



Cloning and characterization of the *UBC* gene from lotus (*Nelumbo nucifera*)

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ABSTRACT. Protein ubiquitination is extensively involved in the regulation of a considerable number of physiological processes in plant cells. E2 (ubiquitin-conjugating enzyme, UBC), one of the essential enzymes of eukaryotic ubiquitination, catalyzes protein ubiquitination together with E1 and E3. In this study, we cloned four full-length cDNA

NnUBCs of *Nelumbo nucifera*. With the same coding sequence length of 459 bp and coding 153 amino acids, these four genes are highly homologous with the *AtUBC1* and *AtUBC2* of *Arabidopsis thaliana*. Quantitative fluorescence polymerase chain reaction showed that these four genes exhibited different expression patterns in different tissues of *N. nucifera*. Overall, the expression of *NnUBC3* was the highest in all plant tissues. Tests of different stress treatments showed that *NnUBC3* plays an important role in response to heat, salt, and drought stresses in *N. nucifera*. Moreover, transgenic *Arabidopsis* plants (*Atubc1-1Atubc2-1* mutant) expressing *NnUBC3* presented a wild-type phenotype, indicating that *NnUBC3* performs the same function as *AtUBC1* and *AtUBC2*.

Key words: *Nelumbo nucifera*; *UBC* gene; cDNA; Expression; Transformation experiment

INTRODUCTION

Protein ubiquitination is extensively involved in the regulation of a considerable number of physiological processes in plant cells. These include hormonal signal transduction, flower development, photoperiodism, photomorphogenesis, aging, immunity, and plant self-incompatibility (Smalle and Vierstra, 2004; Liu et al., 2007; Bartel and Citovsky, 2012; Cheng and Li, 2012; Maier et al., 2013). These processes are mainly catalyzed by a cascade of enzymes, including E1 (ubiquitin-activating enzyme, UBA), E2 (ubiquitin-conjugating enzyme, UBC), and E3 (ubiquitin ligase, UBL) (Scheffner et al., 1995). UBC has the same active site as UBA and UBL; thus, UBC plays the role of a bridge between UBA and UBL in the protein ubiquitination reaction (Pickart, 2001). UBC is a large family of proteins with several members. UBC carries ubiquitin, and identifies the target protein in the ubiquitin chain. Different UBCs mediate different ubiquitination roles. Current studies on UBC have mainly focused on model organisms. For example, 11 *UBC* genes have been found in the yeast genome (Bachmair et al., 2001); at least 37 *UBC* genes have been reported in *Arabidopsis thaliana* that are divided into 12 subgroups (Kraft et al., 2005). In addition, there are 48 putative UBCs in rice and the mode of ubiquitination of SPL11 UBL is critically influenced by individual UBCs (Bae and Kim, 2014).

Lotus (*Nelumbo nucifera* L.) belongs to Nelumbonaceae, Nymphaeales, and is a perennial aquatic plant. It is one of the oldest dicotyledonous plants and has a number of monocotyledon characteristics (Esau and Kosakai, 1975). Lotus is native to tropical and temperate regions of Asia (Borsch and Barthlott, 1994). According to historical records, the lotus has been cultivated as an ornamental plant and vegetable since the Chow Dynasty in China (Sridhar and Bhat, 2007). Moreover, nearly each part of lotus has been used in Chinese traditional medicine, including the seeds, roots, leaves, flowers, and embryos. *N. nucifera* propagates by seed or rhizome. Notably, its seeds can survive for thousands of years, making it the oldest viable seed for which an age has been directly determined (Priestley and Posthumus, 1982). To date, the entire genomic sequence of *N. nucifera* has been published (Ming et al., 2013); however, the *UBC* gene has not yet been reported. Therefore, in this study, the *UBC* gene was cloned and characterized in *N. nucifera*. We used over-expression of the lotus *UBC* gene in *A. thaliana* to understand its biological functions in *N. nucifera*.

MATERIAL AND METHODS

Plant materials and stress treatments

The experimental material was *N. nucifera* cv. Honghua Jianlian, which was planted in a greenhouse at Wuhan University. Seedlings were allowed to grow in the greenhouse for 10 days at 24°C and were then treated with cold stress (0, 1.5, 3.0, and 6.0 h at 4°C), heat stress (0, 1.5, 3.0, and 6.0 h at 42°C), mechanical damage (leaves were scratched with blades and samples of the damaged leaves were taken 0, 1.5, 3.0, and 6 h later), and three chemical stress treatments (6.0 h in 200 mM NaCl, 20% PEG, or 50 μM ABA). Fresh leaves were collected for further studies. When flower buds had appeared, after 2 weeks, the leaves, rhizomes, terminal buds, and infant flowers were collected for further analysis.

Seeds of *A. thaliana* were kindly provided by Prof. Shen Wenhui (the National Center for Scientific Research of France), including the wild type (Columbia Col-0) and the double mutant *Atubc1-1Atubc2-1*. The defining phenotype is a dramatically reduced number of rosette leaves and an early-flowering phenotype (Xu et al., 2009). All *A. thaliana* materials used in this study were grown under long-day conditions (16-h light, 8-h dark) in a greenhouse at 24°C.

Extraction of RNA and synthesis of cDNA

Total RNA was isolated from fresh materials using the RNAPrep Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China). For synthesis of first-strand cDNA, the RNA was first incubated for 5 min at 70°C with oligo(dT)-m13 (Table 1), and then reversed to cDNA using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). The cDNA was diluted and stored at -20°C until further use.

Table 1. Primers used in this study.

Primer	Sequence
oligo(dT)-m13	5'-GTTTCCAGTCACGACTTTTTTTTTTTTTTTTTTTTTV-N-3'
F1	5'-ATGTCRACBCCNGCWMGG-3'
R1	5'-CTAGTCNGCHGTCCARCT-3'
AAP	5'-GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGGIIIG-3'
GSP1	5'-GTTCAACAATTTCACG-3'
AUAP	5'-GGCCACGCGTCGACTAGTAC-3'
M13	5'-GTTTCCAGTCACGAC-3'
GPS2	5'-ATGTTTAGTGAGAACAA-3'
NnUBC1 F	5'-ACTGATGACAATTAACGC-3'
NnUBC1 R	5'-CTCAAGGATAAGAAACGTG-3'
NnUBC2 F	5'-CATCAATTCATCATCTTGATG-3'
NnUBC2 R	5'-CATAAAAAGATGCCAACATAC-3'
NnUBC3 F	5'-GCTATGTAGTATGTACATG-3'
NnUBC3 R	5'-GAAGTTAAAGGGAGTTAGC-3'
NnUBC4 F	5'-TTGAGAATGCCGAGTAGTGT-3'
NnUBC4 R	5'-AAGCATCCAAAACACCCTACC-3'
NnActin F	5'-TGCAGGAATCCATGAGACTACC-3'
NnActin R	5'-GGTCAGCAATACCAGGGAACAT-3'
AtActin F	5'-GGAAGGATCTGTACGGTAAAC-3'
AtActin R	5'-TGTGAACGATTCTGGACCT-3'

Full-length cDNA isolation by real-time polymerase chain reaction (RT-PCR) and RACE

The full-length cDNA was divided into three amplifiable fragments. Based on the

conservative region of the plant *UBC* genes, a degenerate primer pair F1 and R1 (Table 1) was designed to amplify partial cDNA of the lotus *UBC* gene (*NnUBC*), using the first-strand cDNA as template. SMART-RACE technology was used for amplification of 3'- and 5'-RACE. The 5'-RACE was generated by nested PCR using the primary primer set of AAP and GSP1, and the second set of the 5'-nested primer AUAP and GSP1 (Table 1). Similarly, the 3'-RACE was generated by the 3'-primer set M13 and GPS2 (Table 1). The PCR product was ligated into pGEM-T vector (Promega Corporation), transformed into *Escherichia coli* DH5 α , and then sequenced.

Analysis of gene expression

Total RNA was extracted from different tissues (leaf, rhizome, terminal bud, and flower) and reversed to cDNA. Specific primer pairs of each *NnUBC* gene was designed based on their 3'-UTR sequences, which were *NnUBC1* F and *NnUBC1* R for *NnUBC1*; *NnUBC2* F and *NnUBC2* R for *NnUBC2*; *NnUBC3* F and *NnUBC3* R for *NnUBC3*; and *NnUBC4* F and *NnUBC4* R for *NnUBC4* (Table 1). The β -actin of *N. nucifera* (EU131153) was used as a reference gene, in which the designed primers were *NnActin* F and *NnActin* R (Table 1). RT-PCR was carried out in a fluorimetric thermal cycler (Rotor Gene 2000; Corbett Research, Sydney, Australia) using the DNA-binding dye SYBR Green I (Toyobo Co., Ltd., Osaka, Japan) for detection of PCR products. The reaction was conducted with SYBR Green real-time PCR Master Mix according to the manufacturer instructions. The PCR procedure was as follows: initial denaturation at 94°C for 3 min; followed by 50 cycles at 94°C for 10 s and 60°C for 20 s. Each reaction included three replicates, and the trials were repeated three times. Semi-quantitative data analysis was carried out according to the $2^{-\Delta\Delta Ct}$ method published by Livak and Schmittgen (2001).

Agrobacterium-mediated transformation to *A. thaliana*

The *Agrobacterium* GV3101 containing the expression vector was used for the transformation experiments. The expression vector was transferred to *A. thaliana* (*Atubc1-1Atubc2-1* mutant) using the floral dip method (Clough and Bent, 1998). The transformed *A. thaliana* plants and wild types were continuously cultured in the greenhouse at 22°C under long-day conditions (16-h light: 8-h dark). The concentration of the selected antibiotic, hygromycin, was 50 mg/L. Individuals from the T3-generation were used in further tests. RNA of 2-week-old seedlings was extracted from the mutant and T3 plant. The expression level of *NnUBC3* was detected by qRT-PCR, with *Actin* as reference gene (Table 1).

RESULTS

Characterization of UBC cDNA in *N. nucifera*

cDNA of four *UBC* genes in *N. nucifera* were obtained with full-length coding sequence (CDS) and 3'-UTR sequence, using RACE technology. The CDS regions of the four cDNAs had the same length of 459 bp, coding for 153 amino acids. The four cDNAs had the conserved catalytic site of UBC activity C88. Moreover, the similarity of these four genes was 97-99%. However, the 3'-UTR sequences were significantly different among the four

cDNAs, which indicated that they originated from four different genes. These four genes were defined as *NnUBC*1-4, respectively (accession Nos. JF970922.1, JF970923.1, JF970924.1, and JF970925.1).

The evolutionary relationship between *NnUBC* and the 37 genes of *A. thaliana* *UBC* family was assessed by a phylogenetic amino acid-based sequence analysis using MEGA (Figure 1A). The four *NnUBCs* clustered together, as expected, and they clustered with *AtUBC*1 and *AtUBC*2. The amino acid sequence of lotus *UBCs* had a significant homology with *AtUBC*1 and *AtUBC*2 (Figure 1B). The similarity between lotus *UBCs* and *AtUBC*1 was 94-97%, while the similarity between lotus *UBCs* and *AtUBC*2 was 95-98%. These results indicate that the four *NnUBCs* probably have a function similar to *AtUBC*1 and *AtUBC*2.

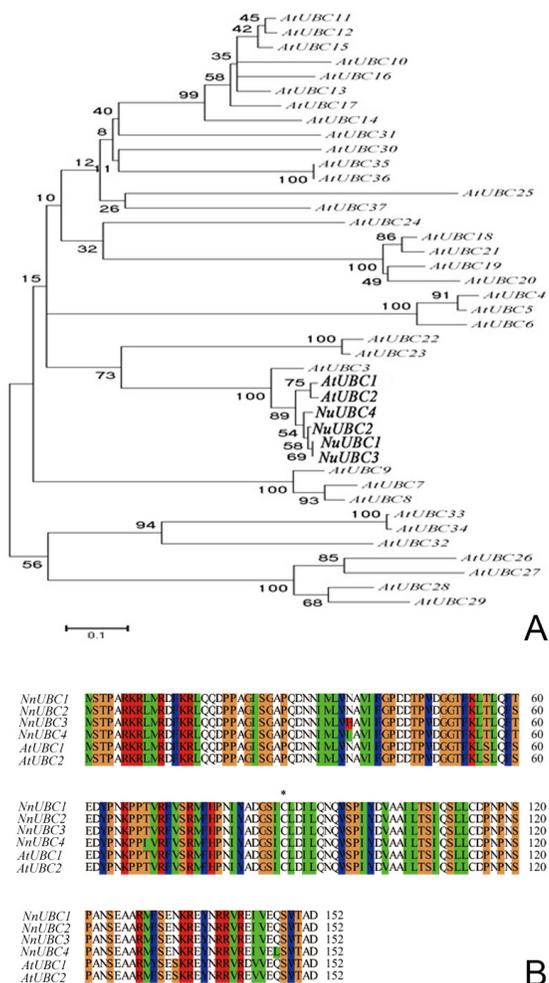


Figure 1. Phylogenetic analysis of *NnUBCs* with *Arabidopsis* *UBCs*. **A.** Phylogenetic tree. Numbers on branches indicate the percentage of 1000 bootstrap replicates, which support the adjacent node. The unit branch length is 0.1 substitutions per site, as indicated by the scale bar. **B.** Amino acid sequence comparison of lotus *UBCs* with *AtUBC*1 and *AtUBC*2.

Expression analysis of UBC genes in lotus

The expression levels of the NnUBC genes were detected in four organs; the leaves, stems, apical buds, and flowers (Figure 2). Although, these four genes were expressed in all four organs, their expression levels in leaves and flowers appeared significantly higher than those in stems and terminal buds, except in the case of *NnUBC2*. Moreover, the expression of each UBC gene varied in different tissues. The expression of *NnUBC1* in flowers was higher than in all other tissues. The expression of *NnUBC2* in leaves was higher than its expressions in other tissues. Both *NnUBC3* and *NnUBC4* showed the highest expression levels in leaves and flowers. In general, *NnUBC3* showed higher expression in all tissues compared to the other homologs.

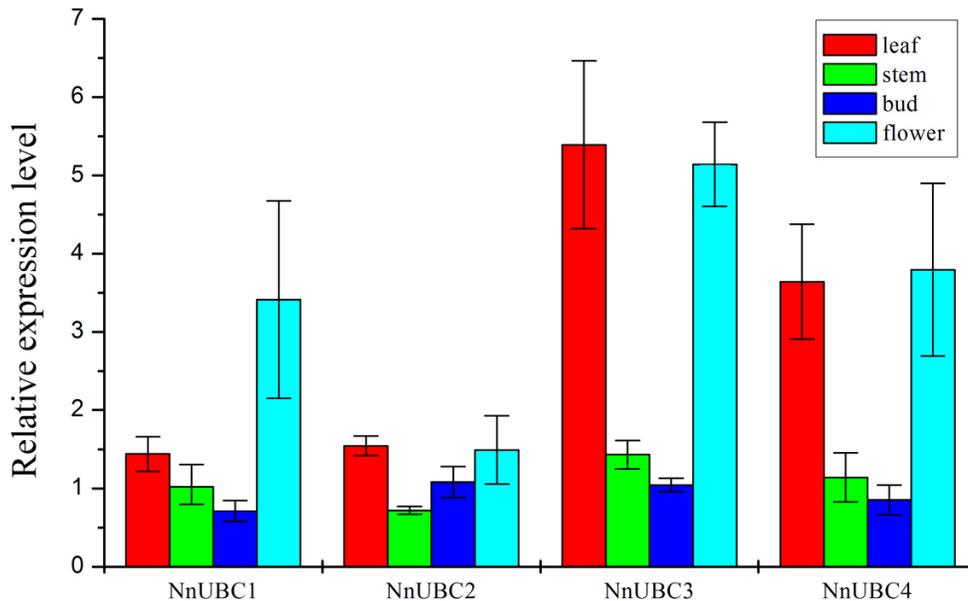


Figure 2. Expression level analysis of NnUBCs in different tissues. Error bars represent the standard deviation of the mean of three replicates.

Structure of the NnUBC3 gene

Because of the high expression level of *NnUBC3* in various tissues, the gene organization of *NnUBC3* was further studied. Our results showed that this gene contained four introns and five exons (Figure 3). The gene structure of *NnUBC3* was similar to both *AtUBC1* and *AtUBC2*; it had the same number of introns and exons, as well as the insert sites of introns. However, the length of each intron differed between *NnUBC3* and *AtUBC1,2*. For example, the second and third introns in *NnUBC3* were more than 600 bp long, whereas those in *AtUBC1,2* were around 200 bp.



Figure 3. Structure of the *Nelumbo nucifera* *UBC3* gene. Red boxes represented the coding sequences (exons); green boxes represented the noncoding sequences (introns), and blue boxes indicate the 5'- and 3'-untranslated regions. The start (ATG) and the stop (TAA) codons are also indicated.

Expression analysis of *NnUBC3* under different stress conditions

The expression level of *NnUBC3* was the highest among the four identified *UBC* genes. Therefore, we further analyzed the expression model of *NnUBC3* under different stress conditions. After treatment at 4°C for 1.5 h, the expression level of *NnUBC3* was down-regulated approximately 10X with respect to the negative control, similar to the observed level after treatment for 6.0 h (Figure 4A). After treatment at 42°C for 1.5, 3.0, and 6.0 h, the expression level was down-regulated 10X, down-regulated 5X, and up-regulated 2X, respectively (Figure 4B). The expression of *NnUBC3* maintained a low level within 3 h and recovered after 6 h in the mechanical damage stress test (Figure 4C). After the treatment of *N. nucifera* with 200 mM NaCl, 20% PEG, or 50 μM ABA, the expression levels of *NnUBC3* were significantly up-regulated by 120, 20, and 8X, respectively (Figure 4D).

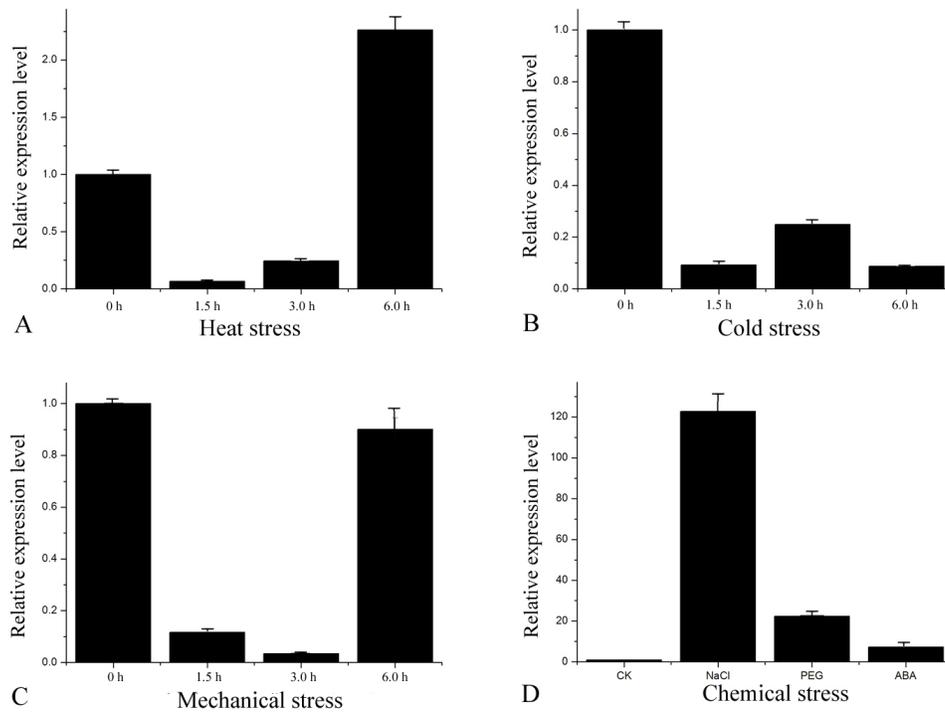


Figure 4. Expression patterns of *NnUBC3* under different stress conditions: **A.** Heat stress. **B.** Cold stress. **C.** Mechanical stress. **D.** Chemical stress.

Expression of NnUBC3 in transgenic *Arabidopsis*

To determine whether the exogenous *NnUBC3* gene was integrated into the mutant genome, T3 seedlings of transgenic *A. thaliana* were analyzed by RT-PCR. As expected, the transgenic plant had an *NnUBC3* PCR product, indicating that the *NnUBC3* gene had been transferred to the *Atubc1-1Atubc2-1* mutant and was successfully expressed (Figure 5A). Under long day conditions, the flowering times of the transgenic, wild-type, and mutant plants were approximately 28, 29, and 20 days, respectively. As expected, after transferring the *NnUBC3*, the mutant returned to normal flowering time, approaching that of the wild type (Figures 5B and C). The number of leaves at flowering of the wild-type, mutant, and transgenic plants was approximately 15, 8, and 15, respectively (Figure 5D and E).

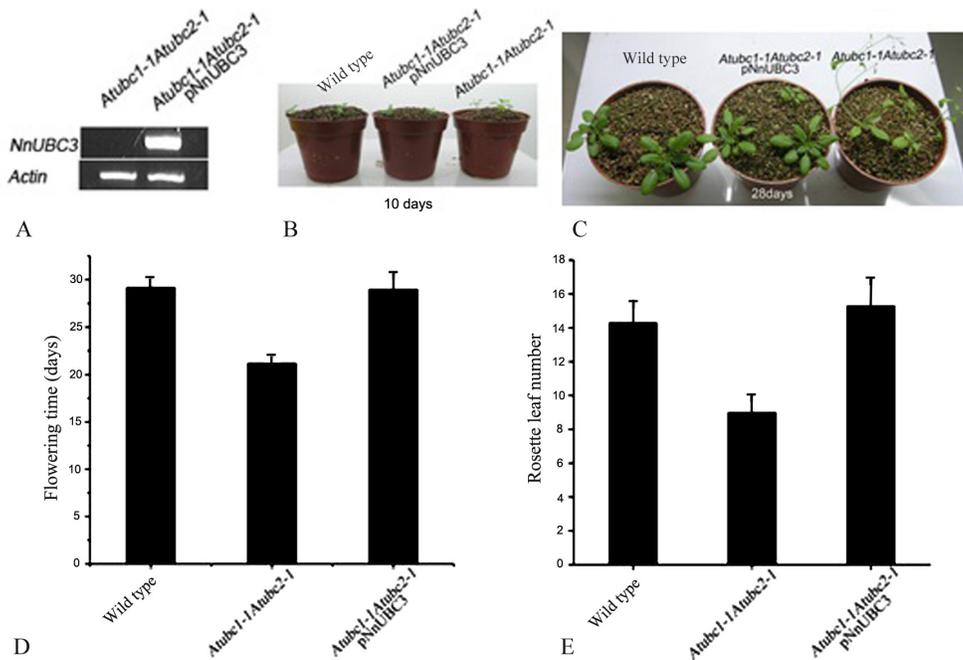


Figure 5. Comparison of wild-type, *Atubc1-1Atubc2-1* mutant and transgenic *NnUBC3* *Arabidopsis* plants. **A.** Identification of mutant and transgenic plants by PCR amplification. **B.** Wild-type, mutant, and transgenic plants after 10 days growth. **C.** Wild-type, mutant, and transgenic plants after 28 days growth. **D.** Flowering time (mean \pm SE, N = 3) of wild-type, mutant, and transgenic plants. **E.** Rosette leaf number (means \pm SE, N = 3) of wild-type, mutant, and transgenic plants at flowering.

DISCUSSION

The UBC proteins play an important role in cell activities, and constitute a protein family with conservative structure, including one UBC domain and one highly conservative catalytic site of cysteine (Dikic et al., 2009; van Wijk and Timmers, 2010). In *Arabidopsis*, *AtUBC1* and *AtUBC2* are two highly homologous genes. Previous studies have verified that E2s (*AtUBC1* and *AtUBC2*) and E3s (*AtHUB1* and *AtHUB2*), used for catalyzing H2B

mono-ubiquitination, have important roles in controlling the flowering time of *A. thaliana* (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009). Detection of H2B ubiquitination of single mutants *Atubc1-1*, *Atubc2-1*, and *Atubc1-1Atubc2-1* double mutant, revealed that *AtUBC1* and *AtUBC2* are two functionally redundant genes. The four identified UBC genes in *N. nucifera* are homologous with the *AtUBC1* and *AtUBC2* genes of *A. thaliana*. However, expression analysis revealed that the four *N. nucifera* genes were expressed in all tissues, indicating that ubiquitination is a basic biological event. However, the expression levels of the genes differed and were significantly tissue-specific, suggesting that the functions of these four UBC genes in *N. nucifera* may not be redundant as the *AtUBC1* and *AtUBC2* genes are in *A. thaliana*.

The *Atubc1-1Atubc2-1* mutant showed significant defects during both vegetative and reproductive growth. The transgenic line carrying *NnUBC3* exhibited the restoration of the *Atubc1-1Atubc2-1* phenotype. This indicates that *NnUBC3* may execute the UBC function of *Atubc1* and *Atubc2*, mediating H2B ubiquitination to activate floral repressor genes and other processes, as indicated by the pleiotropic phenotypes of the mutants (Xu et al., 2009).

Proteins are damaged when plants are under stress conditions. These damaged proteins pose a significant threat to the plant if not degraded by protein-degrading enzymes in time. Ubiquitin degrades abnormal proteins through the ubiquitin pathway, in response to high temperature, drought, cold damage, radiation, and other forms of stress (Ingvarsdén and Veierskov, 2001; Moon et al., 2004; Dreher and Callis, 2007; Shi et al., 2010). Abiotic low-temperature stress on *N. nucifera* resulted in down-regulated *NnUBC3* expression. In contrast, the expression level of *NnUBC3* was significantly up-regulated when plants were exposed to high temperature, high salt, and drought conditions. This phenomenon indicates that *NnUBC3* may be involved in the response of *N. nucifera* to high temperature, high salt, and drought conditions. The expression of this gene was inhibited under low-temperature stress. There may be other ubiquitin-conjugating enzyme genes participating in ubiquitination of abnormal proteins under low-temperature stress. Moreover, when plants are under high salt and drought stress, endogenous ABA levels increase and a series of responses relying on ABA are activated (Christmann et al., 2006; Zhang et al., 2007; Santiago et al., 2009; Saavedra et al., 2010). In the ABA treatment, *NnUBC3* showed up-regulated expression, indicating that UBC has an important role in the ABA signal pathway. In *A. thaliana*, UBL has been shown to play an important role under high salt, drought, and ABA treatments (Zhang et al., 2007). Therefore, the role of UBC in *N. nucifera*, whether assisting the UBL degradation of proteins or mono-ubiquitination of the gene at a certain site to activate the ABA signal pathway, needs to be further studied.

In this study, we cloned four UBC genes in lotus. These genes were widely expressed in the lotus organism, indicating that they contribute to multiple biological processes. Among them, *NnUBC3* was shown to take part in the control of flowering time, plant development, and response to drought and salt stress. Because the lotus is a basal dicotyledonous plant, these results will be helpful to further dissect the function of UBCs in lotus as well as the evolution of UBC genes in plants.

Conflicts of interest

The authors declare no conflict of interest.

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