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Full Length Article

Expression, Cloning and Characterization of ACC Synthase and ACC Oxidase Genes in *Paeonia lactiflora*

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Abstract

Ethylene plays an essential role in the regulation of flower senescence and abscission. 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) are key enzymes in the ethylene production. The 3'-end fragment of *Paeonia lactiflora ACS* gene (*PlACS*), the full-length cDNA and genomic sequence of *P. lactiflora ACO* gene (*PlACO*) were isolated from *Paeonia lactiflora* for the first time. Sequence analysis indicated that *PlACS* was 1126 bp in length which contained a part open reading frame (ORF) of 998 bp, encoding 338 amino acid residues. The full-length of *PlACO* cDNA was 1097 bp, containing one whole ORF of 937 bp and encoding 312 amino acid residues. The genomic sequence was 1460 bp containing four exons and three introns. Sequence alignment revealed that *PlACS* and *PlACO* shared high nucleotides similarities with previously reported *ACS* and *ACO* genes in other plant species. Temporal and spatial expression analysis revealed that the expression level of *PlACS* increased during petals development, and the expression levels in young tissues were higher than that in other tissues. Nevertheless, the expression pattern of *PlACO* differed with *PlACS*, and its highest expression level was in leaves tissue. *PlACS* might be induced by the developmental signal, and *PlACO* could be regulated by other environmental signals.

Keywords: *Paeonia lactiflora*; ACS; ACO; Gene expression

Introduction

Herbaceous peony (*Paeonia lactiflora* Pall.), belonging to the Paeoniaceae family, originated in temperate Eurasia (Eason *et al*., 2002) and is widely cultivated in many countries and areas, such as China, New Zealand, Europe, North America and so on (Jia *et al*., 2008; Waltona *et al*., 2010). *P. lactiflora* is a perennial herbaceous plant, and widely appreciated by people because of its important ornamental and medicinal values (Kim *et al*., 2006). In China, it is also a traditional famous flower, which has been paid more attention with the advancement in flower industry. However, flowering time of single *P. lactiflora* flower is very short, which is between 5 to 7 days. Moreover, its concentrated natural florescence and immature post-harvest storage preservation technology all severely constrain its development. So researches on the regulation of florescence are of extraordinary importance.

Ethylene, one of the most important plant hormones, plays a crucial role in the regulation of plant growth and development, including seed germination, root, stem, petiole and fruit peduncle elongation, flower and leaf senescence and abscission, and fruit ripening (Bleecker and Kende, 2000; Miho, 2010). In tree peony, Jia *et al.* (2006) observed that the patterns of ethylene evolution in cut flowers differed among varieties. In *P. lactiflora*, although cut flowers were insensitive to exogenous ethylene (Hoffman *et al*., 2010), further researches demonstrated that the sensitivity to ethylene of cut flower varied with cultivars and silver thiosulfate (STS) could obviously delay senescence of 'Qihualushuang' and 'Linghuachenyu' (Li *et al*., 2007; Zhang and Guo, 2010). All these results suggested that ethylene could play an essential role in flower senescence of *P. lactiflora*.

In higher plants, the biosynthetic pathway of ethylene has been well understood, which begins with the conversion of methionine to S-adenosylmethionine (SAM) catalyzed by SAM synthase (EC 2.5.1.6), and then SAM is converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS) (EC 4.4.1.14); and finally, ethylene is produced from ACC by ACC oxidase (ACO) (EC 1.4.3) (Adams and Yang, 1979; Yang and Hoffman, 1984). Further researches on the function of related genes in the ethylene biosynthesis pathway, *ACS* and *ACO* were widely accepted as the rate-limiting genes (Arjen and Emst, 1997; Wang *et al*., 2002). In ornamental plants, it is reported that flower senescence and abscission are related with transcriptional regulation of *ACS* and *ACO* genes (Fernández-Otero, 2006). These two genes have been exploited in the breeding of ornamental plants; such as transgenic carnation with *ACS* and *ACO* genes delays carnation petals senescence, respectively (Savin *et al*., 1995; Iwazaki *et al*., 2004).

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To date, these two genes have been isolated from many plant species, but not in *P. lactiflora*.

In order to investigate the roles of *ACS* and *ACO* genes in flower senescence of *P. lactiflora*, we isolated the 3'-end fragment of *PlACS*, the full-length cDNA and genomic DNA sequence of *PlACO*, and then examined their temporal and spatial expression levels. These results could provide some information for the regulation of *P. lactiflora* florescence mediated by ethylene.

Materials and Methods

Plant Materials

P. lactiflora cv. Hongyanzhenghui was grown in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, P. R. China (32°30′ N, 119°25′ E). All materials were sampled from March to May, 2011, among which, the young leaves were used as gene isolation. For expression analysis, four different developmental petals (Fig. 1) and different tissues including roots, stems, leaves and petals were sampled; and the flowering petals of diurnal variation were taken every 3 h from 6:00 to 21:00. The sampling conditions were listed in Fig. 2, which was measured by LI-6400 (LI-COR, USA). All samples were immediately frozen in liquid nitrogen, and then stored at -80°C until analysis.

RNA and DNA Extraction

Total RNA and DNA were extracted according to a modified CTAB extraction protocol used in our laboratory.

Isolation of cDNA and Genomic Sequences

Isolation of cDNA was performed by 3' and 5' full RACE Core Set Ver. 2.0 (TaKaRa, Japan), and the specific operations were performed according to the manufacture's guidelines. Genomic DNA sequence was amplified in a total volume of 25 µL containing 250 ng total DNA, 10×PCR Buffer, 5 pM dNTP Mixture, 1 unit TaKaRa Taq™ (TaKaRa, Japan), 0.25 pM PCR primers. PCR conditions were 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 51°C, and 120 s at 72°C, with a final extension at 72°C for 10 min. All primers are listed in Table 1.

Sequence Analysis

Sequence retrieve was using the GenBank BLAST (http://www.ncbi.nlm.nih.gov/blast/). Sequence analysis and splicing were performed by DNAMAN 5.0 software. Physical and chemical parameters of proteins were detected using ProtParam tool (http://us.expasy.org/tools/protparam.html).

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).

Table 1: Primers used for isolation and expression analysis of *ACS* and *ACO* genes in *P. lactiflora*

Primer	Oligonucleotide sequence (5′-3′)	Application
*ACS*1	TGTCAAAGATGGCAACCG	1st of 3′ RACE
*ACS*2	TTGAGATGGCGAACAGGA	2nd of 3′ RACE
*ACO*1	GTTGACAAAGGAGCATTACA	1st of 3′ RACE
*ACO*2	TGGAGCAGAAGTTCAAGG	2nd of 3′ RACE
*ACO*3	TTCAACCCAGCATACAGC	1st of 5′ RACE
*ACO*4	TGACGCATAGGAGGAACA	2nd of 5′ RACE
ACO_{F1}	GAGAGAAAGAGAGAGAATG	genomic sequence
ACO_{R1}	AACACCTCTCAAGCAGTT	isolation
$Actin_F$	GCAGTGTTCCCCAGTATT	
$Actin_R$	TCTTTTCCATGTCATCCC	
ACS_F	ATTGGCAGAAAGGCAGAG	expression patternd
ACS_R	AACCAGGTGAAACATTGAGT	analysis
ACO_{F2}	GCCTCATCTTACTCTTCCAA	
ACO_{R2}	GCAAGCGACATCCTATTACC	

Fig. 1: Four different developmental stages of petals in *P. lactiflora* including a flower-bud stage (S1), an initiating bloom stage (S2), a full-bloom stage (S3) and a withering stage (S4)

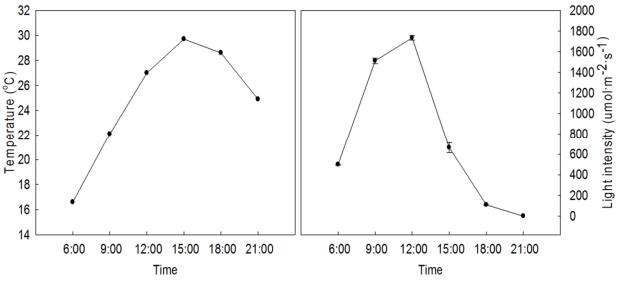

Fig. 2: Sampling conditions of *P. lactiflora* flowering petals on a clear day

The RNA samples were quantified by spectrophotometer (Eppendorf, Germany) at the wavelength of 260 nm. The cDNA was synthesized from 1 µg RNA using PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Japan). *P. lactiflora Actin* (JN105299) was used as an internal control (Zhao *et al*., 2012). Q-PCR was carried out using the SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa, Japan). Gene relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method.

Results

Isolation and Sequence Analysis of cDNAs

Isolation of cDNAs was accomplished by RACE method based on the RNA of young leaves. Two *ACO* bands approximately 850 and 680 bp were obtained with

amplification of 3' and 5' cDNA ends, respectively but only an *ACS* band about 1.1 kb was obtained. After purifying, cloning, sequencing and splicing, the full-length of *ACO* and 3'-end fragment of *ACS* were obtained. Sequence analysis displayed that *ACS* was 1126 bp in length, which contained a part open reading frame (ORF) of 998 bp, an un-translated region (UTR) in 3' of 128 bp and a full poly-A tail. The full-length cDNA sequence of *ACO* was 1097 bp with a whole ORF of 939 bp, an UTR of 62 bp in 5' end, a 3'-UTR of 96 bp and a complete poly-A tail. These two sequence, which were designated as *PlACS* and *PlACO* had been submitted to GenBank with the accession number JN639532 and JN132109, respectively.

Sequence alignment revealed that *PlACS* and *PlACO* shared high nucleotide similarities with previously reported *ACS* and *ACO* genes in other plants, such as *PlACS* shared 96% with *P. suffruticosa* (DQ337250), 75% with *Camellia sinensis* (EF205149) and *Citrus sinensis* (AJ012551), 73% with *Malus domestica* (AB034993), *Eriobotrya japonica* (GU180353), *Prunus salicina* (EU034654) and so on. For *PlACO*, it was 95% similarity to *P. suffruticosa* (DQ337251), 80% to *Camellia sinensis* (DQ904328), *Actinidia chinensis* (HQ293210), *Populus euramericana* (AB033504) and *Vitis vinifera* (AY211549), together with 79% to *Pelargonium hortorum* (U07953) and *Hevea brasiliensis* (AM743172).

Isolation and Structure Analysis of *PlACO* Genomic Sequence

Genomic sequence was performed using the PCR technology, and one approximately 1300 bp band was obtained. After cloning, sequencing and alignment with cDNA, the length of genomic sequence was 1460 bp. The comparison between gene and cDNA sequence indicated that *PlACO* included four exons interrupted by three introns, and the exons sequence was in common with the cDNA. The size and number of the exons and introns were showed in Fig. 3. The G + C contents of the three introns were 26.13%, 31.13% and 23.76%, respectively.

Amino Acid Sequence and Phylogenetic Analysis

Amino acid sequence was deduced from the cDNA sequence. For *PlACS*, the deduced protein contained 338 amino acid residues, while *Pl*ACO had 312 amino acid residues, with predicted molecular weight of about 35.23 kDa and isoelectric point (pI) of 5.32. Physical and chemical parameters showed that formula of *PlACO* was $C_{1587}H_{2481}N_{407}O_{466}S_{16}$, with total number of atoms 4957, the estimated half-life 30 h, instability index 31.87, which was classified as a stable protein. Predicted by SignalP, TMHMM and ProtScale, the protein had no transmembrane and signal peptide, and was a soluble protein. Secondary structure prediction showed that the protein consisted of 39.74% alpha helix, 15.06% extended strand, 45.19% random coil, which in turn spread in the entire protein.

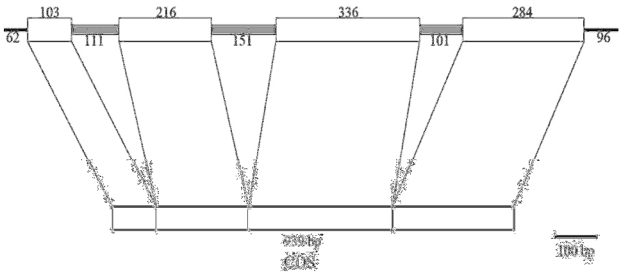

Fig. 3: Genomic sequence structure of ACO gene in *P. lactiflora*. Exons were represented as white boxes, introns as gray lines and UTR as black lines

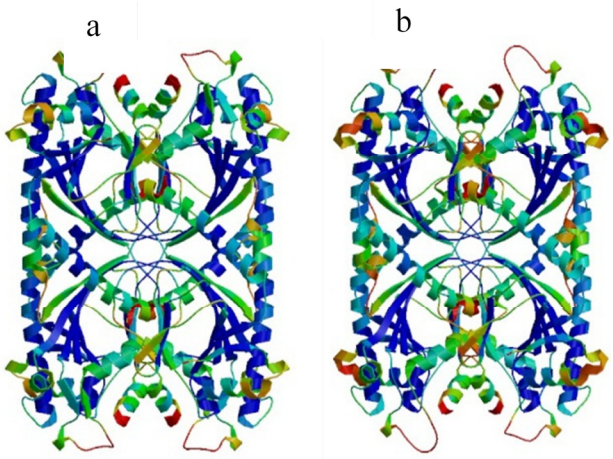

Fig. 4: Protein three-dimensional structure of ACO gene in *P. lactiflora* (a) and *P. suffruticosa* (b)

Using Swiss-Model to construct three-dimensional structure homology modeling of *ACO* amino acid sequence in *P. lactiflora* and *P. suffruticosa*, the result showed they were very similar (Fig. 4). Homology analysis of *ACO* gene amino acid sequence that isolated from *P. lactiflora* and other plants was explored via BLAST of GenBank. The result indicated that the amino acid sequence of *PlACO* had broad homology to other plants with the identity ranged from 82 to 98% (Table 2). Meanwhile, PredictProtein analysis displayed that *PlACS* had N-glycosylation site, cAMP- and cGMP-dependent protein kinase phosphorylation site, Protein kinase C phosphorylation site, Casein kinase II phosphorylation site, N-myristoylation site, Aminotransferases class-I pyridoxal-phosphate attachment site. For *PlACO*, it had one protein kinase C phosphorylation site, two casein kinase II phosphorylation sites, and three N-myristoylation sites.

Phylogenetic tree of *ACO* gene in *P. lactiflora* and other plants was constructed using MEGA4.1 software with the neighbor-joining method (Fig. 5). The results showed that *P. lactiflora* was close to *P. suffruticosa* firstly, and then together with the dicotyledons, such as the Rosaceae family species (*Prunus persica*, *P. armeniaca* and *P. mume*) and the Liliaceae family specie (*Lilium* and *Tulipa gesneriana*),

Table 2: Comparison of amino acid sequence homology and biochemical properties

Plants (GenBank Accession Number)	Biochemical properties		Amino acid	
	Molecular weight (kDa)	pI	Number	Identity (%)
Paeonia lactiflora (AEK70335)	35.23	5.32	312	100
Paeonia suffruticosa (ACO91560)	35.29	5.25	312	98
Actinidia deliciosa (AEM62884)	35.96	5.34	318	84
Hevea brasiliensis (AAP41850)	36.03	5.57	318	84
Petunia x hybrida (AAA33697)	36.27	5.26	320	83
Manihot esculenta (ABK59094)	35.42	5.27	312	83
Camellia sinensis (ABI33224)	36.19	5.29	319	83
Nicotiana attenuate (AAR99394)	35.95	5.25	319	82
Carica papaya (AAC98808)	36.15	5.15	318	82
Ziziphus jujube (ABW91146)	36.24	5.65	319	82

finally with the monocotyledons (Orchidaceae family: *Dendrobium crumenatum*, *Cattleya bicolor* and *Laelia anceps*; Poaceae family: *Sorghum bicolor*, *Saccharum arundinaceum*, *Oryza sativa* and *Phyllostachys pubescens*).

Gene Expression Analysis of *PlACS* and *PlACO*

Before expression analysis, the cDNA of plant samples (including roots, stems, leaves and petals, four different developmental stages and six different times on a clear day of petals) were used to examine its integrity and amplification efficiency of *PlActin*, *PlACS* and *PlACO* gene-specific primers. The amplified band was single, bright and no DNA contamination, which could be used for Q-PCR (Fig. 6). Although the expressions of both the genes were detected in all tissues, their expression levels were different (Fig. 7). In four different organs, the highest expression level of *PlACS* was in petals, and the lowest in leaves. However, the expression level of *PlACO* in leaves was highest which was about 4 times of that in petals. At different developmental stages of petals, the expression level of *PlACS* gradually increased about 28-fold from S1 to S4. Nevertheless, the *PlACO* was higher in S1 and S2, but when the flowers transited from S3 to S4, its expression was also growing. The trend of *PlACS* and *PlACO* expression levels was similar in the diurnal variation of petals, which displayed 'W' model, and the former was always higher than that of *PlACO*. Meanwhile, the two expression levels were higher in 6:00 am, but they decreased with the lapse of time, and reached the second peak in 12:00 and 15:00 h, respectively. In addition, the last peak of the two expression levels appeared at 21:00 at the same time.

Discussion

Ethylene is the major regulator of senescence in many flowers (Wagstaff *et al*., 2005), which can enhance their postharvest respiration, promote peroxidase, lipoxygenase, polyphenol oxidase activities and membrane permeability, loss cell regional role and eventually lead to flower wither (Zhang and Guo, 2010). In biotechnology, it is mainly utilizing the two rate-limiting genes *ACS* and *ACO* in the ethylene biosynthesis pathway to regulate ethylene production (Fernández-Otero *et al*., 2006).

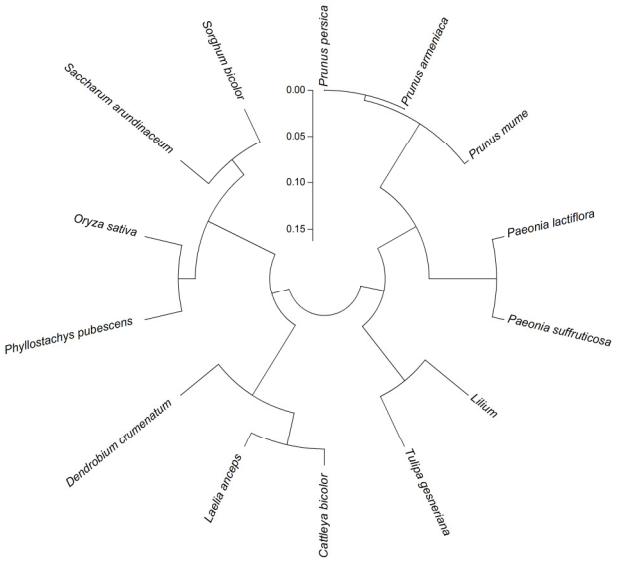

Fig. 5: Phylogenetic tree of ACO amino acid sequence from *P. lactiflora* and some other species. The amino acid sequence were obtained from GenBank: *Paeonia suffruticosa* (ABC69167), *Prunus persica* (AAL26910), *P. mume* (Q9MB94), *P. armeniaca* (AAC33524), *Zea mays* (NP_001130227), *Sorghum bicolor* (XP_002462474), *Oryza sativa* (NP_001063330), *Saccharum arundinaceum* (ABM74187), *Phyllostachys pubescens* (BAB32502), *Lilium* (EU296623), *Tulipa gesneriana* (BAE20195), *Laelia anceps* (AAT02194), *Cattleya bicolor* (AAT02192) and *Dendrobium crumenatum* (AAD02104)

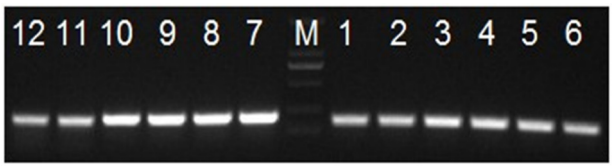

Fig. 6: Detection of primer efficiency and cDNA integrity. M: DL2000; 1-6: Amplification production of *PlActin*; 7-9: Amplification production of *PlACS*; 10-12: Amplification production of *PlACO*

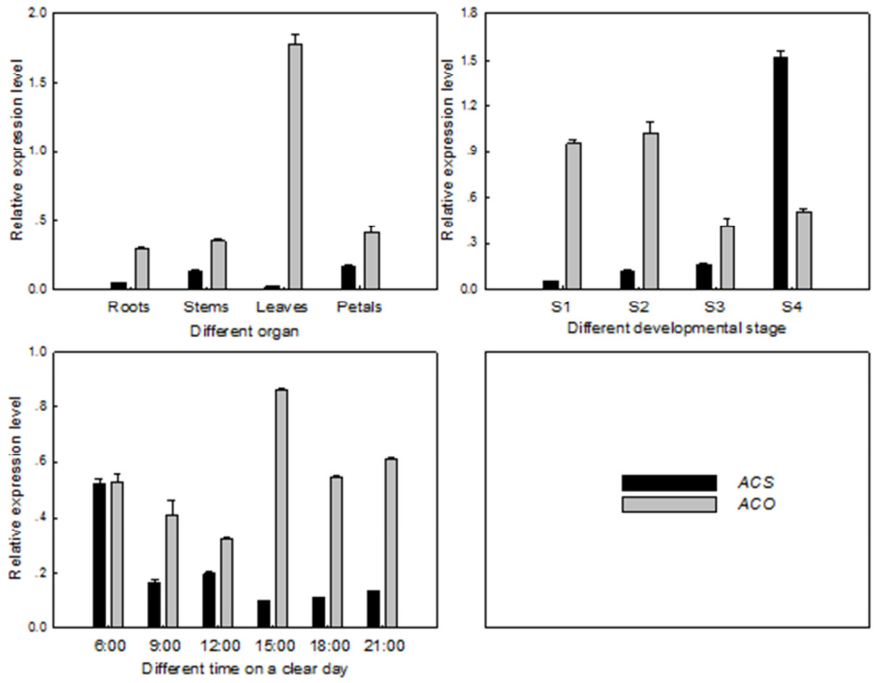

Fig. 7: Expression analysis of *ACS* and *ACO* genes in *P. lactiflora*

Some reports implicated that these two genes were multi-gene family (Nakatsuka *et al*., 1997), but only one member was obtained in this study according to RACE technology, which was effective means of obtaining a complete cDNA based on the known sequence (Wang *et al*., 2011). Nevertheless, only a 3'-end fragment of *PlACS* was obtained, which might arise from the primers design and some special structures in this gene. So, further study was needed to get the full-length cDNA sequence of *PlACS* and other gene families.

In this paper, the number of isolated *PlACO* ORF and amino acid residues was the same as reported *ACO*1 in tree peony, and homology of amino acid was 97.44%. Meanwhile, the number of introns was in accordance with *ACO*1 gene in tree peony, but they were clearly different in 3' UTR (Zhou and Dong, 2008), which revealed that the gene was certainly conservative, and might belong to different family members. Phylogenetic tree of *ACO* coincided with the traditional plant taxonomy, and these plants were divided into two categories firstly, dicotyledons and monocotyledons, and then the same family clustered together. Since *P. lactiflora* and *P. suffruticosa* belonging to the same family, both showed closer relationship. Additionally, there was some correlation between phylogenetic tree and homology analysis, which also demonstrated different evolutionary levels of *ACO* in different species.

These enzymes are encoded by small multigene families and their expressions are differentially regulated by various developmental, environmental and hormonal signals. For example, in model plant tomato, at least eight *ACS* and five *ACO* genes have been identified, and their expression patterns differ from each other (Lucille and Don, 2002). In ornamental plants, many *ACS* and *ACO* genes also have been reported. The expression level of isolated *ACS* increases with the development and senescence of flower, and the young tissues expression levels are higher than that of other tissues, which are also correlated positively with ACC contents and ethylene levels (Rodrigues-Pousada *et al*., 1993; Wang *et al*., 2004; Nagtong *et al*., 2010).

In our study, the increasing expression level of *PlACS* during petals development was consistent with previous reports in other plants (Rodrigues-Pousada *et al*., 1993; Wang *et al*., 2004; Nagtong *et al*., 2010). Meanwhile, the young organs including petals and stems expressions were higher than that of roots and leaves, as reported in *Arabidopsis thaliana*, but the *PlACS* expression levels between roots and leaves were inconsistent with that in *A. thaliana* (Rodrigues-Pousada *et al*.,. 1993). This might have resulted from the degree of leaf senescence. Together with expression pattern of *PlACS* diurnal variation, we suggested that the expression of *PlACS* might be induced by the developmental signal. In addition, the expression pattern of *PlACO* differed with *PlACS*, and its expression level was highest in leaf tissue. Therefore, we opined that *PlACO* might not be induced by the developmental signal, but regulated by other environmental signals, and its function needed further study.

Acknowledgements

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