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Cloning and expression of an *APETALA1*-like gene from *Nelumbo nucifera*

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ABSTRACT. The objective of this study was to clone the full-length cDNA of the *APETALA1* (*AP1*) gene from lotus and analyze its sequence and expression pattern. The full-length cDNA sequence of the *NnAP1* gene was amplified from the petals of *Nelumbo nucifera* 'Hongxia' using RT-PCR and rapid amplification of cDNA ends. Bioinformatic methods were used to analyze the sequence characteristics of the gene. Quantitative real-time PCR methods were used to investigate the expression pattern of *NnAP1* in various organs and during different developmental stages. The cloned full-length *NnAP1* cDNA (GenBank accession No. KF361315) was 902 bp, containing a 795-bp open reading frame encoding 264 amino acids with a relative molecular mass of 30,288.4 and an isoelectric point of 9.13. *NnAP1* had a MADS-box domain and a K-box domain, which is typical of the *SQUA*/*AP1* gene family. A protein sequence identity search showed that NnAP1 was 75-96% similar to other plant AP1s. Phylogenetic tree analysis indicated that *NnAP1* was very closely related to *AP1* of *Glycine max*, suggesting that they shared the same protein ancestor. Quantitative real-time PCR analysis showed that *NnAP1* was expressed in various organs during different developmental stages; it had the highest expression in blooming

flowers and had trace expression in the young vegetative and flower senescence stages. Our analysis suggests that *NnAP1* plays an important role in controlling floral meristem identity and floral organ formation.

Key words: *Nelumbo nucifera*; *NnAP1*; Gene cloning; Expression analysis

INTRODUCTION

Nelumbo nucifera Gaertn. is an aquatic perennial herb of Nymphaeaceae. It is an important aquatic ornamental plant and economic crop, and it flowers from June to September. At present, flower breeding in this ornamental plant is limited to traditional methods. With improvements in gene cloning, the study of temporal and spatial gene expression patterns provides a theoretical basis to reveal the molecular mechanisms of ornamental plant flower development, and it also enables flower engineering using many methods, such as transgenic technology. Flower development involves complex gene expression and regulation as part of a very complicated biological phenomenon. One class of genes involved in this process is the flower-timing genes, which influences flowering time and can control the morphology of flowers and inflorescence meristems (Guo et al., 1998). Research on the molecular mechanism and genetic control of flowering in *Arabidopsis* has shown that *AP1* is a floral meristem identity gene. This gene not only controls the transition from vegetative to inflorescence and floral meristems in plants, but it also controls the growth of the two external whorls in floral organs, sepals, and petals (Irish and Sussex, 1990).

In the classical floral organ ABC model, the *AP1* gene belongs to class A and can activate the expression of class B genes (Mandel and Yanofsky, 1995; Jack, 2004). Research has shown that *APETALA1* (*AP1*) genes are highly conserved (Peña et al., 2001). To date, researchers have identified *AP1*-homologous genes from lily, jujube, apple, longan and *Cymbidium ensifolium*, (Gao et al., 2006; Chen et al., 2008; Sun et al., 2009; Meng et al., 2010; Wu et al., 2013). Flowering of the inflorescence was accelerated when an *AP1*-homologous gene was transformed into the chrysanthemum "Yumianren" (Lü et al., 2007), and three *AP1*-homologous genes were shown to accelerate flowering in transgenic *Arabidopsis* (Chen et al., 2008). However, research on lotus flowering genes has been relatively limited. In a previous study, a lotus *AP2* flowering gene was cloned and its expression characteristics were explored (Liu et al., 2012). In addition, we cloned and examined the expression of *LEAFY* (*LFY*) genes from *N. nucifera* in our previous research. The *LFY* gene regulates the expression of the downstream meristem identity gene *AP1* and one of its effects is to activate the *AP1* gene. *AP1* has a feedback effect on *LFY* (Peña et al., 2001), thus, *AP1* and *LFY* have a close relationship. To date, there has been no report of research on the *AP1* gene in lotus.

The objective of the current study was to clone the full-length cDNA sequence of the *NnAP1* gene. Using the published sequences of homologous genes, we designed degenerate primers for *N. nucifera* 'Hongxia' and combined this with rapid amplification of cDNA ends (RACE). Bioinformatic methods were used to analyze the sequence characteristics of the gene and the sequence information was registered in GenBank. RT-PCR and quantitative real-time PCR methods were used to analyze the expression pattern of *NnAP1* in various organs and during different developmental stages (Lenartowski et al., 2014). This research provides a basis for control of flowering time and the mechanism of floral formation using genetic engineering technology.

MATERIAL AND METHODS

Plant materials

The plant material used was *N. nucifera* Gaertn. 'Hongxiamantian', which is maintained in the landscape plant molecular biology laboratory of Henan Agriculture University. We used roots, stems, leaves, and flowers from young vegetative, early flowering, full-bloom, and flower senescence stage plants as material. All plant samples were immediately frozen in liquid nitrogen and stored at -80°C for later use.

Chemicals for DNA isolation and cloning

A RevertAid™ First Strand cDNA synthesis kit was purchased from Fermentas (Lithuania), a SMARTer™ RACE cDNA amplification kit was purchased from TaKaRa (Japan), and the pUCm-T cloning vector was purchased from Shanghai Shenggong Biological Engineering (China). A Gel DNA extraction kit was purchased from Tiangen Bio-tech (China) and Taq enzymes and DL1000 markers were purchased from TaKaRa. *Nicotiana tabacum* seeds and *Escherichia coli* strain DH5α are maintained in our laboratory. We purchased 2X Power Taq PCR MasterMix and Power 2X SYBR Real-time PCR Premixture from BioTeke Corporation (China).

Total RNA isolation and cDNA synthesis

RNA was extracted from young petals of lotus using a modified CTAB method (Yang et al., 2009). The RNA showed typical absorption curves at A_{260}/A_{280} and A_{260}/A_{230}, demonstrating its purity, and it was then subjected to agarose gel electrophoresis. The Alpha Imager PE system was used to photograph the agarose gel and detect the quality and concentration of DNA. We used 2 μg total RNA for reverse transcription to obtain first-strand cDNA.

Cloning of the complete *NnAP1* gene cDNA sequence

Using BLASTn, we searched for *AP1*-homologous nucleotide sequences in GenBank and chose sequences from species that were closely related to lotus and analyzed their homology. From this, we designed the specific primers F1 and F2 in conserved regions (Table 1). We also designed and synthesized the 3'-RACE primers F1S and F2S and the 5'-RACE primers R1A and R2A (Table 1), which were based on the amplified conserved fragments. The downstream primers were B26 (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3') and UPM (5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'). The middle fragment and the template cDNA of 3'-RACE and 5'-RACE were obtained following protocols for the RevertAid™ First Strand cDNA synthesis and SMARTer™ RACE cDNA amplification kits (Lenartowski et al., 2014).

PCRs were performed in a 20 μL volume including 10 μL 2X MasterMix, 1 μL upstream primer, 1 μL downstream primer, 1 μL template, and 7 μL ddH_2O. The PCR program used was as follows: pre-denaturing at 94°C for 5 min, followed by 94°C for 50 s, annealing (temperatures and times are shown in Table 1), and 72°C for 1 min for 30 cycles, and finally 10 min of extension at 72°C. The PCR products were separated on a 1.0% agarose gel. The

fragments were ligated into the vector pUCm-T after being recovered and purified, and were then transformed into *E. coli* DH5α. The PCR products were sequenced from both directions by the Shanghai Biotechnology Company, China.

Using the DNAMAN software (http://www.lynnon.com/), we combined the 5'-RACE fragment, the middle fragment, and the 3'-RACE fragment. The full coding region was analyzed on the NCBI website using Open Reading Frame Finder. We then designed the primers AP1-S and AP1-A on either side of the coding region (Table 1) to verify the result using PCR. The methods were the same as above, including the PCR program. The PCR product was recovered, purified, and sequenced to obtain the full cDNA sequence.

Table 1. Primer sequences for cloning and annealing conditions of *NnAP1* from *Nelumbo nucifera*.

Primer	Primer sequence	Annealing temperatures and times
Conserved primer		
F1	5'-TGTGATGCTGAAGTCGCT-3'	50°C, 30 s
R1	5'-CCTTTGGTTCCGCTGTAA-3'	
3'RACE primer		
F1S	5'-GTCGCTGTGATCGTCTTCTCCAC-3'	59°C, 30 s
F2S	5'-GAATCACAGGGAAGCTGGTCTCT-3'	55°C, 30 s
5'RACE primer		
R1A	5'-GGTTCCGCTGTAAAATCTCA-3'	55°C, 30 s
R2A	5'-GCTTTCCCTTGGTGGAGAAGACGAT-3'	60°C, 30 s
NnAP1 cDNA amplified primer		
AP1-S	5'-ATGGGGAGAGGTAGGGT-3'	58°C, 30 s
AP1-A	5'-TCAGATGGTGGAACATGCGAGGCCG-3'	

Bioinformatic analysis of the *NnAP1* gene

Using the DNAMAN 6.0 software (http://www.lynnon.com/), we compared the nucleotide and amino acid sequences. A molecular phylogenetic tree was created using MEGA5.0 software (http://www.megasoftware.net/) with homologous amino acid sequences from GenBank obtained using the BLASt program. Protein prediction was performed on the NCBI website (http://www.ncbi.nlm.nih.gov/). The conserved regions were analyzed using a specialized BLAST search (CDD search). The physicochemical protein characteristics were analyzed using the ProtParam software (http://web.expasy.org/protparam/). Analysis of transmembrane domains and transmembrane orientation was performed by TMbase using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). Signal peptide prediction was performed using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Subcellular localization prediction was done using PSORT (http://psort.hgc.jp/form.html) and three-dimensional structure modeling was performed using Swiss-Model Workspace (http://swissmodel.expasy.org/).

Analysis of *NnAP1* expression

NnAP1 expression in different lotus growth stages was examined by quantitative real-time PCR. We used roots, stems, leaves, and flowers from young vegetative stage, early flowering stage, full-bloom stage, and flower senescence stage plants as material. RNA was extracted and stored as above, and reverse transcribed into cDNA.

Primers were designed to analyze gene expression using the open reading frame (ORF)

sequence of *NnAP1* (*NnAP1*-F1, 5'-GCAGGAGCAAAACAACATAC-3'; *NnAP1*-R1, 5'-TAGC AGGAAGGACGGTGA-3'). The amplified fragment length was 133 bp. The lotus *18S-rRNA* gene was used as a reference to analyze expression by RT-PCR; the primer sequences were 18S-rRNAF 5'-CCATAAACGATGCCGAC-3' and 18S-rRNAR 5'-CACCACCCATAGAATCA AGA-3'. RT-PCR was performed following BioTeke Company protocols for the Power 2X SYBR Real-time PCR Premixture kit. PCRs were performed on a Bio-Rad iCycler iQ. The PCR program used was as follows: pre-denaturing at 95°C for 20 s, and then denaturing at 58°C for 20 s, annealing (temperatures and times are shown in Table 1), and extension at 72°C for 20 s for 40 cycles, followed by melting curves analysis. Every sample was run in triplicate and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

RESULTS

NnAP1 cloning and sequencing

From lotus petal tissue we cloned a 216 bp conserved region fragment, a 647-bp 3'-RACE fragment, and a 434-bp 5'-RACE fragment by RT-PCR and 3'/5'-RACE. The total *AP1* cDNA length was 902 bp and was obtained by combining the sequence fragments with DNA-MAN6.0. A 795-bp ORF fragment was obtained using PCR by designing primers from the combined total sequence (Figure 1). Sequencing of the ORF fragment confirmed the combined sequence was correct. The resulting sequence was named *NnAP1* and deposited in GenBank under accession No. KF361315 (Figure 2).

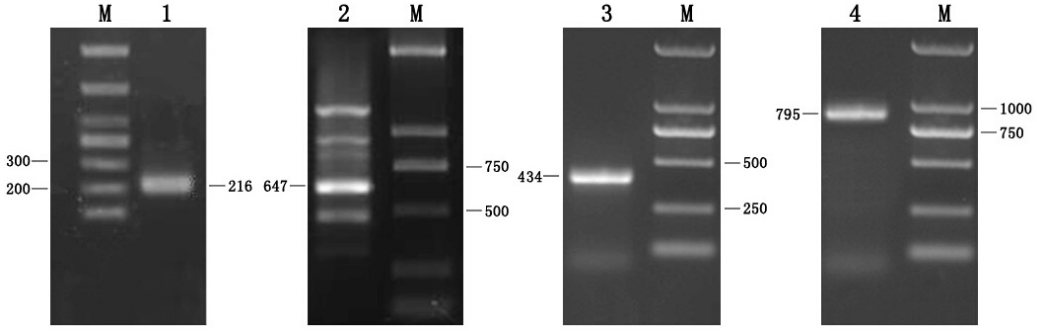

Figure 1. cDNA amplification of the *NnAP1* gene in *Nelumbo nucifera*. *Lane 1* = conserved fragment; *lane 2* = 3'-RACE product; *lane 3* = 5'-RACE product; *lane 4* = complete open reading frame; *lane M* = DNA size marker (units are bp).

Amino acid sequence comparison and phylogenetic analysis

Using the BLAST program, we searched for homologous amino acid sequences, which showed that NnAP1 was 75-96% similar to AP1s from other plant species sequences (Figure 3).

NnAP1 was most homologous to a sequence from *Trochodendron aralioides* (approximately 96% similar). The phylogenetic tree suggested that the homologous proteins shared the same ancestor (Figure 4).

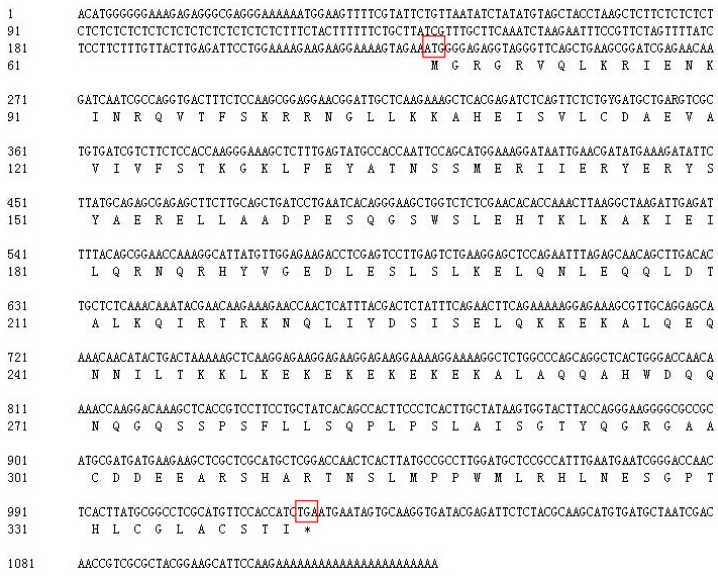

Figure 2. Full cDNA nucleotide and deduced amino sequences of the *NnAP1* gene in *Nelumbo nucifera*. The open reading frame of *NnAP1* was 795 bp, encoding 264 amino acids. The start codon was ATG, the stop codon was TGA, and the 3′-non-coding region was 110 bp, which included 23 adenosine residues (poly A). Red boxes denote position of start and stop codons.

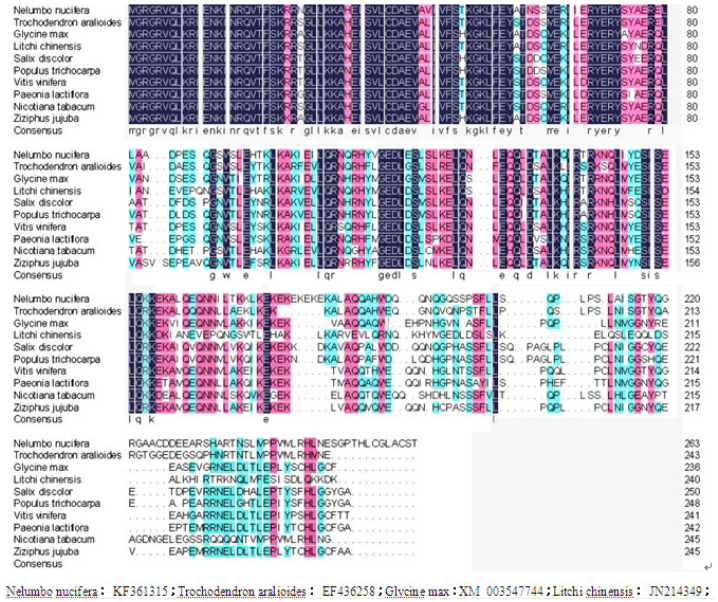

Figure 3. Alignment of the deduced NnAP1 amino acid sequence from *Nelumbo nucifera* and its homologous amino acid sequences from other species. Accession Nos. are: *Nelumbo nucifera*, KF361315; *Trochodendron aralioides*, EF436258; *Glycine max*, XM_003547744; *Litchi chinensis*, JN214349; *Salix discolor*, DQ068268; *Populus trichocarpa*, AY515153; *Vitis vinifera*, AY538746; *Vitis vinifera*, KC354376; *Nicotiana tabacum*, AF009127; and *Ziziphus jujuba*, EU916199.

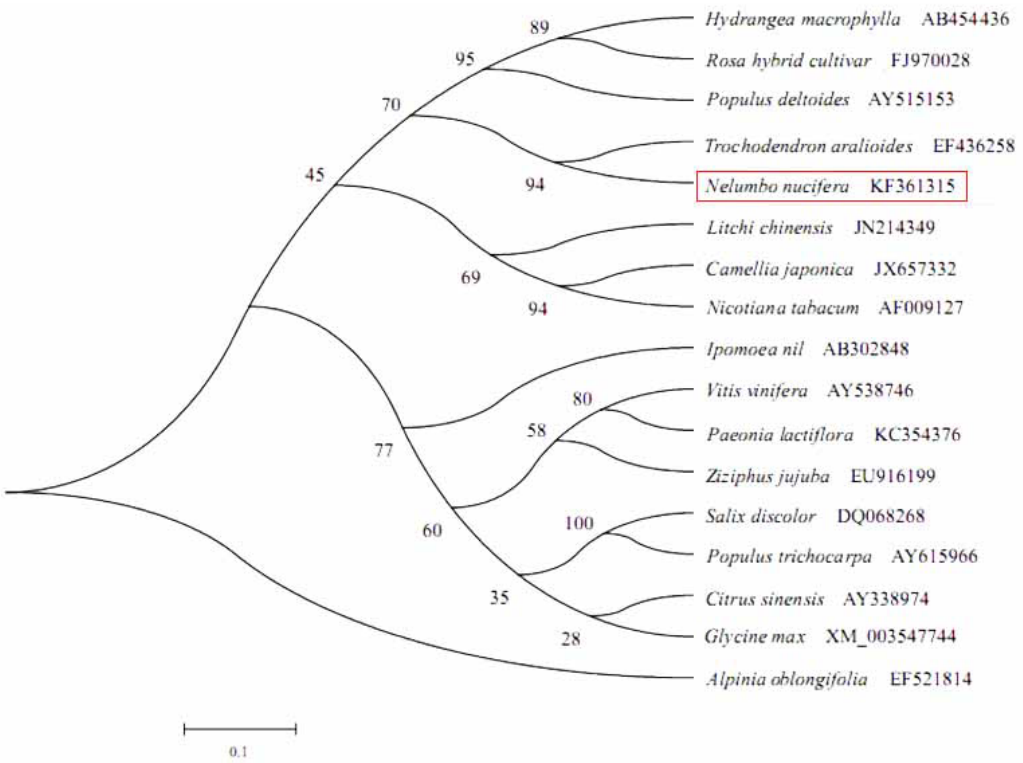

Figure 4. Phylogenetic tree of the NnAP1 protein from *Nelumbo nucifera* and other AP1s. NnAP1 had the closest protein relationships with *Hydrangea macrophylla*, *Rosa rugosa*, *Populus deltoides*, and *Trochodendron aralioides*. It was closest to AP1/FUL of *T. aralioides*. Accession Nos. provided alongside species names. Figures along branches denote level of similarity (%).

Bioinformatic analysis of the NnAP1 protein

The physicochemical characteristics of the NnAP1 protein were analyzed using the ProtParam program. The encoded protein had 264 amino acids, a relative molecular mass of 30,288.4, a chemical formula of $C_{1318}H_{2150}N_{392}O_{409}S_8$, and an isoelectric point of 9.13. The instability coefficient of the protein was 56.39, indicating it was unstable. The fat coefficient was 82.34 and the average hydrophilic coefficient was -0.803, indicating it was hydrophilic. Its extinction coefficient was 27,180 at a depth of 280 nm in aqueous solution. The protein was composed of 20 types of amino acid, with Leu being the most abundant (12.1%). The total number of negatively charged amino acids (Asp + Glu) was 35 and the total number of positively charged amino acids (Arg + Lys) was 42.

We used TMpred to predict the transmembrane domains and transmembrane orientation. The protein had two internal to external spiral regions (located at residues 35-59 and 202-218) and one external to internal spiral region (residues 38-58). Thus, NnAP1 was deduced to be a transmembrane protein. Signal peptide prediction showed that NnAP1 did not have a clear signal peptide, so it was not a secretory protein (Tanaka et al., 1990). PSORT predicted that it was most likely located in the cell nucleus (76% probability), but it also predicted that it could

be located in the cytoplasmic membrane (60%), the mitochondrial plastid (36%), or the chloroplast membrane (10%). Thus, lotus NnAP1 is likely to be mainly located within cell nuclei.

After protein analysis and prediction, the sequence was submitted to the Prosite database to analyze and predict its protein domains. The results showed that NnAP1 has a conserved MADS region (aa 1-61) and a sub-conserved K region (aa 88-178). Its 3D structure was predicted by SWISS MODEL homology modeling. In6jA was identified as a template in the protein database (Liu et al., 2014) and the similarity between NnAP1 and In6jA was 50.69% according to comparison with x-ray crystal diffraction (E value = 3.40e-28; Figure 5).

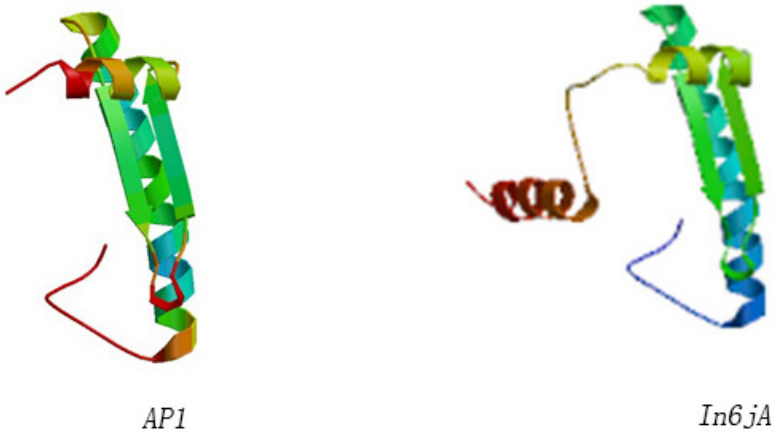

Figure 5. Three-dimensional structure of the NnAP1 protein from *Nelumbo nucifera* and the In6jA template predicted by SWISS-MODEL.

Analysis of *NnAP1* expression in lotus during different developmental stages

The *18S-rRNA* gene was used as a reference gene to normalize *AP1* expression in lotus at different developmental stages. The results showed that *NnAP1* was expressed in the roots, stems, leaves, and flowers and had differential expression during the young vegetative, early flowering, and full-bloom stages (Figure 6). *NnAP1* had limited expression in roots, stems, and leaves during the young vegetative stage, with the highest expression observed in roots (Figure 6 I). During the young vegetative stage, the expression level of *NnAP1* in leaves and stems was 0.77 and 0.25 times that observed in roots, respectively. *NnAP1* expression level was higher during the early flowering stage (Figure 6 II) than in the young vegetative stage (Figure 6 I). During the early flowering stage, *NnAP1* expression was highest in flowers, followed by leaves, stems, and roots, with the expression level in flowers, leaves, and stems being 11, 7, and 6 times that observed in roots, respectively (Figure 6 II). During the full-bloom stage (Figure 6 III), the highest expression of *NnAP1* was observed in flowers, with lower expression recorded in the roots and stems. Thus, the gene has a close relationship with organ formation. During the flower senescence stage (Figure 6 IV), *AP1* expression fell to trace levels in every tissue. Comparatively, *NnAP1* expression was lower in the young vegetative and senescence stages (Figure 6 I and IV, respectively) than it was in the early flowering and full-bloom stages (Figure 6 II and III, respectively). Thus, *NnAP1* is differentially expressed during different developmental stages.

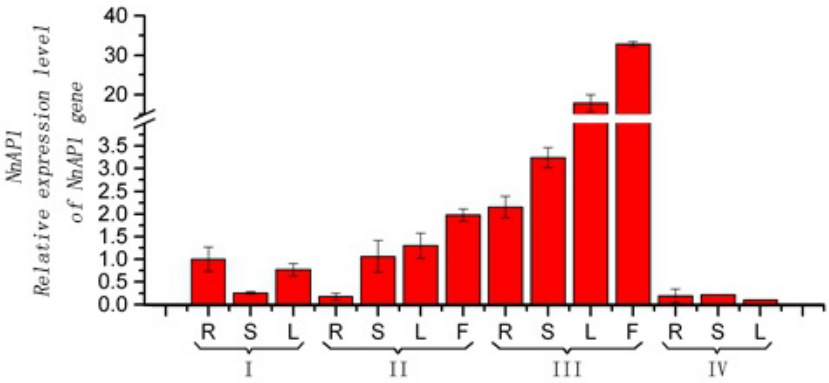

Figure 6. Relative expression of the *NnAP1* gene in different organs and during different growth stages of *Nelumbo nucifera*. I represents the young vegetative stage, II represents the early flowering stage, III represents the full-bloom stage, and IV represents the flower senescence stage. R, root; S, stem; L, leaf; F, flower.

DISCUSSION

In recent years, *AP1* genes have been cloned from many plants. AP1 is essential for the regulation of flower development, which has been proven in the model plants snapdragon and *Arabidopsis* (Mandel and Yanofsky, 1995; Liu et al., 2009). In this study, we cloned and obtained the complete sequence of an AP1-homologous gene from lotus and analyzed its sequence characteristics and expression. The amino acid sequence of NnAP1 included specific MADS-box and K-box domains, suggesting it belongs to the *SQUA/AP1* gene family, the same as was observed for Shijing mango and jujube genes (Jack, 2004). The cloned AP1 amino acid sequence was queried in GenBank with the BLAST program and NnAP1 and AP1s from other plant species showed 75-96% similarity. The MADS-box region had the highest homology and was highly conserved. AP1 belongs to the MADS-box gene family, which participates in flowering transition, flower development, and organ formation (Theißen et al., 1995). NnAP1 included a MADS-box region, which can combine with DNA in a sequence-specific way and function in transcriptional regulation (Suarez-Lopez et al., 2001; Melzer et al., 2010). MADS-box proteins can also form dimers and perform other functions (Jack et al., 1992; Shore and Sharrocks, 1995). Protein dimers can increase the variety of DNA binding proteins and the affinity between the complex and the point of DNA binding. In analysis of *Arabidopsis* MAD-box dimer proteins, AP1 and AGLI were observed to form homogeneous protein dimers (Riechmann et al., 1996); AP1 and CAL can also form protein dimers (Pelaz et al., 2001). Theißen and Saedler (2001) proposed the quartet molecular model: two MADS-box protein dimers can combine with the CArG box to form a quartet molecule via protein interactions and ensure the development of floral organs. Thus, in *Arabidopsis*, the homogeneous dimers AP1/AP2 and SEP/AP1 can form a tetramer, which controls the formation of sepals, and the heterodimers AP3/P1 and SEP/AP1 can form a tetramer that controls petal development (Honma and Goto, 2001; Favaro et al., 2003). Thus, protein polymerization is important for controlling gene expression, and MADS-box genes have an essential role in flower development. Here, we found a MADS-box sequence, which is involved in developmental regulation. Sequence analysis showed that it had high homology to AP1s of other plants, thus, we can

infer similar functions from its similar structure (Xu et al., 2011).

We used quantitative real-time PCR to analyze the expression of the *NnAP1* gene during different developmental stages and in various organs, and found that the different organs had differential expression of *NnAP1* during different developmental stages (Khan et al., 2012). *NnAP1* was expressed in different organ tissues during the young vegetative stage, but the expression level was low, which may be related to flower induction (Kyozuka et al., 1997). As we expected, *NnAP1* had higher expression levels in leaves and stems, with low expression in roots. Additionally, *NnAP1* expression was clearly higher during the full-bloom stage. Wu et al. (2013) found that a homologous gene had higher expression levels in *C. ensifolium*, and the *ZjAP1* gene was found to be expressed during flowering development (Sun et al., 2009; Meng et al., 2010); thus, they share the same expression patterns. Expression analysis showed that *AP1* was expressed stably in stamen primordia and floral organ differentiation. Thus, *AP1* has an important role in controlling floral meristem identity and the formation of floral organs. In research on *Arabidopsis* flowering, *LFY* was found to be an important gene for determining floral meristem identity, like *AP1*. The two genes were not completely independent and expression of *LFY* preceded that of *AP1* (Sessions et al., 2000; Grandi et al., 2012). Thus, *AP1* is involved in the determination of meristem identity as a downstream gene of *LFY* (Ng and Yanofsky, 2001), and *AP1* affects floral meristem identity genes during its early expression, which in turn is affected by the induction of *LFY*. *AP1* also functions in determining floral organ characteristics in the late stage, which is affected by *LFY* indirectly (Mandel, 1992; Liljegren et al., 1999). However, there has been little research on the regulation of expression between *AP1* and *LFY* in lotus and its significance in the lotus bud differentiation regulation mechanism.

In this study, we identified an *AP1* gene that was expressed in different organs and participated in the whole process of floral bud differentiation in lotus during different periods. However, it remains unclear how *NnAP1* functions in lotus. The relationship between the expression characteristics of this *AP1* gene and the regulation of flower development in lotus should be further explored in depth.

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