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Transgene-Mediated and Elicitor-Induced Perturbation of Metabolic Channeling at the Entry Point into the Phenylpropanoid Pathway

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^{3}H–L-Phenylalanine is incorporated into a range of phenylpropanoid compounds when fed to tobacco cell cultures. A significant proportion of ^{3}H–*trans*-cinnamic acid formed from ^{3}H–L-phenylalanine did not equilibrate with exogenous *trans*-cinnamic acid and therefore may be rapidly channeled through the cinnamate 4-hydroxylase (C4H) reaction to 4-coumaric acid. Such compartmentalization of *trans*-cinnamic acid was not observed after elicitation or in cell cultures constitutively expressing a bean phenylalanine ammonia–lyase (*PAL*) transgene. Channeling between PAL and C4H was confirmed in vitro in isolated microsomes from tobacco stems or cell suspension cultures. This channeling was strongly reduced in microsomes from stems or cell cultures of transgenic *PAL*-overexpressing plants or after elicitation of wild-type cell cultures. Protein gel blot analysis showed that tobacco PAL1 and bean PAL were localized in both soluble and microsomal fractions, whereas tobacco PAL2 was found only in the soluble fraction. We propose that metabolic channeling of *trans*-cinnamic acid requires the close association of specific forms of PAL with C4H on microsomal membranes.

INTRODUCTION

The phenylpropanoid pathway is involved in the biosynthesis of a wide variety of natural products from plants. Many of these products have important functions in plant development and in interactions of the plant with its environment (Hahlbrock and Grisebach, 1979; Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). Many studies have addressed the transcriptional regulation of genes encoding enzymes of the phenylpropanoid pathway and subsequent changes in extractable enzyme activities in response to developmental and environmental cues (Cramer et al., 1985; Lawton and Lamb, 1987; Hahlbrock and Scheel, 1989). Far less attention has been paid to how the cell regulates flux into different end products of the pathway once all of the enzymatic machinery is assembled.

The first committed step in the biosynthesis of phenylpropanoid compounds is the conversion of L-phenylalanine (Phe) to *trans*-cinnamic acid by L-Phe ammonia–lyase (PAL; EC 4.3.1.5; Figure 1). PAL is a tetrameric enzyme whose subunits are encoded by a multigene family in most species that have been studied (Cramer et al., 1989; Nagai et al., 1994; Wanner et al., 1995; Fukasawa-Akada et al., 1996). *PAL* genes are transcriptionally activated after microbial infection or treatment of plant cells with microbial elicitors (Edwards et al., 1985; Lawton and Lamb, 1987). The second step in the phenylpropanoid pathway, the hydroxylation of *trans*-cinnamic acid to 4-coumaric acid, is catalyzed by a cytochrome P450 monooxygenase, cinnamic acid 4-hydroxylase (C4H; EC 1.14.13.11; Russell and Conn, 1967; Fahrendorf and Dixon, 1993; Teutsch et al., 1993). C4H is induced by light, elicitors, and wounding (Fahrendorf and Dixon, 1993; Buell and Somerville, 1995; Batard et al., 1997; Bell-Lelong et al., 1997), and its induction often is closely coordinated with PAL induction (Mizutani et al., 1997).

Enzymes of complex metabolic pathways may be present in the cell in arrays of consecutive, physically associated enzymes that are assembled on membranes or other physical structures (Subramanian et al., 1973; Margna and Margna, 1978; Cutler and Conn, 1981; Cutler et al., 1981; Srere, 1987). Such enzyme organization can result in the channeling of pathway intermediates without their release into general metabolic pools (Stafford, 1981; Hrazdina, 1992). Cytochrome P450 enzymes, such as C4H, are anchored to the external surface of the endoplasmic reticulum (Chapple, 1998), but PAL has been regarded generally as an operationally soluble enzyme. However, studies performed with microsomes isolated from potato (Czichi and Kindl, 1975) and cucumber cotyledons (Hrazdina and Wagner, 1985)

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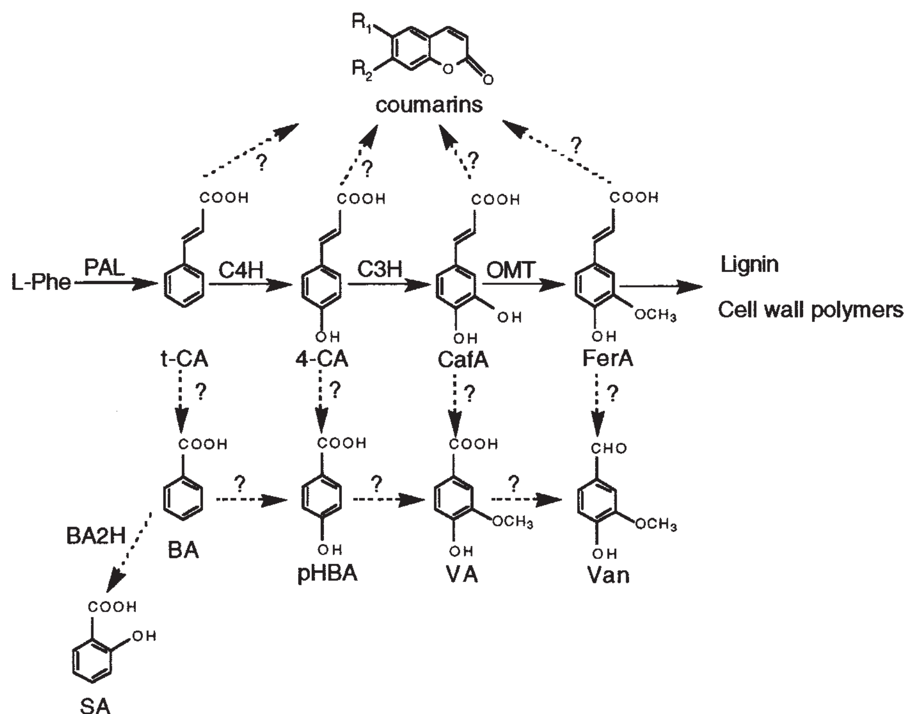

Figure 1. Biosynthesis of Phenolic Compounds in Tobacco.

L-Phe is deaminated by PAL to yield *trans*-cinnamic acid (t-CA), which is converted by C4H to 4-coumaric acid (4-CA). 4-Coumaric acid can be hydroxylated by coumarate 3-hydroxylase (C3H) to caffeic acid (CafA), which is acted upon by an *O*-methyltransferase (OMT) to yield ferulic acid (FerA). Salicylic acid (SA) and pHBA are formed via chain shortening of *trans*-cinnamic acid and 4-coumaric acid, respectively, although the specific mechanisms are not clear. Similarly, the exact biosynthetic origins of vanillin (Van), vanillic acid (VA), and coumarins (such as scopoletin in tobacco) have not been determined definitively; these pathways are indicated by dotted lines and question marks. Benzoic acid (BA) is most probably the precursor of salicylic acid, which is formed by the action of a benzoic acid 2-hydroxylase (BA2H). R_1 and R_2 are commonly substituted positions in coumarins. In scopoletin, $R_1 = OCH_3$ and $R_2 = OH$.

have suggested that PAL and C4H activities are colocalized on membranes of the endoplasmic reticulum. Furthermore, *trans*-cinnamic acid formed endogenously via the PAL reaction is a better substrate for C4H than externally added *trans*-cinnamic acid in in vitro assays. This finding has been interpreted as evidence for channeling of *trans*-cinnamic acid in the conversion of Phe to 4-coumaric acid (Czichi and Kindl, 1975, 1977; Hrazdina and Wagner, 1985; Hrazdina and Jensen, 1992).

To gain further insight into the phenomenon of metabolic channeling, it is necessary to develop a model system that allows for comparison of metabolic compartmentalization/channeling in vivo and in vitro. This system must be amenable to manipulation to increase or decrease various components of the potential channel, and molecular information on the gene products involved in channeling must be available. Tobacco represents such a system. We have generated a series of transgenic tobacco plants containing a heterologous bean *PAL2* gene, in which the levels of extractable PAL activity are increased or decreased when compared with wild-type plants (Elkind et al., 1990; Howles et al., 1996; Sewalt et al., 1997). These plants allow assessment of the effects of quantitative and qualitative changes in a component of a potential metabolic channel on the operation of that channel. Furthermore, it is now possible to design probes to distinguish between different members of the *PAL* gene family and therefore to address which forms of PAL may be associated with channeling.

Here, we present evidence, from in vivo and in vitro labeling experiments with ^{3}H–L-Phe and ^{14}C–*trans*-cinnamic acid, for metabolic channeling that involves coupling of PAL and C4H in tobacco stem tissue and cell suspension cultures. We demonstrate that specific forms of PAL are associated with tobacco microsomes and that microsomal association of a heterologous PAL enzyme in transgenic plants can perturb channeling. We also show that metabolic channeling is no longer measurable after activation of the phenylpropanoid pathway by elicitation.

RESULTS

Metabolic Compartmentalization of *trans*-Cinnamic Acid in Tobacco Cell Suspension Cultures

If a biosynthetic intermediate is channeled, it will not equilibrate freely with an externally added intermediate, and the endogenously formed and externally supplied compound will exist in the cell in different pools. In vivo labeling experiments can show the existence of different metabolic pools, although these pools may or may not result from metabolic channeling. Conversely, demonstration of a single pool of a metabolite that fully equilibrates with the externally added compound would constitute evidence against channeling. Therefore, we used tobacco cell suspension cultures to confirm the existence of more than one pool of *trans*-cinnamic acid in tobacco as a precursor to further studies on channeling between PAL and C4H that make use of transgenic plants and in vitro assays. When fed ^{3}H–L-Phe, such cultures accumulate a range of labeled phenolic compounds, including the conjugates of *trans*-cinnamic acid, 4-coumaric acid, caffeic acid, and ferulic acid, as shown in Figure 2A. These compounds are derived from Phe via *trans*-cinnamic acid and 4-coumaric acid, as shown in Figure 1.

If suspension-cultured tobacco cells possessed only a single pool of *trans*-cinnamic acid with which ^{3}H–*trans*-cinnamic acid formed by the PAL reaction from ^{3}H-Phe readily equilibrated, simultaneous feeding of ^{3}H-Phe and unlabeled *trans*-cinnamic acid would result in a lowering of the specific activity of tritium in the various phenylpropanoid compounds derived from *trans*-cinnamic acid when compared with the values obtained in the absence of unlabeled *trans*-cinnamic acid. At the end of the labeling period, the specific activity of reisolated *trans*-cinnamic acid would represent the minimum specific activity of the ^{3}H–*trans*-cinnamic acid precursor pool, assuming no metabolic compartmentalization, and the final specific activity of the various phenylpropanoid compounds would be equal to or lower than this value, depending on the sizes of their pools and the pools of the intermediates of the pathway during the period of labeling. Thus, assuming complete equilibration of endogenously formed ^{3}H–*trans*-cinnamic acid with endogenous unlabeled internal pools and externally applied unlabeled *trans*-cinnamic acid, the ratio of the specific activity of a particular product to the specific activity of the total extractable *trans*-cinnamic acid should be 1.0 or $<$1.0.

Figure 3 shows the results from a series of such isotope dilution experiments with tobacco cell cultures. In the absence of externally applied unlabeled *trans*-cinnamic acid, the product/precursor specific activity ratio was between 0.2 and 0.99 for 4-coumaric acid, caffeic acid, and ferulic acid (Figure 3A). Small but not statistically significant increases in the product/precursor specific activity ratios were observed for all compounds after simultaneous feeding of 10^{-5} M unlabeled *trans*-cinnamic acid and ^{3}H-Phe. However,

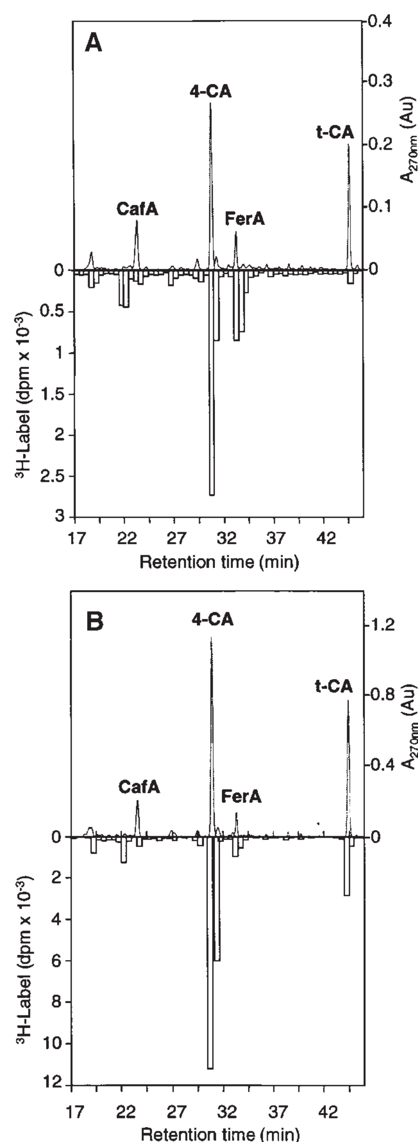

Figure 2. HPLC Traces Showing Incorporation of Tritium from ^{3}H–L-Phe into Phenylpropanoid Compounds in Tobacco Cell Suspension Cultures.

(A) Wild-type tobacco cell suspension cultures.

(B) Tobacco cell suspension cultures overexpressing PAL from the bean *PAL2* gene (OX434).

Cultures were treated simultaneously with 10^{-4} M ^{3}H–L-Phe and unlabeled *trans*-cinnamic acid (10^{-4} M). Cells were harvested 24 hr after the addition of the precursors. Extracts of soluble phenolics were treated with esterase and separated by HPLC. Fractions were collected, and radioactivity in *trans*-cinnamic acid (t-CA), 4-coumaric acid (4-CA), caffeic acid (CafA), and ferulic acid (FerA) was determined by liquid scintillation counting. Au, absorbance units.

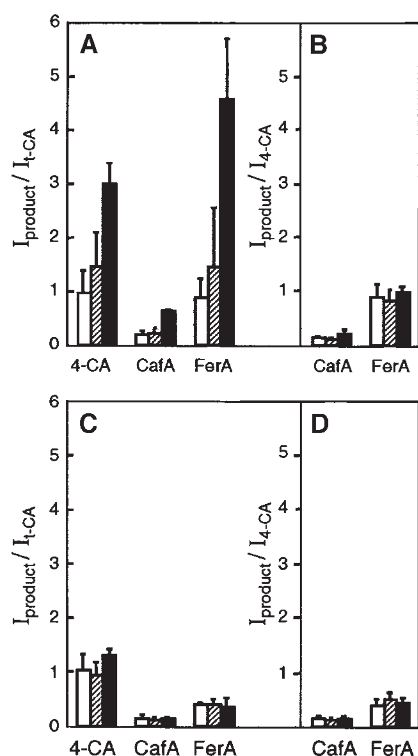

Figure 3. In Vivo Labeling Experiments Demonstrate Metabolic Compartmentalization of *trans*-Cinnamic Acid in Tobacco Cell Suspension Cultures.

Results are expressed as the ratio of incorporation of ^{3}H–L-Phe (determined as specific activity) in a particular phenolic product ($I_{product}$) to that in reextracted *trans*-cinnamic acid ($I_{t\text{-}CA}$) or 4-coumaric acid ($I_{4\text{-}CA}$). Effects of the addition of unlabeled *trans*-cinnamic acid and 4-coumaric acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ and to $I_{4\text{-}CA}$ are shown. Results shown are the means ±SD of two independent experiments. Compounds were extracted 24 hr after treatment.

(A) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in wild-type tobacco cell suspension cultures.

(B) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in wild-type tobacco cell suspension cultures.

(C) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in *PAL*-overexpressing tobacco cell suspension cultures.

(D) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in *PAL*-overexpressing tobacco cell suspension cultures.

Open bars, no unlabeled *trans*-cinnamic acid or 4-coumaric acid; hatched bars, 10^{-5} M *trans*-cinnamic acid or 4-coumaric acid; filled bars, 10^{-4} M *trans*-cinnamic acid or 4-coumaric acid. Extracts of soluble phenolics were treated with esterase and separated by HPLC. Fractions were collected and radioactivity determined by liquid scintillation counting. CafA, caffeic acid; FerA, ferulic acid; 4-CA, 4-coumaric acid.

much larger increases (up to fivefold for ferulic acid) were obtained after feeding of 10^{-4} M unlabeled *trans*-cinnamic acid, due to a very large decrease in the specific activity of *trans*-cinnamic acid, whereas the specific activity of reisolated 4-coumaric acid remained in the same range as that of the downstream products. In a similar experiment in which unlabeled 4-coumaric acid was fed simultaneously with ^{3}H-Phe, no increase in the ratios of product/4-coumaric acid precursor specific activities were observed (Figure 3B). The same results were obtained when incorporation of the coumarin scopoletin or ^{3}H into *p*-hydroxybenzoic acid (pHBA) was measured in the presence or absence of unlabeled *trans*-cinnamic acid or 4-coumaric acid (data not shown). These results indicate that externally added unlabeled *trans*-cinnamic acid does not dilute isotope incorporation from ^{3}H-Phe into phenylpropanoid compounds in tobacco cell cultures, whereas externally added unlabeled 4-coumaric acid does, suggesting a specific metabolic compartmentalization of *trans*-cinnamic acid. This does not directly prove channeling or necessarily indicate the presence of more than one endogenous pool of *trans*-cinnamic acid in the absence of an externally fed compound.

PAL-overexpressing tobacco lines, resulting from constitutive expression of a bean *PAL2* gene, overproduce phenylpropanoid compounds (Howles et al., 1996), whereas underexpressing lines, resulting from epigenetic gene silencing (Elkind et al., 1990; Bate et al., 1994), have reduced levels of phenylpropanoid compounds. We initiated callus and then cell suspension cultures from *PAL*-overexpressing transgenic tobacco; constitutive PAL activities in the cell suspension cultures were at least threefold higher than in comparable cultures derived from wild-type plants. We then repeated the above-mentioned in vivo labeling experiments to determine whether upregulation of PAL activity affects metabolic compartmentalization of *trans*-cinnamic acid.

Figure 2B shows the pattern of phenylpropanoid compounds and the incorporation of tritium from ^{3}H–L-Phe into these compounds in *PAL*-overexpressing cultures. As shown in Figures 3C and 3D, expression of the bean *PAL2* transgene results in a loss of metabolic compartmentalization of *trans*-cinnamic acid. Thus, in contrast to the situation with wild-type cultures (Figure 3A), the addition of unlabeled *trans*-cinnamic acid had no effect on the ratios of product/precursor specific activities for the various phenylpropanoid compounds analyzed (Figure 3C). This indicates full equilibration of endogenously formed ^{3}H–*trans*-cinnamic acid with the pool of externally added compound in *PAL*-overexpressing cultures. As occurs with the wild-type cultures (Figure 3B), unlabeled 4-coumaric acid had no effect on the product/precursor specific activity ratio in *PAL*-overexpressing cultures (Figure 3D). Again, the same results were obtained when incorporation of ^{3}H into scopoletin or pHBA was measured in the presence or absence of unlabeled *trans*-cinnamic acid or 4-coumaric acid (data not shown).

In *PAL*-overexpressing transgenic tobacco plants, constitutive PAL activity is increased without a corresponding in-

crease in constitutive C4H activity (Howles et al., 1999). In contrast, elicitation induces a coordinated increase in both activities (Howles et al., 1999). We repeated the above-mentioned in vivo labeling experiments with suspension cultures of wild-type tobacco that had been exposed to a yeast elicitor for 6 hr before the addition of an isotopic label. The addition of unlabeled *trans*-cinnamic acid had little effect on the ratios of product/precursor specific activities in the various phenylpropanoid compounds analyzed (Figure 4C), in contrast to the situation with unelicited cultures shown in Figure 4A. This indicates full equilibration of endogenously formed ^{3}H–*trans*-cinnamic acid with the pool of externally added compound in elicited cultures. As occurs with the unelicited cultures (Figure 4B), unlabeled 4-coumaric acid had no effect on the product/precursor specific activity ratio in elicited cultures (Figure 4D). These results indicate that coordinated activation of the phenylpropanoid pathway as a result of elicitation results in a loss of the metabolic compartmentalization of *trans*-cinnamic acid observed in unelicited cultures.

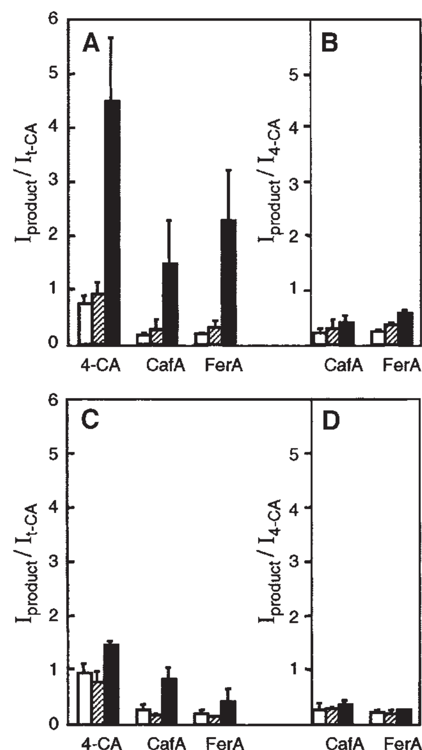

Figure 4. Effects of Elicitation on Metabolic Compartmentalization of *trans*-Cinnamic Acid in Tobacco Cell Suspension Cultures.

Results show the ratio of the $I_{product}$ to $I_{t\text{-}CA}$ and to $I_{4\text{-}CA}$ (see legend to Figure 3) in a particular phenolic compound in cells with or without elicitation. The experiment was conducted in the same way as described in the legend to Figure 3, except that 6 hr before the addition of ^{3}H–L-Phe and unlabeled *trans*-cinnamic acid or 4-coumaric acid, cells were treated either with water or the yeast elicitor, and cells were incubated with the precursors for only 6 hr before being harvested.
(A) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in unelicited tobacco cell suspension cultures.
(B) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in unelicited tobacco cell suspension cultures.
(C) Effects of the addition of unlabeled *trans*-cinnamic acid on the ratio of $I_{product}$ to $I_{t\text{-}CA}$ in elicited tobacco cell suspension cultures.
(D) Effects of the addition of unlabeled 4-coumaric acid on the ratio of $I_{product}$ to $I_{4\text{-}CA}$ in elicited tobacco cell suspension cultures.
Open bars, no unlabeled cinnamic acids; hatched bars, 10^{-5} M *trans*-cinnamic acid or 4-coumaric acid; filled bars, 10^{-4} M *trans*-cinnamic acid or 4-coumaric acid. Results shown are the mean ±SD of two independent experiments. CafA, caffeic acid; FerA, ferulic acid; 4-CA, 4-coumaric acid.

Changes in Phenolic Metabolites as a Result of Elicitation or *PAL* Overexpression

To determine whether metabolic compartmentalization of *trans*-cinnamic acid is associated with accumulation of specific products of phenylpropanoid biosynthesis, we compared the patterns and amounts of phenolic metabolites resolved by HPLC from extracts of wild-type and *PAL*-overexpressing cell suspension cultures by using the cell samples analyzed in Figures 2 and 3. As shown in Table 1, there was a twofold increase in *trans*-cinnamic acid levels in *PAL*-overexpressing cultures compared with wild-type cell cultures, presumably due to the higher PAL activity in these cells. *PAL* overexpression led to a very strong accumulation (up to 10-fold) of a compound that we tentatively identified as a vanillin derivative on the basis of its UV spectrum and chromatographic properties (λ_{max} of 204, 228, 276, and 304 nm; λ_{min} of 216 and 246 nm; 97% spectral identity to vanillin; HPLC retention time of 31.4 min, eluting at 24% acetonitrile) as well as a twofold increase in scopoletin. No significant difference in the accumulation of 4-coumaric acid, ferulic acid, pHBA, salicylic acid, or vanillic acid could be observed in the two different cell lines, although caffeic acid levels were reduced by 50% in the *PAL*-overexpressing cells. The addition of *trans*-cinnamic acid resulted in higher levels of all of the above-mentioned metabolites, except for salicylic acid, in *PAL*-overexpressing cells when compared with wild-type cells. This indicates that the perturbation of channeling as a result of *PAL* overexpression leads to increased metabolism of exogenous *trans*-cinnamic acid.

Surprisingly, elicitation of wild-type cultures resulted in lower levels of most of the extractable phenolics, as shown in Table 2. The addition of 10^{-4} M *trans*-cinnamic acid led to threefold and 5.5-fold increases in 4-coumaric acid levels and 20-fold and 10-fold increases in *trans*-cinnamic acid levels in unelicited and elicited cells, respectively, but the total amounts of 4-coumaric acid, caffeic acid, ferulic acid, and scopoletin were ~50% lower in the elicited cells. This may be due to more rapid metabolism of these compounds and

Table 1. Effects of *PAL* Overexpression on Phenolic Compounds in Tobacco Cell Suspension Cultures

Unlabeled Precursor	Plant Line[a]	t-CA[b]	4-CA[b]	CafA[b]	FerA[b]	pHBA[b]	Scop[b]	SA[b]	VA[b]	Van-D[b]
None	WT	5	93	103	42	10	92	110	159	19
None	OX	12	109	52	56	8	185	73	182	209
10^{-4} M t-CA	WT	60	227	136	64	18	123	137	164	90
10^{-4} M t-CA	OX	170	618	201	219	18	478	139	263	426

[a] Soluble phenolic compounds were isolated from wild-type (WT) and *PAL*-overexpressing (OX) tobacco cell suspension cultures 24 hr after feeding with ^{3}H–L-Phe (10^{-4} M) in the presence or absence of unlabeled *trans*-cinnamic acid (t-CA).
[b] Amounts of phenolic compounds, quantified after enzymatic hydrolysis, are given as nanomoles per gram fresh weight. The compounds are *trans*-cinnamic acid, 4-coumaric acid (4-CA), caffeic acid (CafA), ferulic acid (FerA), pHBA, scopoletin (Scop), salicylic acid (SA), vanillic acid (VA), and vanillin derivative (Van-D). The experiments were conducted as described in the legend to Figure 3, and a duplicate experiment gave essentially the same results.

their subsequent deposition in the insoluble cell wall fraction. No significant amounts of these compounds could be detected in the culture medium or in the soluble cell wall fraction (data not shown).

Channeling of *trans*-Cinnamic Acid in Tobacco Stem Microsomes

Plant microsomes can convert L-Phe to 4-coumaric acid, which is not metabolized further to any significant degree (Czichi and Kindl, 1975, 1977; Hrazdina and Wagner, 1985). The value of the coupling factor, as defined by Czichi and Kindl (1977) and Kindl (1979), is a rigorous criterion for the coupling of enzymatic reactions in vitro. The coupling factor is used to compare the ratios of tritium to carbon-14 in the product (in this case, 4-coumaric acid) with the ratios of tritium to carbon-14 in the intermediate (in this case, *trans*-cinnamic acid) in dual labeling experiments. It thereby gives an estimation of the level of coupling between consecutive enzymes. The use of the ^{3}H-labeled primary substrate (L-Phe) and ^{14}C-labeled secondary substrate (*trans*-cinnamic acid) by enzymes located on isolated microsomes will result in the formation of labeled 4-coumaric acid with a specific tritium/carbon-14 ratio. If the value of the ratio in the product is higher than the respective value in the reisolated intermediate (i.e., a coupling factor >1.0), the bound or channeled form of the intermediate does not freely exchange with the external pool of the intermediate, indicating channeling between L-Phe and 4-coumaric acid.

We initially chose tobacco stem tissue for in vitro channeling assays because of the high levels of PAL and C4H activities in this tissue associated with lignification (Sewalt et al., 1997). We first confirmed the presence of PAL activity in microsomes isolated from tobacco stem tissue. The total activity of PAL in washed microsomes isolated from wild-type tobacco plants amounted to 5 to 10% of the total activity of PAL in the soluble enzyme fraction. As shown in Figure 5A, the specific activities of microsomal PAL were between 30 and 40% of the specific activities of soluble PAL. Microsomal PAL is relatively tightly associated with the microsomes and is not simply cytoplasmic contamination, as shown below.

Microsomal fractions were incubated simultaneously with ^{3}H–L-Phe and ^{14}C–*trans*-cinnamic acid, and the ratios of tritium to carbon-14 in reisolated *trans*-cinnamic acid and in 4-coumaric acid were compared. Coupling factors were between 5 and 11 in microsomes from wild-type plants,

Table 2. Effects of Elicitation on Phenolic Compounds in Tobacco Cell Suspension Cultures

Unlabeled Precursor	Treatment[a]	t-CA[b]	4-CA[b]	CafA[b]	FerA[b]	pHBA[b]	Scop[b]	SA[b]	VA[b]	Van-D[b]
None	Con	4	64	47	81	6	284	114	413	76
None	Eli	7	15	24	41	3	105	107	404	116
10^{-4} M t-CA	Con	86	195	44	101	7	372	97	476	118
10^{-4} M t-CA	Eli	69	79	23	48	7	164	130	357	95

[a] Soluble phenolic compounds were isolated from unelicited (Con) and elicited (Eli) tobacco cell suspension cultures after feeding with ^{3}H–L-Phe (10^{-4} M) in the presence or absence of unlabeled *trans*-cinnamic acid (t-CA).
[b] Amounts of phenolic compounds, quantified after enzymatic hydrolysis, are given as nanomoles per gram fresh weight. The compounds are *trans*-cinnamic acid, 4-coumaric acid (4-CA), caffeic acid (CafA), ferulic acid (FerA), pHBA, scopoletin (Scop), salicylic acid (SA), vanillic acid (VA), and vanillin derivative (Van-D). The experiments were conducted as described in the legend to Figure 4, with the compounds being isolated 12 hr after elicitation. A duplicate experiment gave essentially the same results.

suggesting significant channeling between PAL and C4H (Figure 5B). When soluble PAL from the cytoplasmic supernatant was added to the channeling assays, the ratio of tritium to carbon-14 in 4-coumaric acid remained almost the same, but the tritium/carbon-14 ratio in the reisolated *trans*-cinnamic acid intermediate was strongly increased, resulting in reduced coupling factors, as shown in Figure 5B. This is presumably due to an excess of ^{3}H–*trans*-cinnamic acid formed from ^{3}H–L-Phe, which is not converted to ^{3}H–4-coumaric acid because of the preference of the channeled system for ^{3}H–*trans*-cinnamic acid originating via the microsomal PAL reaction.

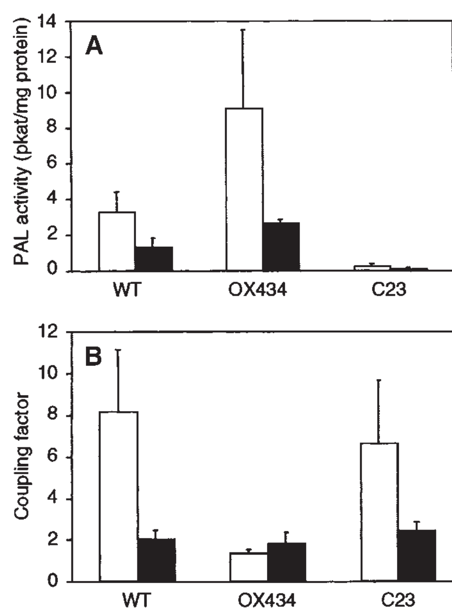

Figure 5. Specific Activities of PAL in the Soluble and Microsomal Protein Fractions and Metabolic Channeling between PAL and C4H in Microsomes from Stems of Wild-Type and Transgenic Tobacco Plants.

Plants were wild-type (WT), *PAL* overexpressers (OX434), or gene-silenced *PAL* underexpressers (C23).

(A) Open bars, soluble (105,000*g* supernatant) PAL activity; filled bars, microsomal (105,000*g* pellet) PAL activity. Total activities of microsomal PAL were 5 to 10% of total soluble PAL activity. pkat, picokatals.

(B) Washed and resuspended microsomes (250 to 500 μg of protein per assay) were incubated simultaneously with 500 nmol ^{3}H–L-Phe (5 nCi/nmol) and 30 nmol ^{14}C–*trans*-cinnamic acid (2 nCi/nmol). The coupling factor is defined as the tritium/carbon-14 ratio in the product, 4-coumaric acid, divided by the tritium/carbon-14 ratio in the reisolated intermediate, *trans*-cinnamic acid. Open bars, coupling factors in microsomes alone; filled bars, coupling factors in microsomes with 100 μL of the soluble PAL fraction added.

Data shown are the means ±SD of six (wild-type) or three (OX434 and C23) independent preparations.

We next studied the effects of transgenic modification of PAL activity on channeling in tobacco stem microsomes. *PAL*-overexpressing plants (OX434) had a two- to threefold higher specific activity of PAL, and PAL sense-suppressed plants (C23) a four- to sixfold lower specific activity of PAL in both soluble and microsomal fractions, when compared with wild-type plants (Figure 5A). Total microsomal activities were five to 10% of that detected in the soluble fraction, as was the case for wild-type tobacco plants. In microsomes from *PAL*-suppressed plants, coupling factors were in the same range as previously demonstrated for wild-type plants (Figure 5B). In contrast, the coupling factors for microsomes from *PAL*-overexpressing plants were reduced significantly to values of 1 to 2. These values are similar to those obtained on adding soluble PAL to wild-type microsomes. These results indicate that either higher PAL activity associated with the microsomes from *PAL*-overexpressing plants or the presence of a heterologous PAL species in the microsomes (see below) leads to a reduction in the extent of coupling between microsomal PAL and C4H.

Channeling between PAL and C4H in Microsomes from Tobacco Cell Suspension Cultures

If the changes in apparent metabolic compartmentalization of *trans*-cinnamic acid observed in in vivo labeling experiments indeed reflect changes in channeling rather than some in vivo labeling artifact, channeling should be demonstrable in microsomes from unelicited tobacco cell cultures but not in microsomes from elicited or *PAL*-overexpressing cultures. Such correlations between in vivo and in vitro labeling results would strengthen the validity and physiological relevance of the in vitro channeling assays.

The specific activity of soluble PAL in unelicited cell suspension cultures overexpressing bean *PAL2* was approximately sixfold higher than in wild-type cell suspension cultures, as shown by comparing time zero values in Figures 6A and 6C. The coupling factor between PAL and C4H in microsomes from unelicited wild-type cells (0 hr after elicitation) was ~12 (Figure 6B) and is similar to that observed in microsomes from stem tissue (Figure 5B). In agreement with the previous results obtained using stem tissue, microsomes from cell cultures overexpressing bean *PAL* had much reduced coupling factors, with a coupling factor below 1.0 and therefore indicative of no channeling (Figure 6D).

We next determined the effects of elicitation on the in vitro microsomal coupling of PAL and C4H in the wild-type and *PAL*-overexpressing cell suspension cultures. Treatment with crude yeast elicitor led to an approximately three- to fivefold increase in soluble PAL activity in wild-type cultures by 12 hr after elicitation (Figure 6A) but to a much smaller fold increase in the *PAL*-overexpressing cultures (Figure 6C). Microsomal PAL activity in elicited wild-type cells reached its maximum, an approximately fivefold increase, 9 hr after elicitation, as shown in Figure 6B. However, the coupling factors

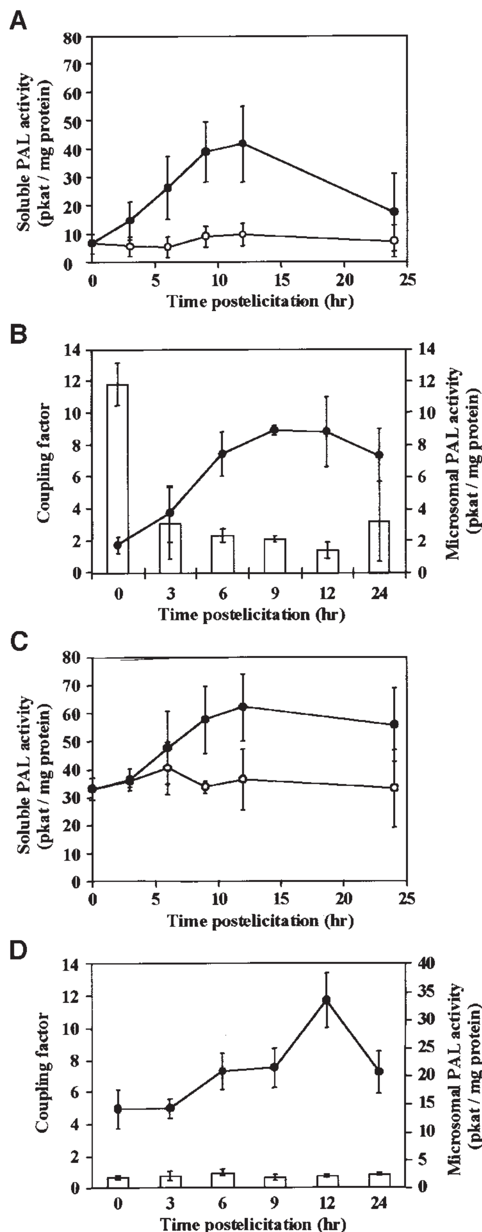

Figure 6. Specific Activities of PAL in the Soluble and Microsomal Protein Fractions, and Metabolic Channeling between PAL and C4H in Microsomes of Elicited Wild-Type and *PAL*-Overexpressing Tobacco Cell Suspension Cultures.

Isolation of microsomal proteins and determination of coupling factors were conducted as described in the legend to Figure 5. Wild-type and *PAL*-overexpressing cultures were treated with the yeast elicitor or an equal volume of water (unelicited) and then harvested at the times shown. pkat, picokatals.

(A) Soluble PAL activity in the unelicited (open circles) and elicited (filled circles) wild-type line.

(B) Microsomal PAL activity (filled circles) and coupling factors (open bars) in the elicited wild-type line.

(C) Soluble PAL activity in the unelicited (open circles) and elicited (filled circles) *PAL*-overexpressing lines.

(D) Microsomal PAL activity (filled circles) and coupling factors (open bars) in the elicited *PAL*-overexpressing lines.

Data shown are the means ±SD of four (wild-type) or three (*PAL*-overexpressing) independent experiments.

in microsomes from elicited wild-type cells decreased significantly by 3 hr after elicitation, to reach minimum values of ~1.4 at 12 hr after elicitation. These results demonstrate that the elicitation of PAL activity results in the perturbation of coupling between PAL and C4H. Elicitation did not strongly enhance microsomal PAL activity in *PAL*-overexpressing tobacco cells, and it had no effect on the coupling factors in microsomes from these cells, with the values being in the same range as in unelicited cells—near or below 1.0 (Figure 6D). Therefore, these data indicate a correlation between metabolic compartmentalization of *trans*-cinnamic acid in vivo (Figures 3 and 4) and coupling of the PAL and C4H reactions on microsomes in vitro.

The Nature of PAL Associated with Microsomal Membranes

The above-mentioned results suggest that PAL, or one or more specific forms of PAL, may be closely associated with C4H as an enzyme complex on microsomal membranes. Cytochrome P450s, such as C4H, are associated with the endoplasmic reticulum by way of a membrane anchor region at the N terminus (Chapple, 1998). The catalytic region of cytochrome P450 is in the cytoplasm. To obtain more information on the association of PAL with microsomal membranes, we fractionated tobacco stem homogenates by ultracentrifugation into soluble and microsomal fractions. Both fractions were assayed for PAL and C4H activity with or without a 20-min pretreatment with trypsin. The results in Table 3 indicate that as expected, no PAL activity could be detected in the soluble fraction after trypsin treatment. In contrast, 19% of the PAL activity in the microsomal fraction was retained. All microsomal C4H activity was destroyed by trypsin treatment. Because microsomal PAL activity is partially protected from the hydrolytic action of trypsin, a small fraction probably is located in the lumen of the endoplasmic reticulum or, alternatively, inside membrane vesicles formed during the preparation of microsomes. In this respect, the addition of the detergent Triton X-100 to the microsomal preparation before trypsin treatment resulted in an almost complete loss of microsomal PAL activity. The twofold increase in soluble PAL activity after the addition of detergent may indicate activation of the enzyme.

Tobacco PAL is encoded by two gene families, each of which contains two very closely related members. The two

Table 3. Effects of Trypsin on Soluble and Microsomal PAL Activity and Microsomal C4H Activity in Extracts from Wild-Type Tobacco Plants

	Total Activity (picokatals)[a]					
	Control		0.1 % Triton X-100		0.6 % Triton X-100	
Enzyme	−Trypsin	+Trypsin	−Trypsin	+Trypsin	−Trypsin	+Trypsin
Soluble PAL	392	0	696	0	668	0
Microsomal PAL	21	4	21	1	21	1
C4H	3	0	3	0	0	0

[a] Two grams of plant material was homogenized in 4 mL of extraction buffer and ultracentrifuged; the supernatant was assayed directly, and the pellet was resuspended in 250 μL of homogenization buffer. Fractions were incubated for 30 min at 4°C in the presence or absence of Triton X-100 before trypsin treatment (+Trypsin) or further incubation in the absence of trypsin (−Trypsin). Enzyme assays were started by the addition of substrate.

families are represented by single-copy *PAL* genes in the two progenitor species, *Nicotiana sylvestris* and *N. tomentosiformis* (Fukasawa-Akada et al., 1996). In this study, PAL1 refers to the product encoded by the *PAL* gene of family I, described by Fukasawa-Akada et al. (1996), and PAL2 refers to the product of the *PAL* gene from family II, as reported by Nagai et al. (1994). To determine whether specific forms of PAL are associated with tobacco microsomes, we raised antibodies against synthetic peptide sequences specific for tobacco PAL1, tobacco PAL2, and bean PAL2 (Howles et al., 1996). Soluble and microsomal proteins were isolated from wild-type, *PAL*-overexpressing (from the bean *PAL2* transgene), and *PAL*-suppressed tobacco stem tissues, subjected to SDS-PAGE, and probed with these antibodies. As shown in Figure 7A, antibodies generated against tobacco PAL1 cross-reacted with a protein band of just under 86 kD (the size of the native PAL subunit), which was present in both the soluble and microsomal protein fractions. By contrast, antibodies specific for tobacco PAL2 cross-reacted with a more diffuse protein band of similar size found only in the soluble protein fraction (Figure 7B). Note that the levels of tobacco PAL proteins were similar in wild-type, *PAL*-overexpressing, and *PAL*-suppressed plants, despite the different levels of PAL activity in these plants; this may be due to post-translational regulation.

Probing the protein gel blots with antibodies specific for bean PAL2 protein (Figure 7C) revealed that a very high amount of bean PAL2 protein is present in the soluble protein fraction, but a substantial amount of the heterologous gene product also is located in the microsomes of *PAL*-overexpressing plants. The ~50-kD band in the microsomal fraction cross-reacting with all three antibodies is most probably the result of nonspecific binding. Essentially identical results were obtained by protein gel blot analysis of soluble and microsomal fractions from cell suspension cultures of wild-type and *PAL*-overexpressing tobacco (data not shown). Furthermore, we could not detect any significant change in the subcellular localization of the various PAL forms after elicitation of the cell cultures (data not shown).

DISCUSSION

Relation of Metabolic Compartmentalization of *trans*-Cinnamic Acid in Vivo to Metabolic Channeling as Revealed by Coupling Assays in Vitro

As discussed by Srere (1987), objections have been raised to the idea that isotopic data can unequivocally indicate more than one pool of a particular intermediate. The major argument is that multiple pools arise due to heterogeneity of the cell population. De-differentiated tobacco cell suspension cultures are highly homogeneous with respect to cell type but somewhat heterogeneous with respect to cell size and degree of aggregation of small cell clusters. We cannot rule out totally the presence of different cell types containing different pools of intermediates that are differentially accessible to an endogenously formed or externally applied intermediate. However, this is unlikely because the isotopic labeling results are reproducible between independent cell culture batches, the isotope dilution experiments reveal at least two pools of *trans*-cinnamic acid but only a single pool of 4-coumaric acid, and more importantly, the effects on compartmentalization of transgenic or elicitor-mediated perturbation of the pathway can be reproduced by using in vitro channeling assays. The difference in isotope dilution behavior of *trans*-cinnamic acid compared with 4-coumaric acid potentially could result from uptake of *trans*-cinnamic acid into an inaccessible compartment, such as the cell wall, whereas all of the 4-coumaric acid may be taken into the cytoplasm. This appears not to be the case because if *trans*-cinnamic acid were taken up in this way, isotope dilution experiments with *PAL*-overexpressing transgenic cell cultures

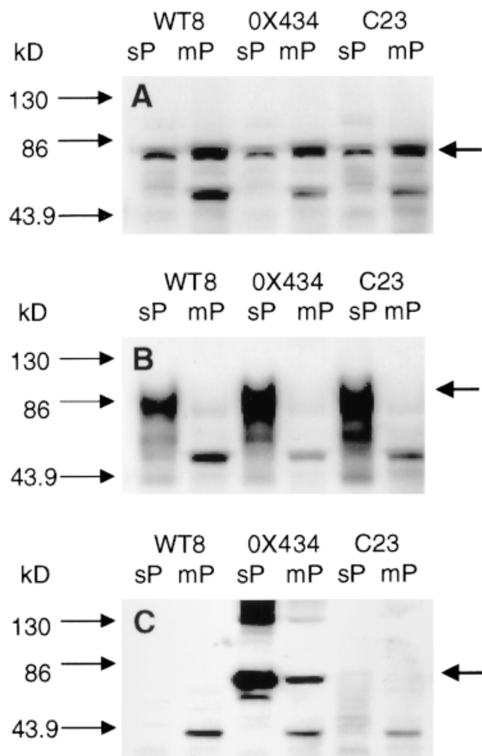

Figure 7. Protein Gel Blot Analysis of PAL Protein Levels in the Soluble and Microsomal Fractions from Wild-Type and Transgenic Tobacco Plants.

WT8 is a wild-type plant; OX434 is a *PAL*-overexpressing line; C23 is a *PAL*-suppressed line. Soluble PAL (sP) represents the PAL protein in the 130,000*g* supernatant, and microsomal PAL (mP) the PAL protein in the 130,000*g* pellet. Proteins (10 μg per lane) were separated by SDS-PAGE.
(A) Tobacco PAL1 protein detected using the anti-tobacco PAL1 antiserum.
(B) Tobacco PAL2 protein detected using the anti-tobacco PAL2 antiserum.
(C) Bean PAL2 protein detected using the anti-bean PAL2 antiserum.
Positions of the molecular mass markers are indicated at left in kilodaltons. The 80-kD PAL subunits are marked with arrows at right.

would artifactually reveal metabolic compartmentalization, which these do not.

This study provides a significant advance over previous work in this area by showing that the alteration of flux into the pathway by transgenic upregulation of the first enzyme, or coinduction of all the enzymes of the putative channel, leads to a loss of channeling, as revealed by both isotope dilution experiments in vivo and microsomal channeling assays in vitro. Thus, the in vitro assays appear to reflect the in vivo situation. This is an important observation in view of the often-cited criticism that in vitro enzyme complexes may not function at the presumed cellular pH and concentration conditions of the cell (Srere, 1987).

Note that the variation we observed in the product/precursor specific activity ratios determined in vivo is quite small, considering the potential biological variation between different cell culture batches, and the values for variation in the in vitro coupling factors are similar. In the latter case, the variation is probably a composite of biological variability and variation caused by sensitivity to disruption of the microsomal association between PAL and C4H. In this respect, it previously has been suggested that reversible inhibition of protein and nucleotide biosynthesis in gently sonicated yeast cells results from disruption of loosely associated enzyme complexes (Burns, 1964).

Previous studies with cucumber hypocotyls showed that hormonal or environmental stimuli could affect channeling between PAL and C4H in vitro. Thus, a coupling factor of ~5.0 was reduced to ~1.5 after exposure of green hypocotyls to ethylene, whereas it was increased to ~25 after irradiation with high-intensity UV light (Czichi and Kindl, 1977). Paradoxically, the latter treatment was reported to induce soluble PAL activity but not to induce microsomal PAL (Czichi and Kindl, 1977). No in vivo labeling data are reported in this study. In contrast, elicitation of tobacco cell cultures results in coordinated induction of both PAL (soluble and microsomal) and C4H activities (Howles et al., 1999) but in a loss of channeling between PAL and C4H, as assessed by both in vivo isotope dilution and in vitro microsomal channeling assays. Note that elicitation induces a different set of defense responses from those induced by UV irradiation (Hahlbrock and Scheel, 1989). The loss of channeling in *PAL*-overexpressing plants and cell cultures could result from two possible mechanisms. Quantitatively, the overexpression of PAL without a corresponding increase in C4H could lead to a spillover of *trans*-cinnamic acid. Alternatively, the qualitative difference in the PAL proteins in the *PAL*-overexpressing suspension cell cultures may reduce channeling if the bean PAL2 protein cannot correctly couple with tobacco C4H.

Association of PAL with Microsomal Membranes

Metabolic channeling may have more than one cellular function. In its simplest form, it might provide for rapid turnover of low concentrations of labile intermediates that have no other metabolic functions (Srere, 1987). However, the phenylpropanoid pathway presents a more complex case. PAL is encoded by small multigene families in all of the plants studied to date, including Arabidopsis (Wanner et al., 1995), and the pathway has several downstream branches leading to functionally distinct end products. It has long been proposed that different forms of PAL may be involved in the synthesis of different end products, primarily on the basis of differential product inhibition of different PAL forms (Alibert et al., 1972; Jones, 1984). Localization of one or more specific PAL isoforms on the surface of the endoplasmic reticulum would provide the necessary structural basis for assembling complexes in which different PAL forms could

channel metabolites into different pathways of phenylpropanoid metabolism.

Previous studies have used immunolocalization and biochemical fractionation techniques to show the association of PAL with endoplasmic reticulum membranes (Czichi and Kindl, 1975, 1977; Wagner and Hrazdina, 1984; Hrazdina and Wagner, 1985). Our results now demonstrate microsomal association of specific forms of PAL. Thus, tobacco PAL1 is found in both soluble and microsomal fractions, whereas tobacco PAL2 is not found in microsomes. PAL activity measured in microsomal preparations from tobacco stem tissues therefore reflects the localization of specific PAL forms on microsomal membranes rather than an artificial entrapment of PAL proteins into microsomal vesicles formed during the isolation process. This suggests that the molecular basis for the channeling of *trans*-cinnamic acid is the coupling of specific PAL forms with C4H located together on the microsomal membranes.

Our data do not suggest a mechanism for the apparent loss of channeling after elicitation. Protein gel blot analysis did not reveal any major difference in the localization of PAL forms after elicitation. The mechanism could therefore be subtle, perhaps involving specific post-translational modifications to PAL or C4H, or might simply reflect a change in the in vivo PAL/C4H activity ratio, with a resultant spillover of *trans*-cinnamic acid.

Implications of Metabolic Channeling for Phenylpropanoid Pathway Regulation

The addition of *trans*-cinnamic acid to bean cell cultures inhibits PAL at the transcriptional level and induces the synthesis of a proteinaceous inactivator of PAL (Bolwell et al., 1986, 1988). Furthermore, downregulation of C4H by antisense gene expression in transgenic tobacco leads to a corresponding decrease in PAL activity, suggesting that *trans*-cinnamic acid is sensed as a metabolic regulator of phenylpropanoid pathway flux in vivo (J. Blount and R.A. Dixon, unpublished results). Tobacco PAL is particularly sensitive to direct inhibition by *trans*-cinnamic acid in vitro (O'Neal and Keller, 1970). Tight coupling between PAL and C4H therefore could maintain, in the microsomal "compartment," a low *trans*-cinnamic acid pool that would avoid feedback inactivation of PAL, as suggested by Noe et al. (1980). Note, however, that the addition of 10^{-4} M *trans*-cinnamic acid in our isotope dilution experiments did not appear to inhibit flux through the PAL reaction in vivo, as assessed by incorporation of tritium from ^{3}H–L-Phe into the various phenylpropanoid compounds (data not shown).

PAL-overexpressing tobacco cells, in which channeling through the C4H reaction is no longer measurable, accumulate twofold higher levels of the potentially antifungal coumarin glycoside scopolin and the corresponding aglycone scopoletin (Ahl Goy et al., 1993; Gutierrez et al., 1995) than do wild-type cells. It is not possible to conclude that this results from a release from channeling of *trans*-cinnamic acid because of uncertainty as to the biosynthetic origin of scopoletin. The levels of scopoletin in tobacco cell cultures treated with a yeast elicitor are much lower than those in untreated cells, which could be due to degradation by induced peroxidases in elicited cells, as has been shown in other systems (Gutierrez et al., 1995; Breton et al., 1997; Edwards et al., 1997). A similar decline of constitutive phenolics associated with cell wall incorporation has been described for isoflavonoids in elicitor-treated cell suspension cultures of *Pueraria lobata* (Park et al., 1995), and we assume that the decline in the levels of most hydroxycinnamic acid derivatives in elicited tobacco cells is associated with such further metabolism rather than being a consequence of changes in metabolic channeling. In contrast, the benzoic acid derivatives pHBA, salicylic acid, vanillic acid, and the partially characterized vanillin derivative accumulated to the same level in elicited and unelicited tobacco cells. There appears to be independent regulation of the metabolic pathways involved in the biosynthesis of hydroxycinnamic acid derivatives, which include lignin precursors, and the biosynthesis of benzoic acid derivatives, which include salicylic acid (Figure 1).

Although the majority of phenylpropanoid compounds derived from the natural products of plants require both PAL and C4H for their synthesis, salicylic acid probably is derived directly from *trans*-cinnamic acid by chain shortening and ring hydroxylation (Lee et al., 1995). It is tempting to speculate that salicylic acid is synthesized via an uncoupled form of PAL, for example, nonmicrosomally associated PAL2 in tobacco. However, tobacco PAL1 could be equally involved in salicylic acid biosynthesis, because overexpression of bean PAL, which, like tobacco PAL1, is localized both cytoplasmically and microsomally in transgenic tobacco stem tissue, results in increased salicylic acid production and corresponding increases in disease resistance in intact tobacco plants (Felton et al., 1999).

Our results obtained in experiments with *PAL*-overexpressing tobacco cell cultures and elicited wild-type cultures do not support the idea that a loss of channeling leads directly to a higher accumulation of salicylic acid produced from unchanneled *trans*-cinnamic acid, because the levels of salicylic acid are the same as those in wild-type tobacco cells. However, a marked difference can be seen in the accumulation of the vanillin derivative, which is 10 times higher in *PAL*-overexpressing tobacco cells when compared with wild-type cells, although it is not significantly induced by elicitation. Currently, we cannot conclude whether the accumulation of this compound in *PAL*-overexpressing cells is due to a perturbation in *trans*-cinnamic acid channeling, because the biosynthetic pathway(s) involved in the formation of benzoic acid derivatives, such as vanillic acid and vanillin, still is to be unequivocally elucidated (Zenk, 1965; Funk and Brodelius, 1990; Yazaki et al., 1991; Schnitzler et al., 1992).

Two important predictions concerning phenylpropanoid pathway organization now can be tested in the model

system of tobacco. The first is that the differential subcellular localization of tobacco PAL1 and PAL2 has functional consequences related to metabolic channeling. This prediction can be addressed by studying the metabolic consequences of differentially downregulating expression of *PAL1* or *PAL2*. The second is that tobacco PAL1, but not PAL2, will be in close physical association with C4H. This prediction can be addressed by immunolocalization studies by using transgenic plants expressing epitope-tagged PAL and C4H species. These experiments are currently in progress.

METHODS

Plant Material

Tobacco (*Nicotiana tabacum* cv Xanthi-nc) plants were either wild type or transformed with the bean phenylalanine (Phe) ammonia–lyase *PAL2* gene (Elkind et al., 1990). The transformed plants displayed either increased PAL activity (Howles et al., 1996) or epigenetic gene silencing with reduced levels of activity. Used as controls were untransformed wild-type plants or plants from which the *PAL2* transgene had segregated. All plants were grown under greenhouse conditions (18°C at night and 27°C by day) and harvested just before flowering. Stem samples were taken from internodes seven to 11, counting from the top, frozen in liquid N_2, and ground in a tissue grinder.

Callus cultures were initiated from leaf discs of wild-type and transformed tobacco plants, as described previously (Bate et al., 1994). Liquid cultures were initiated and maintained in a modified Schenk and Hildebrandt medium, as described previously (Dixon et al., 1981), and subcultured every 7 to 10 days.

Five days after subculturing, dark-grown tobacco cell suspension cultures (75-mL batches) were treated with a yeast elicitor (Schumacher et al., 1987; 75 μg mL^{-1} glucose equivalents) and harvested at various times after elicitation. Control cells were treated with the same amount of distilled water.

Chemicals

^{3}H–L-2,3,4,5,6-Phe (124 Ci/mmol) and uniformly labeled L–^{14}C-Phe (453 mCi/mmol) were supplied by Amersham (Little Chalfont, United Kingdom). ^{14}C–*trans*-Cinnamic acid was synthesized enzymatically from uniformly labeled L–^{14}C-Phe by using PAL from *Rhodotorula glutinis* (14 units per mg of protein; Sigma) as described elsewhere (Edwards and Kessmann, 1992).

Precursor Dilution Experiments

Tobacco cell suspension cultures (75-mL batches) were incubated 4 days after subculturing with 7.5 μmol of ^{3}H–L-Phe (1 μCi/μmol) with or without unlabeled *trans*-cinnamic acid or 4-coumaric acid (0.75 or 7.5 μmol). After 24 hr, the cells were filtered through a nylon mesh and ground in liquid N_2. The soluble phenolics were extracted three times with 8 mL of ice-cold acetone at 4°C in the dark. The extracts were combined and concentrated to dryness under a stream of N_2, and the residue was dissolved in methanol (500 μL per gram fresh weight). Aliquots (20 μL) were separated by HPLC, as described below. Fractions of 500 μL were collected and counted in a liquid scintillation counter.

Enzymatic Hydrolysis of Phenolic Extracts

For the enzymatic hydrolysis of phenolic esters of caffeic acid, 4-coumaric acid, ferulic acid, and *trans*-cinnamic acid, 200 μL of the extracts was concentrated, dissolved in buffer (200 mM Tris-HCl, pH 8.0), and incubated overnight at 37°C with an esterase from rabbit liver (190 units; EC 3.1.1.1; Sigma). Phenolic glucosides of scopoletin, the vanillin derivative, *p*-hydroxybenzaldehyde (pHBA), and salicylic acid were hydrolyzed with almond β-glucosidase (100 units; EC 3.2.1.21; Sigma). Extracts then were processed for analysis of phenolic aglycones as described above.

Separation of Phenolics by Reverse Phase HPLC

Organic extracts from enzyme assays and plant or cell suspension phenolic fractions were applied to an ODS reverse phase HPLC column (5-mm particle size, 4.6 × 250 mm; Metachem Technologies, Inc., Torrance, CA) and eluted in 1% phosphoric acid with an increasing acetonitrile concentration gradient (0 to 5 min, 5% [v/v] acetonitrile; 5 to 10 min, 5 to 10% acetonitrile; 10 to 25 min, 10 to 17% acetonitrile; 25 to 30 min, 17 to 23% acetonitrile; 30 to 65 min, 23 to 50% acetonitrile; 65 to 74 min,100% acetonitrile; and 74 to 85 min, 5% acetonitrile) at a constant flow rate of 1 mL min^{-1}. UV absorbance was monitored with a photodiode array detector (Hewlett Packard, Waldbronn, Germany). Quantification of phenolics was based on calibration curves achieved with authentic standards (Sigma) at 270 and 330 nm.

Preparation of Membrane Fractions

Frozen (−70°C) and ground stem material or suspension cells (4 to 4.5 g fresh weight) were homogenized for 3 × 10 sec in 8 mL of a Tris-HCl buffer (200 mM Tris, pH 8.0, 400 mM sucrose, 1 mM EDTA, 40 mM sodium ascorbate, and 5 mM 2-mercaptoethanol) by using an Ultraturrax blender (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was centrifuged (10,000*g* for 30 min) and filtered through a syringe filled with glass wool. The filtrate was ultracentrifuged (130,000*g* for 1 hr), the supernatant was decanted, and the pellet was blot dried. After resuspending the pellet in 2.5 mL of Pi buffer (200 mM potassium phosphate, pH 8.0, and 3 mM 2-mercaptoethanol) with a rubber spatula, the suspension was subjected to a second ultracentrifugation (130,000*g* for 1 hr). The supernatant was decanted, and the microsomal pellet was blot dried. The microsomes were resuspended carefully in 300 μL of assay buffer (200 mM potassium phosphate, pH 8.0, 6 mM $MgCl_2$, and 3 mM 2-mercaptoethanol). In all cases, 2-mercaptoethanol was added fresh to the buffers, and all steps were conducted on ice or at 4°C.

PAL and Cinnamic Acid 4-Hydroxylase Assays

Soluble PAL activity was determined in the ultracentrifugation supernatants (desalted on a PD-10 column equilibrated with 200 mM boric acid, pH 8.8, and 13 mM 2-mercaptoethanol) by using ^{14}C–L-Phe as a substrate, essentially as described by Legrand et al. (1976). For de-

termination of PAL activity in the microsomal fraction, 25 μL of the microsomal suspension was diluted with 25 μL of boric acid (200 mM, pH 8.8, containing 13 mM 2-mercaptoethanol) before adding the substrate. Cinnamic acid 4-hydroxylase (C4H) activity was determined in the microsomal fraction according to Edwards and Kessmann (1992). Protein concentrations were determined according to Bradford (1976), using BSA as a standard.

In Vitro Channeling Assays

Washed microsomes (200 μL) were preincubated with 245 μL of C4H assay buffer for 5 min, and the reactions were started with 500 nmol of ^{3}H–L-Phe (5 mCi/mmol), 30 nmol of ^{14}C–*trans*-cinnamic acid (2 mCi/mmol), and 1 mmol of NADPH in a total volume of 155 μL at 30°C. The reactions were stopped after 10 min with 50 μL of 6 N HCl.

Channeling assays in the presence of soluble PAL were conducted with 200 μL of the microsomal suspension, 100 μL of the soluble enzyme fraction, 145 μL of the C4H assay buffer, and the substrates, as described above. The assays were extracted with 3 × 700 μL of ethyl acetate, and the extracts were concentrated to dryness and dissolved in 30 μL methanol. Caffeic acid (1 μg) was added to the assays as an internal standard before extraction. Products were separated by reverse phase HPLC and monitored by UV absorbance at 270 nm, and the fractions (250 μL) were collected. The radioactivity in fractions containing 4-coumaric acid and *trans*-cinnamic acid was determined by liquid scintillation counting, by using the automatic quench compensation for tritium/carbon-14 dual label counting on an LS 1701 scintillation counter (Beckmann Instruments, Fullerton, CA).

Treatment of Plant Extracts with Trypsin

Soluble and microsomal enzyme fractions were prepared as described above, incubated with Triton X-100 (Sigma) (final concentration of 0.1 or 0.6%) or homogenization buffer (controls) for 30 min at 4°C, and then treated with 1 mg mL^{-1} trypsin (10,200 units per mg of protein; Sigma) for 20 min at 30°C. PAL and C4H enzyme assays were started by the addition of substrates, incubated for 20, 40, and 60 min, and stopped with HCl as described above.

Generation of Antibodies Specific for Tobacco and Bean PAL Forms

For raising antibodies specific to tobacco PAL proteins, the following amino acid sequences, derived from the available tobacco PAL sequences (Nagai et al., 1994; Pellegrini et al., 1994; Fukasawa-Akada et al., 1996), were used: VRDKSANG (positions 69 to 76 from tobacco PAL1) and VAQNGHQEMDFCVKV (positions 4 to 18 from tobacco PAL2). These sequences represent the few stretches that are unique between the different tobacco PAL forms. Synthetic peptides were coupled to the carrier protein keyhole limpet hemocyanin, and antibodies were raised in rabbits (Genosys Biotechnologies, Inc., The Woodlands, TX). Antibodies specific for bean PAL2 have been described previously (Howles et al., 1996).

Protein Gel Blot Analysis

Soluble and microsomal proteins (10 μg) were subjected to denaturing SDS-PAGE on precast 8 to 16% Tris-glycine gels (Novex, San Diego, CA) and transferred to nitrocellulose membranes (Trans-Blot; Bio-Rad). The membranes were blocked and probed with the primary antibody in 5% fat-free milk powder (Carnation, Glendale, CA) dissolved in 0.2% Tween 20 (Sigma) in Tris-buffered saline (TBST; Ausubel et al., 1994). A goat anti-rabbit IgG–horseradish peroxidase conjugate (Bio-Rad) was used as secondary antibody at a 1:10,000 dilution in TBST. Bands were visualized on a film by using a chemiluminescence assay (ECL; Amersham). Kaleidoscope prestained standards (Bio-Rad) were used as molecular weight markers.

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