Transcriptional Control of Monolignol Biosynthesis in *Pinus taeda*

**Factors Affecting Monolignol Ratios and Carbon Allocation in Phenylpropanoid Metabolism**

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The phenylpropanoid pathway in vascular plants, which ultimately leads to important natural products such as the lignins, lignans, and flavonoids (1), has attracted considerable attention in recent years. One rationale for this interest is that the lignins are, next to cellulose, nature’s second most abundant products, and it is considered that genetically altering lignin amounts and/or composition may be beneficial in both pulp and paper processing (2) and in achieving more readily digestible animal feed (3). Another rationale is in establishing the factors fundamentally controlling lignin assembly and structure and identifying what effects occur when this process is perturbed through genetic manipulation (4). Thus, various strategies, using transgenic plants, have focused upon either of the following: (i) quantitatively reducing lignin contents and/or altering the monomeric compositions of same to render them more readily removable and/or animal feedstock-degradable (5–7), or (ii) examining the effects of disturbing/perturbing lignin assembly on plant cell wall integrity (e.g. for water/nutrient conduction and vasculature strength) and related metabolism (4). However, this worldwide effort, directed toward either up- or down-regulation of each of the monolignol (lignin precursors) forming steps, has often resulted in phenotypes that cannot solely be explained simply on the basis of a direct effect on lignin formation itself (4).

The “normal” xylem lignin of the gymnosperm lobolly pine (*Pinus taeda*) is derived from the two monolignols, p-coumaryl and coniferyl alcohols, these being in ratios of ~1:8, respectively (Fig. 1) (8). (Angiosperm lignins, on the other hand, have a third monolignol, sinapyl alcohol, with an additional methoxyl group (9).) In addition to this normal lignified xylem, the main stem and branches of gymnosperms contain certain cell types that produce a p-coumaryl alcohol-enriched lignin leading to so-called reaction (compression) wood (10). Such specialized tissues are involved in buttressing stem and branches to ensure that the desired alignments/orientations relative to the gravitational vector are attained (11).

At present, there is a very incomplete understanding of how both monolignol formation and lignin biosynthesis in developing vascular plants are regulated, particularly as to how metabolic flux (carbon allocation) in the pathway and lignin compositions are controlled (12, 13). Furthermore, this lack of detailed knowledge of pathway regulation has led to reports by other investigators (14–18) that each and every enzymatic step in the monolignol pathway to the lignins has either a “key” or regulatory role. One reason for this incomplete understanding is that distinct phenylpropanoid metabolic processes are present (operative) in different cell types (including different subcellular locations) in various tissue types, e.g. leading to production of pathway-related secondary metabolites, such as lignans, flavonoids, and the phenolics of suberized tissues (1). Another reason is that none of the aforementioned studies were...
able to examine modulation of the pathway in toto and hence were constrained to extrapolations and reasonings in the absence of, for example, metabolic flux data and transcriptional profiles of the entire pathway.

We therefore chose to develop a cell suspension culture system, using *P. taeda* that could be induced to differentially form both monolignols and a so-called “extracellular lignin” without interference from such competing and/or related pathways (19). (This system permits the quantitative study of how differential monolignol biosynthesis occurs; such studies cannot readily be done with developing intact plants because of the different cell types involved in distinct metabolic processes, for example.) Thus, when the cell suspension cultures were transferred to a solution containing 8% sucrose, the walls underwent partial secondary thickening, and a “lignin-like” material accumulated in the extracellular medium (20). On the other hand, when *H₂O₂* scavengers, such as potassium iodide (KI), are added to the induction medium, the peroxidase-mediated catalysis leading to the extracellular lignin formation is arrested, and instead only the monolignols (lignin/lignan precursors) are excreted into the culture medium without further conversion (20). This system therefore permits the study of monolignol pathway regulation without additional complications.

The biosynthesis of p-coumaryl and coniferyl alcohols (Fig. 1) is initiated with deamination of Phe by phenylalanine ammonia lyase (PAL)1 to form cinnamic acid, which is then hydroxylated by a P450 enzyme, cinnamate 4-hydroxylase (C4H), to form p-coumaric acid (21). The subsequent conversions to afford p-coumaryl alcohol involve sequential CoA transesterification catalyzed by a 4-coumarate:CoA ligase (4CL) and two distinct reductive steps catalyzed by a cinnamoyl-CoA reductase (CCR) and a cinnamyl alcohol dehydrogenase (CAD), respectively (22). The formation of coniferyl alcohol, on the other hand, requires additional hydroxylation and *O*-methylation steps that introduce a methoxyl group at the 3-position of the aromatic ring. The gene encoding the enzyme for this hydroxylation step (p-coumarate 3-hydroxylase, *C3H*) has recently been identified and characterized in *Arabidopsis thaliana* (23). Significantly, its down-regulation in *Arabidopsis* apparently gave a phenotype whose somewhat reduced lignin content was derived mainly from p-coumaryl alcohol (24), in agreement with a role of *C3H* in coniferyl and sinapyl alcohol formation. Interestingly, the recombinant *Arabidopsis C3H* expressed in yeast appears to favor p-coumarate derivatives, such as p-coumaroyl shikimate and p-coumaryl quinate, over either the free acid or its CoA derivatives (23), the significance of which still remains to be fully understood.

Depending upon the substrate, the *O*-methylation reactions can occur either at the level of the CoA esters or the phenylpropanoid acids/aldehydes/alkohols, i.e. methylation of caffeoyl-CoA by caffeoyl-CoA *O*-methyltransferase (CCOMT) leads to formation of feruloyl-CoA (25), whereas methylation of 5-hydroxyphenylpropanoid derivatives by caffeate *O*-methyltransferase (COMT) appears to be exclusively involved in sinapyl alcohol formation in angiosperms (26). In any event, the feruloyl-CoA undergoes successive reduction reactions catalyzed by CCR and CAD, respectively, to form coniferyl alcohol (27).

In a previous study, metabolic flux analyses using loblolly pine cell suspension cultures had suggested, based on intracellular accumulation of metabolites, that Phe availability, C4H, and C3H activities may be rate-limiting factors that control carbon allocation to the pathway (28). In this contribution, we now report how control of the phenylpropanoid pathway from Phe onward to the monolignols is effectuated. This was achieved through quantitatively determining transcript levels for all known and previously unknown genes in the pathway (by quantitative real time PCR) and integrating these data with our present understanding of rate-limiting steps (28). These data provide new insight into the identification of steps controlling carbon allocation versus others whose down-regulation results in either metabolic build-up and/or shunting into non-lignin products.

**Experimental Procedures**

*P. taeda* Cell Cultures—Cell cultures of *P. taeda* were maintained in Lawrence and Brown medium containing 11 µM 2,4-dichlorophenoxy-acetic acid, as reported previously (19), with various aliquots (2.5 ml of packed cell volume) being next individually transferred to 8% (w/v) sucrose solutions (25 ml) containing 20 mM KI and with zero or saturating levels (40 mM) of exogenously provided Phe (28). Cells, with or without Phe, were harvested after 0, 2, 4, 6, 9, 12, 16, 20, and 24 h of induction period stored at −20°C prior to RNA isolation. Aliquots of 1 ml of each cell culture medium were collected at the same time intervals and analyzed by high performance liquid chromatography to determine levels of excreted p-coumaryl and coniferyl alcohols.

Cloning of *P. taeda* PAL, C3H, 4CL, AEOMT, CCOMT, and CAD—*P. taeda* cells (750 mg fresh weight) were ground in liquid nitrogen using a mortar and pestle, and RNA was extracted using the RNAgents protocol. The yield of RNA was estimated by measuring the absorbance at 260 nm (29). Using 10 µg of total RNA as the initial template, the 3‘ end of *P. taeda C3H* was isolated by 3‘-rapid amplification of cDNA ends (3‘-RACE) using a kit from Roche Molecular Biochemicals. The 5‘ end of the gene was isolated by RNA ligase mediated (RLM-RACE), using the reagents and enzymes in the FirstChoice™ RLM-RACE kit (Ambion), following the manufacturer’s instructions.

First strand cDNA was synthesized from 1 µg of total RNA using 200 units of Superscript II reverse transcriptase (Invitrogen) in a 20-µl reaction mixture containing 2 µM oligo(dT) anchor primers (Roche Molecular Biochemicals), 1 mM PCR-grade dNTPs (Invitrogen), 10 µM dithiothreitol, 5 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂. Denaturation at 95°C for 3 min and a “Hot Start” PCR protocol for the resulting cDNA (1 µl of the 20-µl reverse transcription reaction) was used as a template to amplify *P. taeda* genes encoding PAL, C3H, 4CL, hydroxycinnamic acid/hydroxycinnamoyl-CoA ester *O*-methyltransferase (AEOMT), CCOMT, and CAD using gene-specific primers (Tables I and II) designed from sequences submitted to the GenBank™ or, in the case of *C3H*, from the newly determined sequence of the 5‘ and 3‘ ends.

PCR amplification was performed in an AmpliTaq II Thermocycler using the following conditions: hot start at 95°C for 2 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a 5-min extension step at 72°C and an indefinite hold at 4°C. PCR mixtures consisted of 50 ng of template, 500 nM of each primer, 1 mM dNTPs, 2.6 units of Expand High Fidelity PCR System Enzyme Mix, 2 mM Tris-HCl (pH 7.5), 10 mM KCl, 0.1 mM dithiothreitol, 10 µM EDTA, 0.055% (w/v) Tween 20, 0.05% (w/v) Nonidet P-40, and 5% (w/v) glycerol. PCR fragments were cloned into the pCR2.1 vector and transformed into *Escherichia coli* TOP10 cells (Invitrogen) according to the manufacturer’s instructions. Plasmids were isolated from *E. coli* cells using Wizard® Plus SV Minipreps DNA Purification System (Promega), and the inserted DNA was sequenced. Nucleotide sequences were determined using an automated DNA sequencer (Applied Biosystems 377, PerkinElmer Life Sciences). The plasmids were isolated and used as positive controls and/or standards during the optimization of real time PCR conditions for each gene.

Cloning of *P. taeda* C4H and CCR—By using cDNA as template, a specific region of the P450 genes bordered by the consensus sequence of the P450 genes bordered by the consensus sequence of the 5‘ and 3‘ ends (Table II), with the PCR conditions described above. The amplified region was then cloned into the pCR2.1 vector and sequenced for the other genes examined in this study. Based on the nucleotide sequence of the *C4H* fragment, a specific forward primer (Table II) was designed and used together with a common reverse anchor primer (Roche Molecular Biochemicals) to amplify the 3‘ end of the gene by

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1 The abbreviations used are: PAL, Phe ammonia lyase; AEOMT, hydroxycinnamic acid/hydroxycinnamoyl-CoA ester *O*-methyltransferase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; 4CL, 4-coumarate: CoA ligase; COMT, caffeate *O*-methyltransferase; RACE, 3‘-rapid amplification of cDNA ends; RLM, RNA ligase-mediated; ROX, rhodamine X.
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Figure 1: Monolignol biosynthetic pathway in loblolly pine. a, phenylalanine ammonia lyase; b, cinnamate 4-hydroxylase; c, p-coumarate 3-hydroxylase; d, caffeoyl-CoA O-methyltransferase; e, 4-coumarate-CoA ligase; f, cinnamoyl-CoA reductase; g, cinnamyl alcohol dehydrogenase. (Note: X is OH or quinate/shikimate, with the latter two referring to the corresponding transferase.)

3′-rapid amplification of cDNA ends (RACE). The PCR product was then cloned and sequenced as before. To isolate the 5′ end of C4H, 5′-RACE was performed using a kit from Roche Molecular Biochemicals, following the manufacturer's instructions. The coding region of C4H was amplified from cDNA template using forward and reverse primers that have BamHI and EcoRI sites, respectively (Table I I). The PCR product was next purified from 1% agarose gel using a Qiaquick® gel extraction kit (Qiagen), as recommended by the manufacturer, and digested with BamHI and EcoRI (New England Biolabs) at 37 °C for 1 h. The resulting DNA fragment with flanking overhangs was ligated into a similarly digested pYEDP60 plasmid using T4 DNA ligase (New England Biolabs), following the manufacturer's protocol. The recombinant plasmid was transformed into TOP10 E. coli cells for in vivo re-amplification and recovered by plasmid isolation. The plasmid was transformed into Saccharomyces cerevisiae strain WHT1, using a modified lithium acetate protocol (30), for a subsequent heterologous protein expression and functional assay.

A pair of degenerate primers (Table II) was used to amplify a fragment of the CCR gene from P. taeda cDNA, using the same PCR conditions as for C4H. The 5′ and 3′ regions were isolated by RLM- and 5′-RACE, respectively, as described above for C4H and C5H. The coding region was amplified by PCR, cloned into the pYcHia2 vector (Invitrogen), and transformed into E. coli TOP10 cells, according to the manufacturer’s instructions, and expressed in functional form (data not shown).

Quantitative Real Time PCR—For each time point of harvesting the P. taeda cell suspension cultures, grown with and without exogenously provided Phe (40 μM), an aliquot (50 mg fresh weight) was ground in liquid nitrogen using a mortar and pestle, and RNA was isolated using RNeasy® Plant RNA Isolation kit (Qiagen). The concentration of isolated RNA was estimated by its absorbance at 260 nm (29). An aliquot (2 μl) of the RNA extract was treated with DNase-I (Invitrogen) prior to cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and random hexamers, according to the manufacturer's protocol. RNA isolation and cDNA synthesis were carried out in quadruplicate.

For quantification of each phenylpropanoid pathway mRNA, a 40-fold dilution of cDNA mixture (5 μl) was used as template in a quantitative real time PCR assay (25 μl) performed on the Mx4000 (Stratagene). In addition to the template, the PCR mixture contained 2× Platinum enzyme mix (12.5 μl, Invitrogen), 160 μM rhodamine X (ROX), and optimized concentrations of gene-specific primers and probes (Table I). Primers and probes used for C3H, 4CL, AEOMT, CCOMT, CCR, and CAD were designed following recommended guidelines (31). The predicted secondary structure(s) and melting temperatures of the probes were determined using the mfold program (bioinfo.math.rpi.edu/mfold/dna). The melting temperatures of the primers chosen for each beacon were determined using the Oligo Analyzer 2.0 (Integrated DNA Technologies). Primers were purchased from Invitrogen; TaqMan® probes were synthesized by Applied Biosystems, and molecular beacons were from Integrated DNA Technologies.

Quantitative real time PCR employed the Mx4000 (Stratagene) equipped with four filters for 6-carboxyfluorescein, VIC (Applied Biosystems proprietary dye), rhodamine X (ROX), and Cy5 (Amersham Biosciences proprietary dye). A 2 μl aliquot of each sample was added to a 15 μl reaction volume containing power SYBR Green (Invitrogen), 160 nM rhodamine X (ROX), and 3 μM rhodamine X (ROX), and 3 μM fluorescein (Amersham Biosciences proprietary dye), respectively, with the thermocycler set at a ramp rate of 2.0 °C/s. Fluorescence intensities were normalized against the reference dye, ROX, and were plotted against the number of cycles using an adaptive baseline algorithm provided by the manufacturer. The last three fluorescence readings were collected either at 60 °C (for molecular beacons) or at 55 °C (for molecular beacons) in each cycle, and the mean was taken as the fluorescence data for that cycle.

Cycling parameters for the TaqMan® assays were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. When molecular beacons were used, the reaction mixture was also preincubated at 50 °C for 2 min and 95 °C for 2 min, but the 40 cycles that followed consisted of three steps at 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min. Quantification of the cDNA levels of each gene was performed for each of the 4 replicates of cDNA prepared.

PAGE-purified single-stranded DNA oligonucleotides (Integrated DNA Technologies) were used to generate a standard curve for C3H, 4CL, AEOMT, CCOMT, CCR, and CAD, whereas plasmids harboring the coding sequence of the target gene were used for PAL and C4H. The concentrations of these DNA standards were calculated based on their absorbance at 260 nm (29). At least two no-template controls were included for every quantitative real time PCR assay performed.

RESULTS AND DISCUSSION

Cloning of P. taeda Phenylpropanoid Pathway Genes—At the onset of this investigation, only three loblolly pine genes encoding known enzymes in the phenylpropanoid pathway to the monolignols (Fig. 1) remained to be cloned, i.e. C4H, C3H, and CCR. The others, PAL (32), 4CL (33), CCOMT (34), CAD (35), and a proposed COMT, also known as ACOMT (36) (discussed below), had been reported previously. These studies had claimed that PAL (32), 4CL (37), and CAD (35) were regulatory enzymes in loblolly pine on the basis of their relative positions in the phenylpropanoid pathway (PAL is the entry point, 4CL and CCOMT are putative branch points, and CAD is the last step) and correlation of their expression with lignification. However, these data in actual fact provided no clarification into which steps, if any, were rate-limiting/regulatory.

To isolate genes encoding PAL, 4CL, CCOMT, CAD, and the putative COMT (AEOMT), cells were first preincubated in a sucrose/KI medium for 6 h prior to total RNA isolation. This
time frame was selected because the various phenylpropanoid pathway metabolites from Phe to the monolignols are detectable only after 6 h of incubation in the sucrose/KI medium (28), thereby indicating that their respective mRNAs are expressed during that period. Thus, using cDNA as template and gene-specific primers for each gene (Table I), the coding regions of PAL, 4CL, CCOMT, CAD, and AEOMT were amplified by PCR and individually cloned into the pCR 2.1 vector (see "Experimental Procedures"). The isolated plasmids were then used as positive controls and/or standards in quantitative real time PCR assays.

The genes encoding C4H, C3H, and CCR were also obtained as follows. First, the use of degenerate primers (Table II) originally designed to isolate differentially induced P450 genes in petunia (38) led to the facile isolation of \( P. \) taeda C4H (GenBankTM accession number AF096998). Second, the gene sequences for C3H from \( S. \) indicum (GenBankTM accession number AY064170) and \( A. \) thaliana, \( C. \) arietinum, and \( M. \) sativa, respectively. Only single copies of each gene again appear to be present based on an exhaustive search of homologous sequences in the EST data base/GenBankTM.

The predicted amino acid sequence of \( P. \) taeda C4H is \(-80\%\) identical to five C4H sequences reported in the GenBankTM, namely those from \( C. \) roseus, \( H. \) tuberosus, \( P. \) kitakamiensis, \( Z. \) elegans, and \( V. \) radiata. It is also at least 77\% identical to nine other putative C4H sequences, namely those from \( P. \) tremuloides, \( G. \) echinata, \( G. \) max, \( A. \) thaliana, \( P. \) sativum, \( P. \) crispum, \( C. \) arietinum, \( M. \) sativa, and \( C. \) chinense. Only two putative C4H sequences, those from \( M. \) crystallinum and \( P. \) vulgaris, are below 70\% identity (67 and 62\% respectively) to the predicted amino acid sequence of the putative \( P. \) taeda C4H. In the case of \( P. \) taeda C3H, the encoded protein is 72, 68, and 68\% identical to those in \( A. \) thaliana, \( S. \) bicolor, and \( S. \) indicum, respectively.

Finally, comparison of the deduced \( P. \) taeda CCR protein sequence gave 67–68% identity to those from \( E. \) gunnii, \( N. \) tabacum, and \( A. \) thaliana. The first CCR gene cloned was from \( E. \) gunnii, which was functionally expressed in \( E. \) coli (39). The second was from \( N. \) tabacum, for which in vivo proof of function has been provided via generating antisense plants and examining their phenotypes (e.g., reduced lignin content) (40). Only two other CCR genes thus far, cloned from \( A. \) thaliana, have been functionally expressed in \( E. \) coli (41).
Quantitative Real Time PCR Analysis—Under basal conditions (maintenance on a 2,4-dichlorophenoxyacetic acid containing medium), the phenylpropanoid pathway to the monolignols in the *P. taeda* cells is essentially not induced (19). On the other hand, transfer of the cells to a solution containing 8% sucrose (and 20 mM KI, as H₂O₂ scavenger) results in pathway induction within 2 h, as evidenced by intracellular accumulation of cinnamic and *p*-coumaric acids over a 2–24-h duration (Fig. 2A, solid lines). Moreover, in essentially the same time frame, the monolignols, *p*-coumaryl and coniferyl alcohols, are also both formed and ultimately excreted into the liquid medium (Fig. 2B, solid lines) (28). The addition of 40 mM Phe to the *P. taeda* cell "bathing" medium, however, results in further metabolic change as evidenced by the accompanying large increases in intracellular cinnamic and *p*-coumaric acid levels (Fig. 2A, broken lines) and in the enhanced excretion of *p*-coumaryl and coniferyl alcohols, with ratios of the monolignols changing from 1:8 to 1:1 over 24 h (Fig. 2B, broken lines).

As noted earlier (28), this differential build up of intracellular cinnamic and *p*-coumaric acids in the *P. taeda* cells had suggested that C4H and C3H may be rate-limiting steps (i.e. in terms of controlling differential carbon allocation to the different monolignol-forming pathways). Hence, to examine whether such metabolic profiles might be accompanied by corresponding transcriptional changes for each of the genes in the pathway, the *P. taeda* cells were harvested at various time points (0, 2, 4, 6, 9, 12, 16, 20, and 24 h) with transcription levels for each gene being determined for each time point.

Thus, by using a plant RNA isolation kit (Qiagen), RNA was first extracted from an aliquot (50 mg fresh weight) of each harvest of *P. taeda* cells, over the various time intervals described above, with and without exogenously provided 40 mM Phe. Typically, the yield of each RNA sample ranged from 0.3 to 0.5 μg/mg cells (fresh weight), as determined by the absorbance at 260 nm (29). Each RNA sample was then treated with DNase-I and reverse-transcribed into cDNA using random hexamers; the resulting cDNAs were individually diluted 40-fold and used as templates for subsequent quantitative real time PCR analyses of all of the phenylpropanoid pathway enzymes using the Mx4000 (Stratagene).

To quantify transcript levels of each gene encoding the phenylpropanoid pathway enzymes and the putative COMT (AE-OMT), TaqMan® probes (42) and primers were individually designed using the Primer Express™ Software (Applied Biosystems). Among the TaqMan® probe and primer sets so obtained, only those for *PAL* and *C4H* did not amplify the intended regions, despite optimization of primer/probe concentrations, amounts of MgCl₂, and annealing temperatures. This problem was subsequently overcome by using molecular beacons (31) for *PAL* and *C4H*. The primers and probes used for each gene are listed in Table I.

The initial amounts of cDNA template for each gene were determined for every sample using a standard curve generated by a concurrent quantitative real time PCR assay in separate tubes of 10-fold serial dilutions of cDNA standards for each gene (data not shown). The standards used for *PAL* and *C4H* were plasmids harboring the corresponding coding sequences, whereas for the other genes, synthetic single-stranded DNAs were employed. RNA isolation and cDNA synthesis were each performed four times, with each replicate being subjected to
levels gradually increased from

in P. taeda

in the cell bathing medium (A). Therefore the means of four independent experimental samples, quantitative real time PCR analysis. The data generated are therefore the means of four independent experimental samples, and the results obtained are described below (see Fig. 3).

In the absence of exogenously provided Phe, PAL transcript levels gradually increased from ~100 copies at 0 h to ~800 copies at 9 h, and then decreased slightly after 20 h to ~700 copies at 24 h (Fig. 3A). During this time frame, not only was PAL activity induced (data not shown), but the two monolignols were also excreted into the medium (Fig. 2B, solid lines). Further addition of Phe (40 mM) then markedly increased maximum mRNA levels more than 15-fold, i.e. from ~100 copies at 0 h to as much as ~14,000 copies at 16 h followed by a decrease to ~10,000 copies at 20 h (Fig. 3A). Concomitantly, the monolignol amounts excreted in the medium were also significantly increased (Fig. 2B, broken lines). The mechanism, however, by which Phe addition induces these significant changes in PAL transcript levels is not known, although at least three possibilities can be contemplated as follows: (i) Phe or some downstream product, including that involved in ammonium ion (nitrogen) recycling (43), induces transcription of PAL (44); (ii) Phe prevents degradation of PAL mRNA; and (iii) a combination of both. Therefore Phe may act as a signal (received either inside or outside the cell), triggering a signal transduction pathway, which ultimately results in increased transcription and/or decreased degradation of the PAL message. The latter may, in turn, be effectuated through either activation of a positive regulator or deactivation of a repressor (45).

This induction of PAL transcription in response to increased Phe supply indicates that PAL itself is not a rate-limiting step, because the levels of transcription and enzymatic activity are modulated in response to metabolic demand. These data thus strongly suggest that pathway up-regulation leading to increased carbon allocation occurs upstream of Phe formation. Indeed this presumably also explains why overexpression of PAL activity does not apparently lead to any increase in lignin content in transformed tobacco plants (46).

C4H transcript levels of P. taeda cell suspension cultures were also induced in the 8% sucrose, 20 mM KI solution (see Fig. 3B), i.e. from ~170 copies at 0 h to ~450 copies at 9 h, with this subsequently decreasing gradually to ~70 copies by 24 h. Addition of 40 mM Phe, however, while not affecting overall transcript levels (~500 copies by 12 h), resulted in a sustained higher level of transcription over the 24-h time frame examined. This small increase in transcription presumably provides the experimental basis for the corresponding observed increases in p-coumaric acid accumulation (Fig. 2A).

By comparison, C3H message induction profiles remained essentially unchanged (Fig. 3C) under both treatments (0 and 40 mM Phe), although transfer to the 8% sucrose, 20 mM KI solution resulted in a corresponding increase in the C3H message from basal levels of ~4,000 to ~20,000 copies within 12 h, which then decreased gradually to ~13,000 copies by 20 h. The C3H transcript profile at 40 mM Phe, however, was essentially the same, reaching a maximum level of ~16,000 copies at 9 h and then declining to ~8,000 copies by 24 h.

The differential effects on expression of the PAL, C4H and C3H genes, and the increased intracellular accumulation of cinnamic and p-coumaric acids thus provide both a genetic basis for and experimental support to rate-limiting roles for the 4-hydroxylation and 3-hydroxylation steps in controlling the metabolic flux to, and the ratios of, the monolignols so formed (i.e. in terms of differential carbon allocation to each monolignol). Hence, it is concluded that (i) limited expression of the C4H gene enables control to be exercised over metabolic flux into the pathway, even when other genes such as PAL are significantly up-regulated, and (ii) given that, at best, only a very limited up-regulation of C3H can occur, this differential modulation helps facilitate the corresponding shift in monolignol ratios toward preferentially increasing p-coumaryl alcohol levels over that of coniferyl alcohol. It will be of interest to determine in the future the effect on p-coumaryl alcohol formation (i.e. in terms of amounts formed) when C3H is down-regulated in P. taeda.

This form of metabolic control, which involves modulated transcription of C4H as a major determinant of phenylpropanoid pathway flux, is consistent with a previous report (47) describing the phenotypes of an Arabidopsis mutant lacking the transcription factor, AtMYB4. This transcription factor is thought to function as a repressor of C4H gene expression, which is de-repressed by both UV light and wounding, resulting in the formation of more sinapate esters. As for the current study, their data also suggested that C4H is truly rate-limiting, i.e. C4H gene expression is most likely the mechanism by which 4-hydroxylation is modulated. Interestingly, it was also found in the Arabidopsis study that AtMYB4 acts as a strong negative regulator of C4H but not of the other genes studied, which included PAl, 4CL, COMT, CCOMT, and CAD. It can thus be tentatively proposed that repression of C4H by an AtMYB4 ortholog in P. taeda may be unaffected by increases in Phe levels, and hence the levels of C4H mRNA are only slightly altered (up-regulated). Additionally, as with C4H, the 3-hy-
The analyses of the transcriptional profiles of the remaining phenylpropanoid enzymatic steps (4CL, CCOMT, CCR, and CAD) were also instructive, with these providing additional proof for the transcriptional regulation of the pathway as proposed above. Thus, when the *P. taeda* cells were transferred to the 8% sucrose, 20 mM KI solution, there was a gradual increase in 4CL transcript levels reaching a maximum value of ~12,000 (from ~400 copies at t = 0 h); this then gradually declined to ~5,000 copies by 24 h (Fig. 3D). In the added presence of 40 mM Phe, however, the 4CL message increased much more substantially from ~400 copies at 0 h to ~50,000 copies at 12 h and then declined progressively to ~16,000 copies at 24 h.

This increase in induction levels of the 4CL transcripts, effected by Phe availability, together with the essentially unaltered expression of C3H, is consistent with (i) observations of preferential increase in metabolic flux toward p-coumaryl alcohol rather than coniferyl alcohol and (ii) that 4CL, like PAL, is truly not rate-limiting. Furthermore, the effects of increased Phe availability on enhancing both PAL and 4CL transcript levels may also be consistent with known similarities between PAL and 4CL promoter regions, *i.e.* in terms of their cis-regulatory elements, which include P, A, and L boxes (48). The presence of similar regulatory elements in both of the promoter regions thus strengthens further the hypothesis that transcriptional control is being exercised in a similar manner, enabling a coordinated pattern of induction. Thus, these data establish that 4CL is not a rate-limiting step and/or regulatory enzyme as claimed previously (37), although extensive down-regulation of 4CL in *Arabidopsis*, aspen, and tobacco led to decreases in lignin contents (49–51), *e.g.* in tobacco, a severe reduction (to ~1%) of residual 4CL activity relative to wild type resulted in an ~50% decrease in lignin deposition. However, this only occurred when the 4CL levels were severely repressed.

Additionally, both the CCOMT expression and CCR transcript profiles displayed coordinated induction, following the same general trends as for PAL and 4CL, upon transfer to the 8% sucrose, 20 mM KI solution, and with added Phe (Fig. 3, E and F). In the absence of additional Phe, CCOMT transcript levels increased from ~3,000 to maximum values of ~24,000 copies within ~9 h and then gradually declined to ~12,000 copies at 24 h.
copies. In the presence of 40 mM Phe, however, induction levels increased by about 2-fold, with transcript levels rising steadily from ~4000 to 8000 copies at 12 h, which then decreased to ~4000 copies at 24 h. CCR mRNA expression behaved in a relatively similar manner (Fig. 3F). Furthermore, the stimulatory effect of Phe on the transcription of CCOMT and CCR presumably helps explain our previous observations that neither of the substrates (i.e. the CoA esters) accumulated in the cells, and hence on this basis were not rate-limiting steps (28). As for PAL and 4CL, the transcription of CCOMT and CCR thus seems to be manipulated in order to accommodate the presence of an ample supply of pathway precursor, conceivably so as not to restrict carbon flow.

With CAD there were only small increases noted in expression levels when cells were either provided exogenous Phe or were not (Fig. 3G); these mRNA increases were also accompanied by corresponding increases in CAD activity (data not shown). At 0 mM Phe, the CAD induction message was increased ~2-fold from ~3,000 copies at 0 h to ~7,000 copies at 9 h, leveling off to ~6,000 copies at 24 h (i.e. these being coordinated with those of PAL, 4CL, CCR, and CCOMT). At 40 mM Phe, a small enhancement was also observed, i.e. from ~5,000 copies at 0 h to ~10,000 copies at 9 h, without any further significant changes observed until the end of the 24-h incubation period. However, as for the other non-rate-limiting steps, metabolite analyses revealed that there was essentially no build up of aldehydic intermediates under either condition.

Furthermore, previous work (28) had established that when p-coumaryl and coniferyl aldehydes were added to the P. taeda bathing medium, even at very high (non-physiological) concentrations (at least 300,000-fold the maximum cellular levels), both substrates were still efficiently and rapidly converted into the corresponding alcohols. Taken together, these data suggest that under either condition sufficient CAD activity is present in the cells to accommodate even significant increases in metabolic flux. Indeed, all of the transgenic and mutant plant data for proposed down-regulation of CAD did not result in any decrease in the carbon allocated to the pathway (5). Therefore, this step does not serve as a true regulatory step in monolignol carbon allocation, in contrast to previous reports (18, 52).

It can also be speculated that the lack of any significant increase in the transcript levels of CAD may be due to other transcription factors acting on the CAD promoter, which might be distinct from those affecting C3H and C4H, as well as from those up-regulating the genes encoding non-regulatory steps. Indeed, when the ATMYB4 ortholog from arbutus was overexpressed in tobacco (53), CAD mRNA was significantly down-regulated, whereas the latter was not affected when endogenous ATMYB4 was overexpressed in Arabidopsis (47), i.e. an indication of different modes of transcriptional regulation for CAD in different species.

Finally, the putative COMT (AEOMT) transcripts could also be detected, albeit in very low copy numbers relative to the other phenylpropanoid pathway genes, i.e. having less than ~100 copies per ng of RNA (Fig. 3H). There was, however, essentially no induction of AEOMT mRNA expression with the 8% sucrose, 20 mM KI medium, even in the presence of exogenously provided Phe (Fig. 3H). The incongruously very low levels of the putative COMT (AEOMT), together with the lack of coordinated expression with the other phenylpropanoid genes during induction of the pathway, suggests strongly some other metabolic role rather than in monolignol/lignin biosynthesis. Moreover, the AEOMT gene has a very low sequence identity (34–41%) to all known lignin-related COMTs (36), which generally share at least 70–85% identity with each other. Indeed, because many O-methyltransferases are known to have a wide range of substrate specificity, the reports of recombinant AEOMT being able to catalyze the conversion of caffeic acid and caffeoyl-CoA to ferulic acid and feruloyl-CoA, respectively, may have no physiological relevance. Accordingly, until proven otherwise, the physiological function of AEOMT has to be viewed as being unknown at this point.

Concluding Remarks—In summary, carbon allocation to the phenylpropanoid pathway, and to the two monolignols, is regulated in P. taeda in terms of Phe supply and by differential metabolic flux to each monolignol, and this is controlled by modulation of transcription levels of C4H and C3H, respectively. In contrast, gene expression of the other steps is able to adjust to metabolic demand via up-regulation of their corresponding transcript levels, thereby ultimately enabling increased flux for that particular step. Interestingly, in this way monolignol ratios are manipulated between ~1:8 and ~1:1 for p-coumaryl and coniferyl alcohols, respectively; this in turn is consistent with the changes noted for the monolignol contents of its lignin of normal and “reaction” wood, respectively. These data thus appear to provide an explanation as to how such changes in metabolism are effectuated; however, the underlying physiological reasons for increasing the p-coumaryl alcohol content of lignin in reaction wood need to be more fully understood. Future work will also focus upon how this overall transcriptional regulation is effectuated and how the different pathways (e.g. N recycling, phenylpropanoid and shikimic acid pathway metabolism) are coordinately regulated and interconnected.

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REFERENCES

1. Creteau, R., Kuchta, T. M., and Lewis, N. G. (2000) in Biochemistry and Molecular Biology of Plants (Buchanan, B., Jones, R., and Gruissem, W., eds) pp. 1250–1318, American Society of Plant Physiologists, Rockville, MD
2. Roberts, J. C. (1996) The Chemistry of Paper, pp. 161–175, Royal Society of Chemistry, Cambridge, UK
3. Jung, H. G., and Vogel, K. P. (1986) J. Am. Chem. Soc. 108, 1705–1712
4. Anterola, A. M., and Lewis, N. G. (2002) Phytochemistry.
5. Baucher, M., Moni MK, Van Montagu, M., and Boerjan, W. (1998) Crit. Rev. Plant Sci. 17, 125–197
6. Guo, D., Chen, F., Wheeler, J., Winder, J., Selman, S., Peterson, M., and Dixon, R. A. (2001) Transgenic Res. 10, 457–464
7. Baucher, M., Bernard-Vailhé, M. A., Chambert, B., Besle, J.-M., Opsoner, C., Van Montagu, M., and Bottermann, J. (1999) Plant Mol. Biol. 39, 437–447
8. De Stevens, G., and Nord, F. F. (1953) Proc. Natl. Acad. Sci. U. S. A. 28, 80–84
9. Lewis, N. G., and Yamamoto, E. (1986) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 455–496
10. Westing, A. H. (1965) Bot. Rev. 31, 381–480
11. Sculthorpe, G. (1973) Science 179, 647–655
12. van Rensburg, H., Anterola, A. M., Levine, L. H., Davin, L. B., and Lewis, N. G. (2000) ACS Symp. Ser. 742, 118–144
13. Lewis, N. G., Davin, L. B., and Sarkanen, S. (1999) in Comprehensive Natural Products Chemistry (Barton, D. H. R., Nakani, K., and Meth-Cohn, O., eds) Vol. 3, pp. 617–745, Elsevier Science Publishers B.V., Amsterdam
14. Zucker, M. (1965) Plant Physiol. 40, 779–784
15. Douglas, C. J. (1990) Trends Plant Sci. 1, 171–178
16. Gowri, G., Bugos, R. C., Campbell, W. H., Maxwell, C. A., and Dixon, R. A. (1991) Plant Physiol. 97, 7–14
17. Geffner, D., Campbell, M., Campargue, C., Clastre, M., Borderies, G., Boudet, A., and Boudet, A. M. (1994) Plant Physiol. 106, 625–632
18. Grima-Pettenati, J., Campargue, C., Boudet, A., and Boudet, A. M. (1994) Phytochemistry 37, 941–946
19. Eberhardt, T. L., Bernard, M. A., He, L., Davin, L. B., Wooten, J. B., and Lewis, N. G. (1993) J. Biol. Chem. 268, 21008–21096
20. Nose, M., Bernard, M. A., Furlan, M., Zajicek, J., Eberhardt, T. L., and Lewis, N. G. (1995) Phytochemistry 39, 71–79
21. Russell, D. W., and Conn, E. E. (1967) Arch. Biochem. Biophys. 122, 256–258
22. Gross, G. G., Stockigt, J., Mansell, R. L., and Zenk, M. H. (1973) FEBS Lett. 31, 283–286
23. Schoch, G., Goepert, S., Morant, M., Henn, A., Meyer, D., Ullmann, P., and Werck-Reichhart, D. (2001) J. Biol. Chem. 276, 36566–36574
24. Franke, R., Remm, M. M., Denatull, J. W., Ruegger, M. O., Humphreys, J. M., and Chappel, C. (2002) Plant J. 30, 47–59
25. Ye, Z.-H., Knesel, R. E., Matern, U., and Varner, J. E. (1994) Plant Cell 6, 1427–1439
26. Atanassova, R., Fivet, N., Martz, F., Chabbert, B., Tollier, M.-T., Monties, B.,
Transcriptional Control of Monolignol Biosynthesis in *P. taeda*

Fritig, B., and Legrand, M. (1995) *Plant J.* 8, 465–477
27. Gross, G. G., and Kreiten, W. (1975) *FEBS Lett.* 54, 259–262
28. Anterola, A. M., van Rensburg, H., van Heerden, P. S., Davin, L. B., and Lewis, N. G. (1999) *Biochem. Biophys. Res. Commun.* 261, 652–657
29. Warshaw, M. M., and Cantor, C. R. (1970) *Biopolymers* 9, 1079–1103
30. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* 20, 1425
31. Tyagi, S., and Kramer, F. R. (1996) *Nat. Biotechnol.* 14, 303–308
32. Whetten, R. W., and Sederoff, R. R. (1992) *Plant Physiol.* 98, 380–386
33. O'Malley, D. M., Porter, S., and Sederoff, R. R. (1992) *Plant Mol. Biol.* 20, 1425
34. Moller, S. G., and Chua, N.-H. (1999) *Plant J.* 28, 257–270
35. van Heerden, P. S., Towers, G. H. N., and Lewis, N. G. (1996) *J. Biol. Chem.* 271, 12350–12355
36. Chabannes, M., Barakate, A., Lapierre, C., Marita, J. M., Ralph, J., Pearson, M., Danoun, S., Halpin, C., Grima-Pettenati, J., and Boudet, A. M. (2001) *Plant J.* 23, 257–270
37. Lauvergeat, V., Lacomme, C., Lacombe, E., Lasserre, E., Roby, D., and Grima-Pettenati, J. (2001) *Phytochemistry* 57, 1187–1195
38. Holland, P. M., Abramson, R. D., Watson, R., and Gelfand, D. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 7276–7280
39. van Heerden, P. S., Towers, G. H. N., and Lewis, N. G. (1996) *J. Biol. Chem.* 271, 12350–12355
40. Coruzzi, G. M., and Zhou, L. (2001) *Curr. Opin. Plant Biol.* 4, 247–253
41. Moller, S. G., and Chua, N.-H. (1999) *J. Mol. Biol.* 293, 219–234
42. Sewalt, V. J. H., Ni, W., Blount, J. W., Jung, H. G., Maseoud, S. A., Howles, P. A., Lamb, C., and Dixon, R. A. (1997) *Plant Physiol.* 113, 41–50
43. Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtenz, F., Jones, J., Tonelli, C., Weisshaar, B., and Martin, C. (2000) *EMBO J.* 19, 6150–6161
44. Whithred, J. M., and Schuler, M. A. (2000) *Plant Physiol.* 124, 47–58
45. Lee, D., Meyer, K., Chappelle, C., and Douglas, C. J. (1997) *Plant Cell* 9, 135–154
46. Kajita, S., Katayama, Y., and Omori, S. (1996) *Plant Physiol.* 113, 808–812
47. Tamagnone, L., Merida, A., Parr, A., Mackay, S., Cuiianz-Macia, F. A., Roberts, K., and Martin, C. (1998) *Plant Cell* 10, 135–154
Transcriptional Control of Monolignol Biosynthesis in Pinus taeda: FACTORS AFFECTING MONOLIGNOL RATIOS AND CARBON ALLOCATION IN PHENYLPROPANOID METABOLISM

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