

Full Length Research Paper

Molecular analysis and expression of phenylalanine ammonia-lyase from poinsettia (*Euphorbia pulcherrima* Willd.)

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Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is a key regulatory enzyme that link primary and secondary metabolism in plants by catalyzing the conversion of L-phenylalanine to cinnamic acid. In this study, the cDNA and genomic DNA of PAL (named *EpPAL*) in poinsettia (*Euphorbia pulcherrima* Willd.) were isolated and submitted in GenBank with accession number FJ594466 and FJ943503, respectively. The full-length of cDNA was 2429 bp with a poly (A) tail and contains a 2166-bp open reading frame (ORF) encoding 721 amino acids. The sequence of genomic DNA was 3315 bp, and the transcript was divided into two exons by an 886-bp long intron which located at 416 bp downstream initiation codon. Expression analysis of *EpPAL* in poinsettia revealed that expression levels were higher in roots and bracts, but lower in stems and green leaves. Meanwhile, expression levels increased in the order: green leaves - turning color leaves - bracts, which were consistent with their anthocyanin content during growth and development of bracts. The curve of diurnal variation of *EpPAL* expression level in bracts exhibited two highest peaks at 9:00 and 18:00, respectively, and reached the lowest level at 12:00 in a clear day. With the maturation and senescence of bracts, expression levels reduced gradually in both green leaves and bracts, but decreased more rapidly in bracts than green leaves.

Key words: Cloning, expression, phenylalanine ammonia-lyase, poinsettia.

INTRODUCTION

The color of plant organ, such as fruit, leaf and flower, originates from a blend of chlorophyll, carotenoids and flavonoids (Kim et al., 2003, 2006). Flavonoids can act as antioxidants and pathogen protectants, and have many bioactivities such as anti-cancer, anti-inflammation and anti-atherosclerosis (Harborne and Williams, 2000; Arai et al., 2000; Havsteen, 2002). Anthocyanins belong to a

class of flavonoids which include about 4000 members, and contribute to the red, purple and blue color in flower, fruit and seed (Farzad et al., 2003; Tanaka et al., 2008). During ripening period of apple, strawberry, grape and litchi fruits, there is a rapid accumulation of anthocyanins (Woodward, 1972; Given et al., 1988; Reay et al., 1998; Wang et al., 1998). So far, the anthocyanin biosynthetic pathway and the enzymes included in it have been well studied (Holton and Cornish, 1995; Winkel-Shirley, 2001; Grotewold, 2006). Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is a key enzyme catalyzing the first step of phenylpropanoid pathway among those enzymes (Kervinen et al., 1997; Wang et al., 2007) in plants (Janas, 1993; Kumar and Ellis, 2001; Ritter and Schulz, 2004; Francini et al., 2008), fungi (Kalghatgi et al., 1975; Mushi et al., 1980; Sikora and Marzluff, 1982; Evans et al., 1987; Kupletskaya and Dol'nikova, 1992) and bacteria (Emes

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Abbreviations: PAL, Phenylalanine ammonia-lyase; ORF, open reading frame; 5'-UTR, 5'-untranslated region; RT-PCR, reverse-transcription polymerase chain reaction; CTAB, cetyl trimethyl ammonium bromide; RACE, rapid amplification of cDNA ends; PL, theoretical isoelectric point.

and Vining, 1970; Piel et al., 2000; Xiang and Moore, 2005). It was reported that a positive correlation relationship was found between *PAL* activity and anthocyanin content in the fruits of apple (Steyn et al., 2004a, b), grape (Hrazdina et al., 1984) and strawberry (Given et al., 1988), while negative correlation relationship was observed in litchi fruit (Wang et al., 2004). Until now, *PAL* has been cloned from barley (Kervinen et al., 1997), banana (Wang et al., 2007), tobacco (Nagai et al., 1994), yam (Zhou et al., 2008), salvia (Hu et al., 2009), ginkgo (Chen et al., 2004) and so on.

Poinsettia (*Euphorbia pulcherrima* Willd.), which belongs to the family of Euphorbiaceae, is a major ornamental pot plant in many countries (Clarke et al., 2004). Until 2008, the global production of poinsettia has exceeded hundreds of millions and is still expanding, indicating its economic and market potential for the floral industry (Clarke et al., 2008). Bracts are the prominent ornamental organ of poinsettia, which are born on the top of the plant, and they are a kind of red abnormal leaves rather than flowers. Therefore, the colour formation of bracts determines directly the appearance, quality and also the commodity value.

In this research, to clarify the relationship between *PAL* and anthocyanin content in bracts, the cDNA and genomic DNA of *PAL* in poinsettia were cloned, and then its expression model in different organs, different leaves during development and bracts at different time in a clear day were studied by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative polymerase chain reaction (Q-PCR).

MATERIALS AND METHODS

Plant material

Two poinsettia cultivars, 'Prestige' and 'Early Velvet', were grown in the greenhouse of the Horticultural Institute, Jiangsu Academy of Agricultural Sciences, China (32°02'N, 119°51'E). All the plants were treated in 5 weeks short-day in the normal cultivation conditions from August 5, 2008. Young bracts of 'Prestige' used as materials for gene cloning and roots, stems, green leaves, color-turning leaves and bracts of 'Early Velvet' used as materials for expression analysis of different organs were sampled at about 10:00 am. Red bracts of 'Early Velvet' used as materials for expression analysis of diurnal variation were taken every 3 h from 6:00 to 21:00. To investigate the expression model of different developmental stages, bracts were sampled after short-day treatment of 5, 7 and 9 weeks, respectively. All the samples were immediately frozen in liquid nitrogen, and then stored at -80°C until use.

RNA and DNA extraction

Total RNA was extracted from all frozen samples using a modified cetyl trimethyl ammonium bromide (CTAB) extraction protocol (Xu et al., 2004). Prior to reverse-transcription, RNA samples were treated with DNase using DNase I kit (TaKaRa, Japan), according to the manufacturer's guidelines. The RNA samples were quantified by spectrophotometer (Eppendorf, Germany) at 260 nm.

Total DNA extraction from the young bracts of 'Prestige' was

performed according to the method reported previously (Chen et al., 1997) with some modifications.

Isolation of cDNA

Reverse-transcription polymerase chain reaction was performed on 1 µg total RNA using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) with degenerate primers (Forward primer: 5'-TTGCCTTCAAATCTTACCGC-3'; Reverse primer: 5'-GAGAAGAA TGCTAA-3'), and amplification was performed under the following condition: 94°C denaturation for 5 min, running 33 cycles of 94°C for 40 s; 50°C for 40 s; 72°C for 50 s and 72°C elongation for 10 min. Based on the above sequence, amplification of the 3' and 5' regions were accomplished by applying 3' and 5' RACE strategies according to the manufacturer's guidelines of the 3' and 5' full RACE Core Set Ver.2.0 (TaKaRa, Japan) with specific primers. The outer and inner primers of 3' RACE were 5'-GAAATGGATCCATTG CAGAA-3' and 5'-GATGTTTCGAGGAACAAGGC-3'. The outer and inner primers of 5' RACE were 5'-GCTCCTCCCTCACAACT-3' and 5'-CACGGGTCATCGGCATAG-3'.

Isolation of genomic DNA

Total DNA was used as template for polymerase chain reaction (PCR) to obtain genomic sequence of *PAL*. One pair of PCR primers (Forward primer: 5'-GGAAATTACTTCTCAAG-3'; Reverse primer: 5'-TACATAGAAGGGATTACA-3') were designed based on the cloned *PAL* cDNA. Genomic sequence was amplified in a total volume of 25 µl mixture containing total DNA 2 µl, 10 × PCR Buffer 2.5 µl, dNTP mixture (2.5 mM each) 0.5 µl, TaKaRa Taq™ (5 u / µl) 0.5 µl (TaKaRa, Japan), forward primer (10 µM) 1.5 µl, reverse primer (10 µM) 1.5 µl, ddH₂O 16.75 µl. The programs for PCR were as follows: denaturation at 94°C for 5 min, 30 cycles of amplification (denaturation at 94°C for 40 s, annealing at 50°C for 50 s, extension at 72°C for 2.5 min) and a final extension at 72°C for 10 min.

Cloning and sequencing

PCR products were separated by 1% agarose gel electrophoresis, and the incised gels were purified using TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, Japan). The extracted products were cloned into PMD18-T vector (TaKaRa, Japan) and used to transform competent *Escherichia coli* DH5a cells (Trans, China). The recombinant plasmids were identified with the restriction enzymes *Bam*HI and *Hind*III (TaKaRa, Japan) and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Bioinformatics analysis

The nucleotide sequence analysis, protein analysis and multiple sequence alignments were performed by DNAMAN 5.0. Transmembrane topology prediction was performed using TMHMM Server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Structure of genomic DNA organization was performed with Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>). Additional homology analysis was conducted using BLAST of GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Gene expression analysis

The gene *OsActin* (AB047313) was used as an internal control, and

designed primers were 5'-CTGGGTTCCGCCGAGATGAT-3' (forward primer) and 5'-TGAGATCACGCCAGCAAGG-3' (reverse primer). The primers of gene-specific expression were designed according to the cloned *PAL* cDNA, with forward primer 5'-AACACCAACATCACTCCCTG-3' and reverse primer 5'-GCTGAACCA GAACCGACA-3'. Semi-quantitative RT-PCR and Q-PCR were used to analyze expression model of *PAL*. We performed RT-PCR and Q-PCR first to produce cDNA from total RNA. RT-PCR was performed according to the instruction under the following conditions: 3 min at 94°C, 30 (28 for *OsActin*) cycles of 40 s at 94°C, 40 s at 50°C, 50 s at 72°C, and a final extension step for 10 min at 72°C. Q-PCR was performed using an ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, USA) with the SYBR[®] *Premix Ex Taq*[™] (Perfect Real Time) (TaKaRa, Japan). The amplification was carried out under the following conditions: 50°C for 2 min followed by an initial denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 1 min. The threshold cycle (Ct) values of the triplicate reactions were gathered using the 7500 system sequence detection software version 3.0.

Determination of chlorophyll and anthocyanin content

Chlorophyll content was determined by ethanol extraction method (Zou, 2000), and measurement of anthocyanin content was performed with the method reported by Zhang et al. (2006).

RESULTS

Isolation and sequence analysis of cDNA

Reverse-transcription polymerase chain reaction, 3' and 5' RACE strategies were used to clone the cDNA of *PAL*, and the amplifications obtained an approximate of 500, 1000 and 2000 bp band, respectively. The splicing results showed that the size of cDNA was 2429 bp and contained an ATG start-point at position 133, an open reading frame (ORF) of 2166 bp, a TGA stop codon located at positions 2296-2298, a 5'-untranslated region (5'-UTR) of 132 bp, a 3'-UTR of 119 bp, a poly (A) tail and encoded 721 amino acids (Figure 1). Additionally, it had been submitted in GenBank with accession number FJ594466, which was designated as *EpPAL*.

Protein analysis revealed that molecular mass of *EpPAL* was 78316.7 Da, theoretical isoelectric point (pI) was 6.52. Amino acids analysis of *EpPAL* showed that polar amino acids, hydrophobic amino acids, acidic amino acids and basic amino acids accounted for 49.97, 27.77, 11.36 and 12.90%, respectively. The protein had two transmembrane topological structures each containing 29 amino acids located at positions 267 - 295 and 503 - 531. Alpha helix (52.98%) and random coil (34.12%) constituted interlaced domination of the main part in the secondary structure of protein, while extended strand (6.8%) and beta sheet (6.1%) was spreaded in the whole secondary structure.

The homology analysis was carried out using the nucleotide sequence of *PAL* in poinsettia with that from other plants. The results demonstrated that *EpPAL* had an overall 78 - 87% identity with *PAL* isolated from *Manihot esculenta* (Genbank accession number:

AY036011), *Trifolium pratense* (Genbank accession number: DQ073808), *Medicago sativa* (Genbank accession number: CAA41169), *Prunus avium* (Genbank accession number: AF036948), *Camellia sinensis* (Genbank accession number: D26596), *Populus trichocarpa* (Genbank accession number: EU603320), *Glycine max* (Genbank accession number: X52953) and *Brassica napus* (Genbank accession number: AY795078). Comparison of *PAL* amino acids sequence of poinsettia with other plants showed the identity of *PAL* in the same family was relatively high, for example, the identity of *Euphorbia pulcherrima* (Genbank accession number: ACM44926), *Manihot esculenta* (Genbank accession number: AAK60275) and *Jatropha curcas* (Genbank accession number: ABI33979) was between 87 and 92%, *Pinus sylvestris* (Genbank accession number: AAL74336) and *Pinus taeda* (Genbank accession number: T09777) was as high as 98.2%, while different families and classes were less than 80 and 70%. These results were in agreement with the evolutionary laws of plants (Table 1). Therefore, it could be speculated that such cDNA was phenylalanine ammonia-lyase of poinsettia.

Isolation of genomic DNA

Genomic DNA of *EpPAL* was cloned by PCR method based on the obtained cDNA and extracted DNA, and sequence analysis indicated that its length was 3315 bp. To elucidate the genomic organization, the sequence of genomic DNA and cDNA were aligned by software DNAMAN 5.0. Results indicated that transcript was divided into two exons by one intron, and the exons sequence was consistent with the cDNA which encoded 137 and 584 amino acids, respectively. The intron was 886 bp long, located at 416 bp downstream initiation codon and began with the sequence GT and ended with AG which confirmed the consensus 5' and 3' intron splice sites for mRNA. It was deposited in GenBank with accession number FJ943503.

Expression of *EpPAL* in different organs

We determined anthocyanin and chlorophyll content in different types of leaves at first (Figure 2). The anthocyanin content of bracts was highest among all the leaves which was about 2 and 3 times of turning colour leaves and green leaves, respectively. On the contrary, the chlorophyll content of green leaves was most abundant while that of bracts was the lowest. It revealed that red coloration in poinsettia bracts was caused by anthocyanin accumulation, and green leaves displayed green color because of large amount of chlorophyll existence.

Semi-quantitative RT-PCR and Q-PCR were used to investigate the expression pattern of *EpPAL*. It was found that the expression of *PAL* could be detected in different organs tested at different levels (Figure 3). The higher

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1      CGCGGATCCACAGCCTACTGATGATCAGTCGATGGAAAATTACTTCTCAAGATTTCTCTCTTCAATTCTCTCTCTTC
79     AAAACCTGTTTCTCCATTAATTCGGATCATTTAATTATCTGGGTTCTTCTTCGAAATGGAATCTATCCATCAAAAATGGC
                                     M E S I H Q N G
157    TTCAAAAACGGATCTTTAAACAGTTTGTGTACTGATTCAGAATCAATTCGGAGTCATGACCCGTTGAGTTGGGGTCTG
235    F K N G S L N S L C T D S E S I R S H D P L S W G L
GCTGCTGAGTCGATGAAAAGGTAGCCATCTAGATGAAAGTAAAACGAATGGTGGCTGAATTTAGGAAAGCCCGTGGTCAAG
313    A A E S M K G S H L D E V K R M V A E F R K P V V K
TTAGGCGGTGAGACCTTGACTGTGGCTCAGGTTGCAGCAATTGCAGCTAGCAATGGTTCGGAGAATATCCGAGTTGAG
391    L G G E T L T V A Q V A A I A A S N G S E N I R V E
CTAGCTGAGTCGGCTCGGGCAGGTGTTAAGGCTAGCAGCGATTGGGTTATGGATAGTATGAATAATGGGACTGATAGC
469    L A E S A R A G V K A S S D W V M D S M N N G T D S
TATGGTGTGACTACTGGGTTCCGGTGTACTTCTCATAGAAGAACAAGCAGGGTGGTGTCTTTCAGAAAAGAGCTCATT
547    Y G V T T G F G A T S H R R T K Q G G A L Q K E L I
AGATTCCGTAACCGCTGGAATTTTCGGCAATGGAACAGAAAAGCCACATTTTGCACATTCAGCAACAAAAGCAGCA
625    R F L N A G I F G N G T E T S H I L P H S A T K A A
ATGATTGTAAGAATCAATACACTTCTCCAAGGCTATTTCAGGAATCCGATTTCGAAATCCTTGAAGCAATTACCAAGCTC
703    M I V R I N T L L Q G Y S G I R F E I L E A I T K L
TTAAACACCAACATCACTCCCTGTTTACCCTACGAGGTACAATTACTGCCTCAGGAGATTTAGTCCCATTGTCCTAC
781    L N T N I T P C L P L R G T I T A S G D L V P L S Y
ATTGCTGGACTTCTTACAGGCCGATCAAACTCAAAAGCCACTGGTCCCAACGGCGAATCCCTCGATGCCCAACAAAGCC
859    I A G L L T G R S N S K A T G P N G E S L D G A Q Q A
TTTTCTGAAGCCGGGATTGACTCAGGTCTCTTCGAGTTACAGCCTAAAAGAGGTCTTGCCCTTGTTAATGGGACAGCT
937    F S E A G I D S G L F E L Q P K E G L A L V N G T A
GTCGGTTCGTTTCAGCTCAATGGTCTATTTCGATGCAAAATATCCTTGGCCGTTTTATCGGAAAGTCTCTCAGCGATT
1015   V G S G S A S M V L F D A N I L A V L S E V L S A I
TTCGGGAAAGTTATGAACGGAAAACCCGAAATTTACCGACCATTGACTCATAAGTTAAAGCACCATCCCGGCCAGATC
1093   F A E V M N G K P E F T D H L T H K L K H H P G Q I
GAAGCTGCGGCTATAATGGAACATATTTTATAGTGTAGTGGTTATGTTAAAGCTGCTAAGAAAAGTTCATGAAATGGAT
1171   E A A A I M E H I L D G S G Y V K A A K K V H E M D
CCCTTGCAAAAACCGAAGCAGGATCGATACGCTTTGAGGACTTCTCTCAATGGTTAGGGCTCAAAATGAAAGTGATA
1249   P L Q K P K Q D R Y A L R T S P Q W L G P Q I E V I
AGATTCTCGACGAAATCTATCGAAAAGGAAATCAATTCGGTTAATGATAACCCCTTTGATTGATGTGTCAAGGAACAAA
1327   R F S T K S I E R E I N S V N D N P L I D V S R N K
GCATTACATGGTGGAAAACCTTTCAGGGACCCCTATAGGTGTGCAATGGATAATGCTCGTTTAGCTTTCGATTCGATA
1405   A L H G G N F Q G T P I G V S M D N A R L A I A S I
GGGAAGTTAATGTTTGCCTAATTCAGTGAGCTTGTCATGACTTTTACAACAATGGGTTGCCTTCAAATCTTACCGCG
1483   G K L M F A Q F S E L V N D F Y N N G L P S N L T A
GGCCGAAATCCGAGCCTTGATTATGGCTTAAAGGAGCTGAAATTGCTATGGCTTCATATTGTTCCGAGCTTCAGTAT
1561   G R N P S L D Y G F K G G A E I A M A S Y C S E L Q Y
TTAGCGAATCCTGTTACTAGCCATGTTTCAGAGCGCCGAGCAACATAACCAAGATGGAACGTTGGGTTGATTTCT
1639   L A N P V T S H V Q S A E Q H N Q D V N S L G L I S
TCTAGAAAAGACTCAAGAATCTGTGATATACTGAAACTTATGTCCACTACTTTCTTGGTGGCACTTTGCCAAGCTATT
1717   S R K T Q E S V D I L K L M S T T F L V A L C Q A I
GACTTGAGGCATATGGAGGAAAATTTGAGGCAAAACCGTTAAGAATACAGTGAGCCAGGTGGCGAAAAGGGTTTTAACG
1795   D L R H M E E N L R Q T V K N T V S Q V A K R V L T
ACCGGTGCAAAACGGGAACTCCACCCTTCGAGATTTTGTGAAAAAGAGCTTCTCAAAGTTGTGGATCGCGAGTATGTT
1873   T G A N G E L H P S R F C E K E L L K V V D R E Y V
TTGCTATGCGGATGACCCGTGTAGCTCGACTTATCCATTGATGCAAAAAGCTAAGGCAAGTCATTGTGGATCATGCT
1951   F A Y A D D P C S S T Y P L M Q K L R Q V I V D H A
TTGCTCAATGGTGACAATGAGAAGATGCTAACACTTCGATTTTCCAAAAGATTTGGCTTTTGGAGCCGAATTGAAG
2029   L L N G D N E K N A N T S I F Q K I S A F E A E L K
ACCCTTTTGCCTAAAAGAGTGGAGGCTGCTAGAGCTGCTTATGAGAGTGGAAAATGCTCCAATTCCTAATAGGATTATG
2107   T L L P K E V E A A R A A Y E S G N A P I P N R I M
GAGTGATGATCTTATCCACTTTTACAAGTTTGTGAGGGAGGAGCAAGGGACCGGAATCTCACCGGTGACAAAAGTGGCG
2185   E C R S Y P L Y K F V R E E Q G T G I L T G D K V R
TCACCGGGGGAGGAATTCGATAAGGTGTTTACTGCTATGTCCAAAGGGAAGATTATTGATCCATTGATGGATTGTCTC
2263   S P G E E F D K V F T A M C Q G K I I D P L M D C L
AAGGAATGGAATGGAGCTCCTCTTCTTATTGTTGATTAATTAAGAGTTTTTAATAAATTTGATTTTGTTCGTATC
2341   K E W N G A P L P I C *
2419   AAAATTTTGATGATGATTGCTTGTGTAATCCCTTCTATGTATTTAAAACATTTTGAAGGGATGTTTATTTCCCA
AAAAA

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Figure 1. Nucleotide sequence of full-length cDNA and the deduced amino acid sequence of *EpPAL*. The deduced amino acids sequence was shown underneath the corresponding nucleotides sequence, others were un-coding region; stop code was indicated with*.

Table 1. Homology analysis and distance matrix of *PAL* amino acids sequence between poinsettia and other plants.

Plant	<i>Euphorbia pulcherrima</i>	<i>Jatropha curcas</i>	<i>Manihot esculenta</i>	<i>Pinus sylvestris</i>	<i>Pinus taeda</i>	<i>Camellia sinensis</i>	<i>Cicer arietinum</i>	<i>Vigna unguiculata</i>	<i>Oryza sativa</i>	<i>Triticum aestivum</i>
<i>Euphorbia pulcherrima</i>	*	0.122	0.110	0.347	0.373	0.178	0.196	0.176	0.282	0.274
<i>Jatropha curcas</i>	87.8	*	0.083	0.326	0.351	0.164	0.176	0.153	0.257	0.220
<i>Manihot esculenta</i>	89.0	91.7	*	0.327	0.354	0.169	0.172	0.152	0.266	0.227
<i>Pinus sylvestris</i>	65.3	67.4	67.3	*	0.018	0.329	0.348	0.308	0.357	0.323
<i>Pinus taeda</i>	62.7	64.9	64.6	98.2	*	0.357	0.374	0.330	0.370	0.348
<i>Camellia sinensis</i>	82.2	83.6	83.1	67.1	64.3	*	0.184	0.173	0.277	0.245
<i>Cicer arietinum</i>	80.4	82.4	82.8	65.2	62.6	82.6	*	0.128	0.288	0.258
<i>Vigna unguiculata</i>	82.4	84.7	84.8	69.2	67.0	82.7	87.2	*	0.263	0.240
<i>Oryza sativa</i>	71.8	74.3	73.4	64.3	63.0	72.3	71.2	73.7	*	0.160
<i>Triticum aestivum</i>	75.3	78.0	77.3	67.7	65.2	75.5	74.2	76.0	84.0	*

Upper right triangle was phylip distance; Lower left triangle was percent identity. The plants GenBank accession numbers were listed as follows: *Euphorbia pulcherrima* (ACM44926), *Jatropha curcas* (ABI33979), *Manihot esculenta* (AAK60275), *Pinus sylvestris* (AAL74336), *Pinus taeda* (T09777), *Camellia sinensis* (BAA05643), *Cicer arietinum* (CAB60719), *Vigna unguiculata* (AAD45384), *Oryza sativa* (S66313) and *Triticum aestivum* (T06545).

expression levels were found in roots and bracts, lowest in stems, while expression levels increased gradually in green leaves, turning color leaves and bracts.

Expression of *EpPAL* in diurnal variation of bracts

The curve of diurnal variation of *EpPAL* expression level in bracts was bimodal model (Figure 4). It demonstrated two highest peaks at 9:00 and 18:00, respectively, and reached the lowest peak at 12:00 in a clear day. The expression level of two highest peaks was more than 3 times of the

lowest peak at 12:00.

Expression of *EpPAL* in the leaves at different developmental stages

With the extension of short-day treatment, bracts and green leaves continued to mature and senescence. In this process, the anthocyanin content in bracts and green leaves had little change but showed a downward trend, while chlorophyll content reduced significantly both in bracts and green leaves (Figure 5).

Expression levels of *EpPAL* in the leaves at different developmental stages were different.

With the maturation and senescence of bracts, the expression levels of *EpPAL* were gradually reduced in both green leaves and bracts, but decreased rapidly in bracts than green leaves. The expression level in 5 weeks short-day treatment bracts was about 5 and 72 times of those in 7 and 9 weeks short-day treatment ones, respectively, but there was no significant difference in the expression levels of green leaves (Figure 6).

DISCUSSION

Phenylalanine ammonia-lyase was a key regulatory

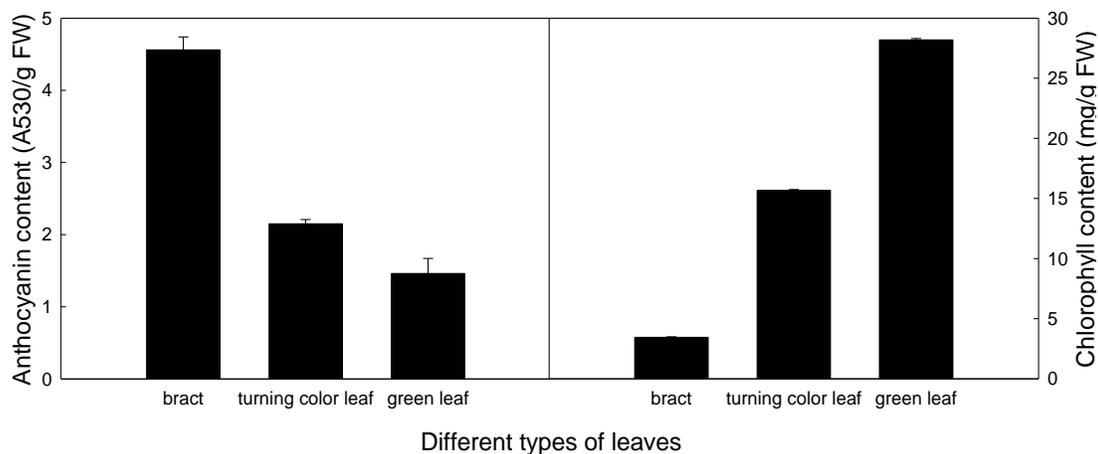


Figure 2. Anthocyanin and chlorophyll content in different types of leaves.

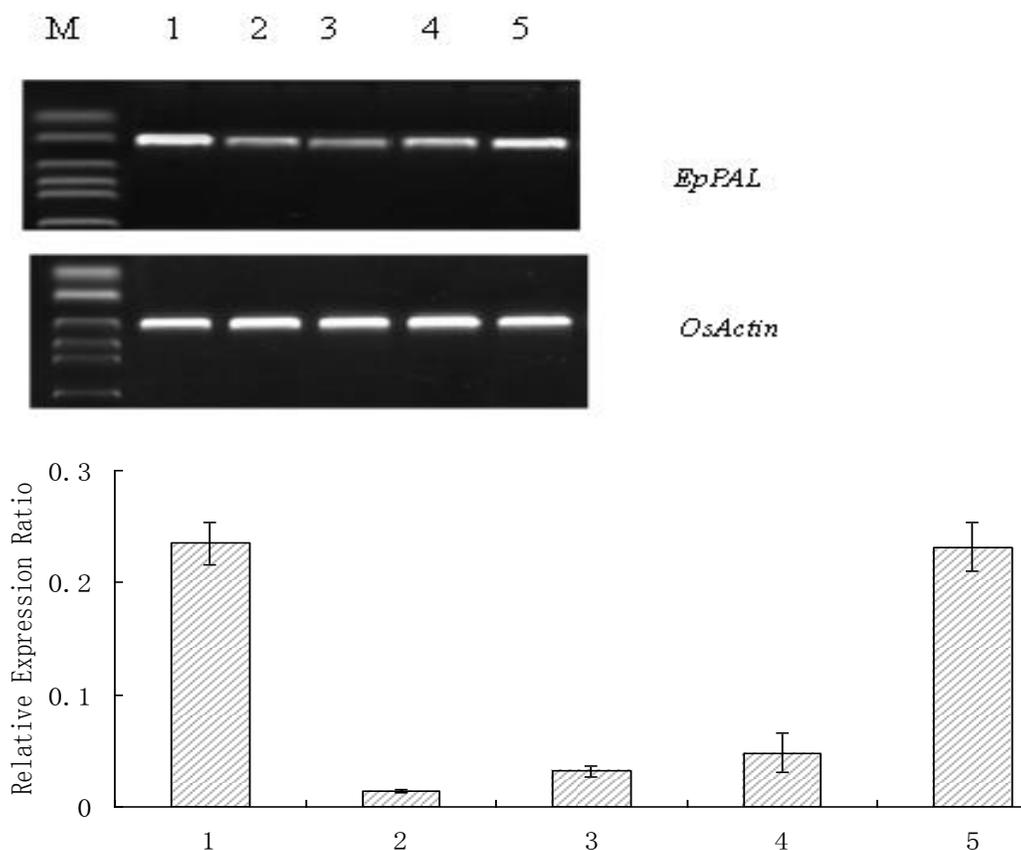


Figure 3. Expression of *EpPAL* in different organs. It was assessed by Semi-quantitative RT-PCR and Q-PCR. M, DL2000; 1, root; 2, stem; 3, green leaf; 4, turning color leaf; 5, bract.

enzyme linking primary and secondary meta-bolism in plant by catalyzing the conversion of l-phenylalanine to cinnamic acid (Hu et al., 2009). It was usually encoded by a multi-gene family (Cramer et al., 1989; Wanner et al., 1995; Francini et al., 2008), and very conservative in the

structure which generally contained only one intron and two exons, such as *PsPAL1* and *PsPAL2* in pea (Yamada et al., 1992), *PAL5* in tomato (Lee et al., 1992), together with *PAL1* and *PAL2* in Arabidopsis (Wanner et al., 1995). In this research, only one *PAL* in poinsettia

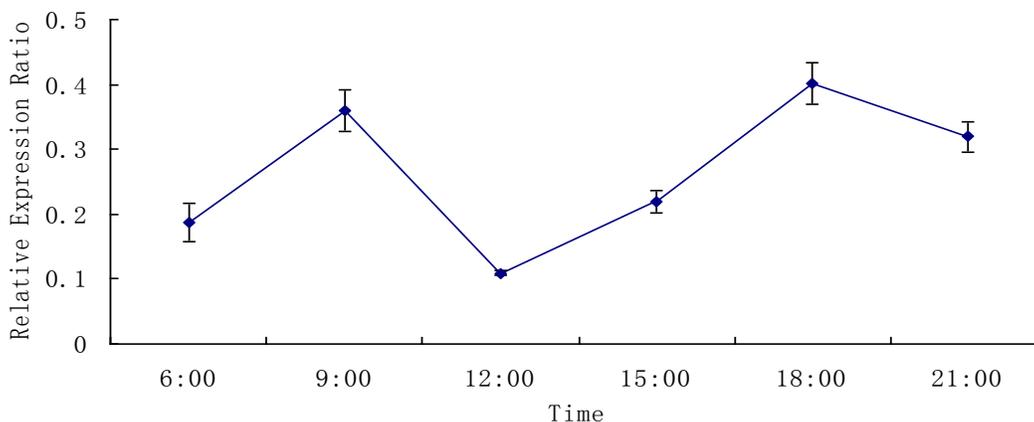


Figure 4. Expression of *EpPAL* in bracts on a clear day. It was assessed by Semi-quantitative RT-PCR and Q-PCR. M, DL2000.

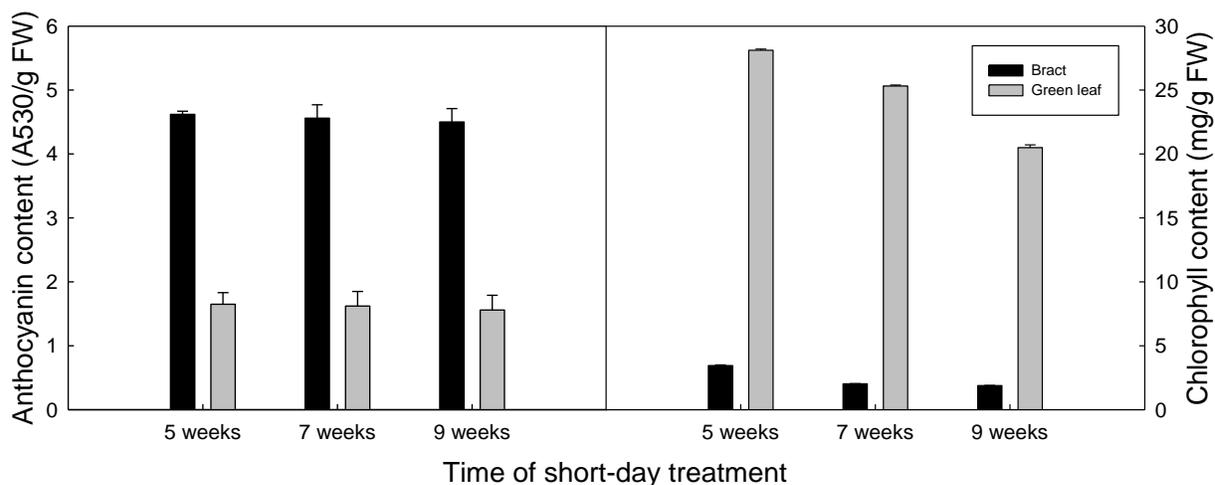


Figure 5. Anthocyanin and chlorophyll content in leaves at different developmental stages.

was isolated, which might be related to the species or the amplification depended on specific primers designed. Meanwhile, the genomic DNA of *EpPAL* had one intron, whose number was basically consistent with *M. esculenta* (Genbank accession number: AF383152), *Nicotiana tabacum* (Genbank accession number: AB008200), *J. curcas* (Genbank accession number: DQ883805) and so on, but the length of intron varied in different plants, which might be the results of long-term evolution of species. Tissue-specific expression of *PAL* existed in plants (Cramer et al., 1989). In this study, the expression levels of *EpPAL* were higher in roots and bracts, but lower in stems and green leaves. Meanwhile, the expression level of *EpPAL* increased in the order: green leaves-turning color leaves-bracts, which was consistent with their anthocyanin content. The above result suggested that *EpPAL* might play an important role in the anthocyanin biosynthetic pathway of poinsettia bracts.

However, there was no anthocyanin accumulation in white roots, but the relative expression level of *EpPAL* was similar to that of red bracts, which apparently showed that *EpPAL* did not play a role for the biosynthesis of anthocyanin in roots, and might synthesize some metabolites in the anthocyanin biosynthesis pathway, such as phenolics and flavonoids.

PAL was an inducible enzyme which could be stimulated by many external factors, light was one of them (MacLean et al., 2007). The activity of *PAL* changed periodically under natural sunlight and continuous illumination of the light. But in the continuous darkness, the *PAL* activity was maintained at a steady level with a low value. Yang and Hou, (1997) and Liu and Cheng, (2003) speculated that light could induce the activity of *PAL* or synthesize *PAL*-I. In this study, the curve of diurnal variation of *EpPAL* expression level in bracts demonstrated two highest peaks at 9:00 and 18:00,

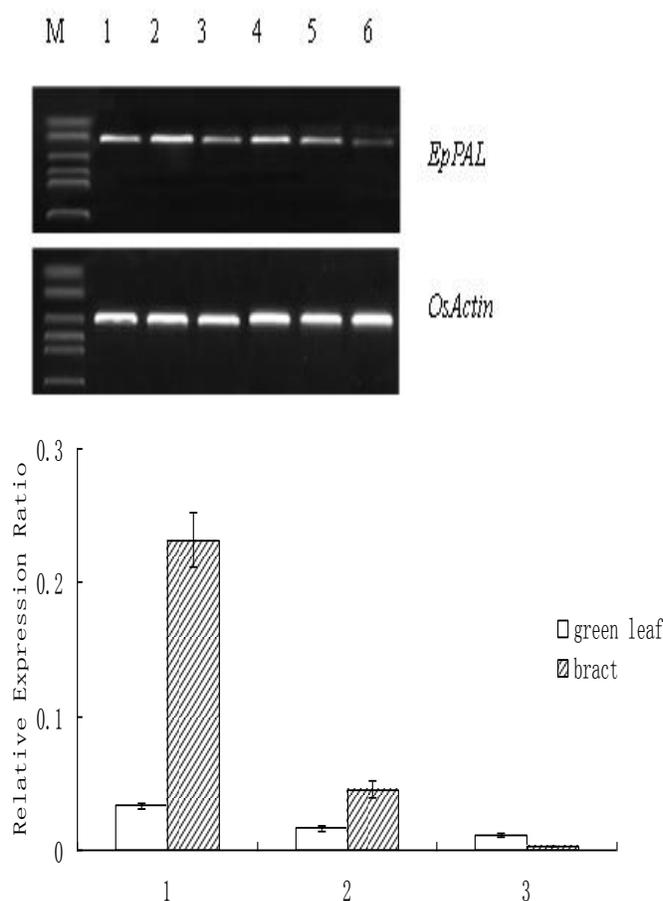


Figure 6. Expression of *EpPAL* in leaves at different developmental stages. It was assessed by Semi-quantitative RT-PCR and Q-PCR. In Semi-quantitative RT-PCR, M: DL2000; 1, green leaf of 5 weeks short-day treatment; 2, bract of 5 weeks short-day treatment; 3, green leaf of 7 weeks short-day treatment; 4, bract of 7 weeks short-day treatment; 5, green leaf of 9 weeks short-day treatment; 6, bract of 5 weeks short-day treatment. In Q-PCR: 1, 5 weeks short-day treatment; 2: 7 weeks short-day treatment; 3: 9 weeks short-day treatment.

respectively, and reached the lowest peak at 12:00 in a clear day. This was in accordance with that above-mentioned, so we deduced that *EpPAL* expression might also be induced by light.

Previous studies revealed that there was a positive or negative correlation relationship between *PAL* activity and anthocyanin content during the development of maturation in peel of apple fruit (Dong et al., 1995; Ju et al., 1995). In this research, with the maturation and senescence of bracts, the expression levels of *PAL* were gradually reduced in both green leaves and bracts, but decreased rapidly in bracts than green leaves. But anthocyanin content in bracts and green leaves had little change but showed a downward trend, while chlorophyll content was significantly reduced. Therefore, we deduced that *EpPAL* activity was relevant to anthocyanin biosynthesis in the stage of turning color poinsettia bracts, in

which precursor substances synthesized and accumulated quickly. With the maturation and senescence of bracts, although the expression of *EpPAL* had decreased gradually together with its small changed activity, anthocyanin continued to synthesize with the previous accumulated precursors, so the downstream enzyme in the phenylpropanoid pathway might be more important than *PAL*.

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