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Phenylalanine ammonia-lyase (PAL) from tobacco (*Nicotiana tabacum*): characterization of the four tobacco *PAL* genes and active heterotetrameric enzymes[1]

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PAL (L-phenylalanine ammonia-lyase), the first enzyme of phenylpropanoid biosynthesis, is often encoded by multigene families in plants. A PCR-based approach was used to isolate cDNA clones corresponding to the four *PAL* genes of tobacco (*Nicotiana tabacum*). By careful comparison of cDNA and genomic clones, a new *PAL* gene (*PAL4*) was defined. PCR amplification of *PAL* sequences from cDNA led to the generation of chimaeric clones between *PAL1* and *PAL4*, and incorrect annotation of *PAL4* ESTs (expressed sequence tags) as *PAL1* in the EST database has given rise to a randomly shuffled tentative consensus sequence. The *PAL2* previously described in the literature was shown, by domain swapping experiments with *PAL1*, to possess a single nucleotide substitution leading to an inactive enzyme. The altered amino acid resulting from this substitution maps to the base of the active site pocket in the three-dimensional structure of PAL. The inactive *PAL2* allele could not be recovered from 13 different tobacco cultivars examined. PALs 1–4 were co-expressed in multiple plant organs, and were also co-induced following exposure of cell cultures to yeast elicitor or methyl jasmonate. All four tobacco PAL proteins expressed in *Escherichia coli* displayed normal Michaelis–Menten kinetics, with K_m values between 36 and 60 μM. Co-expression of different PAL proteins in *E. coli* resulted in formation of heterotetramers, which possessed kinetic properties within the same range as those of the individual homotetramers. The potential physiological function of heterotetrameric PAL forms is discussed.

Key words: domain swapping, gene family, heterotetramer, L-phenylalanine ammonia-lyase (PAL), phenylpropanoid metabolism, tobacco (*Nicotiana tabacum*).

INTRODUCTION

The phenylpropanoid pathway leads to the biosynthesis of a wide range of plant natural products including flavonoids, hydroxycinnamic acids, coumarins, stilbenes, lignin and condensed tannins, which collectively have diverse biological functions as phytoalexins, signal molecules, structural components, flower pigments or UV protectants [1]. PAL (L-phenylalanine ammonia-lyase; EC 4.3.1.24) plays a crucial role at the interface between plant primary and secondary metabolism by catalysing the deamination of L-phenylalanine to form *trans*-cinnamic acid.

The properties, regulation, expression and cellular distributions of PAL have been extensively studied [2], and the crystal structures of PAL from parsley [3] and the yeast *Rhodosporidium toruloides* [4] have been determined. The number of PAL genes ranges from one to five in most plant species studied [5–7]. The various proteins encoded are assumed to be involved in different secondary metabolic pathways, for example for formation of structural components or plant defence, although in many cases this remains to be experimentally determined. In the French bean (*Phaseolus vulgaris*), *PAL1* is expressed in roots, leaves and shoots, *PAL2* is expressed in roots, shoots and petals, and *PAL3* is expressed only in roots. All three bean *PAL* genes are activated by wounding, whereas only *PAL1* and *PAL3* are induced by fungal infection [8]. In quaking aspen, one PAL form may be associated with formation of condensed tannins, the other with lignin production [9].

Although the phenylpropanoid pathway in tobacco (*Nicotiana tabacum*) has been the subject of several studies, and PAL has been partially purified from tobacco leaves and suspension cultures [10,11], information about tobacco PAL genes/enzymes is limited as well as misleading. Fukasawa-Akada et al. [12] identified four *PAL* genes in *N. tabacum* by genomic Southern blot hybridization. They could be divided into two subfamilies, with a member of each family originating from each of the two progenitor species *Nicotiana tomentosiformis* and *Nicotiana sylvestris* (Table 1). The existence of another tobacco *PAL* gene (named *PAL2*) with high sequence similarity to *PAL1* [TC (Tentative Consensus) 7464 in The Institute for Genomic Research Gene Index (http://compbio.dfci.harvard.edu/tgi/)] and belonging to the same subfamily, was predicted [12]. However, different designations for the *PAL* family in tobacco had previously been established, and the *PAL*' gene identified by Nagai et al. [13] had also been named *PAL2* (TC3936) (designated *PAL3* by Fukasawa-Akada et al. [12]). A *PAL3* was characterized by Pellegrini et al. [14] (TC4054), but designated *PAL4* by Fukasawa-Akada et al. [12]. To date, none of the corresponding PAL enzymes has been shown to be catalytically active *in vitro*, and the fourth tobacco *PAL* gene has yet to be described.

Abbreviations used: C4H, cinnamate 4-hydroxylase; DTT, dithiothreitol; EST, expressed sequence tag; HA, haemagglutinin; IEF, isoelectric focusing; 2-ME, 2-mercaptoethanol; MIO, methylidine imidazolone; MJ, methyl jasmonate; NtGI, *Nicotiana tabacum* Gene Index; PAL, L-phenylalanine ammonia-lyase; RT–PCR, reverse transcription–PCR; TC, tentative consensus; TCEP, tris-(carboxyethyl)-phosphine; UTR, untranslated region; YE, yeast elicitor.

[1] This paper is dedicated to the memory of Chris Lamb FRS, CBE (1950–2009).

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The nucleotide sequence data reported will appear in GenBank®, DDBJ, EMBL and GSDB Nucleotide Sequence Databases under the accession numbers EU883669 and EU883670.

Table 1 The four tobacco *PAL* genes

The current annotations are listed in comparison with their original designation by Fukasawa-Akada et al. [12].

PAL gene	Progenitor species	Original designation	TC number	GenBank® number	Reference
Subfamily I					
PAL1	*N. tomentosiformis*	*PAL1*	TC7464	M84466	[12]
PAL4	*N. sylvestris*	*PAL2*		EU883669/70	The present study
Subfamily II					
PAL2	*N. tomentosiformis*	*PAL3*	TC3936	D17467	[13]
PAL3	*N. sylvestris*	*PAL4*	TC4054	X78269	[14]

PAL is highly regulated at both the transcriptional and post-transcriptional levels, and metabolic channelling involving enzyme co-localization is proposed to play a major role in flux control through this enzyme [15]. In this respect, tobacco PAL1 was found to co-localize with the membrane-bound C4H (cinnamate 4-hydroxylase; EC 1.14.13.11) cytochrome P450, whereas PAL2 was localized in the cytosol [16,17]. Characterization of all four PAL enzymes in tobacco will help determine the structural requirements for metabolic channelling.

In view of the discrepancies in the nomenclature used in different studies, and the incomplete description of the tobacco *PAL* gene family, a re-evaluation of the molecular biology and biochemistry of tobacco PALs is warranted. In the present paper, we report the molecular characterization of the full complement of PAL enzymes from tobacco, including PAL4. All four PAL enzymes were biochemically characterized and their expression profiles studied. Surprisingly, *PAL2* sequences previously reported in the literature encode an inactive PAL protein as the result of a single nucleotide polymorphism which changes an amino acid at the base of the active site pocket. The origins of this and other confusing features of the tobacco *PAL* sequences represented in current gene databases are discussed. We also provide direct evidence that PAL is able to form heterotetramers when co-expressed in *Escherichia coli*, and discuss the physiological implications of this finding.

EXPERIMENTAL

Plant material

Tobacco plants were of cultivar *N. tabacum* cv Xanthi nc for all experiments reported in this paper, except the screening for inactive *PAL2* alleles. This was performed with leaf material from cultivars Xanthi nc, Kt19, KDH-960, TN 90, TND 94, MD 40, MD 402, MD A30, KY 908, Oxford 207, Metacomet, Poquonock and SCANTIC. The last 11 cultivars were obtained from the USDA National Plant Germplasm System (http://www.ars-grin.gov/npgs/). Xanthi and Kt19 were from our own seed collection. Plants were grown in soil or Turface MVP™ (Profile Products) under greenhouse conditions (16 h day length). Plant tissues were collected, frozen in liquid nitrogen and pulverized in a tissue grinder (6770-Freezer/Mill, SPEX CertiPrep). *N. tabacum* NT-1 cell suspension cultures were maintained by regular subculture into fresh culture medium (Murashige and Skoog Basal Medium) and incubated at 24 °C with shaking at 130 rev./min. At 3 days after subculture, cultures (40 ml batches) were treated with YE (yeast elicitor; 50 μg/ml glucose equivalents) or 25 μM MJ (methyl jasmonate) in ethanol [16,18]. Control cells received the same amounts of distilled water or ethanol.

Chemicals

Unless specified, chemicals were obtained from Sigma–Aldrich.

Isolation of *PAL* genes

Total RNA was extracted from frozen tissue using either TRI Reagent (Molecular Research Center) according to the manufacturer's protocol, or the RNeasy Plant Mini Kit (Qiagen). DNA was extracted with a DNeasy Plant Kit (Qiagen).

RNA and DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and RNA quality was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies). First strand cDNA synthesis was performed with Superscript III reverse transcriptase (Invitrogen).

PCR primers were designed using either the Lasergene sequence analysis software DNASTAR or Primer3 software (http://frodo.wi.mit.edu/primer3/). Specific primers for the 5′- and 3′-UTRs (untranslated regions) were employed to obtain near-full-length cDNA clones for the four *PAL* genes and, in the case of *PAL4*, also a near-full-length genomic clone. For a list of primers see Supplementary Table S1 (at http://www.BiochemJ.org/bj/424/bj4240233add.htm).

PAL4 was amplified from genomic DNA with TaKaRa LA Taq DNA polymerase (TaKaRa Bio), and from cDNA with KOD Hot Start DNA Polymerase (Novagen), according to the manufacturers' protocols. PCR products were purified, cloned into the pGEM-T Easy vector system (Promega) and used for transformation of *E. coli* (MAX Efficiency DH5α competent cells, Invitrogen). The clones were sequenced, and, in a second round of PCR, NdeI and XhoI restriction sites were introduced at the ends of the coding regions using Cloned Pfu DNA polymerase (Stratagene).

Semi-quantitative RT–PCR (reverse transcription–PCR) was performed with specific primers for the four *PAL* genes (Supplementary Table S1) using GoTaq DNA polymerase (Promega). PCR products were visualized with SybrSafe DNA Gel Stain (Invitrogen). Analysis of agarose gels was performed with ImageQuantTL. The 1 kb band of the DNA ladder (Promega) was used for calibration (=100).

Plasmid construction and generation of PAL variants

For protein expression, the *PAL* sequences were cloned into the expression vector pET15b with an N-terminal His-tag and a thrombin cleavage site or, with the stop codon removed, into pET29a(+) with a C-terminal His-tag (Novagen).

Hybrid PALs were generated using the gene splicing overlap extension approach as described previously [19]. Gene fragments were recombined by PCR using Cloned Pfu DNA polymerase (Stratagene). Single-exchange site-directed mutagenesis was performed with the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol.

The N-terminal HA-epitope (amino acid residues 98–106 of human influenza virus haemagglutinin: YPYDVPDYA) was introduced into the *PAL2* and *PAL4* sequences by PCR with primers coding for the HA nucleotide sequence (see Supplementary Table S1).

For testing of the formation of PAL heterotetramers, two different *PAL* genes were co-expressed in the pETDuet-1 vector system (Novagen). *PAL1* with SacI/NotI restricition sites was cloned into MCS1 for expression with an N-terminal His-tag. HA-tagged *PAL2* or *PAL4* with NdeI/XhoI restriction sites were

cloned into MCS2. The control vectors for these experiments contained only one *PAL* sequence.

Expression and purification of recombinant PAL

Transformed *E. coli* Rosetta 2 (DE3) pLysS cells (Novagen) were grown in LB (Luria–Bertani) broth containing 0.1 mg/ml carbenicillin (pET15b, pETDuet-1) or 0.05 mg/ml kanamycin (pET29a) and 34 μg/ml chloramphenicol at 37 °C. Expression of recombinant protein was induced by addition of 0.25 mM IPTG (isopropyl β-D-thiogalactoside). Cells were harvested after 6 h of incubation at 28 °C. Recombinant PAL proteins were purified using the MagneHis Protein Purification System (Promega). Purified protein was stored in 50 mM Tris/HCl, pH 8.0, 30 % glycerol and 1 mM TCEP-HCl [tris-(2-carboxyethyl)-phosphine hydrochloride; Hampton Research] at − 80 °C.

Analyses were carried out with tagged PAL preparations unless otherwise stated. The N-terminal His-tag was removed where indicated with thrombin protease according to the manufacturer's recommendations (Novagen). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories) using BSA as standard.

In experiments investigating PAL heterotetramer formation, PAL proteins were expressed in *E. coli* and the cells were then lysed in BugBuster Master Mix (Novagen) in the presence of protease inhibitor cocktail (EDTA-free, Roche Applied Science, Mannheim, Germany) and 1 mM TCEP-HCl. The supernatant was incubated for 30 min at 4 °C in 20 mM Tris/HCl, pH 7.5. In control experiments, PAL proteins with His-tags or HA-tags were expressed separately, and then incubated together at comparable concentrations. Purification was performed with the MagneHis Protein Purification System.

Protein analysis

Size-exclusion chromatography was performed with Superdex 200 (10/30) on an Äkta purifier (GE Healthcare) in 50 mM Tris/HCl, pH 8.5, and 150 mM NaCl, at a flow rate of 0.5 ml/min. Gel Filtration Standard (Bio-Rad Laboratories) was used for calculating the molecular mass of the PAL tetramer. Homogeneity of the purified protein was verified by analysis on a denaturing NuPAGE 4–12 % Bis-Tris gel (Invitrogen). Gels were stained with SimplyBlue Safe Stain (Invitrogen). IEF (isoelectric focusing) was performed with vertical Novex IEF gels pH 3–10 (Invitrogen) using non-denaturating Novex IEF sample buffer (Invitrogen). IEF Marker (SERVA Electrophoresis, Heidelberg, Germany) was used for determining pI values. Gels were stained with Coomassie Blue.

PAL proteins (homo- and hetero-tetramers) were identified by protein gel blot analysis. Proteins were transferred on to nitrocellulose using an XCell II Blot Module (Invitrogen). His-tagged PAL was identified with His-tag monoclonal antibody from mouse (Novagen), and PAL with HA-epitope was identified with anti-HA antibody produced in rabbit. The secondary antibody used was either goat anti-mouse or goat anti-rabbit IgG–alkaline phosphatase conjugate. Colour reaction was performed with Western Blue stabilized substrate for alkaline phosphatase (Promega).

Assay of PAL activity

PAL activity was determined spectrophotometrically as described by Edwards and Kessmann [20]. Formation of the product *trans*-cinnamic acid was monitored at 290 nm with a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). The reaction was routinely carried out at 30 °C with 0.1 μg/ml protein in 50 mM Tris/HCl, pH 8.5, containing 12 mM L-phenylalanine; the control contained the same amount of D-phenylalanine. Kinetic data analysis was performed using the computer program HYPER32.exe, Version 1.0.0.

RESULTS

PAL4 is a new *PAL* gene closely related to *PAL1*

Specific primers for the 5′- and 3′-UTRs of *PAL1* [12], *PAL2* [13] and *PAL3* [14] (Supplementary Table S1) were initially used to clone *PAL1–PAL3* from cDNA generated using RNA from yeast elicited tobacco NT-1 cell suspension cultures.

The sequences of the PCR-amplified products of *PAL1* were mostly identical with the published sequence, but some of the clones contained a *PAL1* sequence with different 5′-UTR and N-terminal sequences. The considerable differences in the 5′-UTR suggested that the two *PAL1* sequences we had amplified might actually be from different genes rather than representing different alleles of the same gene.

In view of the equivocal results with amplification of *PAL1*, we set about acquiring the complete sequence of the published *PAL1* gene and the new *PAL* gene (which we named *PAL4*). New specific primers (Supplementary Table S1) were designed for the 5′-UTRs of *PAL1* and *PAL4* and the 3′-UTR of *PAL1*, and both cDNA and genomic DNA were used as templates. These primers amplified two different *PAL* sequences from genomic DNA extracted from tobacco leaves. Of six clones sequenced, two were identical with the published *PAL1* sequence [12], and four represented the new *PAL4* sequence.

These results allowed the design of a specific 3′-UTR primer for *PAL4* (Supplementary Table S1), which was used to amplify *PAL4* from cDNA derived from elicited tobacco cell culture, confirming our assumption that *PAL4* is a separate gene and is expressed in tobacco. *PAL4* sequences derived from cDNA and genomic DNA were identical, except for the intron contained in the genomic sequence. Both nucleotide sequences were deposited in the GenBank® database (EU883669 and EU883670).

For comparison, *PAL1* was amplified in parallel from cDNA with specific 5′- and 3′-UTR primers (Supplementary Table S1). In addition to amplicons identical with the published *PAL1* sequence [12], this also resulted in amplification of chimaeric *PALs*, with the 5′-UTR and N-terminus matching the newly identified *PAL4* and the C-terminus matching *PAL1*, although the sequence switch occurred in different places in different clones. Furthermore, comparison of the *PAL4* sequence with the TC sequence of *PAL1* derived from EST (expressed sequence tag) sequencing [TC7464, DFCI NtGI (*N. tabacum* Gene Index)] showed that the assembled TC is in fact a mixed *PAL1*/*PAL4* sequence. Alignment of *PAL1* and *PAL4* with seven of the ESTs annotated as *PAL1* and used for establishing the *PAL1* TC-sequence revealed that several of the ESTs are, in fact, *PAL4* sequences (EB443260, EB447219 and DW004402). Thus the C-terminus and 3′-UTR of TC7464 is a random mixture of *PAL1* and *PAL4* (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/424/bj4240233add.htm).

PAL4 has high nucleotide sequence identity with *PAL1* in the coding region (97.3 %, giving 98.3 % identity at the protein level) (Supplementary Table S2 at http://www.BiochemJ.org/bj/424/bj4240233add.htm). Similarity of the nucleotide sequence of *PAL4* to those of *PAL2* and *PAL3*, which both belong to subfamily II [12], is slightly lower, at 83.3 % and 83.5 % respectively. The above results suggest that the very close sequence identity

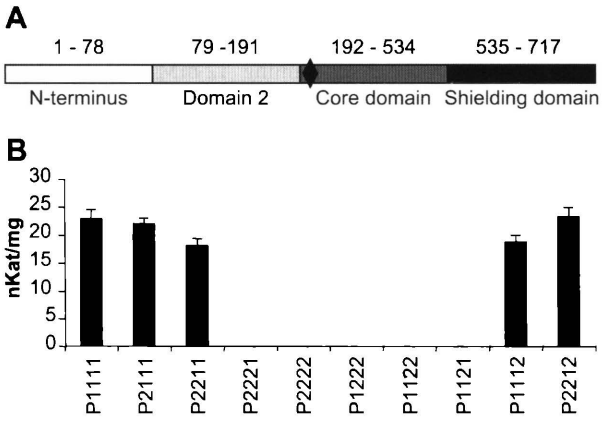

Figure 1 Effects of domain swapping on PAL activity

(**A**) The four domains of PAL. The active site (◆) is situated at the beginning of domain 3 (core domain, residues 203–205, numbering for PAL4). (**B**) Specific activity of hybrid PALs. PAL constructs with an N-terminal His-tag were purified with the MagneHis Protein Purification System. Assays were carried out at 30 °C with 0.2–2 μg/ml protein in 50 mM Tris/HCl, pH 8.5, containing 12 mM L-phenylalanine. Hybrid PALs are designated according to the origin of the four domains, where PAL1=P1111 (domains 1–4 originating from PAL1), PAL2=P2222 (domains 1–4 originate from PAL2), P1121 (domains 1, 2 and 4 originate from PAL1, domain 3 from PAL2), etc. Results are means ± S.D. for triplicate determinations.

between the open reading frames of *PAL1* and *PAL4* can lead to the generation of chimaeric *PAL1:PAL4* amplicons when using PCR, as has been described previously for target sequences of other multigene family members [21,22], and to incorrect TC assembly from ESTs.

With a calculated molecular mass of 78 kDa (DNASTAR), PAL4 encodes the largest of the four tobacco PAL proteins. The *gPAL4* genomic clone consists of 4656 nucleotides with an open reading frame encoding a polypeptide of 717 amino acids. The intron separates exon I (406 bp) and exon II (1748 bp) at the same position as in *gPAL1*; this position for the intron appears to be characteristic of all *PAL* genes [14]. The intron in *PAL4* is 2214 nucleotides compared with 1932 nucleotides in *PAL1*, and the two introns share 52.9 % sequence identity. As predicted by Fukasawa-Akada et al. [12], *PAL4* (designated *PAL2* in their work) has an EcoRI restriction site (which is absent from *PAL1*) in its intron close to the 5′ end of exon 2.

The published tobacco *PAL2* sequence encodes an inactive enzyme

A *PAL2* clone matching the exact sequence described previously [13,23] was obtained from the authors and expressed in *E. coli*. Surprisingly, the purified protein showed no catalytic activity, although it was soluble and formed a tetramer as verified by gel filtration (Supplementary Figure S2 at http://www.BiochemJ.org/bj/424/bj4240233add.htm). The cause of the inactivity was investigated by domain-swapping experiments between PAL1 and PAL2.

The PAL sequence was divided into four segments approximately conforming to the previously described domains [3]; domain 1 (the N-terminus; residues 1–78, numbering according to PAL4), domain 2 (residues 79–191), domain 3 [the core domain including the MIO (methylidene imidazolone) group; residues 192–534)] and domain 4 (the shielding domain; residues 535–717) (Figure 1A). Single domains in the nucleotide sequence were exchanged by gene splicing overlap extension, and the specific activity of the purified recombinant proteins was determined. The hybrid PALs were designated according to the origin of

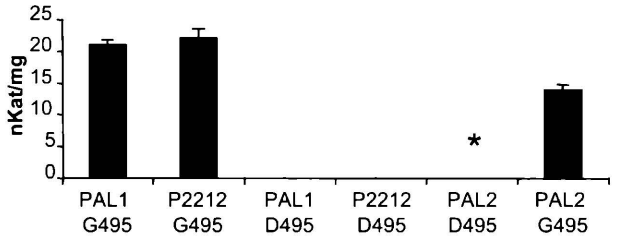

Figure 2 Specific activity of tobacco PAL2 and its D495G mutant

Exchange of glycine with an aspartate residue in PAL1 and P2212 was carried out by single-exchange site-directed mutagenesis. *Recombinant PAL2 with an aspartate residue at position 495 corresponds to the protein sequence reported by Nagai et al. [13]. PAL constructs with an N-terminal His-tag were purified with the MagneHis Protein Purification System. Assays were carried out at 30 °C with 0.2–1 μg/ml of protein in 50 mM Tris/HCl, pH 8.5, containing 12 mM L-phenylalanine. Results are means ± S.D. for triplicate determinations.

the four domains (PAL1=P1111, PAL2=P2222). All wild-type and hybrid PALs gave similar SDS/PAGE protein profiles when expressed in *E. coli*, with the enzyme migrating as a major band of approx. 70 kDa, somewhat smaller than the calculated molecular mass of approx. 80 kDa (Supplementary Figure S3 at http://www.BiochemJ.org/bj/424/bj4240233add.htm). All hybrid PALs containing the core domain 3 of PAL2 were inactive, but all were active when they contained the core domain of PAL1 with a similar specific activity (Figure 1B). P1121 and P2212 differ in ten residues, one of which (an aspartate residue at position 495 which is a glycine in all other PALs) seemed most likely to cause a difference in enzyme activity. This residue was therefore introduced into the sequences of PAL1 and P2212 by single-exchange site-directed mutagenesis. The exchange of G495D in PAL1 and P2212 resulted in an inactive enzyme (Figure 2). Thus the aspartate residue at position 495, which arises due to a single nucleotide polymorphism at nucleotide position 1484 (1469 in the *PAL2* sequence), is responsible for the lack of activity of the PAL2 protein as defined by Nagai et al. [13].

PCR of genomic DNA (from tobacco leaves from 13 different varieties, see the Experimental section) and cDNA (derived from RNA from tobacco cell suspension cultures) with specific primers for the 5′- and 3′-UTRs of *PAL2* (P2UTR5, P2/3UTR3) (Supplementary Table S1) exclusively yielded (active) PAL2 enzyme with a glycine residue in position 495; both this and the G459D mutant were biochemically characterized as described below.

Tobacco *PAL3*

The sequence of the PCR-amplified product of tobacco *PAL3* differed from the sequence published by Pellegrini et al. [14] in only one amino acid, an alanine residue in place of an arginine at position 578 (PAL4 numbering). The TC4054 sequence from the NtGI Gene Index contains an additional nucleotide in this position, causing a frame shift. Since the three other tobacco PALs all contain an alanine in this position, our PAL3 sequence is probably the correct one.

A new classification of the tobacco PAL family

Supplementary Figure S4 (at http://www.BiochemJ.org/bj/424/bj4240233add.htm) shows an alignment of the four PAL protein sequences characterized in this work, and the sequence similarity between their coding regions is summarized in Supplementary Table S2. On the basis of these studies, we propose a new classification of the four tobacco *PAL* genes (Table 1).

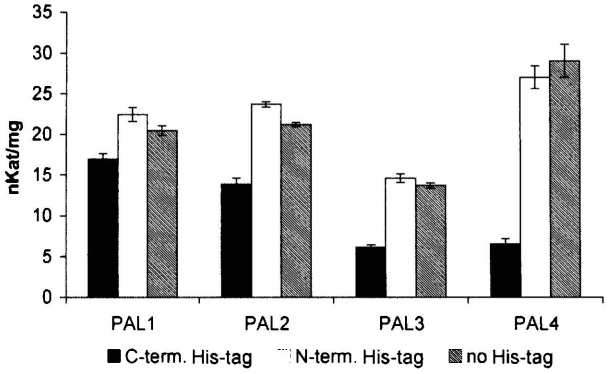

Figure 3 Specific activities of purified recombinant PAL proteins from tobacco

PAL proteins were purified with the MagneHis Protein Purification System. The N-terminal His-tag was removed with thrombin protease where indicated. Assays were carried out at 30 °C with 0.1 μg/ml protein in 50 mM Tris/HCl, pH 8.5, containing 12 mM L-phenylalanine. Results are means ± S.D. for triplicate determinations from duplicate preparations.

Expression and purification of tobacco PAL proteins

The four PAL proteins were expressed in *E. coli* with either C-terminal or N-terminal His-tags. Protein expression levels and yields were comparable for all four PAL proteins independent of the tag used for purification, with approx. 0.4 mg of purified protein obtained per 10 ml of culture. However, PAL3 and PAL4 with C-terminal His-tags had a significantly lower specific activity (approx. 6 nkat · mg^{-1}) than the corresponding PAL1 and PAL2 C-terminal His-tag fusions (17 and 15 nkat · mg^{-1} respectively) (Figure 3). Preparations of all PAL proteins with C-terminal His-tags contained more degradation products that were retained during the purification process than did preparations of N-His-tagged proteins (Supplementary Figure S3), suggesting that the presence of a tag at the C-terminus can destabilize the protein structure leading to degradation and the loss of activity seen in Figure 3. PAL4 without a His-tag exhibited the highest specific activity (29 nkat · mg^{-1}).

Size-exclusion chromatography of PAL1 and PAL2-D495 (both with a C-terminal His-tag) indicated molecular masses of 262 and 270 kDa respectively, consistent with the enzymes existing as tetramers (Supplementary Figures S2 and S5A at http://www.BiochemJ.org/bj/424/bj4240233add.htm). Increasing the protein loading resulted in the presence of an additional peak of approx. 1552 kDa, representing an aggregated hexameric version of the PAL1 tetramer (Supplementary Figure S5B). However, PAL enzymatic activity was primarily associated with the peak of protein representing the PAL tetramer (Supplementary Figure S5C).

All purified recombinant proteins were stable without loss of activity for several months in storage buffer at − 80 °C.

Effects of reducing agents on PAL activity

Sulfhydryl-group-specific reagents can strongly affect PAL activity. Thus tobacco PAL in a relatively crude preparation was competitively inhibited by 2-ME (2-mercaptoethanol) [11], and parsley PAL was irreversibly inhibited by DTT (dithiothreitol) after incubation for several hours [3]. On the other hand, increased enzymatic activity of tobacco PAL was observed in the presence of some reducing agents [10]. This apparent contradiction prompted us to re-investigate the effects of reducing agents on individual recombinant tobacco PAL proteins.

Table 2 Kinetic parameters for purified recombinant PAL proteins from tobacco

Assays were carried out at 30 °C in 50 mM Tris/HCl, pH 8.5. The N-terminal His-tag was removed by digestion with thrombin. The values are the average of triplicate measurement with PAL purified from two independent cell colonies. k_{cat} was calculated assuming four active sites per homotetramer with a subunit size of 78000 Da.

PAL protein	V_{max} (nkat·mg^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
PAL1	14.0 ± 0.6	59.8 ± 7.0	1.09 ± 0.04	18280
PAL2	14.7 ± 0.2	39.5 ± 1.2	1.14 ± 0.02	28933
PAL3	9.8 ± 0.2	36.4 ± 1.5	0.78 ± 0.02	21100
PAL4	19.6 ± 0.3	52.4 ± 2.5	1.53 ± 0.02	29090

All of the recombinant tobacco PAL proteins were competitively inhibited by 2-ME, with a K_i of 3 mM determined for PAL1. However, purification of recombinant PAL1 from *E. coli* in the absence of reducing agents resulted in lower specific activity (16 nkat · mg^{-1}), which could be increased to 22 nkat · mg^{-1} with inclusion of 1 mM TCEP-HCl, a reducing agent which lacks sulfhydryl groups. It is likely that reducing agents are required for stabilizing the enzyme in an active conformation [24], or alternatively preventing the formation of disulfides [4]. Because PALs from several sources appear to be sensitive to sulfhydryl-group-specific reagents [24], TCEP-HCl was added to the storage solution of all PAL proteins at a final concentration of 1 mM. It had no effect on the catalytic properties of the enzyme.

Kinetic properties of tobacco PALs

To determine whether the four different tobacco *PAL* genes might have different functions in plants, we first determined the kinetic properties of the encoded enzymes. Removal of the N-terminal His-tag by cleavage with thrombin did not result in significant changes in the specific activities compared with those of the N-terminal His-tagged protein (Figure 3). However, as the presence of a C-terminal His-tag appeared to affect the stability of the enzyme, particularly for PAL3 and PAL4 (see above), the k_{cat}/K_m ratios for these proteins (9090 and 10290 $s^{-1} \cdot M^{-1}$ respectively) were reduced compared with those of PAL1 and PAL2 His-tag fusions (18650 and 21324 $s^{-1} \cdot M^{-1}$ respectively). Thus for the kinetic studies reported below, all enzymes were expressed as N-terminal His-tag fusions, with the tag subsequently removed by thrombin cleavage.

All four PAL proteins exhibited Michaelis–Menten kinetics with comparable apparent K_m values, ranging from 36.4 μM for PAL3 to 59.8 μM for PAL1 (Table 2). The K_m reported for PAL from tobacco cell suspension cultures (presumably a mixture of the PAL proteins) was 30 μM [11]. In contrast, a K_m value of 220 μM was reported for partially purified tobacco PAL in an assay containing 4 mM 2-ME, a competitive inhibitor [10] (see above). In the present study, comparable values of between 150 μM and 200 μM were measured with recombinant PAL proteins in the presence of 8.6 mM 2-ME.

Near identical turnover numbers of 1.09 s^{-1} and 1.14 s^{-1} were determined for PAL1 and PAL2 respectively, whereas PAL3 had the lowest k_{cat} value of 0.78 s^{-1} and PAL4 the highest (1.53 s^{-1}), assuming four active sites per holoenzyme (Table 2). PAL2 and PAL4 possess similar k_{cat}/K_m ratios, which are slightly higher than those of PAL1 and PAL3 (Table 2). These values are within the same range as the k_{cat}/K_m ratios of between 25 500 and 51 200 $s^{-1} \cdot M^{-1}$ determined for recombinant PAL from *Arabidopsis thaliana* [25]. Overall, our studies indicate that the kinetic properties of the four PAL forms are relatively similar.

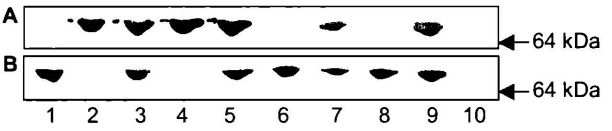

Figure 4 Protein gel blot analysis of PAL1–PAL2 and PAL1–PAL4 heterotetramers

Proteins were purified by affinity binding (to MagneHis beads) specific for the His-tag attached to the PAL1 subunits. The samples were split in two. Each was subjected to denaturing SDS/PAGE, and transferred on to nitrocellulose. One was then incubated with anti-HA antibodies, the other with anti-His antibodies. *E. coli* supernatant (5 μg prior to affinity purification) or 1 μg of purified protein was loaded per lane. (**A**) Detection with anti-HA antibody. (**B**) Detection with anti-His antibody. Lane 1–5, supernatants of His–PAL1, HA–PAL2, PAL1–PAL2, HA–PAL4 and PAL1–PAL4 respectively, to demonstrate antibody specificity; lane 6, purified control for His–PAL1 and HA–PAL2 (proteins expressed independently in *E. coli* and then mixed and co-incubated); lane 7, purified PAL1–PAL2 tetramer; lane 8, purified co-incubation control of His–PAL1 and HA–PAL4; lane 9, purified PAL1–PAL4 tetramer; lane 10, molecular mass standard (Invitrogen), with mass indicated to the right.

Formation of PAL heterotetramers

PAL is generally assumed to form homotetramers, but the appearance of multiple forms of native PAL with similar molecular masses but different pI values has been observed on several occasions [26,27] and the possibility that these may represent heterotetramers has been discussed [28]. We therefore developed an approach to address the ability of tobacco PAL forms to assemble into heterotetramers *in vitro* following co-expression in *E. coli* using the pETDuet-1 vector system.

PAL1 was cloned with an N-terminal His-tag (to be used for purification), whereas a second PAL protein, either PAL2 or PAL4, was co-expressed (in the same *E. coli* cells) with PAL1 as a protein fusion with an N-terminal HA-epitope [17] to allow identification by immunoblotting. Co-expressed PAL forms were isolated from the soluble protein fraction of the bacterial cell lysate with MagneHis Ni-Particles that bind the polyhistidine-tag but not the HA-tag (as checked with individual His–PAL1 and HA–PAL2 prior to analysis of co-expressed proteins). SDS/PAGE analysis of the resulting proteins revealed major PAL subunits of approx. 70 kDa, but this technique is not able to resolve the small differences in molecular mass between the different PAL subunit forms (Supplementary Figure S6 at http://www.BiochemJ.org/bj/424/bj4240233add.htm). However, gel blot analysis of the purified protein with specific antibodies against His-tag and HA-epitope detected the presence of equal signals for both His-tagged PAL1 and HA-tagged PAL2 or PAL4 (Figure 4). In the critical control experiment, the two different PAL proteins were expressed separately in *E. coli*, and the extracts were then mixed, pre-incubated, and purified under the same conditions as above. In this case, gel blot analysis identified only His-tagged PAL1 as being bound to the MagneHis particles; HA-tagged PAL2 was eluted from the beads because it does not contain a His-tag, and was therefore not detected (Figure 4). Thus only co-expression *in vivo* leads to a PAL complex in which the HA-tagged PAL2 can be retained on the MagneHis beads through association with His-tagged PAL1. These data indicate the formation of heteromers (presumably heterotetramers) between PAL1 and PAL2 or PAL4.

IEF and subsequent gel blot analysis of PAL purified from *E. coli* expressing both PAL1 and PAL2 also indicated the formation of heteromers, as PAL1–His mono(tetra)mers were not detected (Figure 5). The pI of PAL1–His was determined to be 6.77 (calculated 6.67), and PAL2–HA determined to be 6.17 (calculated 6.23). The main band for the protein obtained after co-expression of PAL1 and PAL2 in *E. coli* had an estimated pI of 6.38, in good agreement with the calculated pI of 6.44 (DNASTAR) for a PAL1–PAL2 heterotetramer with His- and HA-tags.

Table 3 Kinetic parameters for purified recombinant PAL hetero- and homotetramers

Assays were performed at 30 °C in 50 mM Tris/HCl, pH 8.5. K_{cat} was calculated assuming four active sites per tetramer with a subunit size of 78000 Da. PAL subunits contained either an N-terminal His-tag (PAL1 and PAL2 in homotetramers) or an HA-tag (PAL2 in heterotetramers). Results are means ± S.D. of triplicate measurements with PAL purified from two independent cell colonies.

PAL protein	V_{max} (nkat·mg^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} · M^{-1})
PAL1–His	14.5 ± 0.7	56.2 ± 5.3	1.12 ± 0.06	20000
PAL2–His	17.4 ± 0.9	46.6 ± 2.6	1.35 ± 0.06	28940
PAL1–PAL2	16.2 ± 0.6	51.0 ± 1.5	1.26 ± 0.05	24680
PAL1–PAL2D495	7.4 ± 0.2	46.5 ± 2.9	0.58 ± 0.02	12470

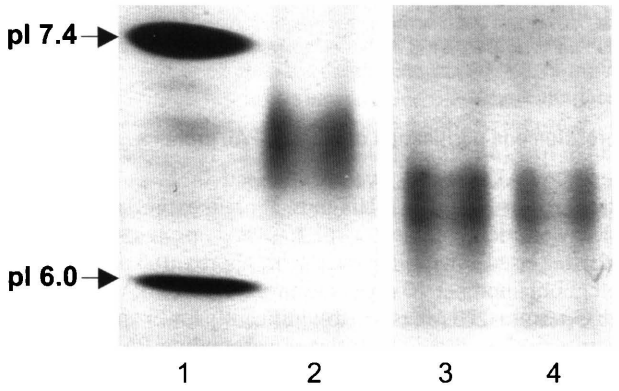

Figure 5 IEF analysis of PAL heterotetramers

PAL forms were separated on a single pre-cast vertical gel (NOVEX pH 3–10). Protein (4 μg) was loaded per lane. Lane 1, IEF markers (with pI values to the left); lane 2, His–PAL1; lane 3, His–PAL1/HA–PAL2; lane 4, His–PAL1/HA–PAL2-inactive. Gel staining was performed with Commassie Blue. The gel was cut vertically to remove additional lanes.

Finally, size-exclusion chromatography of the PAL1–PAL2 heteromer revealed exactly the same molecular mass as seen previously when individual PAL1 and PAL2 preparations were examined, namely a major peak of active PAL protein corresponding to the tetramer, with a catalytically inactive higher-molecular-mass aggregate also being present at high protein loadings (compare Supplementary Figure S7 with Supplementary Figures S5 and S2 at http://www.BiochemJ.org/bj/424/bj4240233add.htm).

We then determined the kinetic properties of the purified PAL1–PAL2 heterotetramer, and compared them with those of a PAL1–PAL2 heterotetramer formed with inactive PAL2 (PAL1–PAL2D495) (Table 3). PAL1–PAL2 exhibited kinetic properties between those of PAL1 and PAL2 homotetramers with N-terminal His-tags. In comparison, PAL1–PAL2D495 heterotetramers had a V_{max} of almost exactly half that of PAL1 with an N-terminal His-tag, although the K_m value was similar to that of PAL2–His. This provides additional evidence for the formation of heterotetrameric forms of PAL, in this case containing both active and inactive subunits.

Negative co-operativity, as has been described for PALs from various plants [26,29,30], was not observed on kinetic analysis of either PAL heterotetramers or mixtures of individual PAL proteins (results not shown).

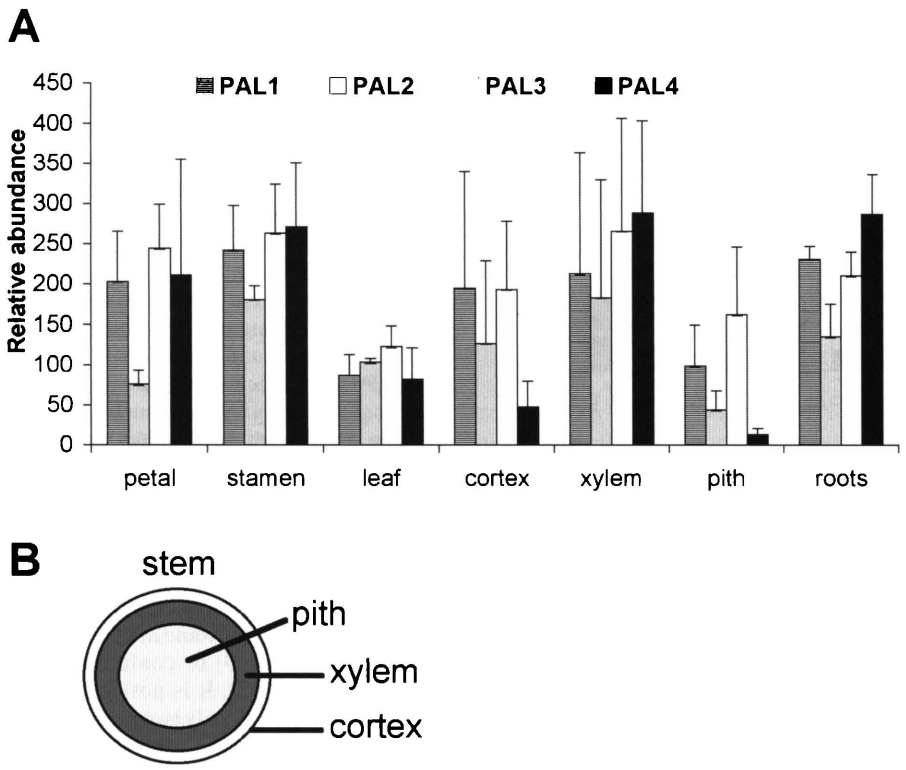

Figure 6 Relative abundance of PAL mRNAs in different tobacco tissues

(**A**) Total RNA extracted from frozen tissues was used for RT–PCR, and cDNA was amplified with specific primers for the four PAL proteins. Quantitative evaluations were based on signal intensity analysed with ImageQuantTL, and the 1 kb band of the DNA ladder (Promega) was used for calibration (=100). Results are means ± S.D. for three biological replicates. (**B**) Cross-section of the stem.

Tissue-specific expression of *PAL* genes

For formation of PAL heterotetramers to have physiological significance *in planta*, the different *PAL* genes must be expressed in the same tissues at the same times. Transcript levels of the different PAL proteins in different plant tissues were therefore investigated by semi-quantitative RT–PCR with *PAL* gene-specific primers for each of the different PAL transcripts. Transcripts of all PALs were found in every tissue investigated (Figure 6) at approximately comparable levels, with some exceptions. *PAL4* was readily detected in stamen, xylem and roots, but was barely detectable in stem pith tissue, suggesting a specific role in lignifying cells. *PAL2* transcript levels in all tissues were lower than those of *PAL1* and *PAL3*, except in xylem and leaves. Transcript levels of most PALs were low in mature leaves and pith.

Elicitation of PAL transcripts

Overall *PAL* expression is induced following exposure of tobacco cell cultures to YE or MJ [23,31]. To determine how this response operates at the level of induction of individual PAL forms, tobacco NT-1 cell suspension cultures were induced with YE or MJ and harvested at different time points. Semi-quantitative RT–PCR was performed with specific primers for the four PAL genes.

PAL1 and *PAL4* were expressed constitutively at higher levels than PAL2 and PAL3 in cell suspension cultures; however, statistical analysis indicated that their apparent induction by YE and MJ was in most cases not significant, due to the high values in unelicited controls at 4 h (Figure 7). This suggests that PAL1 and PAL 4 transcription is very sensitive to handling of the cultures, i.e. during addition of water or dilute ethanol to the controls. *PAL3* was strongly induced within 1 h of exposure to YE, but *PAL2* induction was weaker and delayed by approx. 2 h (results not shown). A previous study had suggested that only *PAL2* (designated PAL A) was elicitor-inducible [23]. Expression of *PAL* transcripts peaked 4 h after exposure to YE, whereas MJ induction of *PAL* transcripts was slower (results not shown), in agreement with earlier results [31].

DISCUSSION

A complete description of the tobacco *PAL* gene family

Fukasawa-Akada et al. [12] proposed the presence of four single-copy *PAL* genes in the tobacco genome belonging to a small multigene family consisting of two subfamilies. *N. tabacum* is amphidiploid and assumed to have arisen by interspecies hybridization between *N. tomentosiformis* and *N. sylvestris* [12,32].

The previously elusive *PAL4* sequence had been wrongly annotated as PAL1 due to the formation of chimaeric *PAL* sequences. However, the identification of the *PAL4* gene in the present study now makes it possible to complete the characterization of the tobacco *PAL* gene family. Here we show that all four *PAL* genes encode functional isoenzymes and that all are expressed in multiple tobacco tissues. *PAL4* corresponds to a gene previously designated as *PAL2* by Fukasawa-Akada et al. [12]. As predicted by this group, *PAL4* has a high similarity to *PAL1* and both belong to subfamily I; however, they derive from different progenitor species.

Biochemical properties of tobacco PAL proteins

PAL1 and PAL4 have comparable enzymatic activities, but vary in the catalytic impacts of their C-termini, as seen by the significant difference in specific activity between PAL1 and PAL4 with

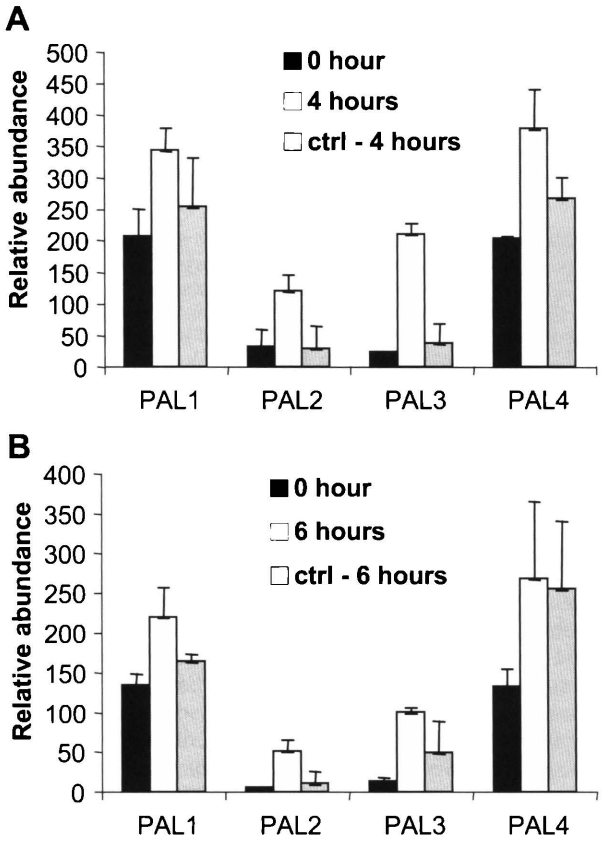

Figure 7 Relative abundance of PAL mRNAs in tobacco NT-1 cell cultures after elicitation with YE (A) or MJ (B)

Cells were elicited at zero time (0 h) and harvested at the indicated time points. The 0 h samples contain no additions, the control samples contained the same amount of water (YE) or ethanol (MJ). Total RNA extracted from frozen tissues was used for RT–PCR, and cDNA was amplified with specific primers for the four *PAL* genes. Quantitative evaluations were based on signal intensity analysed with ImageQuantTL, and the 1 kb band of the DNA ladder (Promega) was used for calibration (=100). Results are means ± S.D. for three biological replicates.

C-terminal His-tags. Although the two proteins share 98.3% similarity at the protein level, PAL4 without a tag has a higher V_{max} and turnover rate than PAL1. The same effect of the C-terminus can be observed among the members of subfamily II. PAL3 with a C-terminal His-tag, similar to the PAL4 derived from *N. sylvestris*, has a 50% lower specific activity than PAL2. Pilbák et al. [33] proposed that the C-terminal multi-helix extension in eukaryotic PALs plays an important role in regulation of the enzyme activity by destabilizing the active conformation of the Tyr^{110}-loop. The highly flexible N-terminus, which shows the highest divergence among the tobacco PAL proteins as well as among PAL sequences from other sources [34], seems to have less impact than the C-terminus on enzymatic properties, as seen from the similar kinetic values for PAL with or without an N-terminal His-tag; the N-terminus may be involved instead in interactions with other cellular components [3].

PAL4 exhibited the highest specific activity of the tobacco PAL proteins (29 nkat · mg^{-1}). This value compares with 40 nkat · mg^{-1} for recombinant PAL from parsley [6], and is slightly higher than that for PALs purified from plant tissues: e.g. rice, 4.6 nkat · mg^{-1} [30]; bean, 5.7 nkat · mg^{-1} [26]; and tomato, 0.5 nkat · mg^{-1} [35]. The k_{cat} data indicate that tobacco PALs have slightly lower turnover rates than PALs from other sources such as *A. thaliana* (1.8–3.2 s^{-1}) [25]; *Nostoc punctiforme* (1.96 s^{-1}); *Anabaena variabilis* (4.3 s^{-1}) [36]; maize (10.6 s^{-1}) [37]; and parsley (13.5 s^{-1}) [38].

Overall, our data are in agreement with published K_m values for recombinant PALs from other plant sources such as parsley (15–24.5 μM) [6], *A. thaliana* (68–71 μM) [25] and French bean (52 μM) [39]. Purified PAL from tomato exhibited slightly higher K_m values with higher deviation between different gene products (121 μM for PAL2 and 840 μM for PAL1) [35], recombinant maize PAL had a K_m of 270 μM [37] and rice PAL had a K_m of 500 μM [30].

As verified in the present study, 2-ME is an inhibitor of PAL. However, reducing agents stabilize the enzyme activity. Because DTT has been found to irreversibly inhibit PAL [3], it is advisable that PAL is purified and stored in the presence of reducing agents without sulfhydryl groups, such as TCEP.

The number of active sites per PAL tetramer has been the subject of debate [6]. Results of early labelling experiments suggested two active sites per homotetramer [24], and Bolwell et al. [26] assumed two active sites per tetrameric enzyme for their calculations with bean PAL. However, the crystal structures of PAL from parsley and *R. toruloides* indicate four functional active sites [3,4]. Regulatory processes have been described for PAL that may render some active sites inaccessible; these include phosphorylation [39] or conformational changes of two highly mobile loops [3,33]. It is possible that in PAL3 and PAL4 with C-terminal His-tags only two active sites are accessible; this could explain why their k_{cat}/K_m ratios are only approx. 50% those of PAL1 and PAL2 with C-terminal His-tags.

Negative co-operativity of PAL preparations has been observed when analysing the kinetics of PAL during early stages of purification [6,26], and the formation of heterotetramers has been suggested as an explanation for this phenomenon. However, we show in the present study that tobacco PAL heterotetramers do not exhibit negative co-operativity, and neither do mixtures of different recombinant tobacco PAL proteins. Altered kinetic properties could conceivably arise as a result of differential post-translational modifications or interactions with specific proteins only present in plant extracts.

Origin and lack of activity of PAL2 G459D

The present study shows that the previously published PAL2 sequence encodes an inactive enzyme. Although the physiological significance of this observation cannot be explained at present, a regulatory function for an apparently inactive *PAL2* allele is possible if the encoded protein can associate into heterotetrameric forms of the enzyme. However, screening of multiple tobacco cultivars failed to recover the inactive *PAL2* allele, suggesting that it is either a rare event in tobacco germplasm, or else an artefact. It is also interesting to note that the sequence of a bean *PAL1* has been published with an aspartate residue in the corresponding position to Asp^{459} in inactive tobacco PAL2, whereas PAL2 and PAL3 both contain a glycine [5]. However, the catalytic activity of this PAL1 enzyme was not determined.

Alignment of tobacco *PAL* sequences with the *PAL* sequences from parsley and the yeast *R. toruloides*, whose crystal structures have been elucidated [3,4], revealed that Gly^{495} (Gly^{493} in parsley) is the first residue of the α-helix α17, one of the three central α-helices forming an electropositive platform for the catalytic MIO group, which is anchored to the three helices through non-covalent bonding [4]. In *R. toruloides* PAL, this residue corresponds to Ala^{506}, whose amide group shares a hydrogen bond with the hydroxy group of Ser^{210} (Thr^{202} in all tobacco PALs), and the hydroxy group of Ser^{275} (most likely Ser^{267} of helix α11 in tobacco PAL). Exchange of Gly^{495} with the negatively charged aspartic

acid apparently disrupts the coherence of the MIO environment in a way that renders the enzyme inactive.

Cell- and organ-specific expression of tobacco *PAL* genes

In plants possessing *PAL* multigene families, attempts have been made to assign different metabolic functions to the individual PAL proteins. In quaking aspen (*Populus tremuloides*), differential expression of two *PAL* genes was observed, one associated with formation of condensed tannins, the other with lignin production [9]. In French bean, PAL3 is expressed only in roots [8], and this may reflect a function in the formation of root-specific secondary metabolites.

Our initial attempts to determine whether there was differential expression of *PAL* transcripts in tobacco organs using real-time quantitative RT–PCR were abandoned due to difficulties in designing primers that met the requirements for real-time quantitative RT–PCR while at the same time being specific for each of the four *PAL* genes which share a relatively low G/C content and high sequence similarity. The results obtained instead with semiquantitative PCR are in agreement with earlier results of Fukasawa-Akada et al. [12] who found high *PAL* transcript levels in roots and flowers and lower levels in stem and leaves. All four PAL genes were expressed in every tissue investigated, as also observed in parsley [6]. *PAL1* and *PAL4* were expressed at similar levels in most tissues, with the greatest differences detected in stems of mature tobacco plants, where *PAL4* transcript levels were high in the xylem layer but very low in cortex and pith tissue, a very different pattern from that of *PAL1* transcript levels. This suggests a specific role of *PAL4* in lignification.

PALs from different sources have been shown to exhibit different expression patterns in response to environmental cues [8]. Of the four *PAL* genes in *Arabidopsis*, only *PAL1* and *PAL2* were induced after a decrease in nitrogen supply and temperature [40]; these genes, together with *PAL4*, play a role in lignin synthesis [41].

In tobacco NT-1 cell suspension cultures, *PAL1* and *PAL4* were expressed constitutively, and their transcript levels increased within 1 h of elicitation with YE or MJ, whereas *PAL2* and *PAL3* were induced at later stages post-elicitation. In contrast, Taguchi et al. [23] found induction of *PAL2* (*PAL A*) with both fungal elicitor and MJ in tobacco cell cultures; *PAL1* (*PAL B*) was not induced. These findings suggest that tobacco *PAL2* and *PAL3* are linked primarily to inducible stress response reactions.

PAL can exist as a heterotetrameric enzyme

Using three different and partially independent approaches, namely affinity pull-down followed by immunoblot with epitope-specific antibodies, IEF and activity assays, we provide evidence in the present study for the formation of heterotetramers between PAL1 and PAL2 or PAL4 following co-expression in a bacterial system. The fact that no homotetramers were found in the purified fraction suggests preferential formation of heterotetramers. Heterotetramers between PAL1 and inactive PAL2 (PAL2D495) had a specific activity that was 50% that of PAL1–PAL2 heterotetramers, suggesting the presence of only 50% of the active sites. Although a mixed population of heterotetramers with different PAL1–PAL2D495 ratios cannot be excluded, the V_{max} data are consistent with the presence of four active sites in fully active PAL. The autocatalytic formation of the prosthetic MIO group has been described for histidine ammonia-lyase, and the mechanism is assumed to be valid for PAL as well [42]. Since residues from three subunits participate in the formation of each active site, it is perhaps remarkable that heterotetramers can form active enzymes. Formation of functional heterodimers *in vitro* has been shown for *O*-methyltransferases from *Thalictrum tuberosum* [43]. Different dimer combinations of the four proteins *in vitro* resulted in heterodimeric enzymes with substrate specificities that deviate from those of the native homodimeric forms.

Constitutive co-expression of PAL1 and PAL4 in a single cell type (e.g. cell suspension cultures) raises the question of whether formation of PAL heterotetramers occurs *in planta*, or whether plants possess mechanisms to prevent heterotetramer formation. Metabolic channelling has been proposed to play a role in regulating the phenylpropanoid pathway and controlling flux into different branch pathways [15]. Evidence exists for differential subcellular localizations of distinct PAL proteins in tobacco, and the association of at least one specific PAL protein with C4H, the membrane-associated cytochrome P450 enzyme that catalyses the second step in phenylpropanoid biosynthesis [16,17]. We speculate that, rather than generating variation in catalytic activity, the formation of PAL heterotetramers may direct forms of PAL to alternative subcellular localizations associated with metabolic channelling. This would constitute an additional regulatory mechanism for the formation of phenylpropanoid end-products [44,45]. The next step will be to evaluate whether PAL heterotetramers can be generated in transgenic tobacco plants expressing differentially tagged PAL proteins.

AUTHOR CONTRIBUTION

Angelika I. Reichert performed experiments and helped to write the paper, Xian-Zhi He performed experiments, and Richard A. Dixon conceived the work and helped to write the paper.

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