Dirigent Proteins and Dirigent Sites Explain the Mystery of Specificity of Radical Precursor Coupling in Lignan and Lignin Biosynthesis[1]

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The recent discovery of dirigent proteins (Davin and Lewis, 1995; Davin et al., 1997; Gang et al., 1999) gives a new perspective into how free radical coupling of monolignol plant phenols is controlled in planta to yield lignans and lignins. With the biochemical pathways to the precursor monolignols essentially fully established (Lewis et al., 1999), this new insight for formation of the lignans and lignins now resolves many if not all of the earlier enigmas associated with phenoxy free radical biochemistry; hitherto, these were considered to lack any defined control in terms of stipulating the outcome of their coupling.

In older textbooks (Sarkanen and Ludwig, 1971), for example, it was suggested that lignin assembly occurs through the passage of monolignol monomers into the cell wall, with polymer formation only requiring oxidative enzymes (such as laccases or peroxidases) to generate the corresponding free radicals, which would then undergo random coupling. If this were correct, then formation of approximately 20% to 30% of all plant organic matter would have been left essentially to chance. This perspective could not, however, explain many biological aspects of lignification, including targeting of specific monolignols into discrete regions within the lignifying cell wall and the observed regiospecificity in coupling resulting in approximately 50% to 70% of all interunit linkages being 8-*O*-4′ bonded. Nor could it explain the optical activities of many of the dimeric lignans (Ayres and Loike, 1990). These observations suggested that some coupling specificity was being exercised in planta, the basis of which was not understood until the discovery of dirigent proteins.

From even the very beginnings of the evolution of life, some biochemical mechanisms might have been in place to help "manage" both the generation of free radicals and their ultimate fate in the assembly, repair, and degradation of living systems. In 1997 (Davin et al., 1997), we described the discovery of a truly unique protein from *Forsythia intermedia* that stipulated precisely the biochemical outcome of phenoxy radical coupling. The term "dirigent," from the Latin "dirigere" (to guide or align) was coined to define this first example of an apparently new class of proteins. In this *Update*, we report that such proteins are present in all of the major land plant groupings examined to date. The important question to contemplate in land plant colonization is how did the function of dirigents evolve? This *Update* also addresses the current understanding of how such biochemical coupling processes are controlled in vivo.

LAND PLANT ADAPTATION AND LIGNIN/LIGNAN PATHWAY EVOLUTION

Unlike most other biochemical pathways, which are operative in both aquatic and land plants, establishment of the phenylpropanoid pathway, from which lignans and lignins derive, appeared to be manifested only during land plant adaptation. This view does not rest solely on scrutiny of fossil evidence, which thus far is very incomplete, but instead relies more heavily on our current knowledge of operative biochemical processes in planta and on chemotaxonomical considerations of extant species (Lewis and Davin, 1994, 1999).

Lignins and lignans are derived mainly via differential partitioning of the monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1) into their respective pathways (Lewis and Yamamoto, 1990; Lewis and Davin, 1999; Lewis et al., 1999). These metabolites, however, have markedly different physiological roles: The heterogeneous lignins, as described later, are structural cell wall components of vascular tissues, whereas the ubiquitous but structurally diverse lignans are involved in plant defense (antioxidants, biocides, etc.). The lignans are found in seed coats, flowers, stems (sapwood and heartwood), leaves, bark, roots, etc. They are typically dimers, although higher oligomers exist and many have important roles in human use (for review, see Lewis and Davin, 1999): For example (Fig. 2), sesamin/

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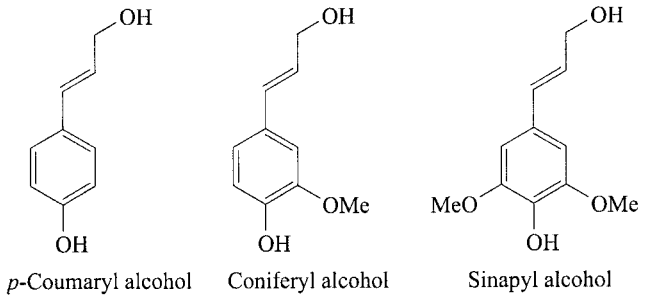

Figure 1. The monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols.

sesamolin and nordihydroguaiaretic acid are antioxidants in seed oil from the sesame plant (*Sesamum indicum*) (Fukuda et al., 1986) and the creosote bush (*Larrea tridentata*) (Oliveto, 1972), respectively; podophyllotoxin from *Podophyllum* species is used in the treatment of venereal warts, whereas its derivatives, etoposide and teniposide, are employed for treatment of testicular cancers (Ayres and Loike, 1990). Secoisolariciresinol, secoisolariciresinol diglucoside, and matairesinol are the "chemopreventive agents" of many edible plants and their metabolism in the gut helps protect against the onset of breast and prostate cancers (Adlercreutz, 1996; Thompson et al., 1996). Others, such as gomisin A from *Schisandra chinensis*, are used in the treatment of liver disorders (Nagai et al., 1989; Nomura et al., 1994), and kadsurenone from *Piper futokadsura* is a platelet-activating factor (Shen et al., 1985).

DISCOVERY OF DIRIGENT PROTEINS

At the onset of our studies, the central question was where to begin in a search for proteins/enzymes involved in stipulating the outcome of phenoxy radical-radical coupling. It was by then well known that at least five distinct plant oxidases possessed both high redox potentials and very broad substrate specificities (for review, see Lewis and Davin, 1998; Lewis et al., 1998, 1999), with each being able to oxidize monolignols into their respective free radical forms. Curiously, all were arbitrarily implicated in cell wall lignin biosynthesis, even though no other biochemical process before or since had described different enzymes for the same step. However, in the presence of monolignols, each of these oxidative enzymes in vitro gave preparations composed of or derived from racemic products, the overall compositions of which failed to adequately reflect lignin structure in vivo in any meaningful way (e.g. the 8-*O*-4′ linkage frequency in the artificial preparations was much lower than in lignin proper). At that time, it was also essentially ignored that monolignols had different metabolic fates, even though they were clearly being differentially partitioned into various product classes such as lignins and lignans (for review, see Lewis and Davin, 1998, 1999; Lewis et al., 1999). Oxidases were also implicated in other specific phenoxy radical-radical coupling processes such as that leading to suberized tissue; in this case, the aromatic building blocks appear to be largely derived from *p*-coumaroyl/feruloyl tyramine and/or hydroxycinnamate esters (Bernards et al., 1995, 1999; Negrel et al., 1996; Bernards and Lewis, 1998). How, then, were all of these processes to be distinguished biochemically and what control, other than compartmentalization of the proteins/enzymes and substrates involved, was there in place?

We considered that a productive line of inquiry might be obtained by first studying the biosynthesis of the simplest phenylpropanoid coupling products, these being the dimeric optically active lignans such as those present in *Forsythia intermedia*, a representa-

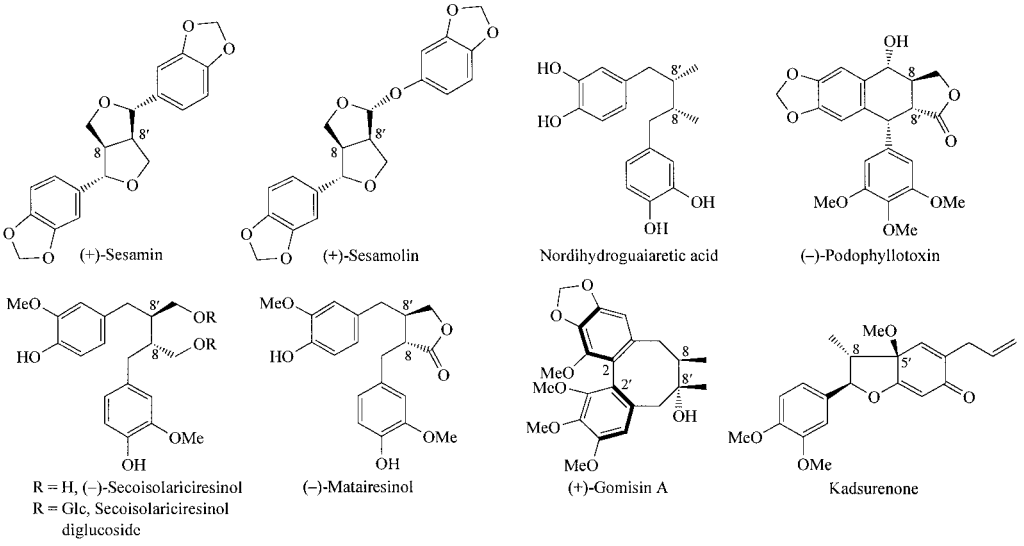

Figure 2. Selected lignans with distinctive medicinal/human health roles.

tive of the Oleaceae. This species contains a series of optically pure 8-8′-linked lignans, such as (+)-pinoresinol and (−)-secoisolariciresinol (Fig. 3), whose presence suggested stereoselective control over monomer-monomer coupling. We therefore rationalized that proteins or enzymes must exist in vivo that are capable of binding differentially the various monolignol-derived substrates, and which would be able to dictate the outcome of coupling. Radio- and stable-isotope tracer and enzymatic assay studies next revealed that the initial coupling step involved stereoselective coupling (control of both regio- and stereochemistry) of two molecules of the monolignol, coniferyl alcohol, to yield the lignan (+)-pinoresinol (Umezawa et al., 1990), and that this in turn served as the precursor of both (−)-secoisolariciresinol and (−)-matairesinol (Umezawa et al., 1991; Katayama et al., 1992, 1993; Chu et al., 1993; Dinkova-Kostova et al., 1996). This work resulted in two important clues: First, a biochemical system was indeed in place that stipulated the outcome of radical coupling, and secondly, the monolignols were in fact differentially employed for both lignin and lignan biosynthesis. Moreover, this represented the first example, in either chemistry or biochemistry, in which the outcome of phenoxy radical-radical coupling could be controlled when the substrate molecule possessed more than one possible coupling site.

Identifying the proteins involved in controlling the outcome of coupling proved not to be an easy task. The soluble proteins, readily removed from *F. intermedia* stem tissue, failed to engender stereoselective coupling, and only non-specific racemic coupling was observed when, for example, H_2O_2 was added as a cofactor.

Next, we turned our attention to the so-called insoluble cell wall portion of the plant material, and quickly established that the stereoselective coupling system was present in this fraction (Davin et al., 1992). Eventually, the corresponding biochemical machinery was solubilized and fractionated into a series of components (Davin and Lewis, 1995; Davin et al., 1997). The dirigent was isolated as an approximately 78-kD native protein, as evidenced by ultracentrifugation and gel permeation chromatography. On its own or with a suite of potential cofactors, the dirigent displayed no catalytic activity; however, in the presence of oxidases such as laccase/O_2 or peroxidase/H_2O_2, the protein was capable of engendering stereoselective coupling. Initial kinetic studies also suggested that the protein functioned in a very unique manner, whereby the oxidase first generates the free-radical intermediates, which are then presumed to be captured by the dirigent. These are bound and orientated in such a manner that coupling can only provide the product (+)-pinoresinol (Fig. 4). Significantly, neither *p*-coumaryl nor sinapyl alcohols (Fig. 1), which differ only in the degree of methoxylation of the aromatic ring, served as substrates for stereoselective coupling: This in turn revealed that the dirigent selectively bound only coniferyl alcohol-derived substrates; therefore, proteins had evolved with distinct monolignol-derived binding sites.

The dirigent was established to be a glycoprotein whose corresponding gene encoded a protein of only about 18 to 19 kD (Fig. 5) (Gang et al., 1999); the corresponding native subunit was found to be glycosylated with a subunit size of approximately 26 to 27 and 21 to 23 kD as determined by SDS-PAGE (Davin et al., 1992) and MALDI-TOF, respectively. Fully functional recombinant dirigents were also obtained in both *Spodoptera*/baculovirus and *Drosophila* expression systems. To date, three closely related dirigent genes have been obtained from *F. intermedia* (Lewis et al., 1997; Gang et al., 1999), indicative of a multigene family. Importantly, database comparisons revealed that the genes encoding dirigents had no homology with any other proteins of known function, this being in accordance with their unique roles.

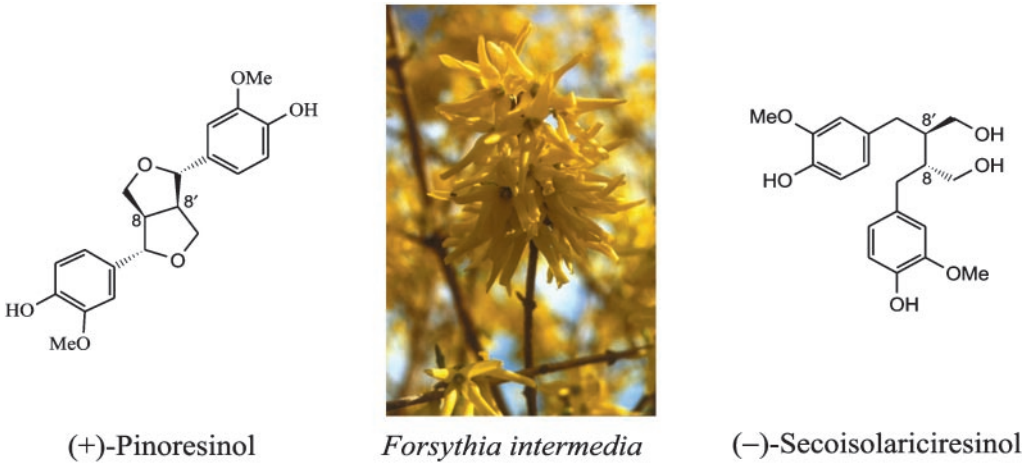

Figure 3. Two representative lignans present in *F. intermedia*.

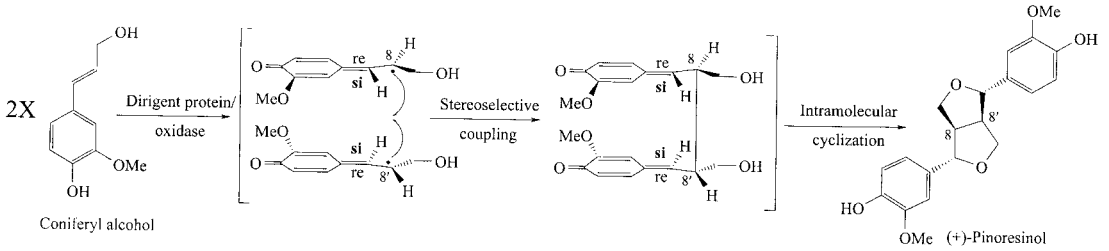

Figure 4. Dirigent-mediated formation of (+)-pinoresinol in *F. intermedia*.

DIRIGENT PROTEINS GALORE

Was the discovery of the (+)-pinoresinol-forming dirigent in *Forsythia* an unusual exception in phenoxy radical coupling, or had the first clue been uncovered as to how such processes are actually controlled in vivo? We anticipated the latter for the following reasons: First, the several thousand lignans known, which are differentially distributed throughout land plants (liverworts, ferns, gymnosperms, and angiosperms), are linked specifically through various yet often quite distinct types of interunit linkages (for examples, see Fig. 2; for review, see Lewis and Davin, 1999); they can also occur in different optical forms depending upon the species. For example, pinoresinol exists as the (+)-enantiomer in *Forsythia* (Lewis and Davin, 1999), whereas its (−)-antipode is present in *Xanthoxylum ailanthoides* (Ishii et al., 1983). This suggested, therefore, that a class of such proteins existed that stipulated the formation of lignans of different optical activities and different interunit linkages. Second, there was no satisfactory explanation as to how lignin (and suberized tissue) formation occurs in vivo. For example, previous suppositions could not account for either the interunit regioselectivity of bond linkages noted in the lignins, or the constraints imposed by the cell itself during lignification (for discussion, see Lewis and Davin, 1998; Lewis, 1999; Lewis et al., 1999; and described later).

Therefore, our strategy was to next ascertain—via judicious selection of appropriate plant species—whether a new class of dirigents and their encoding genes did in fact exist, and whether any general correlation might emerge that provided an explanation for both lignan and lignin-forming processes.

SPECIES-SPECIFIC DIRIGENTS CAN AFFORD DIFFERENT OPTICAL FORMS

The may apple (*Podophyllum peltatum*) accumulates in its rhizomes the lignan podophyllotoxin (Fig. 2). Using various radiolabeling tracer and stable isotope experiments, we have established that podophyllotoxin is also derived from (+)-pinoresinol. The gene encoding the corresponding dirigent was obtained and has approximately 68% similarity and approximately 60% identity to that of the psd Fi1 gene (GenBank accession no. AF210061) from *Forsythia*. (+)-Pinoresinol also serves as a precursor of the antioxidant lignans (+)-sesamin and (+)-sesamolin in *S. indicum* (Jiao et al., 1998). In flax (*Linum usitatissimum*) seeds, the cancer-preventing lignan secoisolariciresinol diglucoside accumulates to a very high percentage (2%–4% by weight). However, unlike the (−)-secoisolariciresinol, which is present in *Forsythia* (Lewis and Davin, 1999), the corresponding (+)-antipode (>99% optically pure) accumulates in flaxseed. Furthermore, radiolabeled experiments revealed that (−)-pinoresinol is converted into (+)-secoisolariciresinol in flaxseed (Ford et al., 1999). Two flaxseed dirigent protein genes (approximately 82% similarity and approximately 79% identity to that of the psd Fi1 gene) have since been obtained. Therefore, biochemical processes are in place in planta not just for producing (+)-pinoresinol, but the corresponding (−)-enantiomer as well. It will be very instructive to learn how such closely related dirigents bind and orientate their substrates such that the opposite stereoselectivity is attained in each case.

DIRIGENT PROTEINS: A PLETHORA OF COUPLING MODES AND SUBSTRATES?

From our own studies, together with analyses of available EST databases, gene banks, and genomic sequences, it is now evident that multiple forms of dirigents and their homologs abound throughout the plant kingdom. In our laboratory, they (or their encoding genes) have been detected in all species examined, which include *Forsythia* species, loblolly pine (*Pinus taeda*), western red cedar (*Thuja plicata*), western hemlock (*Tsuga heterophylla*), eucommia (*Eucommia ulmoides*), Manchurian ash (*Fraxinus mandschurica*), quaking aspen (*Populus tremuloides*), sesame (*S. indicum*), rice (*Oryza sativa*), Arabidopsis, *Schisandra chinensis*, creosote bush (*Larrea tridentata*), *Piper fu-*

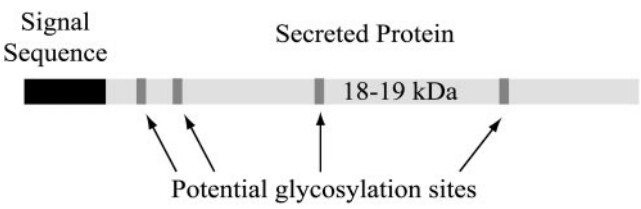

Figure 5. Schematic representation of dirigent gene from *F. intermedia*.

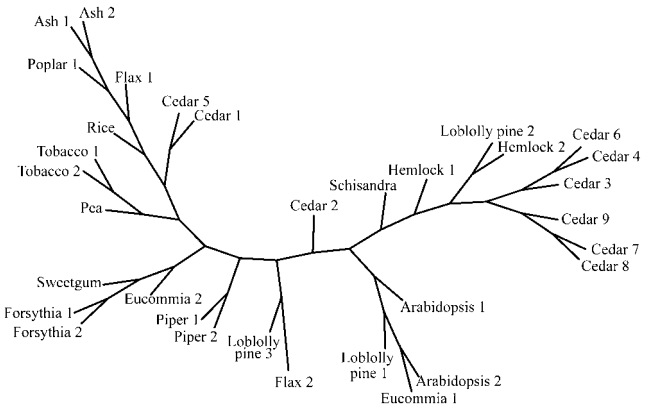

Figure 6. Dirigent and dirigent homolog phylogenetic tree. The PROTOPARS program (Felsenstein, 1998) was employed for tree construction.

tokadsura, flax (*L. usitatissimum*), and tobacco (*Nicotiana tabacum*). Additionally, scrutiny of EST databases and gene banks suggest their presence in *Populus tremula* L. × *tremuloides* Michx (Sterky et al., 1998). A defense-inducible gene encoding a dirigent homolog of unknown function has also been identified in pea (*Pisum sativum*) (Fristensky et al., 1988).

Figure 6 illustrates a phylogenetic tree (Felsenstein, 1998) derived from a sampling of dirigents of established function and those of corresponding homologs. Although its full significance awaits a comprehensive determination of the biochemical function of each homolog, the tree nevertheless displays some interesting—albeit preliminary—correlations. It should be noted that the percentage of similarity of amino acid in each dirigent homolog ranges from 30% to 80%, which may reflect a functional divergence during the course of evolution. Although there are some outliers, the right side of the tree mainly groups the gymnosperm (western red cedar, western hemlock, and loblolly pine) dirigents, whereas the angiosperms (poplar, ash, flax, sweet gum, *Eucommia*, *Forsythia*, pea, Arabidopsis, rice, and tobacco) are more closely grouped toward the left side.

A significant fraction of our ongoing work is now directed toward establishing further the role of dirigents, particularly as regards the substrate specificities and the different modes of interunit coupling encountered. A few examples will suffice for illustrative purposes only: Many lignans are linked through other specific coupling modes, such as the 8-3′-linked kadsurenone (Fig. 2), the platelet-activating factor from *P. futokadsura*. Others, such as syringaresinol (Fig. 7) in *E. ulmoides*, gomisin A from *S. chinensis*, and (nor)dihydroguaiaretic acid (Fig. 2) from the creosote bush (*L. tridentata*) are presumed to be derived not from coniferyl alcohol, but instead from sinapyl alcohol and (iso)eugenol, respectively. In this context, corresponding genes encoding dirigent homologs have been obtained from *P. futokadsura*, *S. chinensis*, and *L. tridentata*. These in turn are believed to encode dirigents involved in the initial coupling steps leading to kadsurenone, gomisin A, and nordihydroguaiaretic acid biosynthesis, respectively, with the corresponding genes displaying approximately 72%, 73%, and 80% similarity and approximately 61%, 70%, and 73% identity to that of the *Forsythia* psd Fi1 gene. Additionally, in *E. ulmoides* two distinct dirigent gene homologs have been obtained with approximately 63% and 76% similarity and approximately 52% and 67% identity to the psd Fi1 gene. With essentially each of the resulting recombinant proteins now in hand, their precise biochemical functions are being determined and will be described elsewhere. However, it can be concluded that a large class of dirigents is present in nature, which in total can both engender distinct coupling modes and use various specific substrates.

TEMPORAL AND SPATIAL CONSTRAINTS DURING LIGNIFICATION: A PROPOSED ROLE FOR DIRIGENT SITES

During cell division in the active cambial layer of (woody) plants, totipotent cells attain specific metabolic functions and fates. This includes designation to form xylem elements (tracheids or vessels depending upon the species), as well as to afford phloem fibers, etc; however, the signals that result in such specialized cells are as yet unknown. On the other hand, the gross ultrastructural and biochemical changes associated with the onset of lignified secondary cell wall development are reasonably well understood, as summarized below.

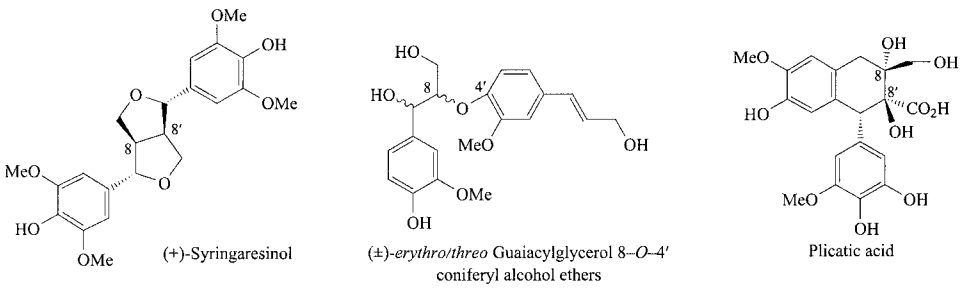

Figure 7. Lignans, syringaresinol, guaiacylglycerol 8-*O*-4′ coniferyl alcohol ethers, and plicatic acid.

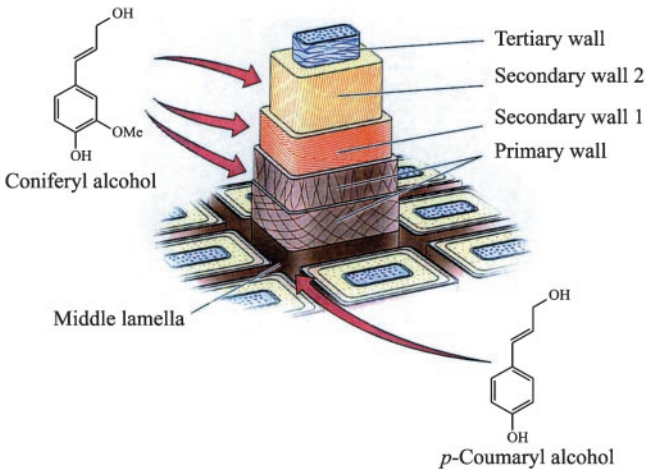

Figure 8. A schematic idealized model of the cell wall structure of softwood tracheids and hardwood libriform fibers (Croteau et al., 2000).

Lignified Sapwood Tracheary Elements

Cells that become either lignified tracheids (in gymnosperms) or vessels (in angiosperms) undergo the following changes. The plasma membranes of such designated cells, which possess so-called primary walls, significantly expand. Prior to the onset of lignification, the plasma membrane next goes through a programmed contraction phase, during which the cellulosic, hemicellulosic, and proteinaceous components are differentially laid down during the ordered deposition of the so-called S1, S2, and S3 layers of the secondary xylem cell wall (Fig. 8). In this way, each cell controls, both temporally and spatially, the manner in which its cell wall components are deposited.

With the cell architecture thus determined, lignification now begins, but, again, in a way whereby the cell retains full control of its wall-forming processes occurring outside of the plasma membrane. Thus, the lignin-forming precursors (*p*-coumaryl, coniferyl, and sinapyl alcohols) first differentially make their way through the plasma membrane and into the cell wall (Lewis et al., 1999). Lignification does not immediately follow upon passage, however, otherwise only a thin layer of lignin would be observed adjacent to the plasma membrane. Instead, each monolignol is differentially targeted to precise sites (so-called lignin initiation sites) at the outermost reaches of the preformed cell wall (Donaldson, 1994). Lignification then begins at these initiation sites and extends back toward the plasma membrane, whereupon lignin biosynthesis is completed and the cell dies. Sarkanen (Guan et al., 1997) has proposed that the subsequent stages of lignin polymerization, i.e. extending from the initiation sites back to the plasma membrane, occurs via a template-mediated replication process. Significantly, the initiation sites appear to differ for each monolignol: *p*-coumaryl alcohol is targeted toward regions within the middle lamella, whereas coniferyl alcohol is initially deposited in the S1 sublayer and cell corners (Fig. 8).

Dirigent Protein (Coniferyl Alcohol Binding) Sites and Lignan/Lignin Biosynthesis

Given that the *Forsythia* dirigent proteins examined thus far only possessed coniferyl alcohol (radical) binding sites, we next considered the possibility that dirigent sites would be present in the lignification initiation sites (i.e. those which employ coniferyl alcohol-derived substrate), as well as in those cell (compartments) undergoing lignan biosynthesis. In this context, all dirigent protein genes isolated to date possess a signal sequence associated with the secretory pathway (Gang et al., 1999). To investigate this possibility, both tissue printing and in situ hybridization experiments were carried out (Kwon et al., 1999), and it was established that the corresponding dirigent protein mRNA was present in the actively dividing cambial cell regions of *F. intermedia* stems; only the results of the in situ hybridization are included in this *Update*, where the signal is observable in the cambial cells (Fig. 9a, antisense) relative to that of the control (Fig. 9b, sense).

Immunolocalization of dirigents, on the other hand, using dirigent protein antibodies, revealed two presumed sites. A strong signal was observed in the cambial region of the actively dividing cells (Fig. 9c) relative to that of the preimmune serum (Fig. 9d), and a second was in the lignified tracheary elements. Closer examination of the lignified tracheary elements, however, revealed that the bulk of the label was in the S1 layer of the secondary wall, coincident with the region associated with coniferyl alcohol targeting to the lignin initiation sites. A small amount of immunolabeling was also detected in the S3 layer (Fig. 9e), which may have originally been associated with lignan biosynthesis nearing cell death.

The localization of the dirigent sites would seem to imply that they (or some homolog thereof) are involved in initial binding of the incoming monolignol-derived substrates, thereby enabling specific targeting of the monolignols to regions far removed from the plasma membrane. These lignin initiation sites would presumably be arranged (ordered) in such a manner whereby interunit linkages (predominantly 8-*O*-4′ aryl, see Fig. 7) are preferentially engendered during polymerization. The lignin template thus formed can replicate and/or cross-link according to Sarkanen's (Guan et al., 1997) template-mediated process. Indeed, such a mechanism could even account for the reported lack of optical activity of the polymeric lignins (Lewis and Davin, 1998). Moreover, it is perhaps significant that Hatakeyama's thermal analyses of lignified wood (Hatakeyama et al., 1999) suggests that lignin in situ behaves more like a polystyrene molecule than as an extensively cross-linked polymer.

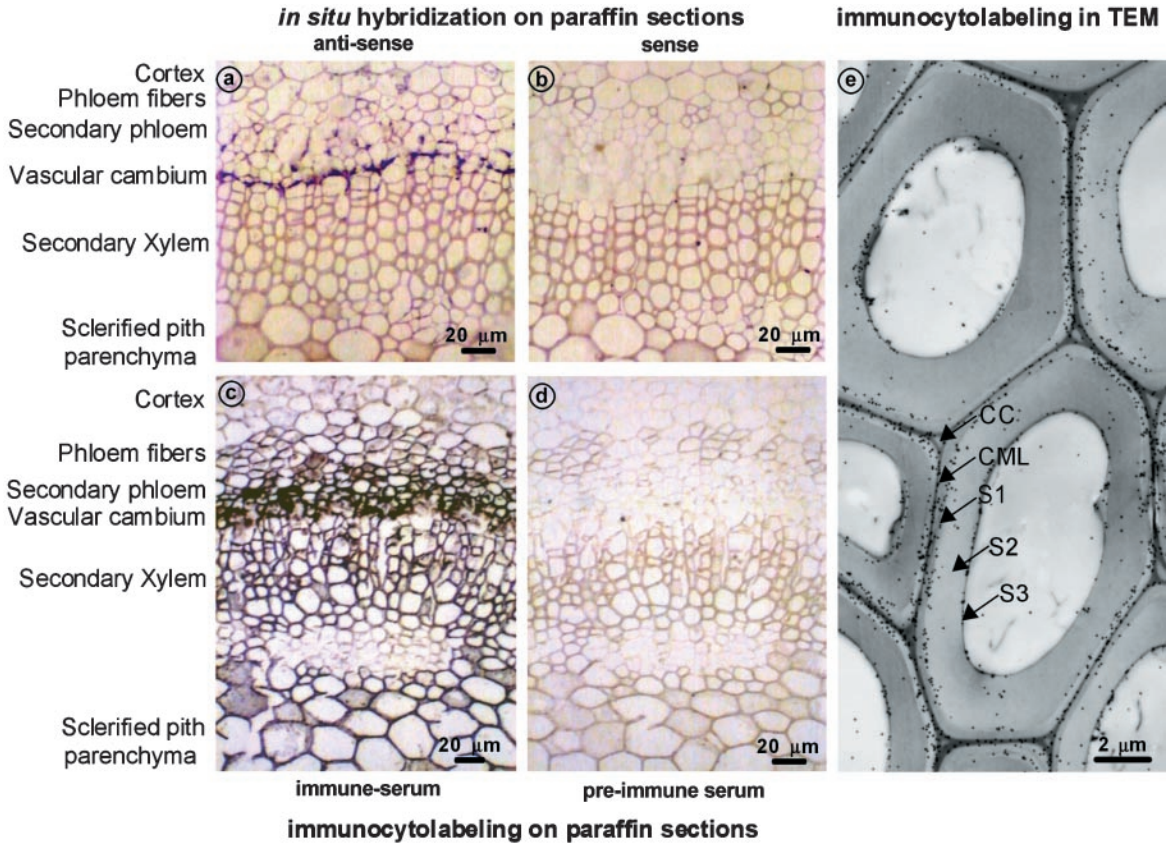

Figure 9. Tissue-specific expression of dirigent gene and subcellular localization of protein epitopes in *F. intermedia* stem sections. a and b, In situ hybridization of dirigent mRNA with digoxigenin-labeled riboprobes in mature stem internode. a, Section labeled with antisense probes; b, section labeled with a sense probe as a negative control. c and d, Immunocytochemical labeling of *F. intermedia* mature stem internode embedded in paraffin using a gold-coupled secondary antibody and further silver enhancement. c, Labeling with the immune serum; d, control with the preimmune serum. e, Immunogold labeling of *F. intermedia* mature stems using polyclonal antiserum raised against the recombinant dirigent at the transmission electron microscopy level.

The second major region of dirigent epitope localization in the stem sections (Fig. 9c) is associated with the cambial region, an area typically described as being solely involved in lignification. In that region, monolignol partitioning may occur to produce both the lignans (which presumably accumulate in the vacuoles of specialized cells [Henges et al., 1996]) and the biopolymeric lignins.

Heartwood Formation

In most long-lived woody species, such as western red cedar (*T. plicata*), another type of woody tissue is generated at some point in the plant's lifetime following sapwood formation, i.e. the subsequent transformation of sapwood into heartwood. In western red cedar, this occurs via massive deposition of the dirigent-derived plicatic acid (Fig. 7) lignans, which have M_r values up to approximately 10,000. These substances are first laid down near the pith and are the result of metabolic activity in the neighboring sapwood-heartwood (forming) transition zone. This zone then extends out radially over time until essentially approximately 95% of the original sapwood is encompassed. In western red cedar, some 15% to 20% of its stem weight is due to plicatic acid-derived lignans. During heartwood formation, the western red cedar essentially shuts off non-productive water and nutrient transport in the heartwood-forming zones, with concomitant lignan impregnation thus providing protection (via infusion) to the prelignified sapwood.

Some of the genes involved in heartwood formation have only now been identified. These genes include those encoding both dirigents and other enzymes involved in downstream metabolic events leading to plicatic acid in western red cedar (Fujita et al., 1999; Gang et al., 1999). It is now evident that the living parenchyma cells within pre-lignified sapwood play a most important role in heartwood formation (Chattaway, 1952; Gang et al., 1998). It is currently envisaged that the metabolites affording the heartwood-forming substances are transported along the ray cells, ultimately undergoing conversion into their species-specific heartwood metabolites.

CONCLUSIONS AND FUTURE OUTLOOK

The discovery of dirigents, and their widespread abundance in land plants, provides the badly needed insight that phenoxy-radical coupling processes can be stipulated precisely and controlled in planta. The involvement of such proteins—and/or comparable homologs/analogs thereof—harboring specific monolignol (radical)-binding sites thus gives a reasonable explanation not only for stereoselective coupling that produces the lignans, but also the nature of lignin initiation sites. Moreover, the results obtained through in situ hybridization and immunolabeling of the corresponding dirigent site(s) underscore the need for caution in interpreting results in the vascular cambium regions, since both lignan and lignin biosynthesis can occur. That is, monolignol metabolites, as with all other products of metabolism, can be differentially partitioned into different pathways, even in the same tissues. The discovery of lignin initiation sites containing putative monolignol (radical)-binding regions, together with Hatakeyama's findings that lignin in situ has properties akin to polystyrene, provide further support to Sarkanen's hypothesis of a template-guided polymerization process in situ.

How dirigents evolved their function is as yet unknown, as is whether they only evolved during land plant adaptation. However, the phylogenetic tree obtained to date appears to reveal a separation into both gymnosperm and angiosperm families; undoubtedly, this phylogenetic relationship will be refined as biochemical functions are determined.

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