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Full Length Article

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_n-His ($HxDx_nH$) 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 monocotyledon. 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.

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; Abdullah *et al.*, 2018). Additionally, flavonols were involved in the ethylene-signaling pathway (Lewis *et al.*, 2011).

Three common types of flavonols including myricetin, quercetin and kaempferol are generally converted from the dihydromyricetin, dihydroquercetin or dihydrokaempferol by flavonol synthase (FLS), which is usually classified as bifunctional dioxgenase and defined as having hydroxylation and desaturation activity (Akita *et al.*, 2018). This process requires coorination of gene expression in the flavonoid pathway including chalcone synthase (CHS), flavanone 3β-hydroxylase (F3H), anthocyanidin 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 catalyze 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; Ferreyra *et al.*, 2010; Kim *et al.*, 2010). Overexpression of *ANS* accumulated a mixture of flavonoids in transgenic rice (Reddy *et al.*, 2007). Healthy phytochemicals treatment 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 curing 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

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biosynthetic synthase of flavonoids is still unveiled in *N. nucifera*. 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 < $1e^{-6}$). Another search for NnFLS was performed using the key word "flavonol synthase" in the Sacred lotus (*N. nucifera*) genome database (http://lotus-db.wbgcas.cn/). The putative NnFLS was further searched in the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.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. Protcomp 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 Clustral W software. Additionally, the phylogenetic relationship of FLS family in high plants was drawn using the neighbor-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 $2^{-\Delta\Delta Ct}$ 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/m^2 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 seedlings 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 hpw, 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.

Statistical Analysis

Three independent biological replicates were performed to caculate 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 cytoplasme (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

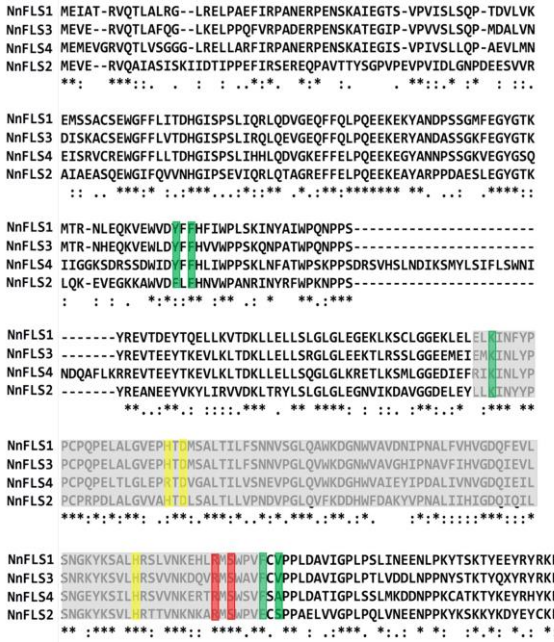

Fig. 1: Amino acid sequence alignment and characteristics of NnFLS. The 2-ODD superfamily conserved region was shadowed by grey. The conserved residues binding with a ferrous iron II were represented by yellow. The putative 2-oxoglutarate binding motif consisting of Arg and Ser was described by red.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

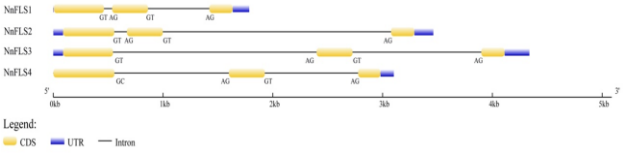

Fig. 2: Exons-intron architecture of *NnFLS* genes, CDS, intron and UTR were labeled. The exon-intron junctions of *NnFLS* genes were examined

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).

Second Structures and Homology Model of NnFLSs

Second structures indicated that both NnFLS1 and NnFLS3 had 11 α-helix and 9 β-strands. NnFLS2 composed of 10 α-helix and 8 β-strands, with 10 α-helix and 10 β-strands detected in NnFLS4. The putative three-dimensional structures of NnFLSs were constructed using crystal structure of *A. thaliana* ANS (PDB. 1gp6_A) as homology template, which shared high degree of homology with NnFLSs. Four NnFLSs had the similar three-dimensional structures, consisting of α-helix and β-strands in N-terminal and C-terminal of proteins (Fig. 4).

Table 1: identified NnFLSs and their sequence characteristics

Gene name	NCBI accession no	Exon1	Intron1	Exon2	Intron2	Exon3	ORF	Amino acids	MW (KD)	pI	GRAVY	location
NnFLS1	XP_010256287.1	462	73	328	558	364	999	332	37.82	5.18	-0.344	Cytoplasmic
NnFLS2	XP_010272845.1	556	114	328	2078	386	1008	335	38.08	5.56	-0.410	Cytoplasmic
NnFLS3	XP_010274342.1	544	1854	328	1172	439	996	331	37.70	5.41	-0.456	Cytoplasmic
NnFLS4	XP_019055362.1	557	1042	328	849	326	1089	362	41.03	5.85	-0.340	Cytoplasmic

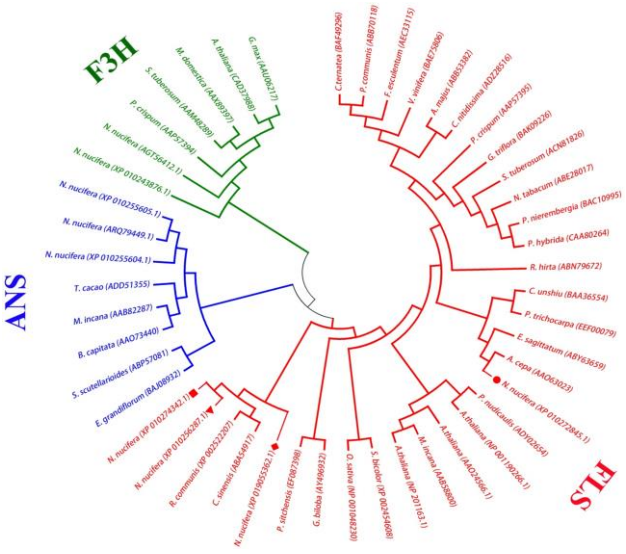

Fig. 3: Phylogenetic analysis of NnFLS and other 2-ODD superfamily including ANS as well as F3H by neighbour-joining method using MEGA software version 4

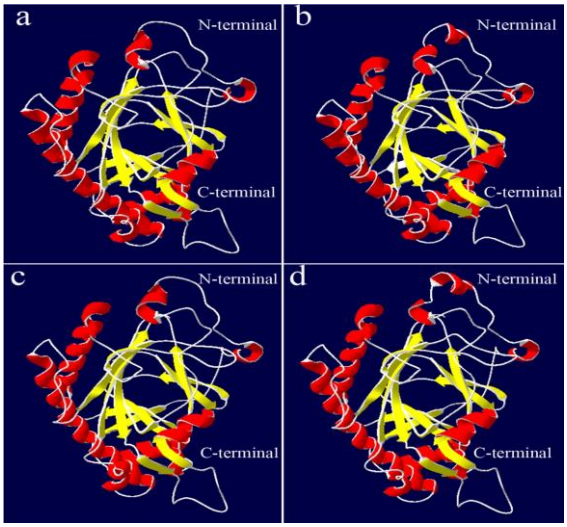

Fig. 4: The homology model of NnFLS1 (a), NnFLS2 (b), NnFLS3 (c) and NnFLS4 (d)

Expression of *NnFLS* in Various Tissues

NnFLS mRNAs were examined in different tissues of *N. nucifera* including young leaves, roots, stems and embryo. The expression pattern of *NnFLS2*, *NnFLS3* and *NnFLS4* was simillar. 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 (4ºC) 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). Interesting, only NnFLS2 was grouped into

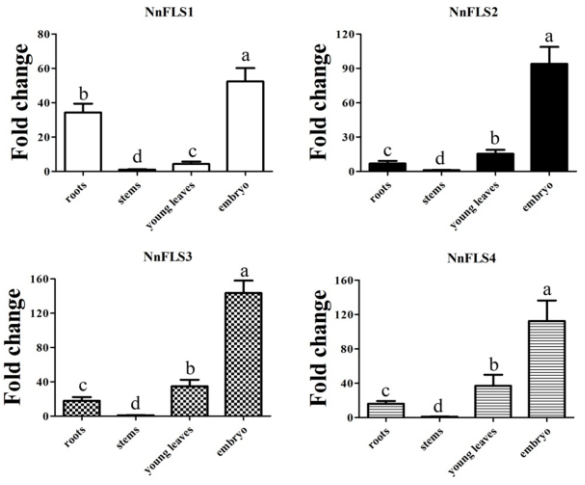

Fig. 5: The expression patterns of *NnFLS* mRNAs in different tissues. Fold change of *NnFLS* mRNAs in different tissues. Total RNAs from roots, young leaves, embryo, and stems were isolated. *NnFLS* mRNAs were detected using *β-actin* as a reference gene by Real-time PCR

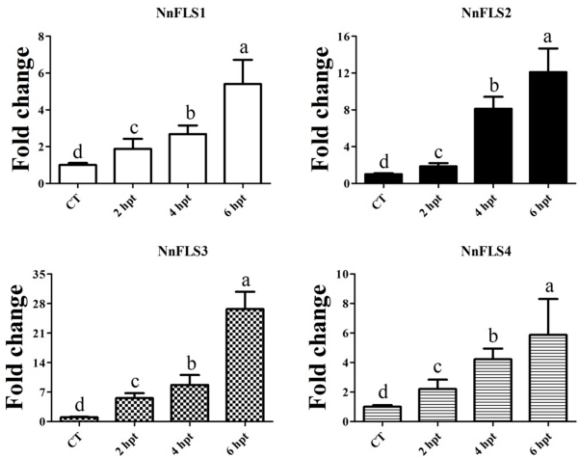

Fig. 6: The mRNA level of *NnFLS* in response to UV-B treament. The seedings in a closed chamber were irradiated under 1000 uJ/m^2 UV-B, and the plants placed in a dark closed chamber were used as control. Relative quantities of *NnFLSs* mRNA at various hours post treament (hpt) were caculated. Different letters were used to represent significant difference at $p<0.05$

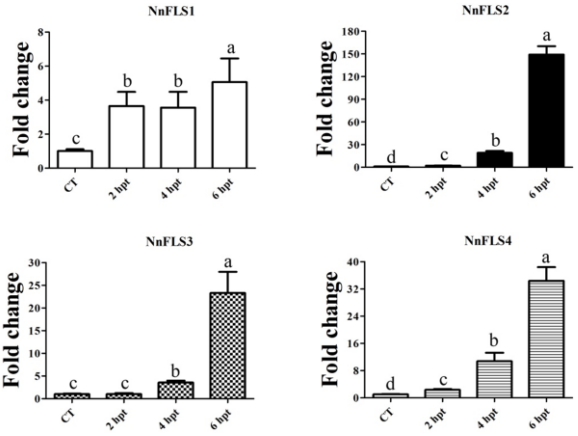

Fig. 7: The mRNA level of *NnFLS* during chilling stress. For chilling stress, the seedings were transferred to a cold (4ºC) growth chamber for 2 h, 4 h and 6 h respectively, and the control plants continued to grow at room temperature (25ºC) in normal condition. The relative expression of NnFLSs was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

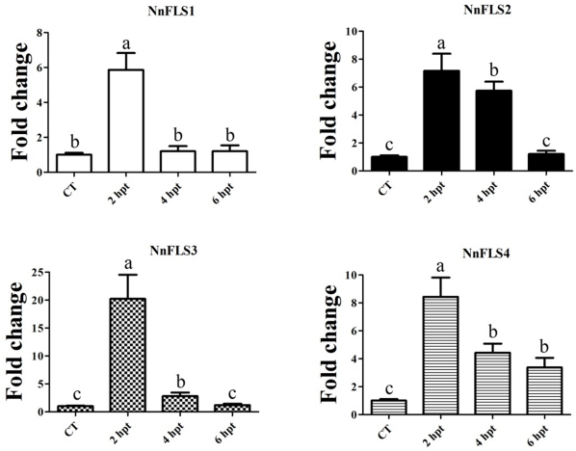

Fig. 8: The mRNA level of *NnFLS* in term of short-time mechanical wounding. The leaves of *N. nucifera* were treated by pressure-stress with a needle puncher when the leaves had unfolded for one week, using the intact leaves as the control. The relative expression of NnFLSs was examined by Real-time PCR

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.

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 stimualted by transcription factor such as MYB11, MYB12 and MYB111 in *A. thaliana*, 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 (Ferreyra *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 defferent plants. Our study indicated *NnFLS* mRNAs were stimulated by mechanical wounding, chilling and UV-B treament (Fig. 6, 7 and 8). All of these results proved that NnFLS played imperative role in defense against enviromental stresses.

Conclusion

Although four *NnFLSs* were induced by environmental stresses including wounding, chilling and UV-B treament, 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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