Journal of Plant Physiology 175 (2015) 26–36

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Molecular Biology

Positive feedback regulation of a *Lycium chinense*-derived VDE gene by drought-induced endogenous ABA, and over-expression of this VDE gene improve drought-induced photo-damage in *Arabidopsis*

Chunfeng Guan[a], Jing Ji[a,*], Xuqiang Zhang[a], Xiaozhou Li[c], Chao Jin[a], Wenzhu Guan[b], Gang Wang[a,*]

[a] School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, People's Republic of China
[b] School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China
[c] Department of Medical Genetics, Tianjin Medical University General Hospital, Tianjin 300072, People's Republic of China

ARTICLE INFO

Article history:
Received 22 March 2014
Received in revised form 4 June 2014
Accepted 24 June 2014
Available online 18 November 2014

Keywords:
Photochemical quenching
Endogenous ABA
L. chinense
VDE
Drought stress

ABSTRACT

Violaxanthin de-epoxidase (VDE) plays an important role in protecting the photosynthetic apparatus from photo-damage by dissipating excessively absorbed light energy as heat, via the conversion of violaxanthin (V) to intermediate product antheraxanthin (A) and final product zeaxanthin (Z) under light stress. We have cloned a VDE gene (*LcVDE*) from *Lycium chinense*, a deciduous woody perennial halophyte, which can grow in a large variety of soil types. The amino acid sequence of *LcVDE* has high homology with VDEs in other plants. Under drought stress, relative expression of *LcVDE* and the de-epoxidation ratio (Z + 0.5 A)/(V + A + Z) increased rapidly, and non-photochemical quenching (NPQ) also rose. Interestingly, these elevations induced by drought stress were reduced by the topical administration of abamine SG, a potent ABA inhibitor via inhibition of NCED in the ABA synthesis pathway. Until now, little has been done to explore the relationship between endogenous ABA and the expression of VDE genes. Since V serves as a common precursor for ABA, these data support the possible involvement of endogenous ABA in the positive feedback regulation of *LcVDE* gene expression in *L. chinense* under drought stress. Moreover, the LcVDE may be involved in modulating the level of photosynthesis damage caused by drought stress. Furthermore, the ratio of (Z + 0.5 A)/(V + A + Z) and NPQ increased more in transgenic *Arabidopsis* over-expressing *LcVDE* gene than the wild types under drought stress. The maximum quantum yield of primary photochemistry of PSII (Fv/Fm) in transgenic *Arabidopsis* decreased more slowly during the stressed period than that in wild types under the same conditions. Furthermore, transgenic Arabidopsis over-expressing *LcVDE* showed increased tolerance to drought stress.

Introduction

Plants can absorb light energy for the purpose of converting them to chemical energy through photosynthesis, but the absorption of too much light that exceeds the photosynthetic capacity can be deleterious (Pinheiro and Chaves, 2011). Plants have evolved a series of photo-protective mechanisms to reduce light damage, including the non-photochemical quenching of fluorescence (NPQ) and redox-regulated changes in gene expression in response to excess light. NPQ mediates the thermal dissipation of excess light energy absorbed by the light-harvesting antenna complex (LHC) of photo-system II (PS II) (Niyogi and Truong, 2013). NPQ is mainly modulated by the inter-conversion of epoxidized to de-epoxidized forms of xanthophyll carotenoids during the so-called xanthophyll cycle (XC). Zeaxanthin (Z), the product of the XC, is formed by de-epoxidation of violaxanthin (V) via antheraxanthin (A), and is accumulated in plants that are exposed to excess light. This process is catalyzed by violaxanthin de-epoxidase (VDE) under high light conditions. Under low light conditions, Z is converted back to V by zeaxanthin epoxidase (ZEP) (Jahns and Holzwarth, 2012).

VDE is classified as a lipocalin-like protein (Frenette Charron et al., 2005) and the central lipocalin catalytic domain was considered to be the binding site for the V substrate (Arnoux et al., 2009). VDE homologs have a conserved C-terminal tail that contains a high number of glutamate residues and an N-terminal peptide that targets the protein to chloroplast. The VDE activities are regulated by the lumen pH and the process requires ascorbate as a cofactor (Arnoux et al., 2009). Since the cDNA of VDE was first cloned from

* Corresponding authors. Tel.: +86 22 87402200; fax: +86 22 87402171.
E-mail addresses: jijing@tju.edu.cn (J. Ji), gangwang@tju.edu.cn (G. Wang).

http://dx.doi.org/10.1016/j.jplph.2014.06.022
0176-1617/

romaine lettuce (Bugos and Yamamoto, 1996), the VDE genes have been characterized from several plants (Bouvier et al., 1996; Huang et al., 2007; Li et al., 2013). The regulation of VDE could take place at transcriptional or post-transcriptional levels. It has been considered that the VDE expression pattern is influenced by light and other environmental factors (Facella et al., 2008; North et al., 2005; Zhao et al., 2012).

Drought is one of the most common environmental stresses that limit agricultural production worldwide. The cellular and molecular responses in plants to environmental drought stress have been studied intensively (Farooq et al., 2012). It has been proposed that, under water deficit, plants experience drought stress that stimulates a series of physiological and biochemical responses including stomata closure, inhibition of cell growth, repression of photosynthesis and activation of respiration (Shinozaki and Yamaguchi-Shinozaki, 2007). Drought can inhibit leaf photosynthesis in most green plants, and cause generally a considerable reduction in contents of important photosynthetic pigments, particularly chlorophyll (Chl). This reduction may occur due to stress-induced impairment in pigment biosynthetic pathways or in pigment degradation (Farooq et al., 2012). However, the extent of these phenomena depends on the species, variety, duration of plant exposure, and tolerance to the stress. A number of earlier studies have shown that drought stress adversely affected the functionality of both PSII and PSI, particularly PSII (Ashraf and Harris, 2013).

Abscisic acid (ABA) is one of the important plant hormones, which plays a critical role in triggering plant responses to adverse environmental stimuli including drought stress. The signals during drought stress appear to be mediated by ABA (Zhang et al., 2006). Since V serves as a common precursor for ABA and Z is involved in energy dissipation (Kalituho et al., 2007), it is reasonable to assume that plants supplied with exogenous ABA should show better protection against the damage from high light stress, because more of the violaxanthin pool would be available for the formation of Z, and thus enhance xanthophyll cycle activities (Zhu et al., 2011). It has been reported that ABA may protect photosynthetic apparatus against photo-damage through an enhanced xanthophyll cycle (Ivanov et al., 1995) and an acceleration of the recovery of the PS II complex from the photo-inactivated state (Saradhi et al., 2000). However, the roles of ABA in modulating the VDE activities remain to be elucidated.

Due to the positive association between drought stress and stress-induced endogenous ABA, and the possible correlation between ABA treatment and the VDE activity, it is assumed that there is a positive link between drought stress and the VDE activity through an ABA-dependent signaling pathway. However, until now, little has been done to explore the relationship between drought stress, endogenous ABA, and the expression of VDE genes and we will investigate these link. In the present study, a novel VDE gene, from leaves of *Lycium chinense*, was isolated and characterized, designated as *LcVDE*. The transcriptional expression of *LcVDE* was detected in all plant organs. The correlation between *LcVDE* transcript expression and drought induced-endogenous ABA accumulation was explored in this study. We evaluated if the increase in endogenous ABA levels observed was a consequence of drought stress required for the induction of *LcVDE* gene expression, and if the over-expression of *LcVDE* gene enhances plant tolerance to drought stress-induced photosynthesis damage in *Arabidopsis*.

Materials and methods

Plant growth and drought stress or abamine SG treatment

Lycium chinense seedlings were grown in a controlled-growth chamber under a 12-h photoperiod at temperatures ranging from about 17 to 25 °C, the photosynthetic photon flux density of 500 μmol photons m^{-2} s^{-1} and the relative humidity of 70–75% in the growth chamber. Experiments were carried out using 15 weeks old plants with 35–40 leaves. Different tissues, including flowers, leaves, stems, and roots were harvested separately from the same *L. chinense* plants, which were not treated with any stress. All samples were immediately frozen in liquid nitrogen before being used.

Drought stress was imposed on 15 weeks old plants and these plants were maintained at 15% soil moisture level for 4 weeks. Throughout this period, soil moisture levels were monitored using a moisture meter HH2 (Delta T devices). The remaining batch of plants was used as controls and watered frequently. Since the beginning of drought stress, the younger fully expanded leaves were used for QF-PCR, endogenous ABA content, Fv/Fm, NPQ and de-epoxidation ratio assays on days 0, 7, 14, 21, and 28 from control and stressed plants. Five replications were maintained by growing them independently.

Treatments with ABA were performed by spraying cis-, trans-ABA (Sigma) solution, at a concentration of 100 μM, onto the plants without drought stress treatment until fully soaked. Abamine SG (Sigma) is a potent ABA inhibitor via inhibition of nine-cisepoxycarotenoid dioxygenase (NCED) in the ABA synthesis pathway (Kitahata et al., 2006). Ten micromolar was sprayed topically onto leaves of unstressed or drought stressed seedlings once a day (approximately 250 μL/plant) with at least 10 pots of plants per treatment. At the same time, at least 10 pots of plant were used as control without any treatments.

Cloning of the LcVDE gene

The *L. chinense* plants used for the investigation were cultivated at 25/18 °C in a controlled-growth chamber (16 h light/8 h dark). The fresh leaves were collected, frozen immediately in liquid nitrogen and stored at −80 °C prior to total RNA extraction. Total RNA was extracted by TRIzol™ reagent according to manufacturer's instructions (Tiangen, China).

Primers of the VDE gene were designed based on the EST unigene of *L. chinense* sequenced by BGI (Beijing Genomics Institute). A gene specific upstream primer LcVDEF1 5′-ATGGCTCTCGCCCCTTATTC-3′ was designed and synthesized for 3′ RACE analysis according to manufacturer's instructions. An aliquot of 1 μg total RNA from leaves was reversely transcribed to get the 1st strand cDNA with reverse transcriptase M-MLV and 3′-adaptor primer A using the 3′-Full RACE Core Set Ver.2.0 Kit (Takara, Japan). Subsequently, LcVDEF1 and 3′RACE Outer Primer (provided in the kit) were used for PCR amplification of target gene. The amplified product was purified, ligated into pMD18-T vector and cloned into *Escherichia coli* strain *DH5α* followed by sequencing.

Bioinformatic and molecular evolution analysis

The obtained DNA sequence and deduced protein were analyzed using bioinformatic tools provided by the following websites www.ncbi.nlm.nih.gov and www.expasy.org. The multiple sequence alignment was conducted with Vector NTI 8.0. Phylogenetic analysis of LcVDE was aligned with CLUSTAL W and evolutionary analysis was constructed with the neighbor-joining (NJ) method using MEGA 2 software (Tamura et al., 2007).

Arabidopsis transformation and drought treatment in transgenic Arabidopsis

To produce transgenic plants, the coding region of the *LcVDE* cDNA was cloned into the pCAMBIA2300 vector, which contains the CaMV35S promoter by using a gene specific upstream primer LcVDEF2: CGCGGATCCATGGCTCTCGCCCCTTATTC, and

downstream primer LcVDER2: ACGCGTCGACATGGCTGAATAATACTCTC. *Arabidopsis* plants (Col-0 ecotype) were transformed by the floral-dip method using *Agrobacterium tumefaciens* strain LBA4404 (Bechtold and Pelletier, 1998). Transformants were selected on 50 mg L^{-1} kanamycin, and T2 lines were used for the experiments.

Arabidopsis transgenic lines 5, 9 and 13 expressing the CaMV35S::*LcVDE* construct were selected and grown side by side with the wild type for comparison. Seeds were sown on petri dishes containing half-strength Murashige and Skoog salts, 0.8% (w/v) phytoagar, and appropriate antibiotics (50 mg L^{-1} kanamycin). Two-week-old seedlings were transplanted into 4-in. standard pots filled with mixed soils (3:2:1 peat moss:vermiculite:perlite). Plants were grown at 23 °C, 60% relative humidity under a 12-h photoperiod light (500 μmol m^{-2} s^{-1}).

For the drought stress evaluation, the *Arabidopsis* transgenic lines 5, 9 and 13 expressing the CaMV*35S*::*LcVDE* construct or wile-type lines were grown in pots for 7 days with constant watering before water was withheld. After 14 days without water, all the pots were re-watered simultaneously, and the plant re-growth was scored 7 days later. Plants were considered dead if all the leaves were brown and there was no re-growth 7 days after re-watering.

During the first 8 days of treatment, 20 pots of transgenic *Arabidopsis* plants (lines 13) and 10 pots of wile-type lines were used for Fv/Fm, NPQ and de-epoxidation ratio assays. Since the beginning of drought stress, the youngest fully expanded leaves from transgenic or wile-type plants were used for assays on days 0, 2, 4, 6, and 8.

Quantitative fluorescent PCR (QF-PCR) analysis

Total RNA was extracted by TRIzol™ reagent according to manufacturer's instructions (Tiangen, China). The primers used for the expression analysis of *LcVDE* were: 5′-CCTGCATGTGCAGCTAATGTTG-3′ (F) and 5′-GTATGGAACTCATGCAATTG-3′ (R). The primers were designed to yield fragments of approximately 300 bp. QF-PCR reactions were carried out using the first strand cDNA from leaf tissues as a template, specific primers, and SYBR Green Master Mix (PE BioSystems) in a final volume of 25 μL. All PCR experiments were performed on a Bio-Rad IQ-5 thermo-cycler with 40 cycles and an annealing temperature of 55 °C was used. Cycle threshold values were determined by IQ-5 Bio-Rad software assuming 100% primer efficiency. Three mRNA samples from three independently harvested leaf samples (biological replicates) were analyzed. The constitutively expressed β-actin gene was used as an internal control to show the normalization of the amount of templates in the PCR reaction (Actin-F1: GGAAACATAGTGCTCAGTGGTG, Actin-R1: GCTGAGGGAAGCCAAGATAG).

RNA sample (1 mg) was also used for semi-quantitative reverse transcription PCR (RT-PCR) analysis. RT-PCR product (15 μL) was loaded on each lane and separated by electrophoresis on 1% (w/v) agarose gel. The actin product was used as a control.

Endogenous ABA determination

The samples of leaf dry matter were extracted in 80% (v/v) acetone containing BHT (butylated hydroxy-toluene) at 0.45 mM and BHA (butylated hydroxy-anisole) (Sigma chemicals) at 0.55 mM, during 7 days under 4 °C, according to Ozfidan et al. (2012) with minor modifications. Subsequently the extract was incubated in saline buffer (Tris–NaCl at 50 mM pH 7.5) (Sigma Reagents), centrifuged at 8000 × *g* for 10 min at 10 °C, and the supernatant removed. The quantification was carried out using Phytodetek-ABA-kit (Agdia, Elkhart, IN). Precaution was also taken to select plants and leaves with similar age and size.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence induction was measured from the adaxial side of leaf segments using a pulse amplitude modulation (PAM-2000) portable fluorometer (Walz, Germany) (Demmig-Adams and Adams, 1996). Before each measurement, the plants were adapted in the dark for 30 min at 22 °C. The minimal fluorescence level (Fo) was measured by measuring light which was sufficiently low that it could not induce any significant variable fluorescence (1.6 kHz, 10 μmol photons m^{-2} s^{-1}). A single saturating pulse light (0.8 s, 3000 μmol photons m^{-2} s^{-1}) was then applied and the maximal fluorescence intensity (Fm) was measured during the fluorescence induction. The maximal photochemical efficiency (Fv/Fm) was calculated as (Fm − Fo)/Fm (Genty et al., 1989). After the decline of the signal from Fm to its initial Fo level, white actinic light (500 μmol photons m^{-2} s^{-1}) which was provided by arrays of 635-nm LEDs was switched on for 5 min and the Fm′ was measured during the fluorescence induction. After switching off the actinic light, a recovery of the Fm″ level was followed in the dark for 5 min. The maximum fluorescence in the course of actinic illumination (Fm′) and in the darkness post-illumination (Fm″) were all determined using a 0.8-s saturating light pulse (3000 μmol photons m^{-2} s^{-1}). The NPQ was defined as Fm/Fm′ − 1. The reversible component of NPQ (relaxing within 5 min) was assigned to energy-dependent NPQ (qE) and calculated as Fm″/Fm′ − , where Fm″ is the maximal yield of fluorescence after 5 min of dark relaxation following the actinic illumination (Strasser et al., 1997). All measurements were repeated three times for each treatment.

Quantitative analysis of pigments

The *L. chinense* and *Arabidopsis* plants were grown under a photon flux density of 500 μmol photons m^{-2} s^{-1} (12-h photoperiod) with different treatment as indicated above. Since the beginning of water deficit stress, the youngest fully expanded leaves were harvested on day-0, 7, 14, 21, and 28 from control and stressed plants. Leaves were immersed in liquid N_2 immediately and stored at −80 °C until use. The carotenoids of the xanthophyll cycle were determined according to the procedures described by Thayer and Björkman (1990). Briefly, samples were harvested at an indicated time and extracted in 100% ice-cold acetone. The pigment extracts were then filtered through a 0.45 μm membrane filter and separated and quantified by an HPLC system (Agilent 1100 series; USA) at room temperature. The de-epoxidation state of the xanthophyll cycle carotenoids was calculated as a (Z + 0.5 A)/(V + A + Z) ratio (Müller et al., 2006). All measurements were repeated three times for each treatment.

Chlorophyll content

The fresh leaf disks were harvested as indicated above, and the Chl content was determined according to our previous study (Guan et al., 2014).

Statistical analysis

The significance of the differences between control and treated groups was analyzed using Student's *t*-test or one-way ANOVA. Asterisks indicated significant differences between control and treated-group by Student's *t* test (*$P < 0.05$). Values marked with different letters represented significant differences between the treatments (one-way ANOVA, $P < 0.05$). All experiments were repeated at least two times. Data were expressed in means ± SE.

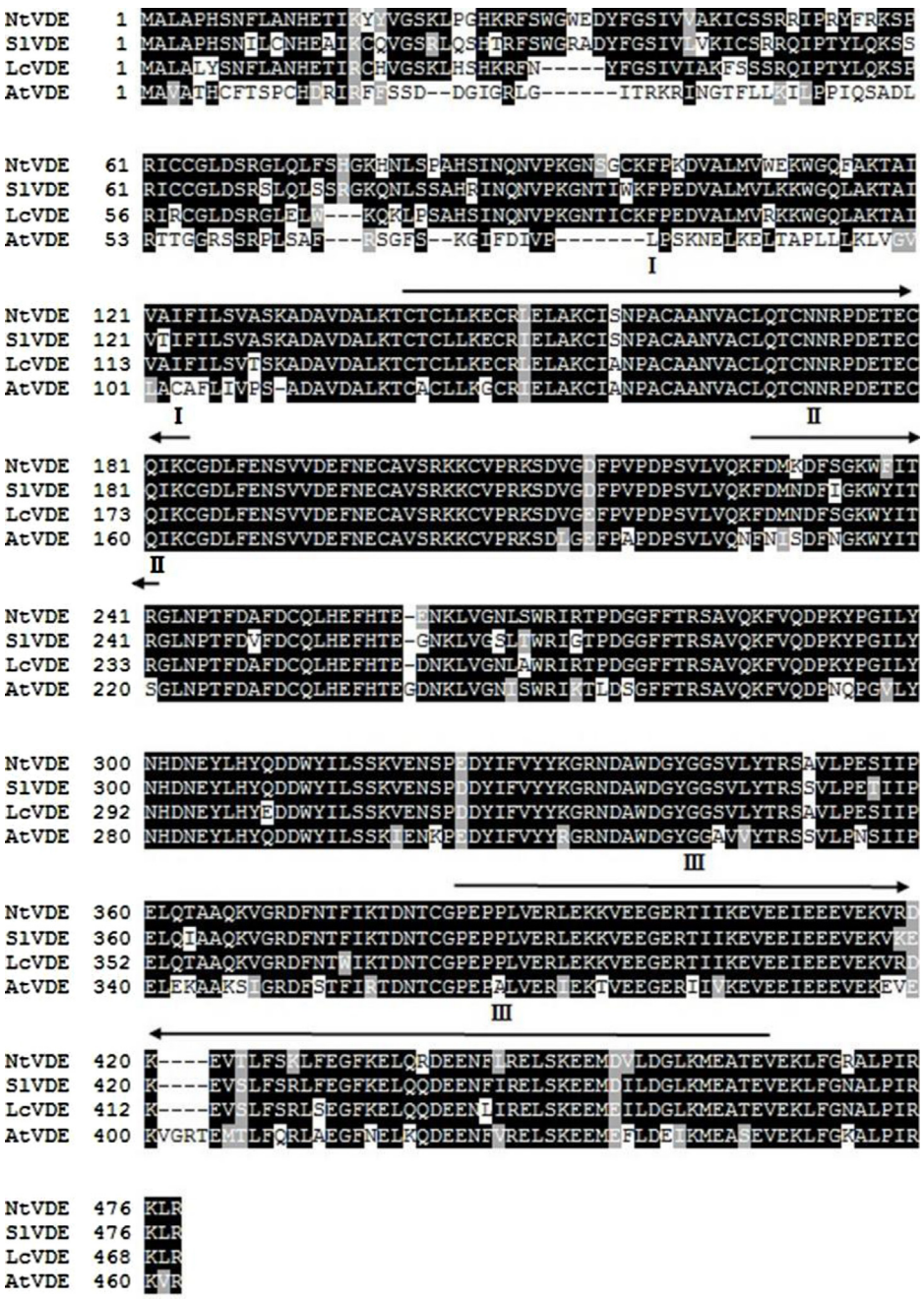

Fig. 1. Alignment LcVDE (*L. chinense*, Accession no. KJ486598) with SlVDE (*S. lycopersicum*, Accession no. ACM92036.1), AtVDE (*A. thaliana*, Accession no. NP172331.1), and NtVDE (*N. tabacum*, Accession no. AFP5768.1.1) were presented here.

Results

Sequence analysis and of the phylogenetic tree construction

The full-length cDNA of VDE was isolated from *L. chinense* and was given the name LcVDE (GenBank accession No. KJ486598), encoding a 470 amino acid polypeptide. The deduced amino acid sequence of the cDNA showed that it encoded a polypeptide of approximately 53.6 kDa. Early studies on various VDEs from different species showed that there were three strong sequence homology regions (blocks I–III) in this member of the lipocalin family (Bugos et al., 1998). Amino acid sequence alignment revealed that the LcVDE contained previously defined regions (Fig. 1), including the cysteine-rich domain in block I, the lipocalin signature domain in block II and the C-terminal glutamate-rich domain in block III, all of which have been shown to form a catalytically essential site in VDE and are overall conserved. The cysteine-rich domain in block I was suggested to be the active site of VDE. The lipocalin signature domain could be a possible binding site for violaxanthin and monogalactosyldiacylglycerol (MGDG), which is the most effective lipid for supporting VDE activity. At the C-terminal region, the glutamate-rich domain in block III was thought to be involved in binding of VDE to the thylakoid membrane.

Amino acid sequence alignment of LcVDE with VDEs of other species was performed and a phylogenetic tree was constructed (Fig. 2). LcVDE can be grouped into the VDE family and it is closely linked to VDEs of *Solanum lycopersicum* and *Nicotiana tabacum* species.

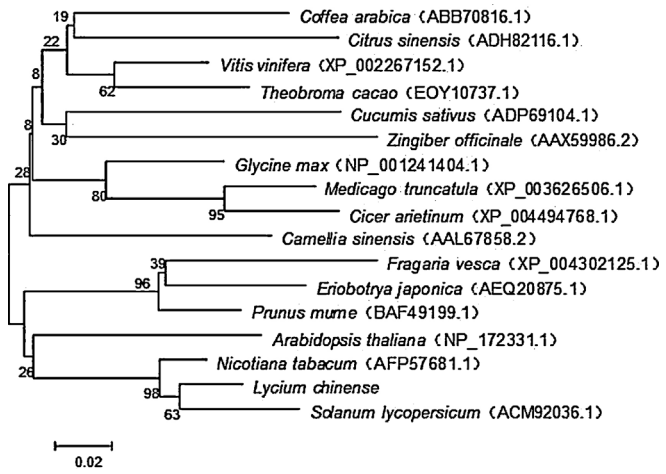

Fig. 2. Phylogenetic relationships of LcVDE with other plants VDE as indicated.

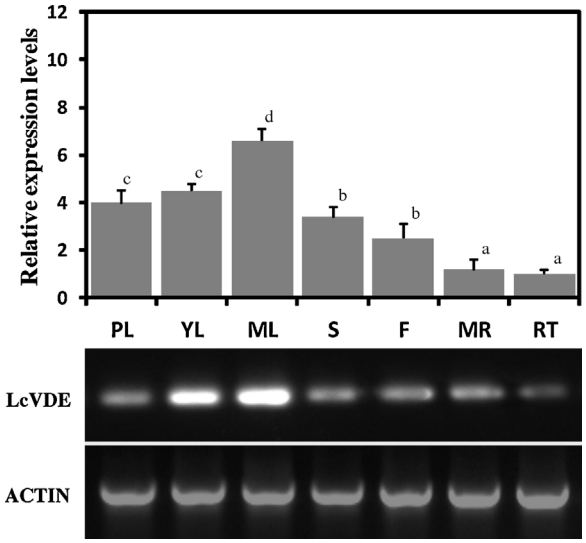

Fig. 3. Semi-quantitative RT-PCR and QF-PCR analysis of the *LcVDE* transcripts in different tissues. PL, primordial leaf; YL, young leaf; ML, mature leaf; MR, mature roots; RT, root tips; F, flower; S, stem. Actin gene was used as an internal control to show the normalization of the amount of templates in the PCR reaction. The relative changes of *LcVDE* transcripts were calculated as X-fold changes relative to the root tips samples. Data shows the average ± SE of five-independent experiments. Values marked with different letters represent significant differences between different tissues (one-way ANOVA, $P < 0.05$). Error bars indicate the standard error of the means.

Expression patterns of LcVDE in different tissues

The expression of the *LcVDE* gene was analyzed in various plant organs (Fig. 3). As shown by the relative expression data, this gene was active in all analyzed tissues including roots, stems, flowers and leaves. The transcript level in leaves and stem were higher than that in roots and flowers. Furthermore, the transcript level of *LcVDE* in mature leaves was higher than in primordial and young leaves. The lowest transcript level of *LcVDE* was detected in root tips. These results suggested that the expression of *LcVDE* was not tissue specific but varied greatly among different organs.

Chlorophyll contents and morphology observation of L. chinensis plants under drought stress

Drought stress induces a range of physiological and biochemical responses in plants, including stomatal closure and repression of plant growth and photosynthesis, which consequently affect biomass and seed productivity. The result of this study shown that no visible damage was observed in the drought stressed young *L. chinense* plants, although the drought treatment caused an increase in the number of yellow leaves (Fig. 4). When *L. chinense* plants were co-treated with drought stress and abamine SG, more yellow leaves were observed than the plant treated by drought stress alone. Control plants and plants treated by abamine SG alone were all growing well except that the abamine SG treatment caused a slight increase in the number of yellow leaves.

Chlorophyll contents of *L. chinensis* plants under drought stress were in accordance with the morphology observation as indicated above. As shown in Table 1, drought stress resulted in significantly lower Chla and Chla/b in *L. chinense* than controls. When *L. chinense* plants co-treated with drought stress and abamine SG, more decrease in chlorophyll content was observed than the plant treated by drought stress alone. There were not significant changes in plants treated by abamine SG alone compared to control (Table 1).

The accumulation of endogenous ABA in L. chinense under drought stress

The accumulation of endogenous ABA in the leaves of *L. chinense* grown under different days of drought treatments was measured. As shown in Fig. 5, leaf ABA content in control was about 6.6 nmol g^{-1} DW (dry weight) in *L. chinense*, while it was increased fast on day 7 of the drought treatment to reach a value about 23.5 nmol g^{-1} DW. Then the ABA level was increased slowly from day 7 to day 14 and reached to the highest value (about 27 nmol g^{-1} DW) on day 14, before decreased to 21 nmol g^{-1} DW on day 21.

To confirm that the application of abamine SG causes a reduction in the ABA content in the leaves of *L. chinense* grown under

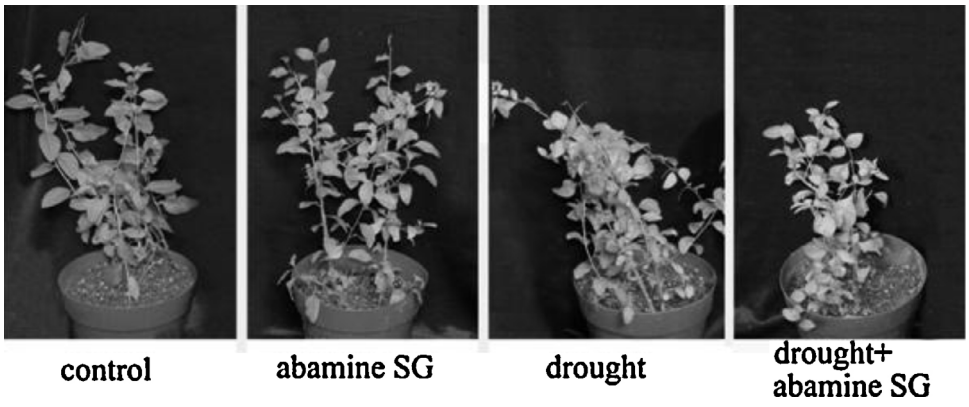

Fig. 4. The representative photographs of *L. chinensis* plants under control, 28 days of abamine SG, drought stress or drought stress + abamine SG treatment.

Table 1
Effect of drought stress on the contents of Chla, Chla b and the ratio Chla/b in leaves *L. chinense* plants.

Treatment/day	0	7	14	21	28
Control (mg g^{-1} FW)					
Chla	0.92 ± 0.13	0.90 ± 0.08	0.88 ± 0.07	0.87 ± 0.12	0.89 ± 0.09
Chlb	0.34 ± 0.05	0.32 ± 0.08	0.31 ± 0.09	0.30 ± 0.10	0.32 ± 0.08
Chla/b	2.71 ± 0.35	2.81 ± 0.21	2.84 ± 0.36	2.9 ± 0.25	2.78 ± 0.29
Abamine SG (mg g^{-1} FW)					
Chla	0.90 ± 0.13	0.88 ± 0.08	0.86 ± 0.07	0.85 ± 0.12	0.84 ± 0.09
Chlb	0.33 ± 0.06	0.32 ± 0.09	0.31 ± 0.07	0.31 ± 0.08	0.30 ± 0.06
Chla/b	2.73 ± 0.41	2.75 ± 0.39	2.87 ± 0.37	2.83 ± 0.29	2.89 ± 0.26
Drought (mg g^{-1} FW)					
Chla	0.90 ± 0.13	0.83 ± 0.12*	0.77 ± 0.09*	0.72 ± 0.08*	0.65 ± 0.08*
Chlb	0.32 ± 0.08	0.30 ± 0.07	0.28 ± 0.06	0.28 ± 0.08	0.27 ± 0.06*
Chla/b	2.81 ± 0.34	2.77 ± 0.38*	2.65 ± 0.28*	2.57 ± 0.43*	2.5 ± 0.46*
Drought + abamine SG (mg g^{-1} FW)					
Chla	0.90 ± 0.13	0.80 ± 0.12*	0.74 ± 0.09*	0.68 ± 0.08*	0.62 ± 0.08*
Chlb	0.32 ± 0.07	0.29 ± 0.09	0.28 ± 0.08	0.27 ± 0.07	0.26 ± 0.09*
Chla/b	2.81 ± 0.32	2.76 ± 0.29*	2.64 ± 0.38*	2.52 ± 0.41*	2.38 ± 0.26*

Values are means (n = 3) with SD, and a significant difference between treatment and control in a column is indicated by * (P < 0.05).

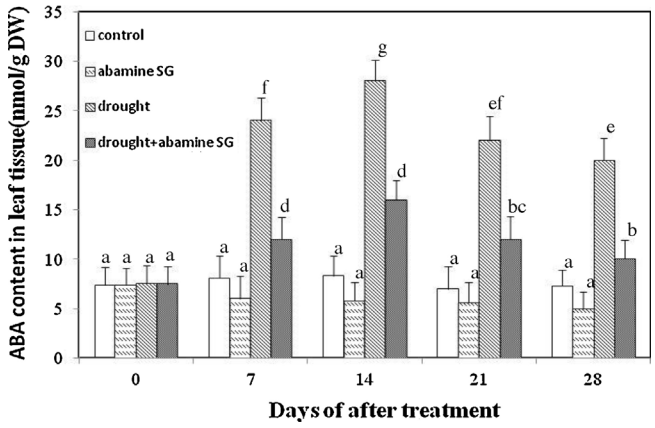

Fig. 5. The endogenous ABA accumulation in the leaves of *L. chinense* grown under unstressed or stressed conditions as indicated in Fig. 4 legend. Data showed the average ± SE of five-independent experiments. Values marked with different letters represent significant differences between the treatments (one-way ANOVA, P < 0.05).

drought treatment, the ABA content in leaves of *L. chinense* co-treated by drought and abamine SG was evaluated. As shown in Fig. 5, application of abamine SG caused an approximately 2-fold reduction in the ABA content of leaf tissues than those treated by drought stress alone after 7 days of treatment. The similar reduction in the ABA content of leaf tissues was observed during the next 21 days of co-treatment with drought stress and abamine SG. In summary, these data support the application of abamine SG causes a reduction in the ABA content in the leaves of *L. chinense* grown under drought stress treatment. It should be noted that the effect of abamine SG on ABA content of *L. chinense* plants under unstressed conditions was not statistically significant compared with control. It may be because that the concentration of abamine SG was so low that it cannot reduce the basic amount of ABA content of *L. chinense* plants under unstressed conditions. However, for a long-term experiment, we have to choose this low concentration of abamine SG, for that *L. chinense* plants subjected to a high concentration of abamine SG application cannot survive for a long time according to our pre-experiments.

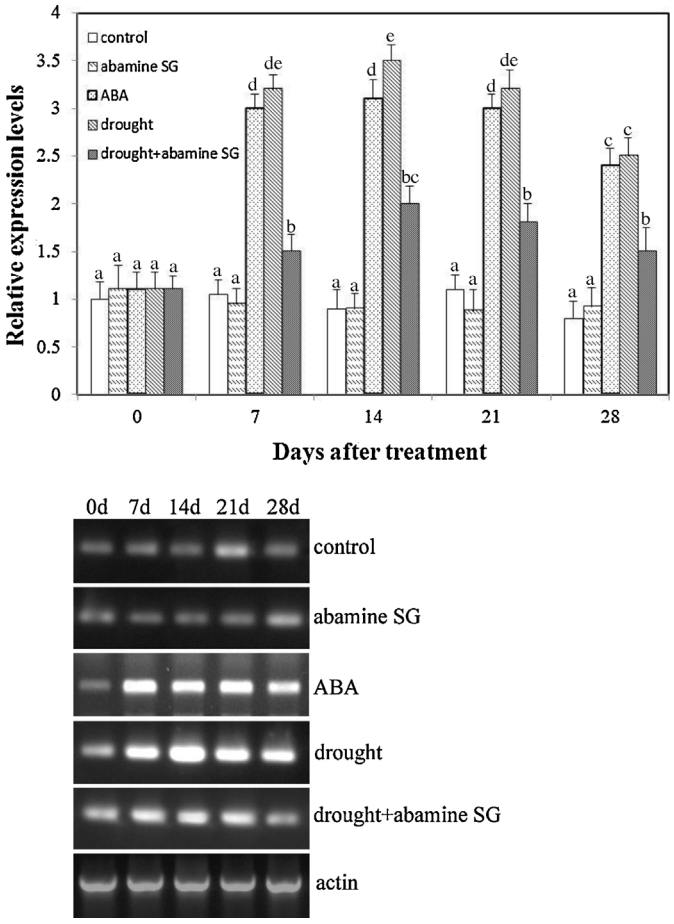

Fig. 6. The accumulation of *LcVDE* transcripts was determined using QF-PCR (upper) and semi-quantitative PCR (lower). Total RNA was isolated from young leaves of *L. chinense* plants subjected to conditions of control, abamine SG treatment, ABA treatment, drought stress treatment or drought stress + abamine SG application as mentioned during 0, 7, 14, 21 and 28 days. Data showed the average ± SE of five-independent experiments. Values marked with different letters represent significant differences between the treatments (one-way ANOVA, P < 0.05).

LcVDE transcript expression in leaves of L. chinense under drought stress

As shown in Fig. 6, a significant induction of *LcVDE* transcript levels was detected in *L. chinense* seedlings after 7 days of drought stress treatment. The expression levels showed highly elevated transcript levels after 14 days of drought stress treatment. Also, the transcript expression levels decreased after 21 days of drought tress treatment. The *LcVDE* transcript of *L. chinense* plant under control condition was always maintained at low level from 0 to 28 days.

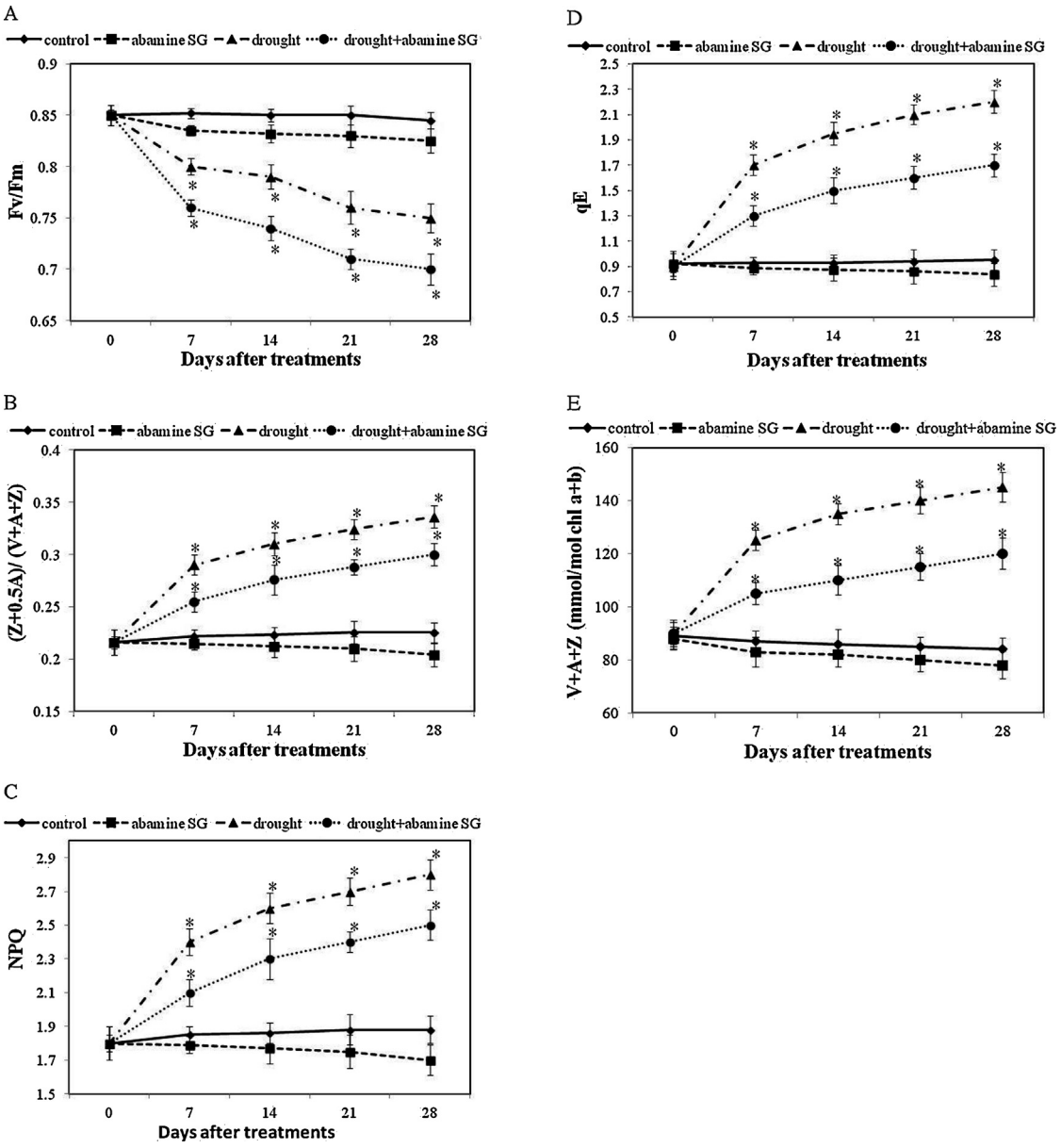

Fig. 7. Effects of drought stress on leaf maximal quantum yield of PSII photochemistry (Fv/Fm) (A), the de-epoxidized fraction of xanthophylls (V + 0.5 A/V + A + Z) (B), the NPQ (C), the qE (D) and the V + A + Z pool (E) under conditions as indicated above. Data showed the average ± SE of five-independent experiments. Pigment composition of leaves was determined in *L. chinense* exposed to a photon flux density of 500 μmol photons m^{-2} s^{-1} (12-h photoperiod) with different treatment as indicated. Fv/Fm was measured after the leaf discs were adapted in the dark for 30 min. The NPQ induction was initiated by the onset of white light (500 μmol photons m^{-2} s^{-1}) for 5 min and defined as Fm/Fm′ − . The reversible component of NPQ (relaxing within 5 min) was assigned to energy-dependent NPQ (qE) and calculated as Fm″/Fm′ − . The maximum fluorescence in the dark-adapted state (Fm), during the course of actinic illumination (Fm′) and in the darkness post-illumination (Fm″) were all determined using a 0.8-s saturating light pulse (3000 μmol photons m^{-2} s^{-1}). Asterisks indicated significant differences between control and treated-group by Student's *t* test (*$P < 0.05$).

The potential involvement of endogenous ABA in modulating the *LcVDE* transcript levels was investigated by following the *LcVDE* transcript levels after topical exogenous application of 10 μM abamine SG (Fig. 6). The *LcVDE* transcript appeared to be significantly down-regulated when the plants were treated by exogenous abamine SG application together with drought stress than those treated by drought stress alone from day 7 to day 28 during the treatments.

As shown in Fig. 6, the expression of LcVDE gene can also be induced by exogenous ABA treatment from day 7 to day 28. ABA may protect photosynthetic apparatus against photodamage through an enhanced xanthophyll cycle (Ivanov et al., 1995). Also, it was reported that the addition of exogenous ABA resulted in enhancement of the size of the xanthophyll cycle pool and promotion of de-epoxidation of the xanthophyll cycle components (Zhu et al., 2011).

Effect of drought on photosynthetic parameters in L. chinense

The maximal photochemical efficiency of PSII (Fv/Fm) has been widely used as an indicator of photo-inhibition. As shown in Fig. 7A, the Fv/Fm was not significantly changed in both control and abamine SG-treated plants for 28 days. Upon exposure to drought stress or drought stress + abamine SG, Fv/Fm was decreased in both treatments. The decrease in Fv/Fm was more evident in drought stress + abamine SG-treated plants than in drought stress alone-treated plants. At the end of the stress, Fv/Fm in drought stress + abamine SG- and drought stress alone-treated

plants decreased by approximately 18% and 12% of their initial values, respectively. The results showed that neither controls nor abamine SG-treated plants showed obvious photo-inhibition, however, drought stress + abamine SG-treated plants and drought stress alone-treated plants showed more severe PSII photo-inhibition than controls.

As shown in Fig. 7B, the de-epoxidized ratio of the xanthophyll cycle pigments, (Z + 0.5 A)/(V + A + Z), was not significantly changed in both control and abamine SG-treated plants for 28 days. The increase of (Z + 0.5 A)/(V + A + Z) was observed in drought stress- or drought stress + abamine SG-treated plants. The increase in the de-epoxidized ratio in drought stress-treated plants was more pronounced than that in drought stress + abamine SG-treated plants.

The capability for excess energy dissipation in PSII was investigated by determining NPQ. As shown in Fig. 7C, neither control nor abamine SG-treated plants showed obvious NPQ; however, drought stress + abamine SG-treated plants and drought stress alone-treated plants lead to more NPQ than control. In general, the accumulation of the NPQ was consistent with the values of (Z + 0.5 A)/(V + A + Z) as indicated above in *L. chinense* under different treatments.

The level of qE showed a similar trend with the value of NPQ as indicated above in *L. chinense* under different treatments (Fig. 7D). The high value of NPQ in plants treated with drought stress may due to an increase of qE level which was observed as the major component (about 75–80%) of the total NPQ (Fig. 7D). The major component of NPQ relaxing within few minutes is the pH- or energy-dependent component (qE) related to a build-up of a thylakoid ΔpH generated by the photosynthetic electron transport. Simultaneously, the ΔpH activates the xanthophylls cycle involved in protection mechanisms of the photosynthetic apparatus (Arnoux et al., 2009). The result presented here suggested the close relationship between qE and the accumulation of de-epoxidized xanthophylls, which was similar to the previous study (Ocampo-Alvarez et al., 2013).

As shown in Fig. 7E, the V + A + Z pool was not significantly changed in both control and abamine SG-treated plants for 28 days. The up-regulation of V + A + Z pool was observed in drought stress- or drought stress + abamine SG-treated plants. The increase in the V + A + Z pool in drought stress-treated plants was more pronounced than that in drought stress + abamine SG-treated plants.

Enhanced drought stress-induced photosynthesis damage tolerance in transgenic Arabidopsis expressing CaMV35S::LcVDE

The involvement of LcVDE in modulating the level of photosynthesis damage caused by the drought stress was further conferred by transgenic *Arabidopsis* expressing CaMV35S::*LcP5CS*. As shown in Fig. 8, the *LcVDE* gene was constitutively expressed in transgenic *Arabidopsis* plants. To determine the effect of *LcVDE* over-expression on drought stress-induced photosynthesis damage tolerance, *Arabidopsis* grown on soil were not watered for 2 weeks and then watered and grown under normal conditions for 7 days. After watering was restarted, transgenic plants showed a stronger growth recovery phenotype than wild-type plants. Only 24% of the wild-type plants survived this treatment. In contrast, more than 38% of *LcVDE*-over-expressed plants survived (Table 2), which suggests that the over-expression of *LcVDE* in transgenic *Arabidopsis* results in greater tolerance to drought stress than in the wild type.

The drought stress-induced photosynthesis damage tolerance phenotype of transgenic plants over-expressing *LcVDE* was consistent with photosynthetic parameters as compared with the wild type. As shown in Fig. 9A, upon exposure to drought stress, Fv/Fm decreased in both wild-type and transgenic seedling leaves. The decrease in Fv/Fm was more dramatic in wild-type than in

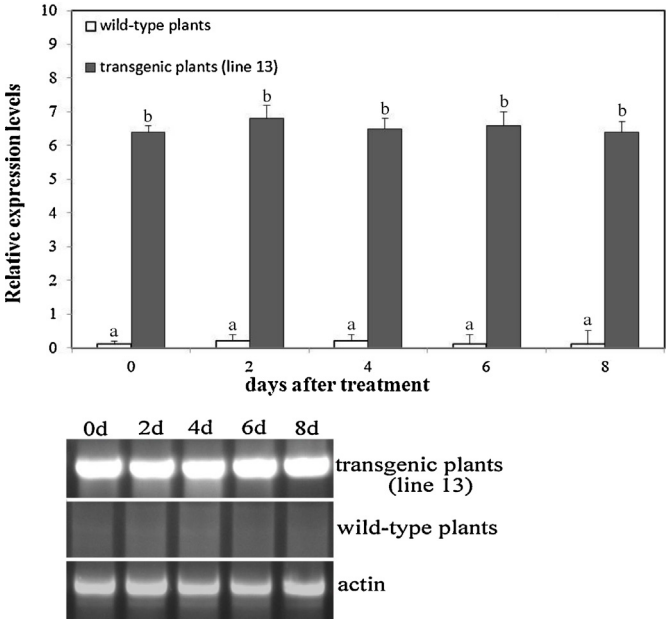

Fig. 8. The accumulation of *LcVDE* transcripts was determined using QF-PCR (upper) and semi-quantitative PCR (lower). Total RNA was isolated from seedlings of wild-type or transgenic *Arabidopsis* plants expressing CaMV35S::*LcVDE* construct subjected to 8 days of drought stress treatment. Data showed the average ± SE of five-independent experiments. Asterisks indicated significant differences between wild-type and transgenic plants by Student's *t* test (*$P < 0.05$).

Table 2
Survival rates of transgenic *Arabidopsis* under drought stress conditions.

	Survival[a]	Total[b]	Survival[c] (%)
Wild-type lines	12	50	24
LcVDE-over-expressed line 5	22	50	44
LcVDE-over-expressed line 9	19	50	38
LcVDE-over-expressed line 13	24	50	48

[a] Number of surviving plants.
[b] Total plants used in drought assay.
[c] Percentage of surviving plants.

transgenic seedlings. The de-epoxidized ratio of the xanthophyll cycle pigments, (A + Z)/(V + A + Z), was higher in transgenic than in wild-type seedlings (Fig. 9B). In both wild-type and transgenic seedling leaves, NPQ significantly increased when plants were exposed to drought stress. However, NPQ increased more rapidly in transgenic than in wild-type seedlings (Fig. 9C). The level of qE was consistent with the values of NPQ as indicated above in *Arabidopsis* under drought stress treatment (Fig. 9D) which also indicated that the qE was the major component of NPQ detected in this study. The V + A + Z pool in drought stress-treated transgenic *Arabidopsis* was higher than that in wild-type plants from day 0 to day 8 (Fig. 9E).

Discussion

Since the cDNA of VDE was first cloned from romaine lettuce (Bugos and Yamamoto, 1996), the VDE genes have been characterized from several plants (Bouvier et al., 1996; Huang et al., 2007; Li et al., 2013). Here, we report the isolation of a novel *L. chinense* VDE gene, named *LcVDE*, which allowed extended functional characterization of the VDE gene from a species of woody plants. Early studies on various VDEs from different species showed that there were three strong sequence homology regions in this member of the lipocalin family (Bugos et al., 1998). In the present study, amino acid sequence alignment revealed that the LcVDE contained the previously defined regions (Fig. 1). The cysteine-rich

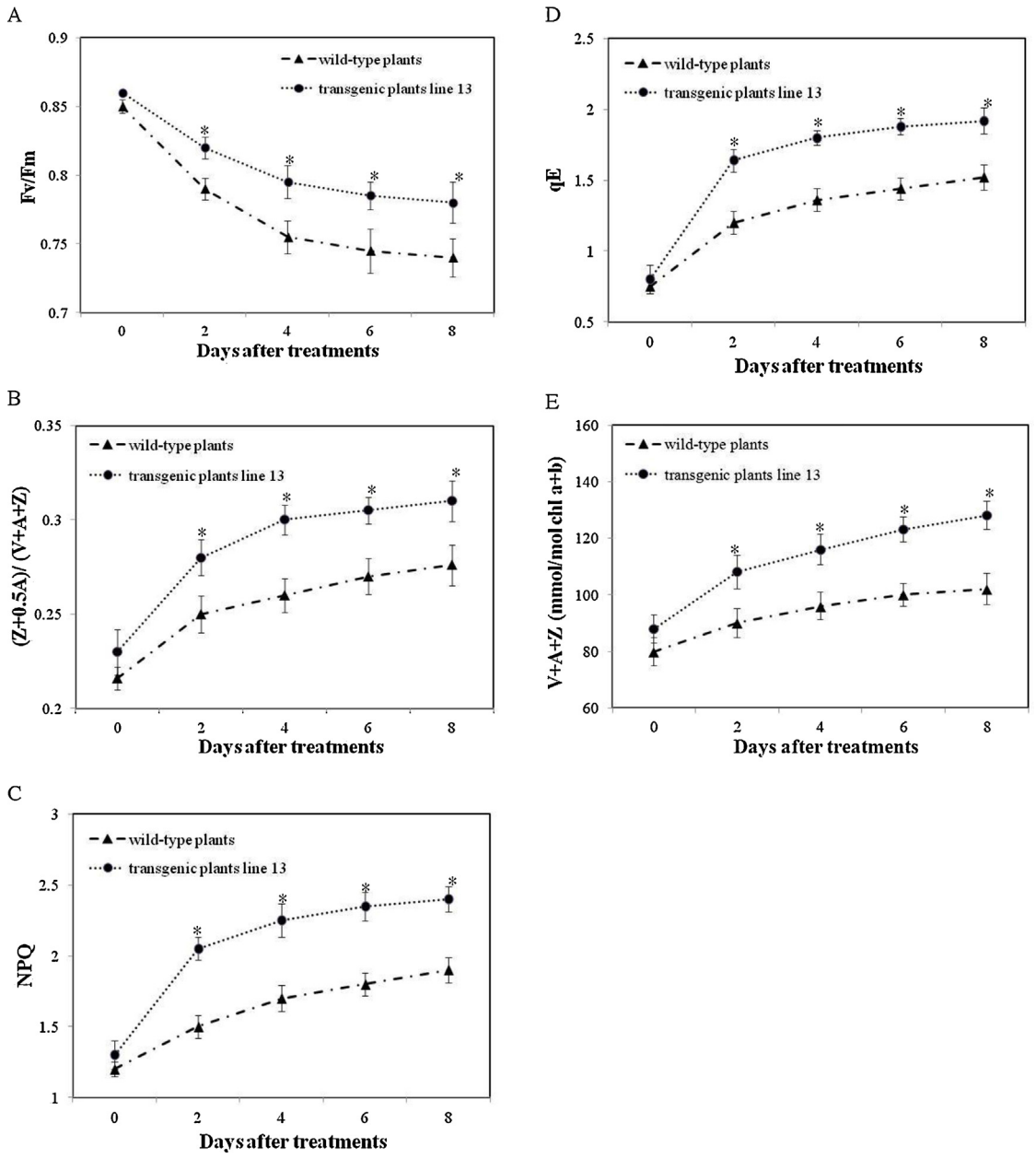

Fig. 9. Effects of drought stress on leaf maximal quantum yield of PSII photochemistry (Fv/Fm) (A), the de-epoxidized fraction of xanthophylls (V + 0.5 A/V + A + Z) (B), the NPQ (C), the qE (D) and the V + A + Z pool (E) under conditions of 8 days drought stress treatment. Data showed the average ± SE of five-independent experiments. Pigment composition of leaves was determined in transgenic and wild-type *Arabidopsis* plants grown under a photon flux density of 500 μmol photons m^{-2} s^{-1} (12-h photoperiod) with different treatment as indicated. Fv/Fm was measured after the leaf discs were adapted in the dark for 30 min. The NPQ induction was initiated by the onset of white light (500 μmol photons m^{-2} s^{-1}) for 5 min and defined as Fm/Fm′ − . The reversible component of NPQ (relaxing within 5 min) was assigned to energy-dependent NPQ (qE) and calculated as Fm″/Fm′ − . The maximum fluorescence in the dark-adapted state (Fm), during the course of actinic illumination (Fm′) and in the darkness post-illumination (Fm″) were all determined using a 0.8-s saturating light pulse (3000 μmol photons m^{-2} s^{-1}). Asterisks indicated significant differences between wild-type and transgenic plants by Student's *t* test (*$P < 0.05$).

domain in block I, the lipocalin signature domain in block II and the C-terminal glutamate-rich domain in block III, all of which have been shown to form a catalytically essential site in VDE, are overall conserved. The regulation of VDE could take place at transcriptional or post-transcriptional levels. It has been considered that the VDE expression pattern is influenced by light and other environmental factors (Facella et al., 2008; North et al., 2005; Zhao et al., 2012).

ABA plays a critical role in several plants developmental processes including mediates plant responses to various environmental stresses, particularly drought and salinity (Cutler et al., 2010; Zhu, 2002). Under drought or salinity stress, the endogenous ABA was quickly synthesized (Zeevaart and Creelman, 1988), which facilitates stomata closure and expression of ABA responsive genes to protect plants from further water loss and damage. ABA is synthesized by oxidative cleavage of epoxy-carotenoids in plants (Zeevaart and Creelman, 1988). Key steps in the pathway have been identified in some plant species (Nambara and Marion-Poll, 2005). The epoxidation of Z and A to form V and neoxanthin is catalyzed by zeaxanthin epoxidase (Lichtenthaler, 1987). The products are isomerized to produce 9-cis isomers which are cleaved by NCED to form xanthoxin (Qin and Zeevaart, 1999). Xanthoxin is oxidized to ABA-aldehyde, which is then converted to ABA by ABA-aldehyde oxidase (Cheng et al., 2002; Seo et al., 2000). Although the pathway is clear, the regulation of ABA synthesis remains poorly understood.

In this study, the accumulation of endogenous ABA in the leaves of *L. chinense* grown under different days of drought treatment was

confirmed (Fig. 5), which is in accordance with previous report (Zhu, 2002). Interestingly, a significant induction of *LcVDE* transcript levels was also detected in *L. chinense* after drought stress treatment. It has been considered that the VDE expression pattern is influenced by several environmental factors (Facella et al., 2008; North et al., 2005; Zhao et al., 2012). For example, under high light stress, relative expression of VDE from cucumber (CsVDE) was increased rapidly. Furthermore, CsVDE was quickly induced by cold and drought stress, reaching maximum levels at the 2nd hour and the 9th day, respectively (Li et al., 2013). However, since V serves as a common precursor for ABA, the up-regulation of VDE activity upon drought stress (and hence converts V to A and Z) in parallel with an increased endogenous ABA biosynthesis seems a little bit contradictory, due to that both processes require V as substrate and thus may compete for the same substrate. To explain this phenomenon, the V + A + Z content in leaves of *L. chinense* under different treatments was evaluated. As shown in Fig. 7E, the up-regulation of V + A + Z pool was observed in drought stress- or drought stress + abamine SG-treated plants. The high content of xanthophylls under stress conditions may serve as a protection mechanism in leaves. The activation of the xanthophyll cycle has also been detected in other plant subjected to drought stress (Fini et al., 2012). Seo and Koshiba (2002) pointed out that Z might be an intermediate substrate for the synthesis of ABA. Thus, regulating the synthesis of ABA may be another function of xanthophyll cycle. In excessive light energy, VDE converts V to Z and then maybe reduces the synthesis of ABA. Under low or limiting light, ZEP converts Z to V and then maybe enhances the synthesis of ABA (Jahns and Holzwarth, 2012). In tobacco and tomato, ZEP or VDE expression fluctuates according to a diurnal rhythm during a time lapse consisting of light and dark periods (Audran et al., 1998; Thompson et al., 2000). The diurnal oscillation of ZEP or VDE expression in leaves of tobacco and tomato are probably related to its role in the xanthophyll cycle. In this case, the fluctuation of ZEP or VDE expression and the quantities of each xanthophyll might not be limiting for ABA synthesis (Du et al., 2010). For the up-regulation of V + A + Z pool was observed in drought stress-treated plants, the V + A + Z pool may be large enough to meet concurrently the Z and ABA biosynthesis even though both processes use V as substrate and thus may compete for the same substrate. Therefore, the up-regulation of VDE activity upon drought stress in parallel with an increased endogenous ABA biosynthesis in this study was reasonable.

Since V serves as a common precursor for ABA, these data support the involvement of ABA in the positive feedback regulation of *LcVDE* gene expression in *L. chinense* plants under drought stress. The LcVDE may be involved in modulating the drought stress-induced photosynthesis damage. Indeed, some previous results already demonstrated that the positive feedback regulation exists in ABA biosynthesis, which contributes to ABA accumulation under drought stress. For example, recent evidence indicates that exogenous ABA significantly enhanced the expression of ABA1 and ABA3 (Xiong et al., 2001, 2002). Furthermore, in the ABA deficient mutant aba1, the transcript of all the inducible ABA biosynthetic genes under stress were significantly lower than those in the wild-type (Xiong et al., 2001, 2002). In addition, AtNCED3 transcript were dramatically reduced in ABA insensitive mutants snrk2.2/2.3/2.6 and pyr1; pyl1; pyl2; pyl4 in comparison to the wild-type under ABA treatment (Fujii and Zhu, 2009; Park et al., 2009).

The measurement of photosynthetic parameters in *L. chinense* supported the above assumption that ABA may positive feedback regulate the expression of *LcVDE* gene in *L. chinense* plants under drought stress. As shown in Fig. 7B, the increase of de-epoxidized ratio of the xanthophyll cycle pigments, (Z + 0.5 A)/(V + A + Z), was observed in drought stress- or drought stress + abamine SG-treated plants. The increase in the de-epoxidized ratio was more pronounced in drought stress-treated plants than in drought stress + abamine SG-treated plants. Also, the increase of NPQ was consistent with the values of (Z + 0.5 A)/(V + A + Z) (Fig. 7C) (Kotabová et al., 2011) as indicated above. Although unstressed plants was not affected too much by photon flux density of 500 μmol photons m^{-2} s^{-1} used in growth chamber (Song et al., 2011), the NPQ also formed due to that drought stress resulted in significantly lower Chla and Chla/b in *L. chinense* than control (Table 1) (Pascal et al., 2005). These data indicated that, drought stress-induced endogenous ABA may regulate the expression of *LcVDE* gene in *L. chinense* with positive feedback manner, and the expression of *LcVDE* gene leads to the increase in (Z + 0.5 A)/(V + A + Z), then the rise in NPQ levels can be detected in *L. chinense* (Li et al., 2013).

The maximal photochemical efficiency of PSII (Fv/Fm) has been widely used as an indicator of photo-inhibition. As shown in Fig. 7A, upon exposure to drought stress or drought stress + abamine SG, Fv/Fm was decreased in both treatments. The decrease in Fv/Fm was more evident in drought stress + abamine SG-treated plants than in drought stress alone-treated plants. This result is in agreement with the previous study, in which exogenously supplied ABA alleviated the decrease in actual efficiency of PS II photochemistry, induced a lower reduction state of PS II, and caused higher capacity of NPQ in ABA-fed plants than in non-ABA-fed plants (Zhu et al., 2011).

The involvement of LcVDE in modulating the level of photosynthesis damage caused by the drought stress was further conferred by transgenic *Arabidopsis* expressing CaMV35S::*LcVDE*. As shown in Table 2, the over-expression of *LcVDE* in transgenic *Arabidopsis* resulted in greater tolerance to drought stress than in the wild type under photon flux density of 500 μmol photons m^{-2} s^{-1}. Furthermore, the drought stress-induced photosynthesis damage tolerance phenotype of transgenic plants over-expressing *LcVDE* was consistent with photosynthetic parameters as compared with the wild type (Fig. 9). Such a positive relationship of photosynthetic capacity to drought tolerance may be a general rule as shown in other studies. For example, a transgenic line showed an enhanced activity of the C3 enzyme sedoheptulose-l, 7-bisphosphatase, which was involved in the enhanced photosynthetic capacity as well as the increased tolerance to salt stress (Feng et al., 2007). It was reported that the NPK1 expression considerably improved drought tolerance in transgenic maize, which was associated with the enhanced rate of photosynthesis, suggesting the effective role of NPK1 in protecting the photosynthetic machinery from the drought-induced injurious effects (Shou et al., 2004). In another study (Yu et al., 2008), an *Arabidopsis* mutant with improved drought tolerance was identified. The mutant showed significantly reduced leaf stomata density, but enhanced photosynthetic capacity (Yu et al., 2008).

Conclusions

Here, we report the isolation of a novel *LcVDE* gene from *L. chinense*. The involvement of ABA in the positive feedback regulation of *LcVDE* gene expression was suggested in *L. chinense* plants under drought stress. Furthermore, the LcVDE may be involved in modulating the level of photosynthesis damage caused by the drought stress in *L. chinense*, which was further conferred by transgenic *Arabidopsis* expressing CaMV35S::*LcVDE*.

Acknowledgments

This work was supported by National Natural Science Foundation of China (31401391) and the National Genetically Modified Organism Major Projects of China (2014ZX0800302B).

References

Arnoux P, Morosinotto T, Saga G, Bassi R, Pignol D. A structural basis for the pH-dependent xanthophyll cycle in *Arabidopsis thaliana*. Plant Cell 2009;21:2036–44.

Audran C, Borel C, Frey A, Sotta B, Meyer C, Simonneau T, et al. Expression studies of the zeaxanthin epoxidase gene in *Nicotiana plumbaginifolia*. Plant Physiol 1998;118:1021–8.

Ashraf M, Harris P. Photosynthesis under stressful environments: an overview. Photosynthetica 2013;51:163–90.

Bechtold N, Pelletier G. In planta Agrobacterium mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. In: Martinez-Zapater J, Salinas J, editors. Arabidopsis protocols. Humana Press; 1998. p. 259–66.

Bouvier F, d'Harlingue A, Hugueney P, Marin E, Marion-Poll A, Camara B. Xanthophyll biosynthesis cloning, expression, functional reconstitution, and regulation of β-cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). J Biol Chem 1996;271:28861–7.

Bugos R, Yamamoto H. Molecular cloning of violaxathin de-epoxidase from romaine lettuce and expression in *Escherichia coli*. Proc Natl Acad Sci USA 1996.

Bugos RC, Hieber AD, Yamamoto HY. Xanthophyll cycle enzymes are members of the lipocalin family, the first identified from plants. J Biol Chem 1998;273:15321–4.

Cheng W-H, Endo A, Zhou L, Penney J, Chen H-C, Arroyo A, et al. A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. Plant Cell 2002;14:2723–43.

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol 2010;61:651–79.

Demmig-Adams B, Adams WW. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci 1996;1:21–6.

Du H, Wang N, Cui F, Li X, Xiao J, Xiong L. Characterization of the β-carotene hydroxylase gene DSM2 conferring drought and oxidative stress resistance by increasing xanthophylls and abscisic acid synthesis in rice. Plant Physiol 2010;154:1304–18.

Facella P, Lopez L, Carbone F, Galbraith DW, Giuliano G, Perrotta G. Diurnal and circadian rhythms in the tomato transcriptome and their modulation by cryptochrome photoreceptors. PLoS ONE 2008;3:e2798.

Farooq M, Hussain M, Wahid A, Siddique KHM. Drought stress in plants: an overview. In: Aroca R, editor. Plant responses to drought stress. Berlin, Heidelberg: Springer; 2012. p. 1–33.

Feng L, Han Y, Liu G, An B, Yang J, Yang G, et al. Overexpression of sedoheptulose-1,7-bisphosphatase enhances photosynthesis and growth under salt stress in transgenic rice plants. Funct Plant Biol 2007;34:822–34.

Fini A, Guidi L, Ferrini F, Brunetti C, Di Ferdinando M, Biricolti S, et al. Drought stress has contrasting effects on antioxidant enzymes activity and phenylpropanoid biosynthesis in *Fraxinus ornus* leaves: an excess light stress affair. J Plant Physiol 2012;169:929–39.

Frenette Charron J-B, Ouellet F, Pelletier M, Danyluk J, Chauve C, Sarhan F. Identification, expression, and evolutionary analyses of plant lipocalins. Plant Physiol 2005;139:2017–28.

Fujii H, Zhu J-K. Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc Natl Acad Sci 2009;106:8380–5.

Genty B, Briantais J-M, Baker NR. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 1989;990:87–92.

Guan C, Liu X, Song X, Wang G, Ji J, Jin C. Overexpression of a peroxiredoxin Q gene, SsPrxQ, in *Eustoma grandiflorum* Shinn enhances its tolerance to salt and high light intensity. Mol Breed 2014;33:657–67.

Huang J-L, Cheng L-L, Zhang Z-X. Molecular cloning and characterization of violaxanthin de-epoxidase (VDE) in *Zingiber officinale*. Plant Sci 2007;172:228–35.

Ivanov A, Krol M, Maxwell D, Huner N. Abscisic acid induced protection against photoinhibition of PSII correlates with enhanced activity of the xanthophyll cycle. FEBS Lett 1995;371:61–4.

Jahns P, Holzwarth AR. The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. Biochim Biophys Acta 2012;1817:182–93.

Kalituho L, Beran KC, Jahns P. The transiently generated nonphotochemical quenching of excitation energy in Arabidopsis leaves is modulated by zeaxanthin. Plant Physiol 2007;143:1861–70.

Kitahata N, Han S-Y, Noji N, Saito T, Kobayashi M, Nakano T, et al. A 9-cis-epoxycarotenoid dioxygenase inhibitor for use in the elucidation of abscisic acid action mechanisms. Bioorg Med Chem 2006;14:5555–61.

Kotabová E, Kaňa R, Jarešová J, Prášil O. Non-photochemical fluorescence quenching in *Chromera velia* is enabled by fast violaxanthin de-epoxidation. FEBS Lett 2011;585:1941–5.

Li X, Zhao W, Sun X, Huang H, Kong L, Niu D, et al. Molecular cloning and characterization of violaxanthin de-epoxidase (CsVDE) in cucumber. PLoS ONE 2013;8:e64383.

Lichtenthaler HK. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 1987;148:350–82.

Müller M, Hernández I, Alegre L, Munné-Bosch S. Enhanced α-tocopherol quinone levels and xanthophyll cycle de-epoxidation in rosemary plants exposed to water deficit during a Mediterranean winter. J Plant Physiol 2006;163:601–6.

Nambara E, Marion-Poll A. Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 2005;56:165–85.

Niyogi KK, Truong TB. Evolution of flexible non-photochemical quenching mechanisms that regulate light harvesting in oxygenic photosynthesis. Curr Opin Plant Biol 2013;16:307–14.

North HM, Frey A, Boutin J-P, Sotta B, Marion-Poll A. Analysis of xanthophyll cycle gene expression during the adaptation of Arabidopsis to excess light and drought stress: changes in RNA steady-state levels do not contribute to short-term responses. Plant Sci 2005;169:115–24.

Ocampo-Alvarez H, García-Mendoza E, Govindjee. Antagonist effect between violaxanthin and de-epoxidated pigments in nonphotochemical quenching induction in the qE deficient brown alga *Macrocystis pyrifera*. Biochim Biophys Acta 2013;1827:427–37.

Ozfidan C, Turkan I, Sekmen AH, Seckin B. Abscisic acid-regulated responses of aba2-1 under osmotic stress: the abscisic acid-inducible antioxidant defence system and reactive oxygen species production. Plant Biol 2012;14:337–46.

Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, et al. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science 2009;324:1068–71.

Pascal AA, Liu Z, Broess K, van Oort B, van Amerongen H, Wang C, et al. Molecular basis of photoprotection and control of photosynthetic light-harvesting. Nature 2005;436:134–7.

Pinheiro C, Chaves MM. Photosynthesis and drought: can we make metabolic connections from available data. J Exp Bot 2011;62:869–82.

Qin X, Zeevaart JA. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proc Natl Acad Sci 1999;96:15354–61.

Saradhi PP, Suzuki I, Katoh A, Sakamoto A, Sharmila P, Shi DJ, et al. Protection against the photo-induced inactivation of the photosystem II complex by abscisic acid. Plant Cell Environ 2000;23:711–8.

Seo M, Koshiba T. Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 2002;7:41–8.

Seo M, Peeters AJ, Koiwai H, Oritani T, Marion-Poll A, Zeevaart JA, et al. The Arabidopsis aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc Natl Acad Sci 2000;97:12908–13.

Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. J Exp Bot 2007;58:221–7.

Shou H, Bordallo P, Wang K. Expression of the Nicotiana protein kinase (NPK1) enhanced drought tolerance in transgenic maize. J Exp Bot 2004;55:1013–9.

Song X, Shang Z, Yin Z, Ren J, Sun M, Ma X. Mechanism of xanthophyll-cycle-mediated photoprotection in *Cerasus humilis* seedlings under water stress and subsequent recovery. Photosynthetica 2011;49:523–30.

Strasser R, Tsimilli-Michael M, Greppin H. How excitation energy distribution indicates the complexity of a developing and to the environment adapting photosynthetic apparatus. Travel Shot Plant Dev 1997;9:9–129.

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol Biol Evol 2007;24:1596–9.

Thayer SS, Björkman O. Leaf xanthophyll content and composition in sun and shade determined by HPLC. Photosynth Res 1990;23:331–43.

Thompson AJ, Jackson AC, Parker RA, Morpeth DR, Burbidge A, Taylor IB. 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 2000;42:833–45.

Xiong L, Ishitani M, Lee H, Zhu J-K. The *Arabidopsis* LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. Plant Cell Online 2001;13:2063–83.

Xiong L, Lee H, Ishitani M, Zhu J-K. Regulation of osmotic stress-responsive gene expression by theLOS6/ABA1 locus in *Arabidopsis*. J Biol Chem 2002;277:8588–96.

Yu H, Chen X, Hong Y-Y, Wang Y, Xu P, Ke S-D, et al. Activated expression of an Arabidopsis HD-START protein confers drought tolerance with improved root system and reduced stomatal density. Plant Cell Online 2008;20:1134–51.

Zeevaart J, Creelman R. Metabolism and physiology of abscisic acid. Annu Rev Plant Physiol Plant Mol Biol 1988;39:439–73.

Zhang J, Jia W, Yang J, Ismail AM. Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res 2006;97:111–9.

Zhao W, Wang S, Li X, Huang H, Sui X, Zhang Z. Molecular cloning and characterization of the light-regulation and circadian-rhythm of the VDE gene promoter from *Zingiber officinale*. Plant Cell Rep 2012;31:1381–92.

Zhu J. Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 2002;53:247.

Zhu S-Q, Chen M-W, Ji B-H, Jiao D-M, Liang J-S. Roles of xanthophylls and exogenous ABA in protection against NaCl-induced photodamage in rice (*Oryza sativa* L) and cabbage (*Brassica campestris*). J Exp Bot 2011;62:4617–25.