Phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and the C4H redox partner cytochrome P450 reductase (CPR) are important in allocating significant amounts of carbon from phenylalanine into phenylpropanoid biosynthesis in plants. It has been proposed that multienzyme complexes (MECs) containing PAL and C4H are functionally important at this entry point into phenylpropanoid metabolism. To evaluate the MEC model, two poplar PAL isoforms presumed to be involved in either flavonoid (PAL2) or in lignin biosynthesis (PAL4) were independently expressed together with C4H and CPR in Saccharomyces cerevisiae, creating two yeast strains expressing either PAL2, C4H and CPR or PAL4, C4H and CPR. When [3H]Phe was fed, the majority of metabolized [3H]Phe was incorporated into p-[3H]coumarate, and Phe metabolism was highly reduced by inhibiting C4H activity. PAL alone expressers metabolized very little phenylalanine into cinnamic acid. To test for intermediate channeling between PAL and C4H, we fed [3H]Phe and [14C]cinnamate simultaneously to the triple expressers, but found no evidence for channeling of the endogenously synthesized [3H]cinnamate into p-coumarate. Therefore, efficient carbon flux from Phe to p-coumarate via reactions catalyzed by PAL and C4H does not appear to require channeling through a MEC in yeast, and instead biochemical coupling of PAL and C4H is sufficient to drive carbon flux into the phenylpropanoid pathway. This may be the primary mechanism by which carbon allocation into phenylpropanoid metabolism is controlled in plants.

Carbon flux into the phenylpropanoid pathway in plants is mediated by a key gateway enzyme, phenylalanine ammonia lyase (PAL) that catalyzes the non-oxidative deamination of phenylalanine (Phe) to produce trans-cinnamic acid. Trans-cinnamic acid is hydroxylated at the para-position by a cytochrome P450 monooxygenase enzyme, cinnamate-4-hydroxylase (C4H), in conjunction with NADPH:cytochrome P450 reductase (CPR). The coordinated reactions catalyzed by these enzymes account for a large fraction of the carbon flow in some specialized plant tissues. For example, in the secondary xylem of woody plants, 15–36% of assimilated carbon is channeled to lignin, a polymer of hydroxycinnamic acids derived from phenylpropanoid metabolism (1). Similarly, in vegetative tissues, soluble flavonoid or phenylpropanoid derivatives such as sinapoyl malate in Arabidopsis, or rutin and chlorogenic acid in tobacco, are known to accumulate in abundance (2–4). Thus, PAL and subsequent enzymes in the phenylpropanoid pathway can direct significant amount of carbon into many different phenylpropanoid metabolic endproducts, as dictated by developmental programs.

Since PAL resides at a metabolically important position, linking the phenylpropanoid secondary pathway to primary metabolism, the regulation of overall flux into phenylpropanoid metabolism has been suggested to be modulated by PAL as a rate-limiting enzyme (5). How this regulation is accomplished, however, is not completely clear. Feedback inhibitory regulation of PAL activity by its own product, trans-cinnamate, has been demonstrated in vitro (6–8), and trans-cinnamic acid was proposed to modify transcription of PAL genes in vivo (9, 10). In tobacco with suppressed C4H expression, reduced C4H activity was counter-intuitively correlated with a decrease in intracellular cinnamate levels, suggesting feedback inhibition (i.e. autoregulation) of PAL at a certain threshold level of endogenous cinnamate (11).

It has also been postulated that PAL can form multienzyme complexes (MECs) that include downstream enzymes such as C4H, facilitating efficient channeling of carbon into phenylpropanoid metabolism. It has been shown that a specific PAL isoform (PAL1) is physically associated with both isolated microsomes and the cytoplasmic fraction in fractionated tobacco cell extracts, supporting a model in which PAL associates with MECs in an isoform-specific manner to modulate a carbon flux into specific downstream pathways (12). Other data from experiments employing classical approaches such as double radioactive labeling assays (13–15), enzyme co-purification (15, 16), and immunohistochemical localization (17) support the presence of MECs among enzymes in the entry point or downstream pathways. Investigation of possible MECs in phenylpropanoid metabolism has been further pursued in recent years using various molecular techniques, revealing direct protein-protein interactions among enzymes in the flavonoid pathway in Arabidopsis (18) and the elicitor-dependent endoplasmic reticulum (ER)-localization of an otherwise cytosolic isoflavone O-methyltransferase in alfalfa (19). However, there is still no direct evidence for PAL-C4H interaction to form an MEC.

In our laboratory, poplar (Populus trichocarpa X Populus
Phenylpropanoid Pathway Reconstruction in Yeast

In all induction procedures, 500 μl medium as recommended by the manufacturer was used to culture and store the transformants according to Gietz et al. (28). The P. kitakamiensis and P. tremuloides contain divergent members of the PAL gene family whose expression correlates with secondary xylem development (PttPAL2, 23), cDNAs were used to transform yeast strains, where they could, in principle, help anchor a MEC that is closely related to the yeast Saccharomyces cerevisiae in different combinations in an attempt to reconstitute the enzyme point of phenylpropanoid metabolism in a phenylpropanoid-free eukaryotic cell. Using simple and novel analytical tools, we have used this system to evaluate the proposed MEC model and to explore how the enzyme point enzymes of phenylpropanoid metabolism redirect carbon flow from primary to secondary metabolism.

EXPERIMENTAL PROCEDURES

**Yeast Strains, Culture, and Gene Induction—S. cerevisiae strain, YPH499 (MATa, ura3-52, lys2-801, ade2-101, trpl-1,63, his3-D200, leu2-3,11) and plasmids for yeast dual expression (pESC-Leu and pECC-His) were purchased from Stratagene. Transformation and the screening of transformants were performed according to Gietz et al. (28). The YPH499 and transformed yeast strains were cultured and stored in a medium as recommended by the manufacturer’s protocol (Stratagene). In all induction procedures, 500 μl of start culture overnight was added to 50 ml of selective medium supplemented with 2% dextrose (S.D.), and cultured at 28 °C with 150 rpm shaking for 12 h to 15 h. Yeast cells were centrifuged at 4,000 g for 5 min, washed once with 20 ml of distilled water, and recentrifuged. The cell pellet was resuspended in the appropriate selective medium for C4H and CPR, and cell pellets and culture medium were separately extracted by organic solvents. The culture medium was acidified by addition of 500 μl of 60% H2SO4 and extracted twice with 2 ml of hexane containing 10% of total) of 4 M H2SO4 and extracted twice with 2 ml of methanol and 15 μg of lyticase (Sigma). Cell suspensions were incubated at 25 °C for 30 min with a gentle shaking. The resulting yeast protoplasts were centrifuged for 3 min at 4,000 x g, and the cells were ruptured by addition of 1 ml of ice-cold extracting buffer containing 50 mM Tris (pH 7.4) and 14 mM β-mercaptoethanol for 10 min in ice with periodic vortexing. The yeast extract was centrifuged at 10,000 x g for 10 min and inserted into the buffer containing 0.2 mM of 60–300 μg of total protein were used for the PAL enzyme assays, carried out for 30 min at 50 °C in 0.1 M H2O/KOH (pH 8.8) buffer solution containing 3 μM phenylalanine in 170 μl total volume. The reactions were terminated by addition of 40 μl of 4 M H2SO4, and the reaction solutions were extracted twice with 300 μl of ethyl acetate. The reaction products were dissolved in methanol after the organic phase had been evaporated under vacuum. The presence of cinnamic acid was identified and quantified using HPLC analysis and authentic cinnamate.

**Analysis of PAL or Triple Gene (PAL, C4H, and CPR)-expressing Yeast Strains—After the standard culture and induction in 25 ml of methanol containing 1 ml of organic solvents and 15 mg each of PAL and CPR, cell pellets and culture medium were separately extracted by organic solvents. The culture medium was acidified by addition of 500 μl of 4 M H2SO4, and the reaction solutions were extracted twice with 2 ml of methanol and 15 μg of lyticase (Sigma). Cell suspensions were incubated at 25 °C for 30 min with a gentle shaking. The resulting yeast protoplasts were centrifuged for 3 min at 4,000 x g, and the cells were ruptured by addition of 1 ml of ice-cold extracting buffer containing 50 mM Tris (pH 7.4) and 14 mM β-mercaptoethanol for 10 min in ice with periodic vortexing. The yeast extract was centrifuged at 10,000 x g for 10 min and inserted into the buffer containing 0.2 mM of 60–300 μg of total protein were used for the PAL enzyme assays, carried out for 30 min at 50 °C in 0.1 M H2O/KOH (pH 8.8) buffer solution containing 3 μM phenylalanine in 170 μl total volume. The reactions were terminated by addition of 40 μl of 4 M H2SO4, and the reaction solutions were extracted twice with 300 μl of ethyl acetate. The reaction products were dissolved in methanol after the organic phase had been evaporated under vacuum. The presence of cinnamic acid was identified and quantified using HPLC analysis and authentic cinnamate.

**Cloning of PAL Genes in the pESC-His Vector—Coding sequences for PAL2 and PAL4 were PCR-amplified from PAL7 and PAL18 cDNAs, respectively. For the PAL2 ORF, a forward primer 5'-AGCTCTAGA-AAAATGGAATTTTGGCTAGATTGCAC-3' and a reverse primer, 5'-AGCTCTAGAAGCTTAGCAAATAGGAAGGAGC-3' were used, and for the PAL4 ORF, a forward primer, 5'-AGCTCTAGAGAAATTGGGACAGCTCACCAGAAT-3' and a reverse primer, 5'-AGCTCTAGACTTAACAGATAGGAGGGGAAGG-3', were used. The amplified fragments were digested with XmaI and inserted into the pESC-HIS vector in the correct orientation. The sequences of clones were confirmed by sequencing analysis.

**PAL Enzyme Assay—For PAL assays, 50 ml cell suspensions of PAL alone or triple-expressing yeast strains were cultured and induced as described above. Yeast cells were resuspended in a solution containing 1 M sorbitol, 0.1 mM EDTA, 0.1% β-mercaptoethanol, and 15 μg of lyticase (Sigma). Cell suspensions were incubated at 25 °C for 30 min with a gentle shaking. The resulting yeast protoplasts were centrifuged for 3 min at 4,000 x g, and the cells were ruptured by addition of 1 ml of ice-cold extracting buffer containing 50 mM Tris (pH 7.4) and 14 mM β-mercaptoethanol for 10 min in ice with periodic vortexing. The yeast extract was centrifuged at 10,000 x g for 10 min and inserted into the buffer containing 0.2 mM of 60–300 μg of total protein were used for the PAL enzyme assays, carried out for 30 min at 50 °C in 0.1 M H2O/KOH (pH 8.8) buffer solution containing 3 μM phenylalanine in 170 μl total volume. The reactions were terminated by addition of 40 μl of 4 M H2SO4, and the reaction solutions were extracted twice with 300 μl of ethyl acetate. The reaction products were dissolved in methanol after the organic phase had been evaporated under vacuum. The presence of cinnamic acid was identified and quantified using HPLC analysis and authentic cinnamate.

**Northern Blot Analysis—For isolation of total RNA, the modified Nucleo PhytoPure method (29) was used, except that ~300 mg starting material was used, and a repeated Nucleo PhytoPure (50 μl) wash step was incorporated. 10 μg of total RNA was resolved on 1.5% agarose gels. Resolved RNA was transferred onto Hybond-XL membranes (Amersham Biosciences), according to standard methods (30). Radioactive probes were prepared using appropriate templates using a random priming kit (Invitrogen Life Technologies, Inc.). Probe hybridization was performed overnight at 65 °C in a buffer containing 1% bovine serum albumin, 7% SDS, 50 mM sodium phosphate (pH 7.5), and 1 M NaCl, washed twice for 30 min at 65 °C in 2× SSC with 0.1% SDS. The final wash was performed at 65 °C in 0.2× SSC and 0.1% SDS for 1 h. Radioactive signals were detected using a phosphorimager screen and a Molecular Dynamics Storm Phosphorimager.

**SDS-PAGE and Immunoblot Analysis—SDS-PAGE and immunoblot for PAL, and CPR was performed as previously described (24). For recombinant PAL2 detection, primary polyclonal antibody (31) at 1:5,000 dilution was used with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences) at 1:2,500 dilution.

**Phenylalanine Feeding Assay—The yeast vector control, PAL only, and triple-expressing strains were pre-cultured and induced (16 h) in standard conditions. The cell densities after induction varied be-
between 7.2 × 10⁴ and 9.0 × 10⁴ cells ml⁻¹, but each batch of the transformed yeast strains was adjusted to a density of 6.0 × 10⁷ cells ml⁻¹ in 25 ml of His/Phe-dropout SG medium (for PAL2/4 expressor) or His/Phe/Leu-dropout SG medium (for vector control and triple expressor). Two additional 25-ml batches were prepared each for the T2 and T4 strains, and the C4H inhibitor piperonyl acid was added to one of the batches for each strain at a final concentration of 10 μM. A final concentration of 500 μM [2-6-¹³C] phenylalanine (56.9 μCi μmol⁻¹, Amersham Biosciences) was added to the 25-ml culture. These yeast strains were then cultured in standard conditions for 4 h. The accumulated phenolic metabolites from the medium and the cells were extracted as described before. The extracted metabolites were fractionated on a reverse phase HPLC column in a gradient of 0.85% phosphoric acid with an increasing proportion of acetonitrile (5–30 min, 5% acetonitrile; 30–40 min, 5–15% acetonitrile; 40–65 min, 15–56% acetonitrile) at a constant flow rate of 1.0 ml min⁻¹. Fractions (500 μl) were collected between 23 and 65 min, and the radioactivity of each fraction was measured by scintillation counting in a LS6000 liquid scintillation counter (Beckman). Major ³H-labeled metabolites detected were: phenylethanol, 33.0 min; phenylacetic acid, 39.5 min; p-coumarate, 45.2 min; cinnamate, 54.5 min; phenylpyruvate, 55.9; syrlyne, 63.1. In T2 and T4 triple expressers, two unknown ³H-labeled metabolites detected were: phenylethanol, 33.0 min; phenylacetic acid, 39.5 min; p-coumarate, 45.2 min; cinnamate, 54.5 min; phenylpyruvate, 55.9; syrlyne, 63.1. In T2 and T4 triple expressers, two unknown ³H-labeled metabolites, each constituting ~0.5% of total ³H activity initially added, were eluted at ~43 and 53 min. In all yeast strains including vector-transformed control, unidentified minor ³H-labeled metabolites (~0.2% of total ³H activity) were eluted at 27 and 50 min. [¹³C]phenylalanine remaining aqueous phase after ether extraction was independently measured by HPLC (retention time, 8.2 min) using the same conditions.

Double Labeling Assay—T2 and T4 yeast strains were induced by addition of 2% galactose for 15 h in standard conditions. These induced cells were resuspended in 50 ml of fresh His/Leu/Phe-dropout SG medium supplemented with 500 μM [2-6-¹⁴C]-high phenylalanine (56.8 μCi μmol⁻¹) together with 10 μM [47.7 μCi μmol⁻¹]-trans-[U-¹⁴C]cinnamate (a gift from Dr. G. H. N. Towers). [¹⁴C]Cinnamate was purified before use by silica gel thin layer chromatography using mobile phase containing toluene and acetic acid in four to one ratio (RF = 0.45). These yeast cell batches were cultured for 30 min at 28 °C. Culture medium, and cells as described before, were centrifuged and the radioactively labeled metabolites were separated by the HPLC, using the program described in the [³H]phenylalanine feeding assay. Fractions (200 μl) were collected that corresponded to the elution times of p-coumarate, cinnamate, and syrlyne. The ³H and ¹⁴C radioactivities in the fractions were measured by scintillation counting in a LS6000 liquid scintillation counter (Beckman) with automatic quench compensation for ³H and ¹⁴C dual label counting.

Analysis of Mixed PAL Alone- and C4H/CPR Alone-expressing Yeast Strains—PAL alone and C4H/CPR expressers were independently cultured, induced for 15–18 h, and the cells were washed with 20 ml of distilled water. Approximately 3 × 10⁶ cells each of the PAL alone and C4H/CPR expressers (total 6 × 10⁶ cells) were mixed in 80 ml of SG medium containing 500 μM Phe. The T2 or T4 strains were also induced, washed, and cultured in the same conditions as a reference, but half the number of total cells (3 × 10⁶ cells) were resuspended in 80 ml SG medium (500 μM Phe) so that the two systems had similar numbers of PAL-expressing yeast cells. 1 ml aliquots of culture medium were collected in a time course, and cells were removed by centrifugation at 6,000 × g for 2 min. Supernatants were filtered through a 0.45-μm filter and the quantity of cinnamate, and p-coumarate was measured by HPLC fractionation.

RESULTS

Cloning of a Xylem Expressed Poplar PAL Gene—Since it has been shown that different PAL isoforms may differentially associate with C4H in vivo (12), it was desirable to employ cDNAs encoding divergent PAL isoforms with potentially distinct roles in phenylpropanoid metabolism to study the interactions between PAL and C4H in yeast. Applying P. kitakakensis PALg2b gene-specific primers (26) on a cDNA pool of P. trichocarpa X P. deltoides (TD) xylem mRNA allowed us to isolate and sequence an additional poplar cDNA (PAL18) whose deduced amino acid was 85% identical to the poplar PAL7 cDNA derived from PAL2 (22, 25). The corresponding gene was designated PAL4 to distinguish the gene represented by this cDNA from previously identified TD PAL genes (22, 23, 25). In Northern blot analysis, predominant expression of the PAL4 gene was detected in secondary xylem and to a lesser extent in green stem, and this PAL4 gene was highly inducible by elicitor-treatment (Fig. 1). To corroborate this finding, the same blot was re-probed with the PAL7 cDNA. As predicted, the expression pattern of PAL2 detected by this probe was very similar to that previously reported (25), i.e., abundant transcripts in young leaf and elicitor-treated cell culture but little in xylem tissue. Therefore, the PAL4 gene clearly showed a predominant expression pattern in secondary xylem, while PAL2 is mainly expressed in young shoots.

Simultaneous Expression of PAL, C4H, and CPR in Yeast—The cDNAs for PAL2 and PAL4 were used together with those for poplar C4H and CPR genes to reconstruct the entry point of the phenylpropanoid pathway in yeast cells. We previously cloned epitope-tagged versions of the C4H and CPR2 ORFs in the pESC-LEU yeast dual expression vector (pESC-LEU::C4H/CPR), where they are expressed under control of the Gal1 and Gal10 promoters and together support high levels of C4H activity in transgenic yeast strains (24). To create yeast strains expressing different combinations of PAL, C4H, and CPR, the ORFs of the PAL7 cDNA (PAL2) or PAL18 cDNA (PAL4) were cloned into the pESC-HIS vector under the control of the Gal10 promoter. Yeast was transformed with the pESC-HIS::PAL2 or pESC-HIS::PAL4 construct alone, or co-transformed with these constructs together with pESC-LEU::C4H/CPR2, to generate four different yeast strains: two strains expressing PAL2 or PAL4 alone, and two triple-expressing strains expressing PAL2/C4H/CPR2 or PAL4/C4H/CPR2, referred as T2 and T4 strains, respectively. In addition, empty vectors (pESC-LEU and pESC-HIS) were co-transformed to generate a vector-transformed control strain.

Functional expression of the three genes in yeast was verified by immunoblot analysis, using monoclonal anti-FLAG and anti-c-Myc antibody to detect epitope-tagged C4H and CPR, respectively, (Fig. 2A) or using polyclonal anti-PAL2 antibody to detect PAL2 and PAL4 recombinant protein (Fig. 2B). Surprisingly, the PAL4 recombinant protein was not detected in either single or triple expressers using this antibody. As an alternative method, PAL enzyme assays were performed using cytosolic fractions from transformed yeast strains. The cytosolic fractions of all four PAL-expressing yeast strains contained an activity that efficiently catalyzed the demamination of Phe to produce authentic cinnamate as judged by HPLC analysis, while this activity was absent in the vector-transformed control strain (data not shown). PAL activities in PAL2 and T2 strains were between 70 and 90 pkat mg⁻¹, while those in PAL4 and T4 strains were between 20 and 35 pkat mg⁻¹ and there were no significant differences in PAL activities between single and triple expressers (data not shown). Therefore, it is clear that the PAL4 enzyme was present in PAL4 and T4 yeast strains.
cinnamate and the above experiments, the maximum amount of cinnamate convert Phe to cinnamate with much lower efficiency. In all of the above experiments, the maximum amount of cinnamate recovered from cell pellets was less than one-tenth of that recovered from media. Thus, the bulk of the cinnamate and p-coumarate produced by the yeast strains was secreted into the culture media.

To determine whether triple-expressing yeast strains T2 and T4 could produce p-coumarate from endogenous Phe (the yeast host YPH499 is prototrophic for Phe), these strains were cultured in minimal media lacking Phe following galactose induction. For comparison, yeast cells from the same induced cultures were also cultured in media supplemented with 0.5 mM or 5 mM Phe. The results of these experiments are summarized in Fig. 4. Even in the absence of added Phe, significant amounts of p-coumarate were synthesized by strains T2 and T4, yet cinnamate production was below the level of detection in all yeast strains under these conditions. Thus, PAL and C4H in the triple-expressing strains efficiently redirected carbon from a pool of endogenous Phe to the secondary product p-coumarate. Strains expressing PAL alone, on the other hand, did not have the capability to divert significant amounts of carbon from endogenous Phe into the phenylpropanoid pathway. Feeding of increasing amounts of Phe resulted in corresponding increases in the levels of both cinnamate and p-coumarate produced by the triple expressers (Fig. 4). In PAL-alone expressers, the amounts of cinnamate increased from undetectable levels to levels only slightly lower than that of p-coumarate when media was supplemented with 5 mM Phe. These data suggest that Phe availability (concentration) is a limiting factor in vivo for cinnamate production by strains expressing PAL alone, but that triple expressers are able to catalyze the conversion of Phe to p-coumarate very efficiently even at the low Phe concentrations produced endogenously by yeast.

Radiotracer Analysis of Phe Metabolism in Yeast Strains—It is known that S. cerevisiae can use Phe as a sole nitrogen source (33) using a catabolic pathway involving the transamination of Phe to phenylpyruvate, subsequent decarboxylation.

Fig. 2. Recombinant proteins detected by immunoblots in transgenic yeast strains. A, immunoblot analysis of microsomal proteins from the yeast strains indicated, reacted with a cMyc monoclonal antibody to detect CPR and a FLAG monoclonal antibody to detect C4H. B, immunoblot analysis of total protein from the yeast strains indicated, reacted with a polyclonal anti-PAL2 antibody. Microsomal proteins (2 µg; A) or total proteins (5 µg; B) were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunodetected by ECL (Amersham Biosciences).

Phenolic Product Accumulation in Yeast Cultures—To determine the efficiency with which the engineered yeast strains could re-direct Phe to phenylpropanoid metabolite production, PAL, C4H, and CPR expression was induced for 15 - 20 h, after which the induced yeast cells were cultured in fresh media containing 0.5 mM Phe for 2 h. Combined extracts of the culture media and cell pellets were fractionated by HPLC and absorption at 290 nm was monitored. Analysis of vector-transformed control cells revealed no major phenolic metabolites (Fig. 3), although a few small peaks were present. Based on comparison to the authentic standard (retention time and UV spectrum), one of these appeared to be phenylpyruvate, likely derived from Phe by the reversible reaction catalyzed by a transaminase in Phe biosynthesis (32) (see Fig. 5). Consistent with this, there was a rapid increase in the amount of this compound in cells fed high Phe concentrations (data not shown). HPLC analysis of extracts from PAL2 or PAL4 strains showed small amounts of an additional compound (Fig. 3) identified as cinnamate, showing that yeast strains expressing PAL only accumulate small amounts of cinnamate.

In contrast to the low levels of cinnamate produced in the PAL2 and PAL4 strains, strains T2 and T4 produced large amounts of p-coumarate, accompanied by a small amount of cinnamate (Fig. 3). These results were consistently observed in repeated experiments. Thus, the simultaneous expression of PAL, C4H, and CPR in yeast cells resulted in efficient production of p-coumarate, whereas PAL-alone expressers seemed to convert Phe to cinnamate with much lower efficiency. In all of the above experiments, the maximum amount of cinnamate and p-coumarate recovered from cell pellets was less than one-tenth of that recovered from media. Thus, the bulk of the cinnamate and p-coumarate produced by the yeast strains was secreted into the culture media.

Fig. 3. HPLC fractionation of phenolic metabolites produced by vector control, PAL alone, and triple-expressing yeast strains. HPLC chromatograms of extracts from different yeast strains, with absorbance at 290 nm. Insets show peaks eluting between 30 and 40 min in an expanded scale. Yeast strains were grown to mid-log phase in glucose media, transferred to inductive galactose media for 18 h. After 2 h incubation in fresh media supplemented with 500 µM Phe, media and cells were extracted by ether and acetone, respectively, and both fractions were pooled together for HPLC analysis. All chromatograms are at the same scale. Arrowhead indicates phenylpyruvate in all strains with retention time at 36.88 ± 0.09 min and maximum absorption at 290.6 nm, arrow indicates cinnamate in PAL2/4 and T2/4 strains with retention time at 35.15 ± 0.08 min and maximum absorption peak at 281.1 nm, and the filled circle indicates p-coumarate in T2/4 strain with retention time of 35.84 ± 0.10 and maximum absorption peak at 309.6 nm. Retention times and ultraviolet spectra of these phenolic compounds were confirmed with authentic standards.
elution profile was quantified. [3H]Phe was independently and the radioactivity associated with each fraction across the cells and culture media of [3H]Phe-fed yeast cultures (con- genic strains. Labeled metabolites extracted from greater detail carbon flux into Phe-derived compounds in the transgenic yeast strains. The potential pathways and metabolites are illus-
nol, respectively, by a single phenylacrylic acid decarboxylase (PAD). These potential pathways and metabolites are illus-
mediates.

In parallel, we used piperonylic acid (PA), an effective and specific inhibitor of C4H with a \( K_i \) of 17 \( \mu \)M (37), to independ-
ance by HPLC. Cells were induced for 18–20 h were resuspended in fresh media containing 0, 0.5, or 5 mM Phe for 2 h. P2 and P4 indicate PAL2- or PAL4-expressing yeast strains, and T2 and T4 indicate PAL2, C4H, and CPR2, or PAL4, C4H, and CPR2 triple-expressing yeast strains, respectively. Levels of p-coumarate and cinnamate in media and cells were determined by HPLC. Data are mean ± S.E. from at least three independent cultures.

to phenylacetaldehyde, and reduction of phenylacetaldehyde to phenylethanol (34). Additional phenolic compounds such as phenylacetate could be synthesized from these catabolic inter-
ates. S. cerevisiae is also reported to metabolize various (hydroxy)cinnamic acids (35, 36), for example the decarboxyla-
tion of cinnamate and p-coumarate to styrene and 4- vinylphenol, respectively, by a single phenylacrylic acid decarboxylase (PAD). These potential pathways and metabolites are illustrated in Fig. 5.

We used [3H]Phe-radiotracer assays to characterize in greater detail carbon flux into Phe-derived compounds in the transgenic yeast strains. Labeled metabolites extracted from the cells and culture media of [3H]Phe-fed yeast cultures (control, PAL or triple gene expressers) were fractionated by HPLC, and the radioactivity associated with each fraction across the elution profile was quantified. [3H]Phe was independently measured by HPLC fractionation of the aqueous medium fraction remaining after ether extraction. Authentic standards for all potential metabolites described above, except 4-vinylphenol, were used to verify complete separation of these compounds and guide product identification.

The results of these metabolic profiling experiments from vector control, PAL alone, or triple-expressing strains are shown in Table I. In agreement with data in Fig. 3, only a small amount of [3H]cinnamate (0.5–1.6% of added label) was found in PAL alone and triple-expressing strains, and a large amount of p-[3H]coumarate (~8.9–10.2% of added label) was found only in the triple expressers. However, additional radioactive metabolites that escaped detection by the previous HPLC analyses were also identified in these yeast strains. [3H]Styrene was detected in all four PAL-containing yeast strains (~1% of added label) but not in the vector control, indicating that cinnamate is further metabolized to this compound, probably by the endogenous enzyme PAD. Due to the lack of an authentic chemical standard, 4-vinylphenol, which could be formed from p-coumarate by the same PAD, could not be definitively identified in triple expressers. However, two additional small radio-
aic peaks specific to triple expressers were identified in fractions collected at 43 and 53 min, each with ~0.5% of the added label, that may be 4-vinylphenol or its metabolic deriv-
atives. The low levels of [3H]styrene and putative 4-[3H]vinyl-
phenol in PAL-only expressers and triple expressers, relative to p-[3H]cinnamate levels in triple expressers, suggests that metabolism of cinnamate and p-coumarate to these compounds is not a major metabolic route in these strains.

Radioactive metabolites common to all the yeast strains should represent metabolites synthesized from Phe by endo-
ogenous yeast enzymes. A major Phe-derived metabolite appeared to be phenylethanol, a known endproduct of the Phe catabolic pathway (Fig. 5). Radioactive metabolites with reten-
tion times similar to phenylpyruvate and phenylacetate were also detected. The catabolic intermediate phenylacetaldehyde was expected to be present but would have eluted before the first fraction (23 min) in the HPLC program and could not be analyzed further. However, the total radioactivity eluted before 23 min was insignificant (~1%), and thus this chemical prob-
ably constitutes only a minor portion of the Phe-derived metab-
olites. Barely detectable levels of radioactivity were eluted at around 27 and 50 min, each with less than 0.2% radioactivity, in all yeast strains, but these were not further identified.

Approximately 30% of the radioactivity from [3H]Phe supplied to the yeast cells was not recovered and was presumed to be incorporated into proteins.

About 50–60% of the Phe label was recovered as unmetabo-
ized Phe in all strains, but this varied according to the strain. An important finding was that Phe utilization was significantly higher in triple-expressing strains T2 and T4 relative to PAL alone expressing strains or the vector control strain. The differ-
ence between the level of residual [3H]Phe recovered from triple-expressing strains T2 and T4 (about 50% of added label) and that found in PAL only expressing strains or in the vector control strain was roughly comparable to the amount of p-[3H]coumarate that accumulated in the triple expressers.

In parallel, we used piperonylic acid (PA), an effective and specific inhibitor of C4H with a \( K_i \) of 17 \( \mu \)M (37), to independ-
ently re-access the role of C4H activity in establishing this metabolic channel in triple expressers. When fed to C4H expres-
ing yeast strains, the minimum concentration of PA re-
quired for the complete inhibition of C4H activity in vivo was found to be 10 \( \mu \)M, and at this concentration, PA had no effect on PAL activity (data not shown). For precise comparison, PA-treated and non-treated triple expressers in Table I were derived from the same batches of yeast cultures. When 10 \( \mu \)M PA was added to yeast strains T2 and T4, [3H]Phe incorporation into p-coumarate was virtually blocked (Table I). In both triple expressers, PA inhibition of C4H activity resulted in small increases not only in cinnamate and its metabolite sty-
rene, but also in phenylethanol, the final product of an inde-
pendent metabolic pathway that shares Phe as an initial sub-
strate. PA-treated strains were also reduced in their ability to more efficiently use Phe, as evidenced by increases in residual [3H]Phe found in PA-treated triple expressers relative to the non-treated triple expressers (Table I). These results, together with those in Fig. 3, indicate that C4H enzyme activity is a strong driver of the metabolic channel into phenylpropanoid metabolism in yeast created by co-expression of PAL and C4H.
Investigation of a Potential PAL/C4H Multienzyme Complex in Yeast—As postulated in plant systems, it is possible that a tight MEC between PAL and C4H could promote metabolic flux in transgenic yeast strains. Our attempts to use yeast two-hybrid assays to demonstrate protein-protein interactions between PAL2 or PAL4 and C4H derivatives in which the membrane-anchoring domain was deleted provided no evidence for PAL-C4H interaction (data not shown). Subsequently, we used a double labeling strategy to examine whether [3H]cinnamate formed endogenously from PAL is preferentially used by C4H, relative to [14C]cinnamate introduced externally in triple expressers. TABLE II

**TABLE II**

| Product          | T2       | Strain | T4       |
|------------------|----------|--------|----------|
| p-Coumarate      | 0.58, 0.50 | 0.47, 0.54 |
| Styrene          | 1.37, 1.71 | 1.63, 1.55 |

**TABLE I**

Major [3H]-labeled phenolic metabolites

| Strains | 10µM PA | PET | PAC | p-CA | t-CA | PPA | Styrene | Phe |
|---------|--------|-----|-----|------|------|-----|---------|-----|
| Vector  | −      | 7.5 | 2.4 | 0    | 0    | 1.2 | 0       | 62.8|
| PAL2    | +      | 2.9 | 2.2 | 0    | 1.6  | 0.4 | 1.0     | 60.1|
| T2      | +      | 3.6 | 2.3 | 0.2  | 1.2  | 0.3 | 1.4     | 56.4|
| T2      | −      | 2.9 | 2.3 | 10.2 | 0.6  | 0.8 | 1.0     | 50.5|
| PAL4    | −      | 3.0 | 2.1 | 0    | 0.6  | 0.7 | 1.1     | 61.3|
| T4      | +      | 4.8 | 2.4 | 0    | 0.8  | 0.6 | 1.4     | 59.5|
| T4      | −      | 3.3 | 2.2 | 8.9  | 0.5  | 0.8 | 0.8     | 51.7|

**Fig. 5.** Phenylalanine metabolism in yeast triple-expressing strains (transformed by PAL, C4H, and CPR), showing reconstruction of the entry point into phenylpropanoid metabolism in this host. Endogenous yeast reactions involved in Phe catabolism are shaded in gray. The new pathway generated by introduction of PAL, C4H, and CPR genes is boxed in black, and endogenous pathways that metabolize cinnamate and p-coumarate are circled. ADH, alcohol dehydrogenase; DC, decarboxylase; PAD, phenylacrylic acid decarboxylase; TA, transaminase. Width of arrows is proportional to carbon flux, and potentially reversible reactions are indicated by double arrows. Question marks indicate potential but uncharacterized reactions or products.

**TABLE II**

Ratio of incorporation of [3H]Phe and [14C]cinnamate into p-coumarate or styrene in yeast strains T2 and T4

| Product          | T2       | Strain | T4       |
|------------------|----------|--------|----------|
| p-Coumarate      | [3H]55, [3H]50 | 0.47, 0.54 |
| Styrene          | 1.37, 1.71 | 1.63, 1.55 |

Numbers represent the percentage of [3H] recovered from cells and media by HPLC fractionation, out of initial [3H]Phe feeding. **Abbreviations:** PET, phenylethanol; PAC, phenylacetate; pCA, p-coumarate; tCA, trans-cinnamate; PPA, phenylpyruvate.

**Numbers** represent the percentage of [3H] recovered from cells and media by HPLC fractionation, out of initial [3H]Phe feeding.

**Investigation of a Potential PAL/C4H Multienzyme Complex in Yeast**—As postulated in plant systems, it is possible that a tight MEC between PAL and C4H could promote metabolic flux in transgenic yeast strains. Our attempts to use yeast two-hybrid assays to demonstrate protein-protein interactions between PAL2 or PAL4 and C4H derivatives in which the membrane-anchoring domain was deleted provided no evidence for PAL-C4H interaction (data not shown). Subsequently, we used a double labeling strategy to examine whether [3H]cinnamate formed endogenously from PAL is preferentially used by C4H, relative to [14C]cinnamate introduced externally in triple expressers. The [3H]Phe and [14C]cinnamate fed simultaneously to the triple-expressing strains T2 and T4 would be incorporated into p-coumarate by way of the PAL and C4H reactions.
and also, though to a much lower extent, incorporated into styrene via the coupled reactions of PAL and PAD (Fig. 5). Assuming that the endogenous yeast PAD and plant PAL enzymes do not have the ability to associate in a multienzyme complex, the ratio of $^{3}H$]/stimyrene to $^{14}C$]styrene can be used as an internal control for the case of two physically separated enzymes in a linear pathway. If PAL and C4H were organized in a functional multienzyme complex in the yeast cells, significant amounts of cinnamate endogenously made by PAL will be directly and preferentially transferred to C4H, and thus the ratio of $^{3}H$/to $^{14}C$ in p-coumarate should be significantly higher than that in styrene.

In two repetitions of the experiment, the $^{3}H$/$^{14}C$ ratios of p-coumarate did not exceed those of styrene in either triple-expressing strain T2 or T4 (Table II) but in fact were lower. The low ratio of p-$^{3}H$]coumarate/p-$^{14}C$]coumarate relative to the $^{3}H$/$^{14}C$ ratio for styrene indicates that $^{3}H$]cinnamate endogenously synthesized from PAL was not preferentially used by C4H in the yeast strains. These results strongly argue against the concept that a MEC readily forms between PAL and C4H when they co-occur in one cell, and suggest that such a complex is not required for efficient channeling of Phe into p-coumarate.

If this metabolic channel does not depend on physical proximity of PAL and C4H in yeast, we reasoned that it might not even be necessary to express PAL and C4H in a single cell to achieve efficient conversion of Phe to p-coumarate. To test whether efficient conversion could occur even with physically separated PAL and C4H/CPR enzymes, we independently induced and then mixed either strain P2 or P4 with a C4H/CPR dual expressing strain (24). Fig. 6 shows the accumulation of cinnamate and p-coumarate accumulation in these two mixed strains, M2 and M4. In parallel, product accumulation in pure cultures of triple-expressing strains T2 and T4 was measured. The accumulation of cinnamate and p-coumarate in M2 and M4 mimicked that of triple-expressing strains T2 and T4, although the amount of p-coumarate that accumulated in the mixed cultures was about half of that in the triple expressers (Fig. 6).

**DISCUSSION**

We engineered *S. cerevisiae* to express key phenylpropanoid entry point enzymes, PAL, C4H, and CPR, to understand their coordinated roles in redirecting metabolic flux and to investigate the proposed MEC in this heterologous host. Although efficient metabolic flux from Phe into p-coumarate was readily accomplished by co-expression of these enzymes, a key finding was that biochemical coupling of PAL and C4H appears sufficient to efficiently redirect and promote metabolic flux from Phe to p-coumarate without physical interaction between the two enzymes. This is supported by data from $^{3}H$]Phe feeding, double labeling assays, and mixed culture assays.

In the double labeling assay (Table II), it is difficult to explain why $^{3}H$/$^{14}C$ ratio of styrene is higher than that of p-coumarate, but perhaps intermediate transfer from cytosolic to cytosolic enzyme (coupled PAL-PAD reaction) is preferred to that from cytosol to ER (coupled PAL-C4H reaction). As previously proposed (6–8), tight feedback inhibition by cinnamate in the entry point of the pathway could explain the impeded carbon flux in PAL alone or C4H-inhibited expressers. The intracellular cinnamate pools in PAL alone and C4H-inhibited expressers might rapidly inhibit PAL activity, but in triple expressers this metabolic inhibition could be released by efficient conversion of cinnamate to p-coumarate. With the poplar recombinant PAL2 enzyme, the $K_{i}$ for cinnamate inhibition was determined to be ~0.4 mM (31), a level unlikely to be reached in any of the engineered yeast strains, making the *in vivo* importance of feedback inhibition questionable. Also, our data show that increasing the external Phe concentration to 5 mM in PAL-alone expressing strains resulted in an enhanced...
consistent with the finding that carbon allocation into monolignol products such as xylem, or leaf epidermal cells. Involvement of MEC formation, if it occurs at all, may require plant-specific factors and may be restricted to cells producing a more diversified pattern of phenylpropanoid metabolites that require additional regulatory mechanisms to partition the fluxes.

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