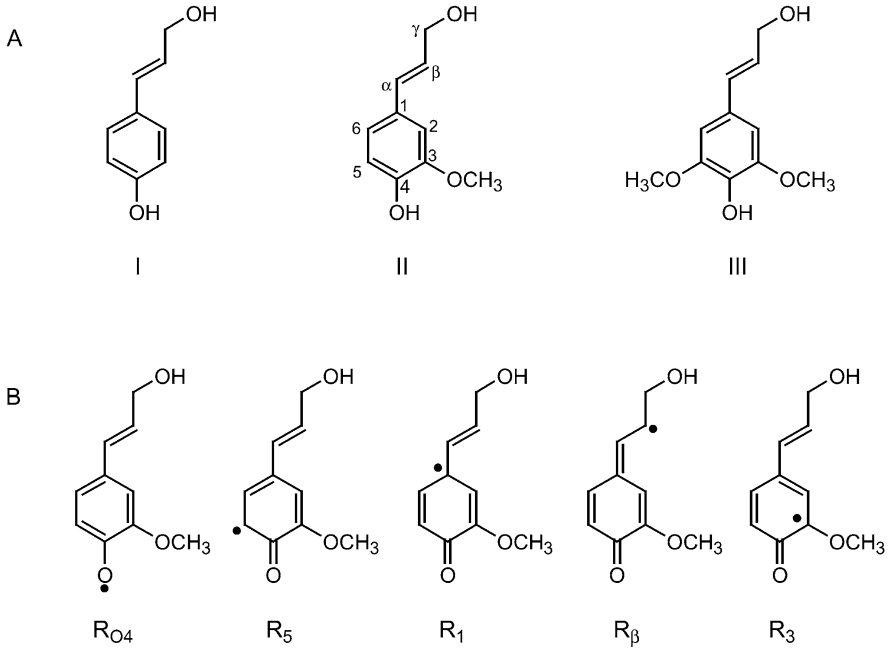


*Figure 1.* A) Structures of *p*-coumaryl (I), coniferyl (II) and sinapyl (III) alcohols. B) Main mesomeric forms of coniferyl alcohol radical resulting from oxidation of coniferyl alcohol by peroxidase.

## The lignin biosynthetic pathway

The biosynthesis of lignins proceeds through a long sequence of reactions that involve (i) the shikimate pathway, which provides L-phenylalanine and L-tyrosine, (ii) the common phenylpropanoid pathway from L-phenylalanine (and/or L-tyrosine) to the *p*-hydroxycinnamoyl CoAs, and (iii) the lignin-specific pathway, which channels the *p*-hydroxycinnamoyl CoAs toward the synthesis of monolignols, and their later polymerization in cell walls (see Whetten and Sederoff, 1995 and Boerjan et al., 2003, for review), in a reaction catalyzed by peroxidases. Plants are the only living organisms capable of channeling carbon from the primary metabolism toward lignin biosynthesis and it is widely accepted that the evolutionary acquisition of the phenylpropanoid pathway has played a key role in the ability of plants to colonize land, not only because one of the pathway's products (the lignins) serves to strengthen the aerial organs of the plant, but also because other products (the flavonoids) act as protectors against dangerous UV radiation. To integrate this novel evolutionary pathway and the general aromatic amino acid biosynthesis pathway in an efficient metabolic highway in vascular plants, the activities of the enzymes of the shikimate pathway are closely coordinated with the activities of the enzymes of the phenylpropanoid pathway (Whetten and Sederoff, 1995).

### *The polymerization step*

The last reaction in the pathway is the oxidative polymerization of the three monolignols to yield lignins in a reaction catalyzed by class III plant peroxidases (POD, hydrogen donor: $H_2O_2$ oxidoreductase, EC 1.11.1.7) (Figure 2). Peroxidases are heme-containing enzymes which catalyze the one-electron oxidation of the three monolignols (RH) at the expense of $H_2O_2$ yielding phenoxy radicals ($R^{\cdot}$) and water:

$$2RH + H_2O_2 \rightarrow 2R^{\cdot} + 2H_2O$$

The catalytic cycle for peroxidase (Yamazaki and Yokota, 1973) may be described as follows. Hydrogen peroxide oxidizes the ferric form of the enzyme (FeIII) in a two electron oxidation step to yield the enzyme intermediate compound I (CoI). CoI, described as an oxyferryl porphyrin $\pi$ cation radical containing Fe in the formal oxidation state, Fe(IV) (Banci, 1997), accepts one electron and one proton from the monolignol to yield its corresponding radical and the oxyferryl heme intermediate known as compound II (CoII). The subsequent one electron reduction of CoII by a second molecule of monolignol yields the ferric form of per-

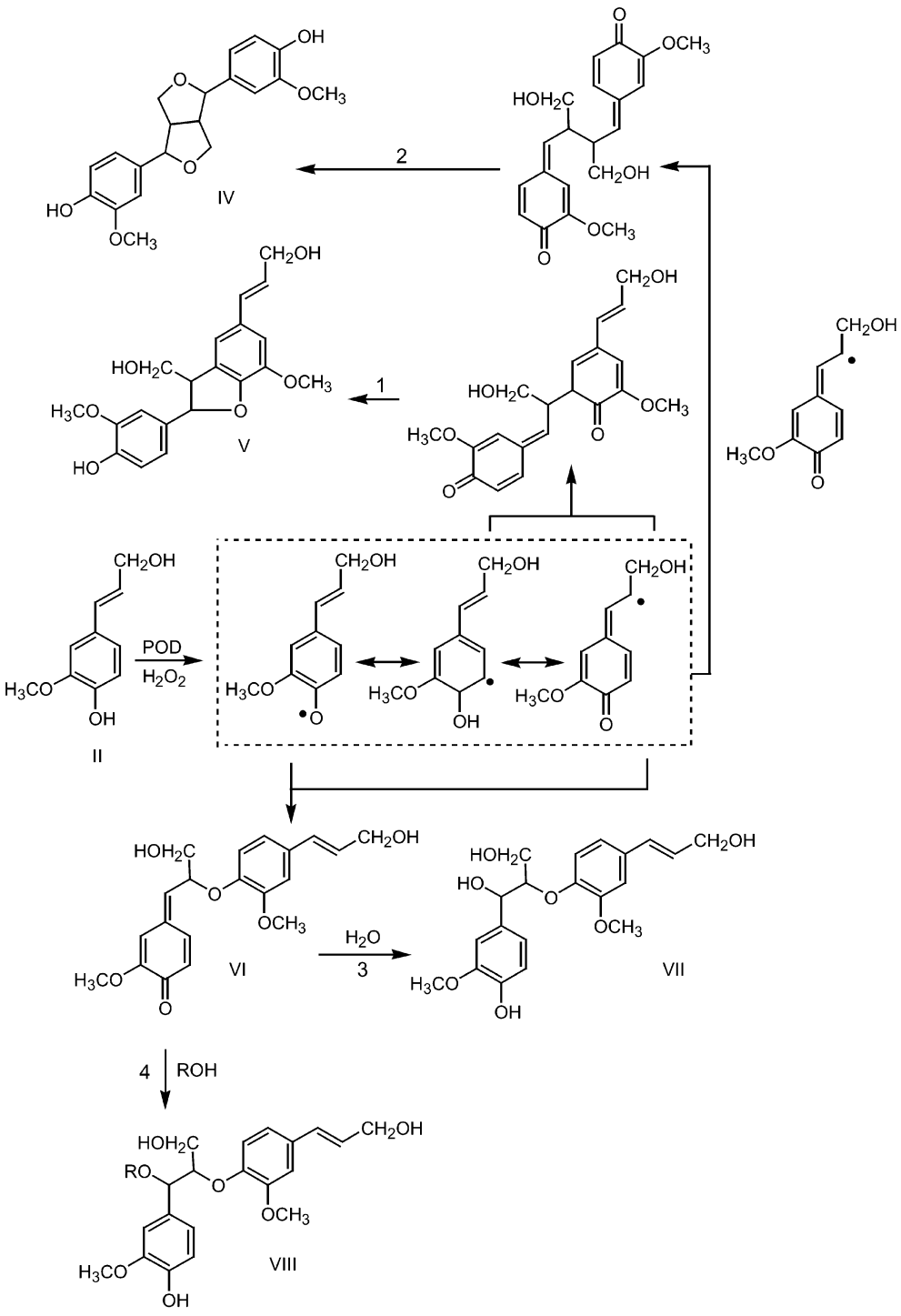


*Figure 2.* Phenoxy radicals formed by oxidation of coniferyl alcohol (II) by peroxidase, and formation *via* reactions 1-4 of the dimers, pinoresinol (IV), dehydrodiconiferyl alcohol (V) and guaiacyl-glycerol-$\beta$-*O*-coniferyl alcohol ether (VII and VIII) via quinone methides (VI).

oxidase, FeIII, thus completing the enzyme catalytic cycle.

As one may expect from the peroxidase reaction mechanism described above, the reactivity of the enzyme intermediates towards a particular monolignol may be estimated *a priori* on the basis of the thermodynamic driving force of these two electron-transfer reactions, which is directly related with the difference between the oxidation/reduction potentials of both the enzyme active intermediates (i.e., CoI and CoII) and the substrate radicals. Thus, the thermodynamic driving force for the reaction of CoI (or CoII) with monolignols is the difference between the mid-point potentials of the CoI/CoII (or CoII/FeIII) and the monolignol radical/monolignol ($R^{\cdot}$,$H^{+}$/RH) redox couples:

$$\Delta E = E(CoI/CoII) - E(R^{\cdot}, H^{+}/RH)$$

The mid-point reduction potential for the enzyme active intermediates, CoI and CoII, has been estimated from stopped-flow measurements (He et al., 1996), giving the values of *E*(CoI/CoII) = + 879 mV and*E*(CoII/FeIII) = + 903 mV at pH 7.0, both relative to the Standard Hydrogen Electrode (SHE). These values illustrate that CoII may be regarded as a slightly stronger oxidant than CoI. However, the order of reactivity of both compounds is somewhat different. In fact, and for a given substrate, CoI reacts several-times faster than CoII (Yamazaki and Yokota, 1973; Folkes and Candeias, 1997) with, in most cases, $k_2 \gg k_3$ (Hayashi and Yamazaki, 1979). Hayashi and Yamazaki (1979) initially attributed this apparent contradiction to the suspected higher mobility of a porphyrin $\pi$-electron than of an iron valence electron. More recently, Candeias et al. (1997) have pointed out that the different reactivities of the two enzyme states can be ascribed to a higher apparent rate of activationless electron-transfer in CoI reactions, which may, in turn, be attributed to the shorter electron-tunneling distance involved in the electron-transfer to the porphyrin radical cation in CoI, compared with the electron-transfer to the iron ion in CoII. In fact, the most important difference between the reduction of CoI and CoII is that, in the former case, the electron is transferred to the porphyrin radical cation, whereas in the latter, it is the iron that changes its oxidation state.

Class III plant peroxidases are usually classified into acidic (isoelectric point below 7.0) and basic (isoelectric point above 7.0) peroxidases, both of which are capable of oxidizing *p*-coumaryl and coniferyl alcohol (i.e., both 4-hydroxyphenyl and guiacyl moieties). However, this situation is not so clear as regards sinapyl alcohol, which possesses a syringyl moiety, for which acidic peroxidases are generally regarded as poor catalysts (Ros Barceló et al., 1987; Dean et al., 1994; Tsutsumi et al., 1994; Takahama 1995; Bernards et al., 1999). This is surprising since sinapyl alcohol is more prone to oxidation than either coniferyl alcohol or *p*-coumaryl alcohol, a behavior which is strongly supported by molecular orbital calculations (Russell et al., 1996). These results suggest that, although peroxidase-catalyzed reactions are driven by redox thermodynamic forces, substrate accommodation in the catalytic center of the enzyme determines the real role played by each peroxidase isoenzyme in lignin biosynthesis. Thus, and although it has occasionally been observed that some acidic peroxidases are capable of oxidizing syringyl moieties, such as that present in syringaldazine (Quiroga et al., 2000; Christensen et al., 2001), it became clear from X-ray crystallographic studies that oxidation of sinapyl alcohol by certain acidic peroxidases is sterically hindered due to unfavorable hydrophobic interactions between the sinapyl alcohol methoxy atoms and the conserved I-138 and P-139 residues at the substrate binding site (Østergaard et al.., 2000) of the enzyme. This overlap apparently does not occur at the substrate binding site of syringaldazine-oxidizing acidic peroxidases (Christensen et al., 2001) nor, of course, in most basic peroxidases, where the I-138 residue is substituted by a L-138. Regardless of whether this is the only factor that conditions the substrate preference of basic peroxidases, the capacity of these enzymes for oxidizing syringyl moieties is universally accepted (Tsutsumi et al., 1994; Bernards et al., 1999; Quiroga et al., 2000; Ros Barceló et al., 2000; Ros Barceló and Pomar, 2001; Aoyama et al., 2002), which would explain why antisense suppression in transgenic plants of basic peroxidases produces decreased levels of both guaiacyl and syringyl lignins (Blee et al., 2003), while antisense suppression of certain acidic peroxidases produces only decreased levels of guaiacyl lignins (Li et al., 2003).

Both *p*-coumaryl alcohol and coniferyl alcohol (in the case of most acidic peroxidases), and *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (in the case of most basic peroxidases), are thus dimerized and polymerized in xylem cell walls in a reaction dependent on $H_2O_2$ (Nose et al., 1995; Ros Barceló and Pomar, 2001) delivered by an NADPH-oxidase-like enzyme (Ros Barceló, 1998). In the case of coniferyl alcohol (Figure 2, II), the main products of con-

iferyl alcohol dimerization by peroxidase (reactions 1-3, Figure 2) are the neolignans, pinoresinol (IV), dehydrodiconiferyl alcohol (V) and guaiacylglycerol-$\beta$-*O*-coniferyl alcohol ether (VII) (Fournand et al., 2003), which are considered as intermediates in macromolecular lignin assembly (Nose et al., 1995). *p*-Hydroxycinnamyl alcohol dimers (dilignols), therefore, are not the end products of the pathway, and they may be oxidized by peroxidases to yield a growing lignin polymer that remains anchored to the cell wall. Lignins can be anchored to cell wall polysaccharides (Figure 2, VIII) by the nucleophilic addition of hydroxyl groups of polysaccharides (Figure 2, reaction 4) to the quinone methide structure (Figure 2, VI), resulting from the $\beta$-*O*-4 coupling mode of two monolignol radicals. Through this mechanism, lignins impart strength to plant cell walls, facilitate water transport and impede the degradation of wall polysaccharides, thus acting as a major line of defense against vertebrate herbivores, insects and fungi.

*Rate limiting steps*

The spatial and temporal control of lignin biosynthesis is extremely important since lignification is a metabolically costly process that requires large quantities of carbon skeletons and reducing equivalents (Amthor, 2003). Plants do not possess a mechanism to degrade lignins (Lewis and Yamamoto, 1990), so any carbon invested in lignin biosynthesis is not recoverable. Consequently, lignified cells represent a significant, non-recoverable carbon sink (Patzlaff et al., 2003) and, as such, plants must carefully balance the synthesis of lignin polymers against the availability of resources, the monolignol biosynthetic pathway therefore being strongly regulated (Patzlaff et al., 2003).

It has long been proposed by several authors that the metabolic flux (carbon allocation) in the phenylpropanoid pathway is controlled at multiple enzymatic levels (see Ros Barceló 1997, and references cited therein). Most recent studies using lignifying cell cultures of *Pinus taeda*, a gymnosperm, have established (Anterola et al., 1999 and 2002) that both the carbon allocation to the pathway, and its differential distribution into the two monolignols, *p*-coumaryl and coniferyl alcohol, is controlled by the rate of phenylalanine supply and the differential modulation of cinnamate-4-hydroxylase (C4H) and *p*-coumarate-3-hydroxylase (C3H) respectively. In these studies, a coordinated up-regulation of phenylalanine ammonia-lyase (PAL), *p*-hydroxycinnamate CoA ligase (4CL), caffeoyl-CoA-*O*-methyl-transferase (CCoOMT), *p*-hydroxycinnamoyl-CoA reductase (CCR) and coniferyl alcohol dehydrogenase (CAD) in respose to phenylalanine supply was also found, which indicates that these steps are not truly rate-limiting since they are modulated according to metabolic demand.

C4H introduces the OH group into C4, yielding *p*-hydroxycinnamyl backbones (e.g. *p*-coumaryl alcohol), while C3H introduces the OH group in C3, first yielding *o*-catechols and then guaiacyl backbones (e.g. coniferyl alcohol), which are the precursors of guiacyl lignins. In angiosperms, there is a novel branching point, the step catalyzed by coniferylaldehyde-5-hydroxylase (CAld5H), which diverts guaiacyl backbones for the synthesis of syringyl (i.e. sinapyl alcohol) moieties, the precursors of syringyl lignins. CAld5H has not been studied in *P. taeda* cell cultures since they are derived from a gymnosperm, but one may extrapolate, in the absence of available data, that, like C4H and C3H in gymnosperms, CAld5H might also constitute a rate limiting step in angiosperms. A similar role, as rate limiting step, has recently been proposed for the polymerization of monolignols catalyzed by basic peroxidases (Ipekci et al., 1999; Talas-Ogras et al., 2001; Blee et al., 2003), although, in the case of acidic peroxidases, their role as rate limiting step is not so clear (Lagrimini et al., 1997; Li et al., 2003). Despite this uncertainty, all the branching (also rate limiting) enzymes of the lignin biosynthetic pathway (C4H, C3H and CAld5H) and the lignin assembling enzyme (peroxidase) are hemeproteins. Any possible metabolic controls over these enzymes would lead to the regulation of not only the global monolignol pools in lignifying plant cells and the H/G/S ratio for carbon partitioning, but also of their rates of polymerization.

## Constraints imposed by the polymerization step

*Constraints imposed by the polymerization mechanism*

The polymerization step, as it is known (Freudenberg et al., 1965; Hatfield and Vermerris 2001), imposes certain constraints. If substrates (monolignols and $H_2O_2$) are delivered to xylem cells by neighboring xylem parenchyma cells, as appears probable (Hosokawa et al., 2001; Ros Barceló and Pomar, 2003), one may expect a diffusion gradient of lignin building blocks from the plasma membrane of xylem parenchyma

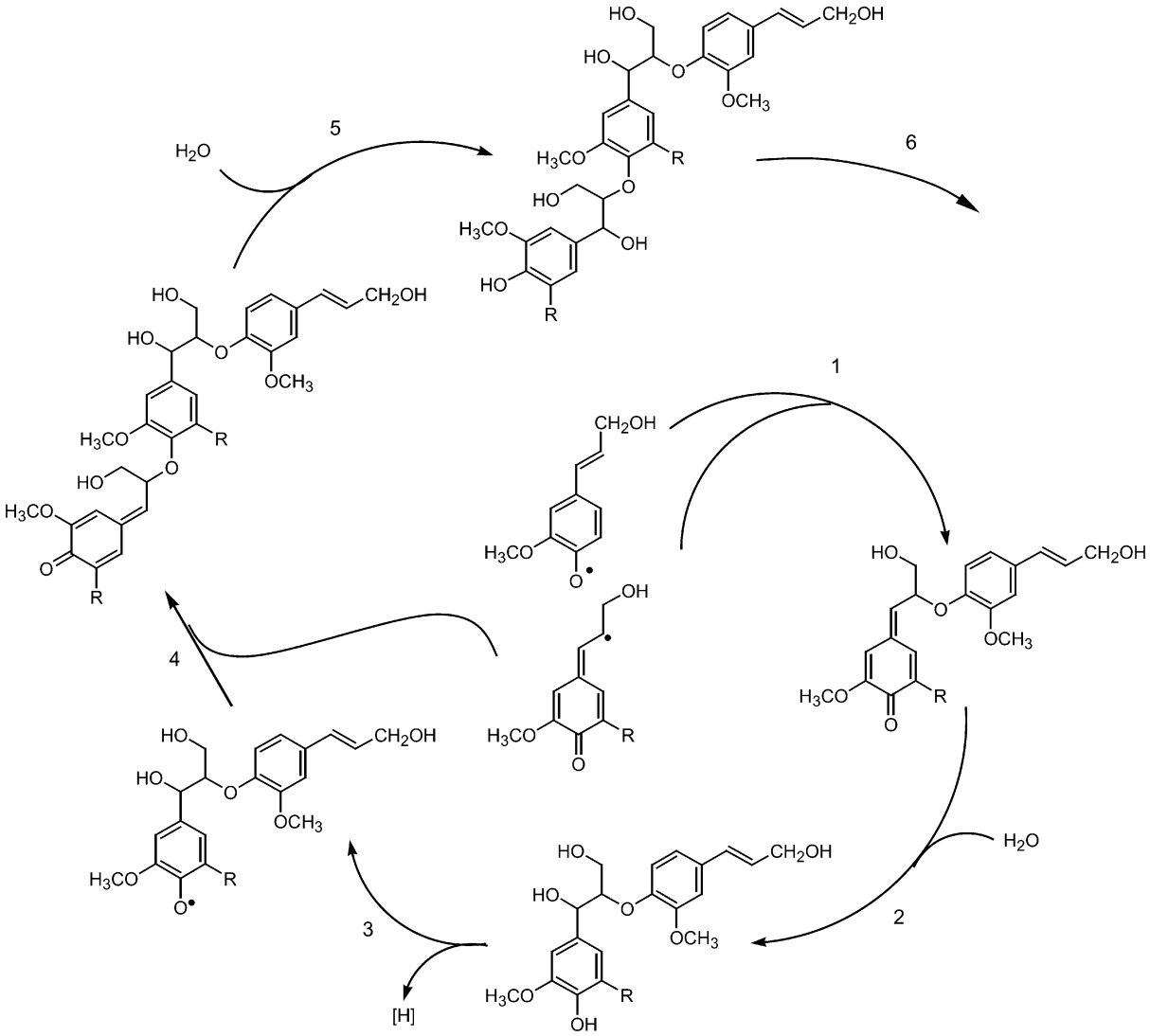


*Figure 3.* Schematic flow for the consecutive addition of coniferyl (R = H) or sinapyl (R = $OCH_3$) radicals at the $\beta$ position to the radical species of coniferyl alcohol at the *O*-4 position. The lignin network arises from the coupling of a monolignol radical at its $\beta$ position with another monolignol radical at its *O*-4 position, so that the last phenolic would act as nucleation (initiation) point, to which monolignol radicals of either coniferyl or sinapyl alcohol will be added in successive steps. This would allow the lignin network to grow by successive radical-radical additions.

cells to the secondary cell walls of xylem vessels. This concentration gradient would probably also occur for peroxidase, which is mainly concentrated in the middle lamella due to its pectin binding properties (Ferrer et al., 1992; Carpin et al., 2001), and whose levels in secondary cell walls are low compared with primary cell walls. This produces two clearly defined topographic zones in lignifying cell walls: one (characterized by high levels of peroxidase and high levels of diffusing substrates) located in the primary cell wall of xylem parenchyma cells and associated xylem vessels, and the other (characterized by lows levels of peroxidase and of unreacted diffusing substrates) located beyond the primary cell walls, i.e., in the secondary cell wall thickenings of xylem vessels. This produces two types of polymerization: one fast (in the primary cell wall) and the other slow (in secondary cell walls). Rapid "bulk" polymerization, as probably occurs in the middle lamella and primary cell walls, favors C-C coupling of monolignols into highly branched polymers, rich in $\beta$-5, $\beta$-1, $\beta$-$\beta$, 5-5 and 5-*O*-4 interunit bonds. In contrast, gradual "end-wise"

polymerization, as may occur in secondary cell walls, favors $\beta$-*O*-4 coupling of monolignols into relatively linear polymers (Lai and Sarkanen, 1975), a process which is favored at pH 5.5 (Grabber et al., 2003), the pH of differentiating (secondary cell wall forming) xylem elements (Roberts and Haigler, 1994).

That these two types of polymerization occurs in lignifying cell walls is supported by both *in situ* and theoretical (modeling) calculations. Thus, lignins rich in *p*-coumaryl residues are mainly deposited in primary cell walls and are strongly cross-linked (Terashima and Fukushima, 1988; Chabannes et al., 2001; Ruel et al., 2002), while lignins rich in coniferyl and sinapyl residues are mainly deposited in secondary cell walls, and the polymer formed is mainly a linear polymer, in which monolignol backbones are linked by $\beta$-*O*-4 bonds (Chabannes et al., 2001; Ruel et al., 2002). Theoretical calculations support these experimental results, since they predict that methoxyl substitution increases unpaired electron density on the phenolic oxygen of monolignol radicals and determines the nature of the bond formed during polymerization, depending on whether it is fast or slow (Russell et al., 1996). Thus, spin density modeling of phenoxy radicals suggests that the existence of these two clearly defined topographic zones for lignin building construction should lead a transition of lignin structure from a preponderance of $\beta$-$\beta$ and 5-5 bonding in the middle lamella, via an approximately equal proportion of $\beta$-5, $\beta$-$\beta$ and $\beta O$-4 linkages in the primary wall layer, to the largely $\beta$-*O*-4 lignins with fewer C-C linkages in the secondary wall (Russell et al., 1996).

The formation of highly branched lignin polymers rich in *p*-coumaryl and coniferyl moieties does not pose special concerns since the ability of plant peroxidases to form them is well known (Tanahashi and Higuchi, 1981). However, the formation of $\beta$-*O*-4 coupled lineal polymers rich in coniferyl and sinapyl alcohols raises certain concerns, especially as regards the inability of certain peroxidases to oxidize syringyl moieties (see above). Thus, taking into consideration that monolignol polymerization in linear polymers occurs by means of an "end-wise" process, monolignol radicals should couple with prepolymer (oligomer) radicals to produce a lignin macromolecule (Figure 3, step 4). Thus, any peroxidase responsible for lignification should be able to catalyze the single electron oxidation of either monomeric (coniferyl and sinapyl alcohols) or oligomeric lignols (Figure 3, step 3). The above mentioned inability of certain peroxidases to oxidize sinapyl alcohol has been skirted by certain authors (Takahama, 1995; Takahama et al., 1996; Fournand et al., 2003; Ralph et al., 2004a) who have elegantly demonstrated that sinapyl (syringyl) moieties may be oxidized through a coniferyl (guaiacyl) alcohol (or *p*-coumaric acid) redox shuttle, in which coniferyl alcohol (guaiacyl) radicals ($G^{\cdot}$) (or *p*-coumaric acid radicals, *p*-$CA^{\cdot}$), formed by the action of either acidic or basic peroxidases on coniferyl alcohol (G) (or *p*-coumaric acid), withdraw an $H^{\cdot}$ atom from sinapyl alcohol (S) to produce the sinapyl alcohol-free radical ($S^{\cdot}$) and regenerating the coniferyl alcohol (G) (or the *p*-coumaric acid) molecule:

$$G^{\cdot}(p-CA^{\cdot}) + S \rightarrow G(p-CA) + S^{\cdot}$$

However, coniferyl alcohols radicals are unable to withdraw a $H^{\cdot}$ atom from sinapyl alcohol oligomers (Aoyama et al., 2002), and only basic peroxidases are able to oxidize them to form highly condensed polymers (Aoyama et al., 2002). This suggest that for lignification to proceed, and for this process to be extensive as occurs *in planta* (Fineran, 1997), a peroxidase capable of oxidizing S oligomers is indispensable, a requirement that is only completely fulfilled by basic peroxidases, such as has been described in poplar (Aoyama et al., 2002; Sasaki et al., 2003).

The oxidation of S oligomers by peroxidases raises special concerns as regards how these high molecular weight compounds may be accommodated in the catalytic active center of the enzyme. To cast light on this, it may be sufficient to mention that in certain peroxidases, particularly in lignin peroxidases from white-rot fungi, an electron transfer from a superficially exposed tryptophan (which allows oxidations at the protein surface via a long-range electron transfer pathway) to the heme pocket of the enzyme has been described as the most probable mechanism able to explain how these enzymes oxidize highly polymerized lignins (Martínez, 2002). That a homologous, or perhaps analogous, mechanism may be developed by peroxidases involved in lignin biosynthesis is probable, since certain plant peroxidases are known to be able to cross-link lignin fragments (Guerra et al., 2000) and to polymerize coniferyl alcohol on a high molecular weight lignin template in the absence of a redox shuttle (Guan et al., 1997).

### *Constraints imposed by the heterogeneity in lignin monomer composition*

From an evolutionary perspective, lignins are uniformly distributed from primitive pteridophytes and

gymnosperms to highly evolved monocotyledons. With very rare exceptions, the monolignol pathway, which affords lignins in pteridophytes and gymnosperms, only utilizes *p*-coumaryl and coniferyl alcohol, whereas in angiosperms the lignin biosynthetic pathway is further branched to use sinapyl alcohol as substrate. In other words, the lignin biosynthetic pathway, at least as far as the lignin precursors, *p*-coumaryl and coniferyl alcohols, is concerned, is highly conserved throughout the evolution of vascular plants and, only in the most recent reproductive group (angiosperms), does sinapyl alcohol enter to form part of the lignin building blocks. However, thioacidolysis and NMR analysis of the lignin monomer composition of the $\beta$-*O*-4 lignin fraction of several gymnosperms and angiosperms has revealed the presence of other minor monomers, besides these monolignols, whose presence in a given plant species depends on its phyletic position. Thus, although most of the building blocks found in natural lignins are mainly derived from *p*-hydroxycinnamyl alcohols, recent NMR evidence has confirmed that *p*-hydroxycinnamaldehydes are also incorporated in the growing lignin polymer, a situation which is especially evident in CAD-depleted mutant or transgenic plants (Kim et al, 2002). In fact, the assembly of *p*-hydroxycinnamaldehydes into preformed lignins is a rather selective process, in which sinapyl aldehyde cross-couples with both guaiacyl and syringyl moieties by means of $\beta$-*O*-4 bonds, whereas coniferyl aldehyde only cross-couples with syringyl units by means of $\beta$-*O*-4 bonds (Kim et al., 2002).

Thioacidolysis is *per se* a non-exhaustive fingerprint analytical method for studying the monomeric composition of lignins (Anterola and Lewis, 2002). However, this method is capable of revealing the presence of further "unusual" terminal phenolic components in lignified cell walls (Lapierre et al., 1995; Ros Barceló et al., 2003). Most of these compounds appear as *O*-4 linked terminal units (Figure 4), suggesting that the heterogeneity in the "unusual" lignin monomer composition appears to be mainly restricted to the 4-*O* ends, while the $\beta$-*O*-4 linear core (Figure 4, VII) is exclusively composed of either $\beta$-O-4-linked coniferyl alcohol (in gymnosperms) or of intercalated units of $\beta$-*O*-4-linked coniferyl and sinapyl alcohol (in angiosperms). However, by far the most exciting results obtained by thioacidolysis concern these terminal lignin units. Thus, gymnosperm lignins were seen to contain 4-*O*-linked coniferyl alcohol (Figure 4, X, R = H), 4-*O*-linked dihydroconiferyl alcohol (XI, R = H), and 4-*O*-linked coniferyl aldehyde (XII, R = H) as terminal units, whereas this heterogeneity was more pronounced in angiosperm lignins, which, although lacking 4-*O*-linked dihydroconiferyl alcohol, show the additional presence of 4-*O*-linked sinapyl aldehyde (XII, R = $OCH_3$), syringaldehyde (XIII, R = $OCH_3$) and *p*-hydroxycinnamic acids (XIV). With the exception of *p*-hydroxybenzaldehydes (Figure 4, XIII), which may arise from *p*-hydroxycinnamaldehydes (XII) through an *in muro* aldol reaction (Kim et al., 2002), the remaining *O*-4-linked phenolic end groups present in this lignin fraction deserve special attention. Most of these unusual structures are *O*-4 terminal units and are thought to act as nucleation (initiation) points for lignin growth (Figure 3). They may only arise from the coupling at the $\beta$ position of a monolignol (coniferyl or sinapyl alcohol) radical with the radical species of the phenolic at the *O*-4 position, in which case the radical of the phenolic would act as a nucleation point, to which radicals of cinamyl alcohols would be added in successive steps (Figure 3 illustrates the case in which coniferyl alcohol itself acts as nucleation/initiation point). This would allow the lignin network to grow by successive radical-radical additions. In other words, these unusual phenolic blocks would fulfill all the requirements to be considered initiation sites (nucleation points) through which cell wall lignification would proceed.

These observations raise further concerns as regards the specificity of the coupling enzyme, since it is known that higher plant peroxidases have evolved by restricting their specificity to guiacyl moieties, such as that present in coniferyl alcohol (Østergaard et al., 2000). In fact, this heterogeneity in lignin monomer composition raises a set of consecutive key questions: Is this plethora of monomer units coupled by a single enzyme or are several enzymes necessary to perform this multiple function? If the first case is true, to what extent has this enzyme been conserved during vascular plant evolution? In the absence of available data, one may expect that such enzymes, like other key enzymes of plant-specific metabolism, such as ribulose-1,5-carboxylase/oxygenase or nitrate reductase, should be strongly conserved during vascular plant evolution, as lignins apparently are.

## The *Zinnia elegans* model

*Zinnia elegans* is a seasonal-cycle flowering plant belonging to the Asteraceae family. This species is commonly used as a model for lignification studies

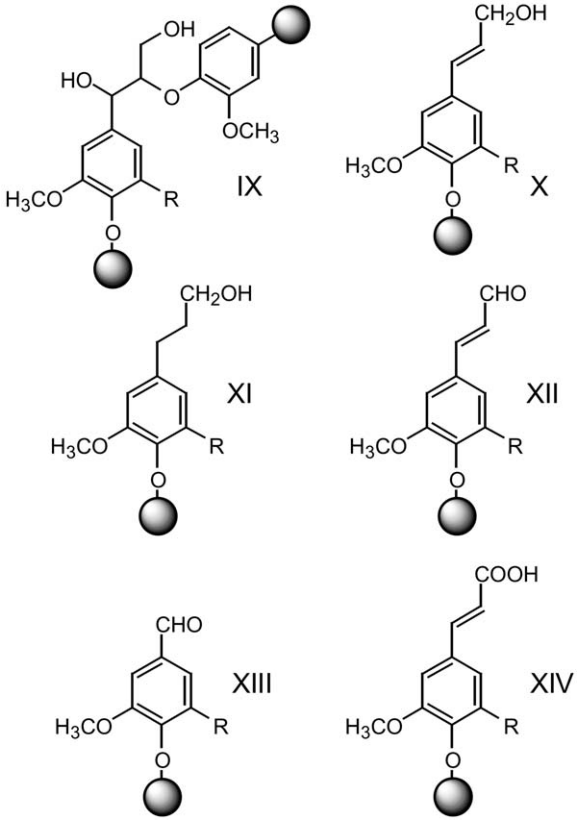


*Figure 4.* Structures of $\beta$-*O*-4 linked monolignols (IX), terminal *O*-4-linked monolignols (X) terminal *O*-4-linked dihydromonolignols (XI), terminal *O*-4-linked *p*-hydroxycinnamaldehydes (XII), terminal *O*-4-linked *p*-hydroxybenzaldehydes (XIII), and terminal *O*-4-linked *p*-hydroxycinnamic acids (XIV) as revealed by thioacidolysis.

(Fukuda, 1996), and has proven a successful model to throw light on the above questions, firstly due to the simplicity and duality of the lignification pattern shown by stems and hypocotyls and, secondly, due to the nature of the peroxidase isoenzyme complement, which is almost completely restricted to the presence of a basic peroxidase isoenzyme. Furthermore, *Z. elegans* offers the unique possibility of working with cell cultures which resemble differentiating xylem cells (Pesquet et al., 2003) and, in fact, they have been used as a model for monitoring the expression of enzymes from the lignin biosynthetic pathway, especially the segment that is concerned with the phenylpropanoid backbones (Demura et al., 2002; Milloni et al., 2002). The reason for this is the fact that the differentiation of mesophyll cells into tracheary elements is synchronously induced in a large number of cells in a relatively homogeneous cell population system, making it possible to study the biochemistry and physiology of xylogenesis free from the complexity which heterogeneous plant tissues impose (Roberts and McCann, 2000; Milloni et al., 2002).

*Lignins*

The lignification pattern of *Z. elegans* seedlings is unique in that, at a certain developmental stage, it offers simultaneously two models of lignification that closely resemble those occurring in gymnosperms and angiosperms. Thus, in 25/30-day-old plants, analysis of the nitrobenzene oxidation products has revealed that the lignin content (H+G+S) of hypocotyls is 11.1-fold greater than the lignin content of the stem (Table 1), data which are predictable since the older hypocotyl is formed two weeks before the youngest stem (first internode) during plant ontogenesis. Furthermore, hypocotyl lignins are mainly composed of guaiacyl/syringyl units in a 41/59 (G/S) ratio (Table 1), while stem lignins also contain significant amounts of *p*-hydroxyphenyl units, in a (H/G/S) ratio of 22/56/22 (Table 1). That is, and unlike hypocotyls, young stem lignins contain H units derived from *p*-coumaryl alcohol, the G/S ratio also being different. In fact, S units predominate in the hypocotyls, while G units predominate in the stem. In this regards, *Z. elegans* hypocotyl lignins are typical of angiosperms, while the lignins of the young stem partially resemble that which occurs in gymnosperms, since (H+G) alone constitute 78% of the lignin building blocks.

Thioacidolysis followed by gas chromatography-mass spectrometry (GC-MS) analyses of both stem and hypocotyl lignified cell walls confirmed these results (Pomar et al., 2002), as well as the heterogeneity of the lignin monomer composition also seen in other species (Lapierre et al., 1995; Ros Barceló et al., 2003). In fact, thioacidolysis of lignified cell walls (Table 2) revealed the presence of the thioethylated monomers arising from the aryl-glycerol-$\beta$-aryl ether structures derived from coniferyl (peak 9) and sinapyl (peak 11) alcohols. The guaiacyl/syringyl (G/S) molar ratio for this $\beta$-*O*-4 lignin fraction was 76/24 in young stems and 43/57 in old hypocotyls, both results being, within experimental error, similar to that found by nitrobenzene oxidation product analysis (71/29 in stems and 41/59 in hypocotyls) (Table 1). These results suggest that the G/S ratio found by thioacidolysis is similar to that found by nitrobenzene oxidation product analyses. Histochemical probes demonstrate that syringyl lignins predominate in the phloem fibers

*Table 1.* Lignin monomer composition, as revealed by nitrobenzene oxidation product analyses, of lignifying *Z. elegans* hypocotyls and stems. nd, not detectable. SE were within 5% of mean values.

| Nitrobenzene oxidation product | Hypocotyl lignins | | Stem lignins | |
|---|---|---|---|---|
| | ng $mg^{-1}$ CW | % | ng $mg^{-1}$ CW | % |
| *p*-hydroxybenzaldehyde (H) | nd | 0 | 72 | 22 |
| Vanillin (G) | 1500 | 41 | 178 | 56 |
| Syringaldehyde (S) | 2090 | 59 | 72 | 22 |
| Total (H + G + S) | 3590 | 100 | 322 | 100 |

*Table 2.* Monomeric degradation products obtained by thioacidolysis of lignifying *Z. elegans* stem and hypocotyls cell walls, and assignment of the original fragment in lignins. TIC: total ionic current; G: guaiacyl; S: syringyl. Tr, trace. nd, not detectable.

| Peak | TIC ($\times 10^8$) | | Original fragment in lignins |
|---|---|---|---|
| | Stem | Hypocotyl | |
| 1 | nd | 0.11 | ester-linked 4-methoxy-3-hydroxybenzoic acid |
| 2 | nd | 0.08 | ester-linked vanillic acid |
| 3 | nd | 0.21 | *O*-4-linked coniferyl alcohol end group |
| 4 | Tr | 0.37 | *O*-4-linked vanillin end group |
| 5 | 4.29 | $< 0.05$ | $C_6C_2$ enol ether structure [G-CH=CHOAr] |
| 6 | nd | 0.89 | *O*-4-linked coniferyl alcohol end group |
| 7 | nd | $< 0.05$ | $C_6C_2$ enol ether structure [S-CH=CHOAr] |
| 8 | 2.10 | Tr | *O*-4-linked coniferyl aldehyde end group |
| 9 | 17.1 | 10.85 | $\beta$-*O*-4-linked coniferyl alcohol |
| 10 | 1.69 | 0.39 | *O*-4-linked coniferyl aldehyde end group |
| 11 | 5.28 | 14.28 | $\beta$-*O*-4-linked sinapyl alcohol |
| 12 | 0.16 | 0.90 | *O*-4-linked sinapyl aldehyde end group |
| 13 | nd | 0.62 | Stilbene structure [G-CH=CH-G] |

of young *Z. elegans* stems (Figures 5A and B), while in *Zinnia elegans* hypocotyls syringyl lignins are mainly associated with the xylem (Figures 5C and D).

In addition to these thioethylated monomers that arise from cleavage of the lineal $\beta$-*O*-4 lignin fraction, thioacidolysis analysis of *Z. elegans* lignins (Table 2) also revealed significant amounts of monomers which arise from ester-linked 4-methoxy-3-hydroxybenzoic acid (peak 1) and vanillic acid (peak 2) (only in hypocotyls), together 4-*O*-linked vanillin (peak 4), the 4-*O*-linked coniferyl aldehyde (peaks 8 and 10) and the 4-*O*-linked sinapyl aldehyde end group (peak 12) (both in stems and hypocotyls). A striking feature of the thioacidolysis analysis of *Z. elegans* stem lignins is the absence of the thioethylated monomer from *p*-coumaryl alcohol, although *p*-hydroxyphenyl units are revealed by nitrobenzene oxidation product analyses (Table 1). However, this result is to be expected since it is known that *p*-coumaryl alcohol is incorporated in the condensed lignin fraction deposited mainly in primary cell walls (Terashima and Fukushima, 1988), which is not susceptible to thioacidolysis cleavage (Lapierre et al., 1995). From the above results (Tables 1 and 2), it may be concluded that *Z. elegans* stem lignins differ from hypocotyl lignins, in which i) they also contain H units derived from *p*-coumaryl alcohol, and ii) the G/S ratio is also different, with a preponderance of S units in hypocotyl lignins and of G units in stem lignins.

These results inevitably raise the following questions: does *Z. elegans* use the same peroxidase isoenzyme complement for lignification of the hypocotyls, whose lignins are typical of angiosperms, as for lignification of the stem, whose lignins resemble those

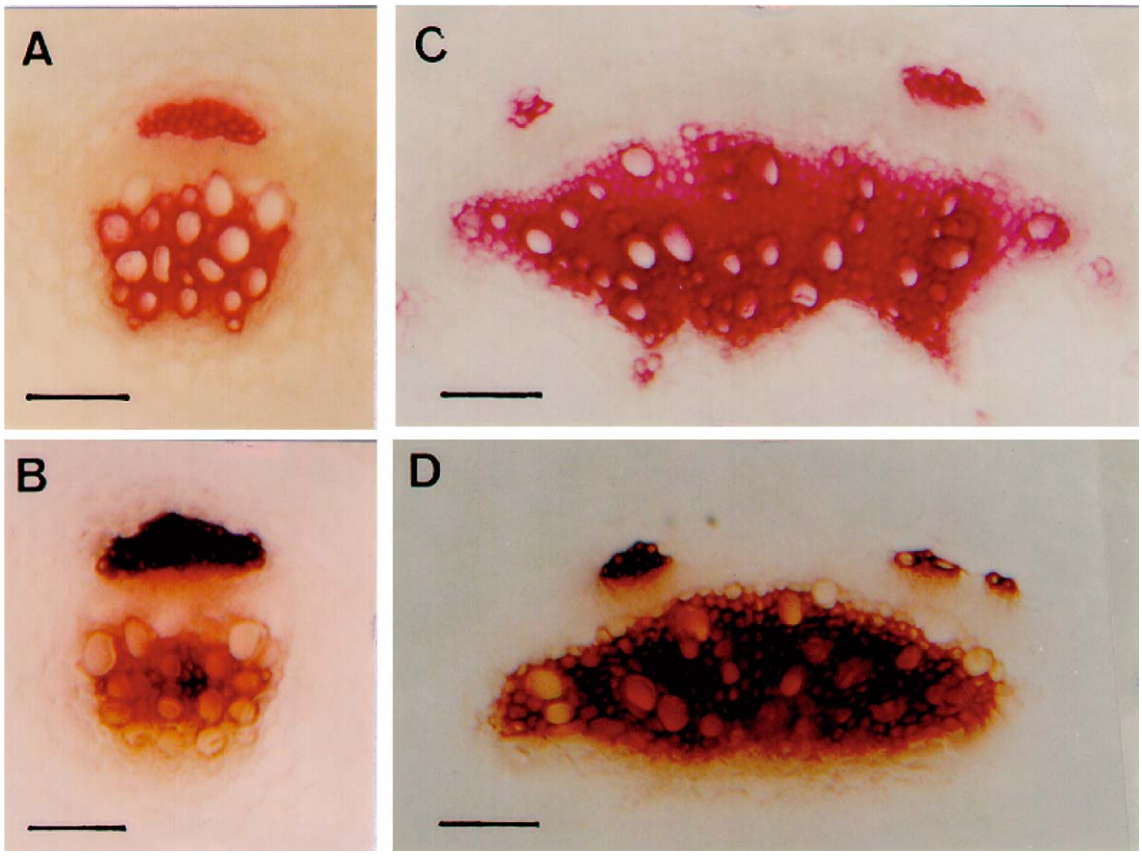


*Figure 5.* Micrographs of a transversal section of the lignifying xylem of the stem (A and B) and hypocotyls (C and D) of *Z. elegans* stained with A,C) the Wiesner reaction which revealed terminal units of *p*-hydroxycinnamaldehydes (Pomar et al., 2002) and B,D) the Maüle reaction which revealed syringyl units (Meshitsuka and Nakano, 1978) Bars = 100 $\mu$m.

of gymnosperms? Are all the monolignols present in *Z. elegans* substrates of one peroxidase isoenzyme, or are several peroxidases isoenzymes required to oxidize the three monolignols? And, finally, does the substrate specificity of peroxidase isoenzyme(s) cover those unusual monomers present in lignins and distinct from the three monolignols?

*Peroxidase*

Both stems and hypocotyls of *Z. elegans* express the same basic peroxidase isoenzyme (Figure 6, lanes a and b) (López-Serrano et al., 2004), which means that, if this peroxidase is the responsible for cell wall lignification in both organs, it should be capable of oxidizing not only coniferyl and sinapyl alcohol, but possibly also their relatives. Results strongly suggest that this is so (Ros Barceló and Pomar, 2001). In fact, the purified peroxidase isoenzyme is capable of oxidizing both coniferyl (Figure 7A) and sinapyl (Figure 7C) alcohol, as well as both coniferyl (Figure 7B) and sinapyl (Figure 7D) aldehyde. In fact, the versatility of this enzyme is such that the substrate preference covers the three *p*-hydroxybenzaldehydes and the three *p*-hydroxycinnamic acids (Ros Barceló et al., 2003). *p*-Hydroxybenzoic acids are, however, poor substrates of the enzyme, and the ability to oxidize them is restricted to syringic acid.

It is worth noting that the affinity of this strongly basic peroxidase for *p*-hydroxycinnamyl alcohols and aldehydes is similar (Ros Barceló and Pomar, 2001) to that shown by the preceding enzymes in the lignin biosynthetic pathway (CAld5H and CAD), which also use *p*-hydroxycinnamyl alcohols and aldehydes as substrates. This indicates that the one-way highway of lignin macromolecule construction has no metabolic "potholes" into which the lignin building blocks might accumulate, as is corroborated from the study of metabolic pools in lignifying cells (Anterola et al., 1999). All these constraints strongly suggest that this per-

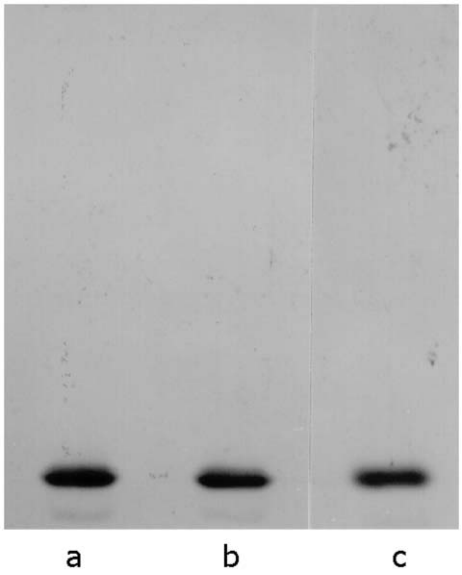


*Figure 6.* Basic peroxidase isoenzyme patterns of hypocotyls (lane a), stems (lane b) and tracheary elements (lane c) of *Z. elegans* obtained by non-equilibrium isoelectric focusing of the apoplastic protein and stained with 4-methoxy-$\alpha$-naphthol and $H_2O_2$ as substrate. For experimental details, see López-Serrano et al. (2004).

oxidase plays a key role in cell wall lignification, a consideration which is supported by its localization in lignifying xylem vessels (Ros Barceló et al., 2000 and 2002). At this point, we should mention that the versatility of certain enzymes is one of the main driving forces in the evolution of land plants and that, to date, there is a general consensus (Boerjan et al., 2003) that such enzymes confer high metabolic plasticity to the lignin biosynthetic pathway. In the case of this basic peroxidase, the real meaning of its versatility may be the following: the existence in plants of this peroxidase, capable of catalyzing with high affinity and high catalytic activity the polymerization of the three *p*-hydroxycinnamyl alcohols and *p*-hydroxycinnamic acids (and of their corresponding derivatives), means that the heterogeneity of lignin precursors should not be prohibitive, nor its metabolic cost terribly expensive for the plant. In fact, the versatility (metabolic plasticity) of this basic peroxidase confers a certain sense to the heterogeneity of the lignin biosynthetic pathway.

A definitive proof for the role of this peroxidase in lignification comes from the use of *Z. elegans* cell cultures undergoing tracheary element differentiation (López-Serrano et al., 2004). Thus, *Z. elegans* tracheary elements (Figure 6, lane c) express the same peroxidase isoenzyme as lignifying stems and hypocotyls (Figure 6, lanes a and b). Western blot analyses (López-Serrano et al., 2004) showed that this isoenzyme is expressed immediately before secondary cell formation starts, protein levels being maintained while lignification is in progress. Western blot analyses (López-Serrano et al., 2004) also showed that the expression of this isoenzyme forms part of the molecular mechanism underlying tracheary element differentiation, since mesophyll cells cultured in a non-inductive medium were unable to express the protein.

The identical nature of the protein expressed in hypocotyls and stems with that expressed in *Z. elegans* suspension cell cultures was confirmed by the total analogy in the N-terminal sequence and identical molecular weights (C. Gabaldón, M. López-Serrano, M.A. Pedreño and A. Ros Barceló, in preparation). The identical nature of the protein expressed in *Z. elegans* suspension cell cultures with that expressed in *Z. elegans* tracheary elements was further supported by purification of the enzyme, followed by trypsin digestion and sequentiation of tryptic peptides by MALDI-TOF/MS. This technique revealed a 14 amino acid-containing peptide located in the C-terminus of the protein, whose sequence (MSIGVVTGTSGIVR) closely matched those contained in a cDNA for peroxidase which is expressed early during tracheary element differentiation (Milloni et al., 2002). These results, finally, suggest that this protein not only fulfills all the catalytic requirements to be involved in lignification, its presence overcoming all the constraints imposed by the polymerization step, but also that its expression is inherent to lignification in *Z. elegans* tracheary elements. The basic pI nature of this peroxidase is not an exceptional frame point in this system, since basic peroxidases are differentially expressed during lignification in other model systems (Quiroga et al., 2000; Holm et al., 2003), they show unusual and unique biochemical properties as the regards oxidation of syringyl moieties (Aoyama et al., 2002), and their down-regulation in transgenic plants leads to a reduction in lignin (G+S) levels (Blee et al., 2003).

## Multifunctional basic peroxidase isoenzymes have been widely conserved during the evolution of land plants

It might be expected that a peroxidase capable of oxidizing coniferyl alcohol (present in both gymnosperm

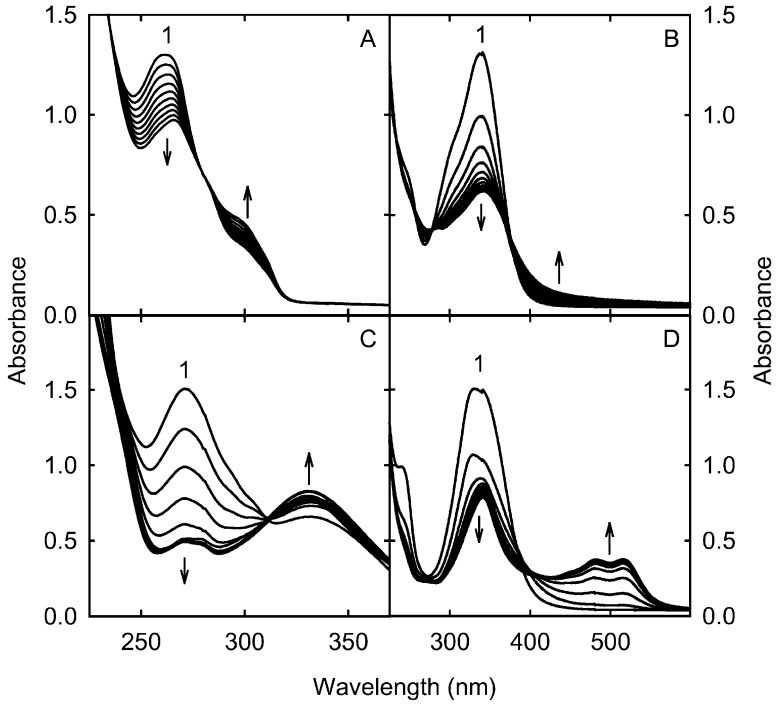


*Figure 7.* Consecutive UV spectra of a reaction medium containing 75 $\mu$M of either coniferyl alcohol (A), coniferyl aldehyde (B), sinapyl alcohol (C), or sinapyl aldehyde (D), 0.5 mM $H_2O_2$ and purified *Z. elegans* peroxidase in 50 mM Na-acetate buffer (pH 5.0) left to react for 10 min at 25°C. Consecutive UV spectra were recorded every minute at a rate of 1000 nm/min. 1, UV spectrum obtained at time zero. Arrows indicates the direction of spectral changes.

and angiosperm lignins) and sinapyl alcohol (present only in angiosperm lignins), as well as all their corresponding (guaiacyl and syringyl) phenolic derivatives, should be highly conserved during the evolution of vascular plants, since its presence would confer a great economy to the expensive process of lignin assembly. To test this hypothesis, a rapid method was developed for screening peroxidase isoenzymes in the apoplast of young stems (or branches) of phylogenetically basal vascular plants. Isoenzymes were analyzed by isoelectric focusing since the isolectric point is determined by the balance of acidic and basic amino acids of the protein, and thus reflects similarities in the amino acid composition of the isoenzymes, as has been successfully demonstrated in the *Z. elegans* model system (see above).

Preliminary results had shown that this peroxidase isoenzyme is universally present in a set of randomly selected species belonging to the Asteraceae family (Ros Barceló and Aznar-Asensio, 2001), homologous proteins also being present in woody angiosperms and evolved monocotyledons, including some common forages (Ros Barceló et al., 2003). These findings suggest that the gene codifying this peroxidase isoenzyme evolved prior to the radiation of angiosperms and, as such, it should be considered an ancestral gene within the monophyletic origin of flowering plants (Qiu and Palmer 1999), specially if we take into account recent molecular studies (Qiu et al., 1999) which support fossil evidence suggesting that monocotyledons are an early branch in angiosperm evolution.

To study the presence of homologous isoenzymes in ancient groups of seed plants, a set of differentially evolved basal plant species was selected (Figure 8). The list includes a member of the order cycadales (exemplified in the species *Cycas revoluta*), which constitutes a relic from the past and represents an old living seed plant. The oldest cycad fossils are dated from the Upper Paleozoic ( 265-290 million years ago) (Brenner et al., 2003). Cycads belong to the earliest groups of living seed plants, and already occurred during the late Carboniferous to Permian periods ( 300 million years ago). With some exceptions, most phylogenies (Qiu et al., 1999; Pryer et al., 2002) place Cycadales as the basal order of gymnosperms, with Ginkgotales and Coniferales as more advanced order (Figure 8). This placement defines cycads as the sister group to all living seed plants. The species selected

also include the ginkgo, or maidenhair tree (*Ginkgo biloba*), which is the sole living member of Ginkgotales, being a truly astonishing survivor. This resembles conifers in its vegetative structure and cycads in its reproduction. The oldest ginkgo-like fossils are dated from late Permian to Triassic ( 260-250 million years ago). Coniferales (pine-like wood), exemplified in the species *Pinus halepensis*, appeared during the mid Triassic period, 240 millions years ago, but the main radiation took place during the Cretaceous and early Tertiary.

Peroxidase isoenzyme patterns in these species are shown in Figure 9. All the species showed the common presence not only of analogues (Figure 9, arrowheads) but also of homologues (Figure 9, arrow) of this basic peroxidase isoenzyme, which were identified as such from its mobility in the focusing gels, and for which we used the enzyme isolated from differentiating *Z. elegans* tracheary elements as marker (Figure 9, lane a). The results obtained suggest that basic peroxidase isoenzymes, which show a special ability to oxidize syringyl moieties (Ros Barceló and Pomar, 2001; Pomar et al., 2002; Aoyama et al., 2002), are strongly conserved during the evolution of vascular plants. In fact, peroxidases capable of oxidizing sinapyl alcohol have been described in gymnosperms (Tsutsumi et al., 1998; McDougall, 2001), which lack syringyl-type lignins, an observation which suggests that the evolutionary gain of the monolignol branch which leads to the biosynthesis of sinapyl alcohol and, of course, to syringyl lignins, was not only possible but was also favored because the enzymes responsible for its polymerization evolved very early during the evolution of land plants. These results also suggest that basic peroxidases should be present in an ancestral plant species, prior to the radiation of seed plants, and that these conserved isoenzymes might have an important specific role. Undoubtedly, these preliminary phylogenetic results support the role of basic peroxidases as a highly conserved evolutionary gateway for lignin monomer polymerization.

The results described above are not surprising since class III plant peroxidases are found in most vascular plant lineages, including early land plants, such as ferns, mosses and liverworts (Duroux and Welinder, 2003), although absent from green algae, or charophytes. Molecular clocks suggest that land plants are monophyletic and that they diverged from green algae about 700 million years ago, the charophytes (freshwater green algae) being the closest lineage to land plants (Qui and Palmer, 1999). Phylogenetic studies

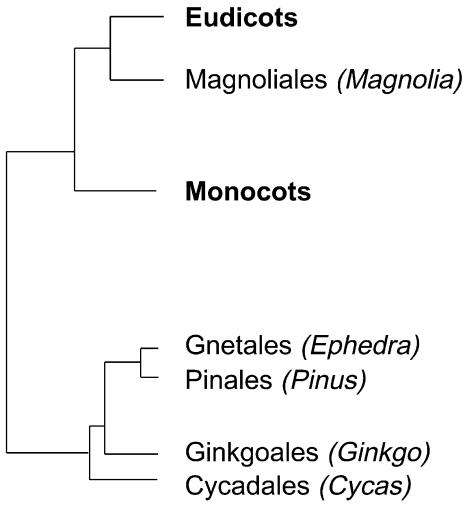


*Figure 8.* Phylogenetic tree of seed plants based on molecular studies (Qui et al., 1999).

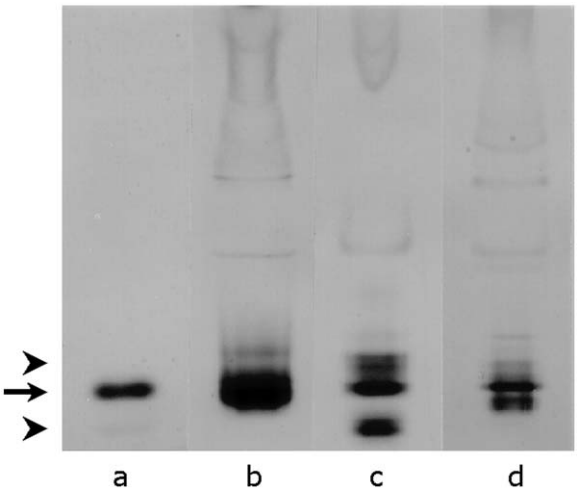


*Figure 9.* Basic peroxidase isoenzyme patterns of tracheary elements of *Z. elegans* (lane a), *Cycas revoluta* (lane b), *Ginkgo biloba* (lane c), and *Pinus halepensis* (lane d) obtained by non-equilibrium isoelectric focusing of the apoplastic protein and stained with 4-methoxy-$\alpha$-naphthol and $H_2O_2$ as substrate. Arrow indicates the position of homologous basic peroxidases.

suggest that class III plant peroxidases appeared with the emergence of land plants (Duroux and Welinder, 2003), and it is likely that this class of enzymes conferred important adaptive traits to plants for their new life on land, the most characteristic gain being the acquisition of vascular tissues coated with lignins. In this scenario, it is not surprising that the enzymes responsible for lignin building construction appeared early in the evolution of land plants, and that these enzymes have been largely conserved during plant evolution.

In this way, despite the absence of molecular data but supported by functional data, multifunctional basic peroxidase isoenzymes appear to be a highly conserved evolutionary gateway for lignin construction and evolution.

## Acknowledgements

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