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The *Arabidopsis* phenylalanine ammonia lyase gene family: kinetic characterization of the four PAL isoforms ☆

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Abstract

In *Arabidopsis thaliana*, four genes have been annotated as provisionally encoding PAL. In this study, recombinant native AtPAL1, 2, and 4 were demonstrated to be catalytically competent for L-phenylalanine deamination, whereas AtPAL3, obtained as a N-terminal His-tagged protein, was of very low activity and only detectable at high substrate concentrations. All four PALs displayed similar pH optima, but not temperature optima; AtPAL3 had a lower temperature optimum than the other three isoforms. AtPAL1, 2 and 4 had similar K_m values (64–71 μM) for L-Phe, with AtPAL2 apparently being slightly more catalytically efficacious due to decreased K_m and higher k_{cat} values, relative to the others. As anticipated, PAL activities with L-tyrosine were either low (AtPAL1, 2, and 4) or undetectable (AtPAL3), thereby establishing that L-Phe is the true physiological substrate. This detailed knowledge of the kinetic and functional properties of the various PAL isoforms now provides the necessary biochemical foundation required for the systematic investigation and dissection of the organization of the PAL metabolic network/gene circuitry involved in numerous aspects of phenylpropanoid metabolism in *A. thaliana* spanning various cell types, tissues and organs.

Keywords: *Arabidopsis thaliana*; Cruciferae; Kinetics; Phenylalanine ammonia lyase; Molecular genomics and proteomics; Recombinant protein; Multigene families

1. Introduction

Phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5) has been extensively studied since it was first described in 1961 (Koukol and Conn, 1961). It catalyses the first step of the phenylpropanoid pathway where L-phenylalanine (L-Phe) (**1**) is non-oxidatively deaminated to form the 7,8-unsaturated *trans*-cinnamic acid (**3**) and an ammonium ion (Fig. 1); the latter compound is recycled via action of GS/GOGAT to ultimately regenerate arogenate (Razal et al., 1996; van Heerden et al., 1996; Singh et al., 1998; Towers et al., 1998), thereby enabling phenylpropanoid metabolism to continue as needed, while cinnamic acid (**3**) is further metabolized to afford various phenylpropanoids and their derivatives (Dixon, 1999; Lewis et al., 1999; Anterola et al., 2002). In monocots, PAL can also utilize L-tyrosine (L-Tyr) (**2**) as substrate, yielding *p*-coumaric acid (**4**) and an ammonium ion (Neish, 1961; Rösler et al., 1997). Typically, PAL is encoded by a small multigene family (Cramer et al., 1989), which is in accordance with the first demonstration of multiple PAL isoforms in *Phaseolus vulgaris* (Bolwell et al., 1985). Potato, however, appears to be an exception with more than 40 copies reported (Joos and Hahlbrock, 1992); by contrast, *Arabidopsis thaliana* (L.) Heynh has four putative PAL isoenzymes.

The extensive in vitro biochemical characterization of PAL has been carried out for several plant species, such

Abbreviations: ABRC, Arabidopsis Biological Resource Center; CAD, cinnamyl alcohol dehydrogenase; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-thiogalactoside; pkat/μg, pmoles of substrate converted to product per second per μg protein; ORF, open reading frame; PAL, phenylalanine ammonia lyase; RT-PCR, reverse transcription-polymerase chain reaction; UTR, untranslated region.

☆ Data Deposition: All sequences reported in this paper have been deposited in the GenBank database [Accession Nos. AY303128 (AtPAL1), AY303129 (AtPAL2), AY528562 (AtPAL3) and AY303130 (AtPAL4)].

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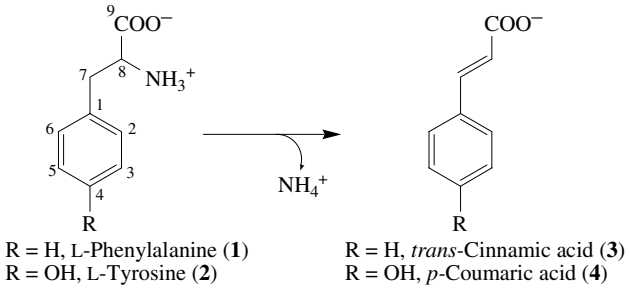

Fig. 1. PAL and TAL conversions, showing substrates and products.

as parsley (*Petroselinum crispum*) (Appert et al., 1994), but not for the crucifer *Arabidopsis*. Accordingly, this particular study was directed to define unambiguously both the biochemical functions and kinetic parameters of the proteins encoded by the putative *pal* genes, as part of a National Science Foundation (USA) initiative ultimately aimed at establishing the true physiological function, or functions, of each *Arabidopsis* gene by 2010.

From a physiological perspective, PAL is expressed constitutively in *Arabidopsis* and can also be induced upon exposure to various stresses. Data from public websites such as MPSS (www.mpss.udel.edu) and TAIR (www.arabidopsis.org) indicate that *Atpal*1 and 2 expression patterns are very similar for various tissues, e.g. callus, inflorescence and roots, but give no definitive indication of physiological function and the metabolic processes involved. As well, expression levels of *Atpal*1 and 2 are higher than that of *Atpal*4 while *Atpal*3 is barely detectable. In *A. thaliana*, *Atpal*1 has been proposed as involved in lignification (Ohl et al., 1990; Mauch-Mani and Slusarenko, 1996; Rookes and Cahill, 2003) although available EST data does not fully support this hypothesis (Costa et al., 2003); however, this latter interpretation is cautionary, because of limited sampling of the aerial (vascular) plant tissue used to create the EST data as well as the limited number of ESTs obtained. Based on analysis of the same EST data, it would appear that either AtPAL2 is as likely a candidate for lignification or that (more likely) overlapping PAL metabolic networks are operative in vascular tissues (e.g. using both AtPAL1 and 2) (Costa et al., 2003). The latter would assure functional capability even if one *pal* gene were impaired in its expression/catalytic properties. Such overlapping metabolic networks leading to functional redundancy have been noted, for example, with the cinnamyl alcohol dehydrogenase (CAD) family during *Arabidopsis* stem vasculature lignification (Kim et al., 2004). By contrast, *Atpal*3 appears to be expressed mainly in roots and leaves, albeit at low levels (Wanner et al., 1995; Raes et al., 2003), while *Atpal*4 is more abundant in developing seed tissue (Costa et al., 2003; Raes et al., 2003). Of the four isoenzymes, *Atpal*1 and 2 are preferentially induced upon treatment with factors such as light, wounding, heavy metals (e.g. mercuric chloride) and pathogen attack (Ohl et al., 1990; Mauch-Mani and Slusarenko, 1996; Rookes and Cahill, 2003).

In this study, all four putative *Arabidopsis pal* genes were cloned, with the corresponding recombinant proteins expressed, purified to apparent homogeneity, and characterized with regard to kinetic and physical parameters. This baseline biochemical information is essential prior to establishing if and how the proposed PAL metabolic networks are functionally organized at the cellular/cell type levels during all stages of growth and development, including identification of the downstream metabolic processes involved and the nature of the defense responses, in *A. thaliana*.

2. Results and discussion

The four putative *pal* genes in *Arabidopsis* encode proteins that share $\geqslant 74\%$ identity and $\geqslant 78\%$ amino acid similarity. ClustalW analysis of the four PAL genomic sequences (i.e. 5′, 3′ UTR regions, ORF including introns) showed that *Atpal*1 and 2 and *Atpal*3 and 4 form two distinct groups (data not shown), in agreement with the phylogenetic analysis carried out by Raes et al. (2003). This 2 + 2 pattern most likely arose from duplication as AtPAL1 and 2 share 90.4% amino acid identity while AtPAL3 and 4 have 83.5% identity. Intron number also differs between the two groups (Wanner et al., 1995) with *Atpal*1 and 2 possessing a single intron whereas *Atpal*3 and 4 have two, which may indicate differential post-transcriptional processing for the two groups. While the significance of chromosome location is as yet unknown, the four PAL genes are distributed over three of the five *Arabidopsis* chromosomes, chromosome II (*Atpal*1), chromosome III (*Atpal*2 and 4) and chromosome V (*Atpal*3). In the latter case, *Atpal*3 is located near the telomere region of chromosome V. Interestingly, *Atpal*3 possesses a non-consensus splice site at the 3′ end of the second exon (Wanner et al., 1995) and, as a result, two distinct amino acid sequences have been reported, with one possessing four additional amino acids i.e. ^{337}DRYVLGYALRTS versus ^{337}DRYALRTS. In this context, we are proposing that this alternative gene splicing may arise as a result of different stresses experienced (Bournay et al., 1996; Marrs and Walbot, 1997). ClustalW alignment of all known PAL isoforms from various other plant species, however, revealed no other examples of additional amino acids in any of the other sequences reported (http://www.ncbi.nih.gov). Indeed, in our hands, the AtPAL3 clone isolated encoded a sequence lacking all four 'extra' amino acids.

Gene specific primers (Table 1) were designed for each *Atpal* cDNA sequence for PCR amplification of the individual target genes. To obtain each PAL clone (except *Atpal*1 which was provided by the *Arabidopsis* Bi-

Table 1
Gene specific primers for amplification of *Arabidopsis* PAL genes *Atpal*1– 4

	Gene locus	Gene size (bp)	Gene specific primers
*Atpal*1	At2g37040	2178	FOR 5′ ATGGAGATTAACGGGGCACA REV 5′ TTAACATATTGGAATGGGAGCTCC
*Atpal*2	At3g53260	2154	FOR 5′ ATGGATCAAATCGAAGCAATG REV 5′ TTAGCAAATCGGAATCGGAGC
*Atpal*3	At5g04230	2085	FOR 5′ ATGGAGTTTCGTCAACCAAAC REV 5′ TTAGCAGATAGAAATCGGAGCA
*Atpal*4	At3g10340	2124	FOR 5′ ATGGAGCTATGCAATCAAAAC REV 5′ TCAACAGATTGAAACCGGAGC

ological Resource Center, ABRC), total RNA was isolated from 8-day-old *Arabidopsis* seedlings with RNA used to synthesize first strand cDNA, this in turn being required to amplify *Atpal*2, 3, and 4 individually. *Atpal*1 cDNA, on the other hand, was amplified using purified plasmid #U10120 as template. Initially, genes encoding AtPAL2 and 4 were cloned into pTrcHis TOPO® expression vectors; however, only low protein yields of AtPAL2 and 4 were obtained (as estimated by SDS–PAGE, data not shown), in spite of a wide variety of IPTG concentrations tested (0.1–1 mM) at different incubation temperatures (20–37 °C) and times (6–24 h). Accordingly, we next employed the pET Blue-1 expression vector system to establish if successful overexpression of the putative AtPAL isoforms in recombinant native form could be obtained instead. Optimal conditions for good overexpression levels of AtPAL1, 2, and 4 (e.g. 2 mg purified AtPAL2 from 2 l culture) were identified by varying temperature (20–30 °C), time (4–20 h) and IPTG concentration (0.1–1 mM). Best conditions found were 22 °C, 14–16 h and 0.35–0.5 mM IPTG; by contrast, none of these conditions resulted in AtPAL3 overexpression.

Initial assays using crude cell lysate to estimate PAL activities utilized L-[U-^{14}C]Phe (**1**; 1 mM, 4 μCi/mmol) with product analysis (i.e. for [U-^{14}C]cinnamic acid (**3**)) involving detection and quantification by HPLC analyses followed by radioactivity measurements. For AtPAL1, 2, and 4, PAL activities were readily detectable after 20 min incubation, whereas with AtPAL3, even overnight assays did not yield detectable radioactive [U-^{14}C]cinnamic acid (**3**) (data not shown).

AtPAL1, 2, and 4 were next individually purified using a combination of acetone precipitation, ultrafiltration, anion exchange (POROS 20HQ) and gel filtration (Sephacryl S-300) chromatographic steps. A small protein (~28 kDa) contaminant was initially present after the first gel filtration step (as revealed by SDS–PAGE analysis), this being removed by repetition of the preceding gel filtration step. In all cases, this yielded essentially homogenous PAL as visualized by silver staining (Fig. 2, AtPAL2 as example; lane 6), but with trace amounts of a larger protein (~160 kDa) being detected in the final purification step; this results from

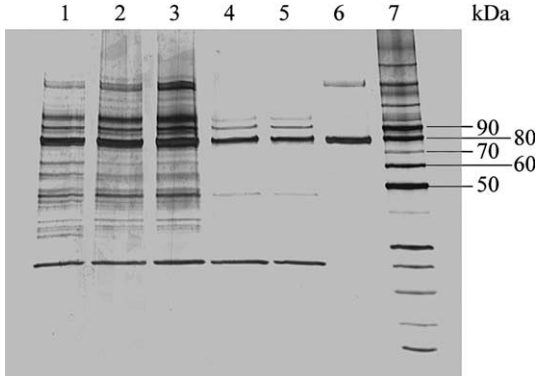

Fig. 2. SDS–PAGE showing steps involved in purification of AtPAL2 from induced (0.5 mM IPTG) *E. coli* cell-free extracts (lysate) with detection by silver staining: crude cell lysate (lane 1) and purity level following acetone precipitation (lane 2), first ultrafiltration (lane 3), anion-exchange column (POROS 20HQ) (lane 4), second ultrafiltration (lane 5) and gel filtration (lane 6) showing both purified PAL (~77.5 kDa) and traces of PAL aggregate (~160 kDa); protein standards (lane 7).

the known ability of PAL to aggregate (Parkhurst and Hodgins, 1971; Schopfer, 1971). SDS–PAGE estimations of apparent molecular masses of each PAL monomer were in close agreement with values calculated for each isoform (78.7, 77.6 and 76.9 kDa for AtPAL1, 2 and 4, respectively).

Since the expression of AtPAL3 as a native enzyme was not detectable above basal levels (as revealed by SDS–PAGE analyses and radioactive assays with radiolabeled L-[U-^{14}C]Phe (**1**), data not shown), we next investigated as to whether a functional AtPAL3 coupled to a His_6-tag could be obtained. Using the pTrc-His TOPO® vector to introduce an in-frame His_6-tag at the N-terminus, the clone was transformed into Tuner cells and AtPAL3 formation was most efficiently induced at 1 mM IPTG (22 °C, 20 h). Soluble protein was purified using immobilized metal affinity chromatography (IMAC) to apparent homogeneity (as estimated by SDS–PAGE, data not shown), this giving circa 0.6 mg purified AtPAL3 from 2 l culture. Its apparent molecular mass (with the attached His_6-tag of ~3–4 kDa) was ~ 79.1 kDa, as determined by SDS–PAGE, this being the expected size since the calculated mass of the native protein is ~76.2 kDa.

Table 2
Kinetic and physical properties of recombinant *Arabidopsis* PAL1–4

	L-Phe (**1**)						L-Tyr (**2**)
	K_m (μM)	V_{max} (pkat/μg protein)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	pH optimum	Temperature optimum (°C)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
AtPAL1	68 ± 5	5.5 ± 0.03	1.8	25,500	8.4–9.2	46–48	75 ± 6
AtPAL2	64 ± 3.5	10.5 ± 0.05	3.2	51,200	8.4–8.9	48	40 ± 12
AtPAL3[a]	2560 ± 340	0.4 ± 0.03	0.1	50	8.7–8.9	31	n.d.
AtPAL4	71 ± 3	9.9 ± 0.04	3.0	43,100	8.4–8.8	46–48	44 ± 1

n.d. = not determined.
[a] *Note*: AtPAL3 overexpression is as the N-terminal His_6-tagged recombinant derivative, whereas AtPAL1, 2, and 4 are overexpressed as recombinant native proteins.

All products of enzyme assays, with either L-Phe (**1**) or L-Tyr (**2**) as potential substrates, were verified by HPLC analyses, using UV, mass spectroscopy (atmospheric pressure chemical ionization, APCI) and radiochemical detection systems as needed. AtPAL1, 2, and 4 each displayed very similar properties for L-Phe (**1**) deamination, as regards to the temperature and pH optima (Table 2). The temperature optima (46–48 °C) obtained for each isoform were quite comparable to native PALs isolated from other Brassicaceae members, such as *Brassica campestris* and *B. juncea* (both 45 °C; www.brenda.uni-koeln.de and references therein). The pH optima, however, ranged from 8.4 to 8.8 (AtPAL4) to 8.9 (AtPAL2) and to 9.2 (AtPAL1), these values being within the ranges known for PAL from *Hordeum vulgare* (Koukol and Conn, 1961), *Sinapis alba* (Gupta and Acton, 1979) and *Petroselinum crispum* (Appert et al., 1994), respectively. Additionally, although AtPAL3 had a similar pH optimum (8.7–8.9), its temperature optimum (31 °C) was lower than that of the other isoforms, an observation which might possibly result from interference by the attached N-terminal His_6-tag with packing of the native tetramer form.

Kinetic parameters of AtPAL1, 2 and 4 were determined (Table 2) with Hanes plots being linear (Fig. 3) indicating that the three homologues followed standard Michaelis–Menten kinetics; calculated kinetic parameters gave values of apparent K_m (64–71 μM), V_{max} (5.5–10.5 pkat/μg protein) and k_{cat} (1.8–3.2 s^{-1}), respectively, for L-Phe (**1**). The K_m values were thus similar to those described for *Glycine max* (Hanson and Havir, 1981) and *Medicago sativa* (Jorrin and Dixon, 1990) PAL (both 70 μM); whereas apparent k_{cat} values (1.8–3.2 s^{-1}) were roughly comparable (only 3–5-fold lower) to maize PAL (10.6 $s^{-1}$with L-Phe (**1**) at pH 8.7; (Rösler et al., 1997)). As well, the AtPAL1, 2, and 4 k_{cat}/K_m values (25,000–51,000 $M^{-1}\,s^{-1}$) were within range of the k_{cat}/K_m value determined for maize PAL (39,000 $M^{-1}\,s^{-1}$; (Rösler et al., 1997)). Comparison of relative enzyme efficacies also showed that AtPAL2 and AtPAL4, when compared with AtPAL1, were catalytically very slightly more efficacious, this being a consequence of a small decrease in K_m (AtPAL2 only) and an increase in k_{cat} (AtPAL2 and 4), respectively. Similar kinetic properties amongst isoenzymes of high homology were also observed for the four parsley PAL proteins (K_m's [L-Phe] from 15–25 μM) (Appert et al., 1994), these sharing very high identity (94.7–99.4%) and possessing nearly indistinguishable catalytic properties. While this correlation of high sequence similarity and comparable kinetic properties has been suggested to arise from relatively

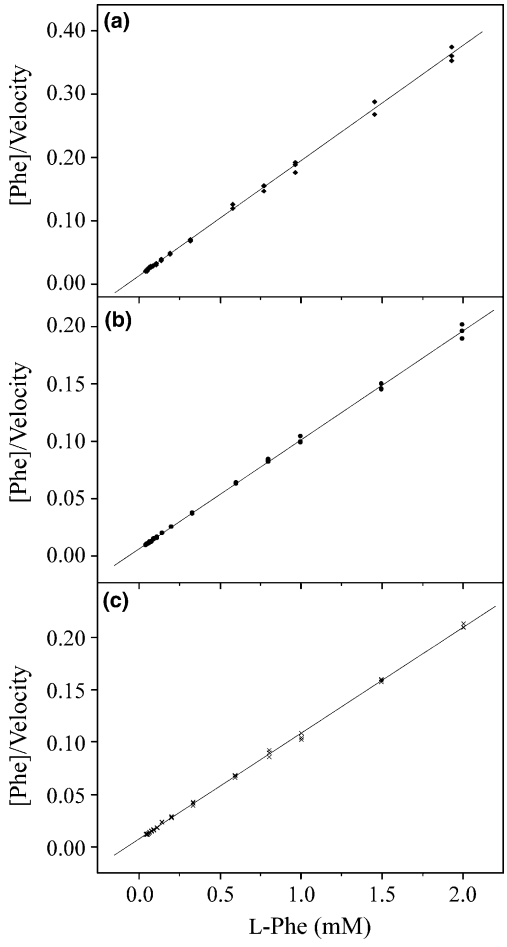

Fig. 3. Hanes plots for L-Phe (**1**) with AtPAL1 (a), AtPAL2 (b) and AtPAL4 (c). [Phe]/velocity is mM μg protein $pkat^{-1}$.

recent divergence of the gene within *Petroselinum* (Kumar and Ellis, 2001), it cannot be extended to *Arabidopsis* where AtPAL4 displays similar kinetic properties to AtPAL1 and 2 yet does not cluster with these two proteins (see above). In contrast to AtPAL1, 2 and 4, the N-terminal His_6-tagged AtPAL3 was of very low catalytic activity with L-Phe (**1**) as substrate, with its Michaelis–Menten plot reaching saturation by ~3.5 mM L-Phe (**1**). In this case, assays required both increased protein amounts ($\geqslant 50$ μg versus ~0.5–1 μg for the other AtPAL isoforms) and an extended incubation period (reaction was still linear at 20 h). Indeed, AtPAL3 was estimated to have a catalytic efficacy 500–1000-fold lower than that of the other PAL isoforms, this resulting from its higher K_m (2.6 mM) and its very low k_{cat} (0.1 s^{-1}) values, respectively. This overall low efficacy in activity may result from either the enzyme being catalytically defective or, as indicated earlier, from introduction of the N-terminal His_6-tag, and thus, may not be a reflection of the true catalytic properties of native AtPAL3.

Additionally, it was deemed instructive to examine whether each of the AtPALs (1–4) were capable of converting L-Tyr (**2**) to *p*-coumaric acid (**4**), given that PAL can utilize both L-Phe (**1**) and L-Tyr (**2**) (Rösler et al., 1997), albeit with L-Phe (**1**) being preferred. However, assays carried out to determine kinetic parameters did not reach enzyme saturation due to solubility limitations with L-Tyr (**2**). Indeed, a high alkaline pH (pH ~10) was required for L-Tyr (**2**) solubilization and only a limited number of substrate concentrations (up to 13 mM L-Tyr (**2**)) were possible. For the AtPAL1, 2, and 4 homologues, enzyme efficacy with L-Tyr (**2**) was greatly reduced as compared to L-Phe (**1**) deamination [340- (AtPAL1), 1200- (AtPAL2) and 970- (AtPAL4) fold decreases, respectively], whereas AtPAL3 was essentially inactive. This finding was not unexpected since dicotyledons such as *Arabidopsis* are not known to effectively employ L-Tyr (**2**) as an alternative starting point for phenylpropanoid biosynthesis; this result is thus similar to that of Appert et al. (1994) who determined that enzyme efficiency of parsley PAL with L-Tyr (**2**) was decreased by four orders of magnitude as compared to L-Phe (**1**).

The establishment of multiple bona fide PAL genes in *Arabidopsis* now raises several important questions, in particular concerning the true physiological function of each PAL isoform. This would include establishing to what extent functionally redundant metabolic networks/gene circuits exist for the PAL family, particularly since most research reported has focused only on *Atpal*1 leaving *Atpal*2, 3, and 4 largely unexplored. In this context, our current understanding of the nature of the metabolic networks leading ultimately to not only different metabolic products, but also involving overlapping networks in specific cell types, is still at a relatively poor level of understanding. Indeed, many studies are carried out at the crude whole tissue level and thus neither address nor give good insight into such aspects: for example, Raes et al. (2003) reported, using RT-PCR, that in *Arabidopsis Atpal*1, 2, and 4 were present in stem tissues, with *Atpal*1 and 2 transcripts apparently increasing during development; however, neither the metabolic significance of this is known, nor the cellular specificities of the tissues involved. Furthermore, the analysis of *Atpal*3 transcripts has given mixed results; Wanner et al. (1995) only detected *Atpal*3 in 10-day-old *Arabidopsis* seedlings and in roots of mature (4–6-weeks old) plants, whereas Mizutani et al. (1997) reported that AtPAL3 mRNA could not be detected in roots of 3-week-old plants; Raes et al. (2003) have since proposed (using RT-PCR), that AtPAL3 is expressed in all tissues but mainly in the leaves.

Accordingly, having now systematically established the catalytic functions of each of the recombinant PAL isoforms in vitro as bona fide PALs, current work is underway using *Arabidopsis* transformants harboring the individual *Atpal* promoter::*GUS* constructs. This, in turn, should permit the unequivocal identification of temporal and spatial patterns of expression of the genes encoding each PAL isoform in the specific cell types during *Arabidopsis* growth and development. Of particular interest is the extent to which the PAL genes are co-expressed in various cell types thereby ensuring functional redundancy, as well as delineation of the precise involvement of each gene in various downstream metabolic processes. These studies, together with analysis of specific PAL isoform "knockouts" and application of RNAi technology (to silence one and/or all of the four PAL isoforms) should provide definitive insight into not only the physiological function of each AtPAL isoform in vivo, but also how the proposed PAL metabolic networks/gene circuits are actually organized in vivo.

3. Experimental

3.1. Materials

All solvents and chemicals were of reagent or high performance liquid chromatography (HPLC) grade unless otherwise specified. Cinnamic (**3**) and *p*-coumaric (**4**) acids as well as L-Phe (**1**) and L-Tyr (**2**) were purchased from Sigma, whereas L-[U-^{14}C]Phe (**1**) (496 mCi/mmol) was from Perkin–Elmer Life Sciences, Inc. RNeasy® Plant Mini and QIAquick Gel Extraction Kits were from Qiagen, while Superscript™ First-Strand Synthesis System for RT-PCR and pTrcHis TOPO® TA Expression Kits were purchased from Invitrogen. A Wizard® *Plus* SV Miniprep kit was obtained from Promega, whereas pETBlue™-1 vector, *Escherichia coli* Tuner (DE3)pLacI cells, Bugbuster® Protein Extraction Re-

agent and Benzonase® were from Novagen and Expand HiFi polymerase was from Roche. Custom oligonucleotide primers for polymerase chain reaction (PCR) and sequencing were synthesized by Invitrogen. DNA concentrations were approximately determined by comparison to a low DNA mass ladder (Invitrogen) in 0.8% agarose gels. Centricon® Plus-80 and Plus-20 microfugal filters, as well as 100,000 MWCO (YM100) filtration membranes were purchased from Amicon, Millipore. POROS 20HQ and POROS 20MC resins were from PerSeptive Biosystems whereas Sephacryl S300 was from Amersham Biosciences.

3.2. Instrumentation

PCR amplifications used an Amplitron®II Thermal cycler (Barnstead/Thermolyne Coop.), whereas RNA and protein measurements utilized a Lambda 6 UV–visible spectrophotometer (Perkin–Elmer). For protein purification, both anion exchange and IMAC steps employed a BioCAD (Perseptive Biosystems), while a Pharmacia LKB FPLC system equipped with a Monitor UV-M unit was used for gel filtration. An Amicon stirred cell (Cell Model No. 8050) with reservoir (RC800) was used for ultrafiltration. Radioactive samples were analyzed in Biodegradable Counting Scintillant (BSC, Amersham Biosciences) and counted using a Liquid Scintillation Analyzer (Tri-Carb 2100TR; Packard). Reversed-phase HPLC analyses were carried out using an HPLC-PDA system that consisted of a Waters™ 600 pump Controller, Waters™ 717 plus autosampler and Waters™ 996 photodiode array detector, all controlled by Millennium 2.1 software. As well, an APCI MS^n, a LCQ mass spectrometer (Thermo-Finnigan), equipped with atmospheric pressure chemical ionization and an ion trap, were used to verify enzyme assay products.

3.3. Cloning of PAL cDNAs

The *Atpal* 1 clone (#U10120) was obtained as a stab culture from the ABRC (Ohio State University, Columbus, Ohio), with plasmid purified from a 5 ml overnight culture, whereas total RNA (to obtain *Atpal*2, 3, and 4) was extracted from 8-day-old *A. thaliana* (L.) Heynh. (ecotype Col.) seedlings using the RNeasy® Plant Mini Kit. For RT-PCR, the RNA was reversely transcribed by Superscript™ First-Strand Synthesis System using 5 μg for each transcription and random hexamers for priming. To amplify the *pal* ORFs 5 μg cDNA (or 80 ng plasmid) was used as template with 10 pmol of each gene-specific primer (Table 1, based on gene sequences available through NCBI; www.ncbi.nlm.nih.gov), 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 1.25 units of Expand HiFi polymerase in 25 μl reactions. PCR conditions were: hot start (94 °C, 3 min), denaturation 94 °C for 1 min, annealing for 2 min at 45 °C and extension for 2.2 min at 72 °C. The final extension time was 10 min at 72 °C. PCR products (~2 kb) were gel-purified using a QIAquick Gel Extraction Kit (according to manufacturer's instructions) and cloned into pETBlue™-1 vectors. Additionally, *Atpal*2, 3, and 4 ORFs were individually cloned into pTrcHis TOPO® vectors. Both the orientation and the sequence of the inserts were checked before transformation of *E. coli* Tuner (DE3)pLacI for expression.

3.4. Overexpression and purification of recombinant PAL

A single colony from each transformed *E. coli* cell line was incubated in 1 ml Luria-Bertani (LB) broth supplemented with carbenicillin (0.1 mg/ml) for 12 h at 37 °C. Inocula were then individually added to 500 ml LB containing carbenicillin (0.1 mg/ml) and grown at 37 °C until an OD_{600} between 0.5 and 0.9 was reached at which time isopropyl thio-β-D-galactoside (IPTG) was added, with PAL expression for each cell line being induced with 0.35 (AtPAL1), 0.5 (AtPAL2 and AtPAL4) or 1 mM (AtPAL3) IPTG, respectively. Cells containing AtPALs 1, 2 and 4 were grown at 22 °C for 14–16 h, while those harboring AtPAL3 were induced at 22 °C for 20 h. Cells were individually harvested by centrifugation at 3000*g* for 20 min and stored frozen at −80 °C until required. Pellets were thawed at room temperature for 5 min, suspended in a 1:1 mixture of 20 mM Tris–HCl (pH 7.5; Buffer A) and BugBuster™ Protein Extraction reagent, then sonicated 10×15 s with 1 min intervals while on ice. Cellular debris from each cell line was removed by centrifugation at 15,000*g* for 30 min. Supernatants containing AtPAL1, 2 and 4 were, respectively, subjected to acetone precipitation, with the 25–55% acetone saturation fraction (also in the 25–55% ammonium sulphate saturation range; data not shown) centrifuged at 15,000*g* for 30 min. Each resulting pellet was next resuspended in Buffer A and filtered through a 100,000 MWCO (YM100) membrane using an Amicon stirred cell. Each retentate was then applied to a POROS 20HQ anion exchange column (100 mm × 4.6 mm; 10 ml/min flow rate) pre-equilibrated in Buffer A. After washing the column with 15 ml Buffer A, each PAL was individually eluted with a linear gradient from 0 to 1 M NaCl in Buffer A with 1 ml fractions collected; PAL isoforms were eluted with 0.37 M NaCl. Fractions containing each PAL were then individually pooled, desalted by ultrafiltration (YM100 membrane), further concentrated using a Centricon® Plus-80 microfugal filter and loaded onto a Sephacryl S-300 gel filtration column (60 cm × 1.6 cm) pre-equilibrated in Buffer B (Buffer A containing 200 mM NaCl) at a flow rate of 0.5 ml/min. Fractions (1 ml) containing each PAL isoform, eluted with 48–58 ml of mobile phase, were pooled, concentrated and reapplied to the column under the same conditions. The resulting PAL fractions were in-

dividually combined and concentrated using Centricon Plus-20 concentrators. Purification levels of AtPALs 1, 2 and 4 were analyzed by SDS–PAGE using Laemmli's buffer system under denaturing and reducing conditions (Laemmli, 1970). Each PAL was separated on a 4–20% gradient gel and visualized by silver staining (Amersham Bioscience Application Note).

Supernatant from sonicated and pelleted *E. coli* containing His-tagged AtPAL3 was filtered (0.45 μm syringe filter Acrodisc®, Gelman Laboratory) prior to purification by IMAC. Isolation of AtPAL3 was carried out at room temperature using a POROS 20MC column (100 mm × 4.6 mm) pre-equilibrated with Buffer C (20 mM Tris–HCl, pH 7.9; 500 mM NaCl; 5 mM imidazole) with a flow rate of 10 ml/min. Aliquots (1.5 ml) of filtrate containing AtPAL3 were applied to the column and, after washing with Buffer D (20 mM Tris–HCl, pH 7.9; 500 mM NaCl; 50 mM imidazole) to remove non-specific proteins, AtPAL3 was eluted with a linear gradient from Buffer D to Buffer E (20 mM Tris–HCl, pH 7.9; 500 mM NaCl; 500 mM imidazole) with 1 ml fractions being collected; recombinant AtPAL3 was eluted with 280 mM imidazole. Aliquots (12 μl) of every other fraction were analyzed by SDS–PAGE as described above. Fractions containing His-tagged AtPAL3 were pooled and concentrated using Centricon® Plus-80 concentrators, diluted with 20 mM Tris–HCl (pH 7.5) and reconcentrated.

All protein concentrations were determined by the Bradford (1976) method using BSA as standard.

3.5. Radiochemical PAL assays – temperature and pH

Temperature and pH optima were individually determined for each PAL protein. For temperature optima, incubations were carried out at a constant pH of 8.2, while varying the temperature (14–70 °C). The assay mixture consisted of Bis–Tris Propane (100 mM, 100 μl, pH 8.2), 10 μg of PAL (post-POROS 20HQ column; AtPAL1, 2, and 4), 20 mM Tris–HCl (pH 7.5) and L-[U-^{14}C]Phe (1 mM, 4 μCi/mmol) in a total volume of 250 μl. Protein and buffers were pre-incubated for 2 min at each temperature prior to assay initiation by addition of substrate. After 20 min, assays were stopped by addition of glacial AcOH (10 μl), with cinnamic acid (**3**; 100 nmol) added as radiochemical carrier before assay mixtures were extracted with EtOAc (2 × 200 μl). The combined EtOAc solubles were washed with distilled water (1 × 200 μl), with an aliquot (200 μl) of the EtOAc layer removed for liquid scintillation counting. Identical assays were carried out for AtPAL3 but with larger amounts of purified protein (50 μg) used and with the incubation times extended to 15 h. For pH optima, assays were performed at 37 °C with Bis–Tris Propane (100 mM; pH 6.5–9.2), TAPS (100 mM; pH 8–9.05) or CAPSO (100 mM; pH 9.2–10.1) buffers as described above. Again, for AtPAL3, an increased protein amount (50 μg) was used, with the assay time also extended to 15 h; assays were carried out at 31 °C. For all data points, including both pH and temperature optima determinations, controls either without enzyme or denatured protein (10 min, 100 °C) were performed. All assays were carried out in triplicate.

3.6. PAL assays – kinetic parameters

Enzyme assays for AtPAL1, 2 and 4 were performed at 37 °C with a 2 min pre-incubation time (whereas AtPAL3 assays were carried out at 31 °C) in a temperature-controlled waterbath. For kinetic analyses, 16 concentrations of L-Phe (**1**) (43 μM to 2 mM) were used for AtPAL1, 2 and 4, while for AtPAL3 they were between 0.25 and 4 mM. Each assay (250 μl) contained 100 mM Bis–Tris Propane (pH 8.4, AtPAL1, 2, and 4; pH 8.8 AtPAL3), purified enzyme (0.39 μg AtPAL1, 0.92 μg AtPAL2, 50 μg AtPAL3 and 0.47 μg AtPAL4) and various amounts of L-Phe (**1**), respectively. Assays were initiated by addition of substrate, incubated for 20 min (AtPAL3, 15 h) and stopped by acidification (50 μl of glacial AcOH). An aliquot (80 μl) of each assay mixture was then subjected to HPLC analysis as described below. K_m and V_{max} values for AtPAL1, 2 and 4 for L-Phe (**1**) were obtained from Hanes plots, while Michaelis–Menten plots were used to determine AtPAL3 kinetic parameters. For K_m determinations using L-Tyr (**2**), concentrations of 0.55–13 mM were used, with each assay (250 μl) containing 100 mM Bis–Tris Propane (pH 8.4), purified enzyme (4.87 μg AtPAL1; 5.40 μg AtPAL2 and 3.92 μg AtPAL4) and various amounts of L-Tyr (**2**). Incubation times were either 30 min (AtPAL4) or 1 h (AtPAL1 and 2). k_{cat}/K_m values were determined from Michaelis–Menten plots.

3.7. Reversed-phase HPLC separation of PAL assays

Cinnamic acid (**3**) detection was accomplished using a reversed-phase column (Nova-Pak® C18, 3.9 × 150 mm, Waters) eluted with a 3% AcOH in H_2O (v/v; Solvent A) and CH_3CN (Solvent B) gradient at a flow rate of 1 ml/min with detection at 278 nm. The column was pre-equilibrated in A:B (23:2), which was held for 2 min after introduction of the sample with a linear gradient to A:B (1:19) applied over 20 min, this being held for 10 min. The gradient was returned to A:B (23:2) over 2 min and held at this ratio for another 18 min for column re-equilibration. The same system was used to separate *p*-coumaric acid (**4**) with detection at 310 nm.

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References

Anterola, A.M., Jeon, J.-H., Davin, L.B., Lewis, N.G., 2002. Transcriptional control of monolignol biosynthesis in *Pinus taeda*: factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. Journal of Biological Chemistry 277, 18272–18280.

Appert, C., Logemann, E., Hahlbrock, K., Schmid, J., Amrhein, N., 1994. Structural and catalytic properties of the four phenylalanine ammonia-lyase isoenzymes from parsley (*Petroselinum crispum* Nym.). European Journal of Biochemistry 225, 491–499.

Bolwell, G.P., Bell, J.N., Cramer, C.L., Schuch, W., Lamb, C.J., Dixon, R.A., 1985. Phenylalanine ammonia-lyase from *Phaseolus vulgaris*. Characterisation and differential induction of multiple forms from elicitor-treated cell suspension cultures. European Journal of Biochemistry 149, 411–419.

Bournay, A.-S., Hedley, P.E., Maddison, A., Waugh, R., Machray, G.C., 1996. Exon skipping induced by cold stress in a potato invertase gene transcript. Nucleic Acids Research 24, 2347–2351.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Costa, M.A., Collins, R.E., Anterola, A.M., Cochrane, F.C., Davin, L.B., Lewis, N.G., 2003. An in silico assessment of gene function and organization of the phenylpropanoid pathway metabolic networks in *Arabidopsis thaliana* and limitations thereof. Phytochemistry 64, 1097–1112.

Cramer, C.L., Edwards, K., Dron, M., Liang, X., Dildine, S.L., Bolwell, G.P., Dixon, R.A., Lamb, C.J., Schuch, W., 1989. Phenylalanine ammonia-lyase gene organization and structure. Plant Molecular Biology 12, 367–383.

Dixon, R.A., 1999. Isoflavonoids: biochemistry, molecular biology, and biological functions. In: Barton, Sir D.H.R., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 1. Elsevier, Oxford, pp. 773–823.

Gupta, S., Acton, G.J., 1979. Purification to homogeneity and some properties of L-phenylalanine ammonia-lyase of irradiated mustard (*Sinapis alba* L.) cotyledons. Biochimica et Biophysica Acta 570, 187–197.

Hanson, K.R., Havir, E.A., 1981. Phenylalanine ammonia lyase. In: Conn, E.E. (Ed.), Biochemistry of Plants. vol. 7. Secondary Plant Products. Academic Press, New York, pp. 577–625.

Joos, H.-J., Hahlbrock, K., 1992. Phenylalanine ammonia-lyase in potato (*Solanum tuberosum* L.). Genomic complexity, structural comparison of two selected genes and modes of expression. European Journal of Biochemistry 204, 621–629.

Jorrin, J., Dixon, R.A., 1990. Stress responses in alfalfa (*Medicago sativa* L.) II. Purification, characterization, and induction of phenylalanine ammonia-lyase isoforms from elicitor-treated cell suspension cultures. Plant Physiology 92, 447–455.

Kim, S.-J., Kim, M.-R., Bedgar, D.L., Moinuddin, S.G.A., Cardenas, C.L., Davin, L.B., Kang, C.-H., Lewis, N.G., 2004. Functional reclassification of the putative cinnamyl alcohol dehydrogenase multigene family in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America 101, 1455–1460.

Koukol, J., Conn, E.E., 1961. The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase of *Hordeum vulgare*. Journal of Biological Chemistry 236, 2692–2698.

Kumar, A., Ellis, B.E., 2001. The phenylalanine ammonia-lyase gene family in raspberry. Structure, expression, and evolution. Plant Physiology 127, 230–239.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277, 680–685.

Lewis, N.G., Davin, L.B., Sarkanen, S., 1999. The nature and function of lignins. In: Barton, Sir D.H.R., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 3. Elsevier, Oxford, pp. 617–745.

Marrs, K.A., Walbot, V., 1997. Expression and RNA splicing of the maize glutathione *S*-transferase *Bronze2* gene is regulated by cadmium and other stresses. Plant Physiology 113, 93–102.

Mauch-Mani, B., Slusarenko, A.J., 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. Plant Cell 8, 203–212.

Mizutani, M., Ohta, D., Sato, R., 1997. Isolation of a cDNA and a genomic clone encoding cinnamate 4-hydroxylase from *Arabidopsis* and its expression manner in planta. Plant Physiology 113, 755–763.

Neish, A.C., 1961. Formation of *m*- and *p*-coumaric acids by enzymatic deamination of the corresponding isomers of tyrosine. Phytochemistry 1, 1–24.

Ohl, S., Hedrick, S.A., Chory, J., Lamb, C.J., 1990. Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2, 837–848.

Parkhurst, J.R., Hodgins, D.S., 1971. Phenylalanine and tyrosine ammonia-lyase activity in *Sporobolomyces pararoseus*. Phytochemistry 10, 2997–3000.

Raes, J., Rohde, A., Holst Christensen, J., Van de Peer, Y., Boerjan, W., 2003. Genome-wide characterization of the lignification toolbox in *Arabidopsis*. Plant Physiology 133, 1051–1071.

Razal, R.A., Ellis, S., Singh, S., Lewis, N.G., Towers, G.H.N., 1996. Nitrogen recycling in phenylpropanoid metabolism. Phytochemistry 41, 31–35.

Rookes, J.E., Cahill, D.M., 2003. A *PAL1* gene promoter-green fluorescent protein reporter system to analyse defence responses in live cells of *Arabidopsis thaliana*. European Journal of Plant Pathology 109, 83–94.

Rösler, J., Krekel, F., Amrhein, N., Schmid, J., 1997. Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. Plant Physiology 113, 175–179.

Schopfer, P., 1971. Die Phenylalaninammoniumlyase (PAL, EC 4.3.1.5) des Senfkeimlings (*Sinapis alba* L.), ein elektrophoretisch einheitliches Enzym. Planta 99, 339–346.

Singh, S., Lewis, N.G., Towers, G.H.N., 1998. Nitrogen recycling during phenylpropanoid metabolism in sweet potato tubers. Journal of Plant Physiology 153, 316–323.

Towers, G.H.N., Singh, S., van Heerden, P.S., Zuiches, J., Lewis, N.G., 1998. Integrating nitrogen and phenylpropanoid metabolic pathways in plants and fungi. In: Lewis, N.G., Sarkanen, S. (Eds.), Lignin and Lignan Biosynthesis. ACS Symposium Series, vol. 697, Washington, DC, pp. 42–54.

van Heerden, P.S., Towers, G.H.N., Lewis, N.G., 1996. Nitrogen metabolism in lignifying *Pinus taeda* cell cultures. Journal of Biological Chemistry 271, 12350–12355.

Wanner, L.A., Li, G., Ware, D., Somssich, I.E., Davis, K.R., 1995. The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Molecular Biology 27, 327–338.