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Research article

Six phenylalanine ammonia-lyases from *Camellia sinensis*: Evolution, expression, and kinetics

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ABSTRACT

Phenylalanine ammonia-lyase (PAL), the branch point enzyme controlling the flow of primary metabolism into second metabolism, converts the L-phenylalanine (L-Phe) to yield cinnamic acid. Based on the sequencing data available from eight transcriptome projects, six *PAL* genes have been screened out, cloned, and designated as *CsPALa-CsPALf*. The phylogenetic tree showed that *Cs*PALs were divided into three subgroups, PALa and PALb, PALc and PALd, and PALe and PALf. All six *Cs*PALs exhibited indiscriminate cytosolic locations in epidermis cells and mesophyll cells. Then, the expression profiles of six *PAL* genes were qualitatively investigated and they displayed tissue-/induced-expression specificity in several tissues or under different exogenous treatments. Furthermore, *in vitro* enzymatic assays showed that all six recombinant proteins were characterized by the strict substrate specificity toward L-Phe, but no activity toward L-Tyr, and they displayed subtle differences in kinetics and enzymatic properties. These results indicate that CsPALs play both distinct and overlapping roles in plant growth and responses to environmental cues.

1. Introduction

Tea plant (*Camellia sinensis*), the raw material of tea beverage, is rich in polyphenolic compounds with all kinds of pharmacological and biological functions. For example, (-)-epifallocatechin-3-gallate (EGCG) can contribute to preventing cancer and coronary heart disease (Cabrera et al., 2006; Mukhtar and Ahmad, 2000). Secoisolariciresinol, a kind of lignan aboundant in camellia oil, is converted by the intestinal microflora into the estrogens to protect against the diseases (Herman, 2007). In terms of biological functions, the synthesis of phenylpropanoid compounds can be induced by various biotic and abiotic stresses, including UV-light, pathogen attack, and wounding (Ra Dixon, 1995). These compounds described above are all derived from cinnamate, the product catalyzed by phenylalanine ammonia lyase (PAL; E.C.4.2.1.5) (Macdonald and D'Cunha, 2007).

In the phenylpropanoid pathway, phenylalanine ammonia lyase, the branch point enzyme controlling the flow of primary metabolism into second metabolism, catalyzes the non-oxidative deamination of ammonia from L-phenylalanine (L-Phe) to yield cinnamic acid (Havir, 1971; Ra Dixon, 1995; Wu et al., 2014). In the last half century, the structure, localization, evolution, catalysis, kinetics, and differential regulation of PALs were well studied in a wide range of organisms, such as *Hordeum vulgare* (Koukol and Conn, 1961), *Arabidopsis thaliana* (Cochrane et al., 2004; Huang et al., 2010; Olsen et al., 2008; Rohde et al., 2004; Wanner et al., 1995; Zhang et al., 2013), *Petroselinum crispum* (Appert et al., 1994; Schulz et al., 1990), *Phaseolus vulgaris* (Bolwell et al., 1985), *Populus* (Chen et al., 2014; Hamberger et al., 2007; Kao and Tsai, 2002; Tsai et al., 2006), *Oryza sativa* (Hamberger et al., 2007), *Nicotiana tabacum* (Achnine et al., 2004; Reichert et al., 2009), *Rhodotorula glutinis* (Fritz et al., 1976), and *Neurospora crassa* (Sikora and Marzluf, 1982).

*Abbreviations used: Cs*PAL, phenylalanine ammonia-lyase from *Camellia sinensis*; L-Phe, L-phenylalanine; L-Tyr, L-tyrosine; MeJA, methyl jasmonate; SA, salicylic acid; ABA, abscisic acid; IBA, indolebutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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PAL is encoded by a multi-gene family. The species, *Nelumbo nucifera* (3 *PALs*), *Arabidopsis* (4 *PALs*), poplar (5 *PALs*), and rice (9 *PALs*) contain several *PAL* gene members (Cochrane et al., 2004; Hamberger et al., 2007; Raes et al., 2003; Tsai et al., 2006; Wu et al., 2014). In *Arabidopsis*, the kinetic and functional properties of these four PALs have been determined (Cochrane et al., 2004). Both *AtPAL1* and *AtPAL2* can strongly respond to abiotic environmental factors, such as nitrogen, temperature, UV-B, and play a redundant role in flavonoid biosynthesis (Hu et al., 2011; Huang et al., 2010).

However, the PALs in tea plants remain unclear in spite of their crucial roles in the formation of catechins, flavonols, and their derivatives, the major contributors to the flavour of tea infusions. A *CsPAL* cDNA (accession number D26596) was first cloned and used as a DNA marker to classify varieties and cultivars of tea plants (Matsumoto et al., 1994, 2002). The gene expression pattern of this gene was well studied under the treatment of drought stress, abscisic acid, and gibberellic acid or in albino tea plants(Singh et al., 2008; Xiong et al., 2013). Another *PAL* gene (GenBank accession number JN944578) was isolated from a species of the same Theaceae family, *Camellia chekiangoleosa*, but the length of open reading frame (ORF) was shorter than ones in other species (Wang et al., 2014b). Deep sequencing of *Camellia sinensis* also indicated that there were 11 *CsPAL* unigenes in the transcriptome (Shi et al., 2010). So far, only these two *CsPAL* genes were reported and incompletely understood. The gene number, differential expression, and kinetic characterization of *Cs*PALs remain uncharacterized.

Here, based on the sequencing data available from eight transcriptome projects, we have screened out six *PAL* genes, designated as *PALa-PALf* in this study. All six PAL isoforms were biochemically identified *in vitro* and the tissue-/induced-expression specificity of *PAL* gene family was also reported in this paper.

2. Materials and methods

2.1. Plant materials

Plant samples (*Camellia sinensis* var. *sinensis* cv. Shuchazao), including bud, first leaf, old leaf, tender stem, root, bark (separated from the 5-month-old stem located at the fourth or fifth internodes), xylem or wood (the rest part of debarked stem), were collected from the horticultural research station of Anhui Agricultural University (Anhui, China) and immediately frozen in liquid nitrogen, then stored at −80 °C until further use.

2.2. Analysis and isolation of CsPAL gene family

Eight transcriptome sequencing data sets in NCBI database (PRJNA283010 for Nongkangzao, PRJNA283013 for Huangjinya, PRJNA283232 for Quntizhong, PRJNA167330 for cultivar 'Fundingdabaicha', PRJNA223181 and PRJNA51793 for cultivar 'Longjing 43', PRJNA170701 for cultivar 'Hongye2', and PRJNA261465 for cultivar 'Teenali') (Cui et al., 2016) were available to analyze the *Cs*PAL family by sequence homology searching or by keyword (phenylalanine ammonia lyase) index. Fragment assembly using DNAMAN (LynnonBiosoft, CA, USA) was applied to eliminate the redundant unigenes after searching. The phylogenetic tree was generated as previously described (Cui et al., 2016). The accession numbers were listed in the diagram and *Pt*PAL sequences were obtained from The *Populus trichocarpa* genome release 1.1 (http://genome.jgi.doe.gov/Poptr1_1/Poptr1_1.home.html) (Tuskan et al., 2006). The phylogenetic tree data sequences were also shown in Supplementary data S1.

Total RNA was extracted from frozen tissue using Fruit-mate (Takara, DaLian, China) and RNAiso Plus (Takara, DaLian, China) according to the manufacturer's protocol. First strand cDNA synthesis was performed using the PrimeScript RT Master Mix (Takara, DaLian, China). Full length cDNA sequences of *CsPAL* genes were amplified through the SMARTer™ RACE cDNA Amplification Kit and RACE-PCR protocol according to the manufacturer's instructions (Clontech, CA, USA). The PCR products were then sent for sequencing (General Biosystems, ChuZhou, China). All primers were designed with Primer Premier 5.0 software (Premier, BC, Canada) and the sequences are listed in Supplementary data S2 Table S1.

2.3. Subcellular localization of CsPALs

For subcellular localization of *Cs*PALs-GFP fusion proteins, the pGWB5 vectors harboring GFP or *Cs*PALs-GFP were electroporated into *Agrobacterium tumefaciens* strain EHA105. These constructs were transfected into 6-week-old *Nicotiana benthamiana* leaves, and GFP signals were observed with FV1000 confocal microscope (Olympus, Tokyo, Japan).

2.4. Expression profiles in different tissues or under exogenous treatments

The expression levels of *CsPAL* genes in various tissues were characterized by semi-quantitative RT-PCR. To insure the accuracy and efficiency (90%–110%), the corresponding primers were designed in non-conserved regions and were used to amplify gene fragments to confirm the accuracy. These gene-specific primers listed in Supplementary data S2 Table S1 were further determined by the optimal standard curves according to the manufacturer's protocol (Bio-Rad, CA, USA). The semi-quantitative RT-PCR reaction was performed as follows: initial denaturation at 94 °C for 3min followed by 32 cycles of denaturation at 94 °C for 30s, annealing at 59 °C for 30s, extension at 72 °C for 30s, with a final extension at 72 °C for 10min. 2 μl products were subjected to electrophoresis on 1.2% (w/v) agarose gel.

For different exogenous treatments, the shoots sprouting around 10 cm were harvested and treated with 100 μM methyl jasmonate (MeJA), 100 μM salicylic acid (SA), 100 μM abscisic acid (ABA), 0.74 μM indolebutyric acid (IBA), 50 mM NaCl, 200 mM mannitol, or 90 mM sucrose for 24 h. The controls were treated with deionized water or 0.02% (v/v) alcohol.

2.5. Heterologous expression and purification of recombinant CsPALs

The ORFs of *CsPALa-PALc* were subcloned into the expression vector pRSFDuet-1 Vector (Novagen, Schwalbach, Germany) and transformed into *E. coli* BL21 competent cells (TransGen Biotech, BeiJing, China). Recombinant histidine-tagged proteins were purified using His-Tag purification system (Roche, Mannheim, Germany). *PALd-PALf* genes were ligated into another kind of expression vector pMAL-c2X (New England Biolabs, MA, USA) encoding water-soluble maltose-binding protein (MBP) upstream the inserted site, and subsequently transformed into *E. coli* Novablue (DE3) competent cells (Novagen, Schwalbach, Germany). Recombinant proteins were purified using amylase resin (New England Biolabs, MA, USA). The purified proteins were used for further enzymatic assays.

2.6. Enzymatic assays of CsPALs

To analyze the activity of *Cs*PALs, reactions were carried out in a 50 μl reaction buffer consisting of 0.8 mM L-Phe or L-Tyr, 200 mM