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Transcription factor NnDREB1 from lotus improved drought tolerance in transgenic *Arabidopsis thaliana*

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Abstract

Dehydration responsive element binding factor (DREB) is believed to be a stress-tolerance enhancer in plants. In the present study, a cold-binding factor (CBF)/DREB homologous gene *NnDREB1* (XP_010242642.1) was isolated from lotus roots using rapid amplification of cDNA ends (RACE) and reverse transcription (RT)-PCR methods. Analysis of the deduced amino acid sequence and phylogeny classified NnDREB1 into the A-1 group of the DREB1 subfamily. Expression profiling using a quantitative PCR method revealed that *NnRDEB1* was significantly induced by NaCl, mannitol, and polyethylene glycol, but not by low temperature and abscisic acid. To evaluate function of NnRDEB1, *Arabidopsis thaliana* was transformed with the *NnDREB1* gene in a binary vector construct. The transgenic plants exhibited higher resistance to drought compared with the wild-type plants in terms of survival rates, dry and fresh masses, and chlorophyll content. In addition, overexpression of *NnDREB1* resulted in higher germination rates compared with the wild type plants on MS medium containing mannitol. The expressions of downstream target stress-related genes, including *cold-regulated15B* (*COR15B*), *rare cold inducible 2B* (*RCI2B*) and *repeat domain 26* (*RD26*), were activated in the transgenic plants. Taken together, the results suggest that NnDREB1 might be an important protein in lotus root drought tolerance.

Additional key words: chlorophyll, gene expression, mannitol, *Nelumbo nucifera*, phylogenetic tree, polyethylene glycol, salinity.

Introduction

Abiotic and biotic stresses affect plant growth and development; especially important crop species are often constrained by water availability (Manavalan *et al.* 2009). Many physiological and biochemical processes are damaged by drought stress (Wang *et al.* 2001), but the severity is unpredictable, because many environmental factors act simultaneously (Wery *et al.* 1994).

To defend against drought stress, plants use several strategies, one of which is manipulating key physiological processes, such as photosynthesis, respiration, water relations, and antioxidant and hormonal metabolism to establish a new homeostasis (Bhargava and Sawant 2013). Another strategy is regulation of stress-responsive genes (Verlotta *et al.* 2013). Genes induced by drought stress could be categorized into two groups according to the functions of their products. The first group comprises functional proteins, such as membrane proteins, key enzymes for osmolyte biosynthesis, the detoxification enzymes, and other proteins for the protection of macromolecules. The second group comprises regulatory proteins, *i.e.*, transcription factors, protein kinases, and proteinases, involved in the regulation of signal transduction and gene expression (Agarwal *et al.* 2006).

Dehydration responsive element binding (DREB) factor belongs to the APETALA2/ethylene response factor (AP2/ERF) family in plants, which is a large group of plant-specific transcription factors that includes

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Abbreviations: cDNA - complementary DNA; *COR* - *cold-regulated*; DRE/CRT - dehydration-responsive element/C-repeat; DREB - dehydration responsive element binding factors; ERF - ethylene response factor; ORF - open reading frame; RACE - rapid amplification of cDNA ends; RCI - rare cold inducible *2B*; RD - repeat domain; RT-PCR - reverse transcription PCR.
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major subfamilies: DREB2A, DREB2B, DREB2C, DREB2D, DREB2E, DREB2F, DREB2G and DREB2H (Mizoi *et al.* 2012). DREB, related to ABI3/VP1 gene (RAV), ERF and the AP2 subfamilies. The DREB subfamily comprises two subgroups, DREB1 and DREB2. DREB1 includes three members, namely DREB1A, DREB1B and DREB1C; and DREB2 includes DREB that could bind to the dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting element to promote expression of downstream stress-responsive genes to improve plant stress tolerance (Yamaguchi-Shinozaki and Shinozaki 1994). For DREB1, a conserved amino acid sequence was identified that could distinguish and bind downstream genes containing DRE/CRT *cis*-acting elements to regulate the stress response (Shinozaki and Yamaguchi-Shinozaki 2007). DREB1 was firstly isolated from *Arabidopsis*, and it shows a high similarity to the ERF/AP2 DNA-binding domain (Liu *et al.* 1998). When *Arabidopsis AtDREB1* is overexpressed in soybean, the transgenic plants show enhanced drought tolerance without any biomass penalty (Reis *et al.* 2014). An *AtDREB1A/C-repeat binding factor* (*CBF3*) was also isolated from *Arabidopsis*, and the plants constitutively expressing this gene have improved tolerance to drought and freezing stress (Zhao *et al.* 2007). Transgenic lines with *OsDREB1F* in rice and *Arabidopsis* exhibit higher survival than wild-type plants by activating downstream genes, including *cold-regulated* (*COR*) and *responsive to desiccation 29B* (*rd29B*), and ras-related proteins Rab-18 (RAB18), when subjected to drought, salt, and low temperature stresses (Wang *et al.* 2008).

Lotus [*Nelumbo nucifera* (L.) Gaertn] is a member of the family *Nymphaeaceae*. Lotus is traditionally cultivated for edible rhizomes rich in nutrients (Liu *et al.* 2010) and stamens and stems are important ingredients in traditional medicine (Borgi *et al.* 2007, Renato *et al.* 2007). However, irregular rainfall usually causes severe drought stress, which affects lotus growth and development. Therefore, developing new genotypes tolerant to drought stress is necessary. Therefore, the isolation and functional characterization of drought-tolerated genes is a prerequisite for improving the resistance of cultivated species. In this study, we identified a *DREB1* from a rhizome of lotus, and analyzed its expression profiles and possible functions in *Arabidopsis*.

Materials and methods

Plants and growth conditions: Lotus [*Nelumbo nucifera* (L.) Gaertn] high salt tolerant cv. YH_5 was obtained from the experimental station of aquatic vegetables in the Yangzhou University. During the growth phase, various plant organs including shoot tips, leaves, petioles, rhizomes, and roots were collected and stored at -80 °C.

Cloning of *NnDREB1*: In a present study, a partial sequence was obtained with transcriptom sequencing (Cheng *et al.* 2013). To amplify full-length gene, rapid amplification of cDNA ends (RACE) was performed using *SMART* ™ RACE mix (*Clontech*, Dalian, China). RNA was extracted from the rhizome tips of seedlings having four leaves by using plant RNA extract mix (*Tiangen*, Beijing, China). DNase was added to remove any DNA contaminations. For the first cDNA strand synthesis, about 2 - 3 μg of RNA was used with RNA first strand mix (*Promega*, Madison, USA). PCR reaction mixtures (0.02 cm^3) consisted of 0.0025 cm^3 of dNTP, 0.002 cm^3 of forward and reverse primers, 0.0025 cm^3 of $MgCl_2$, 0.0002 cm^3 of Taq polymerase (5 U mm^{-3}, *Tiangen*), 0.002 cm^3 of cDNA fragments, and 0.0088 cm^3 of distilled H_2O. Primers for RACE were designed according to known fragment and are listed in Table 1 Suppl. The PCR program consisted of 35 cycles: 94 °C for 1 min; 94 °C for 1 min; 58 °C for 1 min; 72 °C for 1 min, and the final extension at 72 °C for 10 min. Target fragments were extracted on a 1 % (m/v) agarose gel using a *Gel Purification* kit (*TaKaRa*, Dalian, China), TA-ligated into pMD 18-T vector (*TaKaRa*), and transformed into *Escherichia coli* cells DH5α. Plasmid DNA was extracted from the 3 cm^3 of culture using a *Plasmid Purification* kit (*TaKaRa*). DNA sequencing was performed by *Sangon Biotechnology Co*. (Shanghai, China). Sequence analysis was completed using the *DNASTAR* software.

Expression analysis of *NnDREB1* in lotus: Real time quantitative (q)PCR was carried out to analyze *NnDREB1* expression under various treatments. The punched lotus seeds were placed in a container and germinated at a temperature of 28 ± 1 °C in the dark, and then the seedlings were grown at day/night temperatures of 28/22 ± 1 °C, a relative humidity of 70 - 80 %, a 16-h photoperiod, and an irradiance of about 200 μmol m^{-2} s^{-1}. To induce different stresses, the seedlings having four leaves were exposed to 0.25 M NaCl, 0.1 M mannitol, 4 °C, PEG 1 000 (30 %, m/v), or 0.1 mM abscisic acid and stem tips were collected after 0, 6, 12, 18, and 24 h.

For analysis of expression of twenty drought stress-related genes, three transgenic lines and wild-type *Arabidopsis* plants were grown under normal conditions (a temperature of 23 ± 1 °C, a 12-h photoperiod, and an irradiance of about 150 μmol m^{-2} s^{-1}). At four-leaf stage, total RNA was extracted from the leaves, and DNase was added to remove any DNA contaminations. Primers shown in Table 1 Suppl were designed according to lotus *DREB1* and *Arabidopsis* gene sequences derived from the *NCBI* database. *Actin* of lotus and *Arabidopsis* was used as internal standard. The lotus *actin* primer was:

5'-ACGCGTATGAAGTCAGTTGT-3' (forward), and 5'-TTTATGGGGATCAGCTGGT-3' (reverse); the *Arabidopsis actin* primer was 5′-CTTGCACCAAGC AGCATGA-3′ (forward), and 5′-CCGATCCAGACA CTGTACTTCCT-3′ (reverse). Amplification was performed in a 0.025 cm^3 reaction mixture, containing 0.0125 cm^3 of *SYBR Premix Ex Taq* II (*Tli RNaseH Plus*) (2×), 0.001 cm^3 of each of the forward and reverse primers, 0.002 cm^3 of RT reaction solution (cDNA), and 0.0085 cm^3 of distilled water. The PCR program consisted of 94 °C for 30 s, then 40 cycles of 95 °C for 5 s, and 60 °C for 60 s. The qPCR reactions were performed in triplicate. $2^{-\Delta\Delta Ct}$ method was applied for data analysis, and the detail process was referred in Giulietti *et al*. (2001). ΔCt value was obtained according to the $Ct_{(target)}$ and $Ct_{(actin)}$ value in treated plants ($\Delta Ct_{(target)}$) and control ($\Delta Ct_{(normal)}$) , and ΔΔCt value was counted based on the data of $\Delta Ct_{(target)}$ and $\Delta Ct_{(normal)}$. Therefore, $2^{-\Delta\Delta Ct}$, which represented the relative expression was determined.

Vector construction and identification of transgenic *Arabidopsis* plants: The complete open reading frame (ORF) of *NnDREB1* was subcloned into the pGEM-T easy vector. After digestion with *Xba*I and *Kpn*I, the sequence was inserted into the binary transformation vector pSN1301 under the control of the CaMV 35S promoter. The pSN1301::*NnDREB1* plasmid was inserted into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis* transformation was carried out using the floral dip method (Clough and Bent 1998). The plants were then placed in a glasshouse until the seeds were obtained. Seeds of the T_0 generation were grown on MS medium containing 20 μg g^{-1} hygromycin B to identify 'positive' plants. All the plants were cultivated in chamber with the same conditions as above mentioned, and three lines of transgenic plants were used.

Gene function analysis in *Arabidopsis* plants under drought stress: The T_2 generation of the transgenic seedlings and wild-type *Arabidopsis* seedlings grown in pots were at six-leaf stage starved of water for 10 d, and the survival rate was investigated after 5 d of recovery. In another experiment, the seeds of transgenic plants and wild-type plants were sown on MS medium containing 0, 0.1, 0.2, 0.3, and 0.4 M mannitol and their growth was analyzed 7 d after plating.

Comparison of dry and fresh masses, and chlorophyll content in transgenic and wild-type *Arabidopsis* plants: The seeds of transgenic plants (lines 15, 19, and 110) and wild-type plants were surface-sterilized in 30 % (m/v) sodium hypochlorite for 20 min and rinsed six times with sterile water before being placed on MS medium for germination, and then transferred into pots. When they had six leaves, line 15, line 19, line 110, and wild-type plants were starved of water for 10 d, and then recovered under normal conditions for 5 d. The whole plant was collected for dry and fresh mass determination, and chlorophyll analysis. Chlorophyll was isolated and determined using the methods of Hiscox and Israelstam (1979).

Statistics: For statistical analysis, the data were recorded as means ± SEs of three experiments with about 20 seedlings per experiment. Statistical analyses were performed using the *SPSS v. 14.0* software (*SPSS Inc.*, Chicago, IL, USA).

Results

In a previous study, we obtained a lotus partial sequence by transcriptome analysis that showed high sequence similarity with *DREB1* genes from other species. The full-length ORF was amplified by RACE with 660 bp encoding a putative polypeptide of 220 amino acids. The deduced protein contained a conserved AP2 domain of the DREB family. When blasted against *NCBI* database, this gene showed 77, 69, 76, 72, and 72 % sequence similarity with *DREB1* from *Theobroma cacao* (XM_007015865.1), *Adonis amurensis* (HQ889135.1), *Glycine max* (XM_006593208.1), *Ampelopsis brevipedunculata* (EF150893.1), and *Vitis aestivalis* (KF582123.1), respectively (Fig. 1). Based on this similarity, we named it *NnDREB1*. In addition, phylogenetic analysis of NnDREB1 with DREB1 proteins from other species showed that although NnDREB1 was quite distantly related to the other DREB1s, it could be classified into the A-1 group of the DREB1 subfamily (Fig. 2).

The expression of *NnDREB1* was induced in the rhizome tip after exposure to NaCl for 12 h. In addition, exogenous mannitol (0.2 M) significantly induced the *NnDREB1* expression after 6 h of treatment. However, no significant change of expression was found in response to low temperature and abscisic acid (ABA) treatment within 24 h (Fig. 3*A*). PEG was used to treat different organs of lotus, and the results showed that *NnDREB1* expression obviously increased in the petioles, roots, and rhizomes (Fig. 3*B*).

Transgenic *Arabidopsis* plants carrying *NnDREB1* were identified. Three lines (15, 19. and 110) of transgenic *Arabidopsis* plants were selected and exposed to drought stress. The results showed that the survival rates of three lines of transgenic plants after drought treatment were 49, 42, and 52 %, and only 10 % of the wild-type (Fig. 4). This suggested that the transgenic *Arabidopsis* plants overexpressing *NnDREB1* had enhanced drought tolerance. Dry and fresh masses were

significantly higher in the three lines of transgenic plants than in the wild-type plants (Fig. 5*A*,*B*). In addition, the transgenic plants had higher chlorophyll content compared with the wild-type seedlings under drought conditions (Fig. 5*C*). The above experiments further confirmed that the transgenic *Arabidopsis* plants overexpressing *NnDREB1* had enhanced tolerance to drought stress.

For further confirmation, seed germination was investigated on MS medium containing 0, 0.1, 0.2, 0.3, and 0.4 M mannitol. We found that 0.2 M mannitol seriously affected the germination of wild-type plants, but not transgenic plants. For transgenic plants and wild-type plants, only few seeds germinated on MS medium containing 0.4 M mannitol (Fig. 1 Suppl.).

Twenty DREB1 downstream drought stress-responsive genes in *Arabidopsis* were chosen as candidate genes for expression analysis in transgenic *Arabidopsis* plants. Homologous sequences of the selected genes were searched for in the databases for *Arabidopsis*. Most of these genes have been reported to be induced by drought stress in the literature. Total RNA was extracted from the leaves of seedlings (six-leaf stage) and the expression of these genes was analyzed

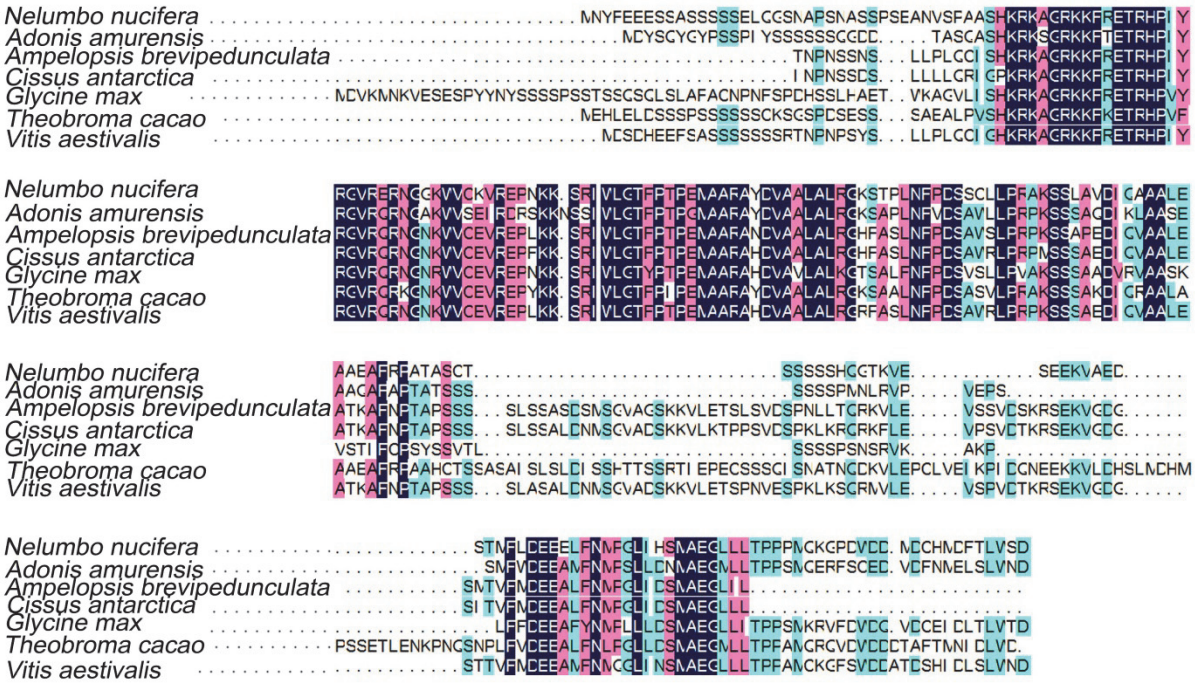

Fig. 1. Alignment of the deduced amino acid sequence of NnDREB1 from *Nelumbo nucifera* (XP_010242642.1) and DREB1s from *Theobroma cacao* (XM_007015865.1), *Adonis amurensis* (HQ889135.1), *Glycine max* (XM_006593208.1), *Ampelopsis brevipedunculata* (EF150893.1), *Cissus antarctica* (gb|ABU55664.1), and *Vitis aestivalis* (KF582123.1) (comparison with the *DNAMAN* software).

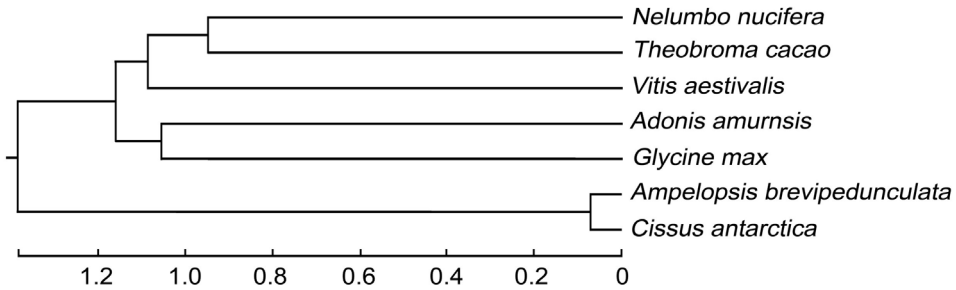

Fig. 2. A phylogenetic tree was constructed with sequence of amino acids using *ClustalW* and *Mega 4.0*. Seven DREB1s were selected from different plants as follows: DREB1 (KF582123.1) from *Vitis aestivalis*, DREB1 (gb|ABU55664.1) from *Cissus antarctica*, DREB1 (EF150893.1) from *Ampelopsis brevipedunculata*, DREB1 (XM_007015865.1) from *Theobroma cacao*, DREB1 (HQ889135.1) from *Adonis amurensis*, DREB1 (XM_006593208.1) from *Glycine max*, and DREB1 (XP_010242642.1) from *Nelumbo nucifera*.

using qPCR. The expressions of three genes: *COR15B*, *RCI2B* and *RD26* increased in the transgenic *Arabidopsis* plants compared with wild-type *Arabidopsis* plants. The expressions of the other 17 genes (*COR4B*, *COR15A*, *COR47*, *ERD7*, *ERD10*, *HB7*, *LEA14*, *LTI30, LTP3*, *NAC019*, *NAC019*, *P5CS2*, *RD29A*, *RDUF1*, *ZF2*, *AtGOIS3*, and *At5G59550*) were not significantly different after transformation with *35S*::*NnDREB1* (Fig. 6).

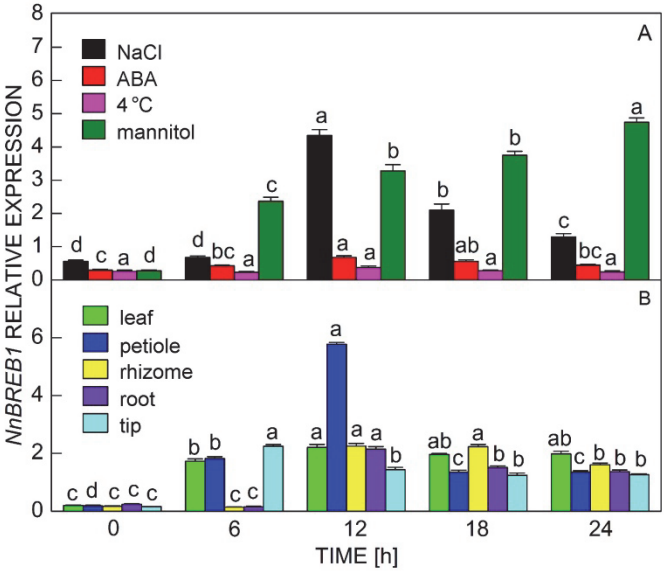

Fig. 3. *NnDREB1* expression patterns in response to various treatments and in different organs of lotus. *A* - Expression of *NnDREB1* in response to NaCl, ABA, mannitol, and low temperature treatments. *B* - Expression of *NnDREB1* in response to PEG treatment in different organs including leaf, petiole, rhizome, root, and stem tip.

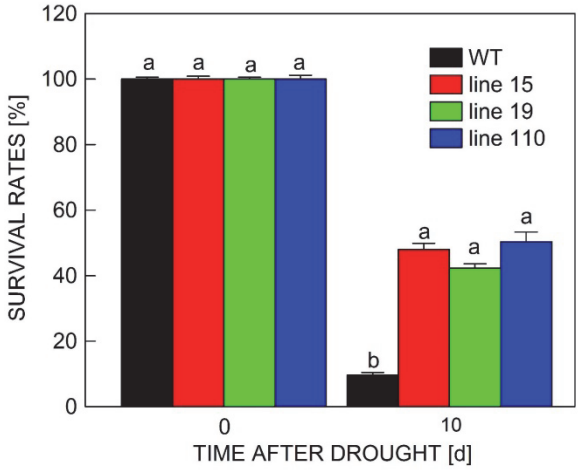

Fig. 4 *Arabidopsis* plants constitutively expressing *NnDREB1* showed enhanced drought tolerance. Survival rates of three transgenic lines (15, 19, and 110) and wild-type plants at six leaf stage determined after 10 d of water starvation followed by 5 d of recovery.

Discussion

Until now, the *DREB* genes had not been analyzed extensively in lotus, even though abiotic stresses were a serious challenge for lotus production in China. In this study, we isolated the *NnaDREB1*, a CBF/DREB homologous gene from lotus. Phylogenetic and amino acid sequence analyses revealed that NnDREB1 belonged to the CBF/DREB subfamily in the AP2/ERF family (Figs. 1,2). NnDREB1 contains an AP2/ERF domain, which is an important site for DNA binding (Sakuma *et al.* 2006). Three members of DREB1s including

DREB1A, DREB1B, and DREB1C have been isolated from many plants since their initial cloning from *Arabidopsis* (Liu *et al.*1998, Mizoi *et al.* 2012).

Most members of the *DREB1* subfamily show increased expression in response to stresses, including salt, drought, cold and heat (Kim *et al.* 2008). However, *DREB1s* are involved in special stresses, despite belonging to the same subfamily. The expression of the *DgDREB1A* group was only responsive to dehydration or salt stress. Dubouzet *et al.* (2003) and Magome *et al.* (2008) found that *DREB1* in *Arabidopsis* and rice is responsive only to salt stress. Wang *et al.* (2008) showed

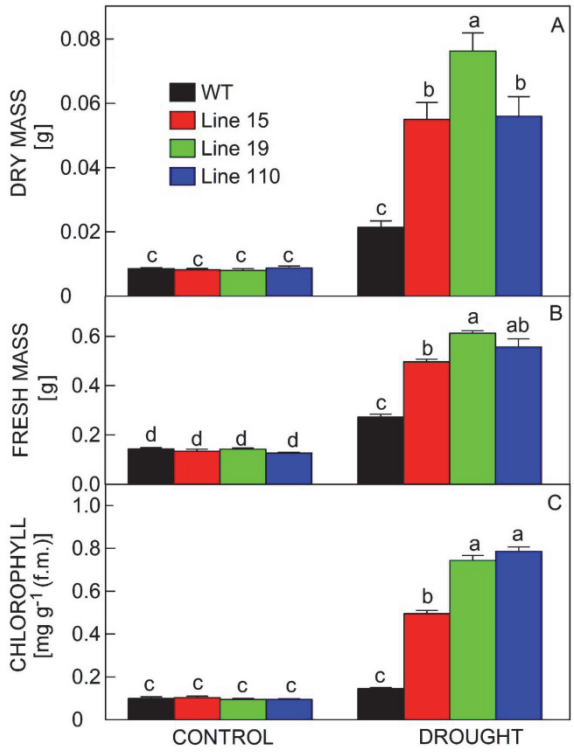

Fig. 5. Dry (*A*) and fresh (*B*) masses and chlorophyll content (*C*) in transgenic plants (lines 15, 19, and 110) and wild-type *Arabidopsis* plants at six-leaf stage after water starvationfor 10 d. Means ± SE, $n = 10$; different letters represent significant differences between transgenic and wild-type *Arabidopsis* plants at $P < 0.01$ (*t*-test).

that expression of *OsDREB1F* was induced by high salinity, drought, cold, and ABA application, but not by oxidative stress, wounding, or pathogen attack. This indicated that *OsDREB1F* participated in ABA-dependent pathway in response to stresses. In this study, we did not detect the expression of *NnDREB1* under normal conditions. *NnDREB1* was responsive to NaCl, mannitol, and PEG treatments, but not to ABA treatment (Fig. 3*A*), which was different from other *DREB1* genes.

In addition, DREB1s also have tissue-specific expression characteristics. For example, *OsDREB1* and *Festuca arundinacea FaDREB1* transcriptions are higher in stem and leaves than in the roots (Tian *et al.* 2005, Tang *et al.* 2005). At the same time, among the three chrysanthemum *DgDREB1* members, the *DgDREB1A* group is predominantly expressed in roots (Tong *et al.* 2009). The same phenomenon is observed in *Arabidopsis* and grape (Haake *et al.* 2002, Xiao *et al.* 2006). We found that plants treated with PEG showed weaker expression in leaves than in the petioles, rhizomes, stem tips, and roots (Fig. 3*B*), suggesting that lotus *NnDREB1* also showed a tissue-specific expression.

The constitutive expression of an *AtDREB1* gene to improve plant adaptation to stress has been demonstrated. Tomato transformed with a *CBF1/DREB1B* gene shows enhanced resistance to drought stress (Hsieh *et al.* 2002). Transgenic rice and *Arabidopsis* plants harbouring *OsDREB1F* gene have improved resistance to multiple stresses, including salt, drought, and low temperature (Wang *et al.* 2008). Overexpression of *MbDREB1* induces high tolerance to drought in *Arabidopsis* (Yang *et al*. 2011). In the present study, the expression of *NnDREB1* in *Arabidopsi*s improved drought adaptation (Fig. 4). However, overexpression of this gene could not enhance freezing and salt tolerance (data not shown). In addition, DREB1s from the different DREB1 groups show different functions in terms of plant growth and development. *Arabidopsis* constitutively expressing *DgDREB1A* exhibits delayed flowering, but not dwarfism. Overexpression of *DgDREB1B* induces dwarfism, but not delayed flowering, although transgenic plants expressing these two genes show enhanced

freezing and drought tolerance (Tong *et al.* 2009). We did not observe any developmental changes in the transgenic plant, suggesting that *DREB1s* have different functions in the regulation of plant growth.

There is evidence that DREB1 improves stress tolerance by activating the expression of downstream genes (Charfeddine *et al.* 2015). FeDREB1 transgenic *Arabidopsis* plants enhance drought stress by upregulating the expressions of *RD29B*, *ATDI21*, *TIP*, *RD26*, *RAS1*, *PEN1*, and *RAB18* (Fang *et al.* 2015). Overexpression of *OsDREB1F* in *Arabidopsis* and rice induces strong resistance to drought stress by increasing the expressions of *COR* genes (Wang *et al.* 2008). The expression of *RD26* is responsive to drought, salt, and ABA, and plants showing constitutive expression of this gene demonstrate that RD26 could activate the promoter

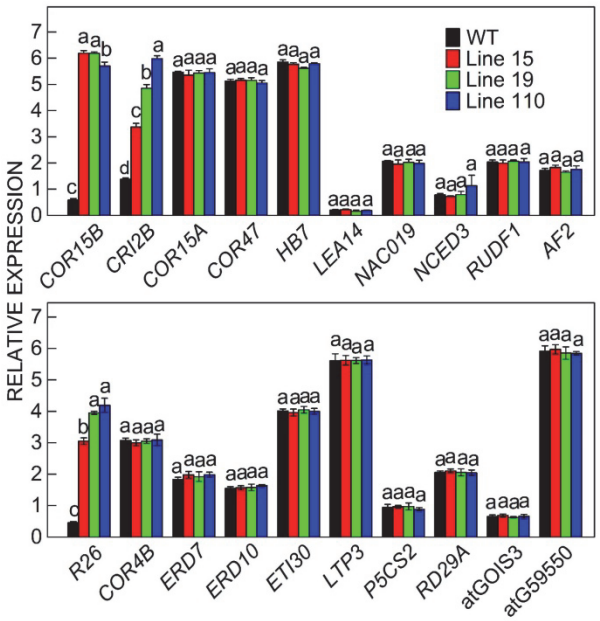

Fig. 6. Expression analysis of 20 drought stress-related genes including *COR15B, RCI2B*, *RD26*, *COR4B*, *COR15A*, *COR47*, *ERD7*, *ERD10*, *HB7*, *LEA14*, *LTI30, LTP3*, *NAC019*, *NAC019*, *P5CS2*, *RD29A*, *RDUF1*, *ZF2*, *AtGOIS3* and *At5G59550* of *Arabidopsis* determined by qPCR in three transgenic lines and wild type *Arabidopsis* plants under normal growth conditions. *Actin* was used as an internal standard.

of its target gene in *Arabidopsis* under abiotic stress (Fujita *et al.* 2004). In this study, 20 drought stress-related genes of *Arabidopsis* were selected for expression analysis in three *NnDREB1* transgenic lines according to their reported functions in stress tolerance in the literature. We found that only *COR15B, RCI2B*, and *RD26* expressions were enhanced under normal growth conditions (Fig. 6). Thus, we believed that *NnDREB1* was an important transcription factor that regulated drought stress responsive signalling in plants. Nevertheless, more work is required for better understanding the contribution of *NnDREB1* in responding to drought stress in lotus.

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