DOI: 10.1007/s10535-013-0373-6 BIOLOGIA PLANTARUM 58 (1): 89-98, 2014

Cloning of 9-*cis*-epoxycarotenoid dioxygenase gene (*TaNCED1*) from wheat and its heterologous expression in tobacco

S.J. ZHANG, G.Q. SONG, Y.L. LI, J. GAO, J.J. LIU, Q.Q. FAN, C.Y. HUANG, X.X. SUI, X.S. CHU, D. GUO, and G.Y. LI*

Crop Research Institute, Shandong Academy of Agricultural Sciences; Key Laboratory of Wheat Biology and Genetic Improvement on North Yellow and Huai River Valley, Ministry of Agriculture; National Engineering Laboratory For Wheat and Maize, Jinan 250100, Shandong , P.R. China

Abstract

Abscisic acid (ABA) regulates plant responses to various environmental stresses. Oxidative cleavage of *cis*-epoxycarotenoids catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) is the critical step in the biosynthesis of ABA in higher plants. Using a homologous cloning approach, a *NCED*-like gene (designated as *TaNCED1)* was isolated from wheat (*Triticum aestivum*). It contained an open reading frame of 1 848 bp and encodes a peptide of 615 amino acids. Multiple sequence alignments showed that *TaNCED1* shared high identity with *NCEDs* from other plants. Phylogenetic analysis revealed that *TaNCED1* was most closely related to a barley *HvNCED1* gene. The predicted 3D structure of *TaNCED1* showed high similarity with other homologues. Southern blot analysis indicated that *TaNCED1* was a single copy in the genome of wheat. *TaNCED1* was differentially expressed in various organs and the expression was up-regulated by low temperature, drought, NaCl, and ABA. Heterologous expression of *TaNCED1* in tobacco (*Nicotiana tabacum*) significantly improved its drought tolerance. Under drought treatment, *TaNCED1*-overexpressing transgenic tobacco plants exhibited higher germination rate, higher relative water content, content of soluble sugars and of ABA when compared with the wild type plants.

Additional key words: abscisic acid, drought tolerance, low temperature, *Nicotiana tabacum*, *Triticum aestivum*

Introduction

Drought, salinity, and low temperature are major environmental factors deterring plant growth and productivity. Upon exposure to abiotic stress conditions, plants adopt appropriate strategies with altered metabolism, growth, and development (Yamaguchi-Shinozaki and Shinozaki 2006). Abscisic acid (ABA) is an essential plant hormone involved in the responses to different environmental stresses and controls stomatal aperture, seed dormancy, and fruit maturation (Zeevaart and Creelman 1988, Davies and Zhang 1991, Leung and Giraudat 1998, Mansouri and Asrar 2012). Cloning genes involved in the ABA biosynthetic pathway cannot only provide a powerful approach for investigating the roles of ABA in responses to dormancy and abiotic stresses, but also make possible to increase ABA accumulation in plants (Taylor *et al*. 2005). Oxidative cleavage of *cis*-epoxycarotenoids violaxanthin and neoxanthin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) is a critical step in regulation of ABA biosynthesis in higher plants (Kende and Zeevaart 1997, Seo and Koshiba 2002, Nambara and Marion-Poll 2005).

The first *NCED* cDNA was obtained from the

Submitted April 2013, *last revision* 24 May 2013, *accepted* 27 May 2013.

Abbreviations: ABA - abscisic acid; CTAB - cetyl trimethylammonium bromide; DIG - digoxigenin; IPTG - isopropyl β-D-1-thiogalactopyranoside; NCED - 9-*cis*-epoxycarotenoid dioxygenase; ORF - open reading frame; pI - isoelectric point; RT-PCR - reverse-transcription polymerase chain reaction; RWC - relative water content.

Acknowledgements: This research was supported by the National Transgenic Major Project (2012ZX08002-004), Shandong Agriculture and Seed Industry (2012), China Agriculture Research System (CARS-03-1-08), Funding for the Post-doctoral Innovative Projects of Shandong Province (201203024), Shandong Agriculture Research System, the National Key Technology R&D program of China (2011BAD35B03), and Outstanding Agricultural Talent and Team. The authors are very grateful to Prof. R.A. McIntosh for reviewing this manuscript.

* Corresponding author; fax: (+86) 531 83178122, e-mail: lgy111@126.com

ABA-deficient *Vp14* mutant of maize (Tan *et al*. 1997). Since then, homologous sequences were cloned and characterized in different plants including tomato (Burbidge *et al*. 1999), bean (Qin and Zeevaart 1999), *Arabidopsis* (Iuchi *et al*. 2001, Tan *et al*. 2003), cowpea (Iuchi *et al*. 2000), avocado (Chernys and Zeevaart 2000), orange (Rodrigo *et al*. 2006), stylo (Yang and Guo 2007), and *Cuscuta reflexa* (Qin *et al*. 2008). In some species, *NCED*-like genes comprise a small multigene family, with only a subgroup involved in stress responses and regulation of ABA biosynthesis (Tan *et al*. 2003, Rodrigo *et al*. 2006).

The expression of *NCED* genes and the subsequent regulation of ABA biosynthesis have been studied extensively in several plant species. In general, water stress is known to induce the expression of *NCED* genes which is accompanied by an accumulation of ABA (Thompson *et al*. 2000, Xiong and Zhu 2003, Yang and Guo 2007) and an increase of the corresponding NCED proteins in leaves (Qin and Zeevaart 1999). Regulation of ABA biosynthesis has mainly been studied in vegetative tissues of several plant species in response to stress conditions. The accumulation of ABA is well correlated with an increased expression of *NCED* genes and accumulation of corresponding NCED proteins (Qin and Zeevaart 1999, Xiong and Zhu 2003, Yang and Guo 2007). In the *Arabidopsis NCED* family, *AtNCED2* and *AtNCED3* are expressed in roots (Tan *et al*. 2003), whereas *AtNCED6* and *AtNCED9* expression is detected in seeds (Lefebvre *et al*. 2006). *VuNCED1* from cowpea is strongly induced by salt stress (Iuchi *et al*. 2000). *SgNCED1* is induced in *Stylosanthes guianensis* leaves and roots under salinity, chilling, and drought (Yang and Guo 2007). Moreover, transgenic plants overexpressing *NCED* genes accumulate large amounts of ABA and are more resistant to stresses (Thompson *et al*. 2000, Iuchi *et al*. 2001, Qin and Zeevaart 2002, Zhang *et al.* 2008).

Common wheat is one of the most important cereal crops in the world; drought and water shortages are the major limiting factors that affect the production. Some genes involved in drought and salt stresses in wheat were previously isolated and characterized. Although several *NCED* genes have been cloned from different species, the homologous genes from wheat have not been studied. In the present study, a *TaNCED1* gene was isolated, its expression under various stress conditions and its overexpression in tobacco were studied.

Materials and methods

Plants and stress treatments: Common wheat (*Triticum aestivum* L.) cv. Jimai21 seedlings were grown in a greenhouse at temperatures from 25 to 30 ºC and irrigated appropriately. At 3 weeks of age, the plants were carefully removed from soil and roots were washed in water overnight. One set of plants was subjected to dehydration on filter papers at room temperature and relative humidity of approximately 40 % for 0, 1, 3, 6, 9, 12, and 24 h. For NaCl and ABA treatments, plants were immersed in a 200 mM NaCl or 100 µM ABA solution for 0, 1, 3, 6, 9, 12 and 24 h. For cold-stress treatment, plants were transferred to an incubator set at 4 ºC and subjected to the stress for 0, 1, 3, 6, 12, and 24 h. Harvested leaves were quickly frozen in liquid nitrogen and stored at -80 ºC until use.

Isolation of the *NCED1* gene from wheat: Genomic DNA was extracted by the CTAB method. PCR assays were performed with primers NCED1-P1 (Table 1) with *TransStart FastPfu* DNA polymerase (*Transgene*, Beijing, China) to amplify the gene. The 50 mm^3 reaction mixture contained 10 mm^3 of 5× buffer, 10 mm^3 of 5× stimulants, 5 mm^3 of dNTPs mix (2.5 mM each), 2 mm^3 of each primer (10 µM), 1 mm^3 of *FastPfu* DNA polymerase (2.5 U/mm^3), 5 mm^3 of DNA template and 15 mm^3 of sterile double distilled (dd) H_2O. PCR amplification was performed as follows: annealing at 94 ºC for 30 s, 11 cycles at 94 ºC for 30 s, at 67 ºC for 30 s, and at 72 ºC for 1 min 20 s, then 26 cycles at 94 ºC for 30 s, at 57 ºC for 30 s, and at 72 ºC for 1 min 20 s, and a final extension at 72 ºC for 10 min. The amplified 1.8 kb fragment was purified and subcloned into a *pEASY-Blunt* cloning kit (*Transgene*) for sequencing. The 3D structure was predicted using the *ExPASy* proteomics server and the *SWISS-MODEL* http://swissmodel.expasy.org/workspace/; Schwede *et al.* 2003, Amoid *et al.* 2006).

Southern blot analysis: Genomic DNA was isolated from leaves by the CTAB method. For Southern blot analysis, 30 µg DNA was digested overnight with *Eco*RI, *Hin*dIII, or *Nco*I with no cut sites in the gene, electrophoresed on 0.8 % (m/v) agarose gels and transferred onto a *Hybond* nylon membrane (*Roche*, Mannheim, Germany). The PCR fragment of *TaNCED1* was labeled with digoxigenin (DIG)-dUTP from a DIG-high prime kit (*Roche*) and used as a probe. Hybridization and detection steps were performed according to the manufacturer's instructions with the DIG nucleic acid detection kit (*Roche*).

RNA isolation, RT-PCR, and real-time PCR: Total RNA was extracted from leaves, stems, and roots of untreated or treated wheat as described above with the total RNA isolation kits (*Sangon*, Shanghai, China). The cDNA synthesis was performed with an RT reagent kit (*Takara*, Dalian, China) according to the manufacture's protocol. The primers for RT-PCR and real-time PCR were the following: *NCED1-P2* (Table 1) for the

TaNCED1 gene and *TaActin* (Table 1) (AB181991) for the common wheat *actin* gene (He *et al*. 2011). The products were loaded on 2 % (m/v) agarose gels. The experiments were repeated three times.

Real-time PCR was performed with an *ABI 7500* sequence-detection system (*Applied Biosystems*, Foster, CA, USA) following the manufacturer's instructions with the *SYBR Green* RT-PCR kit (*Takara*) in a 10 mm^3 reaction volume. The reaction mixtures contained 5 mm^3 of a *SYBR Green* PCR mix, 0.2 μmol of each forward and reverse primers, 1 mm^3 of the diluted cDNA template, and appropriate amounts of sterile ddH_2O. Amplification conditions were 95 ºC for 2 min followed by 40 cycles at 95 ºC for 15 s, at 56 ºC for 30 s, and at 72 ºC for 30 s. Fold changes in the amount of RNA transcript in the plants were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with the wheat *actin* gene as an internal control. Triplicate assays were performed on each cDNA sample.

The expression of the recombinant TaNCED1 protein in *Escherichia coli*: The complete open reading frame (ORF) of *TaNCED1* was subcloned into the pET-28a vector between the *Nde*I and *Eco*RI sites. The successful plasmid constructs formation was confirmed by restriction enzyme analysis and DNA sequencing, and then transformed into *E.coli* BL21 (DE3) cells to express a TaNCED1 fusion protein with a hexahistidine tag at the N-terminus.

Tobacco transformation and selection of transgenic lines: To construct an expression vector for tobacco transformation, the full-length *TaNCED1* gene was ligated into the vector pCAMBIA1301 containing an *hpt* gene conferring resistance to hygromycin under the control of the CaMV35S promoter. For tobacco transformation, leaf discs from *Nicotiana tabacum* L. grown *in vitro* (Horsch *et al.* 1985) were transformed with *Agrobacterium tumefaciens* strain LBA4404. Transformants were selected with 20 mg dm^{-3} hygromycin B (*Roche*) in Murashige and Skoog (MS) medium. Homozygous T2 generation seeds were used for further analysis. After screening for resistance to hygromycin, transgenic plants were verified by PCR. PCR was conducted with a primer *NCED1-P3* (Table 1). RT-PCR was performed with a gene-specific primer *NCED1-P4* (Table 1) to amplify the *TaNCED1* gene. A primer *NtActin* (Table 1) was designed to amplify an *actin* fragment as an internal control.

Evaluation of drought resistance in transgenic tobacco seedlings: For germination experiments, seeds of wild type and of three T2 independent transgenic lines (L1, L2, and L3) were sterilized and placed on filter paper saturated with various concentrations of PEG 6000 (0, 5, 10, and 20 %, m/v) in Petri dishes. The seeds held at 25 ºC for 5 d were considered as germinated when the radicle and green cotyledon emerged. Experiments with each concentration were repeated 3 times using 100 seeds.

After growth on the MS medium for one month, the seedlings of WT and T2 transgenic lines were transferred to pots filled with soil. At 6-leaf stage, the plants were subjected to drought stress for 7 d. During the stress period, soil water content was kept about 12 % of the maximum water capacity by controlling the amount of applied water. Leaf samples were harvested at different times. The physiological parameters measured included leaf relative water content (RWC), total soluble sugar content, and ABA content. Three replications were performed. RWC in leaves was determined as described by Schonfeld *et al* (1988). Total soluble sugars were extracted and estimated by the anthrone reagent using glucose as standard (Yemm and Willis 1954). ABA content was determined following the method of Wang *et al*. (2009).

Statistical analysis: *SPSS* software *v. 11.5* (*SPSS Inc.*, Chicago, USA) was used for statistical analysis. All values were expressed as means ± SD. Data were subjected to analysis of variance (*ANOVA*). Means were subjected to Duncan's multiple comparison tests.

Table 1. PCR primer forward and reverse sequences.

Gene	Primer sequence (5'-3') forward	reverse	Accession No.
NCED1-P1	5'-ATGCAGACTCTGTCGGCGCAGCC-3'	5'-TTAGTGCTGGGCGTCGAGGTCG-3'	JQ772528
NCED1-P2	5'-TCATCGCCGTCCACCAGG-3'	5'-GGGCCGCTCCAGGACATT-3'	JQ772528
TaActin	5'-GCCACACTGTTCCAATCTATGA-3'	5'-TGATGGAATTGTATGTCGCTTC-3'	AB181991
NCED1-P3	5'-CATCCCGCCCTTCATCA-3'	5'-CTCCTCCCACGAGTTCC-3'	JQ772528
NCED1-P4	5'-TCATCGCCGTCCACCAGG-3'	5'- GGGCCGCTCCAGGACATT-3'	JQ772528
NtActin	5'-CTATTCTCCGCTTTGGACTTGGCA-3'	5'-AGGACCTCAGGACAACGGAAACG-3'	JQ256516

Results

Genomic DNA of wheat leaves was extracted, and a homologous PCR-based technique was used for amplification of the expected fragment. The amplified products were separated by electrophoresis on 1.0 % agarose gels, and as expected, a band about 1.8 kb was amplified (Fig. 1*A*). Multiple alignments showed that this sequence shared high identity with *NCED1*s from other species ranging from 74 to 97 % (*e.g.*, barley 97 %, rice 89 %, sorghum 88 %, and maize 87 %). No intron was present. The sequence was submitted to GenBank (accession number JQ772528) and named as *TaNCED1*. *TaNCED1* contained a 1 848 bp ORF encoding a protein of 615 amino acids with predicted molecular mass of 66.7 kDa and an isoelectric point (pI) of 5.51. In Fig. 2, the output of the multiple sequence alignment is shaded according to degree of conservancy. Phylogenetic analysis showed that TaNCED1 shared high identity with other NCEDs and was closest to HvNCED1 (95 %; Fig. 3).

Southern blot analysis was performed to determine the copy number of *TaNCED1* in the wheat genome. The isolated genomic DNA was digested with *Eco*RI, *Hin*dIII, and *Nco*I, respectively. A DIG-labeled PCR fragment of the *TaNCED1* gene was used as a probe. Only one visible band occurred in lanes 1, 2, and 3 after separate digestions by the three restriction enzymes, respectively (Fig. 1*B*).

The tertiary structure prediction was analyzed by comparative protein modeling. The sequence was submitted to the *SWISS-MODEL* server and the automated mode was selected. The program automatically selected maize VP14 as a template which had the sequence similarity of 84.97 %. Therefore, the 3D structure of the TaNCED1 protein was generated using the maize VP14 (PDB ID: 3npeA) as model for *SWISS-MODEL* homology modelling (modelled residue range: 96-614aa). The *QMEAN Z*-score evaluation was -1.68 suggesting that the predicted model was of good quality (Fig. 1*C*).

The complete coding sequence of *TaNCED1* was subcloned into pET28a. After induction by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 ºC for 1, 2, and 3 h, the highest expression occurred with 1 mM of IPTG and 3 h of induction at 37 ºC. SDS-PAGE resulted in the induction and purification of TaNCED1 protein (Fig. 4*A*). A protein with the highest abundance was found in the *E. coli* extracts. The molecular mass of the induced protein was about 66 kDa in accordance with the predicted amino acid sequence (TaNCED1 615 amino acids + His and S tag of 36 amino acids).

RNA was isolated from roots, stems and leaves of wheat grown under normal conditions. The expressions of *TaNCED1* in the different tissues were examined using RT-PCR and qPCR. The highest amount of the *TaNCED1* transcripts was found in leaves, followed by stems and roots, using the a*ctin* gene as an endogenous control (Fig. 4*B, C*).

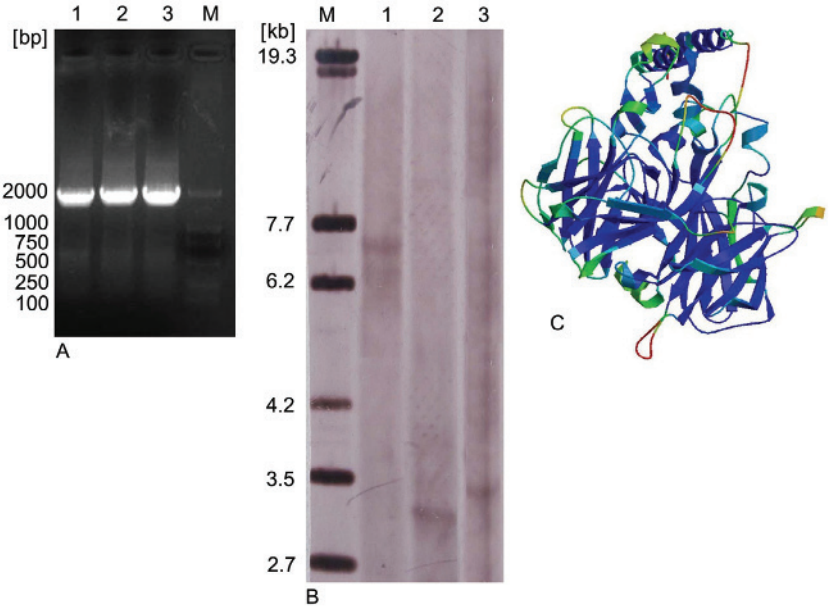

Fig. 1. Molecular characterization of *TaNCED1*. *A* - PCR amplification of *TaNCED1*. *Lanes* 1 to 3 - PCR fragments of *TaNCED1, lane* M - a DNA molecular mass marker DL2000. *B* - Southern blot analysis of the *TaNCED1* gene. Genomic DNA of wheat was digested with restriction enzymes *Eco*RI, *Hin*dIII, and *Nco*I, respectively. *Lane* M - a DNA molecular mass marker EcoT14; *lane* 1 - genomic DNA digested with *Eco*RI, *lane* 2 - genomic DNA digested with *Hin*dIII, *lane* 3 - genomic DNA digested with *Nco*I. *C* - Predicted three-dimensional structure of the TaNCED1 protein.

Fig. 2. Multiple sequence alignment of amino acid sequences of TaNCED1(GenBank JQ772528) with four other species: barley (HvNCED1, AB239297); rice (OsJNCED, NP-001050765); sorghum (SbNCED, xp_002466753.1); maize (ZmNCED, NP001105902.2). Protein sequences were aligned using the *CLUSTALW* alignment algorithm. Sequences were shaded using the *BoxShade* program. Identical and conserved residues are shaded *black* and *grey*, respectively. In the picture, TaNCED1 showed high identity with other NCED1s.

Three-week-old wheat plants were removed from the soil and subjected to various stresses. The plants cultured in water were used as controls. *TaNCED1* was significantly induced by dehydration stress. *TaNCED1* mRNA began to accumulate within 1 h from the start of dehydration, subsequently declined at 3 and 6 h, and again increased at 9 h before declining (Fig. 5*A*). For NaCl stress, increasing transcription was detected at 1 h, and the expression peak was detected at 6 h, and then declined (Fig. 5*B*). For cold stress, transcription was the highest after 3 h, declined quickly after 6 h, but increased again after 9 h before slowly declining at 12 and 24 h (Fig. 5*C*). For ABA application, *TaNCED1* expression was also up-regulated (Fig. 5*D*).

Tobacco leaf discs were transformed *via Agrobacterium tumefaciens* with the pCAMBIA1301-*TaNCED1* (Fig. 6*A*). The transformants were selected by PCR assays using primers designed for the *TaNCED1* gene. Sixteen independent transgenic tobacco lines were identified, and a target band of 826 bp was present in the transgenic plants, whereas no corresponding band was detected in the WT plants (Fig. 6*B*). The expression of *TaNCED1* in the transgenic plants was confirmed by RT-PCR (Fig. 6*C*). The mRNA transcripts of *TaNCED1* in the transgenic lines were different, and no expression of *TaNCED1* was detected in the WT plants. Three independent lines with higher transgene expression were chosen for drought tolerance experiments.

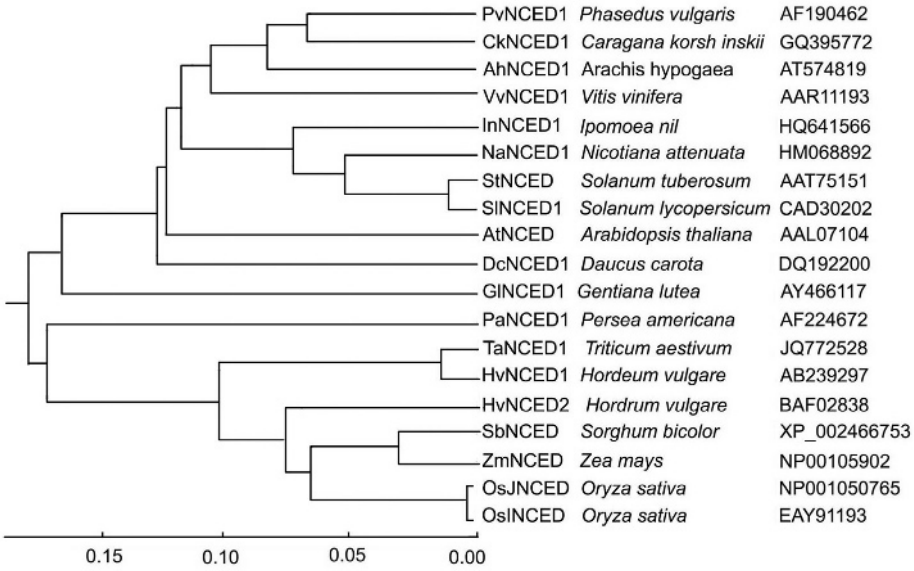

Fig. 3. Phylogenetic analysis of TaNCED1 in common wheat with NCEDs in other plant species. A rooted phylogenetic tree based on the sequence alignment using *MEGA 5.1* software from *CLUSTALW* multiple sequence alignment. The scale represents estimated branch length.

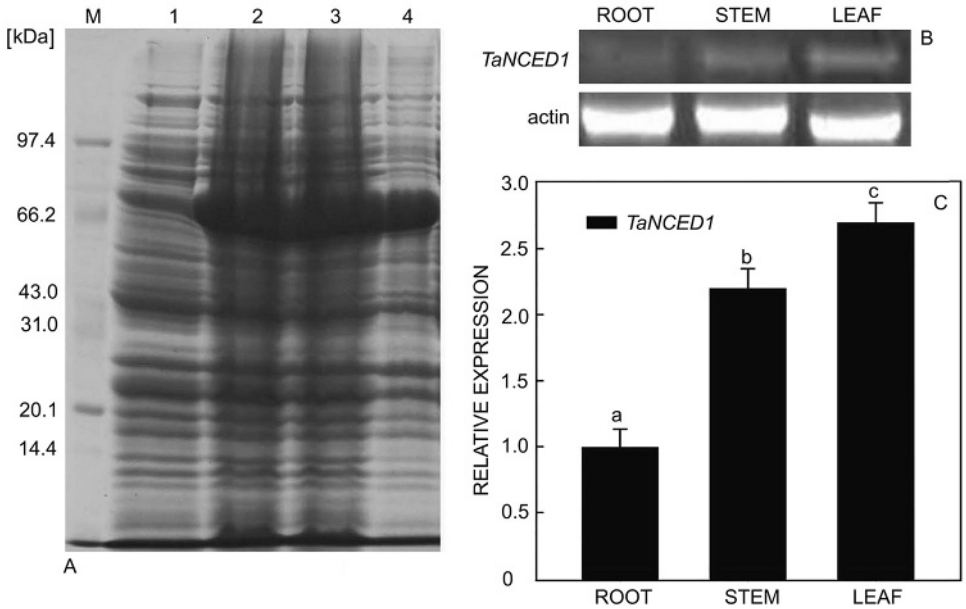

Fig. 4. *A* - Expression and purification of a recombinant TaNCED1 protein in *E.coli* BL21 (DE3). *Lane* M - protein markers, *lane* 1 - bacterial proteins form BL21 (DE3) transformed with pET28a-*TaNCED1* uninduced with IPTG, *lanes* 2 to 4 - bacterial proteins from BL21 (DE3) transformed with pET28a-*TaNCED1* induced with 1 mM IPTG at 37 ºC for 1, 2, and 3 h, respectively. *B* - Detection of *TaNCED1* transcripts in various tissues by RT-PCR. *C* - Detection of *TaNCED1* transcripts by real-time RT-PCR. The common wheat *actin* gene was used as an internal control. Values are means ± SD of three independent replicates. Different small letters represent statistical significance between means for each treatment ($P < 0.05$, Duncan's test).

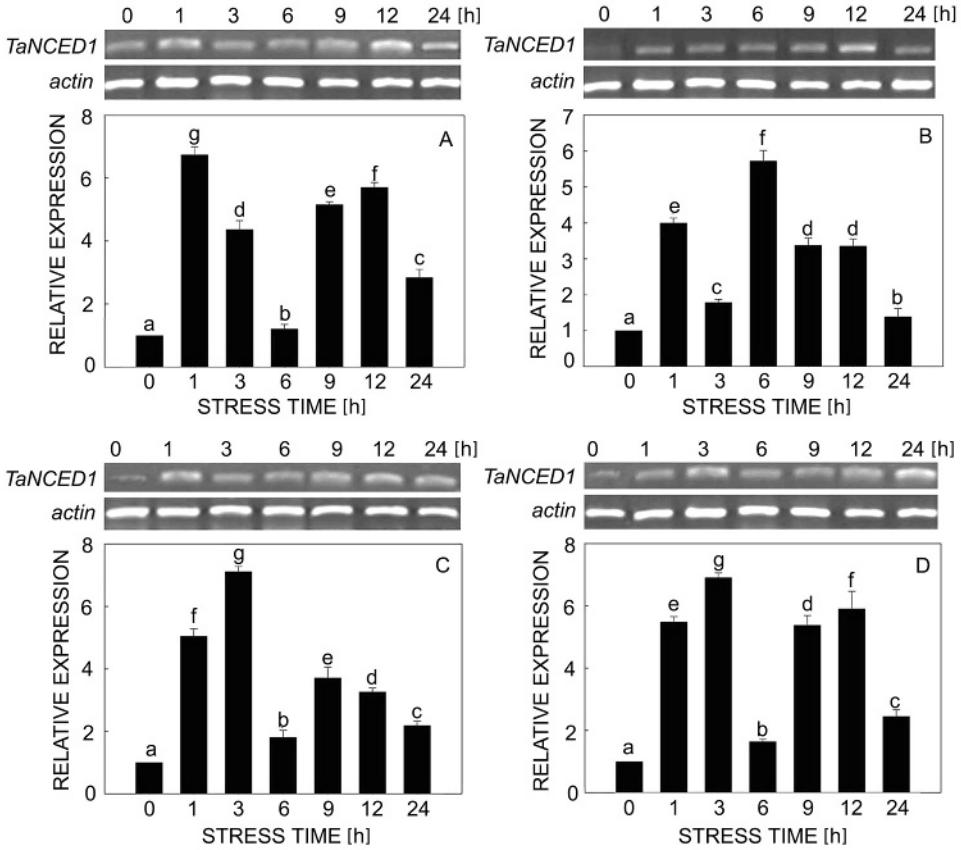

Fig. 5. Expression pattern of the *TaNCED1* gene under different stresses. *TaNCED1* transcript expression under dehydration (*A*), salt (*B*), cold (*C*) and ABA (*D*) stress conditions were analyzed after the treatments for 0, 1, 3, 6, 9, 12, and 24 h. The wheat *actin* gene was used as an internal control. Means ± SD of three independent replicates. Different small letters represent statistical significance between means for each treatment ($P < 0.05$, Duncan's test).

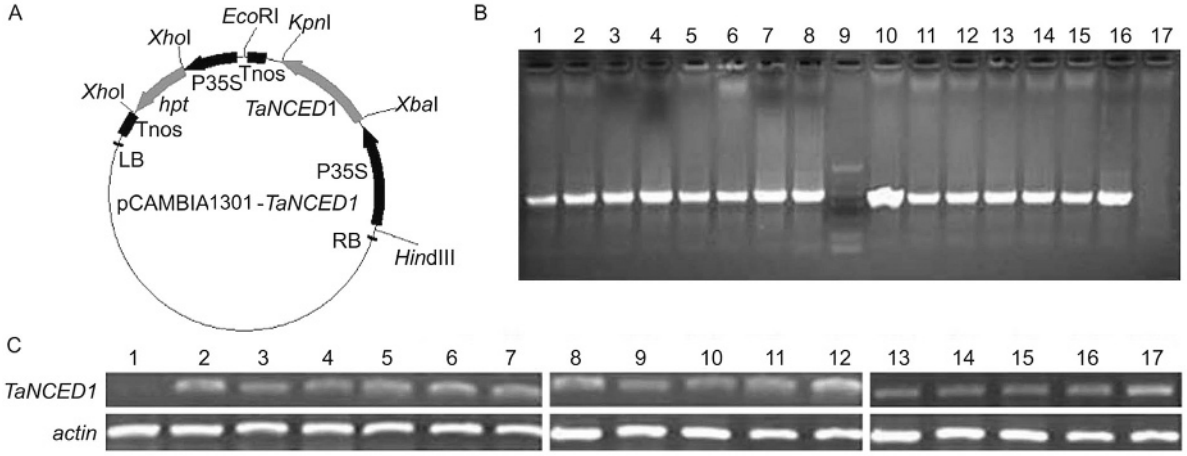

Fig. 6. Overexpression of *TaNCED1* in tobacco. *A* - a T-DNA binary construct used for tobacco transformation. The *TaNCED1* gene was inserted between the CaMV35S promoter (P35S) and NOS terminator (Tnos). *B* - PCR analysis of *TaNCED1*. *Lanes* 1 to 8 and 11 to 16 - DNA from independent transgenic lines, *lane* 9 - DNA marker; *lane* 10 - plasmid. *C* - RT-PCR analysis of transgenic tobacco lines. *Lane* 1 - WT; *lanes* 2 to 17 - independent transgenic lines.

Tobacco seeds were planted on MS media plates supplemented with 0, 1, 5, 15 and 20 % (m/v) PEG. There were no obvious differences in germination rates between the transgenic and WT plants reaching almost 100 % on the MS medium without PEG (Table 2). When the medium was supplemented with PEG, germination rate decreased but germination rates of the transgenic lines were significantly higher than those of WT, the

Table 2. Seed germination [%] of WT and transgenic plants under different PEG concentrations. Means ± SD of three independent replicates from different plants. Means with different small letters in the column are significantly different at $P < 0.05$ (Duncan's test).

Plants	PEG 0 %	PEG 5 %	PEG 10 %	PEG 20 %
WT	100	63.23±3.22a	45.34±3.01a	37.12±3.61a
L1	100	74.37±3.51b	62.16±3.20b	50.32±4.52b
L2	100	77.50±5.41c	62.12±4.81b	64.31±2.69d
L3	100	77.25±4.54c	64.40±3.33b	57.93±4.15c

difference was the highest at 10 % PEG (Table 2). Before the stress, there were no obvious differences in the leaf RWC between the WT and transgenic plants, with a range of 87 - 90 % (Table 3). During the stress treatment, the RWC of all leaves declined, but that of the transgenic plants declined more slowly than that of WT. After 7 d of the drought stress, the RWC of the transgenic plants declined by 21 % compared to 32 % in the WT plants. The content of total soluble sugars in both the WT and transgenic plants was rather low and similar before stress. During the stress treatment, the sugar content increased, and was the highest after 7 d of the drought stress. It was always higher in the transgenic plants than in the WT. ABA was gradually accumulated in leaves of plants under the drought stress in a duration-dependent fashion. The ABA content in the transgenics was higher than that in the WT plants suggesting that *TaNCED1* was responsible for increased ABA biosynthesis during the drought stress.

Table 3 Effect of drought stress treatment on the WT and transgenic tobacco plants. Means ± SD of three independent replicates from different transgenic lines. Means with different small letters in the column are statistical different at $P < 0.05$ (Duncan's test).

Prameter	Plant type	Drought [d] 0	1	3	5	7
RWC [%]	WT	87.19±2.40a	84.23±4.12a	76.14±3.52a	69.32±2.31a	58.40±3.21a
	L1	88.82±3.06a	85.17±4.08a	80.26±3.22c	73.92±3.64b	61.10±2.55b
	L2	86.34±3.52a	84.20±2.51a	81.10±2.30c	72.30±4.12b	63.71±2.60c
	L3	87.10±3.85a	84.45±2.14a	78.42±3.13b	73.47±3.16b	64.16±3.30c
Soluble sugar	WT	2.31±0.03ab	2.71±0.02a	3.46±0.03a	4.03±0.01a	4.68±0.02a
[mg g^{-1}(f.m.)]	L1	2.72±0.02b	3.73±0.01b	4.96±0.01b	6.68±0.03b	7.63±0.03b
	L2	2.45±0.03c	3.81±0.01b	5.52±0.03c	7.09±0.04c	7.67±0.04b
	L3	2.26±0.01a	3.78±0.02b	5.80±0.05d	7.59±0.06d	7.96±0.03c
ABA	WT	0.40±0.02a	1.12±0.01a	1.91±0.06a	2.90±0.02a	3.51±0.02a
[ng g^{-1}(f.m.)]	L1	0.72±0.01b	1.64±0.01b	2.80±0.01bc	3.93±0.02b	5.12±0.02b
	L2	0.91±0.05b	1.82±0.02c	2.73±0.01b	4.15±0.01c	5.54±0.01c
	L3	0.80±0.03c	1.91±0.04c	2.95±0.02c	4.51±0.01c	5.73±0.03d

Discussion

It is well known that ABA plays an important role in mediating responses to environmental stresses. The 9-*cis*-epoxycarotenoid cleavage reaction is thought to be one of the rate-limiting steps in ABA biosynthesis. *NCEDs* are encoded by a gene family in several plant species (Chernys and Zeevaart 2000, Rodrigo *et al*. 2006). The present study describes the first successful isolation of a putative *TaNCED1* gene from common wheat. The predicted TaNCED1 protein shared high sequence identity with other plant NCEDs and contained conserved structural features present in all NCEDs. *In vitro* expression of the full-length coding region of *TaNCED1* indicates that the molecular mass of the TaNCED1 protein was in accordance with the predicted amino acid sequences, similar to the reports for other plant species (Tan *et al*. 1997, 2003, Burbidge *et al*. 1999, Chernys and Zeevaart 2000, Iuchi *et al*. 2000).

Stress-induced *NCED* gene expressions were observed in several plant species, such as maize (Schwartz *et al*. 1997), bean (Qin and Zeevaart 1999), tomato (Thompson *et al*. 2000), cowpea (Iuchi *et al*. 2001), orange (Rodrigo *et al*. 2006), stylo (Yang and Guo, 2007), *Caragana korshinskii* (Wang *et al*. 2009), *etc*. In this study, *TaNCED1* was induced not only by dehydration, salt, or cold, but also by ABA (Fig. 5) suggesting that *TaNCED1* is involved in an intricate network of multi-environmental stress responses. *TaNCED1* expression reached peaks at different time points. In the dehydration stress experiments, the highest expression was induced already after 1 h, whereas under the NaCl stress, the transcription progressively increased and reached the highest expression after 6 h. Under both the cold and ABA treatments, peak levels were reached at 3 h. These differences may suggest that *TaNCED1* is

most sensitive to drought stress.

A functional analysis of *TaNCED1* in drought stress tolerance was carried out using transgenic lines of tobacco overexpressing *TaNCED1*. These transgenic tobacco lines had higher tolerance to the drought stress than the WT. Seed germination of the transgenic tobacco plants in a 10 % PEG solution was significantly higher than that of the WT plants. In drought stress experiments, the RWC and content of soluble sugars and ABA of the transgenic tobacco lines were significantly higher than those of the WT plants. Higher content of soluble sugars, which function as osmolytes, suggests that *TaNCED1* might be involved not only in sugar metabolism but also in osmotic adjustment. Accumulation of soluble sugars is strongly correlated to the acquisition of drought tolerance in plants (Hoekstra *et al.* 2001). Several studies have indicated that overexpression of *NCED* genes in transgenic plants enhance ABA accumulation and increase tolerance to drought (Iuchi *et al.* 2001, Qin and Zeevaart 2002, Aswath *et al.* 2005, Wan and Li 2006, Zhang *et al.* 2008, 2009, Hwang *et al.* 2010), salt stress (Aswath *et al.* 2005, Zhang *et al.* 2008), and cold stress (Yang *et al.* 2007). The transgenic plants maintained a higher ABA content than WT during drought stress indicating that the transgenic lines possessed high stress tolerance. The increased soluble sugar and ABA content facilitated maintenance of the high RWC. Comparison of the RWC between the transgenic and WT plants shows that drought tolerance of the transgenic plants was correlated with a higher RWC. These findings suggest that *TaNCED1* might play an important role in drought stress response.

NCEDs are believed to be involved in ABA signalling, and several members have been identified as positive regulators of ABA signalling and stress tolerance (Aswath *et al*. 2005). For example, *NCED* transcription is induced by abiotic stresses and exogenous ABA (Iuchi *et al.* 2000, Wang *et al.* 2009). These results identified *NCED* as a molecular link between stress- and ABA-induced calcium signalling and gene expression in plant cells. In this study, *TaNCED1* was positively regulated by exogenous ABA, and the *TaNCED1*-overexpressing plants accumulated more ABA under the drought stress treatment. The stress might induce the up-regulation of the *NCED* gene expression, and correspondingly enhanced amount of NCED proteins which led to the accumulation of ABA, which in turn, substantially mediated the expression of genes related to stress tolerance.

It was reported here that the *NCED* genes improved tolerance to the drought, salt, and cold stresses in the transgenic plants. As an ortholog of *NCED*, the *TaNCED1* transcription was elevated by the multiple stresses (Fig. 5). Therefore, *TaNCED1* should be involved in multiple stress responses. The transgenic tobacco plants had a higher drought tolerance than the WT plants. These findings suggest that we obtained a *NCED1*-like gene that encodes 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme involved in ABA biosynthesis, which may be useful for producing transgenic plants with an improved tolerance to abiotic stresses. The results provide useful information for further improving stress tolerance of crops.

References

Amoid, K., Bordoli, L., Kopp, J., Schwede, T.: The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. - Bioinformatics **22**: 195-201, 2006.

Aswath, C.R., Kim, S.H., Mo, S.Y., Kim, D.H.: Transgenic plants of creeping bent grass harboring the stress inducible gene, 9-*cis*-epoxycarotenoid dioxygenase, are highly tolerant to drought and NaCl stress. - J. Plant. Growth. Regul. **47**: 129-139, 2005.

Burbidge, A., Grieve, T.M., Jackson, A., Thompson, A., McCarty, D.R.: Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14*. - Plant J. **17**: 427-431,1999.

Chernys, J.T., Zeevaart, J.A.D.: Characterization of the 9-*cis*-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. - Plant Physiol. **124**: 343-353, 2000.

Davies, W.J., Zhang, J.: Root signals and the regulation of growth and development of plants in drying soil. - Annu. Rev. Plant Physiol. Plant mol. Biol. **42**: 55-76, 1991.

He, C.M., Zhang, W.W., Gao, Q., Yang, A.F., Hu, X.R., Zhang, J.R.: Enhancement of drought resistance and biomass by increasing the amount of glycine betaine in wheat seedlings. - Euphytica **177**: 151-167, 2011.

Hoekstra, F.A., Golovina, E.A., Buitink, J.: Mechanisms of plant desiccation tolerance. - Trends Plant Sci. **6**: 431-438, 2001.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Roger, S.G., Fraley, R.T.: A simple and general method for transferring genes into plants. - Science. **227**: 1229-1231, 1985.

Hwang, S.G., Chen, H.C., Huang, W.Y., Chu, Y.C., Shii, C.T., Cheng, W.H.: Ectopic expression of rice *OsNCED3* in *Arabidopsis* increases ABA level and alters leaf morphology. - Plant Sci. **178**: 12-22, 2010.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K., Shinozaki, K.: Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. - Plant J. **27**: 325-333, 2001.

Iuchi, S., Kobayashi, M., Yamaguchi-Shinozaki, K., Shinozaki, K.: A stress-inducible gene for 9-*cis*-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. - Plant Physiol. **123**: 553-562, 2000.

Kende, H., Zeevaart, J.A.D.: The five classical plant hormones. - Plant Cell **9**: 1197-1210, 1997.

Lefebvre, V., North, H., Frey, A., Sotta, B., Seo, M., Okamoto, M., Nambara, E., Marion-Poll, A.: Functional analysis of *Arabidopsis NCED6* and *NCED9* genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. - Plant J. **45**: 309-319, 2006.

Leung, J., Giraudat, J.: Abscisic acid signal transduction. - Annu Rev. Plant Physiol. Plant mol. Biol. **49**: 199-222, 1998.

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. - Methods **25**: 402-408, 2001.

Mansouri, H., Asrar, Z.: Effects of abscisic acid on content and biosynthesis of terpenoids in *Cannabis sativa* at vegetative stage. - Biol. Plant. **56**: 153-156, 2012.

Nambara, E., Marion-Poll, A.: Abscisic acid biosynthesis and catabolism. - Annu. Rev. Plant. Biol. **56**: 165-185, 2005.

Qin, X., Zeevaart, J.A.D.: The 9-*cis*-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. - Proc. nat. Acad. Sci. USA **96**: 15354-15361, 1999.

Qin, X., Zeevaart, J.A.D.: Overexpression of a 9-*cis*-epoxycarotenoid deoxygenase gene in *Nicotinana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. – Plant Physiol. **128**: 544-551, 2002.

Qin, X.Q., Yang, S.H., Kepsel, A.C., Schwartz, S.H., Zeevaart, J.A.D.: Evidence for abscisic acid biosynthesis in *Cuscuta reflexa*, a parasitic plant lacking neoxanthin. - Plant. Physiol. **147**: 816-822, 2008.

Rodrigo, M.J., Alquezar, B., Zacarías, L.: Cloning and characterization of two 9-*cis*-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). - J. exp. Bot. **57**: 633-643, 2006.

Schonfeld, M.A., Johnson, R.C., Carver, B.F.: Water relations in winter wheat as drought resistance indicator. - Crop. Sci. **28**: 526-531, 1988.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A.D., McCarty, D.R.: Specific oxidative cleavage of carotenoids by VP14 of maize. - Science **276**: 1872-1874, 1997.

Schwede, T., Kopp, J., Guex, N., Peitsch, M.C.: SWISS-MODEL: an automated protein homology-modeling server. - Nucl. Acids Res. **31**: 3381-3385, 2003.

Seo, M., Koshiba, T.: Complex regulation of ABA biosynthesis in plants. - Trends Plant Sci. **7**: 41-48, 2002.

Tan, B.C., Joseph, L.M., Deng, W.T., Liu, L., Cline, K., McCarty, D.R.: Molecular characterization of the *Arabidopsis* 9-*cis*-epoxycarotenoid dioxygenase gene family. - Plant J. **35**: 44-56, 2003.

Tan, B.C., Schwartz, S.H., Zeevaart, J.A.D., McCarty, D.R.: Genetic control of abscisic acid biosynthesis in maize. - Proc. nat. Acad. Sci. USA **94**: 12235-12240, 1997.

Taylor, I.B., Sonneveld, T., Bugg, T.D.H., Thompson, A.J.: Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. - J. Plant Growth Regul. **24**: 253-273, 2005.

Thompson, A.J., Jackson, A.C., Parker, R.A., Morpeth, D.R., Burbidge, A., Taylor, I.B.: Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9-*cis*-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. - Plant mol. Biol. **42**: 833-845, 2000.

Wan, X.R., Li, L.: Regulation of ABA level and water-stress tolerance of *Arabidopsis* by ectopic expression of a peanut 9-*cis*-epoxycarotenoid dioxygenase gene. - Biochem. biophys. Res. Commun. **347**: 1030-1038, 2006.

Wang, X.M., Wang, Z., Dong, J., Wang, M.Z., Gao, H.W.: Cloning of a 9-*cis*-epoxycarotenoid dioxygenase gene and the responses of *Caragana korshinskii* to a variety of abiotic stresses. - Genes Genet. Sys.t **84**: 397-405, 2009.

Xiong, L., Zhu, J.: Regulation of abscisic acid biosynthesis. - Plant. Physiol. **133**: 29-36, 2003.

Yamaguchi-Shinozaki, K., Shinozaki, K.: Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. - Annu. Rev. Plant Biol. **57**: 781-803, 2006.

Yang, J.F., Guo, Z.F.: Cloning of a 9-*cis*-epoxycarotenoid dioxygenase gene (*SgNCED1*) from *Stylosanthes guianensis* and its expression in response to abiotic stresses. - Plant Cell Rep. **26**: 1383-1390, 2007.

Yemn, E.W., Willis A.J.: The estimation of carbohydrates in plant extracts by the anthrone. - Biochem. J. **57**: 508-514, 1954.

Zeevaart, J.A.D., Creelman, R.: Metabolism and physiology of abscisic acid. - Annu. Rev. Plant Physiol. Plant mol. Biol. **39**: 439-473, 1988.

Zhang, Y., Yang, J., Lu, S., Cai, J., Guo, Z.: Overexpressing *SgNCED1* in tobacco increases ABA level, antioxidant enzyme activities, and stress tolerance. - J. Plant Growth Regul. **27**: 151-158, 2008.

Zhang, Y.M., Tan, J.L., Guo, Z.F., Lu, S.Y., He, S.J., S, W., Zhou, B.Y.: Increased abscisic acid levels in transgenic tobacco over-expressing 9-*cis*-epoxycarotenoid dioxygenase influence H_2O_2 and NO production and antioxidant defences. - Plant Cell Environ. **5**: 509-519, 2009.