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Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis

(cosuppression/flux control/lignin/metabolic engineering/transgenic plants)

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ABSTRACT **Phenylalanine ammonia-lyase (PAL) catalyzes the first step in phenylpropanoid synthesis. The role of PAL in pathway regulation was investigated by measurement of product accumulation as a function of enzyme activity in a collection of near-isogenic transgenic tobacco plants exhibiting a range of PAL levels from wild type to 0.2% of wild type. In leaf tissue, PAL level is the dominant factor regulating accumulation of the major product chlorogenic acid and overall flux into the pathway. In stems, PAL at wild-type levels contributes, together with downstream steps, in the regulation of lignin deposition and becomes the dominant, rate-determining step at levels 3- to 4-fold below wild type. The metabolic impact of elevated PAL levels was investigated in transgenic leaf callus that overexpressed PAL. Accumulation of the flavonoid rutin, the major product in wild-type callus, was not increased, but several other products accumulated to similarly high levels. These data indicate that PAL is a key step in the regulation of overall flux into the pathway and, hence, accumulation of major phenylpropanoid products, with the regulatory architecture of the pathway poised so that downstream steps control partitioning into different branch pathways.**

Plants synthesize from phenylalanine a wide variety of natural products based on the phenylpropane skeleton (1). Phenylpropanoid products have important functions in plant defense against pests and predators (1, 2), as UV protectants (3), and as signal molecules both internally and for communication with other organisms (4). Moreover, lignin, which is elaborated from cinnamyl alcohols by oxidative polymerization, is the major structural component of secondarily thickened cell walls of water-conducting xylem elements in the vascular system (5, 6). Lignin is the second most abundant biopolymer after cellulose and hence represents a major fate for fixed carbon in the biosphere.

There is considerable interest in the regulation of phenylpropanoid biosynthesis, both as a model for understanding flux control in a complex biosynthetic pathway and also for the identification of targets for biotechnological manipulation of product accumulation. Phenylpropanoid biosynthesis is initiated by the deamination of phenylalanine to give cinnamic acid, catalyzed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) (7). Successive hydroxylations of cinnamic acid to give *p*-coumaric acid and caffeic (3,4-dihydroxycinnamic) acid are followed by the formation of hydroxycinnamoyl CoA thioesters, which are the substrates for branch pathways for the production of lignin monomers, flavonoids, coumarins, and simple esters (3, 8).

PAL, as the bridge between primary metabolism and natural product biosynthesis, is a potential site for pathway regulation (9), and indeed PAL mRNA and enzyme levels are highly regulated spatially and temporally, associated with the tissue-specific accumulation of phenylpropanoid products, exemplified by selective expression in differentiating xylem cells for production of lignin monomers at the onset of secondary wall deposition (10–13). However, genes encoding subsequent enzymes of phenylpropanoid biosynthesis are often coordinately regulated with PAL (8), and hence these correlations do not delineate whether perturbations in PAL enzyme levels contribute to the control of pathway flux and accumulation of specific phenylpropanoid products. Moreover, while many reports demonstrate a correlation between changes in the levels of phenylpropanoid biosynthetic enzymes and product accumulation (3, 8, 14–17), there is little direct evidence that product accumulation can be quantitatively accounted for by changes in biosynthetic enzyme activity levels integrated over time, and it has been proposed that substrate control through changes in phenylalanine pool size might be the major site of phenylpropanoid regulation (18, 19).

Generation of transgenic plants with altered levels of a specific enzyme provides a new and powerful approach for the analysis of pathway regulation (20). For example, measurement of the rates of carbon fixation in a population of transgenic plants containing a ribulose-bisphosphate carboxylase antisense construct has allowed direct analysis of the role of this enzyme in photosynthetic flux control (21). Transformation of tobacco with the bean PAL2 gene, modified by the inclusion of cauliflower mosaic virus 35S enhancer sequences in its promoter, generates transgenic plants with severely reduced PAL activity and correspondingly lower levels of phenylpropanoid products (22). This paradoxical suppression of PAL activity, which was one of the first examples of sense suppression, or cosuppression, following introduction of transgene copies of plant genes (23, 24), is progressively reversed in succeeding generations homozygous for the PAL2 transgene. Thus PAL sense suppression and subsequent recovery provide the equivalent of an allelic series exhibiting a range of PAL activities. We have used these plants to analyze the quantitative relationship between PAL activity and phenylpropanoid product accumulation in a near-isogenic setting.

MATERIALS AND METHODS

Plant Material. The generation of transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) containing a bean PAL2 gene

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Abbreviations: CGA, chlorogenic acid; CHS, chalcone synthase; PAL, phenylalanine ammonia-lyase.

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with promoter sequences to −550 downstream of the cauliflower mosaic virus 35S enhancer has been described (22). The PAL2 primary transformant YE6-16 and progeny homozygous for the transgene were selfed to generate pools of seed stocks for T_1–T_4 generations. Plants from these seed stocks, as well as wild-type plants from which the transgene had been segregated out, were grown under greenhouse conditions and harvested when the plants were fully mature (20–22 leaf nodes). Leaf tissue was harvested from the 11th nodal leaf and stem sections were removed from the 10th and 11th internodes and stored at −80°C. Small sections of stem were removed for immediate sectioning and staining, and the remainder was stored at −80°C for the determination of enzyme activities, lignin, and soluble phenolics. Calli were generated from leaf tissue of wild-type plants and vegetatively propagated PAL2 primary transformants YE10-6 and YE6-16 by excision of small tissue sections and culture on Murashige and Skoog medium (25) with 1 μM benzyladenine and 2.5 μM naphthylacetic acid in complete darkness.

Phenolic Analysis. Leaf and callus samples were ground in liquid N_2 and stored at −80°C for 3–4 weeks. One hundred nanomoles of isoferulic acid (Aldrich) in 100 μl of methanol plus 2 ml of acetonitrile was added to each sample, the mixture was sonicated, 2 ml of ethyl acetate was added, and the mixture was vortexed and then centrifuged to separate the residue from the extracts. Two further extractions were performed, once with 2 ml of hexane and 2 ml of acetonitrile and once with 3 ml of water and 1 ml of acetonitrile. Extracts were combined and passed through a 0.45-μm filter and evaporated with a N_2 stream. Extraction was shown to be complete by further extraction of a representative sample twice with 5 ml of acetonitrile and calculation of efficiency relative to the yield of the sample prior to reextraction. The dried extracts were combined with 2 ml of acetonitrile/water, 1:1 (vol/vol), and insoluble residues were removed by centrifugation at 12,000 × *g* for 30 min. Twenty microliters of the solution was applied to an octadecylsilane (5-μm particle size) HPLC column (4.6 mm × 250 mm) and was eluted with a gradient of increasing solvent B in solvent A [solvent A, water/formic acid, 98/2; solvent B, acetonitrile/formic acid, 98/2 (vol/vol)]: 0–5 min, 5% B; 5–10 min, 5–10% B; 10–25 min, 10–35% B; and 25–30 min, 35–40% B. The absorbance at 271 nm of the eluate was monitored, and retention times and calibration curves for chlorogenic acid (CGA), rutin, and isoferulic acid were established with authentic samples for the determination of the levels of these products in extracts of tobacco leaf tissue.

Lignin Determination. Stem internode segments were analyzed for lignin by the thioglycolic acid method (26). Stem tissues were ground in liquid N_2 and transferred to a preweighed glass tube for lignin determination. A standard curve was prepared by using commercial lignin (Aldrich) diluted to 10 mg/ml in 2 M HCl and extracted in parallel with the experimental samples.

Histochemistry. Stem tissues were hand sectioned with a model DK-10 sliding microtome (Uchida Yoko) and immediately immersed in 10% phloroglucinol (in 100% ethanol) for 2 min, followed by a 1-min incubation in concentrated HCl. Sections were rinsed and stored in 75% (vol/vol) glycerol prior to dark-field photography with an Olympus OHZ dissecting microscope.

Enzyme Assays. PAL activity was determined in leaf and stem tissue extracts by a radiometric assay (22). The product, *trans*-cinnamic acid, was separated from the reaction mixture by phase partitioning into toluene, and the radioactivity in the organic phase was then measured by liquid scintillation spectrometry. Chalcone synthase (CHS) activity was measured in leaf tissue extracts by a radiometric assay (27).

Chemicals. [2-^{14}C]Malonyl CoA (51 mCi/mmol; 1 mCi = 37 MBq) was obtained from Amersham, and L-[U-^{14}C]phenylalanine (485.7 mCi/mmol) was from Sigma. *p*-Coumaroyl CoA was synthesized as described (27). All other chemicals were obtained from Sigma except where noted.

RESULTS

PAL Activity in PAL2 Transformant Progeny. The bean PAL2 primary transformant YE6-16 shows severely suppressed levels of PAL activity in leaf and stem tissue (22). However, selfed progeny homozygous for the transgene show a progressive recovery in PAL activity. Fig. 1 shows data for the recovery of PAL activity in leaf tissue of a single pedigree of progeny from the T_0 YE6-16 primary transformant, and a similar recovery in PAL levels was also observed in stems and other organs. By examining several different pedigrees arising from the YE6-16 primary transformant, we were able to obtain a large collection of near-isogenic plants with different PAL activity levels from wild type down to 0.2% of wild type.

PAL Activity and Phenylpropanoid Accumulation in Leaf Tissue. CGA (3-caffeoylquinic acid) and rutin (quercitin 3-β-D-rutinoside) are the major soluble phenylpropanoid products in tobacco leaf tissue, accounting for approximately 60% and 10% of total phenolics, respectively (28). CGA is an ester conjugate of caffeic acid, which is an intermediate of the central pathway of phenylpropanoid biosynthesis, whereas rutin is a disaccharide conjugate of quercitin and hence is a product of the flavonoid branch pathway.

Analysis of CGA and rutin levels in the collection of YE6-16 progeny revealed a close relationship between PAL activity and accumulation of these major leaf phenylpropanoid products (Fig. 2 *A* and *B*). The line of best fit through the scatter plots indicates a direct correlation between leaf PAL activity and phenylpropanoid product accumulation, with coefficient of determination (r^2) values of 0.737 and 0.786 for CGA and rutin, respectively. While, as expected, severe suppression of PAL activity resulted in drastically reduced product accumulation, even modest reductions in PAL activity levels below wild type had a proportionate effect on CGA and rutin accumulation. There was also a tight correlation between CGA and rutin accumulation (Fig. 2*C*; r^2 = 0.837), indicating that individual plants did not show selective suppression of the accumulation of the different products. However, the best-fit curves of rutin and CGA accumulation as functions of PAL activity had different slopes, with the latter being closer to unity.

Impact on CHS Activity. The direct relationship between PAL activity and product accumulation implicated perturbations in PAL levels as a key element in the regulation of

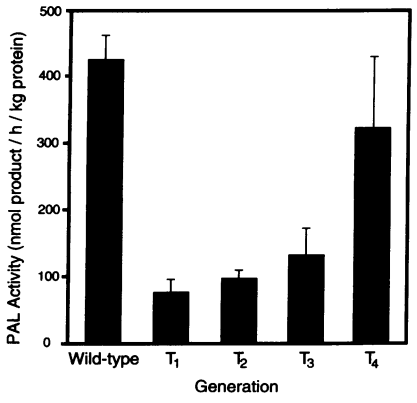

FIG. 1. Recovery of PAL levels in subsequent generations of a pedigree of homozygous progeny of the primary PAL2 transformant YE6-16. Bars denote mean and standard error of replicate PAL activity measurements.

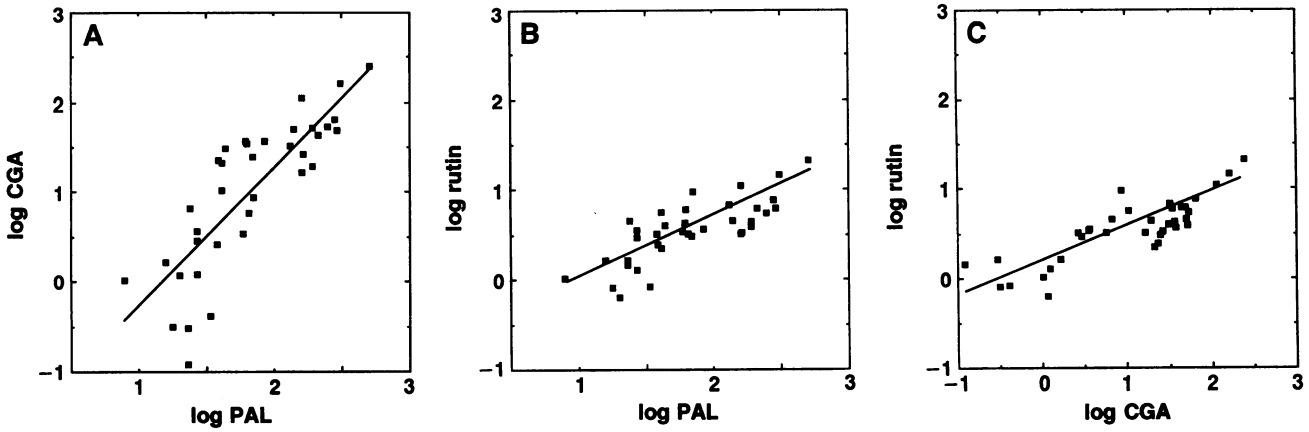

FIG. 2. (*A* and *B*) Relationship between PAL activity and the accumulation of rutin (*A*) and CGA (*B*) in leaf tissue of a collection of near-isogenic plants comprising homozygous T_1–T_4 progeny of various pedigrees originating from the primary PAL2 transformant YE6-16 and in equivalent wild-type plants from which the PAL2 transgene has been segregated out. (*C*) Relationship between CGA and rutin accumulation in this collection of plants. Leaf tissue from individual plants was assayed for PAL activity (nmol of product/hr per kg of protein) and in the same tissue samples rutin and CGA levels (nmol/kg of tissue fresh weight) were determined by HPLC.

phenylpropanoid accumulation in leaf tissue. This regulation might reflect a direct role for PAL as a flux-determining step or an indirect effect in which alterations in PAL activity levels perturbed internal pathway regulatory mechanisms leading to modulation of downstream enzymes which were rate determining. However, the level of activity of CHS, which catalyzes the first committed step of the branch pathway for flavonoid biosynthesis (3), was not correlated with PAL activity level in leaf tissue from this collection of transgenic plants (Fig. 3). Indeed, the best-fit curve had a shallow negative slope, indicating that CHS activity levels tended to rise when PAL activity was severely suppressed.

PAL Activity and Lignin Accumulation in Stems. Lignin, which is the major phenylpropanoid product in stems, is formed by oxidative polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohol monomers (6, 29). Deposition of histochemically detectable lignin was markedly reduced in stems of PAL2 primary transformants showing severe PAL suppression (22). In the present study, phloroglucinol staining of lignin in stem sections demonstrated that in a YE6-16 T_4 plant showing partial suppression of PAL activity, lignin deposition in xylem tissue was intermediate between that in a wild-type plant and in a T_1 plant with severe PAL suppression (Fig. 4). Thus, PAL activity in the T_4 plant was about 6-fold lower than in wild-type plants, and there was significant reduction in the intensity of lignin staining and the size of the strongly stained area. Further suppression of PAL activity in the T_1 plant to 15-fold lower than wild type almost completely blocked the accumulation of histochemically detectable lignin.

Lignin accumulation in stems of this collection of near-isogenic plants was then quantified by the thioglycolate extraction method, which in common with all other methods for lignin determination gives an estimate rather than an absolute value for total lignin content (6). This analysis demonstrated a close quantitative relationship between PAL levels and thioglycolate-extractable, noncondensed lignin (Fig. 5). However, unlike the relationship between PAL activity levels and product accumulation in leaves, the slope of the curve was steeper at lower PAL levels than at near wild-type levels, indicating that modest reductions in PAL activity from wild-type did not have such a great impact on thioglycolate-extractable lignin accumulation as similar fractional changes among the collection of plants showing more severe PAL suppression with activities 3- to 4-fold below wild type.

Product Accumulation in Leaf Callus. Unfortunately, studies with sense-suppressed plants do not allow examination of

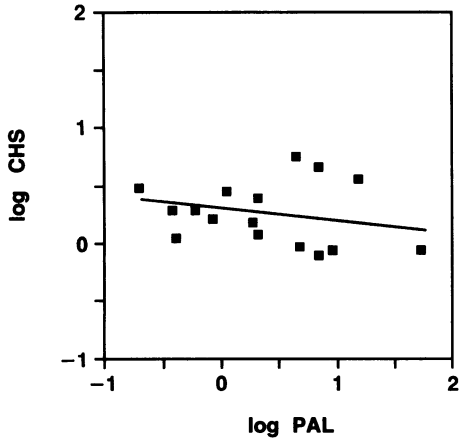

FIG. 3. Relationship between PAL and CHS activity levels (nmol of product/hr per kg of protein) in homozygous T_1–T_4 progeny of the PAL2 transformant YE6-16 and in equivalent wild-type plants.

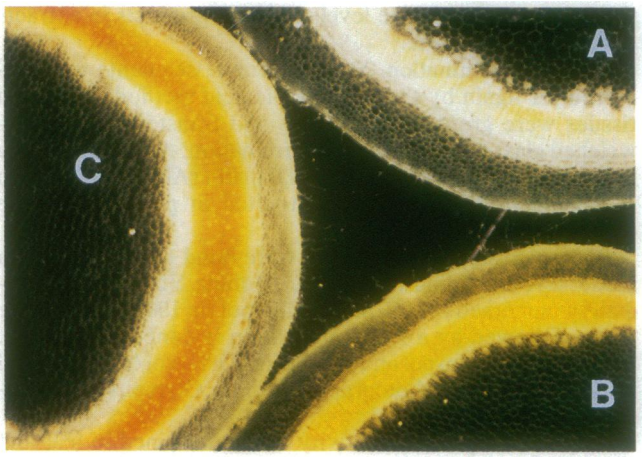

FIG. 4. Phloroglucinol staining of lignin in stem sections from a homozygous T_2 progeny plant of the YE6-16 PAL2 transformant showing severe suppression of PAL (*A*), a homozygous T_4 progeny plant of the YE6-16 PAL2 transformant showing partial suppression of PAL (*B*), and an equivalent wild-type plant from which the PAL2 transgene has been segregated out (*C*). PAL activities were 4×10^3, 9×10^3, and 6×10^4 nmol of product/hr per kg of protein, respectively.

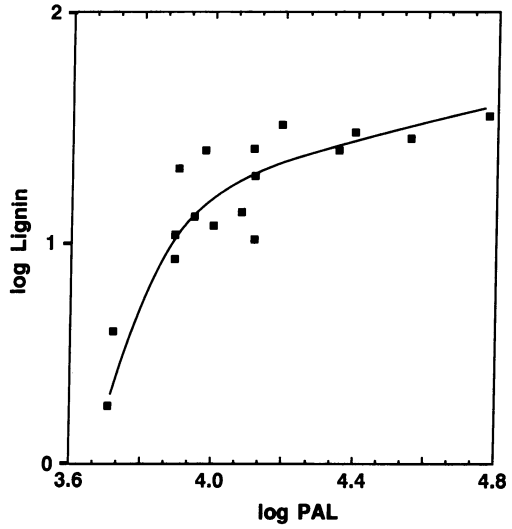

FIG. 5. Relationship between PAL activity and thioglycolate-extractable lignin levels in stem tissue of collections of near-isogenic plants comprising homozygous T_1–T_4 progeny of various pedigrees originating from the primary PAL2 transformant YE6-16 and in equivalent wild-type plants from which the PAL2 transgene has been segregated out. PAL activity is expressed as nmol of product/hr per kg of protein and lignin content as percent dry weight.

the impact on product accumulation of increasing PAL activity above wild-type levels. However, calli generated from T_0 leaf tissue of PAL2 transformants YE6-16 and YE10-6 showed substantially higher levels of PAL activity than equivalent wild-type callus (T.N.-H. and Y.E., unpublished observations). Rutin was the single major product that accumulated in wild-type leaf callus. Interestingly, rutin levels in the PAL2 transgenic callus were not increased, but several other products now accumulated to similarly high levels and the overall accumulation of phenylpropanoid products was substantially greater than in equivalent wild-type leaf callus (Fig. 6).

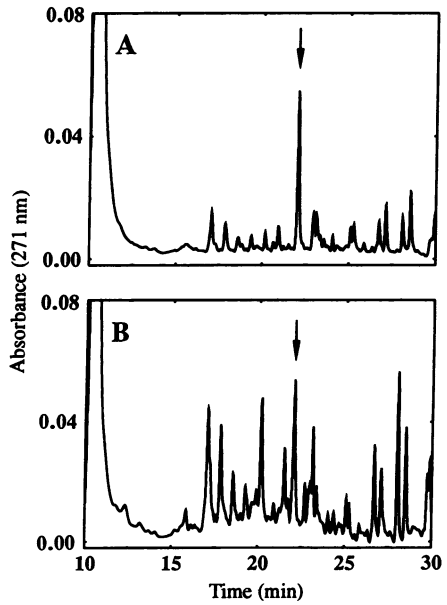

FIG. 6. HPLC analysis of phenylpropanoid accumulation in calli from leaf tissue of vegetatively propagated T_0 PAL2 transformant YE10-6 (*B*) and an equivalent wild-type plant (*A*). PAL activity was 9-fold higher in the transgenic culture than in the wild-type culture. Arrow denotes elution time of rutin.

DISCUSSION

We have exploited the sense suppression of PAL in a PAL2 primary transformant and subsequent progressive recovery in pedigrees of homozygous progeny to evaluate the role of the first committed step of phenylpropanoid biosynthesis in the overall regulation of product accumulation. As in other examples of sense suppression (23, 24, 30, 31), the effect on PAL expression is gene-specific and transcripts encoding other phenylpropanoid biosynthetic enzymes such as CHS and caffeic acid *O*-methyltransferase in the flavonoid and lignin branch pathways, respectively, are not suppressed (N.J.B., unpublished observations). Hence this approach allows direct evaluation of the impact of specific perturbations in PAL levels on phenylpropanoid accumulation within a collection of near-isogenic plants originating from a single primary transformant.

CGA is the major phenylpropanoid product in tobacco leaf tissue, and the direct relationship between PAL activity levels and CGA accumulation in these plants demonstrates that small perturbations in PAL activity have a corresponding effect on accumulation of this product. Hence, PAL appears to be the dominant control point in CGA regulation, so that subsequent steps in this system do not contribute significantly to flux control. In terms of the theoretical analysis of metabolic regulation developed by Kacser and Burns (32), the sensitivity coefficient for modulation of CGA accumulation by small perturbations in the activity of downstream enzymes is very small, whereas that for PAL approaches unity, which is the value for the sum of sensitivity coefficients within a defined system and the theoretical maximum for any one step. The metabolic system for rutin accumulation in leaves behaves slightly differently than that for CGA: although there is a direct relationship between PAL and rutin levels in this collection of plants, the slope of the best-fit curve is lower than that for CGA, which was close to unity. Thus, while PAL almost exclusively controls CGA accumulation, control of rutin accumulation involves contributions to flux control from one or more steps in the flavonoid branch pathway, as well as a major contribution from PAL. Since CGA is the major product, accumulating to severalfold higher levels than rutin in wild-type plants, the data for CGA and rutin taken together indicate that in leaf tissue PAL is the major site for control of overall flux into the pathway.

The slope of the rutin/PAL curve is less than that of the CGA/PAL curve, and hence it appears that at lower PAL levels the flavonoid branch pathway begins to compete more effectively for the diminishing supply of *p*-coumaroyl CoA, the substrate for both CHS and hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase. CHS has a low K_m for *p*-coumaroyl CoA compared to hydroxycinnamoyltransferase, providing a possible mechanism for differential partitioning as a function of overall flux into the pathway (33, 34). In addition, the weak negative relationship between PAL and CHS levels suggests that an internal compensatory mechanism operates for the flavonoid branch pathway. Flooding the central pathway with exogenous supplies of the PAL product cinnamic acid inhibits CHS transcription (35) but induces hydroxycinnamoyltransferase activity (36). Partial derepression of CHS and deinduction of hydroxycinnamoyltransferase as pathway intermediates become progressively more limiting would then ensure some continuing flavonoid synthesis at low PAL levels. Feedback control in the flavonoid branch pathway might also contribute to the switch from CGA to rutin accumulation under conditions of low overall flux into the pathway.

The relationship between PAL levels and phenylpropanoid accumulation in stems is more complex, since small incremental decreases in PAL activity from wild-type levels do not have a corresponding impact on the accumulation of the

major product lignin. Although it is extremely difficult to measure total lignin content in view of the resistance of the polymer to chemical degradation, it nevertheless appears that in wild-type plants PAL makes only a partial contribution to flux control of lignin deposition, with important control sites downstream. The cinnamyl-alcohol lignin monomers often accumulate as glycosylated conjugates in the vacuole, and hence release of the monomers and oxidative polymerization are likely additional control sites (6). However, as PAL levels are decreased below about 20–25% of wild-type levels, PAL becomes the dominant rate-determining step, possibly reflecting depletion of the monomer pool. This regulatory architecture would provide for both substantial flux control at the first committed step, combined with local control of monomer availability and polymerization at sites of secondary wall development during xylem differentiation. Severe suppression of PAL activity led to a corresponding reduction of lignin deposition, with <10% of the thioglycolate-extractable lignin present in equivalent stem tissues from wild-type plants (Figs. 4 and 5).

Overall, this analysis demonstrates that PAL at wild-type levels is a major site of flux control in phenylpropanoid biosynthesis and that in certain contexts PAL is the dominant control point. It remains to be established over what interval PAL can be increased in intact plants before the regulatory architecture of the pathway changes and downstream enzymes become more prominent sites of flux control. However, transgenic callus tissue with higher PAL activity than equivalent wild-type callus exhibits greater overall accumulation of phenylpropanoid products. Interestingly, this higher PAL activity does not result in increased levels of rutin, the major product in wild-type callus, but in the accumulation of other phenylpropanoid products that are present only at trace levels in the wild-type callus. Thus, in this setting at least, while PAL activity levels above wild type lead to an increase in overall pathway flux, the regulatory architecture is poised so that downstream steps in the selected branch pathway rapidly become rate limiting.

Biotechnological manipulation of plant metabolism requires the identification of rate-determining steps as targets for modulation by gene transfer (20), exemplified by recent attempts to suppress lignification in transgenic plants. A burst of lignification in forage crops immediately prior to flowering reduces digestibility by cattle (37), and, likewise, partial inhibition of lignification in tree species would reduce energy costs and solvent use in pulp and paper manufacture (38). It has recently been shown that reduction of the level of caffeic acid *O*-methyltransferase in antisense transgenic plants causes a partial reduction in lignin deposition (39). In contrast, antisense plants with substantially reduced levels of a second lignin branch pathway enzyme, cinnamyl alcohol dehydrogenase, showed no reduction in lignin deposition and relatively minor changes in monomer composition (40), indicating that this enzyme does not contribute greatly to flux control, consistent with observations that dehydrogenases catalyze reactions that are close to equilibrium *in vivo* (41). The present data indicate that selective manipulation of PAL levels should be an effective strategy for modifying lignin content and enhancing the contribution of phenylpropanoid products to plant defense.

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