Overexpression of the *ChVDE* gene, encoding a violaxanthin de-epoxidase, improves tolerance to drought and salt stress in transgenic *Arabidopsis*

Li Na Sun² · Fang Wang² · Jie Wan Wang² · Li Jiao Sun² · Wen Rui Gao² · Xing Shun Song^{1,2}

Received: 28 January 2019 / Accepted: 25 April 2019 / Published online: 3 May 2019
© King Abdulaziz City for Science and Technology 2019

Abstract

To investigate the protective mechanism of violaxanthin de-epoxidase (VDE) zeaxanthin in *Cerasus humilis* under drought and salt-stress conditions, we cloned the entire cDNA sequence of *ChVDE* from *C. humilis* and generated *ChVDE*-overexpression (OE) and *ChVDE*-complementation (CE) *Arabidopsis* plants. The open reading frame of *ChVDE* contained 1,446 bp nucleotides and encoded 481 amino acids. The *ChVDE* showed the highest similarity with those of *Camellia sinensis* and *Citrus sinensis*. Subcellular localization analysis showed that *ChVDE* was located in the chloroplasts. OE plants showed stronger root growth and higher levels of total chlorophyll as compared to WT and *VDE* mutant (*npq1-2*) plants. Moreover, the relative de-epoxidation state of the xanthophyll cycle pigments (A + Z)/(V + A + Z) was higher in OE plants than in the controls. OE plants had enhanced photosynthetic rates, respiration rates, and transpiration rates compared with the WT or *npq1-2* plants after drought or salt treatment. Collectively, our results demonstrate that *ChVDE* plays a positive role in both drought and salt tolerance.

Keywords *Cerasus humilis* · *ChVDE* · Multiple tolerances · Xanthophyll cycle · *Arabidopsis*

Introduction

Plants are often subjected to environmental stressors such as drought, salinity, and extreme temperatures, thereby changing various cellular processes such as photosynthesis, growth, carbon allocation, and carbohydrate and lipid metabolism (Sun et al. 2019). The damage caused by toxic reactive oxygen species (ROS), known as oxidative stress, is one of the main factors that damages plants exposed to environmental stress (Wu et al. 2018). One of the most efficient mechanisms that protects plants under oxidative stress conditions is the xanthophyll cycle (Latowski et al. 2011), which contains three types of carotenoid pigments: violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z). These

carotenoid pigments are catalyzed by two enzymes: violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE). When light energy is excessive, V is catalyzed to Z via A by VDE. When light stress diminishes, the reverse occurs: the epoxidation of Z to A to V, catalyzed by ZE. It has been shown that induced Z improves photoprotection capacity and increases the quenching of excited chlorophyll states (both singlet and triplet) and the scavenging of excessive ROS (Rockholm and Yamamoto 1996; Li et al. 2009).

VDE is the first described putative plant lipocalin (Bugos and Yamamoto 1996) and has been classified as a lipocalin-like protein (Charron et al. 2005). The activation of VDE is related to the conformational change (Kawano and Kuwbara 2000) and the formation of an α -helical coiled structure located at the C-terminal domain (Hallin et al. 2016). Other research has reported that VDE uses ascorbic acid as a secondary substrate, possibly as a monomer. Its active site is occluded with a lipocalin barrel at a neutral pH (Arnoux et al. 2009). The regulation of *VDE* can take place at the transcriptional, post-transcriptional, or translational levels. Extensive studies have revealed that environmental factors influence *VDE* expression patterns (Huang et al. 2007; Facella et al. 2008; Han et al. 2010). For example, Huang

✉ Xing Shun Song
sfandi@163.com

¹ State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University), Harbin 150040, People's Republic of China

² Department of Genetics, College of Life Science, Northeast Forestry University, Harbin 150040, People's Republic of China

et al. (2007) found that VDE is involved in alleviating photoinhibition in tobacco leaves. Overexpression of *LcVDE* in *Arabidopsis* increases plant tolerance to drought stress (Guan et al. 2015).

Chinese dwarf cherry (*Cerasus humilis*), an endemic Chinese species, produces fruit that contains various minerals beneficial to human health. Specifically, the calcium content is higher than that of many other fruits. In recent years, there has been an urgent need to expand the area under cultivation to meet production demand (Song et al. 2011; Yin et al. 2012, 2014). *C. humilis* is tolerant to environmental stressors, such as drought, saline, alkali and cold, all of which adversely affect the growth and yield of plants, including other perennial dwarf shrubs. Our previous study showed that the overexpression of *VDE* confers a great capacity for photoprotection and thus contributes to the survival of *C. humilis* against drought (Song et al. 2011).

In the present study, we cloned the entire cDNA sequence of *ChVDE* from *C. humilis* and introduced the region into *Arabidopsis* plants for further study of its role in the protective mechanism under drought and salt conditions.

Materials and methods

Plant materials and growth conditions

Cuttings of *C. humilis* 'Nongda 4' were used in the present study, and the growth conditions followed those previously described (Yin et al. 2014). Each cutting was transplanted into a container (35 × 35 × 25 cm) filled with organic soil and irrigated regularly with Hoagland solution. Cuttings were grown under a 12-h photoperiod at temperatures ranging from about 17–25 °C, photosynthetic photon flux density (PPFD) of 600 μmol m⁻² s⁻¹, and a relative humidity of 70–75% in the greenhouse. Seedlings with 25–35 leaves were used for RNA extraction.

Arabidopsis seeds were soaked in 75% ethyl alcohol for 1 min and sodium hypochlorite solution (bleach) for 1 min, and then rinsed 5 times with sterilized water. Seedlings were grown in a Murashige–Skoog (MS) medium including vitamins, 2.5% sucrose, and 0.8% agarose (pH 5.8), or in soil in a growth chamber kept at 22 °C with a 16-h photoperiod as described by Kim et al. (2014a).

ChVDE cloning and sequence analysis

Total RNA from *C. humilis* leaves was isolated using Trizol reagent. One microgram of total RNA was reverse-transcribed with Revert Aid Reverse Transcriptase (Fermentas) and Oligod (T) primers (TaKaRa). Reverse transcription conditions for each cDNA amplification were 65 °C for 5 min, 37 °C for 52 min, and 70 °C for 15 min as described

by Yin et al. (2017). Based on the *C. humilis* transcriptome database constructed by our laboratory (NCBI accession number: SAMN11393361), a novel *VDE* was isolated using specific primers (*VDE*-F: 5'-CACCATGGCATTGGCTGGACGTTCA-3' and *VDE*-R: 5'-TCTTAGCTTCCTAAGTGG AAG-3'). The gene sequence was analyzed using the NCBI database. Multiple amino acid sequence alignments were performed using Clustal X (1.81), and a phylogenetic tree was constructed using MEGA6 software.

Vector construction and *Arabidopsis* transformation

To generate the *VDE*-overexpression (OE) and *VDE*-complementation (CE) plants, the full-length coding sequence of *ChVDE* obtained by reverse transcription PCR (RT-PCR) amplification was extended from the CACC at the 5'-end using the proofreading KOD-Plus-Neo enzyme (TOYOBO). The fragment was first cloned into the pENTR™/SD/D-TOPO vector (Invitrogen) and then transferred into the pGWB5 vector using Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen) according to the manufacturer's protocol. The recombinant vector was transformed into both *Arabidopsis* WT and *VDE* mutant (*npq1-2*) plants using *Agrobacterium tumefaciens* strain EHA105 by employing the floral dipping method (Clough and Bent 1998). *Npq1-2* seeds (CS3771) were purchased from the *Arabidopsis* Biological Resource Center (ABRC). The first-generation (T0) seeds of the transgenic plants were collected and screened in MS medium with 50 mg L⁻¹ kanamycin. Kanamycin-resistant plants were transferred into soil pots and grown in a growth chamber with a 16-h photoperiod cycle at 22 °C. Finally, transgenic *Arabidopsis* plants were identified by PCR amplification. The T3 homozygous-positive plants were used for all further experiments.

Subcellular localization of *ChVDE*

Three-week-old OE seedlings were observed under a Zeiss LSM 510 Meta (Carl Zeiss, Jena, Germany) confocal microscope using the 63X oil immersion objective. Excitation wavelengths used were 488 nm with green fluorescent protein (GFP) (Schindelin et al. 2015). Images were analyzed using the LSM 5 Image Browser.

Stress treatment in the transgenic plants

For the germination of transgenic *Arabidopsis* seeds under stress treatment, T3 homozygous seeds were germinated on MS medium supplemented with 10% polyethylene glycol (PEG) and 100 mM NaCl (Zhou et al. 2016). WT and empty vector-transformed plants did not show any difference during any of the experimental phases. Thus, only data from WT plants are presented here.

For seedling experiments, 2-week-old WT, *npq1-2*, and two transgenic (OE and CE) plants in MS medium were transplanted into small plastic pots (6.3 × 5.0 cm) with one seedling per pot for 5 weeks, and subsequently subjected to drought stress for 9 days by withholding water. In another group, seedlings of different types were flooded with 200 mM NaCl for 7 days (Kim et al. 2014a, b).

Quantitative real-time PCR (qRT-PCR) analysis of *ChVDE*

Total RNA from Arabidopsis leaf tissue was isolated using a Plant RNA Kit (OMEGA). DNA-free total RNA was reverse-transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). The cDNA template obtained was diluted tenfold for subsequent quantitative analysis, which was performed with a qRT-PCR system (Roche Light Cycler 480 II, Switzerland) and UltraSYBR Mixture (CWBIO). Equal amounts of cDNA were amplified with gene-specific forward (F) and reverse (R) primers for the quantification of *VDE* gene expression (F: 5'-TGGCTTGAATCCTACC-3'; R: 5'-GGCTGATTGGGTCTT-3'). The reaction was conducted in a total volume of 20 µL, containing 10 µL of Ultra SYBR Mixture, 1 µL of each primer, and 8 µL cDNA. The thermocycler program was set at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 32 s. Values represent the mean of three biological replicates and two technical replicates. The relative gene expression levels were calculated following the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Expression of *AtACTIN* was used for the normalization of amplification curves and gene expression as an internal standard.

Analysis of xanthophyll cycles

Leaf samples were taken after drought or salt treatment and immediately frozen in liquid nitrogen. Pigment compounds in the leaves were extracted using ice-cold 80% acetone. The pigment extracts were filtered through a 0.45-µm membrane and analyzed with an HPLC following a previously published method (Thayer and Björkman 1900). The relative de-epoxidation state of the xanthophyll cycle pigments was calculated as $(A + Z)/(V + A + Z)$ (Yang et al. 2015).

Measurement of chlorophyll content and photosynthesis

The total chlorophyll content was measured using a CCM-200 chlorophyll meter (OPTI-SCIENCES, USA). Gas exchange was measured with an open gas-exchange system (LI-6400, Li-Cor, Lincoln, NE, USA). Measurements of net photosynthetic rate (P_n), transpiration rate, and respiration

rate were performed as previously described (Resmi et al. 2015).

Statistical analysis

Statistical significance of differences between the control and transgenic plants was measured using one-way ANOVAs and Student's *t* tests for each measured parameter. Means were considered to be significantly different when $P \leq 0.05$.

Results

Isolation and sequence analysis of *ChVDE*

The full-length cDNA was obtained from *C. humilis* using RT-PCR, namely *ChVDE* (GenBank accession number: KM504154). The 1,446-bp ORF encoded a putative peptide of 481 amino acids, with a calculated theoretic isoelectric point (pI) of 5.75 and molecular mass of 54.4 Ku.

Multiple alignments of the amino acid sequences for homologous genes from other plant species were conducted using Clustal X (version 1.81) software. The *ChVDE* sequence had 65.64% homology with *VDE* in Arabidopsis (AAC50032.1), 87.94% with *Pyrus × bretschneideri* (XP_009379699.1), 85.86% with *Malus × domestica* (XP_008350656.1), 98.13% with *Prunus persica* (XP_007207430.1), and 98.13% with *Prunus mume* (XP_016651830.1; Fig. 1a). The amino acid sequence alignment revealed that *ChVDE* contained three interesting domains, including a cysteine-rich domain (Block I), a lipocalin signature domain (Block II), and a C-terminal glutamic acid-rich domain (Block III; Fig. 1a), which were all conserved. The phylogenetic analysis showed that *ChVDE* had high similarity to orthologous proteins from some shrubs or small trees such as *Camellia sinensis* and *Citrus sinensis* (Fig. 1b).

Identification of transgenic plants

To functionally characterize the function of *ChVDE*, we overexpressed full-length *ChVDE* under a strong constitutive CaMV 35S promoter in Arabidopsis (Fig. 2a). After the first screening with kanamycin, all transformants infected with *A. tumefaciens* carrying *ChVDE* were confirmed via PCR using specific primers. Three independent OE and four independent CE with individual kanamycin-resistance were obtained, and genomic DNA from the PCR analysis indicated the integration of the *ChVDE* gene by the presence of a 1.4-kb band (Fig. 2b, c). At this time, heterozygous transgenic plants were obtained. Transgenic plants were continuously sown on

Fig. 1 Amino acid sequence alignment of VDE in different plants and phylogenetic trees of VDE isolated from different species. **a** Three conserved domains are indicated below the alignment. Asterisks indicate the conserved amino acid. The accession numbers in GenBank for VDE are as follows: PbVDE (XP_009379699.1), MdVDE (XP_008350656.1), PpVDE (XP_007207430.1), PmVDE (XP_016651830.1), ChVDE (AIZ75647.1), and AtVDE (AAC50032.1). Multiple alignments were performed using Clustal software. **b** The phylogenetic tree was created using the neighbor-joining method in MEGA6 software

the kanamycin-resistance screening medium for three generations to obtain homozygous transgenic plants (Fig. 2d).

Subcellular localization of ChVDE

To determine the subcellular localization of ChVDE, we produced Arabidopsis plants transformed with *ChVDE-GFP* and subjected them to confocal microscopy analysis. In a fresh epidermal cell from *ChVDE-GFP* plants, the signal was located at the chloroplasts (Fig. 3a). Moreover, the fluorescent signal was also enhanced near the inner borders of some guard cells in young plant leaves (Fig. 3b red arrows).

The effect of drought or salt stress on morphology, *ChVDE* expression, and root length in the transgenic plants

We speculated that overexpression of *ChVDE* would enhance both drought and salt stress tolerance. First, we compared the morphological profiles of test plants after drought or salt treatment. The *npq1-2* mutant plants exhibited severe necrosis on day 9 of drought treatment (Fig. 4a) and did not recover after irrigation was resumed (data not shown). However, compared with the *npq1-2* mutant plants, the OE-1 plants showed better growth under drought stress. Moreover, the CE-3 plants had improved growth and stayed greener as compared with the *npq1-2* plants (Fig. 4a). Drought stress

Fig. 2 The identification of transgenic plants. **a** Schematic representation of the *ChVDE* construct driven by constitutive CaMV 35S promoter and nopaline synthase terminator (NOS) in a pGWB5 vector. **b** Genomic PCR analysis of the *ChVDE* gene from putative transgenic T1 WT Arabidopsis plants. *M* maker2000, 1–3 transgenic plants, *WT* wild-type plant, *d* ddH₂O, *P* positive control (plasmid). **c** Genomic PCR analysis of *ChVDE* gene from putative transgenic npq1-2 mutant Arabidopsis plants. *M* maker2000, 1–4 transgenic plants, *Mu* mutant plant, *WT* wild-type plant, *d* ddH₂O, *P* positive control (plasmid). **d** Representative picture of WT, npq1-2, and T3 transgenic plants germinated on MS medium supplemented with kanamycin

resulted in the gradual increase of *ChVDE* transcripts in the OE-1 plants. By the end of the 9-day treatment, the *ChVDE* transcript had increased by 26.6% compared with the control plants (Fig. 4b). In contrast to the drought treatment, we did not observe significant phenotypic differences during NaCl treatment (Fig. 4c). However, the *ChVDE* gene in the OE-1 plants was upregulated and reached a peak on day 7 about 1.6-fold higher as compared to the controls (Fig. 4d). In the drought and salt stress treatments, germinated seedlings were grown in MS medium with 10% PEG or 200 mM NaCl for 14 d. In the PEG treatment, WT seedlings exhibited slower root growth as compared to the OE-1 seedlings. The roots of the WT seedlings grown in PEG were 1.88 cm long, while the roots of the OE-1 seedlings were 2.38 cm long (Fig. 5a, b). Similarly, differences in root growth between the WT and transgenic plants were apparent at 200 mM NaCl. WT and OE-1 roots were 3.94 cm long and 4.76 cm long, respectively (Fig. 5c, d). No difference was observed in root length in all lines without PEG or NaCl (normal conditions) under our current experimental conditions (data not shown). These results indicated that roots of OE plants were more tolerant to drought and high salinity than those of the WT plants.

Xanthophyll cycle changes in transgenic Arabidopsis plants

In the drought stress treatment, the de-epoxidation ratio of the xanthophyll cycle pigments $(A + Z)/(V + A + Z)$ in OE-1 seedlings increased markedly relative to WT plants, while the pigments in the npq1-2 plants were lower than in the WT. Plants

Fig. 3 Subcellular localization of ChVDE in overexpression Arabidopsis chloroplasts (**a**) and guard cells (**b**). (i) GFP (ChVDE-GFP fusion protein) fluorescence imaging, (ii) bright-field image, (iii) chloroplast auto fluorescence imaging, and (iv) overlap image of GFP (green) and chlorophyll (red) fluorescence. Bars = 10 μ m

in the CE-3 plants maintained the same pigment levels as the WT plants. At the end of the 9-day drought treatment, the ratio of $(A + Z)/(V + A + Z)$ was 0.87, 0.59, and 0.62 in the OE-1, CE-3, and WT plants, respectively (Fig. 6a). In plants treated with salt stress, the ratio of $(A + Z)/(V + A + Z)$ increased to 0.76 in OE-1, 0.64 in CE-3, 0.38 in npq1-2, and 0.61 in WT (Fig. 6c). These results demonstrated that overexpression of *ChVDE* could improve the level of de-epoxidation in the xanthophyll cycle when there was excess light energy induced by stressors.

Comparison of chlorophyll content in transgenic Arabidopsis plants

In plants grown under normal conditions, no significant difference in total chlorophyll content was detected among

Fig. 4 Phenotypes of *ChVDE*-overexpression (OE-1), *ChVDE*-complementation (CE-3) and *VDE* mutant (*npq1-2*) Arabidopsis plants grown under different stress treatments. Comparative growth in response to 9 days of withholding water (a) and NaCl treatment (c) in WT, *npq1-2*, CE-3, and OE-1 plants, respectively. The expression levels of *ChVDE* after withholding water (b) and NaCl treatment (d) in OE-1 plants. Different letters indicate significant differences at the $P < 0.05$ level

Fig. 5 Comparative root growth in control and transgenic *ChVDE*-expressing plants after PEG (a, b) and NaCl treatments (c, d). Seeds of independent transgenic lines (WT, *npq1-2*, CE-3, and OE-1) were germinated on MS supplemented with 10% PEG/100 mM NaCl, and the root length was measured after 14 days. Different letters indicate significant differences at the $P < 0.05$ level

the control and other lines. Drought treatment significantly decreased the total chlorophyll content of the WT plants, and hardly any chlorophyll content was detected in CE-3 plants. However, the leaves of OE-1 plants retained a greenish color under drought conditions (Fig. 4b), and the total chlorophyll content was maintained at the same level as in leaves from control WT plants (Fig. 6b). Under the salt stress treatment, the total chlorophyll content in the *npq1-2* plant was slightly lower compared with the other lines when they were grown under optimal conditions. Seven days after treatment with salt stress, the WT and *npq1-2* plants maintained the same level of total chlorophyll content as they had prior to the treatment with salt stress. The total chlorophyll content increased by 200% and 118% in leaves from OE-1 and CE-3 plants, respectively (Fig. 6d).

Effects of drought and salt stress on photosynthetic parameters in transgenic Arabidopsis plants

During drought stress treatment, Pn was significantly inhibited in all lines. However, OE-1 plants had higher Pn as compared to plants in the other lines after nine days of drought stress treatment (Fig. 7a). The transpiration rate change followed a similar pattern as that of Pn (Fig. 7c). Moreover, the respiration rate of OE-1 plants remained at the same level as that of plants grown under optimal conditions. In contrast, the respiration rate decreased by 36% and 66% for WT and CE-3 plants, respectively, compared with plants grown under normal conditions (Fig. 7b). The chlorophyll content was so low in the *Npq1-2* plants after drought treatment that these photosynthetic parameters were hardly detected (Fig. 7a–c).

Before salt stress treatment, no significant difference was observed in Pn among all lines. After growth under salt stress for 7 days, the Pn of WT, *npq1-2*, and CE-3 plants decreased, and this decrease was greatest in *npq1-2* plants.

Fig. 6 Changes in de-epoxidation ratio of the xanthophyll cycle pigments ($A+Z/A+V+Z$) and chlorophyll content in WT, *npq1-2*, and transgenic Arabidopsis plants under water deficit for 9 days (**a, b**, respectively) or salt stress for 7 days (**c, d**, respectively). *nd* not detected. Different letters indicate significant differences at the $P < 0.05$ level

Fig. 7 Changes in net P_n , respiration rate, and transpiration rate in WT, *VDE* mutant (*npq1-2*), and transgenic Arabidopsis plants under water deficit for nine days (**a–c**, respectively) or salt stress for 7 days (**d–f**, respectively). *nd* not detected. Different letters indicate significant differences at the $P < 0.05$ level

However, the Pn of OE-1 plants maintained the same level as that of plants grown under normal conditions (Fig. 7d). Similarly, the OE-1 plants showed significantly higher transpiration rates and respiration rates as compared to the other plants (Fig. 7e, f). These results suggest a positive relationship between *ChVDE* overexpression and increased vigor.

Discussion

During growth, *C. humilis* is affected by unfavorable conditions such as drought or salt stress. Energy dissipation via deoxidization of the xanthophyll cycle is one of the key mechanisms that allow plants to adapt to environmental stress (Demmig-Adams and Adams 1996). VDE plays a key role in the xanthophyll cycle, protecting the photosynthetic apparatus from photodamage (Li et al. 2009; Demmig-Adams et al. 1996). Our previous study indicated that VDE is expressed in *C. humilis* subject to abiotic stress (Song et al. 2011). In the present study, we investigated this further by cloning the entire length of VDE from *C. humilis*, transforming it into Arabidopsis plants, and treating several lines with drought or salt stress.

The amino acid sequence of *ChVDE* shares the most similarities in the middle and C-terminal regions with the VDE protein from other species (Arnoux et al. 2009). These regions contain Cys-rich, lipocalin signature, and Glu-rich domains (Fig. 1a). Previous studies have also identified three domains in VDEs isolated from other species (Han et al. 2010; Bugos et al. 1998; Jahns and Heyde 1999; Yang et al. 2015). The cysteine-rich domain in Block I should be the active site of VDE. The lipocalin signature domain in Block II has been suggested to be a possible binding site for V and monogalactosyldiacylglycerol (MGDG), which is the most effective lipid supporting VDE activity. The glutamate-rich domain (Block III) at the C-terminal region is thought to take part in the binding of VDE to the thylakoid membrane (Arnoux et al. 2009). The cDNA isolated from *C. humilis* encodes a functional, active VDE. The relationship of enzymatic activity of *ChVDE* and residues merits further investigation into the characterization of its function in silico and site-directed mutagenesis in vitro (Gao et al. 2013).

Our phylogenetic tree data (Fig. 1b) indicated that VDEs have a distant genetic relationship among different species, as indicated in some previous phylogenetic studies (Coesel et al. 2008; Li et al. 2013). *ChVDE* proteins were mainly observed in chloroplasts and guard cells in *C. humilis* leaves (Fig. 3), which is in agreement with previous studies on the expression of AtVDE in *Arabidopsis thaliana*, LeVDE in *Lycopersicon esculentum*, GVDE in *Zingiber officinale*, and CsVDE in *Cucumis sativus* (North et al. 2005; Huang et al. 2007; Han et al. 2010; Zhao et al. 2012; Li et al. 2013). Location of the *ChVDE* proteins in the chloroplasts is also in

agreement with their photoprotective role under light stress (Yang et al. 2015; Li et al. 2013).

Transgenic Arabidopsis leaves remained green during drought treatment (Fig. 4a), suggesting that they have higher photosynthetic capacity as compared to WT leaves (Augustine et al. 2015). After exposure to drought or salt stress, the OE plants showed stronger root growth as compared to the respective controls (Fig. 5), indicating that transgenic seedlings were highly tolerant to both high salinity and drought treatments after germination. OE-1 leaves consistently retained their chlorophyll more efficiently than WT leaves subjected to either high salinity or drought stress (Fig. 6b, d). These data demonstrate that the overexpression of *ChVDE* conferred tolerance to salt and drought stresses in Arabidopsis plants. Similar results have also been reported in ginger (Zhao et al. 2012), tomato (Han et al. 2010), tobacco (Gao et al. 2010), cucumber (Li et al. 2013), bamboo (Gao et al. 2013), and peanut (Yang et al. 2015).

Compared with their respective controls, OE-1 and CE-3 plants with *ChVDE* induced a change in the level of de-epoxidation under stressors (Fig. 6a, c). This indicates that by altering the VDE levels in stressed transgenic plants, de-epoxidation affected the components of the xanthophyll cycle (Gao et al. 2010). Xanthophyll cycle-dependent non-photochemical quenching (NPQ) plays an important role in the protection of PSII under excess irradiation (Li et al. 2003). It has been noted that there is a good correlation between NPQ and the amount of Z or the sum of A and Z in *C. humilis* (Song et al. 2011). Thus, the higher de-epoxidation ratio of xanthophyll cycles in OE-1 and CE-3 plants in the present study indicates that the overexpression of *ChVDE* enhanced the xanthophyll cycle-dependent energy dissipation under drought and salt stressors. The limitation of thermal dissipation resulted in the reduction of the de-epoxidation state in *npq1-2* mutant plants subjected to drought or salt stress (Fig. 6a, c).

After drought or salt treatment, OE-1 plants exhibited higher chlorophyll content and Pn (Figs. 6b, d, 7a, d) as well as slight changes in respiration rates compared to WT plants (Fig. 7b, e), indicating that *ChVDE* took part in alleviating the suppression of PSII activity in transgenic Arabidopsis grown under stress conditions. The accumulation of ROS is relevant to the delayed consumption of excess light energy, and ROS are potentially dangerous for plants under drought stress (Peltzer et al. 2002). In our previous studies, we found that *C. humilis* plants under drought stress maintained CO₂ assimilation rates by increasing light reactions and the Calvin cycle to some extent. Additionally, the enhancement of the ascorbate–glutathione cycle and glutathione peroxidase/glutathione s-transferase pathway could minimize oxidative damage by ROS (Ren et al. 2016; Yin et al. 2017). Accordingly, we speculated that enhanced drought or salt tolerance in OE-1 plants was caused partly by a reduction in the excess

accumulation of ROS. In contrast to the Pn changes, the transpiration rate was higher in the transgenic plants than in WT plants (Fig. 7a, c, d, e). These results, along with the localization of ChVDE in guard cells in some epidermal cells (Fig. 3b), implied that the abiotic stress response of transgenic plants might at least partly be due to the stomatal changes that regulate water level in plants. The related mechanism needs to be studied in detail.

It is worth mentioning that *npq1-2* are hypersensitive to drought and salt in the physiological parameters we investigated. However, *ChVDE* was able to complement the drought- and salt-sensitive changes of *npq1-2* mutants to a large extent (Figs. 6, 7), suggesting that the functions of *ChVDE* and *Arabidopsis VDE* are, at least partially, overlapped and can be reciprocally substituted in different plants.

In conclusion, *ChVDE*-OE transgenic *Arabidopsis* plants were found to be more tolerant to drought and salinity stress as compared to WT plants, suggesting a positive role of *ChVDE* from *C. humilis* in response to abiotic stressors.

Acknowledgements This work was supported by Fundamental Research Funds for the Central Universities (2572018CG02), the National Natural Science Foundation of China (31170569) and the Innovation Project of State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University).

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

- Arnoux P, Morosinotto T, Saga G, Bassi R, Pignol D (2009) A structural basis for the pH-dependent xanthophyll cycle in *Arabidopsis thaliana*. *Plant Cell* 21:2036–2044
- Augustine SM, Narayan JA, Syamaladevi DP et al (2015) *Erianthus arundinaceus* HSP70 (EaHSP70) overexpression increases drought and salinity tolerance in sugarcane (*Saccharum* spp. hybrid). *Plant Sci* 232:23–34
- Bugos RC, Yamamoto HY (1996) Molecular cloning of violaxanthin de-epoxidase from romaine lettuce and expression in *Escherichia coli*. *Proc Natl Acad Sci USA* 93:6320–6325
- Bugos RC, Hieber AD, Yamamoto HY (1998) Xanthophyll cycle enzymes are members of the lipocalin family, the first identified from plants. *J Biol Chem* 273:15321–15324
- Charron JBF, Ouellet F, Pelletier M, Danyluk J, Chauve C, Sarhan F (2005) Identification, expression, and evolutionary analyses of plant lipocalins. *Plant Physiol* 139:2017–2028
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743
- Coesel S, Oborník M, Varela J, Falcatore A, Bowler C (2008) Evolutionary origins and functions of the carotenoid biosynthetic pathway in marine diatoms. *PLoS One* 3:e2896
- Demmig-Adams B, Adams WW (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends Plant Sci* 1:21–26
- Demmig-Adams B, Gilmore AM, Adams W (1996) Carotenoids 3: in vivo function of carotenoids in higher plants. *FASEB J* 10:403–412
- Facella P, Lopez L, Carbone F, Galbraith DW, Giuliano G, Perrotta G (2008) Diurnal and circadian rhythms in the tomato transcriptome and their modulation by cryptochrome photoreceptors. *PLoS One* 3:e2798
- Gao S, Han H, Feng HL, Zhao SJ, Meng QW (2010) Overexpression and suppression of violaxanthin de-epoxidase affects the sensitivity of Photosystem II photoinhibition to high light and chilling stress in transgenic tobacco. *J Integr Plant Biol* 52:332–339
- Gao ZM, Liu Q, Zheng B, Chen Y (2013) Molecular characterization and primary functional analysis of PeVDE, a violaxanthin de-epoxidase gene from bamboo (*Phyllostachys edulis*). *Plant Cell Rep* 32:1381–1391
- Guan CF, Ji J, Zhang XQ, Li XZ, Jin C, Guan WZ, Wang G (2015) 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*. *J Plant Physiol* 175:26–36
- Hallin EI, Hasan M, Guo K, Åkerlund HE (2016) Molecular studies on structural changes and oligomerisation of violaxanthin de-epoxidase associated with the pH-dependent activation. *Photosynth Res* 129:29–41
- Han H, Gao S, Li B, Dong XC, Feng HL, Meng QW (2010) Overexpression of violaxanthin de-epoxidase gene alleviates photoinhibition of PSII and PSI in tomato during high light and chilling stress. *J Plant Physiol* 167:176–183
- Huang JL, Cheng LL, Zhang ZX (2007) Molecular cloning and characterization of violaxanthin de-epoxidase (VDE) in *Zingiber officinale*. *Plant Sci* 172:228–235
- Jahns P, Heyde S (1999) Dicyclohexylcarbodiimide alters the pH dependence of violaxanthin de-epoxidation. *Planta* 207:393–400
- Kawano M, Kuwabara T (2000) pH-dependent reversible inhibition of violaxanthin de-epoxidase by pepstatin related to protonation-induced structural change of the enzyme. *FEBS Lett* 481:101–104
- Kim EY, Seo YS, Park KY, Kim SJ, Kim WT (2014a) Overexpression of CaDSR6 increases tolerance to drought and salt stresses in transgenic *Arabidopsis* plants. *Gene* 552:146–154
- Kim Y, Wang M, Bai Y, Zeng Z, Guo F, Han N et al (2014b) Bcl-2 suppresses activation of VPEs by inhibiting cytosolic Ca^{2+} level with elevated K^{+} efflux in NaCl-induced PCD in rice. *Plant Physiol Bioch* 80:168–175
- Latowski D, Kuczyńska P, Strzałka K (2011) Xanthophyll cycle—a mechanism protecting plants against oxidative stress. *Redox Rep* 16:78–90
- Li XG, Meng QW, Jiang GQ, Zou Q (2003) The susceptibility of cucumber and sweet pepper to chilling under low irradiance is related to energy dissipation and water-water cycle. *Photosynthetica* 41:259–265
- Li Z, Wakao S, Fischer BB, Niyogi KK (2009) Sensing and responding to excess light. *Annu Rev Plant Biol* 60:239–260
- Li X, Zhao WC, Sun XY, Huang HY, Kong LC, Niu DD et al (2013) Molecular cloning and characterization of violaxanthin de-epoxidase (*CsVDE*) in cucumber. *PLoS One* 8:e64383
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408
- North HM, Frey A, Boutin J-P, Sotta B, Marion-Poll A (2005) 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* 169(1):115–124
- Peltzer D, Dreyer E, Polle A (2002) Differential temperature dependencies of antioxidative enzymes in two contrasting species: *Fagus sylvatica* and *Coleus blumei*. *Plant Physiol Biochem* 40:141–150
- Ren J, Sun LN, Zhang QY, Song XS (2016) Drought tolerance is correlated with the activity of antioxidant enzymes in *Cerasus humilis* seedlings. *Biomed Res Int* 2016:1–9
- Resmi MS, Vivek PJ, Soniya EV (2015) Over-expression of bael quinolone synthase in tobacco improves plant vigor under favorable conditions, drought, or salt stress. *FEBS Lett* 589:332–341
- Rockholm DC, Yamamoto HY (1996) Violaxanthin de-epoxidase (purification of a 43-kilodalton luminal protein from lettuce by lipid-affinity precipitation with monogalactosyldiacylglyceride). *Plant Physiol* 110:697–703
- Schindelin J, Rueden CT, Hiner MC, Eliceiri KW (2015) The ImageJ ecosystem: an open platform for biomedical image analysis. *Mol Reprod Dev* 82:518–529
- Song XS, Shang ZW, Yin ZP, Ren J, Sun MC, Ma XL (2011) Mechanism of xanthophyll-cycle-mediated photoprotection in *Cerasus humilis* seedlings under water stress and subsequent recovery. *Photosynthetica* 49:523–530
- Sun ZX, Liu RH, Guo B, Huang KS, Wang L, Han YH et al (2019) Ectopic expression of GmZAT4, a putative C2H2-type zinc finger protein, enhances PEG and NaCl stress tolerances in *Arabidopsis thaliana*. *3 Biotech* 9:166
- Thayer SS, Björkman O (1900) Leaf xanthophyll content and composition in sun and shade determined by HPLC. *Photosynth Res* 23:331–343
- Wu J, Ji XD, Tian SH, Wang SX, Liu HR (2018) Ectopic expression of a *Citrus kinokuni* β -carotene hydroxylase gene (*chyb*) promotes UV and oxidative stress resistance by metabolic engineering of zeaxanthin in tobacco. *3 Biotech* 8:450
- Yang S, Meng DY, Hou LL, Li Y, Guo F, Meng JJ et al (2015) Peanut violaxanthin de-epoxidase alleviates the sensitivity of PSII photoinhibition to heat and high irradiance stress in transgenic tobacco. *Plant Cell Rep* 34:1417–1428
- Yin ZP, Shang ZW, Wei C, Ren J, Song XS (2012) Foliar sprays of photosynthetic bacteria improve the growth and anti-oxidative capability on Chinese dwarf cherry seedlings. *J Plant Nutr* 35:840–853
- Yin ZP, Li S, Ren J, Song XS (2014) Role of spermidine and spermine in alleviation of drought-induced oxidative stress and photosynthetic inhibition in Chinese dwarf cherry (*Cerasus humilis*) seedlings. *Plant Growth Regul* 74:209–218
- Yin ZP, Ren J, Zhou LJ, Sun LN, Wang JW, Liu YL et al (2017) Water deficit mechanisms in perennial shrubs *Cerasus humilis* leaves revealed by physiological and proteomic analyses. *Proteome Sci* 15:9–25
- Zhao WC, Wang SH, Li X, Huang HY, Sui XL, Zhang ZX (2012) Molecular cloning and characterization of the light-regulation and circadian-rhythm of the VDE gene promoter from *Zingiber officinale*. *Plant Cell Rep* 31:1381–1392
- Zhou YL, Cheng Y, Yang YQ, Li X, Supriyo B, Sun XD et al (2016) Overexpression of SpCBL6, a calcineurin B-like protein of *Stipa purpurea*, enhanced cold tolerance and reduced drought tolerance in transgenic *Arabidopsis*. *Mol Biol Rep* 43(9):957–966