Reduced Expression of C-Class Genes Is Associated with the Multiple-Petal Phenotype in *Nelumbo nucifera*

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The sacred lotus (*Nelumbo nucifera*) is an aquatic basal eudicot that belongs to the small family Nelumbonaceae. Sacred lotus cultivars are generally one of two types: rhizome lotus and flowering lotus. Rhizome lotus cultivars form only a few simple flowers, but produce an extremely enlarged edible rhizome, which is widely cultivated in East Asian countries. In contrast, flower lotus cultivars form many beautiful flowers in a variety of colors (white, red, pink) and shapes (single, double, proliferating). Since the whole-genome sequence of the sacred lotus ‘China Antique’ was released in 2013, genome-wide analysis tools have been rapidly established, and it is now possible to comprehensively identify genes of interest (Ming et al., 2013; Wang et al., 2013, 2015; Lin et al., 2019b). Despite this progress, the molecular bases of many important processes, including floral organ specification and development, remain unknown.

The ABC model of floral organ development was proposed based on genetic analysis of the core eudicots *Arabidopsis* (*Arabidopsis thaliana*) and snapdragon (*Antirrhinum majus*) (Coen and Meyerowitz, 1991). In this model, the floral organs are arranged in four concentric areas called “whorls” and the combination of ABC-class genes expressed in each whorl determines floral organ identity. A-class gene function specifies sepals in the first whorl, and an A plus B function specifies petals in the second whorl. In the third whorl, a B plus C function specifies stamens, and a C-class gene function alone specifies carpels in the fourth whorl. This model was later expanded to the ABC model to incorporate the function of E-class genes. *APETALA1 (AP1)* and *APETALA2 (AP2)* are A-class genes, *PISTILLATA (PI)* and *APETALA3 (AP3)* are B-class genes, *AGAMOUS (AG)* is a C-class gene, and...
SEPALATA1-4 (SEP1-4) are E-class genes. All of these genes encode MADS-box type transcription factors, except for AP2, which encodes an AP2-ERF-type transcription factor. Recently, another MADS-box gene homolog, AGAMOUS-LIKE 6 (AGL6), was reported to function as an A-class gene in Nigella damascena (Wang et al., 2016).

Although the ABCE model has been used to explain flower development in a variety of species, it is still unclear whether the model is directly applicable to basal angiosperm lineages. The floral organs of many basal angiosperms and early diverging eudicots are arranged spirally rather than whorled, and the flowers of these plants usually have a variable (flexible) number of floral organs (Endress and Doyle, 2007). In basal angiosperms, the transition from sepals to petals (the outer two organ whorls in the ABCE model) is sometimes indistinct. According to Hayes et al. (2000), the perianth of Nelumbo nucifera consists of two sepals and 18 to 30 petals, but as in the case of many basal angiosperms, the sepals are not well differentiated, so the boundary between the first and second whorl is not clear. N. nucifera has a polysymmetric flower structure in which some floral organs are arranged radially (stamens and carpels), while others are arranged spirally (petals). As is the case for flowers of other basal species, the sacred lotus flower bears a variable number of organs (Hayes et al., 2000). The flowers of lotus cultivars are roughly categorized as single-, double-, or proliferating-type, based on the number of petals. The single-petalled flower is thought to be the default type because the ancient lotus variety ‘China Antique’ has single-petalled flowers (Ming et al., 2013). In general, an increase in petal number correlates with an increase in petaloid stamens and a decrease in stamen number, suggesting that a homeotic conversion of floral organs occurs in these cultivars. Although some ABCE-class genes have been reported in N. nucifera (Yoo et al., 2010; Liu et al., 2012; Kong et al., 2015), the molecular mechanisms of floral organ specification and development in N. nucifera are still unclear. In this study, we screened the genome database of N. nucifera for floral organ identity genes and investigated their expression patterns in floral organs of several lotus cultivars with different flower types. This study helps elucidate the molecular mechanisms of floral organ development in early diverging eudicots and provides important insights into the evolutionary relationships among these species.

Materials and Methods

Plant materials

Three flowering lotus cultivars were used in this study: the single-petalled cultivar ‘Maiyoren (MAYR)’, the double-petalled cultivar ‘Judaren (JUDR)’, and the proliferating-petalled ‘Senbenren (SNBN)’ (Fig. 1). For the flowers used in the qRT-PCR experiment, ‘MAYR’ and ‘JUDR’ respectively produced 17–22 and 70–157 total petals from three independent flowers. In contrast, the stamen numbers in ‘MAYR’ (132–153) were dramatically higher than those in the double-petalled cultivar ‘JUDR’, in which 11–35 stamens and 10–28 petaloid stamens formed in the same number of flowers. In ‘JUDR’, most carpels were transformed into petaloid carpels. The proliferating cultivar ‘SNBN’ did not form any stamens, carpels, or receptacles and continued to produce petals, with the total petal number reaching more than 1000 (Fig. 1). The plants were grown at the Institute for Sustainable Agro-ecosystem Services, Graduate School of Agricultural and Life Sciences (Nishi-Tokyo City, Tokyo, Japan), or at the Yayoi campus (Bunkyo-ku, Tokyo, Japan), The University of Tokyo.

Identification of floral organ identity genes and phylogenetic analysis

Based on searches of the public databases LOTUS-DB (<http://lotus-db.wbgcas.cn/>), TAIR (<https://www.arabidopsis.org/>), NCBI (<https://www.ncbi.nlm.nih.gov/>), and UniProt (<http://www.uniprot.org/>), coding sequences of Nelumbo, Arabidopsis (Arabidopsis thaliana), Nigella damascena, Magnolia grandiflora, and other plant species were collected. Using the tblastn search on LOTUS-DB, the contigs that showed highest identity to the known floral genes were regarded as candidate homologs. The MUSCLE program was used for multiple sequence alignment, and
the Maximum Likelihood Method was applied for phylogenetic analysis within the MEGA X software (Kumar et al., 2018).

**RNA extraction, reverse-transcription, and real-time RT-PCR**

Plant samples were frozen in liquid nitrogen and ground using a mortar and pestle. Total RNA was extracted from lotus tissues (ca. 150 mg) by the combining CTAB method and a NucleoSpin RNA Plant Kit (MACHEREY-NAGEL). Genomic DNA was eliminated by treatment with DNase on the column, according to the manufacturer’s instructions. A PrimeScript RT Master Mix (Takara Bio Inc., Japan) was used to synthesize cDNA from 500 ng of total RNA. The synthesized cDNA was diluted 10-fold, and 2 μL was used in 20-μL reactions with a SYBR Premix Ex Taq™ II Tli RNaseH Plus (Takara Bio) for qRT-PCR that was performed on a thermal cycler CFX96 connect (Bio-Rad). The relative expression of each gene was calculated with the ΔCt method using \( NnACTIN \) as the normalization gene. The amplification efficiency of each primer set was confirmed by preliminary experiments using dilution series of plasmids containing the genes of interest. The coding region of \( NnAG1 \) and \( NnAG2 \) shared 89.5% identity at the nucleic acid level. Specific primer pairs for amplifying each gene were designed (Supplementary Fig. 1).

**Gene isolation, cloning, and sequencing**

The coding regions of \( NnAG1 \) and \( NnAG2 \) were amplified by PCR from cDNA libraries of the \( Nelumbo \) cultivars ‘MAYR’ and ‘JUDR’. The PCR products were cloned into the SmaI site of a pUC19 vector by blunt-end ligation, and 4–5 independent plasmid clones for each amplification were sequenced with an ABI 3130 Genetic Analyzer (Thermo Fischer Scientific, USA).

**In situ hybridization**

Flower buds just emerged from the water surface were fixed using fixing solution (4% (w/v) paraformaldehyde-1% (v/v) TritonX-100/100 mM Na-P buffer) at 4°C for two days. Ethanol was used for dehydration and then replaced by permutation solution. The flower buds were embedded in paraffin and then cut into 8 μm sections. The proteins were removed using Proteinase K, and RNA probes were used to detect target nucleic acid sequences. RNA probes were generated using T7 polymerase from vectors carrying coding sequences of \( NnAG1 \) or \( NnAG2 \). The target mRNAs on sections were stained with NBT/BCIP, showing as a brownish-purple, and the spatial expression patterns of target RNAs were observed with a stereomicroscope (S9D; Leica, Germany). All sequences of primers used for in situ hybridization are listed in Supplementary Table 1.

### Results

**Identification of ABCE-class genes from \( Nelumbo nucifera \) genome sequences**

To understand floral organ specification in \( Nelumbo nucifera \), lotus genome databases were screened for ABCE-class floral organ identity genes. The amino acid sequences of Arabidopsis MIKC\(^{C}\)-type MADS-box and AP2/ERF-type AP2 genes were used as queries for tblast searches of LOTUS-DB (Wang et al., 2015). These searches yielded strong candidates for \( N. nucifera \) orthologs in each class of floral organ identity genes: in the A-class, one \( AP1 \)-like (\( NnAP1 \)) and three \( AP2 \)-like (\( NnAP2, NnAP2a, NnAP2b \)) genes; in the B-class, one \( PI \)-like (\( NnPI \)) and two \( AP3 \)-like (\( NnAP3a, NnAP3b \)) genes; in the C-class, two \( AG \)-like (\( NnAG1, NnAG2 \)) genes; and in the E-class, three \( SEP \)-like (\( NnSEP1a, NnSEP1b, NnSEP3 \)) genes (Table 1). Two closely located gene models, NNU_26658-RA and

### Table 1.  Gene list of floral organ identity genes in \( Nelumbo nucifera \).  

| Gene name | Full length (aa) | Clade | Class | Gene ID |
|-----------|------------------|-------|-------|---------|
| \( NnAP1 \) | 188 aa | AP1/FUL | A | NNU_07470-RA |
| \( NnAP2 \) | 512 aa | AP2 | NNU_15241-RA |
| \( NnAP2a \) | 554 aa | AP2 | NNU_17043-RA |
| \( NnAP2b \) | 569 aa | AP2 | NNU_13608-RA |
| \( NnAP3a \) | 210 aa | AP3/DEF | B | NNU_23351-RA |
| \( NnAP3b \) | 230 aa | AP3/DEF | NNU_15351-RA |
| \( NnPI \) | 189 aa | PI/GLO | NNU_02674-RA |
| \( NnAG1 \) | 225 aa | AG/PLE | C | NNU_0192-RA |
| \( NnAG2 \) | 224 aa | AG/PLE | NNU_26656-RA |
| \( NnSEP1a \) | 243 aa | SEP | E | NNU_04431-RA |
| \( NnSEP1b \) | 177 aa | SEP | NNU_07469-RA |
| \( NnSEP3 \) | 143 aa | SEP | NNU_02038-RA |
| \( NnAGL6a \) | 243 aa | AGL6 | A | XP_010272608 |
| \( NnAGL6b \) | 242 aa | AGL6 | XP_010255596 |
NNU_26658-RA, were initially predicted as independent genes, but were later confirmed to encode the C- and N-terminus of NnAG2, respectively. Two additional candidate A-class genes, the AGL6-like genes NnAGL6a and NnAGL6b, were identified in the whole-genome shotgun database at NCBI.

**Phylogenetic analysis of N. nucifera MADS-box type ABCE-class genes**

Phylogenetic analysis of the amino acid sequences of the putative MADS-box ABCE-class genes of *N. nucifera* and several other plant species was carried out using MEGA X software (Kumar et al., 2018). Each gene of *N. nucifera* was categorized into the predicted SEP, AGL, AP1/FUL, AG, and AP3/PI clade (Fig. 2). In each clade, the *N. nucifera* gene was closely associated with the homologs of *Magnolia grandiflora* (Magnoliaceae), a basal angiosperm, or with those of *Nigella damascena* (Ranunculales), an early diverging eudicot, further supporting the orthologous relationships and evolutionary lineage between those species (Chase et al., 2016).

**Comparison of cDNA sequences of C-class genes between single- and double-petalled lotus cultivars**

In many plant species, complete loss of function or reduced function of a C-class AG gene results in the loss of determinacy; stamens and carpels are converted into sepals and petals, leading to an increased number of petals and formation of double flowers (Nitasaka, 2003; Galimba et al., 2012; Tanaka et al., 2013; Noor et al., 2014; Klocko et al., 2016; Wang et al., 2016). To test whether mutation of C-class genes is associated with the double-petalled phenotype in lotus, we compared the gene sequences of a single-petalled lotus cultivar, ‘MAYR’, and a double-petalled cultivar, ‘JUDR’ (Fig. 1). The CDS of two C-class genes, NnAG1 and NnAG2, were PCR-amplified, cloned, and sequenced. We did not detect any mutations that would cause a premature stop codon or amino acid substitution in the coding regions of the NnAGs of either cultivar (Supplementary Fig. 2), suggesting that loss or gain of function of a C-class AG gene is not the cause of the double-petal phenotype in *N. nucifera*.

**Expression analysis of ABCE-class genes in floral organs of single- and double-petalled lotus cultivars**

Because no significant mutations were observed in the coding regions of the C-class genes, we next looked for cultivar differences in the expression of ABCE-class genes at the transcriptional level. Expression of ABCE-class genes in fully developed floral organs of single- and double-petalled lotus cultivars was measured by qRT-PCR (Fig. 1). Among the A-class genes, NnAP1 was mainly expressed in the sepals, petals, receptacles, and leaves, and there were no marked differences between single- and double-petalled cultivars (Fig. 3). Two AGL6-like genes (NnAGL6a and NnAGL6b) were highly expressed in the outer whorls (sepal and petal) of the single-petalled cultivar, and were expressed at a slightly reduced level in the double-petalled cultivar. The three AP2-like genes (NnAP2, NnAP2a, and NnAP2b) were relatively broadly expressed in all floral organs and leaves (Supplementary Fig. 3). Among the E-class genes, NnSEP1a was preferentially expressed in the sepals, petals, and stamens of the single-petalled cultivar, and was expressed at a slightly reduced level in the double-petalled cultivar. The other two E-class genes, NnSEP1b and NnSEP3, were relatively broadly expressed in all floral organs in both the single- and double-petalled cultivars. However, in the double-petalled cultivar, NnSEP3 expression expanded to the inner whorls. Among the B-class genes, NnAP3a was most highly expressed in stamens of the single-petalled cultivar, but in the double-petalled cultivar, it was broadly expressed in the inner petals, petaloid stamens, and stamens. NnPI was expressed mainly in sepal, petals, and stamens of both cultivars. The two C-class genes, NnAG1 and NnAG2, were specifically expressed in stamens, carpels, and receptacles located in the inner...
whorls of single-petalled flowers, and the expression was particularly high in the stamens. This high stamen expression was markedly reduced in the double-petalled cultivar (Fig. 3).

**Localization of C-class gene expression by in situ hybridization**

The spatial expression patterns of *NnAG1* and *NnAG2* during early flower development were analyzed by *in situ* hybridization. Young flower buds just emerged from the water (10.3–12.4 mm in length) were collected from ‘MAYR’ (single-type), ‘JUDR’ (double-type), and ‘SNBN’ (proliferating-type), fixed, and analyzed for *NnAG1* and *NnAG2* expression. The proliferating flower cultivar ‘SNBN’ did not form any stamens or carpels, instead continuing to produce petals (Fig. 1). Microscopic observations confirmed that the collected flower buds had already developed all floral...
organisms. \( \text{NnAG1} \) and \( \text{NnAG2} \) showed very similar expression patterns, with no obvious differences between them (Fig. 4). In the single-petalled cultivar ‘\( \text{MAYR} \)’, strong signals for \( \text{NnAG1} \) and \( \text{NnAG2} \) were detected in the stamens, the stigma, and the upper part of receptacles (Fig. 4A). However, in the double-petalled cultivar ‘\( \text{JUDR} \)’, the signal was weaker and was observed only in some stamens and in parts of the upper receptacle (Fig. 4B). Moreover, in the proliferating-petalled cultivar ‘\( \text{SNBN} \)’, which formed no stamens, carpels, or receptacles, no clear signal of \( \text{NnAG1} \) or \( \text{NnAG2} \) expression was detected (Fig. 4C).

**Discussion**

**Gene duplication of C-class genes and their possible function in floral organ specification and development**

A genome-wide survey of genes revealed that \( \text{N. nucifera} \) has two orthologs of the C-class genes \( \text{AG}, \text{NnAG1} \) and \( \text{NnAG2} \). \( \text{Nigella damascena} \), another early diverging eudicot, also has two paralogs of \( \text{AG} \) (\( \text{NdAG1} \) and \( \text{NdAG2} \)). Silencing of both \( \text{NdAG1} \) and \( \text{NdAG2} \) resulted in transformation of stamens into petals and ‘flower-within-flower’ structures. \( \text{NdAG1} \) is expressed in both stamens and carpels, whereas \( \text{NdAG2} \) is expressed only in carpels (Wang et al., 2016). These results indicate that \( \text{NdAG1} \) controls specification of stamen and carpel identities, whereas \( \text{NdAG2} \) is involved in both specification of carpel identity and determinacy of the floral meristem. In the basal angiosperm \( \text{Nymphaea colorata} \), two paralogs of \( \text{AG} \) (\( \text{AGa} \) and \( \text{AGb} \)) have been identified. Expression of \( \text{AGa} \) was restricted to stamens and carpels, whereas \( \text{AGb} \) was broadly expressed in outer floral organ whorls, suggesting that these genes may have undergone subfunctionalization for flower development (Zhang et al., 2020). In \( \text{N. nucifera} \), both \( \text{NnAG1} \) and \( \text{NnAG2} \) are specifically expressed in stamens and carpels with almost identical spatial expression patterns (Figs. 3 and 4), but qRT-PCR quantification revealed that \( \text{NnAG1} \) transcript levels were slightly (3 to 5 times) higher than those of \( \text{NnAG2} \). Therefore, these two C-class gene may be functionally redundant, but \( \text{NnAG1} \) may act predominantly to regulate floral organ identity in the inner whorls and determine floral meristem determinacy in \( \text{N. nucifera} \).

**Down-regulation of C-class gene expression in the double-petalled cultivar**

A strong correlation between reduced expression of \( \text{AG} \) homologs and a double flower phenotype has been observed in several plant species, such as \( \text{Camellia japonica} \) and \( \text{Tricyrtis macranthopsis} \). In \( \text{C. japonica} \), expression of the \( \text{AG} \) ortholog \( \text{CjAG} \) was dramatically reduced or almost undetectable in formal double flower cultivars (Sun et al., 2014; Li et al., 2017). In \( \text{T. macranthopsis} \), expression of a C function ortholog (\( \text{TrimAG} \)) was reduced in petaloid tepals formed in the two inner reproductive whorls (Sharifi et al., 2015). In these plants, the molecular mechanisms of reduced expression of C-function genes are still unclear. In \( \text{Thalictrum thalictroides} \), a basal eudicot belonging to Ranunculales, researchers reported that the double-petal ornamental cultivar ‘Double White’ has a retrotransposon insertion that results in loss of function of the C-class gene \( \text{ThtAG1} \). Down-regulation of \( \text{ThtAG1} \) by virus-induced gene silencing phenocopied the double-petal cultivar, resulting in homeotic conversion of stamens and carpels into sepaloid organs and an indeterminate flower structure (Galimba et al., 2012). Our results show that in fully developed \( \text{N. nucifera} \) flowers, two C-class genes, \( \text{NnAG1} \) and \( \text{NnAG2} \), are down-regulated in stamens and carpels of the double-petalled cultivar (Fig. 3). Spatial expression analysis of these genes by \( \text{in situ} \) hybridization at an earlier stage of flower development further revealed that \( \text{NnAG1} \) and \( \text{NnAG2} \) expression was high in stamens and carpels of the single-petalled cultivar, but was dramatically lower in the double-petalled cultivar and almost absent in the
proliferating-petalled cultivar (Fig. 4). These results strongly support the idea that down-regulation of C-class genes may be responsible for the increase in petal number and loss of determinacy in double- and proliferating-petalled N. nucifera cultivars. So far, we have not detected any mutations that would affect transcriptional regulation of NnAG1/2 in double-petalled cultivars. Recently, whole transcriptome analysis of N. nucifera flowers detected several MADS-box genes, including AGL6 and AG homologs, as candidate genes for controlling stamen petaloidy (Lin et al., 2018). However, whole-genome bisulfite sequencing did not detect any significant DNA methylation in the transcriptional regulatory regions of these genes (Lin et al., 2019a). In Arabidopsis, spatio-temporal regulation of AG expression in the floral meristem is regulated by a number of transcription factors including a floral meristem identity gene, LEAFY (LFY), and a meristem maintenance gene, WUSCHEL (WUS). LFY and WUS activate AG expression by binding to its cis-regulatory element located in the second intron (Lenhard et al., 2001; Lohmann et al., 2001). AP2, an A-class gene, acts as a negative regulator of AG expression. AP2 expression is post-transcriptionally regulated by a microRNA, miR172 (Wollmann et al., 2010). Thus, it is of interest to test whether changes in expression level, transcriptional activity, or mRNA stability of these transcription factors, as well as mutation in the cis-regulatory element, are responsible for reduced expression of NnAGs and the double flower phenotype. Such analyses are currently underway.

The functions of ABCE-class genes in floral organ formation in Nelumbo nucifera

Based on the expression profiles of ABCE-class genes in floral organs of single- and double-petalled cultivars, we propose a possible model of floral organ formation in N. nucifera. Two AGL6 homologs (NnAGL6a/b) were highly expressed in sepals and petals of the single-petalled cultivar, but in the double-petalled cultivar their expression was expanded to the inner petals and petaloid stamens (Fig. 3), suggesting that NnAGL6a/b may act as A-function genes in N. nucifera (Fig. 5). The classification of NnAP1 as an A-class gene is still tentative because although it was preferentially expressed in sepals and petals, it was also highly expressed in vegetative organs such as leaves (Fig. 3). The E-class genes NnSEP1a and NnSEP3 showed higher expression in the outer whorls of the single-petalled cultivar, but NnSEP3 expression expanded to the inner whorls of the double-petalled cultivar (Fig. 3). Given that functional diversification of E-class genes and a role for these genes in petal identity have been reported in several plant species (Pan et al., 2014; Soza et al., 2016; Zhang et al., 2017), NnSEPs may also specify petal formation in N. nucifera (Fig. 5). The B-class gene NnAP3a was most highly expressed in stamens, but was also detected in other organs, including sepals, petals, and carpels (Figs. 3 and 5). Expression of NnAP3a was highest in the stamens of the single-petalled cultivar, but was also high in inner petals and petaloid stamens of the double-petalled cultivar (Figs. 3 and 5), indicating that petal identity is determined by the combinatorial effect of B- and C-function genes and petal identity is caused by reduced expression of the C-function genes NnAG1/2, and probably by ectopic expression of NnAP3a (Fig. 5). In addition, NnPI was broadly expressed in sepals, petals, and stamens. A broader expression pattern of B-class genes is often seen in basal eudicots, monocots, and basal angiosperms (Soltis et al., 2007; Chanderbali et al., 2016). In nongrass monocots such as lily and tulip, in which petaloid organs called tepals are formed in the outer two whorls, expression of B class genes is extended to whorl 1 (Kanno, 2016). The broader expression of B-function genes observed in N. nucifera is in good agreement with the fact that sepals and petals are not morphologically well differentiated in this species.
Supplements
Supplementary data are available at <http://doi.org/10.2503/hortj.UTD-214>.

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