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Altering Expression of Cinnamic Acid 4-Hydroxylase in Transgenic Plants Provides Evidence for a Feedback Loop at the Entry Point into the Phenylpropanoid Pathway[1]

Jack W. Blount, Kenneth L. Korth[2], Sameer A. Masoud[3], Susanne Rasmussen[4], Chris Lamb, and Richard A. Dixon*

Plant Biology Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, Oklahoma 73402 (J.W.B., K.L.K., S.A.M., S.R., R.A.D.); and Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, Scotland (C.L.)

Pharmacological evidence implicates trans-cinnamic acid as a feedback modulator of the expression and enzymatic activity of the first enzyme in the phenylpropanoid pathway, L-phenylalanine ammonia-lyase (PAL). To test this hypothesis independently of methods that utilize potentially non-specific inhibitors, we generated transgenic tobacco lines with altered activity levels of the second enzyme of the pathway, cinnamic acid 4-hydroxylase (C4H), by sense or antisense expression of an alfalfa C4H cDNA. PAL activity and levels of phenylpropanoid compounds were reduced in leaves and stems of plants in which C4H activity had been genetically down-regulated. However, C4H activity was not reduced in plants in which PAL activity had been down-regulated by gene silencing. In crosses between a tobacco line over-expressing PAL from a bean *PAL* transgene and a C4H antisense line, progeny populations harboring both the bean PAL sense and C4H antisense transgenes had significantly lower extractable PAL activity than progeny populations harboring the PAL transgene alone. Our data provide genetic evidence for a feedback loop at the entry point into the phenylpropanoid pathway that had previously been inferred from potentially artifactual pharmacological experiments.

The levels of enzymes of the phenylpropanoid pathway are tightly and coordinately regulated during responses of plants to changes in their environment (Hahlbrock et al., 1976; Bolwell et al., 1985; Ni et al., 1996). We have previously suggested that trans-cinnamic acid, the product of the first enzyme of the phenylpropanoid pathway, L-Phe ammonia-lyase (PAL), might act as a signal molecule for regulating the flux into the pathway. Thus, exogenously applied cinnamic acid inhibits PAL activity and transcription of *PAL* genes and induces the synthesis of a proteinaceous inhibitor of PAL (Lamb, 1979; Bolwell et al., 1986; Mavandad et al., 1990). In addition, cinnamic acid down-regulates transcription of the flavonoid pathway gene encoding chalcone synthase (CHS) (Loake et al., 1991). Furthermore, chemical inhibition of PAL activity in vivo can superinduce the PAL transcription rate, steady-state transcript level, and extractable enzyme activity, whereas chemical inhibition of cinnamic acid 4-hydroxylase (C4H) activity in vivo has been observed to result in reduced PAL expression (Durst, 1976; Bolwell et al., 1988; Knypl and Janas, 1990; Orr et al., 1993), which is consistent with the product of the PAL reaction being a negative regulator of the phenylpropanoid pathway. However, these essentially pharmacological studies have been criticized because inhibitors of PAL such as L-α-aminooxyacetic acid and L-α-aminooxy-β-phenylpropionic acid, inhibitors of the C4H cytochrome P450 such as tetcyclasis and anaerobiosis (which remove the co-substrate oxygen), and even cinnamic acid itself have potential unknown side effects. It is therefore important to re-address the PAL feedback regulation hypothesis using a method independent of the exogenous application of inhibitors.

PAL is regulated both transcriptionally and post-transcriptionally in response to a wide variety of developmental and environmental signals (Jones, 1984; Dixon and Paiva, 1995). Less is known of the mechanisms regulating expression of C4H in plants, although this cytochrome P450 enzyme has been characterized at the molecular level (Fahrendorf and Dixon, 1993; Mizutani et al., 1993; Teutsch et al., 1993). PAL and C4H activities and/or transcripts are co-induced in wounded Jerusalem artichoke and potato tubers (Durst, 1976; Lamb, 1977), illuminated or elicitor-treated parsley cell suspension cultures (Hahlbrock et al., 1976; Batz et al., 1998), fungally infected wheat and pepper plants (Maule and Ride, 1983; Saimmaime et al., 1991), and elicited alfalfa cell suspension cultures (Kessmann et al., 1990; Ni et al., 1996). It is not known whether expression of these two enzymes is coordinated primarily through environmental-stimulus-dependent transcriptional activation or through sensing of the level of pathway intermediates.

Altering C4H activity in vivo by molecular genetic approaches provides a means of studying the relation between PAL and C4H activities without the reliance on

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[2] Present address: Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701.

[3] Present address: Faculty of Agriculture, Mu'tah University, Karak, Jordan.

[4] Present address: Waksman Institute, Rutgers, State University of New Jersey, 190 Frelinghuysen Road, Piscataway, NJ 08854.

* Corresponding author; e-mail radixon@noble.org; fax 405–221–7380.

potentially artifactual pharmacological approaches. We examined the relation between PAL and C4H activities in transgenic plants modified in expression of C4H and/or PAL. Our results indicate that the level of PAL activity is approximately proportional to the level of C4H activity in plants with reduced C4H expression, whereas reduction in PAL activity does not lead to a significant reduction in C4H activity. Genetic down-regulation of C4H can also reduce PAL activity in plants over-expressing PAL from a bean PAL transgene. These results support the hypothesis that flux into the phenylpropanoid pathway is controlled, at least in part, via feedback regulation of PAL sensed through production of cinnamic acid.

MATERIALS AND METHODS

Vector Construction, Plant Transformation, and Growth of Transgenic Plants

Standard recombinant DNA techniques in *Escherichia coli* strains HB101 or DH5α were performed according to the method of Sambrook et al. (1989). The *Bam*HI/*Sal*I fragments containing the alfalfa C4H cDNA sequence from the yeast expression vector constructs W2A (sense) and S2A (antisense) (Fahrendorf and Dixon, 1993) were cloned in place of the *GUS* gene in the binary vector plasmid pBI121, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator, as shown in Figure 1A. Orientations of the insert were confirmed by restriction mapping, and the 5′ ends of each construct confirmed by sequencing from an oligonucleotide primer from the 35S promoter.

The above binary constructs and an empty vector control plasmid derived from pBI121 were used to transform *Agrobacterium tumefaciens* strain LBA4404 using a freeze-thaw technique (Chen et al., 1994). Leaf disc transformation of tobacco (*Nicotiana tabacum* L. cv Xanthi) was performed as described previously (Masoud et al., 1993) using kanamycin selection. Rooted plantlets were grown in magenta boxes, transferred to soil, and, after acclimation on a mist bench, grown in the greenhouse.

Transgenic tobacco plants either epigenetically co-suppressed for PAL expression or over-expressing PAL have been described elsewhere (Bate et al., 1994; Howles et al., 1996). All plants in individual experiments were grown together under identical environmental conditions and harvested together at the identical physiological stage.

DNA Isolation, PCR, and Southern-Blot Analysis

Tobacco genomic DNA was isolated according to the method of Junghans and Metzlaff (1990), and electrophoresed and blotted onto nylon membranes according to the method of Sambrook et al. (1989). Hybridization of blots with random-primer-labeled probes (Amersham, Arlington Heights, IL) was carried out as described previously (Church and Gilbert, 1984). For analysis of populations segregating *PAL* and *C4H* transgenes, genomic DNA was isolated (Edwards et al., 1991) and analyzed by PCR.

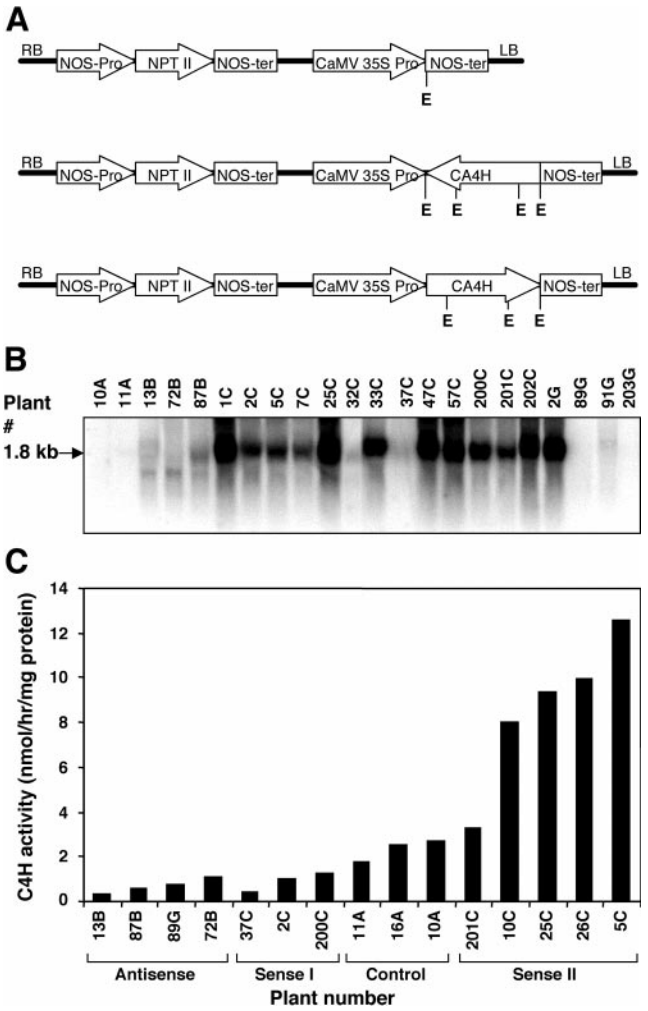

Figure 1. A, Binary vector constructs used to alter expression levels of C4H in transgenic plants. Top, Empty vector control construct; middle, antisense construct; bottom, construct for over-expression. RB and LB, T-DNA right and left borders, respectively; NOS-Pro, NOS promoter sequence; NPT II, neomycin phosphotransferase sequence; NOS-ter, NOS terminator sequence; CaMV 35S Pro, CaMV 35S promoter from positions −800 to +1; E, *Eco*RI cleavage sites; CA4H, alfalfa cinnamic acid 4-hydroxylase cDNA sequence (1,740 bp). B, RNA gel-blot analysis of C4H transcript levels in transgenic tobacco harboring alfalfa C4H gene constructs. Plants shown were transformed with the empty vector construct (A designations), antisense construct (B and G designations), or sense construct (C designations). C, Extractable activities of C4H in young leaves from independent transgenic tobacco lines containing C4H sense or antisense constructs, or empty vector controls. Plants transformed with the sense construct but showing below control levels of C4H activity are classified as operationally co-suppressed.

The presence of the *PAL* transgene in progeny plants was confirmed using standard conditions for PCR with the oligonucleotide primers 5′-GCCTTTGAATTGGCCAACATTGGTTCTGAG-3′ and 5′-TCTCCCTCTCAATTGACTTGGTAGAAAACC-3′; the *C4H* transgene was amplified using primers 5′-GTTCAACAGTATCGTTACGGGTGGGAG-3′ and 5′-GTCTTGGTGGTTCACTAGCTCAGCAATTCC-3′.

RNA Isolation and Northern-Blot Analysis

Tissue (200–400 mg) consisting of frozen leaf material from greenhouse or magenta box plants was ground in microcentrifuge tubes under liquid N_2, and RNA was extracted using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RNA was fractionated on denaturing 1.2% (w/v) agarose gels in MOPS buffer (Lehrach et al., 1977), and equal loading of gel lanes was confirmed by ethidium bromide staining and hybridization to an Arabidopsis 18S ribosomal RNA probe. The alfalfa C4H *Bam*HI/*Sal*I fragment (Fahrendorf and Dixon, 1993), the bean PAL *Bsp*HI-*Nar*I fragment (Howles et al., 1996), and ribosomal probes were labeled with ^{32}P using the random priming technique. Blots were washed at a final stringency of 0.5× SSC at 65°C, and then exposed to x-ray film or phosphorimager analysis.

Enzyme Assays

PAL (cytosolic) and C4H (microsomal) activities were assayed in extracts of leaf or mid-stem material (internodes 8–11) prepared as described previously (Edwards and Kessmann, 1992). Frozen tissues were ground in extraction buffer in a polytron (2 × 30 s on ice). The final microsomal pellet for the C4H assay was resuspended in 100 μL/2 g fresh weight (stems) or 100 μL/4 g fresh weight (leaves), and enzyme assays (using 20–100 μL of microsomal preparation) were performed in 15-mL tubes on a shaker at 180 rpm and 30°C for 30 min. Formation of 4-coumaric acid was determined by HPLC, and peak area values converted to nanomoles using a standard curve constructed with authentic 4-coumaric acid.

PAL was either assayed in the cytosolic supernatant from the microsomal preparation, as described previously (Legrand et al., 1976) with the modifications described in Howles et al. (1996), or, when measuring large numbers of segregating progeny, were determined spectrophotometrically (Edwards and Kessmann, 1992) with the following modifications. The enzyme was assayed using 50 μL of extract in a total volume of 1.0 mL, and the absorbance of 100-μL aliquots was determined as a function of time in a spectrophotometer (UV2401PC, Shimadzu, Columbia, MD) using a 16-sample microcell. Caffeic acid 3-*O*-methyltransferase (COMT) was extracted and assayed as described previously (Sewalt et al., 1997b). All enzyme assays were performed in duplicate. Protein concentrations were determined by the Bradford procedure (Bradford, 1976).

When measuring PAL activities in the segregating population of a PAL over-expressor crossed with a C4H antisense line (which had to be analyzed in three independent batches because of the large sample size), tissue samples from the same nine independent plants covering the full range of PAL activities were included as internal controls with each batch of new samples analyzed, and data were normalized to the average value for a wild-type plant at the particular developmental stage (mature, pre-flowering) at which the plants were analyzed (7.8 nmol h^{-1} mg^{-1} protein).

Analysis of Phenylpropanoid Compounds

Leaf or mid-stem samples (2 g fresh weight) were ground in liquid N_2. Extraction of soluble phenolic compounds and separation and quantification by HPLC were as described previously (Howles et al., 1996). Leaf residues previously extracted for soluble phenolics were washed three times with absolute ethanol, dried under N_2, re-weighed, and subjected to base hydrolysis in 10 mL of 1 N NaOH at room temperature for 18 h in 15-mL tubes. Sixty percent of the supernatant was removed after centrifugation at 8,000*g* for 15 min at 4°C, acidified to pH 1.0 to 2.0 with 2 N HCl, and extracted three times with an equal volume of ethyl acetate. The organic phases were combined, taken to dryness, and resuspended in HPLC-grade methanol to a final concentration equivalent to 200 mg dry weight of original leaf tissue per milliliter of methanol. Twenty microliters of solution was analyzed by HPLC as described previously (Howles et al., 1996), monitoring at 235, 270, and 310 nm.

Mid-stem samples (internodes 8–11) 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 neutral detergent fiber was oven-dried (55°C) and used for quantification of Klason lignin according to the method of Sewalt et al. (1997a).

RESULTS

Generation of Transgenic Tobacco Lines with Altered Expression of Phenylpropanoid Pathway Enzymes

We previously described the generation and analysis of transgenic tobacco plants with reduced PAL activity as a result of gene silencing from a bean *PAL* transgene (Elkind et al., 1990; Bate et al., 1994), and the recovery of such lines to yield PAL-over-expressing plants (Howles et al., 1996). When reduced to approximately 20% of wild-type levels, PAL becomes limiting for lignin accumulation in stems (Bate et al., 1994; Sewalt et al., 1997a). The same PAL-suppressed and PAL-over-expressing lines were used in the present work.

To generate transgenic tobacco plants with altered levels of C4H activity, we utilized binary vector constructs containing the complete alfalfa C4H cDNA sequence (Fahrendorf and Dixon, 1993) in both sense and antisense orientations, under control of the CaMV 35S promoter (Fig. 1A). We generated a total of 32 independent sense-transformed lines and 23 antisense-transformed lines based on PCR analysis using primers specific for the 35S promoter. Southern-blot analysis and kanamycin-resistance segregation tests of T_1 progeny plants revealed that most transformants contained one or two transgene inserts (data not shown). Further details of these plants have been published elsewhere (Sewalt et al., 1997a).

Total RNA from leaves of PCR-positive primary transformants (data not shown) and selfed T_1 progeny (Fig. 1B) was subjected to RNA-blot analysis. No signal was seen in leaves of empty vector control lines at the high stringency used for probing the blots. Several of the C4H sense-

transformed plants expressed very high levels of alfalfa C4H transcripts driven by the constitutive 35S promoter, whereas others had quite low C4H transcript levels (Fig. 1B). The levels of C4H antisense transcripts in the antisense lines (except in line 2G) were lower than the levels of C4H sense transcripts in the over-expressing sense transformants, as revealed by northern-blot analysis using a double-stranded alfalfa C4H probe. Gel-blot analysis revealed a diffuse hybridizing band at the size of the alfalfa C4H transcript plus an additional band of lower molecular size in all antisense transformants (Fig. 1B). The levels of transgene expression were stably inherited in T_1 progeny plants.

Extractable C4H activity was measured in leaf and stem tissue from a range of control and C4H sense/antisense transformed lines. The SD in C4H activity in a population of 15 control, untransformed plants was ±21% of the mean (data not shown). Figure 1C shows C4H activities in young leaves of 15 selected control and C4H antisense and sense transformants, including many used for subsequent analysis as described below. The assays were performed in parallel on leaf material at the identical developmental stage. Several plants transformed with the antisense construct exhibited strong reduction in C4H activity compared with control plants, with the lowest activity (15% of the average control value) in line 13B. Plants transformed with the sense construct fell into two classes on the basis of C4H activity. Over-expressors had elevated activity up to 5-fold the average wild-type value, whereas activity in other plants (such as line 37C) was significantly reduced below control levels (Fig. 1C). We define these latter plants as operationally co-suppressed (gene-silenced), although further studies would be needed to confirm the molecular basis for the reduced C4H activity in some of the sense-transcript-expressing lines. However, these reductions were consistently observed in vegetatively propagated material over a period of more than 12 months and in T_1 progeny plants. Line 201C had the highest C4H activity in stem tissue (32.2 nmol h^{-1} mg^{-1} protein), approximately 2-fold higher than control values. C4H activity was much higher in stem tissue than in leaf tissue due to its involvement in lignin biosynthesis (Sewalt et al., 1997a).

Effects of Altered Expression of C4H on Accumulation of Phenylpropanoid Compounds in Leaves and Stems of Transgenic Tobacco

The levels of soluble and wall-bound phenolic compounds in leaf extracts of C4H transgenic plants (primary transformants) were determined by HPLC analysis, as shown in Figure 2, A through F. Down-regulation of C4H consistently resulted in lower levels of chlorogenic acid (CGA) (Fig. 2G). For example, the levels of CGA were reduced by approximately 50% in line 13B (antisense) (Fig. 2, B and G) and by 88% in line 200C (sense-suppressed) (Fig. 2G) compared with levels in the empty vector control line 10A. Levels of other soluble caffeic acid esters were affected in a similar manner. These changes were associated with approximately 80% and 50% reductions in C4H activity, respectively, in leaves of lines 13B and 200C compared with wild-type plants. In contrast, over-expression of C4H had variable effects on the levels of CGA in different transgenic lines. Thus, CGA increased by about 2-fold in line 201C (Fig. 2C), but did not increase in other lines that expressed alfalfa C4H transcripts and had elevated C4H activity. In this respect, it is interesting that line 25 had a C4H activity in leaves that was nearly three times that of line 201C (Fig. 1C), although the activity of C4H in stem tissue of line 201C was more than double that in line 25.

There were no major qualitative differences in either soluble or wall-bound phenolic profiles in leaves of the various transgenic lines, and levels of most wall-bound phenolics (except for an as-yet-unidentified compound of RT 45 min) were not drastically affected by modification of C4H expression (Fig. 2, A–F). We conclude that down-regulation of C4H results in reduced accumulation of CGA and other caffeic acid esters in the soluble fraction of leaf tissue, but that over-expression of C4H in leaves does not consistently result in increased accumulation of CGA.

Relation between C4H and PAL Activities in Leaf and Stem Tissue of Plants Transformed with Alfalfa C4H Constructs

The effects of down-regulation of C4H activity on phenylpropanoid levels were similar to those previously shown to be associated with reductions in PAL activity (Bate et al., 1994), which is consistent with the model predicting that decreased levels of C4H activity would reduce PAL activity levels due to feedback modulation by cinnamate. The results shown in Figure 3A indicate that there was indeed a positive linear relationship ($r^2 = 0.79$) between C4H and PAL activities in fully expanded leaves of transgenic plants that exhibited below wild-type C4H activity. A similar linear relationship ($r^2 = 0.87$) between PAL and C4H activities was observed with a sample of six independent transgenic plants; activities were measured in young leaves that were not yet fully expanded (data not shown).

In stem internodes of transgenic tobacco with varying levels of C4H activity as a result of transgene expression, there was a positive relation ($r^2 = 0.58$) between the extractable activities of C4H and PAL, as shown in Figure 3B. The SD from the mean for PAL activity in stem tissue of a population of control tobacco plants grown under identical conditions was ±26% ($n = 15$) (Sewalt et al., 1997a), and reduction in C4H activity led to decreases in PAL far in excess of this value. In contrast, although there appeared to be a very weak positive relationship ($r^2 = 0.33$) between C4H and the extractable activity of COMT, an enzyme of the monolignol branch of the phenylpropanoid pathway (Fig. 3C), the SD from the mean for COMT activity in tobacco stem tissue was shown to be ±22% ($n = 20$) (V.J.H. Sewalt and R.A. Dixon, unpublished results), and all values for the population of plants analyzed in Figure 3C fall within that range. We therefore conclude that down-regulation of C4H results in feedback reduction of extractable PAL activity levels but does not significantly affect levels of a lignin branch pathway enzyme.

The levels of free and total (free plus Glc esterified) cinnamic acid were measured in stem tissue extracts of

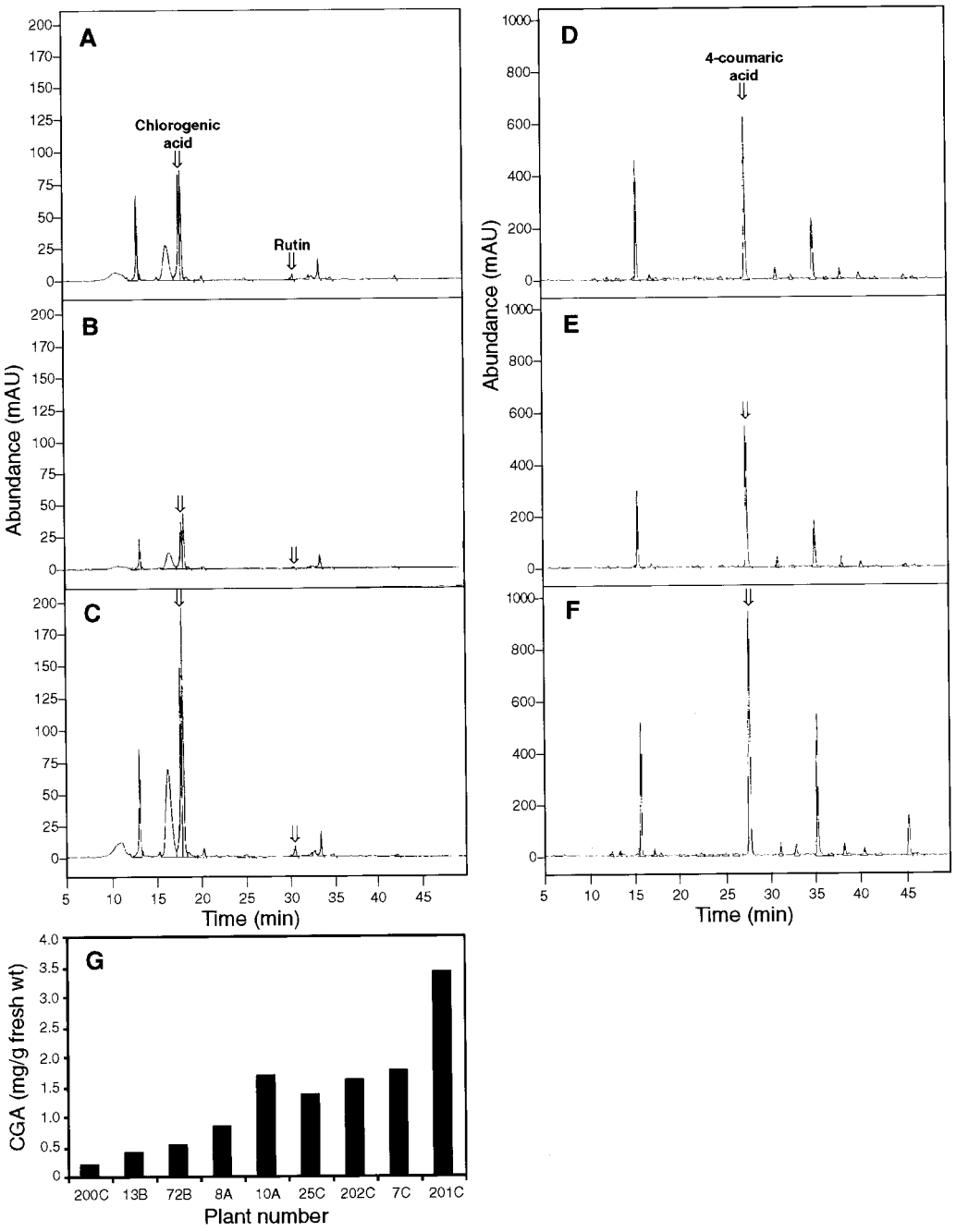

Figure 2. Effects of modification of C4H expression on soluble and wall-bound phenolic compounds in leaves of tobacco primary transformants. A through C, HPLC traces (measured at 310 nm) of soluble phenolic compounds in leaf extracts from tobacco plants transformed with an empty vector construct (A, line 8A), a C4H antisense construct (B, line 13B), and a C4H sense construct leading to over-expression (C, line 201C). D through F, HPLC traces (measured at 270 nm) of solubilized wall-bound phenolic compounds from lines 8C, 13B, and 201C, respectively. G, Levels of CGA in leaves from a range of independent transgenic plants. Major soluble phenolic compounds eluting at around 13 and 16.5 min were identified as esters of caffeic acid. mAU, Milliabsorbance unit.

C4H transgenic plants. The level of glucosylated cinnamic acid was insignificant compared with the free pool in these tissues. Cinnamic acid levels decreased linearly ($r^2 = 0.75$) with decreasing C4H activity (Fig. 4). At first sight, reducing C4H might be predicted to lead to an increase in cinnamate pool size. The fact that this does not occur is consistent with coupling between C4H and PAL activity levels mediated by sensing of the cinnamate pool(s), such that any increase in cinnamate is compensated for by a decrease in input through the PAL reaction.

Although it has been previously shown that cinnamic acid inhibits transcription of a reporter gene driven by the bean

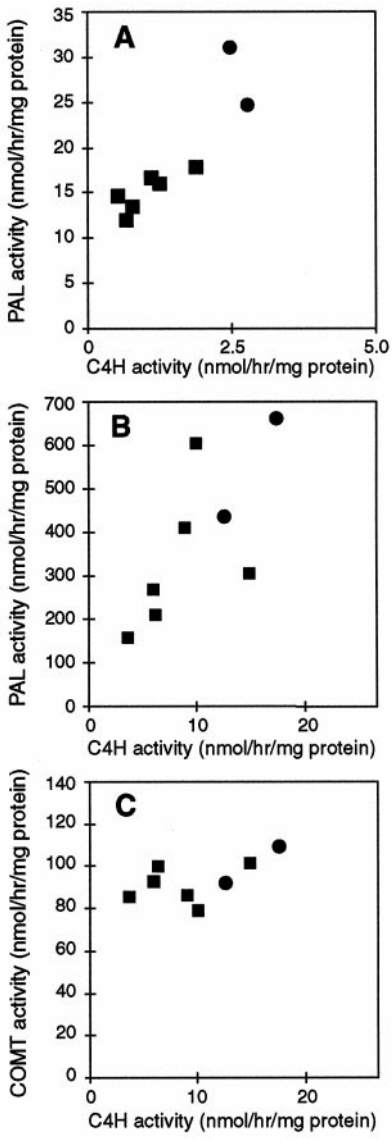

Figure 3. Relation between the activity of C4H and PAL or COMT activities in transgenic tobacco plants with C4H activity levels modified by expression of an alfalfa C4H transgene. A, C4H and PAL activities in young mature leaves; B, C4H and PAL activities in stems; C, C4H and COMT activities in stems. The lines analyzed were control (●) and C4H antisense or sense-suppressed plants (■).

chs-15 promoter in transient protoplast assays (Loake et al., 1991), we were unable to show any relationship between the enzymatic activities of CHS and C4H in the present studies. This is consistent with the observation that there is no relation between PAL and CHS activity in transgenic tobacco plants in which PAL activity has been reduced through epigenetic gene silencing (Bate et al., 1994).

Relation between PAL and C4H Activity Levels in Plants with Genetically Modified PAL Activity

Stem samples from a population of 11 independent lines comprising PAL-suppressed (seven lines) and wild type (four lines), in which PAL activity ranged from 127 to 1,050 nmol h^{-1} mg^{-1} protein, were analyzed to determine the effects of modulation of PAL activity on the level of C4H activity, the reciprocal experiment to those reported above. There was no effect of down-regulation of PAL on the level of C4H activity (r^2 = 0.14; data not shown). Thus, the regulatory loop involving the first two reactions of the phenylpropanoid pathway appears to operate in one direction only to down-regulate PAL activity levels.

Reduction of C4H Activity Can Reduce PAL Activity in Transgenic Tobacco Over-Expressing PAL

We were interested in determining whether the dominant negative effect of reduced C4H expression on PAL activity levels observed above would also operate to overcome the effects of genetically manipulated increases in PAL expression. Therefore, the effects of introduction of a C4H antisense transgene into a tobacco line that was overexpressing PAL from a bean PAL2 transgene were examined. C4H antisense line 13B (which contains three tightly linked copies of the alfalfa C4H transgene in the heterozygous state, and in which endogenous tobacco C4H transcripts are strongly down-regulated, as assessed by northern-blot analysis using a PCR-amplified tobacco C4H probe) was used as the male parent, and homozygous bean PAL over-expressor 10-6 T1 (Howles et al., 1996) as the female parent. The bean PAL transgene in 10-6 T1 is expressed from its own promoter boosted by additional CaMV 35S enhancer elements (Elkind et al., 1990). Progeny from the cross were initially analyzed by PCR to determine if PAL and C4H transgenes were present, and these results were subsequently confirmed by Southern-blot border analysis.

PAL activities were analyzed in leaf extracts from a population of 17 progeny plants that had segregated for the *PAL* transgene but did not have the *C4H* antisense transgene, as shown in Figure 5A (plants indicated by black bars). Except for one plant (no. 47), all values were between the average value for a population of parent PAL overexpressors and the wild-type average. In contrast, a very different distribution of PAL activity was observed in a

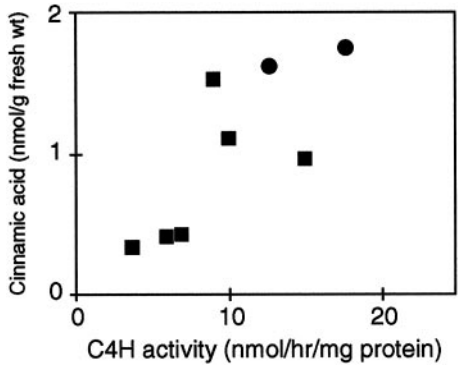

Figure 4. Levels of free cinnamic acid in stem tissue of transgenic plants with altered levels of C4H expression, plotted as a function of C4H activity. The lines analyzed were control (●) and C4H antisense or sense-suppressed plants (■).

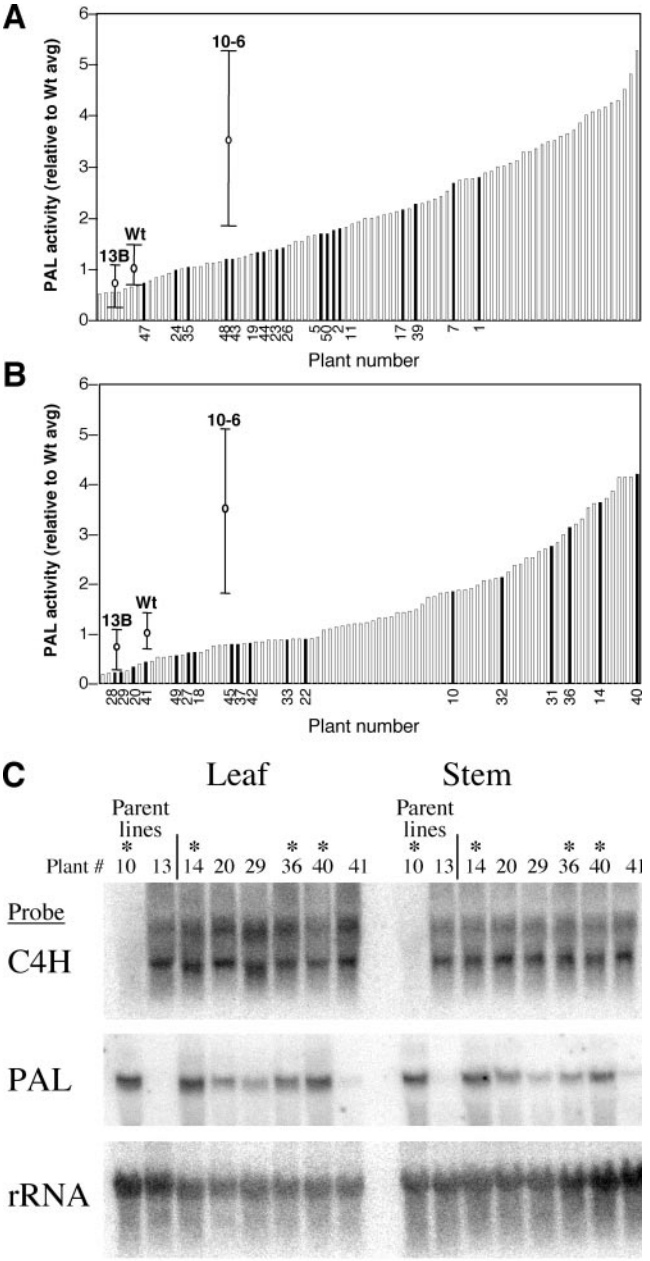

Figure 5. Segregation of PAL activity level in a cross between a PAL over-expressing line and a C4H antisense line. A, PAL activity in leaves from 76 F_1 progeny containing the bean *PAL2* gene but no *C4H* antisense gene. B, PAL activity in leaves from 78 F_1 progeny harboring both the bean *PAL2* gene and the alfalfa *C4H* antisense transgene. The plants designated by black bars and numbered in A and B were analyzed in a preliminary experiment, and the seeds of all the other plants were sown together at a later time. The three sets of error bars indicate the relative PAL activities, plus SD, in populations of independent progeny of the 10-6 PAL over-expressor line ($n = 25$), from vegetatively propagated 13B parent plants ($n = 12$), and from independent wild-type (Wt) plants ($n = 9$). Relative activities are given because of the need to analyze the individual plants in separate batches, each of which included a set of replicated samples to aid normalization (see "Materials and Methods"). The absolute values of PAL enzymatic activity in the two parent lines 10-6 and 13B in the second seed batch analyzed were 5.1 and 26.9 nmol h^{-1} mg^{-1} protein, respectively. C, Northern-blot analysis of alfalfa C4H and total PAL transcripts in parental lines (marked 10 and 13) and lines containing both *PAL* and *C4H* transgenes. Total RNA (15 μg/lane) was isolated from the parental lines and selected progeny from the 10-6/13B cross. Asterisks (*) indicate plant lines with high PAL activity. avg, Average.

population of 18 progeny plants that segregated for both transgenes, with 12 plants having PAL activity very near or significantly below the level of the average value for the C4H-suppressed parent (Fig. 5B, plants indicated by black bars). Two plants from this population had PAL activity higher than that of the average value for the parent PAL over-expressors; Southern border analysis of genomic DNA confirmed that the *C4H* and *PAL* transgenes in these plants had not undergone rearrangements (data not shown).

Alfalfa C4H antisense transcript levels, as determined by northern-blot analysis, were similar in leaves and stems of all progeny tested that harbored the *C4H* transgene (Fig. 5C). However, C4H enzymatic activity was high (0.57 and 0.96 nmol h^{-1} mg^{-1} protein, equal to and higher than, respectively, the value in the PAL over-expressing parent line) in the two C4H antisense-containing progeny plants with the highest PAL activity among the plants designated with the black bars in Figure 5B. C4H activity in the remainder of the progeny designated with black bars in Figure 5B varied from 0.21 nmol h^{-1} mg^{-1} protein (below that of the C4H-suppressed parent line 13B) to 0.55 nmol h^{-1} mg^{-1} protein, with 13 of the plants exhibiting values below 0.4 nmol h^{-1} mg^{-1}. All of these values are below the C4H activity of the 10-6 PAL over-expressing line. In only two of the 18 progeny (nos. 31 and 32) harboring both transgenes was low C4H activity (0.23 and 0.22 nmol h^{-1} mg^{-1} protein) associated with high PAL activity. These data indicate that the majority of the segregants with reduced C4H activity had a corresponding decrease in PAL activity.

We then examined, in a separate experiment (different sowing time of the seeds), a much larger population of plants from the cross between 13B and 10-6 T_1. We selected 62 plants harboring the bean PAL transgene alone (Fig. 5A, plants marked with white bars) and 64 plants with both the bean PAL transgene and the C4H antisense transgene (Fig. 5B, plants marked with white bars). There was considerable variation in PAL activity within each genotype, perhaps because of the now heterozygous state of the bean PAL transgene, and the difference in extractable PAL activity between the two populations was less striking than in the smaller sample of plants identified by black bars in Figure 5. However, the extractable PAL activities of the two populations (PAL transgene plus or minus C4H antisense) remained significantly different at $\alpha = 0.001$ (standard t test). Our data therefore indicate that C4H down-regulation can reduce extractable PAL activity even if PAL is being expressed from an artificially enhanced promoter.

Northern-blot analysis of bean PAL transcript levels in selected lines harboring the C4H antisense and bean *PAL2* transgenes indicated that selected lines with strongly re-

duced PAL activity (Fig. 5B) also exhibited reduced levels of bean PAL transcripts (Fig. 5C). Based on enzyme activities, relative transcript levels, and protein levels determined by western-blot analysis, approximately 70% of the total PAL activity could be accounted for by bean PAL expression in leaves of the 10-6 PAL over-expressing parent line (Howles et al., 1996). Thus, the reduction in PAL enzymatic activity in C4H down-regulated plants most likely occurs, at least in part, by effects on PAL gene expression. Furthermore, it is clear from Figure 5 that some progeny plants, such as numbers 20 and 29, although showing reduced bean PAL transcript levels compared with the parental line, exhibit PAL activity that is lower than wild-type plants that do not contain the bean PAL transgene. Thus, endogenous tobacco PAL gene expression must be strongly down-regulated in these plants. Direct determination of the mechanisms of PAL down-regulation in C4H antisense lines will require the development of molecular probes that discriminate between bean PAL and members of the two tobacco *PAL* gene families.

Leaf samples of plants harboring the C4H and PAL transgenes were analyzed for soluble phenolic compounds. There was a positive correlation between the levels of CGA and either PAL (r^2 = 0.613) or C4H (r^2 = 0.641) for the population of plants designated with black bars in Figure 5B. Plants 31 and 32, the only two with C4H activity around that of the 13B antisense parent coupled with high PAL activity, both exhibited reduced levels of CGA (33% and 44%, respectively) and of the flavonoid rutin (64% and 58%, respectively), suggesting that the reduced C4H activity in these plants could impact flux into downstream metabolites.

DISCUSSION

It has been suggested that flux through the phenylpropanoid pathway may be sensed via the endogenous cinnamic acid pool, with cinnamate causing inhibition of both PAL transcription and enzymatic activity when increased due to its greater production than utilization (Dixon and Lamb, 1990). Supporting evidence for this hypothesis comes from studies in which the chemical inhibition of PAL activity in vivo leads to superinduction of PAL transcription and enzymatic activity (Bolwell et al., 1988; Knypl and Janas, 1990), findings that have been interpreted as indicating that the reduction of the pool of endogenous cinnamic acid formed via the PAL reaction releases the enzyme system from feedback inhibition. Conversely, chemical inhibition of C4H leads to a reduction in PAL expression (Durst, 1976; Orr et al., 1993), which is again consistent with the feedback hypothesis. However, previous studies have failed to demonstrate increases in the endogenous cinnamic acid pool preceding reduction of PAL activity following its induction by elicitor in cell suspension cultures (Orr et al., 1993).

One prediction of the cinnamate regulation hypothesis is that manipulation of C4H activity in transgenic plants would lead to corresponding changes in PAL activity, a prediction confirmed by the present results demonstrating a direct relationship between PAL (driven by its endogenous promoter) and C4H activity in plants in which C4H levels have been reduced by transgene expression. Down-regulation of PAL can even be observed in plants exhibiting abnormally high PAL activities, as a result of expression of a bean *PAL* transgene, when crossed with a C4H antisense line. However, because of biological variation in progeny plants, it is necessary to analyze quite large populations of plants to confirm statistically significant effects.

We propose that reduced C4H activity puts a "brake" on flux into the phenylpropanoid pathway, and that this leads to feedback down-regulation of PAL. If this brake is sensed rapidly, there may be little experimentally measurable increase in the cinnamic acid pool, due to the corresponding decrease in cinnamic acid production through the PAL reaction and further metabolism of cinnamate. Indeed, the present experiments indicate that the cinnamic acid pool in plants with genetically modified C4H activity simply reflects the overall PAL and/or C4H activities, rather than the ratio between the two activities. That the total free cinnamic acid pool is not constant, as might be predicted if there were a threshold concentration that triggered down-regulation of PAL, suggests that there may be some compartmentation of cinnamate in planta, with a subfraction of the total pool involved in the feedback mechanism. This idea is consistent with previous demonstrations of the presence of both soluble and microsomally associated PAL in plants (Czichi and Kindl, 1977; Hrazdina and Jensen, 1992; Rasmussen and Dixon, 1999).

If C4H is to function as a component of a flux sensing mechanism, its own expression should be independent of changes of flux into the phenylpropanoid pathway. This idea is strongly supported by the lack of a reciprocal relationship between C4H and PAL activity in transgenic plants with genetically down-regulated PAL activity.

The mechanism by which PAL is feedback down-regulated may be complex, since endogenous application of cinnamic acid can inhibit the enzyme activity itself, inhibit PAL transcription, and induce the synthesis of a proteinaceous inactivator of PAL (Lamb, 1979; Bolwell et al., 1986; Mavandad et al., 1990). Any or all of these effects could occur in plants with down-regulated C4H. The 10-6 PAL over-expressing line contains the bean PAL transgene under control of its own promoter plus 35S promoter enhancer sequences. It should, therefore, respond to endogenous factors that affect PAL transcription, and transcriptional effects appear likely from the reduced levels of PAL transcripts in PAL over-expressing progeny that also harbor the C4H antisense transgene.

It is perhaps surprising that down-regulation of C4H expression should lead to reduced PAL activity but not to reduced CHS activity, because expression of CHS, like that of PAL, has been shown to be inhibited by cinnamic acid at the transcriptional level (Loake et al., 1991). A possible explanation is that transcription of CHS is stimulated by 4-coumaric acid (Loake et al., 1991), levels of which are, like those of cinnamic acid, proportional to C4H activity in tobacco (J.W. Blount, unpublished results).

Previous studies have shown that PAL is the rate-determining step for the synthesis of CGA in tobacco leaves, with a flux control coefficient (Kacser et al., 1995;

Stitt and Sonnewald, 1995) of 1.0 (Bate et al., 1994; Howles et al., 1996). Furthermore, when reduced below a threshold of 20% to 25% of wild-type activity, PAL becomes a rate-limiting step for lignin synthesis (Bate et al., 1994; Howles et al., 1996). Reduced C4H activity is also correlated with reduced levels of Klason lignin (gravimetrically determined insoluble lignin). For example, lignin levels were shown to be reduced to approximately 20% of the wild type in line 13B, which has an approximately 80% reduction in C4H activity. Indeed, there is a near linear relationship between the level of C4H activity and Klason lignin content for plants in which C4H activity is reduced below wild-type levels (Sewalt et al., 1997a).

At first sight, the present results appear to show, paradoxically, that C4H also has a very high flux control coefficient for synthesis of both CGA and lignin in tobacco, which is inconsistent with flux control theory (Kacser et al., 1995). This implies regulatory cross-talk between the PAL and C4H gene/enzyme systems, and this can be explained by the feedback mechanism proposed above. Increasing the activity of PAL (Howles et al., 1996; Sewalt et al., 1997a) or C4H (Sewalt et al., 1997a) does not lead to increased production of lignin, indicating the presence of downstream flux control points, probably at the level of the reactions specific for monolignol synthesis.

Because of the above feedback phenomenon, it is not easy to determine at what level reduction in C4H activity begins to lead to reduced phenylpropanoid synthesis. However, in the limited number of plants we generated from the PAL over-expressor/C4H antisense cross with reduced C4H but not reduced PAL, there was an apparent reduction in the levels of rutin and CGA compared with those in the PAL over-expressing parent, suggesting that modest reduction in C4H activity can of itself result in reduced phenolic levels.

In summary, the use of transgenic approaches to modify early phenylpropanoid pathway enzyme activity levels provides independent support for the model, previously suggested on the basis of essentially pharmacological experiments, that PAL is regulated by negative feedback control exerted at the level of C4H activity. This feedback may occur at both the transcriptional and post-transcriptional levels (Dixon and Lamb, 1990). Confirmation of the in vivo operation of a feedback regulatory loop for PAL expression sets the stage for future studies aimed at analyzing the molecular mechanisms involved.

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LITERATURE CITED

Bate NJ, Orr J, Ni W, Meroni A, Nadler-Hassar T, Doerner PW, Dixon RA, Lamb CJ, Elkind Y (1994) Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate determining step in natural product synthesis. Proc Natl Acad Sci USA **91:** 7608–7612

Batz O, Logemann E, Reinold, Hahlbrock K (1998) Extensive reprogramming of primary and secondary metabolism by fungal elicitor or infection in parsley cells. Biol Chem **379:** 1127–1135

Bolwell GP, Cramer CL, Lamb CJ, Schuch W, Dixon RA (1986) L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*: modulation of the levels of active enzyme by *trans*-cinnamic acid. Planta **169:** 97–107

Bolwell GP, Mavandad M, Miller DJ, Edwards KH, Schuch W, Dixon RA (1988) Inhibition of mRNA levels and activities by *trans*-cinnamic acid in elicitor-induced bean cells. Phytochemistry **27:** 2109–2117

Bolwell GP, Robbins MP, Dixon RA (1985) Metabolic changes in elicitor-treated bean cells: enzymic responses in relation to rapid changes in cell wall composition. Eur J Biochem **148:** 571–578

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Chen H, Nelson RS, Sherwood JL (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques **16:** 664–670

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem **162:** 156–159

Church GH, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 65–71

Czichi U, Kindl H (1977) Phenylalanine ammonia-lyase and cinnamic acid hydroxylases as assembled consecutive enzymes on microsomal membranes of cucumber cotyledons: cooperation and subcellular distribution. Planta **134:** 133–143

Dixon RA, Lamb CJ (1990) Regulation of secondary metabolism at the biochemical and genetic levels. *In* BV Charlwood, MJC Rhodes, eds, Secondary Products from Plant Tissue Culture. Clarendon Press, Oxford, pp 103–118

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell **7:** 1085–1097

Durst F (1976) The correlation of phenylalanine ammonia-lyase and cinnamic acid-hydroxylase activity changes in Jerusalem artichoke tuber tissues. Planta **132:** 221–227

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res **19:** 1359

Edwards R, Kessmann H (1992) Isoflavonoid phytoalexins and their biosynthetic enzymes. *In* SJ Gurr, MJ McPherson, DJ Bowles, eds, Molecular Plant Pathology: A Practical Approach. IRL Press, Oxford, pp 45–62

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ (1990) Abnormal plant development and down regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc Natl Acad Sci USA **87:** 9057–9061

Fahrendorf T, Dixon RA (1993) Stress responses in alfalfa XVIII (*Medicago sativa* L.): molecular cloning of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. Arch Biochem Biophys **305:** 509–515

Hahlbrock K, Knobloch KH, Kreuzaler F, Potts JRM, Wellmann E (1976) Coordinated induction and subsequent activity changes of two groups of metabolically interrelated enzymes. Eur J Biochem **61:** 199–206

Howles PA, Paiva NL, Sewalt VJH, Elkind NL, Bate Y, Lamb CJ, Dixon RA (1996) Over-expression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. Plant Physiol **112:** 1617–1624

Hrazdina G, Jensen RA (1992) Spatial organization of enzymes in plant metabolic pathways. Annu Rev Plant Physiol Plant Mol Biol **43:** 241–267

Jones DH (1984) Phenylalanine ammonia-lyase: regulation of its induction, and its role in plant development. Phytochemistry **23:** 1349–1359

Junghans H, Metzlaff M (1990) A simple and rapid method for the preparation of total plant DNA. Biotechniques **2:** 176

Kacser H, Burns JA, Fell DA (1995) The control of flux. Biochem Soc Trans **23:** 341–366

Kessmann H, Choudhary AD, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.): III. Induction of medicarpin and cytochrome P450 enzyme activities in elicitor-treated cell suspension cultures and protoplasts. Plant Cell Rep **9:** 38–41

Knypl JS, Janas KM (1990) Stimulated synthesis of phenylalanine ammonia-lyase in *Spirodela oligorrhiza* treated with 1-amino-2-phenylethylphosphonic acid. J Plant Physiol **136:** 750–753

Lamb CJ (1977) Phenylalanine ammonia-lyase and cinnamic acid 4-hydroxylase: characterisation of the concomitant changes in enzyme activities in illuminated potato tuber discs. Planta **135:** 169–175

Lamb CJ (1979) Regulation of enzyme levels in phenylpropanoid biosynthesis: characterization of the modulation by light and pathway intermediates. Arch Biochem Biophys **192:** 311–317

Legrand M, Fritig B, Hirth L (1976) Enzymes of the phenylpropanoid pathway and the necrotic reaction of hypersensitive tobacco to tobacco mosaic virus. Phytochemistry **15:** 1353–1359

Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16:** 4743–4751

Loake G, Choudhary AD, Harrison MJ, Mavandad M, Lamb CJ, Dixon RA (1991) Phenylpropanoid pathway intermediates regulate transient expression of a chalcone synthase gene promoter in electroporated protoplasts. Plant Cell **3:** 829–840

Masoud SA, Johnson LB, White FF, Reeck GR (1993) Expression of a cysteine proteinase inhibitor (oryzacystatin-I) in transgenic tobacco plants. Plant Mol Biol **21:** 655–663

Maule AJ, Ride JP (1983) Cinnamate 4-hydroxylase and hydroxycinnamate:CoA ligase in wheat leaves infected with *Botrytis cinerea*. Phytochemistry **22:** 1113–1116

Mavandad M, Edwards R, Liang X, Lamb CJ, Dixon RA (1990) Effects of *trans*-cinnamic acid on expression of the bean phenylalanine ammonia-lyase gene family. Plant Physiol **94:** 671–680

Mizutani M, Ward E, DiMaio J, Ohta D, Ryals J, Sato R (1993) Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 (P450C4H) possessing cinnamate 4-hydroxylase activity. Biochem Biophys Res Commun **190:** 875–880

Ni W, Fahrendorf T, Ballance GM, Lamb CJ, Dixon RA (1996) Stress responses in alfalfa (*Medicago sativa* L.): XX. Transcriptional activation of phenylpropanoid pathway genes in elicitor-treated cell suspension cultures. Plant Mol Biol **30:** 427–438

Orr JD, Edwards R, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.): XIV. Changes in the levels of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor treated cell suspension cultures. Plant Physiol **101:** 847–856

Rasmussen S, Dixon RA (1999) Metabolic channeling at the entry point into the phenylpropanoid pathway: coupling of phenylalanine ammonia-lyase and cinnamate 4-hydroxylase on microsomal membranes of tobacco (*Nicotiana tabacum* cv. Xanthi). Plant Cell **11:** 1537–1552

Saimmaime I, Coulomb C, Coulomb PJ (1991) *Trans*-cinnamate 4-hydroxylase activity in host-parasite interaction: *Capsicum annuum-Phytophthora capsici*. Plant Physiol Biochem **29:** 481–487

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sewalt VJH, Ni W, Blount JW, Jung HG, Howles PA, Masoud SA, Lamb C, Dixon RA (1997a) Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol **115:** 41–50

Sewalt VJH, Ni W, Jung H, Dixon RA (1997b) Lignin impact on fiber degradation: increased enzymatic digestibility of genetically engineered tobacco (*Nicotiana tabacum*) stems reduced in lignin content. J Agric Food Chem **45:** 1977–1983

Stitt M, Sonnewald U (1995) Regulation of metabolism in transgenic plants. Annu Rev Plant Physiol Plant Mol Biol **46:** 341–368

Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier JM, Jeltsch JM, Durst F, Werck-Reichhart D (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc Natl Acad Sci USA **90:** 4102–4106

Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci **74:** 3583–3597