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DOI: 10.2225/vol15-issue6-fulltext-7 *SHORT COMMUNICATION*

Isolation of a UDP-glucose: Flavonoid 5-*O*-glucosyltransferase gene and expression analysis of anthocyanin biosynthetic genes in herbaceous peony (*Paeonia lactiflora* Pall.)

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Abstract Herbaceous peony (*Paeonia lactiflora* Pall.) is an excellent material for studying the formation of flower colour because of its abundant colour. The full-length cDNA of a UDP-glucose: Flavonoid 5-*O*-glucosyltransferase gene (*UF5GT*) containing 1629 bp nucleotides was obtained from *P. lactiflora*. The expression patterns of nine related anthocyanin biosynthetic genes (*PlPSY*, *PlCHS*, *PlCHI*, *PlF3H*, *PlF3'H*, *PlDFR*, *PlANS*, *PlUF3GT* and *PlUF5GT*) in diurnal variation petals showed that their expression peaks were basically at 15:00 and the expression patterns were consistent with the trend of sampling conditions except individual gene. And the highest expression levels were in *PlCHS*, *PlDFR* and *PlUF3GT*, which could be the candidates to regulate *P. lactiflora* flower colour by means of genetic engineering.

Keywords: anthocyanin, expression, glucosyltransferase, herbaceous peony.

INTRODUCTION

Flower pigmentation is caused by the accumulation of pigments within the epidermal cells, which contains flavonoid, carotenoid and betacyanin (Mol et al. 1998). Among those, flavonoid is comprised of anthoxanthin (flavone and flavonol) and anthocyanin (Qi, 1989). And anthocyanin can assume a wide range of colors, therefore, more attention is paid by biochemists and breeders (Tanaka and Ohmiya, 2008; Tanaka et al. 2010). Till now, hundreds of different forms of anthocyanin have been isolated, and their structures have also been identified (Harborne and Williams, 2000; Fukuchi-Mizutani et al. 2003). Meanwhile, the anthocyanin biosynthetic pathway and related structural genes have been studied in many flower plants, such as azalea (Nakatsuka et al. 2008a), chrysanthemum (Chen et al. 2010), sunflower (Zhang et al. 2009) and so on, which provide part explanations for extensive variation of flower colours.

In anthocyanin biosynthetic pathway, L-phenylalanine is converted to naringenin by phenylalanine ammonialyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI). And then, the next pathway is catalyzed the formation of complex aglycone and anthocyanin composition by flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-glucoside: flavonoid glucosyltransferase (UFGT) and methyl transferase (MT). Among those, *UFGT* is divided into *UF3GT* and *UF5GT*, which are responsible for the glucosylation of anthocyanin to produce stable molecules (Li et al. 2001; Yamazaki et al. 2002). And the best-studied *UFGT* is only *UF3GT*, which is regarded as a key enzyme in anthocyanin biosynthesis (Kobayashi et al. 2001; Castellarin et al. 2007a, Castellarin et al. 2007b; Fukuchi-Mizutani et al. 2011; Hu et al. 2011). In contrast, there are only few reports on *UF5GT*. Studies show that *UF5GT* catalyzes glucosylation at the 5-hydroxyl

position of anthocyanidin 3-O-glycoside, thereafter, it has been isolated from solanaceae (Vogt et al. 1999), asteraceae (Ogata et al. 2001), gentian (Nakatsuka et al. 2008b), etc.

Herbaceous peony (*Paeonia lactiflora* Pall.), a traditional famous flower in China, contains nine flower colours, which makes it an excellent material for studying the formation of flower colours (Wang and Zhang, 2005). In the studies of *P. lactiflora* anthocyanin compositions, Jia et al. (2008a) and Jia et al. (2008b) have identified eight anthocyanin including peonidin-3,5-di-O-glucoside, pelargonidin-3,5-di-O-glucoside, cyanidin-3,5-di-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-O-glucoside, peonidin-3-O-gluco-side-5-O-arabinoside, cyanidin-3-O-glucoside-5-O-galactoside and pelargonidin-3-O-glucoside-5-O-galactoside. These compositions demonstrate that *UF5GT* is absolutely necessary in *P. lactiflora*. In the studies of *P. lactiflora* molecular biology, almost all of the structural genes involved in anthocyanin biosynthetic pathway have been isolated, but an important gene encoding UDP-glucose: anthocyanin 5-*O*-glucosyltransferase remains to be identified. In this study, the full-length sequence of *UF5GT* was isolated, and the diurnal expression patterns of nine anthocyanin biosynthetic genes in petals were examined. These would provide a basis for flower colour modification by engineering of the anthocyanin biosynthetic pathway.

MATERIALS AND METHODS

Plant materials

Herbaceous peony was grown in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, P.R. China (32º30’ N, 119º25’ E). The petals of *P. lactiflora* (cv. ‘Hongyanzhenghui’) were sampled every 3 hrs from 6:00 to 21:00 in the flowering stage to study diurnal expression patterns of anthocyanin biosynthetic genes, and the sampling conditions were illustrated in Figure 1 which was measured by LI-6400 (LI-COR, USA). All samples were immediately frozen in liquid nitrogen, and then stored at -80ºC until analysis.

RNA extraction and purification

Total RNA was extracted according to a modified CTAB extraction protocol (Zhao et al. 2011). Prior to reverse-transcription, RNA samples were treated with DNase using DNase I kit (TaKaRa, Japan), according to the manufacturer’s guidelines.

Primers design

3’ rapid-amplification of cDNA ends (RACE) primers were designed according to the retrieved *UF5GT* cDNA sequences of other plants from GenBank (Outer primer: 5’-CACAAGGCCACATAAACCC-3’; Inner primer: 5’-TGGATTGAACCGGCTAC-3’). And then on the basis of the 3’ cDNA sequence, 5’ RACE primer was designed (5’-TGAGAAACTGAGTCGCCACCGAAAGAA-3’). In gene expression analysis, the *P. lactiflora Actin* was used as an internal control (Zhao et al. 2012), and other gene expression analysis primers were together listed in Table 1.

Table 1. Gene-specific primers sequence for detection by Q-PCR.

Gene	Accession No.	Forward primer (5’-3’)	Reverse primer (5’-3’)
Actin	JN105299	GCAGTGTTCCCCAGTATT	TCTTTTCCATGTCATCCC
PAL	JQ070801	ACATTCTCGCCACTACCA	CTTCCGAAATTCCTCCAC
CHS	JN132108	CACCCACCTTGTTTTCTG	CCCTTTGTTGTTCTCTGC
CHI	JN119872	TCCCACCTGGTTCTTCTA	AACTCTGCTTTGCTTCCG
F3H	JQ070802	AGTTCTTCGCTTTACCGC	CAATCTCGCACAGCCTCT
F3’H	JQ070803	TGGCTACTACATTCCAAAAG	CCAAACGGTATAACCTCAA
DFR	JQ070804	CTTCCTGTGGAAAAGAACC	CCAAAAACAAACCAGAGATC
ANS	JQ070805	AGGAGAAGATCATACTCAAG	ACAAAGAAGCACAAAGGCAC
F3GT	JQ070806	AACACCGAATGCCTAAAC	AGCCACCCATCACTAAAT
F5GT	JQ070807	GAAGCGTCTCTGTTTTACC	CTCCTTGTCTCCATCTCG

Isolation of cDNA sequence

Isolation of cDNA was performed by 3' full RACE Core Set Ver. 2.0 (TaKaRa, Japan) and SMARTer™ RACE cDNA Amplification Kit (Clontech, Japan), and the specific operations were performed according to the manufacture's guidelines. PCR products were cloned and sent Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) to sequence.

Sequence and bioinformatics analysis

Physical and chemical parameters of proteins were detected using ProtParam tool (http://us.expasy.org/tools/protparam.html). Prediction of secondary and Motifs were carried out by GOR4 (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html) and PredictProtein (http://www.predictprotein.org/). Phylogenetic tree was constructed by MEGA 5.05 (Tamura et al. 2011).

Gene expression analysis

Real-time quantitative polymerase chain reaction (Q-PCR) was performed on a BIO-RAD CFX96™ Real-Time System (C1000™ Thermal Cycler) (Bio-Rad, USA). The cDNA was synthesized from 1 μg RNA using PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Japan). Q-PCR was carried out using the SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa, Japan) and gene relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method (Schmittgen and Livak, 2008).

RESULTS AND DISCUSSION

Isolation and sequence analysis of *UF5GT* cDNA

In this study, 3' and 5' RACE strategies using gene-specific primers for *UF5GT* gene resulted in an approximate 850 bp band of 3' and 5' cDNA ends, respectively. The spliced results showed that *UF5GT* cDNA had 1629 bp nucleotides in length which contained a 5'-untranslated region (UTR) of 106 bp, an open reading frame (ORF) of 1398 bp started with an ATG initiation codon and ended with a TGA stop codon, a 3'-UTR of 125 bp and a poly (A) tail, encoding 465 amino acid residues. In addition, this gene had been submitted to GenBank with accession number JQ070807.

Amino acid sequence and phylogenetic analysis

Amino acid sequence analysis of *UF5GT* in *P. lactiflora* demonstrated that the putative molecular weight was 51.92 kDa, theoretical isoelectric point (pI) was 5.40, and total number of negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys) was 64 and 47, respectively. Its instability index (II) was computed to be 50.22 which classified this protein as unstable. This protein was comprised of several phosphorylation sites of cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase II and Tyrosine kinase, other sites of N-glycosylation and N-myristoylation, and a UDP-glucosyltransferase signature. NCBI search in conserved domain database showed that this protein had a conserved domain of Glycosyltransferase_GTB_type superfamily. Meanwhile, homology analysis revealed that the similarities of amino acid sequences between *P. lactiflora* and other plants including *Petunia* x *hybrida* (BAA89009), *Perilla frutescens* (BAA36422.1), *Glandularia* x *hybrida* (BAA36423), *Arabidopsis thaliana* (NP_193146.1), *Solanum melongena* (BAF03079.1) were 56%, 54%, 53%, 51% and 50%, which were in conformity with homology matrix of 6 sequences (Table 2). And low similarity was also presented in other plants (Yamazaki et al. 2002; Nakatsuka et al. 2008b). Therefore, this sequence could be confirmed *UF5GT* gene in *P. lactiflora*, which was appointed *PlUF5GT*.

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Table 2. Homology matrix of amino acid sequences.

	Paeonia lactiflora	***Petunia* x *hybrida***	***Perilla frutescens***	***Glandularia* x *hybrida***	***Arabidopsis thaliana***	***Solanum melongena***
Paeonia lactiflora	100%	-	-	-	-	-
Petunia x *hybrida*	57.2%	100%	-	-	-	-
Perilla frutescens	53.9%	58.2%	100%	-	-	-
Glandularia x *hybrida*	53.0%	57.3%	71.5%	100%	-	-
Arabidopsis thaliana	52.3%	49.3%	49.3%	50.7%	100%	-
Solanum melongena	50.4%	87.2%	55.5%	55.4%	45.7%	100%

Phylogenetic tree shown in Figure 2 was drawn according to multiple sequence alignment of *PlUF5GT* and other *UFGT* genes by MEGA software. These genes could be classified into two types according to species: plant and bacterium, and plant also could be divided into *UF3GT* and *UF5GT* groups. Among those, *PlUF5GT* belonged to *UF5GT* group which had close relationship with *Eustoma grandiflorum*. These results were concordant with traditional classification.

Expression analysis of anthocyanin biosynthetic genes

Previous studies had shown that structural genes in plant anthocyanin biosynthesis pathway were specifically expressed which could be genetically manipulated to modify flower colour (Hu et al. 2009; Tanaka et al. 2010). In present study, in order to examine diurnal variation expression patterns of anthocyanin biosynthetic genes, *i.e. PlPSY*, *PlCHS*, *PlCHI*, *PlF3H*, *PlF3'H*, *PlDFR*, *PlANS*, *PlUF3GT* and *PlUF5GT* which had been submitted to GenBank by our laboratory, Q-PCR technology was performed using total RNA obtained from 'Hongyanzhenghui' petals of 6 time points in the flowering stage. According to the expression patterns, these nine genes could be divided into three groups: the first group (*PlPSY*, *PlCHS*, *PlCHI*, *PlF3H* and *PlANS*) gradually increased with the elapse of time until 15:00, and then declined; the second group (*PlF3'H* and *PlUF5GT*) was throughout the whole day with a low level; the third group (*PlDFR* and *PlUF3GT*) was highly expressed in general, and that of afternoon was higher than that of forepart. When the relative expression level was concerned, the highest levels were in *PlCHS*, *PlDFR* and *PlUF3GT* genes, while the lowest levels were in *PlF3'H* and *PlUF5GT* (Figure 3).

CHS was the first key gene catalyzing the formation of entry point chalcone in plant anthocyanin pathway (Nakatsuka et al. 2008a; Park et al. 2011). Noda et al. (2004) found *CHS* was highly expressed in petals and sepals of lisianthus, Zhou et al. (2010) and Zhou et al. (2011) discovered that the highest transcript abundance of *CHS1* isolated from tree peony was in petals. In present study, *PlCHS* was abundantly expressed which was in conformity with previous reporters (Noda et al. 2004; Zhou et al. 2010; Zhou et al. 2011), these revealed that *PlCHS* played an essential role in the formation of *P. lactiflora* flower colour. In addition, *DFR*, *ANS*, *UF3GT* and *UF5GT* were also the key genes in the anthocyanin pathway. *DFR* catalyzed the reaction from dihydroflavonol to colourless leucoanthocyanidin, which was catalyzed by *ANS* to the coloured anthocyanidin. And they were all glucosylated to produce stable molecules by *UF3GT* and *UF5GT* (Yamazaki et al. 2002; Nakatsuka et al. 2005). In this study, the expression levels of *DFR* and *UF3GT* were higher than that of *ANS*, which was consistent with previous reports in fully opened flowers of gentian plants (Nakatsuka et al. 2005). Meanwhile, the expression level of *UF5GT* was very low. Hence an inference could be drawn from these results that *DFR*, *ANS* and *UF3GT* were more important than *UF5GT*. Considering the nine genes, their expression peaks were basically at 15:00, the expression patterns were consistent with the trend of sampling temperature, and lagged behind the trend of light intensity except individual gene, which indicated that the formation of flower colour was affected by external environmental conditions (Miller et al. 2011).

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Figures

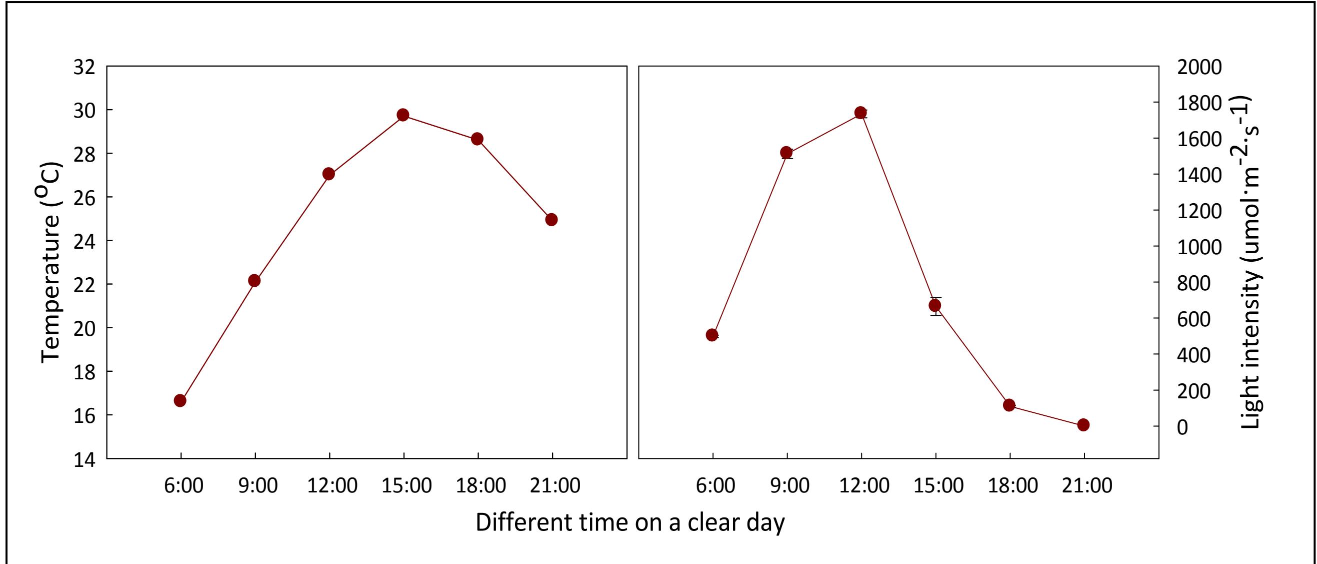

Fig. 1 Sampling conditions of *P. lactiflora* flowering petals on a clear day.

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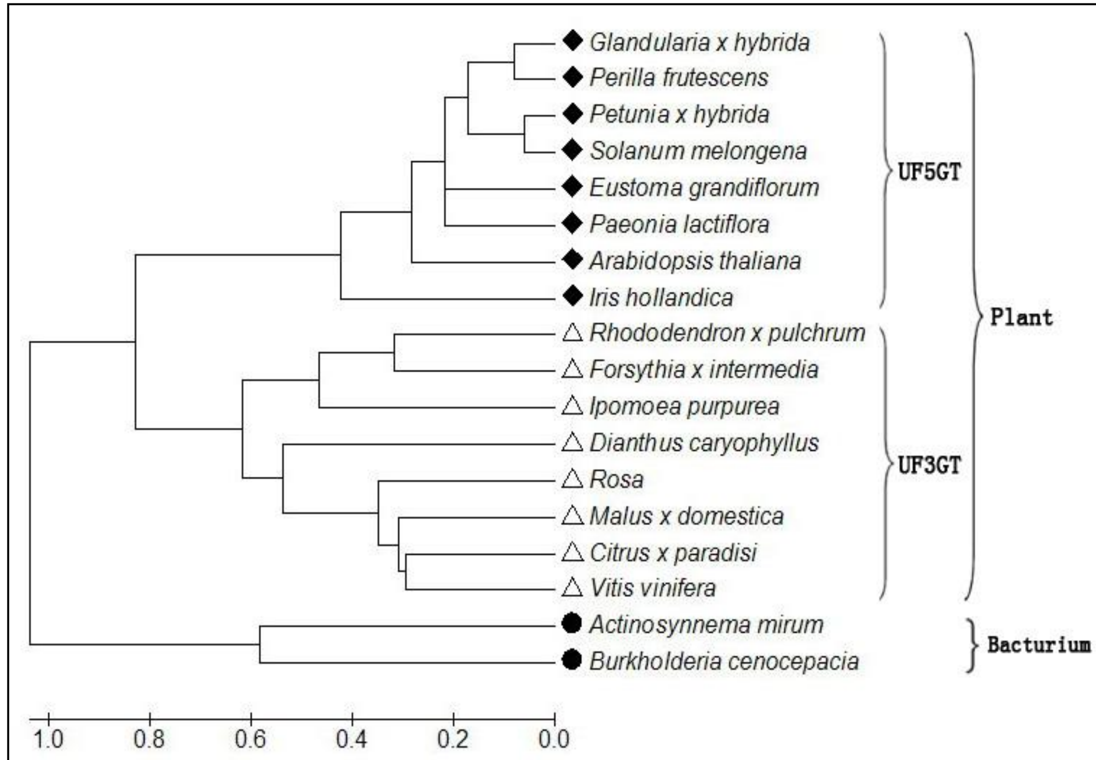

Fig. 2 Phylogenetic tree of *PlUF5GT* and *UFGT* from some other species. The amino acid sequence were obtained from GenBank: *Glandularia* x *hybrida* (BAA36423), *Perilla frutescens* (BAA36422), *Petunia* x *hybrida* (BAA89009), *Solanum melongena* (BAF03079), *Eustoma grandiflorum* (BAF49285), *Arabidopsis thaliana* (NP_193146), *Iris hollandica* (BAD06874), *Rhododendron* x *pulchrum* (BAF96949), *Forsythia* x *intermedia* (AAD21086), *Ipomoea purpurea* (AAB86473), *Dianthus caryophyllus* (BAD52005), *Rosa* (BAE72453), *Malus* x *domestica* (AAZ79375), *Citrus* x *paradise* (ACS15351), *Vitis vinifera* (AAB81683), *Actinosynnema mirum* (YP_003100956), *Burkholderia cenocepacia* (YP_002230965).

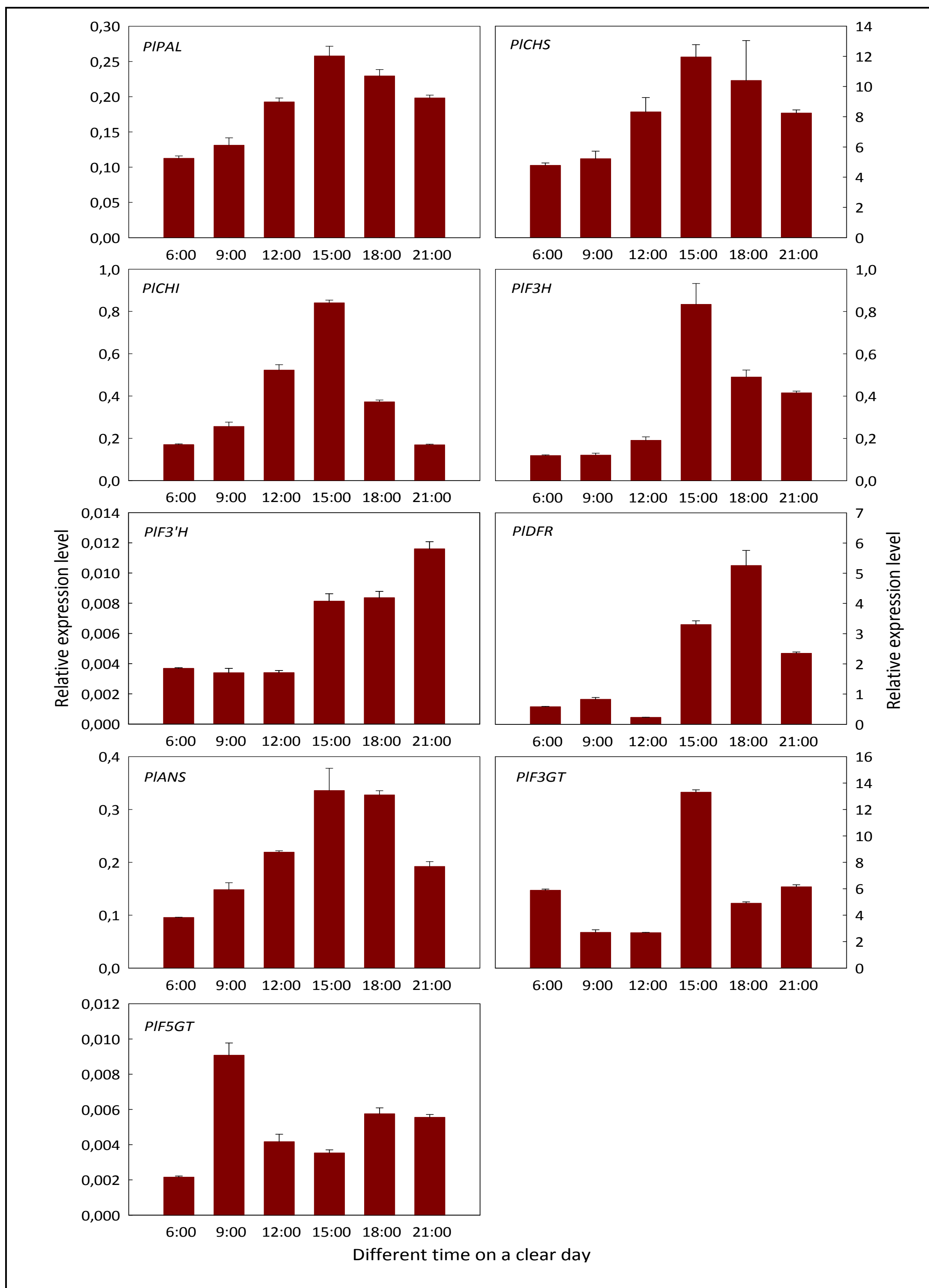

Fig. 3 Expression analysis of anthocyanin biosynthetic genes in *P. lactiflora*.

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