Phytochemistry 71 (2010) 1999–2009

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Cloning, expression, site-directed mutagenesis and immunolocalization of phenylalanine ammonia-lyase in *Bambusa oldhamii*

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ARTICLE INFO

Article history:
Received 27 May 2009
Received in revised form 30 August 2010
Available online 27 October 2010

Keywords:
Phenylalanine ammonia-lyase (PAL)
Bamboo
Bambusa oldhamii
Poaceae
Enzyme purification
Molecular cloning
Gene expression
Immunohistochemistry

ABSTRACT

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) from green bamboo was isolated and cloned from the shell of *Bambusa oldhamii*. The K_m of bamboo shell PAL for L-Phe was 476 μM, and the molecular mass of native PAL was estimated as 275 kDa and the molecular mass of a subunit was about 76 kDa, indicating that PAL from bamboo also exists as a tetramer. The optimum temperature for PAL activity was 50 °C and the optimal pH 9.0. The identity of the purified bamboo shell PAL was confirmed using Q-TOF tandem MS/MS *de novo* sequencing. Four *PAL* genes, designated as *BoPAL1* to *BoPAL4*, were cloned from *B. oldhamii*. The open reading frames of *BoPAL3* and *BoPAL4* were 2142 and 2106 bp in size, respectively: *BoPAL2–4* contained one intron and two exons, but no intron was found in *BoPAL1*. BoPAL4 expressed in *Escherichia coli* possessed both PAL and tyrosine ammonia-lyase activities. While recombinant wild-type PAL proteins had similar biochemical properties to the native bamboo shell PAL, both site-directed mutagenesis of BoPAL1 F133H and BoPAL2 F134H, respectively, showed decreased k_{cat}/K_m values toward L-Phe, whereas BoPAL2 F134H showed a slightly increased k_{cat}/K_m value toward L-Tyr. These data suggest other residues largely control Phe/Tyr substrate specificity. An antibody raised against the purified shell PAL was generated for histochemical studies. In bamboo shell and branch shoots, PAL was localized primarily in sclerenchyma cells.

1. Introduction

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the non-oxidative deamination of phenylalanine to *trans*-cinnamic acid and ammonia, the first step in the biosynthesis of phenylpropanoid metabolites (Koukol and Conn, 1961; Hahlbrock and Scheel, 1989). Many secondary metabolic products in plants, such as flavonoids, anthocyanins, plant hormones, lignins, phytoalexins, and benzoic acid derivatives, are derived from phenylpropanoids (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). PAL plays an important role in plant defense and is also involved in the biosynthesis of the signaling molecule, salicylic acid, which is required for plant systemic resistance (Nugroho et al., 2002; Chaman et al., 2003). PAL is present in all higher plants studied and is also found in some fungi (Kim et al., 1996; Hattori et al., 1999), and cyanobacteria (Moffitt et al., 2007), but has not yet been detected in Eubacteria, Archaea, and animals.

In several plant species, such as parsley (*Petroselinum crispum*; Appert et al., 1994; Logemann et al., 1995), *Arabidopsis thaliana* (Wanner et al., 1995; Cochrane et al., 2004), and raspberry (Kumar and Ellis, 2001), PAL is encoded by a multi-gene family. PAL, purified from French bean (Bolwell et al., 1985) or tomato (Sarma et al., 1998), has been shown to exist as a tetramer. It has been reported that co-expression of different tobacco PAL proteins in *Escherichia coli* could produce functional heterotetrameric enzymes (Reichert et al., 2009).

Some studies have shown that PAL from monocotyledonous plants also have tyrosine ammonia-lyase (TAL) activity and can use tyrosine as substrate (Havir et al., 1971; Rösler et al., 1997), whereas PAL from dicotyledonous plants only utilizes phenylalanine efficiently (Kyndt et al., 2002). Expression of recombinant maize PAL in *E. coli* provided direct evidence that a single polypeptide had both PAL and TAL activities, as its recombinant PAL produced cinnamic acid from phenylalanine and *p*-coumaric acid from tyrosine (Rösler et al., 1997). The TAL activity of PAL in monocotyledonous plants might provide an alternative route by avoiding the reaction catalyzed by cinnamate 4-hydroxylase (Rösler et al., 1997). Pure TAL activity has not been identified in plants and fungi, but has been reported in several bacteria, such as *Rhodobacter capsulatus* (Kyndt et al., 2002). *Rhodobacter sphaeroides*

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doi:10.1016/j.phytochem.2010.09.019

Table 1
Purification of PAL from bamboo shell.

Purification step	Total activity (nkat)[a]	Total protein (mg)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Crude extract	457.9	642.7	0.71	1.0	100
Protamine sulfate	414.6	442.2	0.94	1.3	91
40–60% $(NH_4)_2SO_4$	210.2	112.8	1.86	2.6	46
Sephacryl S-200	147.7	21.4	6.90	9.7	32
Phenyl Sepharose	102.7	6.2	16.56	23	22
Mono Q HR 5/5	47.8	0.5	95.60	135	10

[a] The starting material was 100 g of bamboo shells. One kat of activity was defined as the amount of enzyme required to produce 1 mol of *trans*-cinnamic acid from L-phenylalanine at 37 °C and pH 8.5 per second.

TAL (RsTAL) was employed to analyze the substrate specificity between L-Phe and L-Tyr; site-directed mutagenesis study had demonstrated that His 89 of RsTAL was an important substrate selectivity determinant (Louie et al., 2006; Watts et al., 2006).

Bambusa oldhamii is a monocotyledonous plant and belongs to the Poaceae family which is mainly found in East Asia especially in Taiwan (Hsieh et al., 2010a). When bamboo shoots emerge from the ground, the bamboo shell commences photosynthesis and turns into green tissue; the bamboo shoot is therefore often viewed as an organ with both sink and source properties. Development of the chloroplast leads to biosynthesis of aromatic amino acids via the shikimate pathway (Herrmann, 1995) and PAL activity is then up-regulated by both increased substrate levels and light. PAL is also induced by wounding (Hisaminato et al., 2001). When bamboo shoots are harvested, PAL activity might be induced to play a role in the biosynthesis of phenolic compounds, which causes hardening and browning substance deposition in the shoots and in the development of a bitter taste.

Although PAL is a well-studied enzyme in plants, little is known about its function in bamboo. To better understand the role of PAL in bamboo for further applications, we have characterized this enzyme at both protein and DNA levels for providing background information about bamboo PAL.

2. Results

2.1. Purification of PAL from bamboo shell

Native PAL was purified from bamboo shell using a six-step procedure of buffer extraction, protamine sulfate precipitation, ammonium sulfate fractionation, and Sephacryl S-200, Phenyl-Sepharose, and Mono Q (FPLC) anion exchange chromatographic purification steps. The Mono Q chromatography-purified PAL was eluted as a single protein peak, with a 10% yield and 135-fold purification (Table 1). On native and SDS gels, a major band was observed after purification (Fig. 1A and B). The shell PAL was purified to ~90% purity as judged by Coomassie blue staining. The bamboo shell PAL was stable during purification and could be stored at −20 °C without addition of cryo-protectants for at least 6 months. The specific activity of the purified bamboo shell PAL was 95.6 nkat mg^{-1} protein (Table 1).

To determine the localization of PAL protein in plant tissue, polyclonal mouse antibodies were prepared against purified bamboo shell PAL, with this used at a dilution of 1:1500 in the following Western blotting analysis (Fig. 1C and D). The proteins in the different fractions for the preparation of bamboo shell PAL were separated by native- and SDS–PAGE. By native-gel analysis a single band corresponding to a molecular mass of 275 kDa was detected by the antibody (Fig. 1C, lanes 1–6), while on the SDS gel a single band with a molecular mass of 76 kDa was detected (Fig. 1D, lanes 4–6). Thus, the polyclonal anti-PAL antibodies specifically recognized both native and recombinant forms of bamboo PAL. The antibodies also recognized PALs from other plants, including sweet potato and asparagus (our unpublished data).

2.2. Biochemical properties of bamboo shell PAL

Using gel filtration chromatography on Superose 6, the molecular mass of bamboo shell PAL was estimated to be about 275 kDa (Fig. 2A). By SDS–PAGE, the bamboo shell PAL subunit migrated with an apparent molecular mass of 76 kDa (Figs. 1B and 2B, lane 6), showing that it exists as a tetramer. In several species, the native PAL subunit is inherently unstable *in vitro*, and during purification, can break down to yield partially degraded forms (Sarma et al., 1998; Bolwell et al., 1985). However, degradation was not significant during purification of bamboo shell PAL.

The kinetic parameters of PAL were determined in an enzyme activity assay using L-Phe as substrate. A hyperbolic curve was obtained using native bamboo shell PAL (Fig. 2C). Double reciprocal plots (Lineweaver–Burk plot, Fig. 2D) were generated to calculate the kinetic parameters. The K_m of bamboo shell PAL for L-Phe was calculated as 476 μM, similar to the value of 500 μM for rice PAL (Sarma and Sharma, 1999).

2.3. Amino acid sequencing by Q-Tof

Mono Q chromatography-purified bamboo shell PAL was subjected to protein sequencing (Fig. 1B, lane 6). PAL protein was separated by SDS–PAGE and transferred to a PVDF membrane. Thioglycolic acid (2 mM) was added to the cathode buffer prior to electrophoresis to remove free radicals. After staining with Coomassie blue, the band corresponding to PAL was excised for N-terminal sequencing on a protein sequencer (Applied Biosystem Procise 492), but no sequence was obtained, probably due to a blocked N-terminus.

To verify the identity of the purified PAL, quadruple time-of-flight tandem mass spectrometry (Q-TOF MS/MS) was used. Prior to MS analysis, the protein sample was separated by SDS–PAGE and stained with Coomassie blue, then the main protein band was excised and subjected to in-gel digestion using sequencing-grade trypsin with the recovered peptides subjected to Q-TOF MS/MS analysis. The data were analyzed using the on-line Mascot program (Perkins et al., 1999) and these were used for BLAST analysis using the NCBI database. Five were matched the sequence of *B. oldhamii* PAL (shown in red in Fig. 4)[1] and covered about 10% of the sequence.

2.4. Cloning and analysis of the BoPAL genes

A Lambda ZAPII cDNA library constructed from poly(A)$^+$ RNA isolated from bamboo shoot was screened with a PAL-specific DIG-labeled probe and positive clones were subjected to *in vivo* excision, followed by DNA sequencing. Two full-length *PAL* genes were identified and designated as *BoPAL1* and *BoPAL2*; the sequences have been deposited at GenBank with accession numbers AY450643 (Hsieh et al., 2010a) and FJ715635 (Hsieh et al., 2010b).

[1] For interpretation of color in Fig. 4, the reader is referred to the web version of this article.

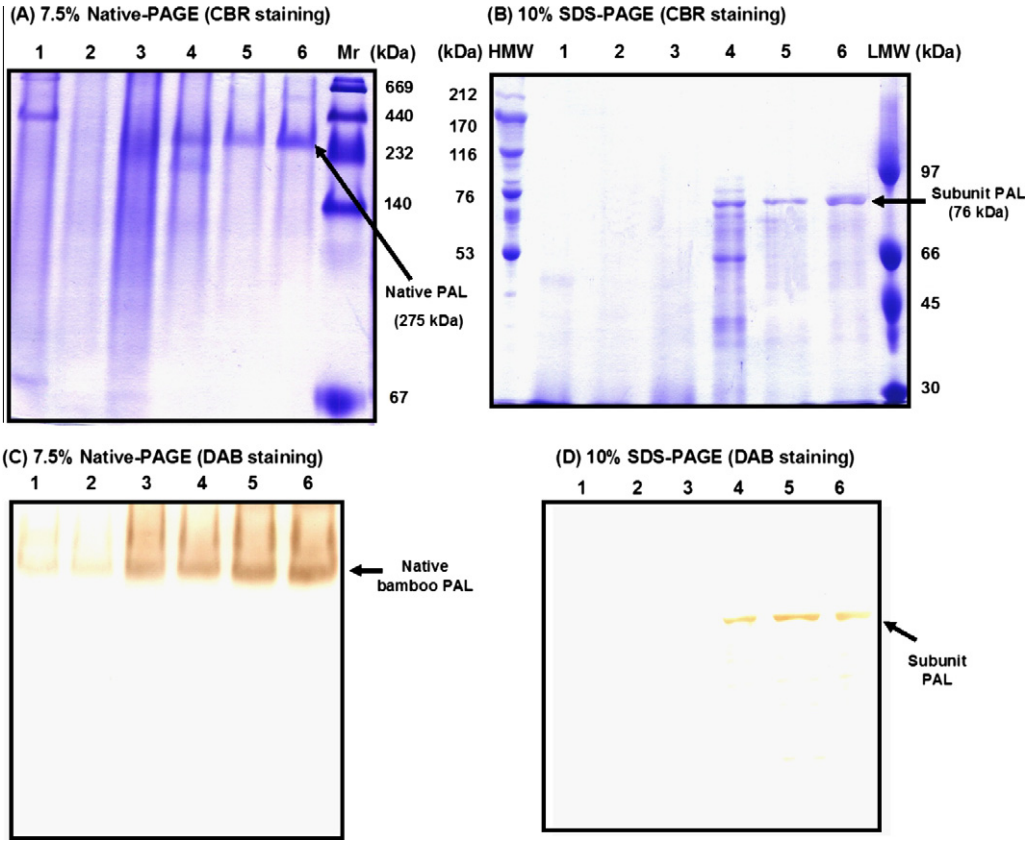


Fig. 1. Polyacrylamide gel electrophoresis of purified PAL protein obtained after purification. Samples (5 μg) of soluble bamboo shell protein at the different purification steps were subjected to electrophoresis on 7.5% native-PAGE (A) and 10% SDS–PAGE (B), and then stained with Coomassie Brilliant Blue R-250. Specificity of the anti-BoPAL antibody was evaluated by Western blotting (DAB staining). Samples (5 μg) were separated by 7.5% native-PAGE (C) or 10% SDS–PAGE (D) and transferred onto PVDF membranes. Secondary antibody is conjugated with horseradish peroxidase and detected with H_2O_2/DAB. Lane 1, crude extract; lane 2, protamine sulfate precipitation; lane 3, ammonium sulfate fractionation; lane 4, Sephacryl S-200 fraction; lane 5, Phenyl-Sepharose fraction; lane 6, Mono Q HR 5/5 fraction (FPLC). Mr, high molecular weight native-PAGE marker; HMW, high molecular weight SDS–PAGE marker; LMW, low molecular weight SDS–PAGE marker.

Another two partial length *PAL* genes were also screened from cDNA library. RACE (rapid amplify cDNA ends) method was utilized to obtain the full length cDNAs of *BoPAL3* and *BoPAL4*; these sequences have been deposited at GenBank with the accession numbers GU338008 and GU592807, respectively.

BoPAL3 contained a 2142 bp ORF and encoded a 713 amino acid polypeptide or a 76.94 kDa protein. *BoPAL4* contained a 2106 bp ORF and encoded a 701 amino acid polypeptide or a 75.71 kDa protein. Protein sequence alignment analysis showed that BoPAL3 and BoPAL2 shared 90% identity; however, BoPAL4 and BoPAL2 only shared 77% identity (Fig. 4; Table 2). All PAL sequences contained a conserved active site motif, a Ala-Ser-Gly triad, which can be converted into a 3,5-dihydro-5-methylidine-4H-imidazol-4-one (MIO) prosthetic group (Rétey, 2003; MacDonald and D'Cunha, 2007).

The genomic sequences containing *BoPAL2*, *BoPAL3*, and *BoPAL4* were amplified by PCR and the sequences of the genomic DNA and cDNA aligned using the Spidey program (NCBI). The intron–exon organization is shown and drawn to scale in Fig. 5A. It was astonishing to find that there was no intron in *BoPAL1* (Hsieh et al., 2010a). However, it had been reported that a *GbPAL* gene in *Ginkgo biloba* did not contain an intron either (Xu et al., 2008). Comparison of the genomic DNA and cDNA sequences indicated that *BoPAL2*, *BoPAL3* and *BoPAL4* contained one intron and two exons (Fig. 5A; Table 2), as also observed for most *PAL* genes characterized to date, such as *AtPAL1* and *AtPAL2* in *A. thaliana* (Wanner et al., 1995), *Salvia miltiorrhiza* (Song and Wang, 2009), *Oryza sativa* (Minami et al., 1989), *Coffea canephora* (Mahesh et al., 2006) and so on. The exon–intron structure of *BoPALs* followed the GT-AG rule. The intron in *BoPAL2* and *BoPAL3* were 135 and 96 bp long, smaller than that in *SmPAL1* (622 bp) and AtPAL1 (458 bp). The intron in *BoPAL4* was 1297 bp long, similar to that in *OsPAL1* (1310 bp).

A phylogenetic tree was generated based on the deduced amino acid sequence of BoPALs and PALs from different plant species (Fig. 5B). BoPAL1 shared 96% amino acid sequence identity with PpPAL1 and BoPAL2 (Table 2). BoPAL1/BoPAL2 shared 89–90% amino acid sequence identity with BoPAL3 (Table 2). BoPAL4 was not only the closest homolog to OsPAL1 with 94% amino acid identity but also shared similar exon–intron organization (Fig. 5A). Furthermore, previous research on ZmPAL showed that the recombinant protein exhibited both PAL and TAL activities (Rösler et al., 1997). BoPAL4 was similar to ZmPAL which was indicated that BoPAL4 might have dual PAL/TAL activities.

2.5. Expression of BoPALs in E. coli

E. coli was employed for production of recombinant BoPALs. The recombinant proteins isolated from *E. coli* were designated as BoPAL1, and BoPAL2 and BoPAL4, respectively. The pTrcHisA vector was used to construct the plasmid for heterologous protein production in *E. coli* (Top10 strain). A $(His)_6$-tag was fused to the N-terminus to facilitate protein purification.

His 89 of RsTAL is an important determinant for substrate specificity (Louie et al., 2006; Watts et al., 2006). The conserved residues of BoPALs were Phe 133 of BoPAL1, Phe 134 of BoPAL2/BoPAL3, and His 124 of BoPAL4 (Fig. 6A). Two mutants, BoPAL1

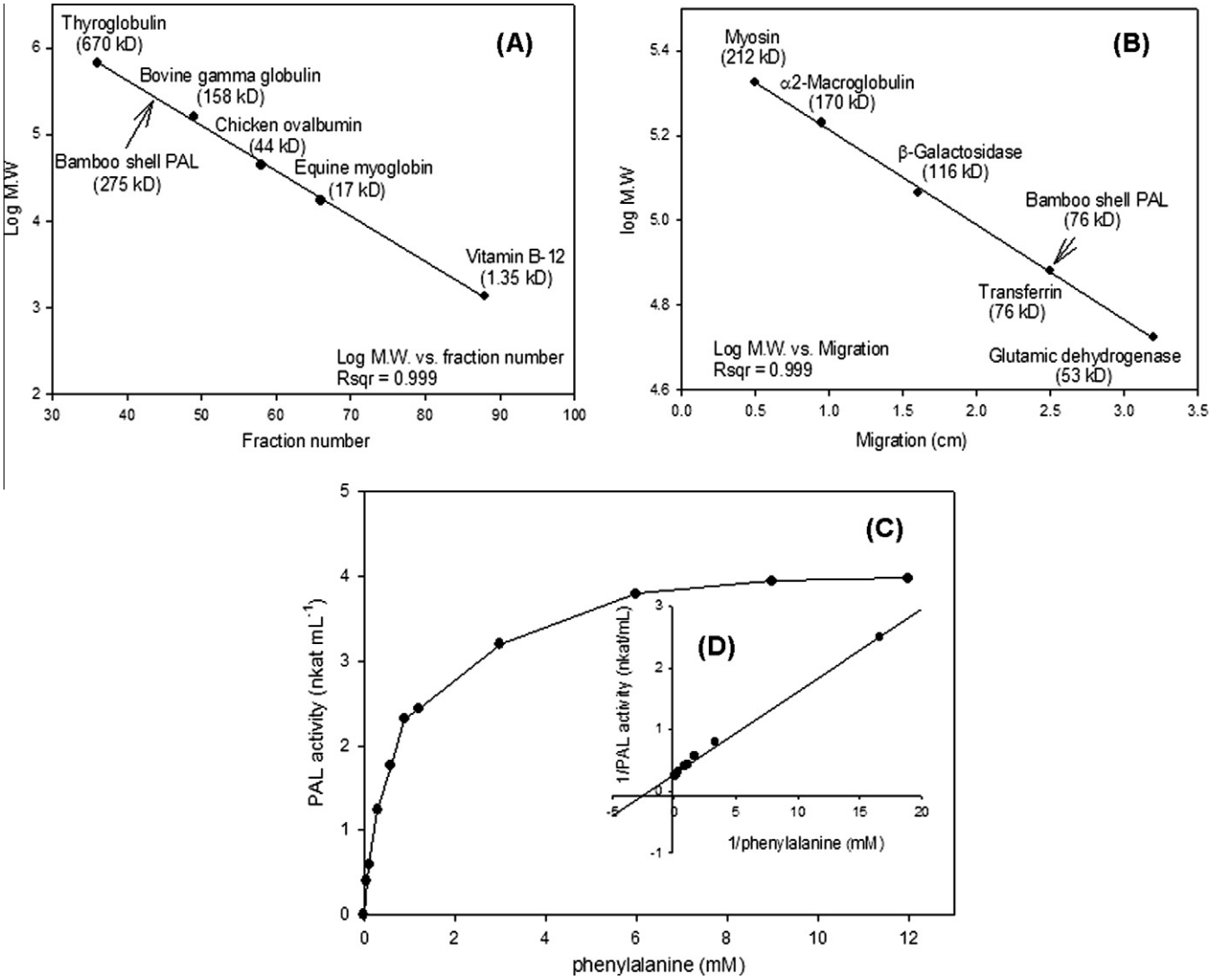


Fig. 2. Biochemical characterizations of purified bamboo shell PAL. (A) Molecular mass determination. (A) Native molecular mass of PAL determined by gel filtration chromatography on a Superose 6 column. (B) Subunit molecular mass of PAL determined by 10% SDS–PAGE. Standard curves were generated using molecular weight markers. (C) Substrate saturation plot and (D) Lineweaver–Burk plot of the initial rate data for PAL. The initial rate was determined as the change in the A_{290} over the 30 min incubation period at various concentrations of phenylalanine.

F133H and BoPAL2 F134H, were created by site-directed mutagenesis method for substrate specificity study.

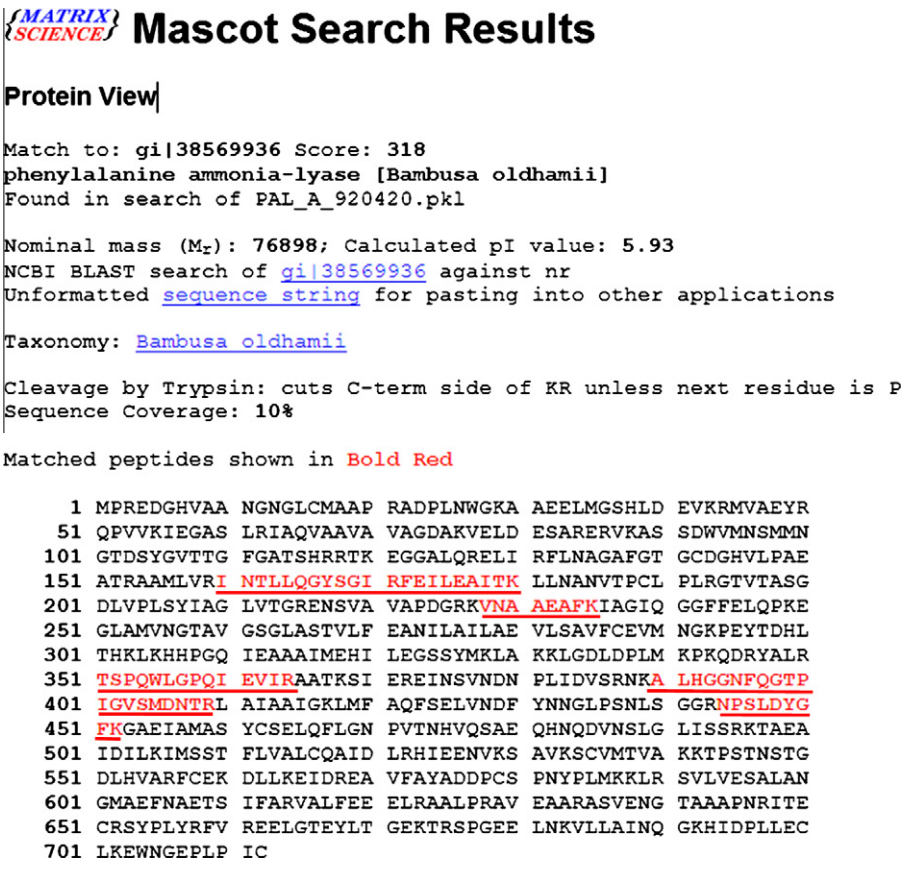
The recombinant $(His)_6$-tagged BoPAL proteins were purified on a Ni-IDA chelating column with Ni^{2+} ions, with recombinant proteins eluted at 250 mM imidazole buffer (Fig. 6B, lanes 1–5). All recombinant proteins could detect PAL activity. In this study, *E. coli* is suitable for recombinant BoPAL proteins expression.

2.6. Substrate specificity study

PAL activity was measured from three wild-type and two mutant BoPALs (Table 3). No TAL activity was measured from BoPAL1, and only a slight TAL activity was detected using BoPAL2 with a K_m of 993 μM. However, BoPAL4 was a potent PAL/TAL enzyme with clear TAL activity. The K_m value of BoPAL4 toward L-Phe was calculated as 2072 μM which was about 6–9 times higher than BoPAL1 (230 μM) and BoPAL2 (333 μM). However, the k_{cat} value of BoPAL4 toward L-Phe was calculated as 16.32 s^{-1}, which was comparable to BoPAL1 (16.29 s^{-1}) and BoPAL2 (21.3 s^{-1}). The K_m values of BoPAL1 F133H and BoPAL2 F134H toward L-Phe were calculated as 4220 and 2288 μM, respectively, which indicated reduced substrate affinity in these mutants; however, the k_{cat} values of the two mutant proteins were comparable or only slightly lower than those values of wild-type proteins (Table 3). Both BoPAL1 F133H and BoPAL2 F134H showed decreased k_{cat}/K_m values toward L-Phe. It is interesting to note that BoPAL2 F134H showed a slightly increased k_{cat}/K_m value toward L-Tyr.

2.7. Comparison of biochemical properties in native and recombinant BoPALs

The biochemical properties of PALs purified from bamboo shell, produced in heterologous host are summarized in Table 4. Gel filtration on Superose 6 was used for molecular mass determinations of the native and recombinant proteins. The results showed that BoPAL1, BoPAL2 and BoPAL4 had an estimated molecular mass of about 290–330 kDa. On SDS–PAGE, the recombinant proteins showed a major band with a molecular mass of about 80 kDa (Fig. 6B, lanes 1, 3, and 5), comparable to the predicted mass (77 kDa). By comparing the molecular mass estimated from gel fil-



MATRIX SCIENCE **Mascot Search Results**

Protein View

Match to: **gi|38569936 Score: 318**
phenylalanine ammonia-lyase [Bambusa oldhamii]
Found in search of PAL_A_920420.pkl

Nominal mass (M_r): **76898**; Calculated pI value: **5.93**
NCBI BLAST search of gi|38569936 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Bambusa oldhamii

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **10%**

Matched peptides shown in Bold Red

```
    1 MPREDGHVAA NGNGLCMAAP RADPLNWGKA AEELMGSHLD EVKRMVAEYR
   51 QPVVKIEGAS LRIAQVAAVA VAGDAKVELD ESARERVKAS SDWVMNSMMN
  101 GTDSYGVTTG FGATSHRRTK EGGALQRELI RFLNAGAFGT GCDGHVLPAE
  151 ATRAAMLVRI NTLLQGYSGI RFEILEAITK LLNANVTPCL PLRGTVTASG
  201 DLVPLSYIAG LVTGRENSVA VAPDGRKVNA AEAFKIAGIQ GGFFELQPKE
  251 GLAMVNGTAV GSGLASTVLF EANILAILAE VLSAVFCEVM NGKPEYTDHL
  301 THKLKHHPGQ IEAAAIMEHI LEGSSYMKLA KKLGDLDPLM KPKQDRYALR
  351 TSPQWLGPQI EVIRAATKSI EREINSVNDN PLIDVSRNKA LHGGNFQGTP
  401 IGVSMDNTRL AIAAIGKLMF AQFSELVNDF YNNGLPSNLS GGRNPSLDYG
  451 FKGAEIAMAS YCSELQFLGN PVTNHVQSAE QHNQDVNSLG LISSRKTAEA
  501 IDILKIMSST FLVALCQAID LRHIEENVKS AVKSCVMTVA KKTPSTNSTG
  551 DLHVARFCEK DLLKEIDREA VFAYADDPCS PNYPLMKKLR SVLVESALAN
  601 GMAEFNAETS IFARVALFEE ELRAALPRAV EAARASVENG TAAAPNRITE
  651 CRSYPLYRFV REELGTEYLT GEKTRSPGEE LNKVLLAINQ GKHIDPLLEC
  701 LKEWNGEPLP IC
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Fig. 3. Mascot search result for bamboo shell PAL. Shell PAL was in-gel digested with trypsin and analyzed by LC-Q-TOF MS/MS. The acquired data were used to search the NCBI protein database.

tration and SDS–PAGE, the recombinant proteins were found to exist as a homotetramer, as noted for most other PALs reported (Appert et al., 1994).

The optimum temperature and pH for bamboo shell PAL were determined to be 50 °C and pH 9.0, respectively. Similar results were observed for the BoPAL4. However, BoPAL1 and BoPAL2 were slightly different, as they had an optimum temperature of 60 °C and an optimum pH of 8.0 (Table 4). PAL is relatively stable at a high temperature. The activation energy (Ea) of bamboo shell PAL was calculated to be 11.8 kcal/mol using an Arrhenius plot, while those of the recombinant proteins were between 9.3 and 24.7 kcal/mol, close to that of native bamboo shell PAL.

The K_m of bamboo shell-purified PAL for L-Phe was calculated as 476 μM. Recombinant BoPAL1 and BoPAL2 had smaller K_m values (230 and 333 μM, respectively), while BoPAL4 had value of 2072 μM. These kinetic parameters implied that PAL purified from bamboo shell might be present as a protein complex formed by different PALs.

2.8. Immunohistochemistry

The anti-PAL antibody was used to determine tissue localization of PAL in sections of bamboo shell (Fig. 7A) and branch shoot (Fig. 7E). Those tissues are suitable for paraffin fixation; its vascular bundles are fully mature and easy to observe. The green shell used would have begun photosynthesis and PAL can be induced by light (Fig. 7A). Branch shoots can grow from any node on bamboo culms (Fig. 7E), green bamboo shell and branch shoots were paraffin-embedded and sectioned transversely. No signal was detected on the section treated with pre-immune serum (Fig. 7B). Using the anti-PAL antibody, PAL was found to be most abundant in sclerenchyma cells, which are cells with thickened lignified walls (Fig. 7C). Lignin staining is shown in Fig. 7D, which also stains the sclerenchyma cells. Lignin is a common component of plant secondary cell walls. The location of PAL correlated with that of lignin-enriched tissue (Sakurai et al., 2001), and PAL is also the entry point into the phenylpropanoid pathway; however, PAL does not act as a rate-limiting step of monolignol biosynthesis (Anterola and Lewis, 2002). In the branch shoots (Fig. 7F), PAL was more abundant in sclerenchyma cells than parenchyma cells (Fig. 7G and H), similar to results obtained with shell sections. A strong PAL signal was also detected from smaller cells near the epidermis of branch shoots. The cell walls of the epidermis contains waxy substances to prevent water loss. Some waxy substances are composed of polyphenolics as structural monomers which are phenylpropanoid derivatives, and the biosynthesis of polyphenolics share similar upstream reactions as for lignin biosynthesis.

3. Discussion

Purification of PAL from different species to near homogeneity has been reported (Bolwell et al., 1985; Kim et al., 1996). In this study, PAL was isolated from bamboo shell with an ~135-fold purification and a 10% yield (Table 1). About 0.5 mg of PAL enzyme could be extracted from 100 g fresh weight of bamboo tissue. These results indicate that PAL is abundant in bamboo shell. During the fast growth of bamboo, PAL may play an important role in supplying precursors for plant secondary metabolites. Purity of purified bamboo PAL was monitored by Coomassie blue staining and further confirmed by Western-blotting (Fig. 1), which corresponded to the band used for Q-TOF MS (Fig. 3).

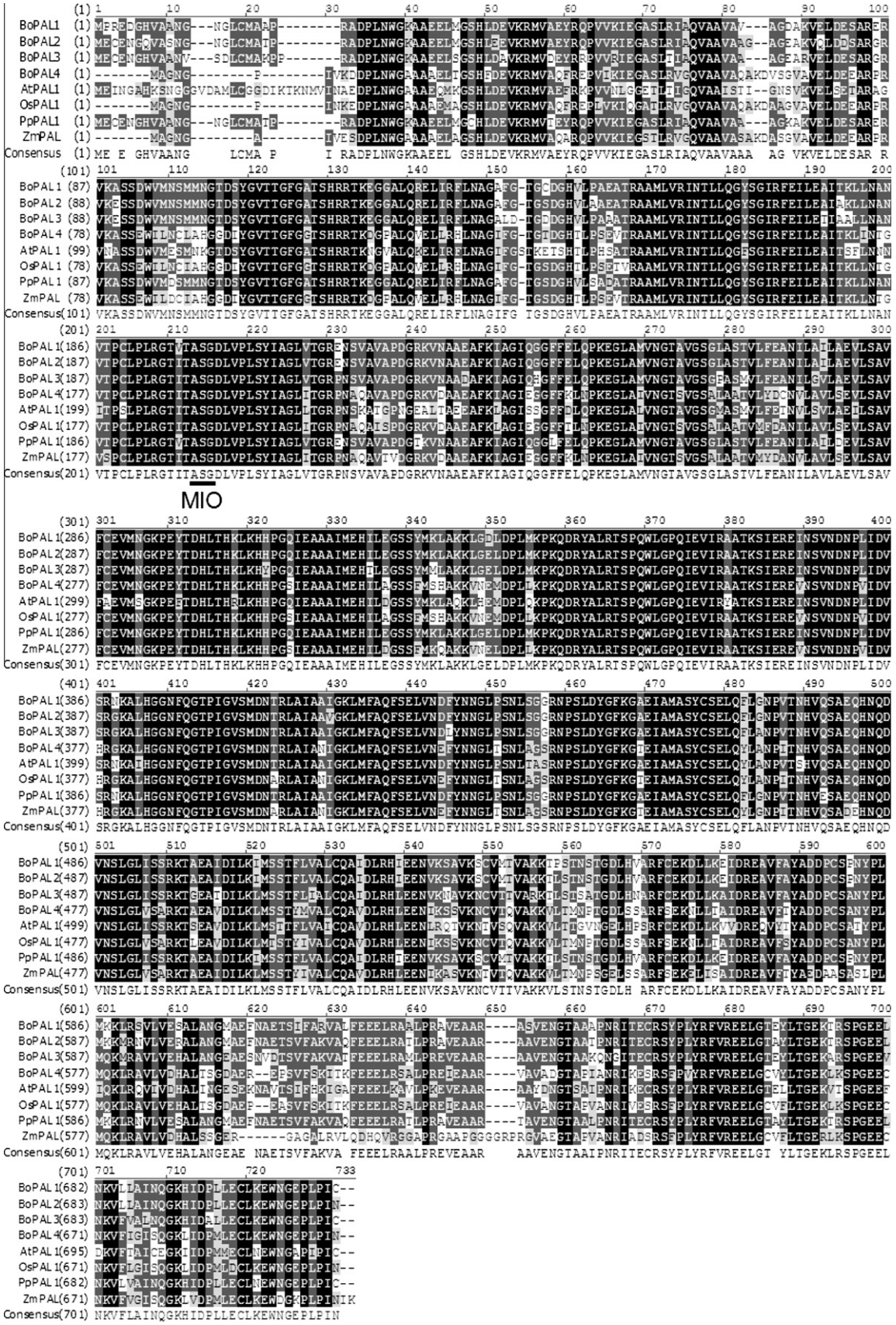

Fig. 4. Multiple sequence alignment of deduced amino acid sequences of PALs from monocot and dicot plants. The sequences compared are from *Bambusa oldhamii* (BoPAL1, AY450643; BoPAL2, FJ715635; BoPAL3, GU338002; BoPAL4, GU592807), *Arabidopsis thaliana* (AtPAL1, AY303128), *Oryza sativa* (OsPAL, AK102817), *Phyllostachys parvifolia* (PpPAL, FJ592178), and *Zea mays* (ZmPAL, EU957015). The conserved active site motif (Ala-Ser-Gly) can be converted into a MIO prosthetic group and is indicated by line under the sequences.

Table 2
Sequence identities among *BoPAL* genes.

Gene name	Number of exons	Length of intron	Number of amino acids	Mass (kDa)	Predicted pI	Identity to BoPAL1 (nt/aa, %)[a]
BoPAL1	1	0	712	76.95	5.9	100/100
BoPAL2	2	135	713	76.97	6.1	91/96
BoPAL3	2	96	713	76.94	5.6	83/89
BoPAL4	2	1271	701	75.71	6.1	79/77

[a] nt: nucleotide; aa: amino acid.

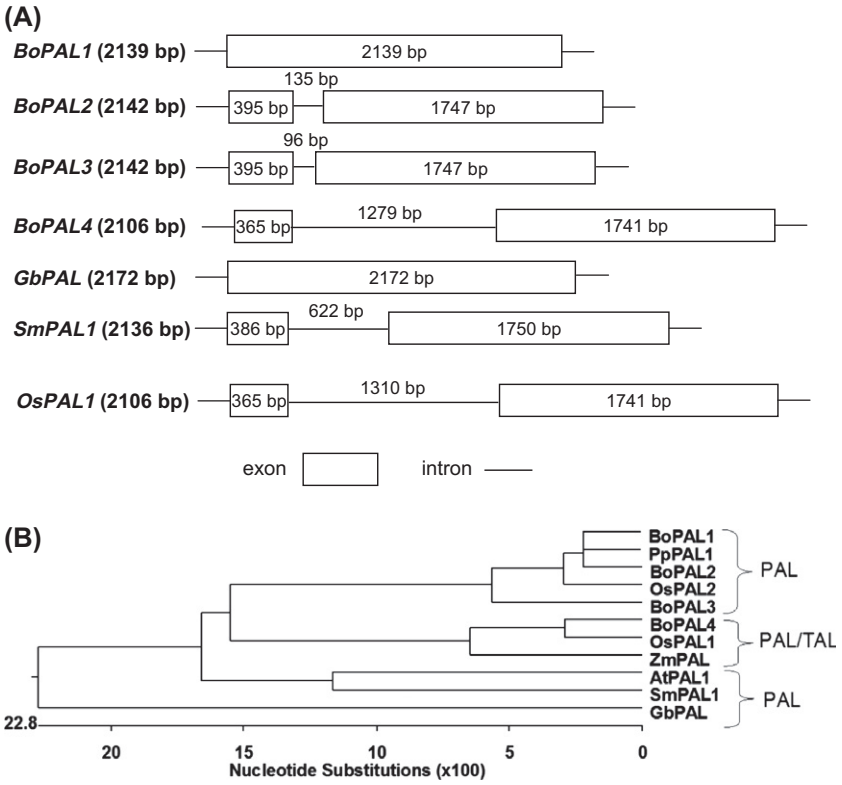

Fig. 5. Genomic organization of the *PAL* genes in various species (A). The exons (rectangles) and introns (lines) are drawn to scale and the number of base pairs (bp) indicated. Sequences compared are from *Bambusa oldhamii* (*BoPAL1*, GQ871752; *BoPAL2*, GQ871753; *BoPAL3*, HM009319; *BoPAL4*, and GU592808), *Arabidopsis thaliana* (AtPAL1, AY303128), *Oryza sativa* (OsPAL, AK102817), *Phyllostachys parvifolia* (*PpPAL*, FJ592178), *Salvia miltiorrhiza* (*SmPAL*, EF462460), *Ginkgo biloba* (*GbPAL*, EU071050) and *Zea mays* (ZmPAL, EU957015). Phylogenetic analysis of PALs from other plant species (B).

Four *PAL* genes were cloned from *B. oldhamii* and three of them expressed three in *E. coli*. All recombinant proteins exhibited PAL activity and existed in homotetrameric form. PALs from higher plants have been reported to be tetramers and encoded by a multiple gene family (Jones, 1984; Appert et al., 1994). Bamboo shell PAL seemed to be a single tetramer when purified, and more evidence is still needed to determine if this tetramer is either an heterotetramer or homotetramer. Wounding, an elicitor, or gamma-irradiation can induce the expression of different PAL isozymes (Sarma et al., 1998; Sarma and Sharma, 1999). Further studies will examine the transcriptional profile of the four *BoPAL* genes.

Although BoPALs share a conserved active site motif, BoPAL4 seems to be different from the other three BoPALs in the substrate selectivity point and the putative phosphorylation site (Fig. 6A). The phosphorylation site of French bean PAL has been identified as a Thr residue in the conserved motif (R/K–X–X–S/T) (Allwood et al., 1999). This Ser/Thr residue is well conserved in the four BoPAL (Fig. 6A), indicated that all BoPALs might be regulated by phosphorylation.

PALs from several monocots have been reported to have TAL activity and to utilize tyrosine as a substrate (Havir et al., 1971; Rösler et al., 1997). PAL purified from bamboo shell could thus possess both PAL and TAL activity (data not shown). However, only BoPAL4 had an higher affinity to catalyze L-Tyr as physiological substrate, although the K_m value and sequence alignment results indicated that PAL purified from shell was closer to BoPAL1 and BoPAL2. As a result, shell PAL is likely to be present as a heterotetramer. Although His 124 might be an important residue for BoPAL4 in substrate specificity, BoPAL2 F134H showed only a slightly increased TAL activity, while both BoPAL1 F133H and BoPAL2 F134H showed decreased affinity for L-Phe (Table 3). A small level of TAL activity was detected using BoPAL2 (Hsieh et al., 2010b), and also the TAL activity of BoPAL2 F134H was higher than that in BoPAL2. Accordingly, it seems more likely that other residues also contribute to the TAL activity of BoPAL2. More site-directed mutagenesis experiments are necessary to fully understand how PAL and TAL activities are actually determined.

The anti-PAL antibody specifically recognizes the protein isolated from bamboo (Fig. 1) and the recombinant proteins. Bamboo shell is an unique tissue which has properties between shoot and leaf. Exposure of bamboo shoots to daylight leads to chloroplast formation, followed by photosynthesis, and the bamboo shell then changes from white to green. Sections of the green shell and branch shoots contained fully developed vascular bundles, including one protoxylem, two xylems, and one phloem, surrounded by sclerenchyma (Fig. 7). The vascular bundle of white bamboo shell

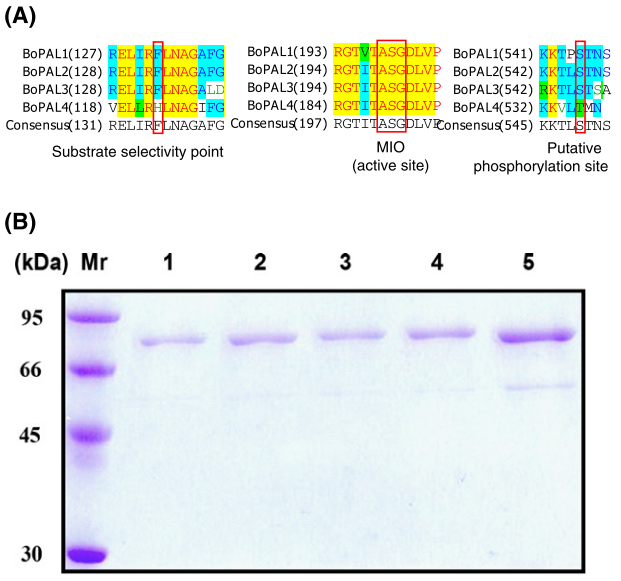

Fig. 6. Expression of three *BoPAL* genes in *E. coli*. (A) Comparison of substrate switching point, active site and putative phosphorylation site in four *BoPAL* genes. (B) Purification of the recombinant proteins by Ni-IDA column chromatography and separated 10% SDS–PAGE then stained with Coomassie Brilliant Blue R-250. Mr, low molecular weight SDS–PAGE marker; lane 1, BoPAL1; lane 2, BoPAL1 F133H; lane 3, BoPAL2; lane 4, BoPAL2 F134H; lane 5, BoPAL4.

Table 3
Kinetic parameters for BoPALs.

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
BoPAL1	L-Tyr	ND	ND	ND
BoPAL1	L-Phe	16.29	230	7.08×10^{-2}
BoPAL1 F133H	L-Tyr	0.03	103	2.66×10^{-4}
BoPAL1 F133H	L-Phe	7.8	4220	1.85×10^{-2}
BoPAL2	L-Tyr	0.03	993	2.75×10^{-5}
BoPAL2	L-Phe	21.3	333	6.39×10^{-2}
BoPAL2 F134H	L-Tyr	0.10	335	3.09×10^{-4}
BoPAL2 F134H	L-Phe	19.2	2288	8.39×10^{-3}
BoPAL4	L-Tyr	0.54	97	5.56×10^{-3}
BoPAL4	L-Phe	16.32	2072	7.88×10^{-3}

ND is not determined.

Table 4
Comparison of biochemical properties of bamboo shell PAL and expressed PALs.

Source	Optimum pH	Optimum temp. (°C)	Ea[a] (kcal/mol)	K_m (μM)	Molecular mass (kDa)
Bamboo shell	9.0	50	11.8	476	275
BoPAL1	8.0	60	9.3	230	320
BoPAL2	8.0	60	10.1	333	330
BoPAL4	9.0	50	24.7	2072	290

[a] Ea: activation energy.

is not fully developed, however, and it was not easy to observe its mature xylem and phloem structure. PAL was most abundantly detected in sclerenchyma cells, which are lignified. During development of vascular bundles, PAL in sclerenchyma cells would participate in phenylpropanoid biosynthesis.

3.1. Concluding remarks

Browning and lignification after harvest are problematic in agriculture (Hisaminato et al., 2001). The accumulation of polyphenolic compounds and lignin biosynthesis induced by wounding are critical factors in post-harvest physiology. During storage of harvested vegetables or fruits, polyphenols are produced by the phenylpropanoid pathway and further oxidized by polyphenol oxidase (EC 1.14.18.1) to produce brown substances. Chemicals have been used to prevent enzymatic browning of cut lettuce (Fujita et al., 2006). To prevent cut lettuce from browning during storage, heat shock treatment at 50–60 °C is used to suppress induction of PAL due to cutting (Murata et al., 2004). Increased PAL activity and ethylene are found in some citrus fruits stored at a chilling temperature (Lafuente et al., 2001). Post-harvested bamboo shoots usually undergo short heat treatment, and then are stored at low temperature. A better understanding of the properties of bamboo PAL might thus help in developing an improved procedure for storage of bamboo shoots. Transgenic bamboo with altered expressed *PAL* could have improved end products of polyphenolics and better quality shoots with less bitter tasting.

4. Experimental

4.1. Plant material

Bamboo (*B. oldhamii*) shoot was harvested between April and September and separated into two major fractions of edible shoot and non-edible shell, which were stored at −80 °C until use.

4.2. Methods

4.2.1. Protein purification

Unless stated otherwise, all purification steps were carried out at 4 °C. Bamboo shells (100 g) were ground in liquid N_2 and homogenized in extraction buffer (200 ml) (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 1% (w/v) polyvinylpolypyrolidone). The homogenate was filtered through four layers of Miracloth, and then centrifuged for 15 min at 10,000*g*. The supernatant was made to 0.2% (w/v) using protamine sulfate and centrifuged for 15 min at 10,000*g*. $(NH_4)_2SO_4$ was then added to the supernatant in steps and PAL was precipitated between 40% and 60% saturation. After centrifugation at 12,000*g* for 30 min, the pellet was dissolved in buffer A (50 mM Tris–HCl, pH 7.5) and dialyzed overnight against buffer A. The sample was then concentrated on an Amicon (Millipore, USA) and applied to a Sephacryl S-200 column (2 × 100 cm) and eluted with buffer A. Fractions with PAL activity were pooled and $(NH_4)_2SO_4$ added to a final concentration of 0.5 M. The sample was then applied to a Phenyl-Sepharose column (5 × 10 cm) previously equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$ and eluted with a linear gradient of 0.5–0 M $(NH_4)_2SO_4$ in buffer A. Fractions with PAL activity were pooled and dialyzed against buffer A to remove $(NH_4)_2SO_4$, then this was applied to a Mono Q HR 5/5 column (FPLC, Pharmacia/LKB) equilibrated with buffer A and eluted with a liner gradient of 0–0.5 M NaCl in buffer A. Fractions with PAL activity were combined and stored at −20 °C for further analysis.

4.2.2. Activity assay

Protein concentrations were determined by a dye-binding method (Bradford, 1976) using bovine serum albumin as the protein standard. PAL activity was assayed by measuring *trans*-cinnamic acid formation by the absorbance increase at 290 nm (Hsieh et al., 2010b). *Trans*-cinnamic acid was used to generate the standard curve. The reaction mixture contained 50 mM Tris–HCl, pH 8.5, 12.1 mM L-phenylalanine, and an aliquot of enzyme in a total volume of 1.0 ml. The reaction was carried out at 37 °C for 30 min and terminated by addition of 6 N HCl (100 μl). As a control, D-phenylalanine was replaced with L-phenylalanine. PAL activity was expressed in nkat (nanomole of *trans*-cinnamic acid formed per second).

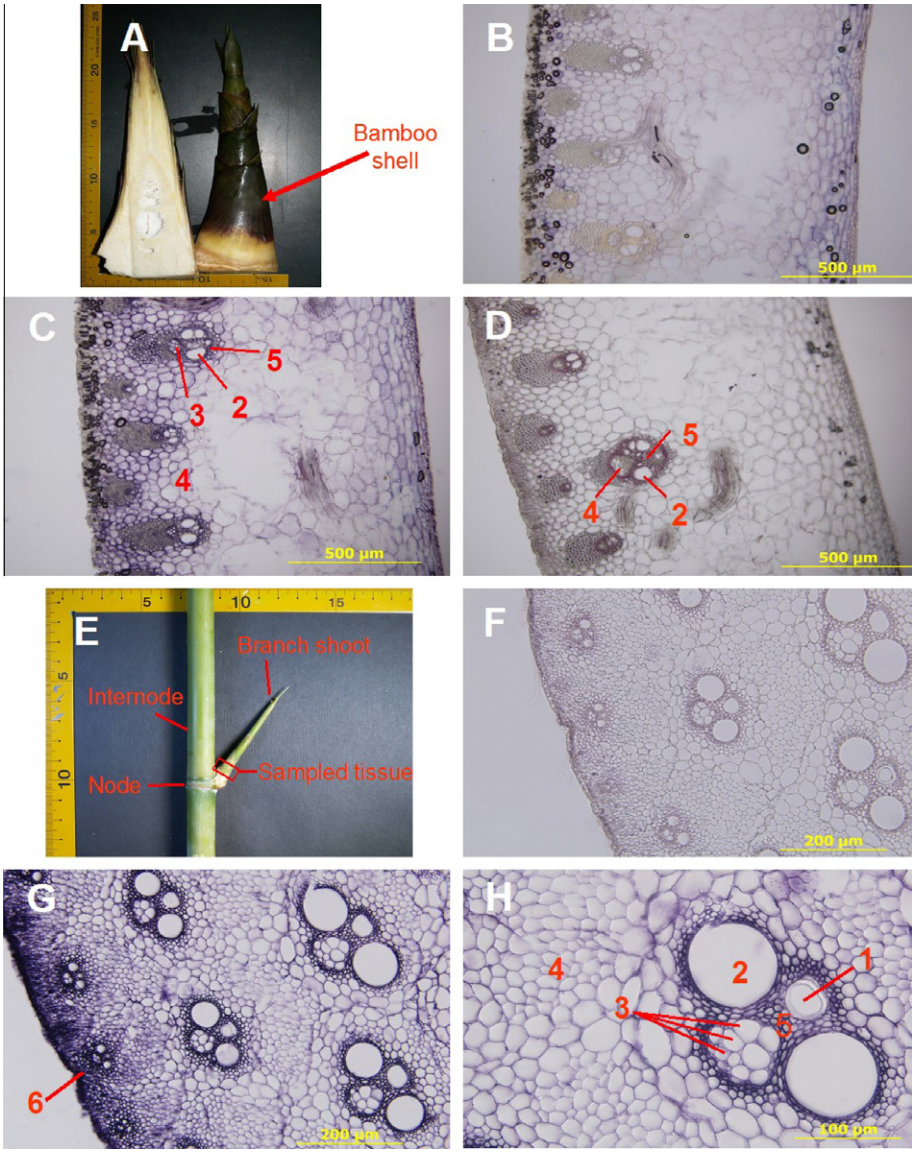

Fig. 7. Localization of BoPAL in the shell (A–D) and branch shoot (E–H) of bamboo. Green bamboo shell (A) and branch shoot (E) were sectioned transversely and stained with pre-immune serum (B and F) or anti-PAL antibody (C, G, and H). Lignin staining using the phloroglucinol-HCl method was visualized as a red color (D). (1) Protoxylem; (2) xylem; (3) phloem sieve element; (4) parenchyma cell; (5) sclerenchyma cell; (6) epidermis. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

TAL activity was assayed by monitoring *p*-coumaric acid formation at 310 nm (Kyndt et al., 2002). The substrate concentration was varied between 0.1 and 1.9 mM. TAL activity was expressed in nkat (nanomole of *p*-coumaric acid formed per second).

To determine the optimum pH, assays were performed at 37 °C for 30 min using a series of universal buffers (pH 4.0, 5.0, 6.0, 7.0, 8.0, 8.5, 9, 10.0, and 11.0). To determine the optimum temperature, assays were performed at pH 8.5 for 30 min with various temperatures (20, 30, 40, 50, 60, 70, and 80 °C). The activation energy was calculated using the Arrhenius equation and is presented as kcal/mol.

To determine the kinetic parameters, a range of concentrations of L-Phe was used. Using the Michaelis–Menten equation, hyperbolic plots were obtained and converted to double reciprocal plots (Lineweaver–Burk plot) to calculate the K_m.

4.2.3. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% native-gels and 10% SDS-gels (Hsieh et al., 2010b). The native protein standards (GE Healthcare, USA) contained thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa). Following electrophoresis, proteins were stained by Coomassie Brilliant Blue R-250.

4.2.4. Antibody production and Western blotting

Bamboo-purified PAL, eluted from a preparative non-denaturing polyacrylamide gel, was used to immunize 4-week-old female BALB/c mice to generate polyclonal antibodies. The protein (50 μg in PBS buffer) was emulsified with an equal volume of Freund's complete adjuvant (Sigma, USA) for the initial intraperitoneal (i.p.) injection and with Freund's incomplete adjuvant (Sigma, USA) for i.p. boosts. After four booster injections at 2-week intervals, the mice were injected i.p. with 2×10^6 SP2 myeloma cells for ascites fluid production. Antibodies from the collected ascites fluid were further purified by ammonium sulfate precipitation, and then the precipitate was resuspended in PBS buffer containing 50% glycerol and stored at −20 °C.

Purified shell PAL was separated on native- and SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (400 mA h, 1 h, 4 °C) using CAPS buffer (10 mM CAPS, pH 11, 10% MeOH). All subsequent steps were at room temperature. The membrane was washed twice with PBS, blocked for 1 h with NET solution (0.25% gelatin, 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 0.05% Tween 20, pH 8.0), washed, incubated for 1 h with anti-PAL antibody diluted in NET (1:1500), then incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (KPL, USA) before bound antibody was detected using diaminobenzidine (DAB, Sigma).

4.2.5. Molecular mass determination

The molecular mass of PAL was estimated by gel filtration chromatography and SDS–PAGE. To determine the molecular mass of the native protein, PAL was applied to a Superose 6 HR 10/20 column and eluted with buffer A on an FPLC system. The column was calibrated using the following standards (Bio-Rad, USA): thyroglobulin (660 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa). A plot of log (molecular mass) vs. mobility was generated to calculate the molecular mass of the purified enzyme. The molecular mass of the PAL subunit was determined by 10% SDS–PAGE. Standard curve was constructed using the following molecular mass markers (GE Healthcare, USA): myosin (212 kDa), α2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa).

4.2.6. Q-TOF mass spectrum

The pure enzyme preparation was subjected to electrophoresis and the gel stained with CBR. The main protein band was excised for in-gel digestion (Kristensen et al., 2000). The gel section was cut into small particles, then 25 mM NH_4HCO_3 (100 μl) (pH 8.0)/CH_3CN–H_2O (1:1, v/v) was added and the suspension mixed by constant vortexing for 10 min. The supernatant was removed and discarded and this wash/dehydration step was repeated up to three times. The gel particles were then covered with 10 mM DTT for 1 h at 56 °C, then the DTT was removed, the same volume of 55 mM iodoacetamide added, and the sample incubated at room temperature for 45 min with occasional vortexing. The gel particles were rehydrated with 25 mM NH_4HCO_3 (100 μl) and dehydrated with 25 mM NH_4HCO_3/CH_3CN–H_2O (1:1, v/v), then the liquid phase was removed and the gel particles dried on a vacuum centrifuge. The rehydrated gel particles were then suspended in an equal volume of 0.1 mg/ml sequencing grade trypsin (Sigma–Aldrich, St. Louis, USA) and the sample incubated at 37 °C for 12 h. The peptides formed were extracted three times by covering the gel particles with 5% TFA/CH_3CN–H_2O (1:1, v/v) and the extracts concentrated to about 25 μl on a vacuum centrifuge. Samples were analyzed on a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanoelectrospray ionization source. Data analysis was performed using the on-line Mascot program (http://www.matrixscience.com/), which is based on the NCBI database.

4.2.7. Gene cloning

cDNA library screening was as for Hsieh et al. (2010b). Hybridization and detection were performed under the optimum conditions described by the manufacturer (Roche, Germany). Positive clones were phage-purified and phagemids were converted into plasmids by *in vivo* excision. The inserts were completely sequenced. Two full-length *PAL* cDNAs were designated as *BoPAL1* (Hsieh et al., 2010a) and *BoPAL2* (Hsieh et al., 2010b). Two partial *PAL* cDNAs were isolated from library. Smart™ RACE kit was performed to synthesis 5′- and 3′-ends of *BoPAL3* and *BoPAL4* cDNAs following the manufacturer's instruction (Clontech, USA).

Sequence alignments were carried out using Vector NTI 10.0 Sequence Analysis Software (Invitrogen, USA). A phylogenetic tree was constructed by MegAlign program in DNAStar software using the ClustalW method (Thompson et al., 1994).

4.2.8. Determination of the PAL genomic DNA sequence by PCR

Total RNA and genomic DNA were extracted from bamboo shoot using the TRIZOL reagent (Invitrogen, USA). The specific primers of *BoPALs* were designed based on the coding sequence of *BoPALs* cDNA. Genomic DNA was used as the template and betaine (trimethylglycine) was added to a final concentration of 2 mM (Spiess and Ivell, 2002), then PCR was performed under the conditions of 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 1 min at 58 °C, and 3 min at 72 °C; and a final extension of 5 min at 72 °C. Genomic DNA to cDNA sequence alignment was performed using the Spidey program on NCBI.

4.2.9. Expression and purification of recombinant BoPALs

BoPAL2 and *BoPAL4* were subcloned into the pTrcHis A vector (Invitrogen, USA) using the *Bam*H I/*Eco*R I and *Kpn* I/*Eco*R I restriction sites, respectively. A single recombinant *E. coli* Top10 colony was used to inoculate 250 ml of LB (Luria–Bertani) medium containing ampicillin (100 μg/ml). IPTG (isopropyl-β-D-thiogalactoside) was added to a final concentration of 1 mM when an OD_{600} of 0.6 was reached and the cells were further incubated at 30 °C with vigorous shaking for 12 h.

Recombinant proteins were purified on a HiTrap Chelating HP column (GE Healthcare, USA), which was charged with nickel (Ni^{2+}) ions and eluted with 10, 100, and 250 mM imidazole buffers.

4.2.10. Site-directed mutagenesis

Constructed pTrcHis PAL1 and pTrcHis PAL2 vectors were utilized for site-directed mutagenesis study by using a QuikChange® kit (Stratagene, Germany). PCR was performed under conditions of 1 min at 95 °C; 16 cycles of 50 s at 95 °C, 50 s at 60 °C, and 13 min at 68 °C (2 min/kb); and a final extension of 7 min at 68 °C. Circular form methylated plasmid was digested by *Dpn* I, and then the linear form PCR product was transformed and expressed in *E. coli* Topo10 cell.

4.2.11. Immunohistochemistry and lignin staining

All steps were for 1 h at room temperature. Bamboo shell and branch shoots were fixed in 4% paraformaldehyde/1% glutaraldehyde in 10 mM phosphate buffer, pH 7.2, and dehydrated with *t*-BuOH and EtOH. Paraffin-embedded tissue was sectioned (10 μm thick) and placed on glass slides pretreated with 0.5% gelatin. Sections were deparaffinized with xylene and rehydrated through a graded series of EtOH, then were washed with TBST buffer (20 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0) and probed using anti-PAL antibody diluted in NET solution (1:250) and alkaline phosphatase-conjugated goat anti-mouse IgG antibodies as secondary antibody. After washing, bound antibodies were detected using NBT/BCIP solution (Roche, Germany).

Lignin was stained by the phloroglucinol-HCl method (Gahan, 1984). Rehydrated sections were treated with EtOH–H_2O (80:20, v/v) (v/v) ethanol, 2.4 M HCl, and 1% (w/v) phloroglucinol. The lignified walls were stained red within a few minutes and the sections were photographed immediately.

Acknowledgements

The authors thank Prof. Rong-Huay Juang for helping in producing the anti-PAL antibody and Prof. Ai-Yu Wang for providing bamboo cDNA library. This work was supported by grants from the National Science Council, Taiwan.

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