Plant Physiol. (1996) 112: 1617–1624

Overexpression of L-Phenylalanine Ammonia-Lyase in Transgenic Tobacco Plants Reveals Control Points for Flux into Phenylpropanoid Biosynthesis[1]

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Transgenic tobacco (*Nicotiana tabacum* L.) plants overexpressing the enzyme L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) were grown from seeds of a primary transformant containing the bean *PAL2* gene, which had shown homology-dependent silencing of the endogenous tobacco *PAL* genes. Analysis of endogenous and transgene-encoded PAL transcripts and protein in the primary transformant (T_0) and first-generation (T_1) overexpressor plants indicated that the transgene-encoded PAL is the cause of the greater than wild-type levels of PAL activity (up to 5- and 2-fold greater in leaf and stem tissue, respectively) in the T_1 plants. Leaves of PAL-overexpressing plants contained increased levels of the hydroxycinnamic acid ester chlorogenic acid but not of the flavonoid rutin, indicating that PAL is the key control point for flux into chlorogenic acid. In addition, levels of the glucoside of 4-coumaric acid increased in the overexpressing plants, suggesting that the 4-coumarate:coenzyme A ligase or coumarate hydroxylase reactions might have become limiting. These results help to define the regulatory architecture of the phenylpropanoid pathway and indicate the possibility of engineering-selective changes in this complex metabolic pathway by overexpression of a single early pathway gene.

The phenylpropanoid pathway is responsible for the synthesis of a large range of natural products in plants, including flavonoids (pigments and UV protectants), the structural polymer lignin, and antimicrobial furanocoumarin and isoflavonoid phytoalexins (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). Salicylic acid, which is involved in the establishment of both local and systemic plant defense responses, is also a product of this pathway (Klessig and Malamy, 1994). Although the importance of phenylpropanoid natural products makes the pathway an obvious target for plant improvement by metabolic engineering, little is known about the control of flux into the various branches of the pathway. Many phenylpropanoid synthesis genes have now been cloned (Dixon and Paiva, 1995), but the roles of the enzymes they encode in determining the pattern and extent of accumulation of phenylpropanoid end products have only been inferred from correlations between changes in extractable activity and product levels (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995).

The first step of phenylpropanoid biosynthesis is catalyzed by the enzyme PAL, which converts L-Phe to *trans*-cinnamic acid. This enzyme and its corresponding genes have been the subject of intense study in a number of plants (Cramer et al., 1989; Logemann et al., 1995; Wanner et al., 1995). To gain a greater understanding of the role of PAL in the regulation of phenylpropanoid metabolism, a *PAL* gene from the French bean (*Phaseolus vulgaris*) was introduced into tobacco to study the consequences of overexpression of this enzyme (Elkind et al., 1990). Paradoxically, two plants from this initial transformation event, YE6-16T_0 and YE10-6T_0, had severely reduced expression of the endogenous *PAL* genes ("sense-suppression") at both the RNA and enzyme activity levels (Elkind et al., 1990). The plants were also phenotypically abnormal, displaying stunted growth and curled leaves. Similar homology-dependent gene silencing has been observed in a number of transgenic studies at both the transcriptional and posttranscriptional levels (Matzke and Matzke, 1995).

In YE6-16T_0, the sense-suppressed state was inherited over a number of generations and gradually became less severe (Bate et al., 1994). However, the characteristics of the plants generated from seeds of the primary transformant YE10-6T_0 have so far not been reported. Here we show that YE10-6T_1 plants have, surprisingly, changed from sense suppression to overexpression of PAL enzyme activity. The increase in PAL activity is probably due to the bean PAL protein, which is absent in the sense-suppressed YE10-6T_0 and YE6-16 lines. These plants provide a unique system for studying the regulatory architecture of the phenylpropanoid pathway. We show that the increased PAL activity

[1] This research was supported by The Samuel Roberts Noble Foundation. P.A.H. is a Noble Foundation/Salk Institute Plant Biology Postdoctoral Fellow.

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Abbreviations: CGA, chlorogenic acid; NDF, neutral detergent fiber; PAL, Phe ammonia-lyase (EC 4.3.1.5); RT, retention time; T_1, first generation; T_2, second generation.

in the YE10-6T_1 plants results in increased accumulation of the hydroxycinnamic acid ester CGA, the major phenolic compound in tobacco leaves, but does not increase accumulation of the flavonoid rutin.

MATERIALS AND METHODS

The generation of the tobacco lines YE6-16 and YE10-6 was described previously (Elkind et al., 1990). These lines contain the bean *PAL2* gene under the control of its own promoter, plus additional cauliflower mosaic virus 35S enhancer sequences. Plants were analyzed when fully grown, just prior to flowering. Leaf samples were taken from internodes 6 to 8 (counting from the top), and stem samples were taken from internodes 8 to 10.

Isolation and Analysis of RNA

RNA was extracted from leaf and stem tissue using guanidinium thiocyanate and phenol-chloroform (Chomczynski and Sacchi, 1987). Northern blot and RNase protection analyses were performed at high stringency by standard procedures (Ausubel et al., 1994). The bean *PAL2* transcript was detected in RNase protection assays by an antisense probe generated from the plasmid pSPP2 (Liang et al., 1989), and the 500-bp *BspHI-NarI* fragment of the bean *PAL2* gene (Cramer et al., 1989) was used as the probe in northern blot analyses; neither probe cross-hybridized with tobacco *PAL* genes. Endogenous tobacco *PAL* transcripts were detected by RNase protection using two nonoverlapping probes: a 360-nucleotide antisense transcript of the *HincII-KpnI* fragment of a tobacco *PAL* cDNA, nucleotides 1279 to 1639 of the coding sequence (Nagai et al., 1994), and an antisense probe transcribed from the plasmid pET3-4 (Elkind et al., 1990; Brederode et al., 1991). The lack of complete sequence conservation between pET3-4 and the corresponding sequence of the tobacco PAL cDNA (Nagai et al., 1994) suggests that the two RNase protection probes recognize different PAL transcripts. Both tobacco probes were specific for tobacco *PAL* genes in RNase protection assays but cross-hybridized with bean *PAL* transcripts on northern blots.

Size markers for RNase protection were formed by filling in the ends of appropriately sized restriction fragments from pBluescript with Klenow fragments and [^{32}P]dATP. The 600-bp *XhoI-SacI* fragment of the constitutively expressed β-ATPase gene (Boutry and Chua, 1985) was used as a loading control for northern blots.

Production of Antisera and Western Blot Analysis

The amino acid sequences used to raise antibodies specific for the bean PAL2 and tobacco PAL proteins were ILNAKEAFELANIGSE and TLNAEEAFRVAGVNGG, respectively; these sequences were derived after examining the bean PAL2 sequence (Cramer et al., 1989) and the available tobacco PAL sequences (Nagai et al., 1994; Pellegrini et al., 1994; Fukasawa-Akada et al., 1996). Synthetic peptides corresponding to these sequences were coupled to the carrier protein keyhole limpet hemocyanin and (primary) antibodies were raised in rabbits (HRP, Denver, PA). Secondary antibody consisted of a 1:7500 dilution of anti-rabbit IgG-alkaline phosphatase conjugate (S373B, Promega). 5-Bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as alkaline phosphatase substrates were used to visualize bands (Ausubel et al., 1994).

Leaf and stem proteins were extracted in 0.2 M borate buffer, pH 8.8, and subjected to denaturing PAGE by standard procedures (Ausubel et al., 1994). Proteins were transferred to membranes (Immobilon-P, Millipore) in 25 mM Tris-HCl, pH 8.3, 192 mM Gly, and 20% methanol (Towbin et al., 1979). Membranes were blocked and probed with primary and secondary antibodies in 3% BSA in 0.1% Tween 20 in Tris-buffered saline (Ausubel et al., 1994).

Enzyme Assays

PAL activity was determined by the conversion of L-[U-^{14}C]Phe to [^{14}C]*trans*-cinnamic acid essentially as described previously (Legrand et al., 1976), except that 50 μL of protein extract was combined with 50 μL of a 1 mM unlabeled Phe solution containing 0.025 nmol [^{14}C]L-Phe (Amersham, 474 mCi/mmol). After incubation for 3 h at 37°C, labeled cinnamic acid was extracted with toluene and combined with scintillation fluid for counting. Protein concentrations were determined by the Bradford procedure, using BSA as the standard (Bradford, 1976).

Analysis of Soluble Phenolics

Leaf samples (2 g fresh weight) were ground in liquid N_2 and divided into two 1-g portions. One of these was assayed for PAL enzyme activity, and the other was analyzed for phenolics. In the latter case, the ground tissue was extracted three times with acetone (8 mL per extraction) and the extracts were combined and concentrated under a stream of N_2. The residue was taken up in 1 mL of methanol by a combination of sonication and vortexing and centrifuged for 10 min to pellet the remaining insoluble material. The supernatant was diluted 1:5 for HPLC analysis of CGA and rutin, and it was left undiluted for analysis of the remaining phenolic compounds. Twenty microliters of each solution was applied to an octadecylsilane HPLC column (5-μm particle size, 4.6 × 250 mm) with elution by increasing the concentration of solution B with respect to solution A (solution A: 1% phosphoric acid; solution B: 100% acetonitrile; 0–5 min of constant 5% B; 5–10 min of 10% B [gradient duration 5 min]; 10–25 min of 15% B [gradient duration 15 min]; 25–45 min of 40% B [gradient duration 20 min]; 45–55 min of 95% B [gradient duration 1 min]; 55–65 min of 5% B [gradient duration 1 min]; flow rate constant at 1 mL/min). UV was monitored at both A_{275} and A_{320}, and the amounts of CGA and rutin were determined by constructing standard curves with authentic standards (Sigma).

Characterization of the Glucoside of 4-Coumaric Acid

The phenolic compound of RT 27 min was isolated by extracting bulk leaf tissue from YE10-6T_1 plants as above and separating the phenolics on a preparative octadecylsilane HPLC column (10-μm particle size, 22.5 × 250 mm, gradient essentially as above but with a flow rate of 20 mL/min). Appropriate fractions were collected, diluted with water, and extracted three times with ethyl acetate. The organic phases were combined and the solvent was removed by rotary evaporation. Recovery and purity were assessed by analytical HPLC. Two aliquots of pure compound were dried under N_2, resuspended in 1 mL each of 16 mM sodium citrate and 50 mM Na_2HPO_4, pH 5.2, and to one of these was added 2 mg of almond β-glucosidase (Sigma); both were incubated overnight at 37°C and extracted with ethyl acetate, and the phases were combined and dried under N_2. Compounds were dissolved in methanol and examined by analytical HPLC with diode array UV scanning, along with authentic samples of 4-coumaric acid.

Determination of Lignin

Stem samples were lyophilized and extracted with boiling neutral detergent (Van Soest et al., 1991) using filter bags in a batch fiber analyzer (ANKOM, Fairport, NY). The residual NDF was oven-dried (55°C) and used for quantitation of Klason lignin according to the method of Kaar et al. (1991), modified for microanalytical scale.

RESULTS

Levels of Endogenous and Transgene-Derived *PAL* Transcripts in YE10-6T_0 Primary Transformants and T_1 Plants

In striking contrast to the abnormal visible characteristics of the parent YE10-6T_0, all selfed-progeny YE10-6T_1 plants exhibited a wild-type phenotype, suggesting that the T_1 plants might have lost the sense-suppressed state of the primary transformant. Although there was a dramatic reduction in the level of endogenous *PAL* transcripts in the YE6-16 line, which is diagnostic of the sense-suppressed state (Fig. 1B), transcripts from the bean transgene were readily detectable (Elkind et al., 1990; Fig. 1A). Northern blot analysis showed that transcripts from the bean *PAL* transgene were likewise expressed at a relatively high level in leaves of the sense-suppressed YE10-6T_0 primary transformant (Fig. 1A), but the endogenous tobacco *PAL* transcripts in this line were almost undetectable by RNase protection (Fig. 1B). However, bean *PAL* transcripts were expressed more strongly in leaves of YE10-6T_1 plants than in the T_0 plants (Fig. 1A), and the level of endogenous *PAL* transcripts in the T_1 plants was as great as that in wild-type plants (Fig. 1B); this restoration of appearance of endogenous *PAL* transcripts, suggesting loss of the sense-suppressed state, was most clearly demonstrated in stem tissue, in which *PAL* was maximally expressed (Fig. 1B).

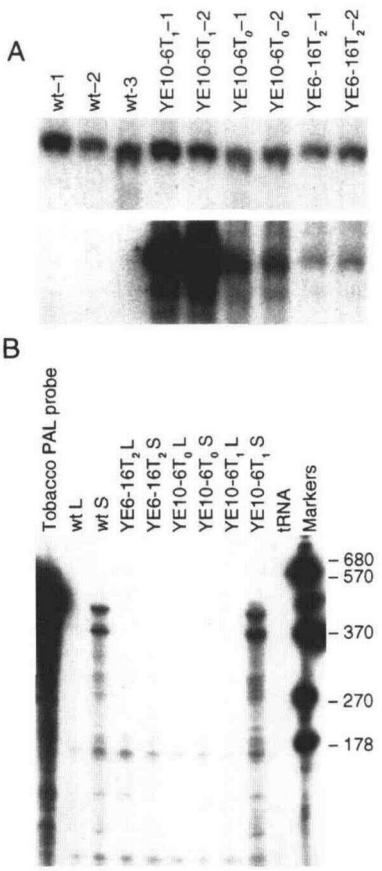

Figure 1. PAL transcript levels in wild-type, PAL sense-suppressed, and PAL-overexpressing tobacco lines. A, Bean *PAL2* transcript levels in leaves as determined by northern blot analysis. Total RNA (10 μg) was electrophoresed, transferred to a membrane (Hybond-N, Amersham), and hybridized with a ^{32}P-labeled probe specific for the bean *PAL2* transcript (bottom). Blots were stripped and rehybridized with a β-ATPase gene probe (top) as a control for loading and transfer efficiency. wt 1 to 3, Three individual wild-type plants; YE10-6T_1, two individual T_1 YE10-6 plants; YE10-6T_0, two loadings from a YE10-6 sense-suppressed primary transformant; YE6-16T_2, two individual T_2 sense-suppressed YE6-16 plants. RNase protection with an antisense probe generated from plasmid pSPP2 gave a similar result (data not shown). In stem tissue, a higher level of expression of the transgene in YE10-6T_1 plants compared with the sense-suppressed plants was also observed (data not shown). B, Endogenous *PAL* transcript levels in leaves (L) and stems (S) of wild-type (wt) and transgenic plants as determined by RNase protection analysis. Total RNA (10 μg) was hybridized with a ^{32}P-labeled antisense transcript synthesized from a plasmid containing a 360-bp *Hin*cII-*Kpn*I fragment of a tobacco *PAL* gene, and fragments protected from RNase digestion were separated on a 6% polyacrylamide gel. Designation of lines was as in A. tRNA was a negative control. Leaf and stem tissue for each plant came from a single individual. The two bands presumably represent transcripts from different tobacco PAL genes. The same relative levels of endogenous *PAL* transcripts in wild-type and transgenic plants was seen when antisense RNA transcribed from plasmid pET3-4 was used in RNase protection assays (data not shown). Sizes of markers (bp) are shown on the right.

Levels of Bean and Tobacco PAL Enzyme Protein in YE10-6 T_0 and T_1 Plants

The purpose of the present work was to study the metabolic consequences of PAL overexpression rather than the mechanisms underlying the shift from epigenetic sense suppression to overexpression. However, knowing whether the changes in overall PAL activity are caused by changes in expression of endogenous PAL, transgene PAL, or both, is important from a metabolic viewpoint.

To determine the relative levels of expression of endogenous and transgene-derived PAL proteins in the YE10-6 T_0 and T_1 lines, we raised two antibodies, one specific for the bean PAL2 protein and the other specific for tobacco PAL proteins. Bean PAL protein was clearly detected in YE10-6T_1 plants but not in sense-suppressed YE10-6T_0 or YE6-16 plants (Fig. 2). Thus, in the sense-suppressed state, either the bean *PAL* transcript was not translated or the level of translation was so low that the bean protein could not be detected under the conditions used. Probing blots with antiserum specific for endogenous tobacco PAL demonstrated that the level of this protein in the YE10-6T_1 plants was similar to that in wild-type plants and that very little if any tobacco PAL protein was present in sense-suppressed YE10-6T_0 or YE6-16 plants, as expected (data not shown).

PAL Activity in YE10-6T_1 Plants

In contrast to the barely detectable activity in YE10-6T_0 plants (Elkind et al., 1990), the loss of the PAL-suppressed phenotype in YE10-6T_1 plants was associated with a higher extractable PAL enzyme activity than in wild-type plants (Fig. 3). PAL activity in the leaf tissue of overexpressing plants was up to 5 times that of wild type, and in stems it approached twice the wild-type level. The wild-type level of endogenous PAL protein in YE10-6T_1 plants, combined with the presence of the bean PAL2 protein, which is not found in the YE10-6T_0 primary transformant, strongly suggests that the overexpression of PAL activity in YE10-6T_1 plants is due to the activity of the protein encoded by the bean *PAL* transgene. These activity levels were reproducible in subsequent assays. However, there was not always a correlation between the level of PAL activity in leaf and stem tissue in individual T_1 transgenic plants; a plant with a high PAL activity in the leaf did not necessarily have a high activity in the stem (Fig. 3). Three individual YE10-6T_1 plants showing high PAL activity levels (at least 3 times wild type) were selfed, and their progeny (T_2) were germinated on kanamycin selection medium. All such plants had PAL activity levels similar to their parents, demonstrating the inheritance of the overexpression phenotype in at least some lines.

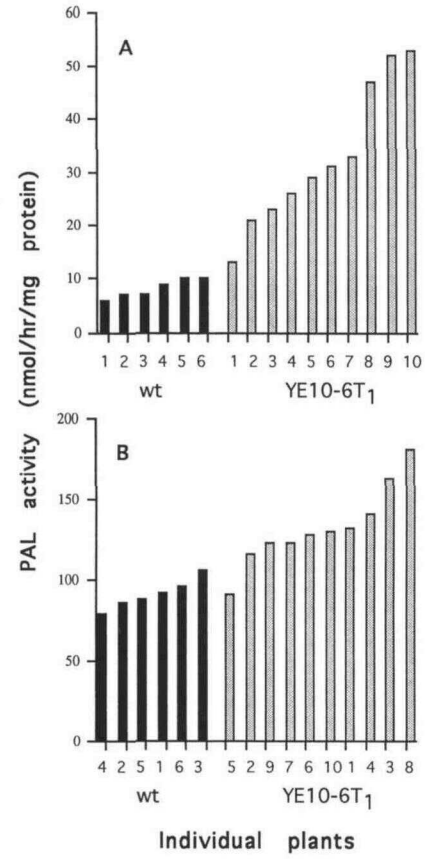

Figure 3. PAL activity in leaf (A) and stem tissue (B) of wild-type (wt, control) and transgenic YE10-6T_1 tobacco plants. Activity levels are shown for 6 individual wild-type plants and 10 individual transgenic plants. The same plants are shown in A and B, arranged from lowest to highest PAL activity. PAL activity in YE10-6T_0 plants was barely detectable (data not shown).

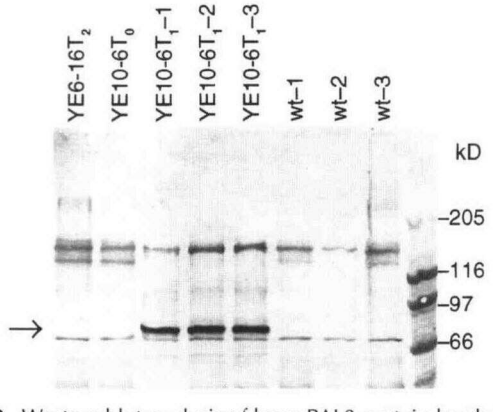

Figure 2. Western blot analysis of bean PAL2 protein levels in wild-type, sense-suppressed, and PAL-overexpressing plants. Leaf proteins (10 μg) were separated by SDS-PAGE and transferred to a membrane (Immobilon P, Millipore), and PAL2 protein (arrow) was detected using anti-bean PAL2 serum. Extracts from wild-type plants (wt, three individuals) served as a negative control. YE10-6T_1, Three individual T_1 YE10-6 plants; YE10-6T_0, YE10-6 sense-suppressed primary transformant; YE6-16T_2, T_2 YE6-16 sense-suppressed plant. Identical results were obtained with stem tissue (data not shown).

Relation between PAL Activity and Phenylpropanoid Accumulation

The YE10-6 T1 tobacco plants overexpressing PAL activity from an introduced bean PAL transgene provided a novel system for studying the metabolic consequences of

increasing flux into the phenylpropanoid pathway, both quantitatively and qualitatively. We therefore analyzed the transgenic plants for the extractable phenolic compounds in tobacco leaf tissue, the major ones being the hydroxycinnamic acid ester CGA and the flavonoid glycoside rutin (Snook et al., 1986). The levels of these two metabolites in sense-suppressed YE6-16 plants are reduced compared with wild-type levels, in parallel to the reduction in PAL activity (Bate et al., 1994).

HPLC analysis of phenolics from leaves of wild-type and YE10-6T_1 PAL-overexpressing plants indicated that increases in PAL activity above wild-type levels result in increased levels of CGA but not rutin (Fig. 4). The relationship between the PAL activity and CGA content of leaves approximated to linear, with an r^2 value of 0.507 by regression analysis (in contrast, the r^2 value for the relationship between PAL and rutin was 0.007). This observation, along with the demonstration that the reduction in CGA levels in sense-suppressed plants was also proportional to PAL activity, indicates that PAL is the key rate-limiting enzyme for CGA biosynthesis but that there are additional flux control points into the flavonoid branch pathway.

In addition to changes in CGA levels, comparison of the HPLC traces of phenolic compounds from wild-type and PAL-overexpressing YE10-6T_1 plants revealed increases in a limited number of other compounds, particularly two with RTs of 27 and 30 min, when PAL activity was above wild-type levels (Fig. 5). The levels of these compounds did not, however, appear to be directly proportional to PAL activity (data not shown). The peak of RT 27 min was purified by preparative HPLC. Treatment of the compound with β-glucosidase resulted in the disappearance of the 27-min peak on analytical HPLC and the appearance of a new peak of RT 31 min. Diode array UV analysis and co-chromatography with authentic standards indicated that the compound of RT 31 min was 4-coumaric acid; the compound of RT 27 min was therefore the β-glucoside of this phenolic acid.

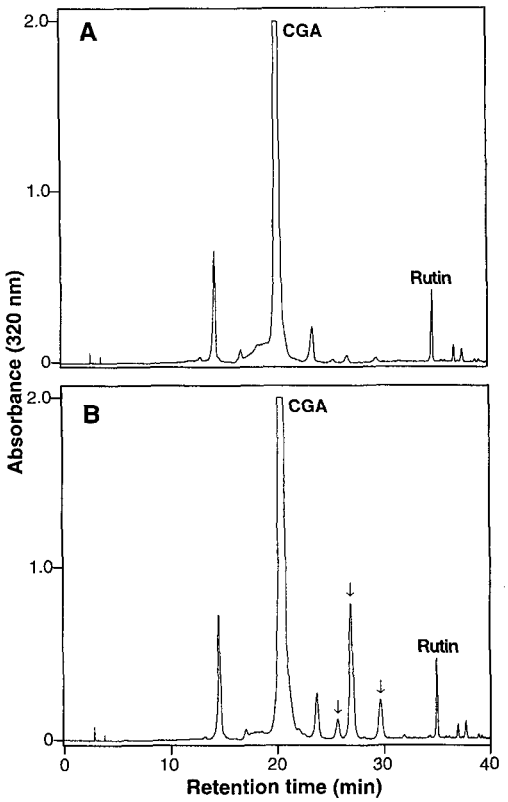

Figure 5. Typical HPLC profiles of phenolic extracts from leaves of a wild-type tobacco plant (A) and a YE10-6T_1 transgenic plant (B). The CGA and rutin peaks are marked, and arrows indicate other peaks that were consistently and markedly increased in the PAL-overexpressing plants. The large peak at RT 27 min was identified as the β-glucoside of 4-coumaric acid.

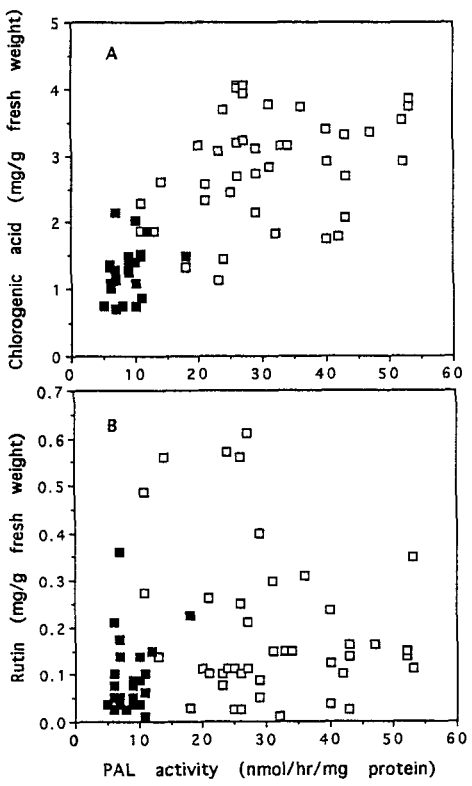

Figure 4. Relationship between PAL enzyme activity and levels of CGA (A) and rutin (B) in leaves of a range of wild-type (■) and YE10-6T_1 transgenic (□) tobacco plants.

Reduction of PAL activity to levels below 10% of wild type are required for a significant reduction in lignin levels in PAL-suppressed tobacco (Bate et al., 1994). To assess whether overexpression of PAL can impact lignin accumulation, we determined NDF and Klason lignin levels in stem samples of four wild-type and four YE10-6T_1 plants. NDF values as percentages of dry matter were 54.9 ± 2.0 for control plants and 58.4 ± 1.6 for PAL overexpressors, and corresponding Klason lignin values as percentages of dry matter were 9.5 ± 1.0 and 10.9 ± 1.2, respectively. A larger sample size would be necessary to confirm whether the small apparent increase in NDF and Klason lignin in PAL-overexpressing plants is significant.

DISCUSSION

YE10-6T_0 Sense-Suppressed Tobacco Change to PAL *Overexpression in a Single Generation*

The sense-suppressed state of the YE6-16 primary transformant, as characterized by severely reduced PAL enzyme activity and endogenous PAL transcript levels, is inherited over a number of generations, gradually decreasing in severity until an essentially wild-type PAL phenotype is obtained (Bate et al., 1994). However, in T_1 plants generated from seeds of YE10-6T_0, PAL enzyme activity is greater than that of wild type, and the level of endogenous *PAL* transcripts has returned to that of wild type. Both lines of plants demonstrate that the silencing phenomenon, although stable in one generation, is not permanent and, indeed, in the case of the YE10-6 plants, can be reversed in the next generation. The reason for the divergent fate of the two sense-suppressed lines is not known, although combinations of overexpression and sense suppression in independently transformed lines, and the reversal of expression between generations, have been observed in previous studies (de Carvalho et al., 1992; Hart et al., 1992; Boerjan et al., 1994). The mechanisms underlying such changes are not known.

Increased PAL Activity in YE10-6T_1 Plants Is the Result of Expression of the Bean *PAL2* Transgene

The high level of expression of the bean *PAL2* transcript in YE10-6T_1 plants results in the production of bean PAL protein (Fig. 2). However, in sense-suppressed YE10-6T_0 and YE6-16 plants, no bean PAL protein can be detected, even though *PAL2* transcripts are clearly present. The presence of transcripts but absence of the corresponding proteins may be due to rapid posttranslational degradation or to defects in polysome recruitment or translational elongation/initiation (Berry et al., 1988, 1990; Seymour et al., 1993; Vayda et al., 1995). It is not clear whether such posttranscriptional events are an associated part of the sense-suppression phenomenon or whether they reflect an aspect of the endogenous mechanisms controlling PAL expression.

The high level of bean PAL protein and the wild-type level of endogenous PAL in leaf tissue of YE10-6T_1 plants implies that the greater than wild-type levels of extractable PAL enzyme activity in these plants is due to the bean PAL protein. Since the native PAL enzyme exists as a tetramer, it is possible that heterotetramers between the bean and tobacco subunits are formed in these transgenic plants. The existence of PAL heterotetramers in bean has been suggested on the basis of the enzyme's broad chromatofocusing profile (Hamdan and Dixon, 1987), although this has yet to be confirmed by molecular techniques. It has recently been demonstrated that heterodimers between different subunits of the flavonoid pathway enzyme chalcone synthase are catalytically active, as are heterodimers of the closely related enzyme stilbene synthase (Tropf et al., 1995). Even if tobacco/bean PAL heterotetramers are catalytically inactive, functionally active bean homotetramers are presumably made, accounting for at least some of the increase in PAL activity in YE10-6T_1 plants.

PAL Activity and the Flux of Carbon in the Phenylpropanoid Pathway

Increasing the activity of PAL above that normally found in tobacco leads to a corresponding increase in CGA, the major phenolic compound in leaf tissue, to no increase in the flavonoid rutin in leaves, and to a small increase in lignin (approximately 1 mg/g fresh weight) in stems. Increased accumulation of other phenolic compounds, one of which was shown to be a conjugate of 4-coumaric acid, suggests that the flux into the CGA pathway had increased to a point at which enzymes other than PAL become rate-limiting. The pathway of CGA biosynthesis in tobacco probably follows the order 4-coumaric acid → 4-coumaroyl CoA → caffeoyl CoA → CGA. Alternatively, 4-coumaric acid may be hydroxylated prior to formation of the thioester. This would imply that 4-coumarate:CoA ligase or coumarate hydroxylase becomes limiting under conditions of PAL overexpression.

Production of phenolic conjugates is often observed when levels of phenylpropanoid pathway intermediates or end products are artificially increased. For example, addition of exogenous cinnamic acid to alfalfa cell cultures leads to the accumulation of two cinnamate conjugates (Orr et al., 1993). Infection of tobacco with tobacco mosaic virus leads to an accumulation of both salicylic acid and its conjugate, tentatively identified as *O*-β-D-glucosyl-salicylic acid (Enyedi et al., 1992), whereas the injection of salicylic acid into tobacco leaves results in most of it being converted to the conjugate (Hennig et al., 1993). Glycosylation of potentially reactive phenolic hydroxyl groups is a common detoxification mechanism in plants (Vickery, 1981).

It has been proposed that there may be channeling of substrate in the initial reactions of the phenylpropanoid pathway (Czichi and Kindl, 1977; Hrazdina and Jensen, 1992). If this is the case, bean PAL homotetramers and/or heterotetramers between bean and tobacco PAL subunits must be able to associate with the microsomal cinnamate 4-hydroxylase for increased PAL activity to result in accumulation of CGA. In this respect, it is not clear whether expression of different PAL genes in transgenic tobacco would give qualitatively similar patterns of phenylpropanoid accumulation.

The observation that plants overexpressing PAL accumulate CGA at greater than wild-type levels suggests that the Phe pool is not a limiting factor for CGA synthesis or that the enzymes involved in the biosynthesis of Phe are concomitantly induced. In tomato cells, elicitation leads to increased transcription of genes encoding PAL and enzymes of the shikimate pathway of aromatic amino acid biosynthesis, such as 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (Gorlach et al., 1995). We could find no difference in the levels of transcripts for this enzyme in leaves of wild-type or YE10-6T_1 plants (data not shown).

In metabolic control analyses (Kacser and Burns, 1973; Kacser and Porteous, 1987), the flux control coefficient of an enzyme is a measure of the response in flux to a change

in concentration of that enzyme, and the sum of the individual enzyme coefficients for a given flux must total 1. Although this theory seeks to describe flux in terms of control being shared by a number of enzymes, the present studies, in combination with our earlier work with PAL-suppressed plants (Bate et al., 1994), suggest that PAL has a control coefficient approaching unity for CGA synthesis over a wide range of activity levels. Accumulation of CGA might therefore be viewed as a "default pathway" for phenylpropanoid synthesis in tobacco leaves. Nevertheless, CGA is not simply a metabolic waste product, since it has antimicrobial activity and plants with reduced levels of CGA and other constitutive phenylpropanoid compounds show increased disease susceptibility (Maher et al., 1994), suggesting an important function for preformed phenolic compounds in quantitative resistance.

A few examples of overexpression of an early pathway enzyme in transgenic plants leading to an increase in downstream metabolites have been recently reported (Chappell et al., 1995; Harms et al., 1995; Schaller et al., 1995). In the case of increasing flux into the isoprenoid pathway by overexpression of hydroxymethylglutaryl-CoA reductase, levels of the intermediate cycloartenol increased along with increased levels of sterols, thus defining a potential downstream flux control point in the pathway (Chappell et al., 1995; Schaller et al., 1995). However, the regulatory architecture of a specific plant secondary metabolic pathway will not necessarily allow such a strategy to succeed, as seen from attempts to increase flux into alkaloid biosynthesis (Nessler, 1994). The PAL-overexpressing lines reported here enable us to address important questions relating to the control and functions of the plant phenylpropanoid pathway in vivo. For example, such plants will be a useful and novel system for investigating the nature and extent of metabolite channeling between PAL and cinnamate 4-hydroxylase and for further investigations into the role of pre-existing phenylpropanoid compounds in disease resistance.

ACKNOWLEDGMENTS

We thank Professor M. Okazaki (Shinshu University, Ueda, Japan) for providing the tobacco PAL cDNA, Dr P. Goldsbrough (Purdue University, West Lafayette, IN) for the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase cDNA, and Drs. Brandt Cassidy and Martin Guttenberger for critical reading of the manuscript.

Received June 26, 1996; accepted August 28, 1996.

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