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Overexpression of wheat *TaNCED* gene in *Arabidopsis* enhances tolerance to drought stress and delays seed germination

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Abstract

Abscisic acid (ABA) regulates various plant physiological processes, especially participates in the plant responses to harsh environments. The 9-*cis*-epoxycarotenoid dioxygenase (NCED) is a key enzyme in ABA biosynthesis pathway. Here, a *TaNCED* with an 1 887-bp open reading frame was cloned from wheat, which encodes a peptide of 628 amino acids. A chloroplast transit peptide sequence was found at the N-terminus of the TaNCED protein. Multiple sequence alignments indicate that the TaNCED protein shared high similarities with other NCEDs from different species. Real-time quantitative PCR analysis shows that expression of *TaNCED* was strongly up-regulated by treatments with ABA, polyethylene glycol, and drought stress, and it was down-regulated during germination of the wheat seeds. Ectopic overexpression of the *TaNCED* gene in *Arabidopsis* resulted in an increase of endogenous ABA and free proline content. A lower water loss rate and stomatal conductance of leaves were found in the transgenic plants in comparison with the wild type. Subsequently, the transgenic plants displayed an enhanced tolerance to drought stress but delayed seed germination. These data provide evidence that the *TaNCED* might play a primary role in regulation of ABA content during water stress and seed dormancy.

Additional key words: abscisic acid, 9-*cis*-epoxycarotenoid dioxygenase, polyethylene glycol, *Triticum aestivum*.

Introduction

The phytohormone ABA plays crucial roles in regulation of growth and development in higher plants such as seed maturation and dormancy, root growth, fruit maturation, as well as responses to multiple environmental stresses including respective gene expressions (Davies and Zhang 1991, Duckham *et al*. 1991, Chandler and Robertson 1994). In plants, ABA content is determined by the balance of ABA biosynthesis and catabolism. To date, the pathway of ABA metabolism has been elucidated, and almost all genes participating in ABA biosynthesis have been isolated from many species (Nambara and Marion-Poll 2005). The 9-*cis*-epoxycarotenoid dioxygenase (NCED), which cleave 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin to produce xanthoxin as precursor of ABA, has been proved to be the rate-limiting enzyme in ABA biosynthesis, and its expression correlates with endogenous ABA content (Kende and Zeevaart 1997, Koornneef *et al*. 1998).

The *NCED* gene was firstly identified in the ABA-deficient mutant *viviparous 14* (*vp14*) of maize (Schwartz *et al*. 1997). Subsequently, *NCED* genes have been isolated from many plant species such as *Arabidopsis* (Iuchi *et al*. 2001), rice (Hwang *et al*. 2010), bean (Qin and Zeevaart 1999), cowpea (Iuchi *et al*. 2000), orange (Rodrigo *et al*. 2006), potato (Destefano-Beltrán *et al*. 2006), and tomato (Thompson *et al*. 2000a). The *NCED* genes appeared as a multiple gene family. There are nine *NCED*-related sequences in *Arabidopsis* and five of them (*AtNCED2, 3, 5, 6,* and *9*) have been found to possess NCED activities. Their organ-specific expression profiles as well as physiological roles have been comprehensively characterized. The *AtNCED2* and *AtNCED3* are

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Abbreviations: ABA - abscisic acid; DEX - dexamethasone; F_v/F_m - variable to maximum chlorophyll fluorescence ratio (maximum efficiency of photosystem II); g_s - stomatal conductance; NCED - 9-*cis*-epoxycarotenoid dioxygenase; ORF - open reading frame; PCR - polymerase chain reaction; PPFD - photosynthetic photon flux density; *vp14* - *viviparous 14*; WT - wild type.
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expressed in roots and flowers, and *AtNCED3* has a prominent function in stress responses (Tan *et al*. 2003). Expressions of *AtNCED6* and *AtNCED9* can be detected in seeds and both of them are involved in ABA biosynthesis during seed development (Lefebvre *et al*. 2006). Furthermore, *AtNCED9* also plays a crucial role in a high temperature-induced ABA synthesis and germination inhibition (Toh *et al*. 2008). Recently, *AtNCED5* has been documented to induce seed dormancy together with *AtNCED6* and *AtNCED9* or to regulate ABA synthesis coupled with *AtNCED3* under water stress (Frey *et al*. 2012).

The *NCED* genes from other species have also been well characterized. A detailed study of *NCED* expression during water stress shows a clear correlation among mRNA expression of *PvNCED1*, content of PvNCED1 protein, and content of ABA in dehydrated leaves and roots of bean (Qin and Zeevaart 1999). Three *NCED*-related cDNAs (*PaNCED1*, *2*, and *3*) have been cloned from avocado, and two products of them (*PaNCED1* and *3*) exhibit NCED enzyme activity (Chernys and Zeevaart 2000). The *PaNCED1 i*s induced by water stress, whereas expression of *PaNCED3* is not detectable in dehydrated leaves. Both *PaNCED1* and *PaNCED3* are also expressed in ripening fruits. Recently, it has been reported that overexpression of the *OsNCED3* gene from rice in *Arabidopsis* could result in accumulation of ABA, a lower relative water loss, delayed seed germination and better tolerance to drought stress than wild type (WT) plants (Hwang *et al*. 2010). Moreover, overexpression of the *NCED* gene has been well documented to enhance ABA accumulation and drought tolerance in transgenic plants (Thompson *et al*. 2000b, Wan and Li 2006, Yang and Guo 2007, Wang *et al*. 2009, Xian *et al.* 2014).

Genetic and physiological studies highlighted the importance of ABA in seed dormancy and germination (Grappin *et al*. 2000, Gubler *et al*. 2005). It has been reported that an ABA-deficient mutant of tobacco shows a non-dormant phenotype (Grappin *et al*. 2000). An enhanced expression of *LeNCED1* in tomato is able to increase ABA synthesis and accumulation, which cause a prolonged dormancy (Thompson *et al*. 2000b). Moreover, *PvNCED1* induced by dexamethasone in tobacco causes a delay in seed germination as a result of dexamethasone (DEX)-induced increase of ABA synthesis (Qin and Zeevaart 2002). Ectopic expression of *GlNCED1* in tobacco displays up to a 2-fold prolonged dormancy and delays germination time by extending the time of radicle formation (Zhu *et al*. 2007).

Wheat is one of the most important crops in the world. A variety of abiotic stresses, such as drought, cold, and salinity, continuously pose negative impacts on its growth, development, and productivity. The abilities of response and tolerance to abiotic stresses are thus important for plants to deal with various environmental stresses. The mechanisms by which plants respond to water stress involved both ABA dependent pathways and ABA independent pathways. Accumulation of ABA is one of the classical traits of plants under water stress. Oxidative cleavage of xanthophylls by NCED is a major regulatory step of ABA accumulation in plants. To our knowledge, few papers have documented the *NCED* gene in wheat (Ji *et al*. 2011, Zhang *et al*. 2014) and it remains an open question whether other wheat *NCED* genes are involved in ABA biosynthesis. In the present study, a novel *TaNCED* from wheat was isolated and characterized. Ectopic overexpression of the *TaNCED* gene in *Arabidopsis* was performed to extensively study the function of the *TaNCED* gene in regulation of ABA biosynthesis, responses to drought stress, and roles in seed germination.

Materials and methods

Plants and stress treatments: The seeds of the wheat (*Triticum aestivum* L.) genotype CAU981 were sterilized and soaked in water at 4 °C in the dark for 24 h and then grown on filter paper in a greenhouse at a temperaure of 23 °C, a 16-h photoperiod, a photosynthetic photon flux density (PPFD) of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and a 60 % relative humidity. At the two-leaf-stage, stress treatments were carried out. Sodium chloride (250 mM), ABA (100 μM), and cold stress (4 °C) treatments were performed as described previously (Tong *et al*. 2007). For osmotic stress, the roots of the seedlings were immersed in 25 % (m/v) polyethylene glycol (PEG_{6000}) or in water for control. The plants were subjected to the stress treatments for 0.5, 1, 2, 6, and 12 h, and then the samples were harvested and frozen in liquid nitrogen for further use. The treatments of drought stress of common wheat seedlings and during germination were done as described by Domínguez *et al*. (2002) and Rampino *et al*. (2006). Briefly, 10-d-old seedlings were placed on dry filter paper for 0.5, 1, 2, 6, and 12 h at room temperature and well-watered seedlings were used as control. At the end of the stress, whole seedlings were taken, frozen in liquid nitrogen and stored at -80 °C. For germination, wheat grains were sterilized in 2 % (m/v) NaClO for 20 min, washed twice with sterile water, once with 0.01 M HCl, and then thoroughly with sterile distilled water. The sterile grains were allowed to germinate for 8, 16, 24, 32, and 40 h at room temperature and whole grains were collected, frozen in liquid nitrogen, and stored at -80 °C.

Arabidopsis thaliana L. was cultivated under a PPFD of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a 8-h photoperiod, a temperature of 23 °C, and a relative humidity of 60 %. Seeds were sown

on a moistened, loosely packed 3:1 mixture of soil and *Vermiculite* and then vernalized at 4 °C in darkness for 72 h before transfer to a growth chamber.

Cloning and sequence analysis of *TaNCED*: The total RNA was extracted from wheat leaves using an RNeasy *Protect Mini* kit *(Qiagen*, Hilden, Germany). Specific *TaNCED* forward and reverse primers (Table 1) were designed and synthesized to amplify the *TaNCED* gene by PCR. The PCR product was purified and ligated to the *pGEM-T Easy* vector (*Promega*, Madison, USA) for sequencing.

Other NCED proteins from different species were searched by using the *BlastP* program in the National Center for Biotechnology Information (*NCBI*). Alignment of TaNCED with homologous proteins was performed using the *ClustalX* program (*v. 1.83*) and viewed by the *GeneDoc* software (*v. 2.5*). Predictions of a transit peptide and cleavage sites of the TaNCED protein were carried out using the online software *ChloroP1.1* (http://www.cbs.dtu.dk/services/ChloroP/). Molecular mass and theoretical isoelectric point were predicted by *ExPASy* (http://web.expasy.org/protparam/).

Expression analysis of *TaNCED* under various stresses: Specific primers for real-time quantitative PCR were designed and synthesized (Table 1) and the reactions were performed as previously described (Tong *et al*. 2007). Reproducible data were obtained from three independent experiments and normalized to the amplification of the *TaActin* internal control. Relative gene expression with respect to *TaActin* was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer names and sequences.

Names	Sequences (5'-3')
For reverse transcription-PCR	
TaNCED-F	ATGTCTGATCTCTACCCCGC
TaNCED-R	TCATTGATGCTGTGACCGGAGCT
For real-time quantitative PCR	
TaActin-F	TATGCCAGCGGTCGAACAAC
TaActin-R	GGAACAGCACCTCAGGGCAC
TaNCED-F	CTCTTCTGCTGCTGCTCCGAAC
TaNCED-R	CGACCAAGTGCTCTTCCGTCTC

***Arabidopsis* transformation:** Plasmids used in transformation of *A. thaliana* were constructed with the coding sequence of *TaNCED*, which was cloned into the pCAMBIA Super-1300 vector. The construct was introduced into *Agrobacterium tumefaciens* L. strain GV3101 and then transferred into wild-type *A. thaliana* L. (Columbia ecotype) by the floral dip transformation method described by Clough and Bent (1998). The transformants were selected with hygromycin in Murashige and Skoog medium, and three homozygous T2 generation seeds (N1, N3, and N19) were randomly screened for further analysis. Expression of *TaNCED* in the transgenic lines was analyzed by real-time quantitative PCR as described above. The products were loaded on a 2 % (m/v) agarose gel for electrophoresis.

Determination of endogenous ABA and free proline content and seed germination assay: Three-week-old rosette leaves were excised from the transgenic or WT *Arabidopsis* plants grown in soil. For drought stress, the leaves lost 30 % of their initial fresh mass and were placed in a sealed plastic bag with wet paper towels for an additional 5 h. Then the tissues were frozen in liquid nitrogen and ground into powder. Exraction of ABA, purification, and subsequent quantification were carried out as previously described (Xiong *et al*. 2001). Briefly, 1 g of the powder was suspended in 15 cm^3 of an extraction solution (80 % methanol, 100 mg dm^{-3} butylated hydroxytoluene, and 0.5 g dm^{-3} citric acid monohydrate.). The suspension was stirred overnight at 4 °C and centrifuged at 1 000 *g* for 20 min. The supernatant was transferred to a new tube and dried under vacuum and the dry residue was dissolved in 0.1 cm^3 of methanol plus 0.9 cm^3 of Tris-buffered saline (50 mM Tris, 0.1 mM $MgCl_2 . 6 H_2O$, and 0.15 M NaCl, pH 7.8).

Free proline content was measured according to Ábrahám *et al*. (2010). Briefly, 0.1 g of the powder was suspended in 2 cm^3 of cold 20 % (v/v) ethanol and vortexed for 5 min. The solution (1 cm^3) was transferred into a new tube and added 120 μM α-aminobutyrate and the mixture was incubated at 70 °C for 5 min and then transferred to 4 °C for 1 h. The mixture was centrifuged at 14 000 *g* and 4 °C for 30 min. The supernatants were collected and dispensed into 0.2 cm^3 aliquots. The aliquots were dried under vacuum and the dry residue was dissolved in 1.2 cm^3 of distilled water for HPLC analysis.

Seeds harvested from the WT and transgenic *Arabidopsis* of the same age were used for seed germination analysis. The seeds were surface sterilized in 5 % (m/v) NaClO for 5 min, then washed 5 - 7 times with sterile water before plating onto Murashige and Skoog media with or without 1 μM ABA (three replicates, $n = 60$). After 4 d of chilling at 4 °C, the seeds were then incubated in a growth chamber at an 8-h photoperiod, a PPFD of 30 - 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$, a temperature of 22 °C, and a relative humidity of 60 %. Germination was assessed as radicle appearance and full development of cotyledons every day.

Measurement of stomatal conductance, water loss, and drought tolerance: The youngest fully expanded leaves were excised from three-week-old rosettes grown in soil to measure stomatal conductance and water loss at growth chamber conditions. Stomatal conductance (g_s) was measured with a portable photosynthetic open-

system *CI-340* (*Walz*, Effeltrich, Germany) between 10:00 and 12:00 after an adaptation to a sufficient irradiance for more than 1 h (Thompson *et al*. 2000a, Li *et al*. 2014). Measurement of leaf relative water loss was performed as described previously (Li *et al*. 2014). The excised rosette leaves were placed on filter paper and the leaf mass was measured at 0.5, 1, 1.5, 2, 2.5, 3, 3.5 h.

Three transgenic lines (N1, N3, and N19) were selected to subject to the drought stress at the seedling stage together with the WT plants as a control. The drought treatment (interruption of watering and subsequent rehydration) was performed as reported by Woo *et al*. (2008). To further evaluate the effect of the drought stress on the transgenic *Arabidopsis* plants, chlorophyll fluorescence parameters were determined by an *IMAGING-PAM* chlorophyll fluorometer (*Maxi version*, *Walz*, *Effeltrich*, *Germany*) with imaging areas up to 10 × 13 cm as previously described (Woo *et al*. 2008).

Statistical analysis. Values are presented as means of three replicates and standard errors (SEs). Student's *t*-test was used to determine the significance of difference.

Results

One single 1 900 bp product was obtained using the specific primer pair TaNCED-F/TaNCED-R by PCR. The sequencing result of the amplified DNA fragment was annotated as the NCED protein by *BlastX* search. The nucleotide sequences reported here were designated as *TaNCED* and have been submitted to GenBank with accession number KP099105.

Sequence analysis indicates that the *TaNCED* gene contained an intact open reading frame of 1 887 bp, which encoded a peptide of 628 amino acids. The deduced molecular mass of TaNCED was 67.5 kDa with the isoelectric point 6.11. A putative chloroplast transit peptide of 49 amino acids was found at the N-terminus of the TaNCED protein by analysis with the online software *ChloroP1.1* (Fig. 1 Suppl.). The amino acid sequences of TaNCED shared high similarities with other plants NCED proteins with one exception of the N-terminus region. The TaNCED had the highest identity (73 %) with *Oryza sativa* (AY838897.1) at the amino acid level.

Under the osmotic (25 %, m/v, PEG_{6000}) and drought stresses, transcripton of *TaNCED* in wheat leaves was up-regulated within 0.5 - 12 h post-treatment and reached a maximum peak at 2 h being 245-fold greater than in the control (Fig.1*A*,*C*). In the ABA treatment, *TaNCED* expression reached a maximum peak at 6 h post-treatment (21-fold of the control; Fig. 1*B*). During germination of the wheat seeds, *TaNCED* expression was down-regulated after 8 h and kept on the same level within 16 - 40 h (Fig. 1*D*). The results imply that the *TaNCED* gene may be involved in signalling pathways mediated by osmotic and drought stresses as well as ABA.

The *TaNCED* gene was transformed into *Arabidopsis* under the control of a strong constitutive promoter CaMV 35S. Three independent homozygous T3 lines of the transgenic plants were selected for further analysis. Transcription of *TaNCED* in the transgenic *Arabidopsis* plants was investigated by real-time quantitative PCR. The results indicate that the transcription level of *TaNCED* was different in the three transgenic lines, and N3 had the highest transcription among the three lines (Fig. 2).

Abscisic acid content of the three transgenic lines was more than 2 - 3 times higher than that of the WT before the drought stress (Fig. 3*A*). This increase of ABA accumulation was associated with an increase in the *TaNCED* mRNA. The drought stress led to an increase of ABA content in both the WT and the transgenic lines, but the elevation in the transgenic lines was more significant than that of the WT. In conclusion, the ABA content of the transgenic lines was dramatically higher than that of the WT before and after drought stress treatment.

Content of proline was slightly higher in the transgenic plants than in the WT plants without the drought stress. However, content of proline increased up to 10-fold in the transgenic plants under the drought stress. However, the enhancement in the WT plants was much less than in the transgenic plants (Fig. 3*B*).

Stomatal conductance was determined in the transgenic and WT plants. There was a reduction in stomatal conductance in the transgenic plants compared to the WT (Fig. 3*C*). The mean g_s values of transgenic lines N19, N1, and N3 were 68.4, 66.7, and 57.6 %, respectively, of g_s in the WT plants. The drought stress caused decreases of g_s in the transgenic plants faster than in the WT plants. To confirm the influence of differences in g_s on transpiration, the rate of water loss from detached leaves was examined. The analysis of the water loss indicates that the transgenic plants were more resistant to water loss than the WT plants: when detached leaves of the WT plants lost 50 % of their water content, the transgenic plants lost only 20 % of their water content (Fig. 3*D*).

The *TaNCED* was a drought-inducible gene (Fig. 1*B*) and, therefore, it was likely to be involved in regulation of drought signalling. To characterize the performance of *TaNCED* transgenic plants under the drought stress, the three transgenic lines and WT plants were subjected to the drought stress at the seedling stage. After about 15 d, leaves of the transgenic and WT plants wilted and showed chlorosis as a result of the severe water stress (Fig. 4*A*). After rehydration for 3 d, the majority of the WT plants did not recover and died; a survival ratio was

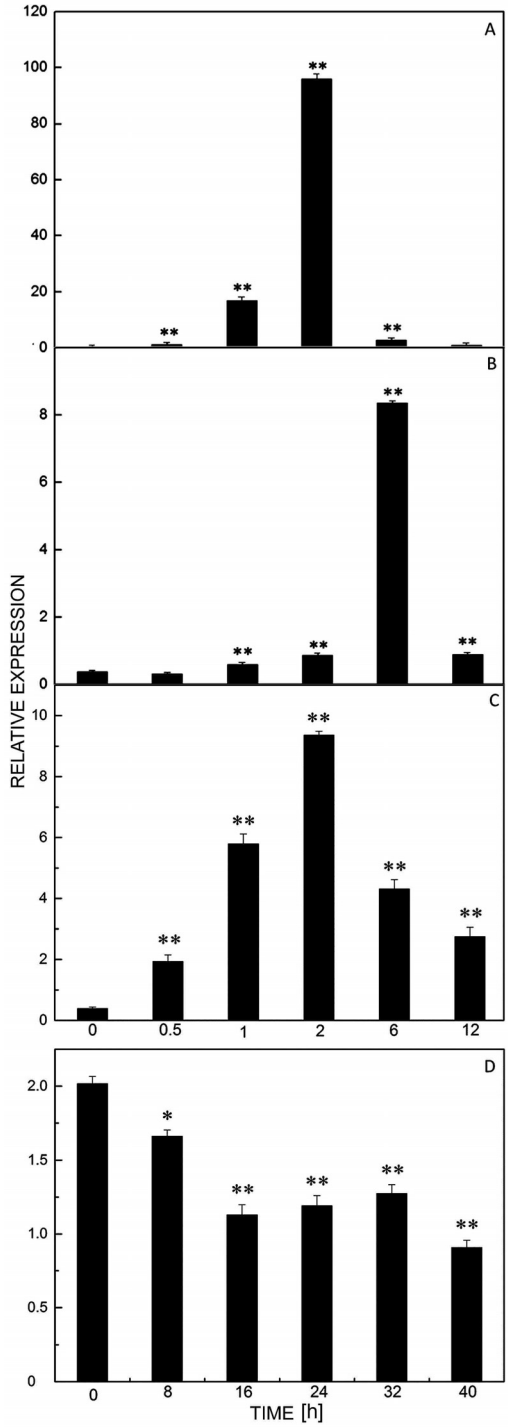

Fig. 1. Expression patterns of *TaNCED* in wheat under different stresses and during germination of wheat seeds: *A* - expression of *TaNCED* under polyethylene glycol treatment; *B* - expression of *TaNCED* under abscisic acid treatment; *C* - expression of *TaNCED* under drought stress, *D* - expression of *TaNCED* during germination. Means ± SEs, n = 3, ** - significantly (t-test, $P < 0.01$) different from the control (0 h treatment).

only 11.1 %. By contrast, all three transgenic lines (N1, N3, and N19) showed higher survival ratios of 66.67, 88.89, and 77.78 %, respectively, and successfully produced seeds (Fig. 4*D*).

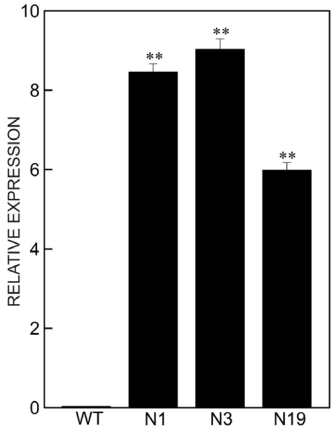

Fig. 2. Expression of *TaNCED* in transgenic *Arabidopsis* lines (N1, N3, and N19) and in the wild type (WT) determined by real-time quantitative PCR. The *actin* gene cloned from *Arabidopsis* was used as an internal reference. Means ± SEs, n = 3, ** - significantly different from the WT (t-test; $P < 0.01$).

The parameters of chlorophyll fluorescence imaging and the maximum efficiency of photosystem II (F_v/F_m) can serve as indicators to monitor damage of plants during drought stress (Woo *et al*. 2008). As shown in Fig. 4*B*,*C*, the F_v/F_m ratios were not significantly different (0.77 - 0.80) between the transgenic and WT plants until 12 d of the treatment. However, within following 3 d, this ratio decreased sharply in the WT plants to very low levels (0.11 - 0.31). After rewatering for 3 d, the majority of the WT plants were not able to recover (Fig. 4B; $F_v/F_m < 0.267$). In contrast, the F_v/F_m ratio declined slowly in the transgenic plants and the majority of the transgenic plants recovered gradually after rewatering (Fig. 4*B*,*C*,*D*). These results indicate that the transgenic plants possessed an enhanced tolerance to the drought stress.

The seed germination experiments of the transgenic and WT plants were carried out by measuring the appearance of a radicle and cotyledon (Fig. 2 Suppl.). Seeds of the WT started to germinate 35 h after seeding (about 50 % of the seeds formed a radicle). However, germination of the transgenic plants started already 30 h after seeding. However, cotyledons were visible approximately 85 h after seeding in all transgenic plants in comparison with 58 h in the WT plants (Fig. 2 Suppl.). However, the appearance of a radicle was delayed significantly under the 1 μM ABA treatment (Fig. 2 Suppl.). In addition, application of 1 μM ABA during germination prolonged the period of seed dormancy and cotyledon development in the transgenic and WT plants.

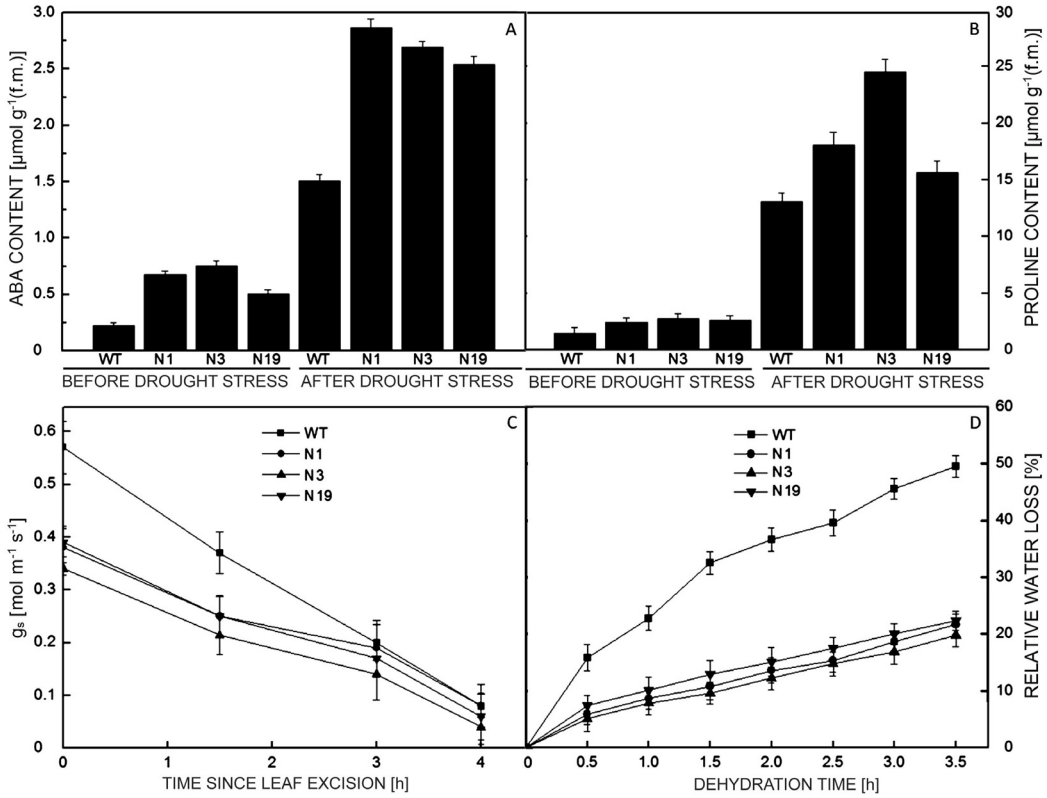

Fig. 3. Characteristics of transgenic *Arabidopsis* and wild type (WT) plants under water stress: *A* - endogenous abscisic acid (ABA) content; *B* - proline content; *C* - stomatal conductance (g_s); *D* - rate of water loss from detached leaves. Means ± SEs, $n = 3$.

Discussion

It is well known that ABA plays an essential role in mediating responses to abiotic stresses in plants. Oxidative cleavage of *cis*-epoxycarotenoids catalyzed by NCED is the rate-limiting step in biosynthesis of ABA. In the present work, a novel *TaNCED* gene from wheat was isolated and the analysis of alignment shows that the TaNCED protein share a high similarity to other plant NCEDs.

It is noteworthy that transcription of *TaNCED* displayed different patterns under the various stresses, which indicates that different regulatory mechanisms or pathways exist in plants to regulate ABA biosynthesis. In this study, expression of *TaNCED* was strongly induced by the PEG_{6000} and drought stress within 2 h (Fig. 2*B*). The results imply that there was a quick response mechanism in plant cells to regulate expression of *TaNCED* and consequently biosynthesis of ABA. Similar results with other plant species, such as maize (Schwartz *et al*. 1997), rice (Hwang *et al*. 2010), tomato (Thompson *et al*. 2000a), peanut (Wan and Li 2006), *Stylosanthes guianensis* (Yang and Guo 2007), and citrus species (Neves *et al*. 2013, Xian *et al*. 2014), were also reported. The data of real-time quantitative PCR also show that expression of *TaNCED* was up-regulated by addition of ABA. It is likely that *cis*-elements, such as ABRE (CACGTG and TACGTG) and C-repeat/DRE (TGGCCGAC) in the promoter region of TaNCED respond to dehydration and ABA. These results demonstrate that expression of *NCED* might be regulated not only by a pathway responding quickly to water stress but also by a pathway depending on ABA. In previous studies, it has been reported that expression of *NCED* from cowpea is strongly induced by salt stress (Iuchi *et al*. 2000). However, an opposite result was observed by Xian *et al* (Xian *et al*. 2014). Our data (not shown in this paper) also indicate that *TaNCED* fails to respond to salt stress. As mentioned in the introduction, the *NCED* genes appear as a multiple gene family. The members of the family are distributedin different tissues or organs and respond to different stresses. The *TaNCED* is the only member of the family which responds to water stress and ABA but not to salt stress as Xian *et al*. (2014) found. There are several studies showing that the *NCED* gene is strongly induced by cold stress (Yang and Guo 2007, Wang *et al*. 2009, Xian *et al*. 2014). Although the C-repeat/DRE and LTR (CCGAAA) *cis*-acting elements responding to a low temperature were found in the promoter region of *TaNCED*, transcriptional expression of *TaNCED* did not respond to the cold stress. The results were in accordance with the results of Qin and Zeevaart

(1999) and Iuchi *et al* (2000) and also implied that the mechanism of regulation is much more complicated as it appeared. The explanation for the differences among our results and those of others suggests further very well designed investigations.

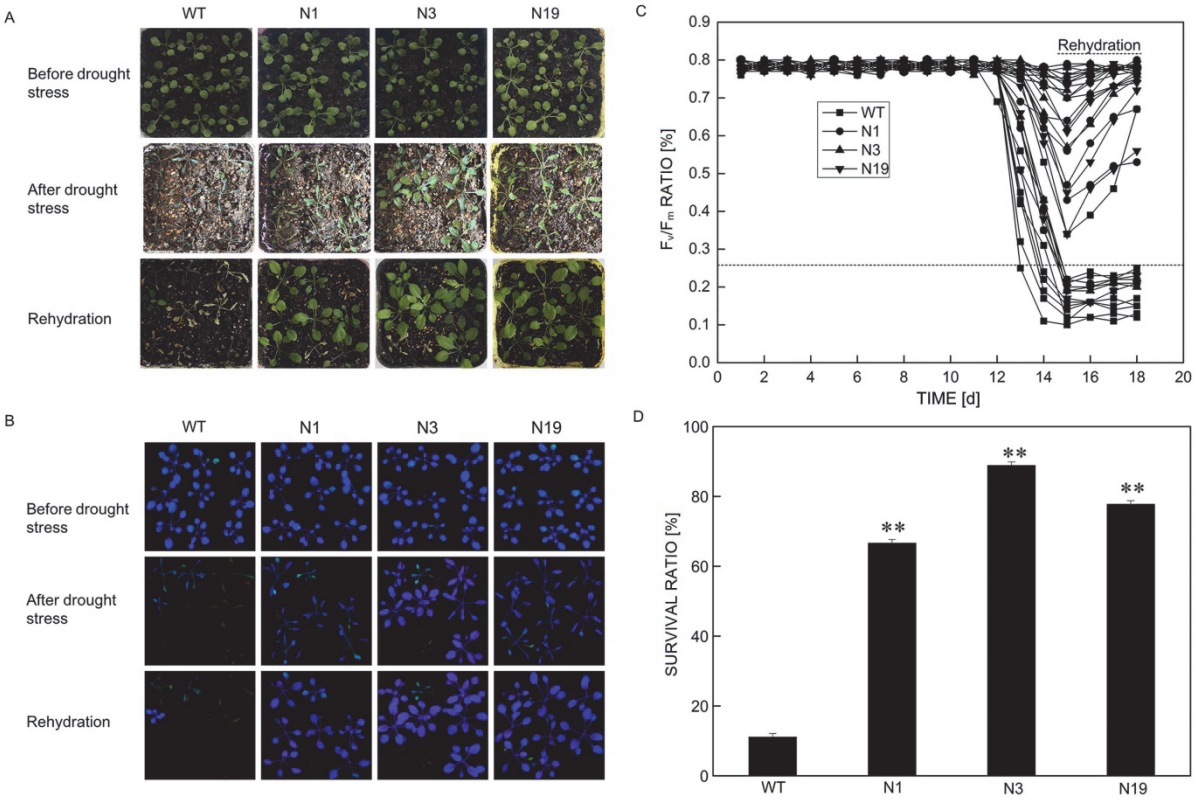

Fig. 4. Drought stress tolerance assay in transgenic (N1, N3, and N19) and wild type (WT) plants: *A* - appearance of plants before stress, after drought stress, and after rewatering; *B* - false-colour images of maximum efficiency of photosystem II (F_v/F_m) measurements obtained before stress, after drought stress, and after rewatering; *C* - changes in F_v/F_m during progression of drought stress and rehydration (33 % threshold for a typical average control F_v/F_m of 0.800 is shown as a *dotted* line); *D* - percentage of survival after rehydration. Means ± SEs, $n = 60$, ** - significantly different from the WT (*t*-test; $P < 0.01$).

The results of real-time quantitative PCR shows that *TaNCED* expressed highly in the transgenic *Arabidopsis* (Fig. 2). Overexpression of *TaNCED* in the transgenic *Arabidopsis* resulted in an obvious increase of ABA not only under the drought stress but also under the normal conditions (Fig. 3*A*). This was direct evidence that *TaNCED* was indeed overexpressed in the transgenic plants and further led to the increase of ABA content. Some previous reports also support our result (Iuchi *et al*. 2001, Lefebvre *et al*. 2006). As a stress responding phytohormone, ABA is well known to function as to resist abiotic stresses, for example, inducing stomata closure to restrict water loss or to induce proline synthesis to balance osmotic stress. To evaluate the influences exerted by increased ABA in the transgenic *Arabidopsis*, g_s, rate of water loss, and content of proline were measured. The results show that the initial g_s was notably lower in the transgenic lines (Fig. 3*C*), and leaves excised from the transgenic plants had strikingly lower water loss rates compared to the WT plants (Fig. 3*D*), which indicates that the transgenic plants had a higher water retention capacity. The measurement of proline content revealed that proline was evidently elevated in the transgenic plants, and proline accumulation was significantly raised under the water stress (Fig. 3*B*). Proline is a major osmolyte in plants to tolerate osmotic stress (Xiong *et al*. 2001). Our results suggest that the transgenic plants had a higher water retention capacity and ability to balance the osmotic equilibrium due to an increased ABA biosynthesis. Supporting evidence were obtained by measurements of the maximum efficiency of photosystem II (the F_v/F_m ratio), which confirmed that the transgenic plants exhibited a significant resistance to water stress (Fig. 4).

It is well known that exogenous ABA could prevent germination (Hilhorst *et al*. 1998). In previous reports, tomato *LeNCED1* overexpressing transformants showed a prolonged seed dormancy (Thompson *et al*. 2000b). Our data show that a delay in seed germination of the transgenic plants was likely caused by overexpression of *TaNCED*, and delays were also induced in the transgenic plants and WT by adding 1 μM ABA. These data suggest

that an enhanced expression of *TaNCED* in the transgenic plants had been able to increase ABA biosynthesis, and ABA accumulation resulted in a prolonged seed dormancy.

In conclusion, *TaNCED* in wheat was induced by the drought stress, exogenous PEG_{6000}, and ABA. Ectopic overexpression of *TaNCED* in *Arabidopsis* could lead to an increase of endogenous ABA, which conferred resistance to the water stress of the transgenic plants. Overexpression of *TaNCED* also resulted in a delayed germination of freshly harvested seeds of the transgenic *Arabidopsis*. These findings suggest that *TaNCED* indeed takes part in biosynthesis of ABA and isevidently involved in drought stress tolerance and seed germination by mediating ABA biosynthesis pathway.

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