INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–1271/2021/25–4–851–858
DOI: 10.17957/IJAB/15.1738
http://www.fspublishers.org

Full Length Article

Molecular Cloning, Characterization and Expression of *SUPPRESSOR of OVEREXPRESSION of CONSTANS 1* (*NnSOC1*) and *NnSOC1-like* in *Nelumbo nucifera*

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Received 09 September 2020; Accepted 24 December 2020; Published 25 March 2021

Abstract

For the aim of unveiling the molecular mechanism of flowering, the MADS-box genes of *SUPPRESSOR of OVEREXPRESSION of CONSTANS 1 (NnSOC1)* and *NnSOC1-like* were isolated in *Nelumbo nucifera*. Seven introns splicing of *NnSOC1* and *NnSOC1-like* strictly followed the GT-AG rule, consisting of all the characteristic motifs of SOC1 family. *NnSOC1* and *NnSOC1-like* were widely distributed in reproductive and vegetative tissues of *N. nucifera*, exhibiting the highest expression in leaves and the lowest level in embryo. Additionally, both genes were expressed in the whole flowering stage, with the highest mRNA level observed in the initiation stage of flowering and the lowest expression in fruit set. Ectopic expression of *NnSOC1* and *NnSOC1-like* advanced the flowering time of transgenic Arabidopsis, and decreased the rosetta leaves production. These results suggested that *NnSOC1* and *NnSOC1-like* were involved in initiation of flowering, which are likely to serve as fundamental research for studying molecular mechanism of flowering in aquatic plants.

Key words: Flower opening; Gene expression; MADS-box; *Nelumbo nucifera*; SOC1

Introduction

Flowering is the floral transformation from vegetative phase to reproductive stage, which is considered as an imperative agronomic trait of crops. The floral initiation is critical for harvesting more products, controlling by combined function of endogenous gene network as well as environmental factors. Unveiling the molecular mechanism of flowering would be benefit for breeding in various surroundings. Various signals were integrated by networks to determine flower transition. Up to date, at least four flowering pathways including long day, autonomous, vernalization and gibberellin pathways were reported to regulate floral induction of high plants (Liu *et al*. 2012). MADS-box genes, encoding a highly conserved domain known as MADS-box, were considered as the key members of gene networks controlling the flowering transition and development.

The SUPPRESSOR of OVEREXPRESSION of CONSTANS 1 (SOC1) was classified as MADS-box type Ⅱ(MIKC^{C}) in high plants (Lee and Lee 2010), and is considered as a critical integrator for flowering activation in Arabidopsis. SOC1 integrated various flowering signals from temperature, photoperiod and hormones. As a key composition of transcription factors, SOC1 was characterized by the domains of MADS-box (M), an intervening region (I), a keratin box (K), as well as a C-terminal domain (C) (Zhong *et al*. 2012; Li *et al*. 2020). *SOC1* genes from higher plants including peony, barley, apricot, soybean and Mango have been studied (Papaefthimiou *et al*. 2012; Zhong *et al*. 2012; Wang *et al*. 2015; Wei *et al*. 2016), which were highly conserved among angiosperms. *SOC1* genes were widely distributed in tissues of leaves, flower and root. Ectopic expression of *SOC1* stimulated the early flowering of tobacco, tree peony and *Pyrus bretschneideri* (Wang *et al*. 2015; Yu *et al*. 2020; Liu *et al*. 2020). Beside flowering time, SOC1 also had other biological roles such as regulation of petal development in *Gerbera hybrida* (Ruokolainen *et al*. 2011), and floral organ senescence in *Camellia sinensis* (Tan and Swain 2007).

Lotus (*Nelumbo nucifera* L.) belongs to Nelumbonaceae family, known as a perennial aquatic plant. *N. nucifera* was famous as imperative ornamental plant as well as economic crop, having colorful flowers and numbers of petals. *N.nucifera* is considered as the species between dicots and monocots (Yang *et al*. 2014). The flowers, leaves, seeds and buds appear at the reproductive stage

To cite this paper: Dong C, F Du, Y Li, N Yang, J Mao, Z Hu (2021). Molecular cloning, characterization and expression of *suppressor of OVEREXPRESSION of CONSTANS 1* (*NnSOC1*) and *NnSOC1-like* in *Nelumbo nucifera*. *Intl J Agric Biol* 25:851–858

of *N. nucifera*. Although SOC1 family was relatively conserved, similar expression pattern was detected in dicots and monocots, their function might be divergent in *N. nucifera*. Therefore, further studies are necessary to unveil the functional divergence of *SOC1* for the aim of understanding the special mechanism controlling initiation of flowering in *N. nucifera*.

In this study, *NnSOC1* and *NnSOC1-like* cDNAs in *N. nucifera* were isolated. Their exon–intron structures, conserved motifs and phylogenetic analysis were performed. The three-dimensional structures were generated by homology modeling. Additionally, the expression pattern of both genes was examined during flower opening. Finally, the function of *NnSOC1* and *NnSOC1-like* were illustrated by transferring into the wild type Arabidopsis.

Materials and Methods

Plant material

Nelumbo nucifera "var. Taikonglian-36" was planted in Henan University of Technology, China. The rhizomes of the same size were taken randomly, and planted in pools with 2 rhizomes in each pool in April, 2018. The size of pools was about 10 m×5 m. All the samples were harvested at reproductive stage in September 2018, and frozen in liquid nitrogen immediately. Seeds of wild-type and transgenic *A. thaliana* were germinated and grown in the growth chamber under long-day conditions at 22℃, with 16 h light/8 h darkness cycles and 60% relative air humidity.

Isolation and characteristic of *NnSOC1* and *NnSOC1-like*

To scan *SOC1* in the genome of *N. nucifera*, the key word "SOC1" was used to search the Sacred lotus (*N. nucifera*) genome database (Ming *et al*. 2013). The putative proteins were further searched in the NCBI conserved domain to confirm the presence of conserved domains of SOC1. The primers were used to isolate the complete open reading frames (ORF) of *NnSOC1* and *NnSOC1-like* respectively (Table 1), based on their nucleotide sequences (XM_010257287; XM_010274299). The first leaf was collected in vegetative stage and frozen in liquid nitrogen immediately. Total RNAs from the first leaf were isolated by RNAprep pure Plant kit (TIANGEN, China). The first strand cDNA was generated by M-MLV transcriptase (Promega, USA) by reverse transcription. The reaction mixture was as follow: 3 µL RNA, 2 µL olig$(dT)_{17}$, 5 µL M-MLV reaction buffer, 3 µL dNTP, 10 µL nuclease-free water and 2 µL M-MLV transcriptase. The target products were harvested and sequenced as described previously (Dong *et al*. 2015).

Characterization and exon-intron structures

The Prot Param program was performed to evaluate the putative molecular weight (MW) and isoelectric point (pI), based on amino acid compositions of NnSOC1 and NnSOC1-like. Protcomp Version 9.0 software was used to predict the Sub-cellular location. Protein structures of NnSOC1 and NnSOC1-like were predicted by SMART online tools. Conserved motifs were indicated using online MEME program. The exon–intron structures of both genes were analyzed by Gene Structure Display Server (GSDS).

Similarity and the homology model of NnSOC1 and NnSOC1-like

Multiple alignment of the putative amino acid sequences of NnSOC1 and NnSOC1-like with other SOC1 from higher plants was performed by CLUSTAL W. Phylogenetic analysis was carried out by the Neighor-Joining method using the software of MEGA version 4 (Tamura *et al*. 2007). In order to further characterize their structures, Myocyte-specific enhancer factor 2B (MEF2B) from *Homo sapiens* (PDB No. 1n6j) was selected as the highest scoring template for homology model (Han *et al*. 2003). The homology models of NnSOC1 and NnSOC1-like were constructed, using phyre2 bath processing (Kelley *et al*. 2015).

The expression profile of *NnSOC1* and *NnSOC1-like*

Eight-weeks old lotus was planted in pool, and the total RNA was isolated from embryo, flower, root, stem and leaves in vegetative stage for investigating their expression in various tissues. Moreover, the leaves were collected during the four stages of flowering with the reported method (Yang *et al*. 2014). Four stages were: Stage 1, floral buds were generated underwater; Stage 2, floral buds emerged from water; Stage 3, floral buds developed into bloom; Stage 4, the flowers were pollinated and seeds were produced. Real-time PCR was performed for detection of *NnSOC1* and *NnSOC1-like*. Relative expression of *NnSOC1* and *NnSOC1-like* was calculated using *β-actin* as the reference gene (Livak and Schmittgen 2001).

Ectopic expression of *NnSOC1* and *NnSOC1-like* in Arabidopsis

For further exploring the roles of *NnSOC1* and *NnSOC1-like* in flower opening, both ORF amplified by PCR using gene-specific primers including attB-sites (Table 1), were inserted into pEarleyGate 101 (Earley *et al*. 2006) using the Gateway LR reaction (Invitrogen). The *Agrobacterium tumefaciens* GV3101 strain electroporated by recombinant plasmids and was transformed into wild type Arabidopsis. Transformed Arabidopsis seeds were selected by spraying a 0.002% (V/V) Basta solution, and T3 homozygote plants were used for further experiments. The flowering phenotype of transgenic Arabidopsis was examined.

Statistical analysis

Three independent experiments were performed to ensure

Table 1: Primers used in the present study

Primer name	Sequence (5'-3')	Experiments
NnSOC1 CF	ATGGTGAGGGGGAAGACCCAGATGA	Isolation *NnSOC1*
NnSOC1 CR	TCATACTGAGCCATCTCCAACCAAT	Isolation *NnSOC1*
NnSOC1-like CF	ATGGTGAGGGGGAAGACGCAGATGA	Isolation *NnSOC1-like*
NnSOC1-like CR	CTAATAGTCCTGTAATGGGTAGCGT	Isolation *NnSOC1-like*
NnSOC1 F	TTATTTAGGGAGCAGATTGCAA	Real-time PCR
NnSOC1 R	TTATTCAGGGAGCAGATTGAGG	Real-time PCR
NnSOC1-like F	GCTCTTTCAGGCCTCCCA	Real-time PCR
NnSOC1-like R	GCTCTTTCAGGCCTCCCT	Real-time PCR
β-actin F	TGATCGGAATGGAAGC	Real-time PCR
β-actin R	CAGCAATACCAGGGAAC	Real-time PCR
NnSOC1 EF	ggggacaagtttgtacaaaaaagcaggctATGGTGAGGGGGAAGACCCA	Ectopic expression of *NnSOC1*
NnSOC1 ER	ggggaccactttgtacaagaaagctgggtaTACTGAGCCATCTCCAACCAAT	Ectopic expression of *NnSOC1*
NnSOC1-like EF	ggggacaagtttgtacaaaaaagcaggctATGGTGAGGGGGAAGACGCA	Ectopic expression of *NnSOC1-like*
NnSOC1-like ER	ggggaccactttgtacaagaaagctgggtaATAGTCCTGTAATGGGTAGC	Ectopic expression of *NnSOC1-like*

reproducibility. Data were expressed as the mean ± SD from three independent biological replicates. Significance was calculated based on one-way analysis of variance (ANOVA) by SPSS 22.0 software. Different letters represent significant differences at $p < 0.05$.

Results

Identification and conserved domains of NnSOC1 and NnSOC1-like

The ORF of *NnSOC1* and *NnSOC1-like* was 675 and 654 bp respectively, consisting of the start codon ATG and stop codon TAG. *NnSOC1* mRNA encoded a putative protein of 224 amino acids, with predicted MW of 25.68 kD and pI of 9.37. The putative protein of NnSOC1-like was composed of 217 amino acids with MW of 25.41 kD and pI of 9.28. Both had the similar structure of the MADS-box family, consisting of highly conserved MADS-box, variable I-box, relative conserved K-box with the size of 93 amino acid residues and variable C-terminal domain (Fig. 1). The SOC1 motif (DVETELFIGRP) was highly conserved in the C-terminal of NnSOC1 and NnSOC1-like.

Exon-intron architectures of *NnSOC1* and *NnSOC1-like* genes

Exon-intron architectures of *NnSOC1* and *NnSOC1-like* genes were almost the same, consisting of 8 exons and 7 introns. Their ORF contained nucleotide sequences of partial exon 2, exon 3, exon 4, exon 5, exon 6, exon 7 and partial exon 8. The sizes of exon 3 (79 bp), exon 4 (62 bp), exon 5 (100 bp), exon 6 (42 bp) and exon 7 (42 bp) were the same in *NnSOC1* and *NnSOC1-like*, with little difference in exon 1 (382 bp for *NnSOC1*; 329 bp for *NnSOC1-like*), exon 2 (190 bp for *NnSOC1*; 252 bp for *NnSOC1-like*) and exon 8 (423 bp for *NnSOC1*; 402 bp for *NnSOC1-like*). The introns splicing of *NnSOC1* and *NnSOC1-like* genes strictly followed the GT-AG rule (Fig. 2).

The phylogenetic analysis of NnSOC1 and NnSOC1-like

A phylogenetic tree was constructed based on the amino acid sequences of high plants, indicating all the SOC1 could be grouped into dicot and monocot clades (Fig. 3). The SOC1 of *Triticum aestivum* (TaSOC1) and *Hordeum vulgare* (HvSOC1) from monocot was grouped into one branch, whereas SOC1 and SOC1-like from higher plants were clustered together. Although NnSOC1 and NnSOC1-like were grouped into dicot (Fig. 3), they had relatively farther relationship with other members in dicot. The special motif (motif 10) known as MEHPNQN was detected in both NnSOC1 and NnSOC1-like (Fig. 3).

The homology model of NnSOC1 and NnSOC1-like

About 41% of residues in NnSOC1 and 42% of residues in NnSOC1-like were modeled with 100% confidence. Both NnSOC1 and NnSOC1-like were consisted of two spatially distinct domains, the αββα structure of the MADS-box (AA 13-71), and the large helical K-box (Fig. 4). About 59% α-helix was widely detected in the amino acid sequence of NnSOC1, which were inlaid by 5% β-strand. Moreover, α-helix in NnSOC1-like was about 57% with 5% β-strand.

Expression profile of *NnSOC1* and *NnSOC1-like* in various tissues

The expression pattern of two transcripts was studied in reproductive and vegetative tissues by Real-time PCR. *NnSOC1* and *NnSOC1-like* mRNAs were widely distributed in root, leave, stem, flower and embryo, exhibiting the similar expression pattern (Fig. 5). The expression of *NnSOC1* mRNA in leave (8.46 fold), stem (7.41 fold), root (5.29 fold) and flower (4.11 fold) was significantly more than embryo. Additionally, the mRNA level of *NnSOC1-like* in leave (3.23 fold), stem (2.91 fold) and root (1.94 fold) was relatively higher than flower and embryo (Fig. 5). The mRNA level of *NnSOC1* was relatively higher than *NnSOC1-like*, suggesting *NnSOC1* was the major transcript in the tissues examined.

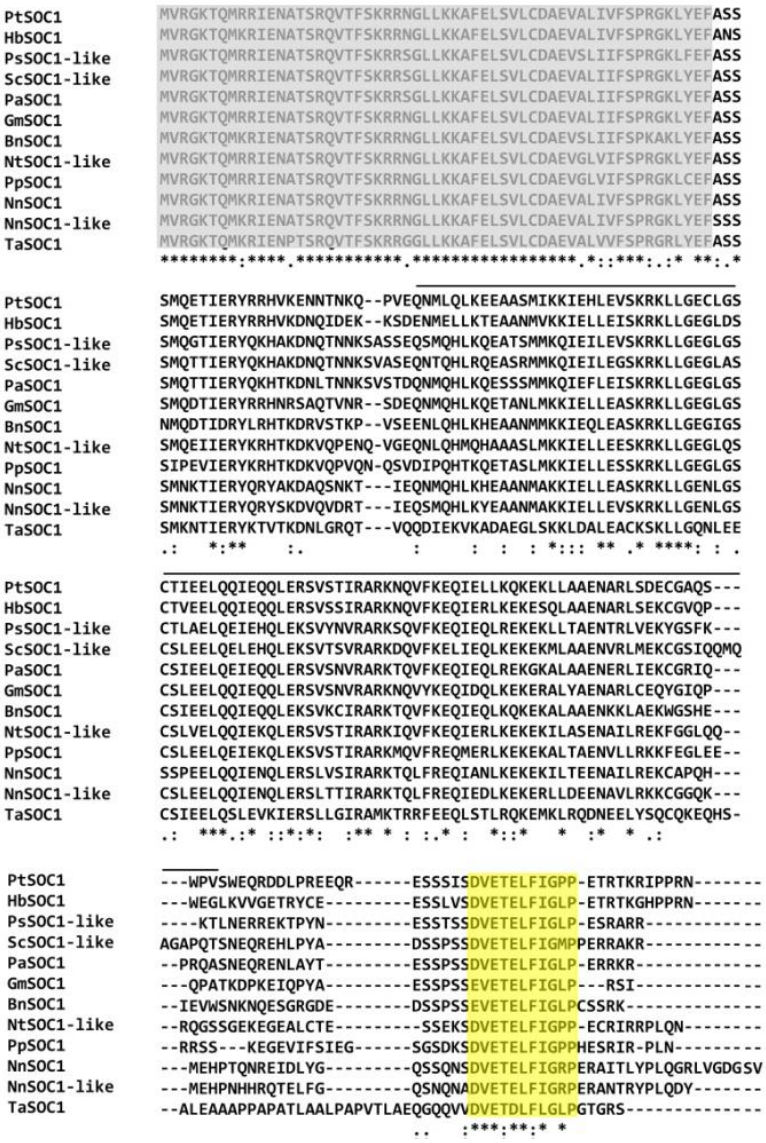

Fig. 1: Amino acid sequence alignment and characteristics of NnSOC1 and NnSOC1-like. The highly conserved MADS-box was shaded in grey, and relative conserved K-box was lined. The highly conserved SOC1 motif (DVETELFIGRP) was represented by yellow in the C-terminal of proteins

The expression pattern of *NnSOC1* and *NnSOC1-like* in flower opening

Real-time PCR indicated that *NnSOC1* and *NnSOC1-like* mRNAs were decreased in the process of flower opening (Fig. 6). Both transcripts showed the highest mRNA level in leaves when floral buds were generated underwater (stage 1). *NnSOC1* started to decrease during the stage of floral buds appearing from water, and it was relatively stable at the stage of developing into bloom (stage 3). Then *NnSOC1* mRNA was further decreased into the lowest mRNA level when the flowers were pollinated and plant yielded fruit (stage 4). Interestingly, *NnSOC1-like* exhibited the similar expression pattern with *NnSOC1*. *NnSOC1-like* mRNA at stage 1was almost the same to stage 2. Then *NnSOC1-like* was significantly down-regulated at stage 3, which was further declined to the lowest level at stage 4. Moreover, *NnSOC1* mRNA exhibited higher expression level than *NnSOC1-like.*

Functional analysis of *NnSOC1* and *NnSOC1-like* in transgenic arabidopsis

To examine the role of *NnSOC1* and *NnSOC1-like* in regulation of flowering, they were overexpressed in Arabidopsis by the CaMV 35 S promoter. The early flowering phenotype was detected in the transgenic lines of *35 S::NnSOC1* and *35 S::NnSOC1-like* (Fig. 7a). *NnSOC1*

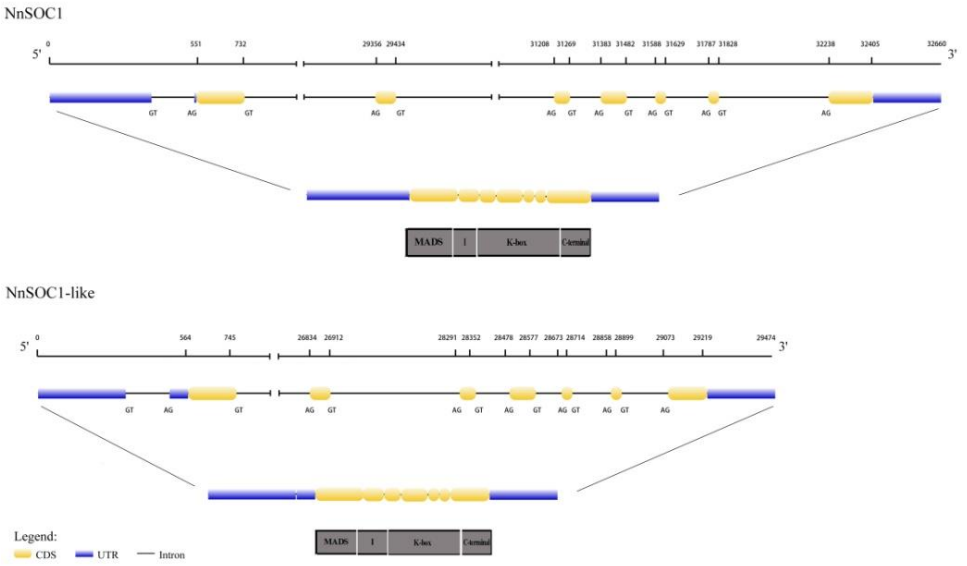

Fig. 2: Exons-intron architecture of *NnSOC1* and *NnSOC1-like* genes. The UTR, CDS and introns were labeled. The highly conserved MADS-box, variable I-box, relative conserved K-box and variable C-terminal domains were represented

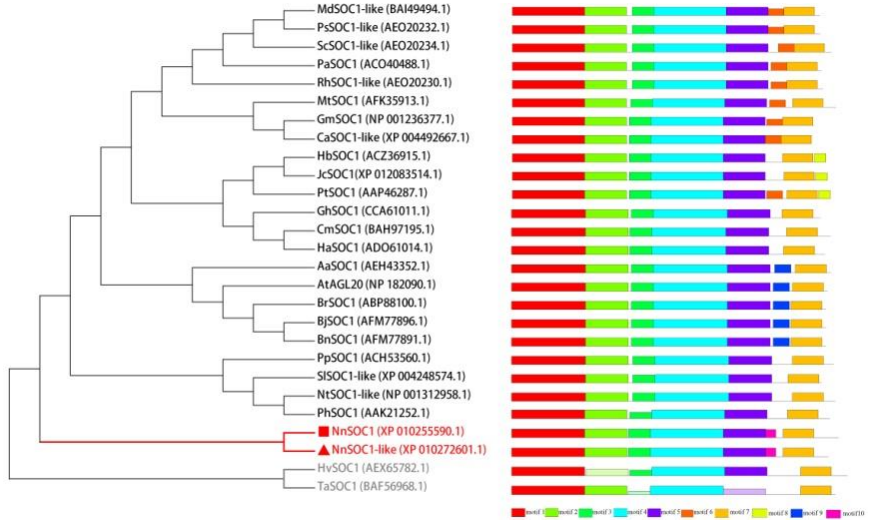

Fig. 3: The conserved motifs and phylogenetic relationship of SOC1 family. Phylogenetic analysis of SOC1 family was performed by MEGA4 software. The special motif (motif 10) known as MEHPNQN was detected in both NnSOC1 and NnSOC1-like

and *NnSOC1-like* advanced the flowering time of transgenic lines, with 21 days earlier than wild type (Fig. 7b). However, ectopic expression of *NnSOC1* and *NnSOC1-like* decreased the number of rosette leaves, and more fewer rosette leaves were detected in *35 S::NnSOC1-like* than *35 S::NnSOC1* (Fig. 7b).

Discussion

Considering as one of the most important aquatic crops widely planted in tropic and subtropic regions, the molecular mechanism of the floral transition in *N. nucifera* are still unveiled. As one of the most important members of MADS-box genes, *NnSOC1* and *NnSOC1-like* were identified and their amino acid sequences were characterized in this study. MADS-box domain and K-box domain were identified in NnSOC1 and NnSOC1-like (Fig. 1), which were considered as two characteristic domains of MADS-box family (Ruokolainen *et al*. 2011; Ding *et al*. 2013; Zheng *et al*. 2020). Therefore, NnSOC1 and NnSOC1-like were classified as the members of MADS-box family (Ding *et al*. 2013). Three amino acid residues (Arg^{24}, Glu^{34} and Gly^{112}) in NnSOC1 and NnSOC1-like were the same with SOC1 from Arabidopsis, and substitution of these residues in Arabidopsis could affect early flowering time (Lee *et al*. 2008).

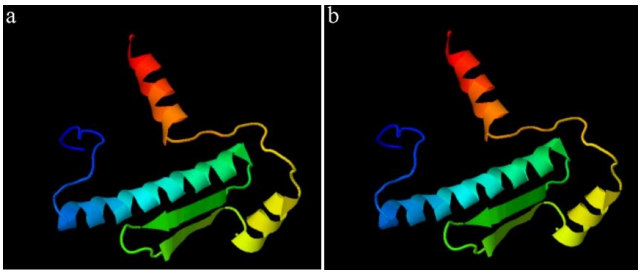

Fig. 4: The three-dimensional structures of MADS-box domains of the NnSOC1 (a) and NnSOC1-like (b)

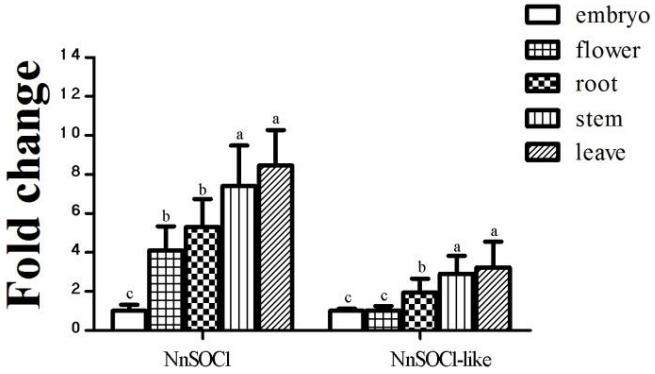

Fig. 5: The expression of *NnSOC1* and *NnSOC1-like* in various tissues. Real-time PCR was performed to test the mRNA level of *NnSOC1* and *NnSOC1-like* in embryo, leaves, flower, stem and root

Data were expressed as the mean ± SD from three independent biological replicates. Significance was calculated based on one-way ANOVA analysis by SPSS 22.0 software. Different letters represent significant differences at $p < 0.05$

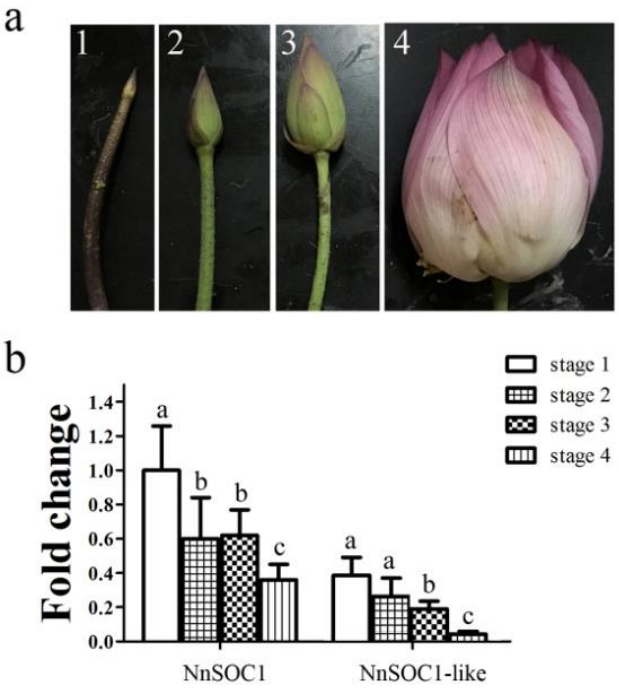

Fig. 6: Expression pattern of *NnSOC1* and *NnSOC1-like* during the four stages of flowering. The total RNAs were extracted from leave during the life cycle of flower (a). And *NnSOC1* and *NnSOC1-like* mRNAs were examined by Real-time PCR (b)

The exons-intron architecture of *NnSOC1* and *NnSOC1-like* genes was almost the same, following the GT-AG rule (Fig. 2). Moreover, SOC1 and SOC1-like in high plants were analyzed by Neighbor-Joining method, which

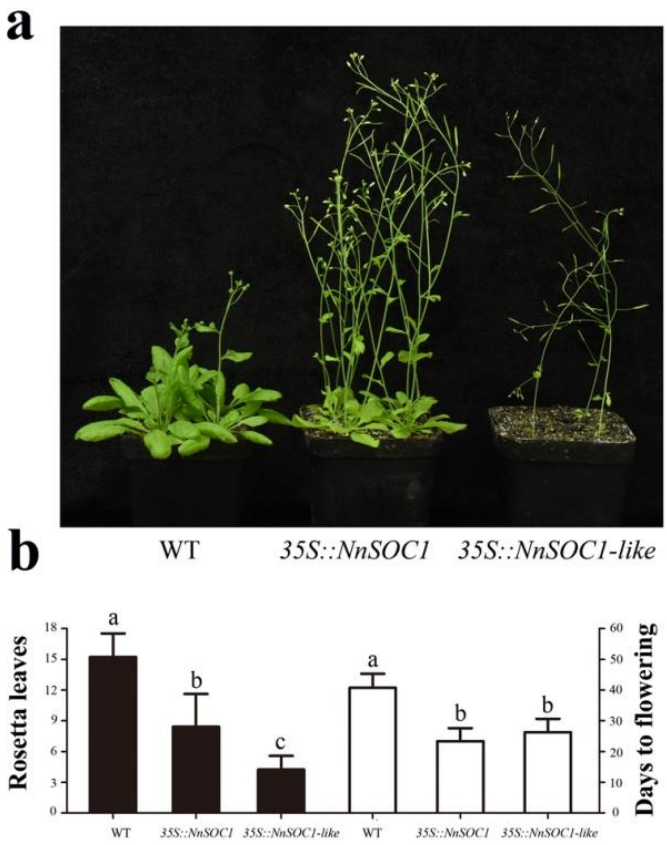

Fig. 7: Ectopic expression of *NnSOC1* and *NnSOC1-like*. The phenotype of *NnSOC1* and *NnSOC1-like* transgenic lines (a). Days to flowering and the counts of rosette leaves for *35S::NnSOC1* and *35S::NnSOC1-like* transgenic lines (b)
Data were calculated from three independent experiments, using different letters represented significant difference ($p<0.05$)

indicated that NnSOC1 and NnSOC1-like had more homology with SOC1 and SOC1-like of dicot (Fig. 3). It suggested that SOC1 of higher plants might have come from the same ancestor before evolving independently in dicots and monocots (Wei *et al*. 2016).

The *SOC1* genes were expressed in distinct tissues, showing various roles in dicot plants (Borner *et al*. 2000). Our results indicated both *NnSOC1* and *NnSOC1-like* were widely expressed in reproductive and vegetative tissues (Fig. 5), while more expression of both genes was found in leave than flower. This was consistent with the study in Arabidopsis, *O. sativa*, *H. vulgare* and *T. aestivum* (Komiya *et al*. 2009; Papaefthimiou *et al*. 2012). The SOC1 was mainly considered as the integrator of multiple flower signals. *NnSOC1* and *NnSOC1-like* mRNAs were decreased in the process of flower opening. The highest expression was detected in the leaves of floral initiation, and the least mRNA level was found at fruit set (Fig. 6). The down-regulation of *SOC1* during flower opening was also reported in Mango (Wei *et al*. 2016).

Overexpression of *NnSOC1* and *NnSOC1-like* significantly promoted early flowering (Fig. 7). This suggested that the role of flowering activator for SOC1 was almost conserved in the high plants (Ding *et al*. 2013; Wang *et al*. 2015). Moreover, the abnormal leaves were observed in *35S::NnSOC1* and *35S::NnSOC1-like* transgenic Arabidopsis, which were also detected in transgenic Arabidopsis by overexpression of *SOC1* genes in mango (Wei *et al*. 2016), *Phyllostachys violascens* (Liu *et al*. 2016) and *Davidia involucrate* (Li *et al*. 2020). Although how MADS-box genes regulate floral initiation remains to be elucidated, nonetheless the results provide valuable information for unveil the molecular mechanism of *NnSOC1* and *NnSOC1-like* for regulation the initiation of flower opening.

Conclusion

Results suggest that *NnSOC1* and *NnSOC1-like* were involved in initiation of flowering in *N. nucifera*. This provides a foundation for breed of this species for horticultural purpose to regulate flowering time in aquatic plants.

Acknowledgments

This work was supported by National Nature Science Foundation of China (no. 31601366), Science and Technology Planning Project of Henan Province, China (no.

192102110120), and Natural Science Foundation of Henan Province (202300410114).

Author Contributions

Chen Dong designed the experiments and wrote the manucript. Fei Du and Ye Li performed Real-time PCR. Ningning Yang isolated SOC1 gene. Jiaqi Mao expressed SOC1 and SOC1-like in Arabidopsis. Zhongli Hu revised the manuscript. All authors have read and approved the final manuscript.

Conflict of Interest

There is no conflict of interest among the authors and the institutions where the research has been conducted

Data Availability Declaration

All data related to this article are in the custody of corresponding author and will be available on request

Ethics Approval

Not applicable

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