Genome-wide Identification, Classification and Expression of Flavonol Synthase from *Nelumbo nucifera* in Defense against Various Stresses

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Abstract

Four full-length cDNA of flavonol synthase (NnFLS) named as NnFLS1, NnFLS2, NnFLS3 and NnFLS4 was identified in *Nelumbo nucifera*, encoding proteins with sizes between 331 and 362 amino acids. His–x–Asp–x–His (HxDxH) domain was found in putative NnFLS1, NnFLS2 and NnFLS3, while the first His was replaced by Arg in NnFLS4. Arg–x–Ser (RxS) domain was highly conserved in all NnFLSs. Four NnFLSs genes comprised of 3 exons and 2 introns, and the different exon-intron junctions of NnFLS4 was investigated. Additionally, phylogenetic analysis indicated NnFLS2 was grouped together with dicotyledons, and other three NnFLSs were classed into monocotyledons. The second structures and homology models indicated that four NnFLSs were highly conserved. Moreover, the highest levels of four NnFLSs mRNAs were detected in embryo with the lowest levels in the stems. Furthermore, the levels of four NnFLSs mRNA were significantly elevated in response to various stresses including chilling, ultraviolet-B radiation (UV-B) and short-time mechanical wounding. These results are likely to serve as fundamental research for future evolutionary and functional characterization studies of the *NnFLS* genes in lotus. © 2019 Friends Science Publishers

Keywords: Flavonol synthase; Flower opening; Gene expression; *Nelumbo nucifera*

Introduction

Flavonoids are well-known types of polyphenols in high plants, exhibiting antioxidant, anti-proliferative and anti-inflammatory functions in defending against cancer and cardiovascular disease (Kaur et al., 2008). Considering as the most widely distributed flavonoids, Flavonols play imperative roles in plant physiology, growth and development including protection from UV damage or change of flower color (Stracke et al., 2010a). Additionally, flavonols were involved in the ethylene-signaling pathway (Lewis et al., 2011).

Three common types of flavonoids including myricetin, quercetin and kaempferol are generally converted from the dihydroquercetin or dihydrokaempferol by flavonol synthase (FLS), which is usually classified as bifunctional dioxygenase and defined as having hydroxylation and desaturation activity (Akita et al., 2018). This process requires coordinanation of gene expression in the flavonoid pathway including chalcone synthase (CHS), flavanone 3β-hydroxylase (F3H), anthocyanin synthase (ANS) and FLS. All of these genes belong to the second largest family of higher plants known as 2-oxoglutarate-dependent dioxygenase (2-ODD), which catalyse the oxidation reaction using ferrous iron or 2-oxoglutarate as cofactor (Akita et al., 2018). *FLS* cDNAs had been isolated from high plants including *Zea mays*, *Oryza sativa*, *Arabidopsis thaliana*, *Petroselinum crispum*, lisianthus, strawberry and soybean (Takahashi et al., 2007). Studies have indicated that *FLS* was encoded by a muticopy gene family in plants, and the different copies were expressed in an organ-specific pattern (Preuß et al., 2009; Ferreya et al., 2010; Kim et al., 2010). Overexpression of *ANS* accumulated a mixture of flavonoids in transgenic rice (Reddy et al., 2007). Healthy phytochemicals treament also led to the production of novel flavonoids in tomato fruit (Schijlen et al., 2006).

As one of the oldest plants surviving from the last ice age, sacred lotus (*Nelumbo nucifera* Gaertn) is considered as a traditional ornamental and medicinal plant in China. Its seeds have exceptional longevity, remaining viable as long as thousands of years. Each parts of sacred lotus could be used in herbal medicines for curation of cancer, depression, diarrhea, heart disease as well as insomnia. It is also well-known as healthy food rich in proteins, lipids, dietary fiber, minerals and flavonoids. Moreover, petals and stamens of lotus rich in flavonoids and alkaloids are widely used in Chinese herbal medicine.

Although flavonoids in lotus were considered as the key composition in Chinese herbal medicine, the biosynthetic synthase of flavonoids is still unveiled in *N. nucifera*. 

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We hypothesize that comprehensive analysis of FLS in *N. nucifera* (NnFLS) is thought to benefit for illustrating the mechanism of biosynthetic pathway of flavonoids in *N. nucifera*. Here, we identified four NnFLSs genes in lotus genome. A systematic analysis of NnFLS genes was performed, including their organization of intron and exon, evolutionary relationship, and expression profile in various tissues as well as stresses. The findings of this study will serve as foundation for functional characterization studies of NnFLS in lotus.

**Materials and Methods**

**Plant Material**

Seed of *N. nucifera* “Taikonglian-36” was collected from Lotus Engineering Research Center of Hubei Province, Wuhan University, and planted in Henan University of Technology, China. Rhizome is the storage organ of *N. nucifera*, which is cultured in soil of experimental ponds for asexual propagation. All the materials were collected and frozen in liquid nitrogen immediately.

**Identification and Bioinformatics Analysis of NnFLS**

To scan NnFLS in the genome of *N. nucifera*, BLASTP searches were conducted using proteins sequences of FLS from *Arabidopsis thaliana* against the NCBI database (E value < 1e−5). Another search for NnFLS was performed using the key word “flavonol synthase” in the Sacred lotus (*N. nucifera*) genome database (http://lotus-db.wbrcas.cn/). The putative NnFLS was further searched in the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/bwrcpd/bwrcpsb.cgi) to confirm the presence of conserved domains of FLS. The ProtParam program (http://web.expasy.org/protparam/) was performed to evaluate the putative molecular weight (MW), isoelectric point (pI) and grand average of hydropathicity (GRAVY) for NnFLS. Procomp Version 9.0 software was used to predict the Sub-cellular location of NnFLS.

**Analysis of Exon–intron Structures and Conserved Motifs**

The exon–intron structures of NnFLS were analyzed by Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn/index.php) (Hu et al., 2014). Protein structures of NnFLS were predicted by SMART online tools (http://smart.embl-heidelberg.de/). Conserved motifs were indicated using online MEME program (http://meme.nbcr.net/meme/cgi-bin/meme.cgi).

**Phylogenetic Analysis**

The putative protein sequences of NnFLS were compared with other FLS of high plants by Clustal W software. Additionally, the phylogenetic relationship of FLS family in high plants was drawn using the neighbour-joining (NJ) method by the MEGA software version 4 (Tamura et al., 2007).

**Secondary Structures and Three Dimensional Models of NnFLS**

The secondary structures of NnFLS were predicted using the PSIPred program (Expasy tools). ANS of *A. thaliana* (PDB.1gp6_A) was selected as the homology template from RSCB protein data bank (Wilmouth et al., 2002). About fifty homology models of NnFLS were produced by the MODELLER software with the default parameters (Sanchez and Sali, 1997), which were further assessed by PROCHECK 3.5 for testing the qualities of the models (Laskowski et al., 1993). The best model was selected and shown by Swiss-pdbviewer 4.1.0.

**Differential Expression of NnFLS in Tissues**

For examining the level of *NnFLS* mRNAs in various tissues, experimental materials such as young leaves, stem, roots and embryo were harvested. β-actin (GenBank accession no. EU131153) was selected as a reference gene. The gene-specific primers for *NnFLS* (NnFLS F and NnFLS R) and β-actin (β-actin F and β-actin R) were synthesized using Primer Premier 5 software (Premier) (Table S1). DNA binding dye SYBR GreenI (TOYOBO) was used for detection of PCR products in Real-time PCR (Dong et al., 2017, 2018). The PCR program included one cycle at 94ºC for 30 s and then 30 cycles of 94ºC for 15 s and 60ºC for 15 s. According to the method published (Livak and Schmittgen, 2001), the fold change of NnFLSs was calculated using β-actin as a reference gene by 2^ΔΔCT method.

**Expression Pattern of NnFLS in Response to Stresses**

To unveil the roles of *NnFLS* in the face of environmental stresses, the levels of *NnFLS* mRNAs were detected by Real-time PCR. The seedings in a closed chamber were irradiated under 1000 μJ/m² ultraviolet-B radiation (UV-B) and separately collected at 2 h post treatment (hpt), 4 hpt and 6 hpt and the plants placed in a dark closed chamber were used as control. For chilling stress, the seedings were transferred to a cold (4ºC) growth chamber for 2, 4 and 6 h, respectively and the control plants continued to grow at room temperature (25ºC) in normal condition. In term of short-time mechanical wounding treatment, the leaves of *N. nucifera* were treated by pressure-stress with a needle puncher when the leaves had unfolded for one week. The leaves were separately collected at 2, 4 and 6 hwp, using the intact leaves as the control. The total RNAs of materials were isolated, and the levels of *NnFLS* mRNA were examined by Real-time PCR as method described above.
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Statistical Analysis

Three independent biological replicates were performed to calculate the fold change of NnFLS by Real-time PCR. Data were expressed as the mean ± SD from three independent biological replicates. Significance was determined based on one-way analysis of variance (ANOVA) and the least significant difference (LSD) was performed to find out differences between groups (p<0.05). The experiments were repeated twice each time to get reproducible data.

Results

Identification and Conserved Domains of NnFLS

Four NnFLSs named as NnFLS1 (XP_010256287.1), NnFLS2 (XP_010272845.1), NnFLS3 (XP_010274342.1) and NnFLS4 (XP_019055362.1) were identified in our study, encoding the putative proteins between 331 and 362 amino acids (Table 1). The MW of different NnFLSs was between 37.70 KD and 41.03 KD, with pI between 5.18 and 5.85. Moreover, all the four NnFLSs were predicted to be located in cytoplasm (Table 1). Alignment of conserved domains and functional analysis illustrated all NnFLSs belonged to the 2OG-FeII_Oxy superfamily. Three highly conserved amino acids (His, Asp and His) were detected in NnFLS1, NnFLS2 and NnFLS3, which were involved in binding with a ferrous iron II while the first His was replaced by Arg in NnFLS4 (Fig. 1). Moreover, two amino acids as Arg and Ser were the putative 2-oxoglutarate binding sites and highly conserved in four NnFLSs. The conserved region of 2-ODD superfamily was also detected in NnFLSs (Fig. 1).

Exon–intron Structures of NnFLS

According published DNA sequence of NnFLS genes in NCBI, the detailed comparisons suggested that all NnFLSs had 3 exons and 2 introns, and the two introns showed different sizes (Fig. 2). The lengths of exon 1 and exon 3 were various, and all the exon 2 of four NnFLSs was 328 bp (Table 1). The exon-intron junctions of NnFLS genes were consistent with the GT-AG rule, except GC-AG splicing way in intron 1 of NnFLS4 (Fig. 2).

Phylogenetic Analysis of NnFLSs

A phylogenetic tree was constructed to investigate the evolution relationship of 2-ODD superfamily. The topology of the phylogenetic tree was an asymmetric clover shape, including FLS, F3H and ANS families. Compared with F3H, FLS family had closer relationship with ANS. Among the 2-ODD superfamily, NnFLSs showed higher identity with other FLS from high plants, and had lower similarity with F3H or ANS (Fig. 3). In subgroup of FLS, NnFLS2 was belonged to dicotyledons. However, other three NnFLSs were grouped together with monocotyledon (Fig. 3).

Expression of NnFLS in Various Tissues

NnFLS mRNAs were examined in different tissues of N. nucifera including young leaves, roots, stems and embryo.

![Fig. 1: Amino acid sequence alignment and characteristics of NnFLS. The 2-ODD superfamily conserved region was shadowed by grey. The conserved residues binding with a ferrous iron II were represented by yellow. The putative 2-oxoglutarate binding motif consisting of Arg and Ser was described by red. Several amino acids (Phe, Phe, Lys, Phe and Ser) involved in binding to substrate were represented by green.](image)

![Fig. 2: Exons-intron architecture of NnFLS genes, CDS, intron and UTR were labeled. The exon-intron junctions of NnFLS genes were examined](image)
The expression pattern of NnFLS2, NnFLS3 and NnFLS4 was similar. The highest level of NnFLS2, NnFLS3 and NnFLS4 mRNA was detected in embryo, with the moderate level of NnFLSs mRNA in young leaves. Additionally, low expression of NnFLS2, NnFLS3 and NnFLS4 mRNA was detected in roots while it was still more than stems as control (Fig. 5). However, different expression profile of NnFLS1 was found. The most expression of NnFLS1 was indicated in embryo and higher level of NnFLS1 mRNA was found in roots than young leaves using stems as control (Fig. 5).

**NnFLS Expression in Response to Various Stresses**

To find out the role of NnFLS in various stresses, Real-time PCR was used to study the expression pattern of NnFLS mRNA. UV-B treatment increased the expression of four NnFLSs mRNA at 2 hpt using Tween 20 treated seeding as control (Fig. 6). The level continued to increase at 4 hpt and reached the highest level at 6 hpt. Additionally, mRNAs level of NnFLSs was also induced by chilling treatment (4°C) using the seeding in room temperature as control (Fig. 7). Their expression started to increase at 2 hpt except NnFLS3 and significantly increased at 4 hpt with the highest expression at 6 hpt. Moreover, short-time mechanical wounding augmented the expression of NnFLS mRNA. Real-time PCR indicated that NnFLS mRNA reached the highest level as soon as 2 hpt using normal seeding as control, but declined at 4 hpt and 6 hpt (Fig. 8).

**Discussion**

Since FLS roles as the major enzyme involved in the flavonoid pathway, and several FLS genes in high plants have been studied at the chemical, genetical or enzymatic levels (Nguyen et al., 2016). However, few reports are available on the identification of FLS genes in aquatic plant. The flavonoids are known as the most imperative medicinal component in N. nucifera, whereas biosynthetic pathway of flavonoids is still unclear. In this study, four NnFLSs were identified and studied in lotus (Table 1), which possessed all the conservative domains and active sites of FLS family, including ferrous iron Ⅱbinding residues and putative 2-oxoglutarate binding residues (Fig. 1). All NnFLS genes contained 3 exons and 2 introns. The exon-intron junctions of NnFLS genes were consistent with the GT-AG rule, except intron 1 of NnFLS4 (Fig. 2). Moreover, phylogenetic tree analysis indicated NnFLS was located in FLS group (Fig. 3). Interestingly, only NnFLS2 was grouped into dicotyledons, and other three NnFLSs were classed into monocotyledon. Considering N. nucifera was the species between dicotyledons and monocotyledon in evolutionary relationship, NnFLS1, NnFLS3 and NnFLS4 were probably evolved from NnFLS2.

**Table 1:** identified NnFLSs and their sequence characteristics

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**Fig. 3:** Phylogenetic analysis of NnFLS and other 2-ODD superfamily including ANS as well as F3H by neighbour-joining method using MEGA software version 4

**Fig. 4:** The homology model of NnFLS1 (a), NnFLS2 (b), NnFLS3 (c) and NnFLS4 (d)

The expression pattern of NnFLS2, NnFLS3 and NnFLS4 was similar. The highest level of NnFLS2, NnFLS3 and NnFLS4 mRNA was detected in embryo, with the moderate level of NnFLSs mRNA in young leaves. Additionally, low expression of NnFLS2, NnFLS3 and NnFLS4 mRNA was detected in roots while it was still more than stems as control (Fig. 5). However, different expression profile of NnFLS1 was found. The most expression of NnFLS1 was indicated in embryo and higher level of NnFLS1 mRNA was found in roots than young leaves using stems as control (Fig. 5).

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NnFLSs contained several β-strands, forming a hydrophobic active center known as jellyroll fold. Additionally, α-helix was detected in both N-terminus and C-terminus of NnFLS (Fig. 4). The absence of the first α-helix in AtFLS1 could cause loss of activity (Li *et al.*, 2012) and the mutation or deletion of this region in other AtFLSs would cause the inactivation of expression products (Owens *et al.*, 2008). Both FLS and ANS belonged to the 2-ODD superfamilies with similar sequences and functions. Previous study illustrated FLS shared the same substrates with ANS *in vitro*, which produced the same products.

Flavonols are normally rich in leaves, flower, branches and heartwood of *A. confusa* (Hsieh and Chang, 2010). AtFLS1 could be stimulated by transcription factor such as MYB11, MYB12 and MYB111 in *A. thaliana*,

Fig. 5: The expression patterns of NnFLS mRNAs in different tissues. Fold change of NnFLS mRNAs in different tissues. Total RNAs from roots, young leaves, embryo, and stems were isolated. NnFLS mRNAs were detected using β-actin as a reference gene by Real-time PCR.

Fig. 6: The mRNA level of NnFLS in response to UV-B treatment. The seedings in a closed chamber were irradiated under 1000 µJ/m² UV-B, and the plants placed in a dark closed chamber were used as control. Relative quantities of NnFLSs mRNA at various hours post treatment (hpt) were calculated. Different letters were used to represent significant difference at p<0.05.

Fig. 7: The mRNA level of NnFLS during chilling stress. For chilling stress, the seedings were transferred to a cold (4°C) growth chamber for 2 h, 4 h and 6 h respectively, and the control plants continued to grow at room temperature (25°C) in normal condition. The relative expression of NnFLSs was calculated by Real-time PCR.

Fig. 8: The mRNA level of NnFLS in term of short-time mechanical wounding. The leaves of *N. nucifera* were treated by pressure-stress with a needle puncher when the leaves had unfolded for one week, using the intact leaves as the control. The relative expression of NnFLSs was examined by Real-time PCR.
which result in various spatial accumulation of specific flavonol derivatives in leaves, stems and roots (Stracke et al., 2010b). A similar expression profile of various NnFLSs was investigated in this study. Significant change of NnFLSs mRNA was detected with the highest level in embryo, while lowest expression of NnFLSs was found in stems (Fig. 5).

Plants have complex mechanisms to cope with environmental factors including UV irradiation, chilling, wounding, nutrient depletion as well as plant hormones, which advanced the accumulation of flavonols in plants (Ferreira et al., 2010; Lewis et al., 2011). It had been reported that FLS in poplar was not stimulated by wounding treatment (Mellway et al., 2009). We therefor concluded that FLS exhibited various expression patterns in different plants. Our study indicated NnFLS mRNAs were stimulated by mechanical wounding, chilling and UV-B treatment (Fig. 6, 7 and 8). All of these results proved that NnFLS played imperative role in defense against environmental stresses.

Conclusion

Although four NnFLSs were induced by environmental stresses including wounding, chilling and UV-B treatment, transgenic plants should be developed for understanding the mechanism of NnFLS in aquatic plant.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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