BIOLOGIA PLANTARUM 59 (2): 298-304, 2015 DOI: 10.1007/s10535-015-0493-2

Characterization of genes coding phenylalanine ammonia lyase and chalcone synthase in four *Pogostemon cablin* cultivars

S. ZENG[1], P. OUYANG[2], X. MO[2], and Y. WANG[1,3]*

Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, P.R. China[1]
Guangdong Food and Drug Vocational College, Guangzhou 510520, P.R. China[2]
Key Laboratory of Plant Germplasm Enhancement and Speciality Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, Hubei 430074, P.R. China[3]

Abstract

Pogostemon cablin (Blanco) Benth is a medicinal plant rich in terpenoids and flavonoids. Currently, the molecular mechanism of flavonoid biosynthesis in *P. cablin* remains unclear. In this study, three copies of the *phenylalanine ammonia lyase* (*PAL*) gene designated as *PcPAL1* - *PcPAL3*, one copy of the *chalcone synthase* (*CHS*) gene referred to as *PcCHS*, and four copies of the *CHS-like* gene referred to as *PcCHSL1* - *PcCHSL4* were isolated from *P. cablin*. *PcPAL1*, *PcPAL2*, and *PcPAL3* comprised 2 136, 2 136, and 2 148 bp full-length open reading frames (ORFs) encoding 711, 711, and 715 amino acids, respectively. *PcCHS*, *PcCHSL1*, *PcCHSL2*, *PcCHSL3*, and *PcCHSL4* contained 1 173, 1 176, 1 179, 1 173, and 1 170 bp ORFs encoding 390, 391, 392, 390, and 389 amino acids, respectively. A phylogenetic analysis indicates that *PcPAL2* was closer to *PcPAL3* than *PcPAL1*, and that *PcCHS* was clustered with other plant *CHS* genes but independently to the *PcCHSLs* subgroup. A sequence analysis shows that PcCHSLs diverged in functional residues when compared to plant CHSs including PcCHS, suggesting that PcCHSLs may be divergent in tertiary structures. A quantitative reverse-transcription polymerase chain reaction analysis indicates that the expression profiles of *PcPALs*, *PcCHS*, and *PcCHSLs* in leaves and stems differed among four cultivars. Our results suggest that divergence in sequence and expression profiles of the tested genes may contribute to the diversity in flavonoids among the four cultivars.

Additional key words: *CHS*, *CHS-like,* flavonoids, *PAL,* phylogenetic tree.

Introduction

Pogostemon cablin (Blanco) Benth (syn. *P. patchouli* Hook.) from *Lamiaceae* family is an essential Chinese medicinal plant (Chen *et al.* 2013) and contains more than 40 major components most of which are sesquiterpenoids and flavonoids. Although some key genes involved in the terpenoid biosynthesis have been isolated and functionally characterized (Deguerry *et al.* 2006, Wu *et al.* 2006), the flavonoid biosynthesis in *P. cablin* is unclear.

Flavonoids are one kind of end products of the phenylpropanoid pathway. Phenylalanine ammonia-lyase (PAL, EC: 4.3.1.5) catalyze the non-oxidative deamination of L-phenylalanine to form trans-cinnamic acid. Subsequently, cinnamic acid is a substrate for cinnamate-4-hydroxylase (C4H, EC: 1.14.13.11), *p*-coumarate-3-hydroxylase (C3H, EC: 1.14.13.), and 4-coumaroyl:CoA-ligase (4CL, EC: 6.2.1.12), and products are used in the lignin pathway. Alternatively, cinnamic acid is successively metabolized by C4H and 4CL and further introduced into the flavonoid pathway. In the flavonoid pathway, the first committed enzyme is chalcone synthase (CHS, EC: 2.3.1.74) which catalyzes

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Submitted 26 June 2014, *last revision* 26 September 2014, *accepted* 1 October 2014.

Abbreviations: 4CL - 4-coumaroyl:CoA-ligase; C4H - cinnamate-4-hydroxylase; CHS - chalcone synthase; ORF - open reading frame; PAL - phenylalanine ammonia lyase; PCR - polymerase chain reaction; RT-qPCR - reverse-transcription quantitative PCR.

Acknowledgements: This study was partially supported by the Natural Science Project (2013J4100076) of the Science and Information Technology of Guangzhou City and the Natural Science Projects (2012YZ002, 2013YZ004) of the Guangdong Food and Drug Vocational College, and the Scientific Research Equipment Project of the Chinese Academy of Sciences (YZ201227). The first two authors contributed equally to this paper.

* Corresponding author; e-mail: yingwang@wbgcas.cn

the stepwise condensation of three molecules of malonyl-CoA to one molecule of 4-coumaroyl-CoA to produce naringenin chalcone. Therefore, PAL and CHS are important enzymes for flavonoid biosynthesis. According to our knowledge, there are some *PAL* genes isolated from medicinal plants, *e.g*., from *Salvia miltiorrhiza* (Song and Wang 2009), *Angelica gigas* (Park e*t al.* 2010), and *Epimedium sagittatum* (Zeng *et al.* 2013). Previous studies demonstrated that the plant *PAL* gene family contains multiple copies (Guo and Wang 2009, Reichert *et al.* 2009, Huang *et al.* 2010). Also, the *CHS* gene function has been characterized, *e.g*., in apple (Dare *et al.* 2013), *Petunia hybrida* (Morita *et al.* 2012), and orchid *Oncidium* (Liu *et al.* 2012). However, the detailed characterization of both *PAL* and *CHS* of *P. cablin* is not known.

In *P. cablin*, a previous study also indicated that the proportion of bioactive compounds in cv. Hainangensis differed from those in cv. YN introduced from Indonesia (Jiao *et al*. 2013). Currently, cvs. Shipaiensis, Gaoyaoensis, and Hainangensis are extensively used in Chinese medicine market. In this study, with the purpose of elucidating the flavonoid biosynthesis in four *P. cablin* cultivars, *PcPAL*, *PcCHS*, and *PcCHS-like* genes were isolated and their expression profiles in leaves and stems were compared.

Materials and methods

All *Pogostemon cablin* (Blanco) Benth. plants used in this study were cultivated in the medicinal garden of the Guangdong Food and Drug Vocational College, Guangzhou, P.R. China. Young leaves of cv. Shipaiensis were harvested and prepared for isolating full-length genes. The samples of four cultivars, Shipaiensis, Gaoyaoensis, Hainangensis, and one introduced from Indonesia (abbreviated as SP, GY, HN, and YN) were also collected to investigate spatio-temporal differences in expression profiles of genes isolated in this study. Young leaves (YL) referred to the first leaf on the shoot tip. Mature leaves (ML) referred to green and fully-expanded leaves, whereas old leaves (OL) referred to leaves coloured light yellow. Young stems (YS) referred to green shoot tips, mature stems (MS) and old stems (OS) referred to non-lignified stems and stems lignified, respectively. Approximate 1 g samples for each specimen were harvested and frozen immediately in liquid nitrogen before storage at -80 °C in a freezer.

Total RNA was isolated using a *TRIzol* reagent (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. The integrity of total RNA was visually checked on a 1.5 % (m/v) agarose gel stained with *Goldenview* (*Sangon Biotech*, Shanghai, China). Quality detection was assessed using a *Nanodrop 2000c* spectrophotometer (*Thermo*, Waltham, USA). RNA samples with the absorbance ratio (A_{260}/A_{230}) higher than 2 were used for gene isolation and expression profile.

About 20 ng of total RNA was reverse-transcribed to single strand cDNA using *SuperScript*™ *II* reverse transcriptase (*Invitrogen*). Sequences homologous to genes *PAL* and *CHS* were retrieved from a *P. cablin* leaf EST database generated on an *Illumina*® *Hiseq2000* platform (Mo *et al*., unpublished data). The primers were designed on the basis of the RNAseq data using *Primer3* (http://bioinfo.ut.ee/primer3-0.4.0/) to amplify the full-length genes (Table 1). PCR was performed on 20 ng of single strand cDNA using 10 μM each primer, 200 μM dNTPs, and 1 U polymerase (*TaKaRa*, Tokyo, Japan) in 2.5 mm^3 of a LA Taq buffer (10×). Cycling conditions were: denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products were extracted using an *E.Z.N.A.* gel extraction kit (*Omega Bio-Tek*, Norcross, USA) and subsequently ligated with *pMD-19T* vector (*TaKaRa*) and transformed into *DH5α* strain *Escherichia coli*. In order to confirm the full-length open reading frames (ORFs) of *PAL*, *CHS*, and *CHS-Like* genes, three clones for each gene were sequenced using *Sanger* sequencing (*Sangon Biotech*).

Sequence alignment was performed using *Clustal X1.83* (Thompson *et al.* 1997) with a manual correction. Phylogenetic analyses were carried out using *MEGA 4.0* (Tamura *et al.* 2007) with default parameters. PAL proteins, CHS, and CHS-like proteins of other species selected for phylogenetic analysis are listed in Table 1 Suppl.

The automatic modelling method of *SWISS-MODEL* (http://swissmodel.expasy.org/workspace/) was used to predict protein tertiary structures. In this study, the tertiary structures of PcCHS and PcCHSLs were constructed based on the model of alfalfa CHS (PDB ID: 1bi5A).

For quantitative reverse-transcription PCR (RT-qPCR), total RNAs were reverse-transcribed after that the residual genomic DNA digested using *PrimeScript* RT reagent kit with gDNA eraser (*TaKaRa*). Transcripts were amplified with a *SYBR Premix ExTaq*$^{\text{TM}}$ *II* kit (*TaKaRa*) and detected on a *LightCycle480* system (*Roche*, Basel, Switzerland). The reaction was in a 20 mm^3 volume containing 10 mm^3 of *SYBR*$^{®}$ *Premix Ex Taq*$^{\text{TM}}$ *II* (2×), 0.8 mm^3 of a forward primer (10 μM), 0.8 mm^3 of a reverse primer (10 μM), 2 mm^3 of a cDNA template (20 ng), and 6.4 mm^3 of deionized H_2O. Cycling conditions were: denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. A dissociation stage of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s was included. Each reaction was performed in triplicate. Primers for RT-qPCR were designed to the unique 3’ untranslated regions (3’UTRs) for gene-specific amplification (Table 1). PCR efficiencies were calculated using standard curves of six serial 5-fold dilutions of pooled cDNAs for each gene and were higher than 90 %. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using *Actin* as reference.

Table 1.Primer names and sequences.

	Primer name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Cloning full-length gene	PcPAL1	CGCCTGAAATGGAAAATGGAAAC	CATCTGATTTGATCAGGAGATTGG
	PcPAL2	ATGGCGCCGGCGGTTGAAAACGG	CTAGCAAATTGGGAGAGGTGCAC
	PcPAL3	ATGGCTGCTGCGACGGAAAATGG	CTAGCAGATAGGCAGGGGAGCAC
	PcCHS	ATAATTTTGCACCGCCTCAC	TCATCCAAAGTCCAAACATGA
	PcCHSL1	GCAACATTTTTCCGGTGGA	TTTCTGGGGGAGGTAAAACC
	PcCHSL2	ACATTCATCTTCATGCAACCA	CCGTAACTCTTTCAGTTTTTCTTGA
	PcCHSL3	GACAGTGCAGCTAGGCAGGT	CCACGAACATAACATAGCATGG
	PcCHSL4	ACAGCGCCAGCTCTGTATTT	AACTGTGTAGCACAGAAGATGGT
RT-qPCR	PcPAL1	CTGGAGCTGGAAAACAGGAG	CTCGTTCGCCCGTAAGTAAG
	PcPAL2	GACAAGGAGAAGAACGCCAG	CCGGTTCCCAATTACTTCCT
	PcPAL3	TGCAGATCGTATCCGCTGTA	TCAATCCCTTACTCAACGCC
	PcCHS	TATGGGAACATGTCGAGTGC	TTGATGGACACGCTATGCAG
	PcCHSL1	CCGGAGTCATTGGATCACTT	TTTCTGGGGGAGGTAAAACC
	PcCHSL2	TCTCCGTTGATACGGTTGTG	AGTGCACCGGTCCATCTTAG
	PcCHSL3	CGCTCTTTGTGATGGATGAA	GGGAAAACTGCGAAGAATGA
	PcCHSL4	GCTGTTCGTGATGGATGAAA	GGTCTCAATGAAGAGGCCC

Results and discussion

Possibly, *P. cablin* herbs originated from different cultivars, and/or producing areas might differ in the composition and proportion of bioactive compounds (He *et al.* 1999). Therefore, it is urgent to elucidate the molecular mechanism of flavonoid biosynthesis with the aim of cultivating cultivars with the high content of bioactive components.

In order to elucidate the flavonoid biosynthesis in *P. cablin*, *PAL* and *CHS* genes were retrieved from the *P. cablin* leaf EST database generated by the *Hiseq2000* sequencing platform and subsequently confirmed by Sanger sequencing. In this study, three copies of *PAL* genes, designated as *PcPAL1*, *PcPAL2*, and *PcPAL3*, were isolated from *P. cablin* cv. Shipaiensis. The *PcPAL1*, *PcPAL2*, and *PcPAL3* genes contained 2 136, 2 136, and 2 148 bp ORFs encoding 711, 711, and 715 amino acids, respectively. *PcPAL1-3* was deposited in GenBank with the accession number of KJ768873-KJ768875. The sequence analysis shows that PcPALs were conserved with other plant PALs in active residues, 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) residues, and residues responsible for substrate selectivity and phosphorylation (Fig. 1*A* Suppl.) suggesting that PcPALs had the bioactivity of the PAL enzyme.

In addition, five *CHS* and *CHS-like* genes were isolated and referred to as *PcCHS*, *PcCHSL1*, *PcCHSL2*, *PcCHSL3*, and *PcCHSL4*. Among those, *PcCHS* contained 1 173 bp ORF encoding 390 amino acids. *PcCHSL1*, *PcCHSL2*, *PcCHSL3*, and *PcCHSL4* had 1 176, 1 179, 1 173, and 1 170 bp ORFs encoding 391, 392, 390, and 389 amino acids, respectively. *PcCHS* (accession number KJ768876) and *PcCHSL1-4* (accession number KJ768877-KJ768880) were deposited in GenBank. PcCHS, PcCHSLs, and other plant CHSs shared conserved residues responsible for binding coumaroyl (Fig. 1*B* Suppl.). Furthermore, PcCHS also conserved residues responsible for a cyclization reaction and a catalysis reaction similarly as other CHSs. However, the substitutions of T132A and D255H (hereafter amino acids numbered according to PcCHS) in PcCHSL1 occurred in and near the residues catalyzing cyclization when compared with PcCHS, respectively. In addition, residues responsible for catalysis were substituted in PcCHSL1 S133V and S338L. In PcCHSL2, substitutions in T194Y, S338F, and S338F were also detected. T194 and S338 catalyze reactions, whereas F265 cyclizes the substrates. In PcCHSL3, residues serving to cyclize the substrate were substituted when compared to PcCHS, for instance, T132I, I254G, and F265H. In addition, the residue responsible for catalysis was substituted in T194Y. In PcCHSL4, residues cyclizing the substrate were also changed when compared to PcCHS, for instance, I254A and F265L. These results suggest that PcCHSLs might functionally diverge when compared to PcCHS, and that the functions of PcCHSLs might also differ between each other.

To further uncover the functional diversity of PcCHSLs, the tertiary structure of PcCHSLs was constructed according to the alfalfa CHS model. In the PcCHSL1 case, it failed to predict the tertiary structure of PcCHSL1 (data not shown). As shown in Fig. 1*A*, on the global view, PcCHS and PcCHSL2-4 were conserved in tertiary structures, although there were small changes in the functional residues of PcCHSL2-4 when compared in pairwise to PcCHS. However, it is noticeable that these changes in or near the functional residues of PcCHSLs may alter the space and shape of the active-site cavity or the capability of catalysis and further determine the substrate selectivity and/or catalytic activity. Finally, these changes possibly led to the functional diversity between PcCHSLs and PcCHS.

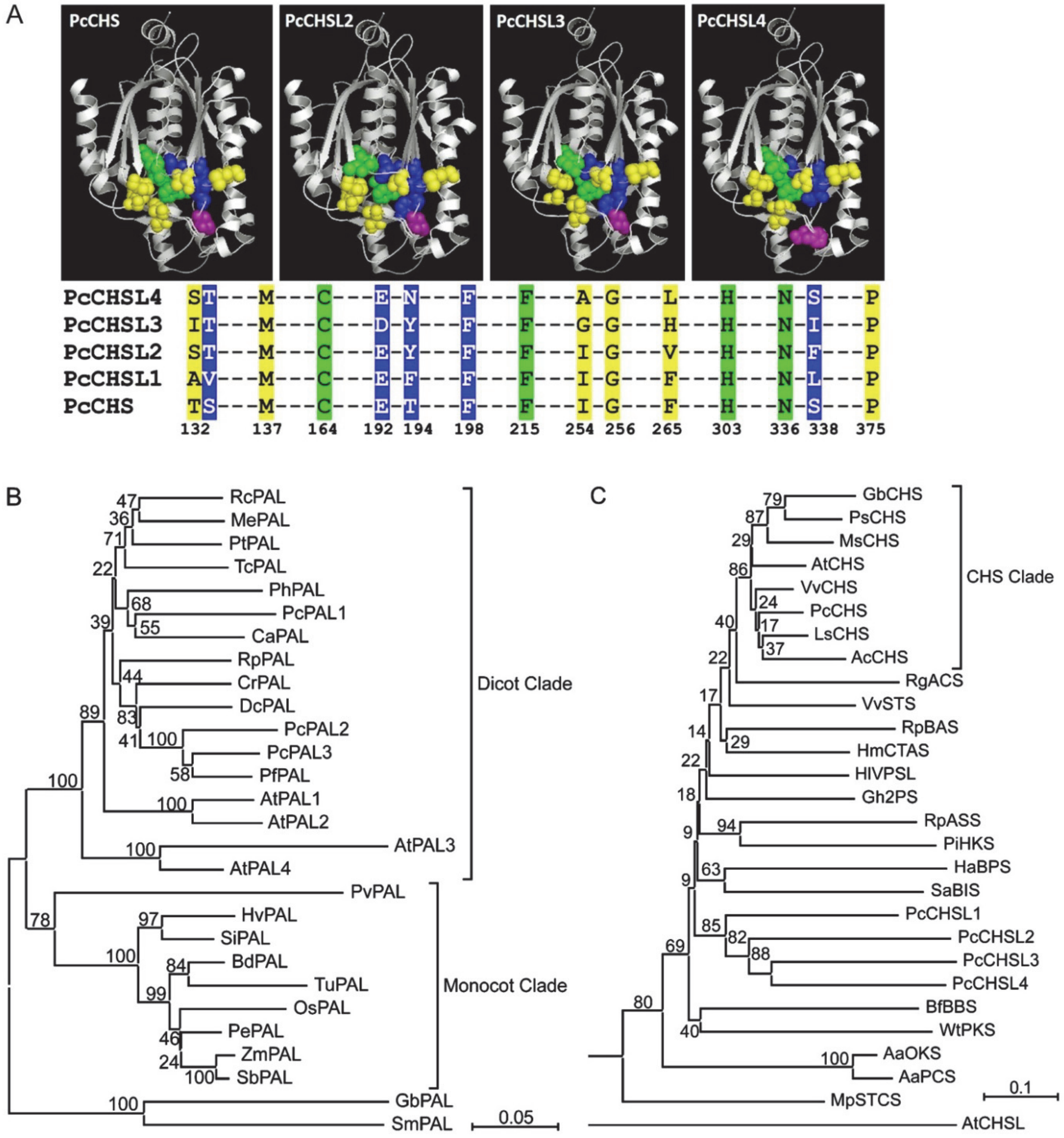

Fig. 1. Characterizations of tertiary structures of PcCHS and PcCHSLs (*A*), and phylogeny of *PcPAL*s (*B*) and *PcCHS*s (*C*). The tertiary structures of PcCHS and PcCHSLs were homology-modelled based on the alfalfa CHS (PDB ID: 1bi5A). Amino acids in *yellow* and *blue* indicate the residues responsible for a cyclization reaction and a catalysis reaction, respectively. The *green spheres* indicate the coumaroyl-binding residues, whereas the *purple spheres* indicate the amino acid (F198) responsible for a cyclization reaction and binding coumaroyl. Neighbor-joining phylogenetic analysis was conducted by *MEGA 4.0* with 2 000 bootstraps.

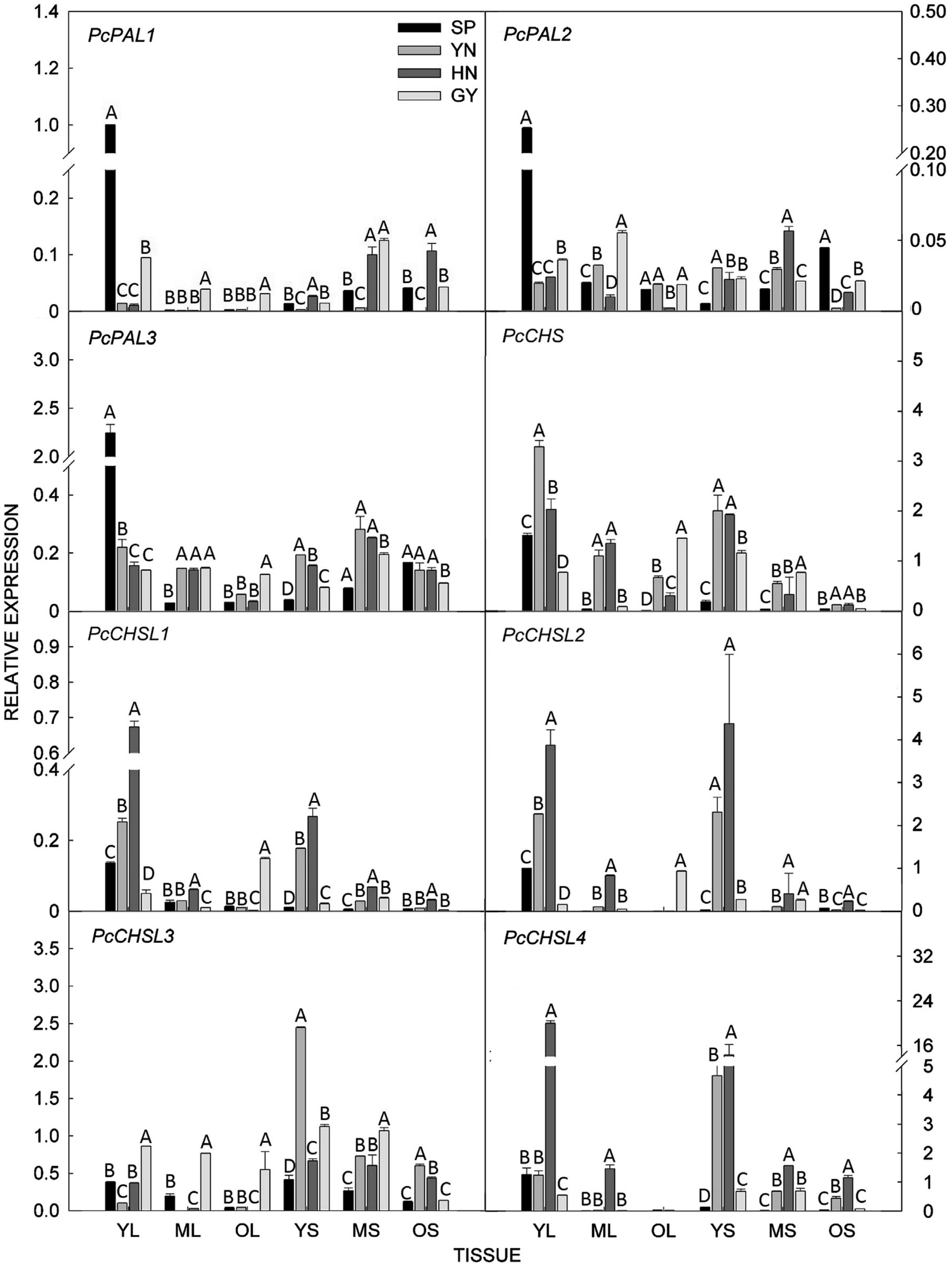

Fig. 2. Expression profiles of *PcPAL*s, *PcCHS*, and *PcCHSLs* in four *P. cablin* cultivars: SP - Shipaiensis, GY - Gaoyaoensis, HN - Hainangensis, and YN - introduced from Indonesia. YL - young leaves, ML - mature leaves, OL - old leaves, YS - young stems, MS - mature stems, OS - old stems. Means ± SD of three replicates. Different letters show significant differences in expression as calculated by the Duncan's multiple range test ($P < 0.01$). The expressions of *PcPAL*s relative to *PcPAL1* in YL of SP, and the relative expressions of *CHS* or *CHS-like* genes to that of *PcCHSL2* in YL of SP.

Previous studies indicated that the functionally divergent CHS-superfamily type III PKSs attribute to the differences in the selection of the starter molecules, the number of malonyl-CoA condensations, and the mechanisms of the cyclization reactions (Austin and Noel 2003, Isono *et al.* 2010). Theoretically, the space and shape of the active-site cavity determine the starter substrate selectivity, the polyketide chain length, and the manner of folding and cycling in the enzyme reaction (Austin and Noel 2003, Isono *et al.* 2010). For instance, benzophenone synthase (BPS), single-mutated in the active site cavity (T135L), converting BPS into functional phenylpyrone synthase (PPS) (Klundt *et al.* 2009). Therefore, a small modification in the active-site architecture may result in a significant diversity in enzyme function (Isono *et al.* 2010). In this study, the tertiary structure of PcCHSL1 was failed to construct using the alfalfa CHS model may attribute to the large differences in sequence, especially in functional residues. Also, PcCHSLs diverged in functional residues when compared to PcCHS. Totally, these results suggest that PcCHSLs functions might differ from each other and from PcCHS because of diversity in residues recognizing and/or catalyzing the substrate.

To study the phylogeny of *PcPALs* and *PcCHSs*, phylogenetic trees were constructed using *MEGA 4.0* (Fig. 1). *PcPAL2* and *PcPAL3* clustered with *PfPAL* in a subgroup independent of *PcPAL1*. However, *PcPAL*s embedded in the dicot clade which is significantly separated from the monocot clade. For the *CHS* gene, *PcCHS* was obviously grouped in the *CHS* clade which included *AtCHS*, *VvCHS*, *MsCHS*, and other plant *CHS* genes. Interestingly, *PcCHSLs* clustered together in an independent subgroup suggesting that the PcCHSLs function might differ from other plant CHS-like genes (Fig. 1*C*).

Previous studies demonstrated that the composition of essential oil in *P. cablin* is tightly related to genotypes, geographic distributions, and environmental factors (Liu *et al.* 2002, Wu *et al.* 2010). In order to evaluate the effects of genetic factors on flavonoid biosynthesis, all the four cultivars were cultivated in the medicinal garden of the Guangdong Food and Drug Vocational College, leaf and stem samples were collected, and the expression profiles of the tested genes were detected using RT-qPCR. *PcPAL1* expressed predominately in stems, especially in old stems, but rarely in leaves of the HN cultivar. In GY, *PcPAL1* significantly expressed in young leaves and mature stems. In SP and YN, *PcPAL1* transcripts were predominant in young leaves (Fig. 2). In SP, *PcPAL2* transcripts were remarkably accumulated in young leaves, whereas rarely in other tissues. In YN, *PcPAL2* transcripts were abundant in all tissues except of old stems. In HN, *PcPAL2* significantly expressed in young leaves and mature stems. In GY, the amount of *PcPAL2* transcript was highest in mature leaves followed by young leaves, and it was stable in remained tissues (Fig. 2). As presented in Fig. 2, the amount of *PcPAL3* transcript was dominant in young leaves of SP and few in remained tissues. In YN, the *PcPAL3* expression decreased during leaf development and increased to maximum in mature stems and then decreased. Taken together, *PcPALs* were more expressed in SP young leaves than in other tissues or cultivars. The *PcPAL2* expression was lower when compared to *PcPAL1* or *PcPAL3*.

PcCHS expressed prominently in young leaves of SP, YN, and HN (Fig. 2). However, the *PcCHS* transcript in GY was highly accumulated in young leaves, old leaves, young stems, and mature stems, whereas much less in mature leaves and old stems (Fig. 2). *PcCHSL1* expressed significantly in young leaves in all the cultivars except that the *PcCHSL1* transcript was highly accumulated in GY old leaves (Fig. 2). The expression profile of *PcCHSL2* was similar to that of *PcCHSL1* with a minor difference of increased transcription in young and mature stems of GY (Fig. 2). The *PcCHSL3* transcript decreased during leaf and stem development. Noticeably, *PcCHSL3* highly expressed in YN stems. Also, the *PcCHSL4* transcription occurred especially in young leaves of all the cultivars and decreased during stem development (Fig. 2). The amount of *PcCHSL1* transcript was lower than the amount of *PcCHSs* or *PcCHSLs*.

In *P. cablin* leaves, *PcPALs*, *PcCHS*, and *PcCHSLs* transcripts were predominant in young leaves suggesting that young leaves possibly synthesized more flavonoids than other samples and were good candidates for the extraction of bioactive compounds. However, in GY, *PcCHS*, *PcCHSL1*, and *PcCHSL2* were highly expressed in old leaves, suggesting the flavonoids biosynthesis differred significantly when compared to the remaining cultivars. Similarly, *PcPALs*, *PcCHS*, and *PcCHSLs* remarkably expressed in young stems.

In conclusion, three *PAL*, one *CHS*, and four *CHS-like* genes were isolated from *P. cablin* and their expression profiles among leaves and stems were characterized in four cultivars in this study. Results presented here indicate that diversity in CHS-like sequences might contribute to a functional diversity among PcCHSLs, and different expression profiles of the tested genes among tissues and/or cultivars were detected. These results indicate the differences in composition and/or proportion of flavonoids among the four cultivars and tissues leading to distinct medicinal effects.

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