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Full Length Research Paper

An *actin* gene as the internal control for gene expression analysis in herbaceous peony (*Paeonia lactiflora* Pall.)

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As an internal control gene, actin plays an important role in gene expression analysis. In this study, the full length cDNA and genomic DNA sequences of an actin gene were isolated from Chinese herbaceous peony (*Paeonia lactiflora* Pall.) for the first time. Sequence analysis indicated that the *full length* of cDNA was 1657 bp, containing one open reading frame (ORF) of 1134 bp that encodes 377 amino acids, and the genomic DNA sequence was 1817 bp, containing three exons and two introns. They all had been submitted to GenBank and obtained the accession number JN105299 and FJ713744, respectively. Homology analysis showed that the cDNA and its deduced protein of *P. lactiflora* actin (*Plactin*) shared high similarities with those from other plants. Protein structure prediction showed that the deduced protein did not have signal peptide and transmembrane topological structure, and was a hydrophilic and stable protein. Temporal and spatial expression analysis of *Plactin*, which was performed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), displayed its stable expression which conformed to the regulation of using as an internal control gene. The results suggested that actin of herbaceous peony could be used as an alternative internal control gene for the study of the targeted genes expression and regulation characteristics in herbaceous peony.

Key words: *Paeonia lactiflora*, actin, internal control gene, gene expression.

INTRODUCTION

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative real-time PCR (Q-PCR) are effective methods to quantify the mRNA from various tissues, and an internal control gene to correct for sample-to-sample variations is necessary for both methods (Bjarnadottir and Jonsson, 2005). At present, the conventional internal control genes contain actin, glyceraldehyde-3-phosphate dehydrogenase, 18SrRNA and tubulin in the gene expression studies (Sturzenbaum and Kille, 2001). Among which, actin is one of the more commonly used internal control genes (Xiao et al., 2011). Moreover, actin is an ubiquitous and highly conserved protein in eukaryotic cells (Wang et al., 2008) which play many important roles in plant growth and developmental processes such as cell shape, cell movement, cell junction, cytoplasmic streaming and cell contraction (Pantaloni et al., 2001). In higher plants, since Yan and Shi put forward the existence of actin for the first time in 1963 (Yan and Shi, 1963), actin*s* are isolated from many plants such as Chinese licorice (Wang et al., 2009), jujube (Meng et al., 2009), ramie (Ma et al., 2010), birch (Chen et al., 2009) etc. However, actin as an internal control gene must be validated because it is a multi-gene family.

Herbaceous peony (*Paeonia lactiflora* Pall.), belonging to the Paeoniaceae family (Hong and Pan, 2001) originates in temperate Eurasia (Eason et al., 2002), and is widely cultivated in many countries and areas such as China, New Zealand, Europe, North America (Yu et al.,

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2006; Waltona et al., 2010). Herbaceous penoy is a perennial herbaceous plant, and is widely appreciated by people because of its important ornamental and medicinal values (Kim et al., 2006). In China, herbaceous penoy is a traditional famous flower which has been paid more attention with the advance of flower industry development. To date, the researches on herbaceous penoy have been focused on the tissue culture (Kim et al., 2006; Tian et al., 2010), postharvest physiology (Eason et al., 2002; Waltona et al., 2010), extraction and pharmacology of bio-active components (Kamiya et al., 1997; Lee et al., 2005; Wang et al., 2009; Baumgartner et al., 2010; Ou et al., 2011; Zhou et al., 2011). However, there are few reports on molecular biology until now, and there is no research that has been investigated on actin of herbaceous peony to our knowledge. In this study, we isolated the full-length cDNA (named as *Plactin*) and genomic DNA sequences of herbaceous peony, and then investigated whether it could stably express using its temporal and spatial expression analysis.

These results could lay the foundation for carrying out the researches on herbaceous peony gene expression and regulation characteristics based on this gene as an internal control.

MATERIALS AND METHODS

Plant materials

Herbaceous peony used in this study was grown in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, China (32° 30′ N, 119° 25′ E). The leaves of herbaceous peony (*P. lactiflora* cv. 'Hongyanzhenghui') were sampled once a month from April to July, 2011, four different tissues including roots, stems, leaves as well as petals were picked in the flowering stage. All samples were immediately frozen in liquid nitrogen, and then stored at -80°C until analysis.

RNA extraction and isolation of cDNA

Total RNA was extracted from different tissues of herbaceous peony according to a modified Cetyl Trimethyl Ammonium Bromide (CTAB) extraction protocol used in our laboratory (Zhao et al; 2011). Isolation of cDNA adopted rapid amplification of cDNA ends (RACE) strategy which was treated with 3′ and 5′ full RACE Core Set Ver.2.0 (TaKaRa, Japan) according to the manufacturer's guidelines. The 3′ ends of actin cDNA were amplified in two rounds of PCR with the gene-specific primers designed according to the actin sequence of other plants (Outer primer: 5'-AAAAGGATGCCTATGTTGG-3'; inner primer: 5'-AGTTTCGTGTTGCTCCTG-3'). Meanwhile, the gene-specific primers of 5′ ends were designed and synthesized according to the above sequenced 3′-region (outer primer: 5'-ATGGCTGGAACAGGACTT-3'; inner primer: 5'-TCACCAGAATCCAGCACA-3').

DNA extraction and isolation of genomic DNA

Total DNA extraction from the leaves was performed according to the method reported (Chen et al., 1997) with some modifications. The extracted total DNA of the leaves was used as template for PCR to obtain genomic sequence of actin. Genomic DNA sequence was amplified in a total volume of 25 µL reaction system containing total of DNA 2 µL, 10 × PCR Buffer 2.5 µL, dNTP mixture (2.5 mM each) 2 µL, TaKaRa Taq^{TM} (5 u/µL) 0.2 µL (TaKaRa, Japan), PCR primers (10 µM) (forward primer: 5'-GCAGTGTTCCCCAGTATT-3'; reverse primer: 5'-AGGAACTCACCATCAAACCC-3') 2.5 µL, ddH_2O 15.8 µL. 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.

Cloning and sequencing

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

Bioinformatics analysis

The nucleotide sequence and multiple alignment analysis were performed by DNAMAN 5.0. Phylogenetic tree was constructed by MEGA 4.1. Physical and chemical parameters of the deduced protein was determined using ProtParam tool (http://us.expasy.org/tools/protparam.html), while the signal peptide was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Hydrophobicity analysis was performed on ProtScale (http://www.expasy.org/cgi-bin/protscale.pl) and transmembrane topology prediction was performed using TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Secondary structure was predicted by GOR4 (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automa.pl?page=/NPSA/npsa_hnn.html).

Gene expression analysis

RT-PCR was used to study expression analysis of *Plactin*. RNA samples were quantified by spectrophotometer (Eppendorf, Germany) at 260 nm. The cDNA was synthesized from 1 µg RNA using PrimeScript® RT reagent Kit with gDNA eraser (TaKaRa, Japan). One pair of primers was used in gene expression analysis (forward primer: 5'-GCAGTGTTCCCCAGTATT-3'; reverse primer: 5'-TCTTTTCCATGTCATCCC-3'), and its amplified product was 169 bp in length. RT-PCR reaction system was the same as that of genomic sequence isolation, and its conditions was as listed follows: 3 min at 94°C, X cycles of 30 s at 94°C, 30 s at 51°C, 30 s at 72°C, and a final extension step for 10 min at 72°C. Among which, appropriate cDNA template amount and cycles of RT-PCR were analyzed.

RESULTS AND DISCUSSION

RNA quality detection and full length cDNA isolation

As shown in Figure 1, the electrophoretic bands of 18 and 28S rRNA was sharp and bright. Meanwhile, the absorbance of samples was measured by spectrophotometer,

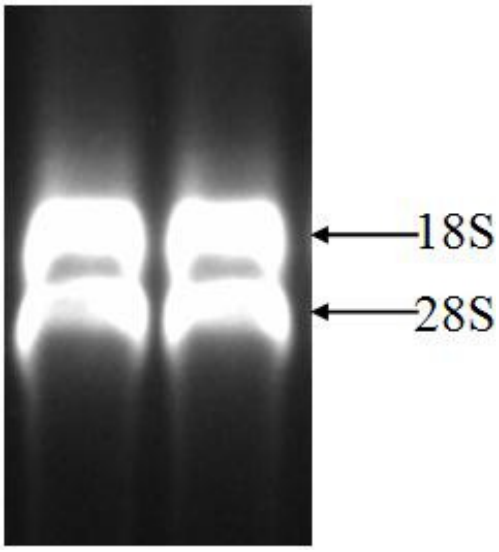

Figure 1. Agarose gel electrophoresis of total RNA extracted from the herbaceous peony leaves.

and A_{260} / A_{280} and A_{260} / A_{230} were 1.93 and 2.07, respectively. These data revealed that the quality of total RNA extracted from the leaves in herbaceous peony was good, which could meet the demand of late experiments. The cloning of actin cDNA was accomplished by applying RACE strategy with specific primers. Two approximate 1200 and 450 bp bands were amplified by 3′ and 5′ RACE, respectively. After cloning, sequencing and splicing, the full length of actin cDNA was obtained. Sequence analysis showed that the cDNA was 1657 bp in length, contained an ATG initiation codon, an open reading frame (ORF) of 1134 bp encoding 377 amino acids, a TAA stop codon, a 5′-untranslated region (5′-UTR) of 151 bp and a 3′-UTR of 372 bp, a poly (A) tail (Figure 2). Sequence homology analysis displayed that it shared high homologies with the actin cDNAs from other plants, and was a highly conserved gene (Wang et al., 2008, 2009). Therefore, it could be speculated that such cDNA was actin of herbaceous peony, which was designated as *Plactin*.

Additionally, *Plactin* had been deposited in GenBank with accession number JN105299. According to previous research results, actin was a multi-gene family (Wang et al., 2010), but in this study only one actin was isolated which might be related with the specificity of primers.

Cloning and sequence analysis of genomic DNA

Genomic DNA was performed using PCR technology, and an approximate 1400 bp band was obtained. After alignment with cDNA sequence, the result displayed that genomic DNA sequence was 1817 bp long, containing three exons and two introns (Figure 3). These introns began with the sequence GT and ended in AG, confirming the consensus 5′ and 3′ intron splice sites for mRNA. However, the number of *Plactin* introns was not in conformity with related reports of other plants (Thangavelu et al., 1993; Wang et al., 2010), the first intron that near 5′ end was lost due to genetic variation occurrence of species. In addition, this genomic DNA sequence had been deposited in GenBank with accession number FJ713744.

Amino acid sequence and phylogenetic analysis

The deduced amino acid sequence from cDNA was predicted by ProtParam, physical and chemical parameters showed that its formula was $C_{1852}H_{2920}N_{492}O_{561}S_{21}$, with total number of atoms 5846, molecular weight 41.72 kDa, theoretical isoelectric point (pI) 5.31, the estimated half-life 30 h, instability index 37.37, and it 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 this protein consisted of 30.87% alpha helix, 21.49% extended strand, and 47.75% random coil which in turn spread to the entire protein (Figure 4A). Conserved domain search revealed that this protein contained ATP, gelsolin and profilin binding sites which belonged to one of ACTIN superfamily (Figure 4B).

Meanwhile, Predict Protein analysis displayed that actin had actins signature (YVGDEAQSKRG, WISKGEYDE), actins and actin-related proteins signature (LLTEAPLNPKANR). Moreover, it also had an N-glycosylation site, a cAMP- and cGMP-dependent protein kinase phosphorylation site, six protein kinase C phosphorylation sites, four Casein kinase II phosphorylation sites, two tyrosine kinase phosphorylation sites, eight N-myristoylation sites, which revealed that this gene could hold complex modification and regulation at the protein level. Homology analysis of amino acid using BLAST of GenBank showed that the deduced amino acid of *Plactin* shared high homology with actin protein sequence in other plants (Figure 5) such as 99% identity with *Gossypium hirsutum* (AAP73457), *Stevia rebaudiana* (AAN40685), *Ricinus communis* (AAR15174), *Actinidia deliciosa* (ABR45727), *Betula platyphylla* (ACB88021) and *Mimosa pudica* (BAA89214), 97% identity with *Camellia sinensis* (ADY38689) and so on, which illustrated that this gene was considerably conservative.

Actin phylogenetic tree of some species including *P. lactiflora* was constructed according to neighbor-joining method (Figure 6). The result showed that actin of all used species was from a common ancestor, but they had some change in response to different selection pressures which could be divided into two groups. One group was fungi, and the other group could be divided into two

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1       GAAAATTTATTTTAGCGTTTTCATCGAGGAGAATCTAGTGTGCGAAGGAAAAAAAAGGAG
61      GGCCAGGCCATCGTCTACGGAGATTGGCTTGGTTGTCTGCGCTTGATCTTCTTCATCTTC
121     TCCTCCTCGCGATCAACAAGGTTTGTAAAAGATGGCCGATGCTGAGGATATCCAGCCCCT
1                                       M  A  D  A  E  D  I  Q  P  L
181     TGTCTGTGACAATGGAACTGGAATGGTCAAGGCTGGTTTTGCTGGTGATGATGCTCCCAG
11        V  C  D  N  G  T  G  M  V  K  A  G  F  A  G  D  D  A  P  R
241     AGCAGTGTTCCCCAGTATTGTTGGTCGACCCAGACACACTGGAGTCATGGTTGGAATGGG
31        A  V  F  P  S  I  V  G  R  P  R  H  T  G  V  M  V  G  M  G
301     CCAAAAGGATGCCTATGTAGGTGATGAAGCACAATCAAAAAGAGGTATTCTTACCTTGAA
51        Q  K  D  A  Y  V  G  D  E  A  Q  S  K  R  G  I  L  T  L  K
361     ATATCCTATTGAGCATGGTATAGTCAGCAACTGGGATGACATGGAAAAGATCTGGCATCA
71        Y  P  I  E  H  G  I  V  S  N  W  D  D  M  E  K  I  W  H  H
421     TACGTTCTACAATGAACTTCGTGTTGCTCCTGAAGAGCACCCAGTGCTCCTCACAGAGGC
91        T  F  Y  N  E  L  R  V  A  P  E  E  H  P  V  L  L  T  E  A
481     ACCCCTTAACCCCAAAGCCAACAGAGAAAAGATGACTCAGATCATGTTTGAGACCTTCAA
111       P  L  N  P  K  A  N  R  E  K  M  T  Q  I  M  F  E  T  F  N
541     TGTGCCTGCAATGTACGTTGCCATCCAGGCCGTGCTGTCTCTATATGCCAGTGGTCGTAC
131       V  P  A  M  Y  V  A  I  Q  A  V  L  S  L  Y  A  S  G  R  T
601     AACTGGTATTGTGCTGGATTCTGGTGATGGTGTGAGTCACACTGTACCTATCTATGAAGG
151       T  G  I  V  L  D  S  G  D  G  V  S  H  T  V  P  I  Y  E  G
661     TTATGCCCTTCCTCACGCTATCCTCCGTCTTGACCTTGCTGGTCGTGATCTCACAGATTC
171       Y  A  L  P  H  A  I  L  R  L  D  L  A  G  R  D  L  T  D  S
721     CTTGATGAAGATCTTGACTGAAAGAGGTTACATGTTTACCACCACTGCTGAACGGGAAAT
191       L  M  K  I  L  T  E  R  G  Y  M  F  T  T  T  A  E  R  E  I
781     TGTCCGTGACATGAAGGAGAAGCTAGCATACGTTGCCCTTGATTACGAGCAGGAACTGGA
211       V  R  D  M  K  E  K  L  A  Y  V  A  L  D  Y  E  Q  E  L  E
841     GACTTCCAAAAGCAGCTCATCGGTTGAGAAGAACTACGAATTGCCTGATGGACAAGTCAT
231       T  S  K  S  S  S  S  V  E  K  N  Y  E  L  P  D  G  Q  V  I
901     TACCATCGGAGCTGAGAGATTCCGTTGCCCAGAAGTCCTGTTCCAGCCATCACTAATCGG
251       T  I  G  A  E  R  F  R  C  P  E  V  L  F  Q  P  S  L  I  G
961     AATGGAAGCTGCTGGAATTCACGAGACTACTTACAATTCTATCATGAAGTGTGATGTGGA
271       M  E  A  A  G  I  H  E  T  T  Y  N  S  I  M  K  C  D  V  D
1021    TATCAGAAAGGATTTATATGGAAACATTGTTCTCAGTGGTGGATCAACTATGTTCCCTGG
291       I  R  K  D  L  Y  G  N  I  V  L  S  G  G  S  T  M  F  P  G
1081    TATTGCAGACAGAATGAGCAAGGAAATCACTGCTCTTGCTCCCAGCAGCATGAAGATTAA
311       I  A  D  R  M  S  K  E  I  T  A  L  A  P  S  S  M  K  I  K
1141    GGTTGTGGCACCGCCTGAGAGAAAATACAGTGTCTGGATTGGAGGGTCTATTCTTGCTTC
331       V  V  A  P  P  E  R  K  Y  S  V  W  I  G  G  S  I  L  A  S
1201    CCTCAGTACCTTCCAGCAGATGTGGATTTCCAAGGGTGAATACGATGAATCTGGTCCATC
351       L  S  T  F  Q  Q  M  W  I  S  K  G  E  Y  D  E  S  G  P  S
1261    CATTGTCCACAGGAAGTGCTTTTAAGTTTTTCTACAAGGAGGGTTTGATGGTGAGTTCCT
371       I  V  H  R  K  C  F  *
1321    TTTCCTTTTCTTTAGTTGGCTTTTCGTGTTATGTGTCAAGAACTCAAGTCTGGTTGGACA
1381    TGGAAAGTGTAGACGCAGAGGGCAATATTGTAGTTTCATCAAAAAGTGATGTCACGTCTC
1441    TGTCTCTATGGATGAGTGAATTACTCATGTAATACACACCTCGATTTGTAGGGGTGGACC
1501    TTTCTGTGTGGTACAACCAGTAGGACGTCTGTAGAAGAAGAGTGATTGTGATGCTTTTAT
1561    TTTTTTCGTTTTTTAATTTTTTTTATTTGGAGTTTTTATTTTTATTTTTTGCTGGGAACA
1621    ATTTATGTTAATATTTATTGTATGAGAAAAAAAAAAA
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Figure 2. Nucleotide sequence and the deduced amino acid sequence of *Plactin* cDNA. The deduced amino acid sequence was showed underneath the corresponding nucleotide sequence, others was un-coding region; stop code was indicated with*.

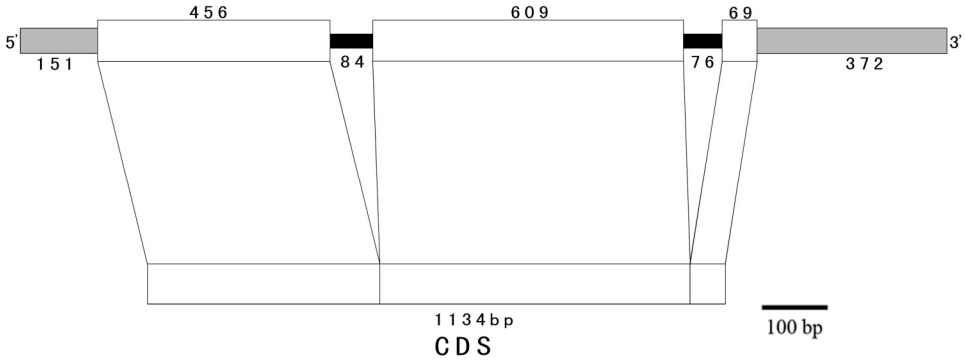

Figure 3. Genomic organization of *Plactin*. Exons were represented as white boxes, introns as lines, and UTR as gray lines.

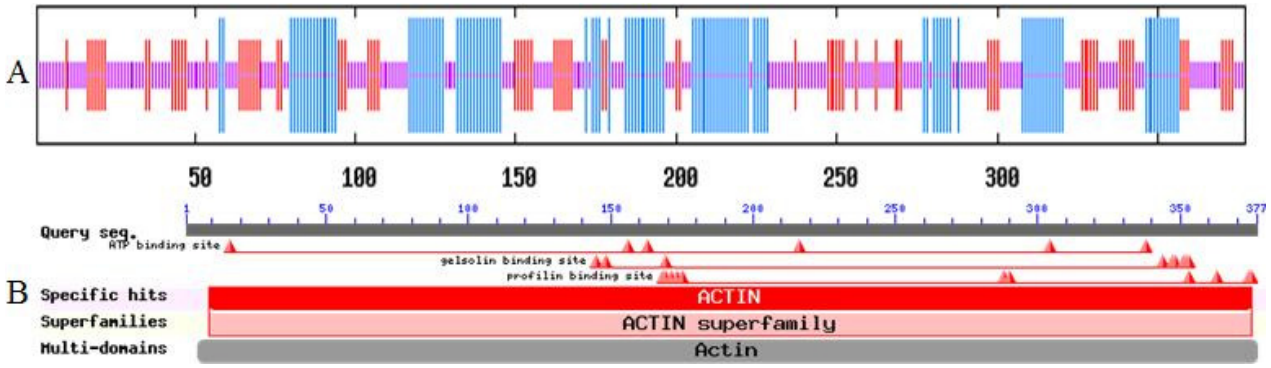

Figure 4. Bioinformatic prediction of *Plactin*. (A) Secondary structure; blue curve line, alpha helix; red curve line, extended strand; purple curve line, random coil. (B) Conserved domain.

Figure 5. Multiple alignment of actin amino acid sequence from *Paeonia lactiflora* and some other species.

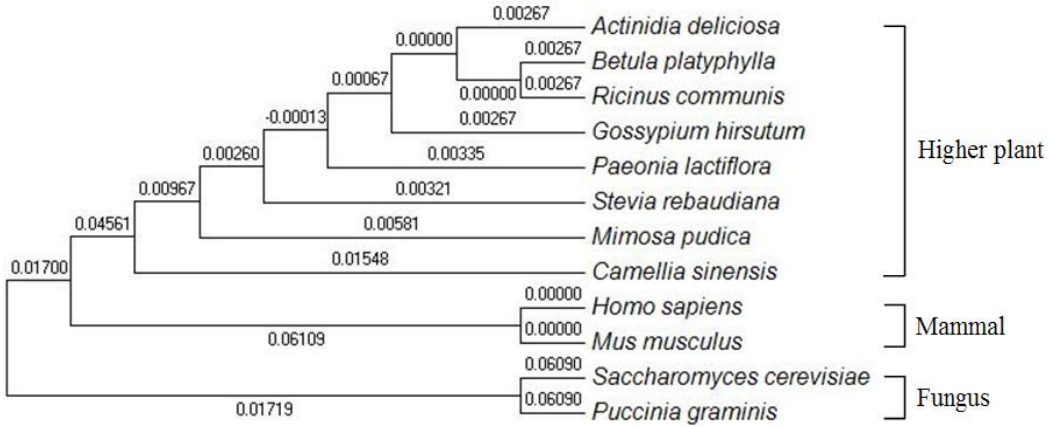

Figure 6. Phylogenetic tree of actin amino acid sequence from *Paeonia lactiflora* and some other species. The amino acid sequence were obtained from GenBank: *Gossypium hirsutum* (AAP73457), *Stevia rebaudiana* (AAN40685), *Ricinus communis* (AAR15174), *Actinidia deliciosa* (ABR45727), *Betula platyphylla* (ACB88021), *Mimosa pudica* (BAA89214), *Camellia sinensis* (ADY38689), *Homo sapiens* (NP_005150), *Mus musculus* (NP_033738), *Saccharomyces cerevisiae* (AAA34391) and *Puccinia graminis* (CAA54848).

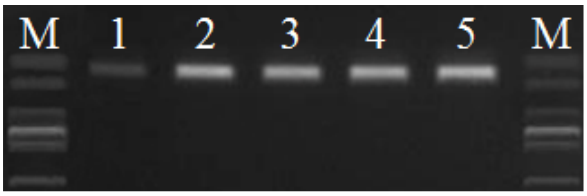

Figure 7. Optimization of RT-PCR template. M, DL2000; 1, 0.2 µL; 2, 0.5 µL; 3, 1.0 µL; 4, 1.5 µL; and 5, 2.0 µL.

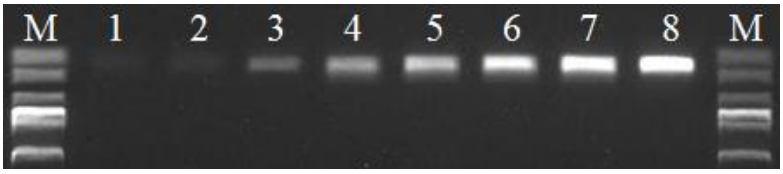

Figure 8. Optimization of RT-PCR cycles. M, DL2000; 1, 17 cycles; 2, 21 cycles; 3, 25 cycles; 4, 27cycles; 5, 29 cycles; 6, 31 cycles; 7, 33 cycles; and 8, 35 cycles.

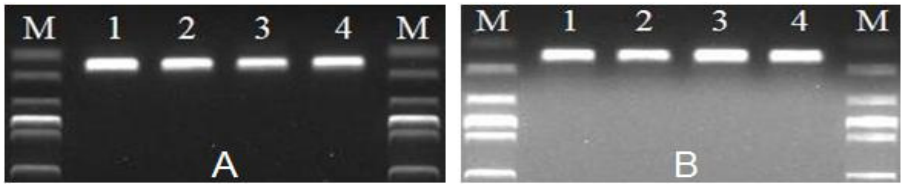

Figure 9. Temporal and spatial expression analysis of *Plactin*. (A) Expression levels of the leaves in different developmental stages. M, DL2000; 1, leaves of April; 2, leaves of May; 3, leaves of June; 4, leaves of July. (B) Expression levels of four different tissues in the flowering stage. M, DL2000; 1, roots; 2, stems; 3, leaves; and 4, petals.

subgroups, that is, higher plants and mammals which were in accordance with the conclusion of plant and animal actin sharing a common ancestor (Wang et al., 2010). In higher plants, the relationship of actin between *P. lactiflora* and *Stevia rebaudiana* was closer than that between *P. lactiflora* and other plants.

Optimization of RT-PCR conditions

To examine whether *Pl*actin could stably express, RT-PCR was optimized for temporal and spatial expression analysis. Total RNA extracted from the leaves in the flowering stage was used to optimize RT-PCR conditions. *Plactin* was amplified when the template volume was 0.2, 0.5, 1.0, 1.5 and 2.0 µL, and the results were showed in Figure 7. When the template volume was 0.2 µL, the amplified band was very weak, and the amplified product was significantly less than those of other template. Furthermore, when the template volume was 0.5, 1.0, 1.5 and 2.0 µL, respectively, the amplified efficiency was almost similar. Therefore, the initial template volume of RT-PCR using 0.5 µL was more appropriate. *Plactin* was amplified under 17, 21, 25, 27, 29, 31, 33 and 35 cycles, but there was almost no amplified bands when the cycles were 17 and 21. When it was 25 cycles, the amplified product could be detected, and then the intensity was increasing with the added cycles. Whereas, the band intensity had almost no change after 31 cycles which revealed that it is reached plateau phase in 31 cycles (Figure 8). RT-PCR should quantify mRNA in the exponential growth phase, therefore, 29 cycles was optimal for RT-PCR.

Temporal and spatial expression analysis of *Plactin*

On the premise of the same cDNA concentrations using optimal RT-PCR conditions to detect the temporal expression patterns of herbaceous peony leaves at different developmental stages (Figure 9A), we found that the expressions of *Plactin* gene could be detected in the leaves of all stages, and the expression levels were same. Meanwhile, the spatial expression patterns of four different herbaceous peony tissues in the flowering stage including roots, stems, leaves and petals were performed,

and the results showed that amplified bands intensity were almost identical (Figure 9B). These results indicated that *Plactin* was very stable in temporal and spatial expression, which was consistent with the condition of using as the internal control gene (Xiao et al., 2011). Therefore, *Plactin* could be used as an alternative internal control gene for gene expression analysis in herbaceous peony.

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