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Mechanism of xanthophyll-cycle-mediated photoprotection in *Cerasus humilis* seedlings under water stress and subsequent recovery

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Abstract

Periodic drought fluctuation is a common phenomenon in Northwest China. We analyzed the response of Chinese dwarf cherry (*Cerasus humilis*) seedlings, a dwarf shrub species with considerably strong adaptabilities, exposed to water stress (WS) by withholding water for 21 d, and subsequent recovery of 7 d. Leaf relative water content (LRWC), net photosynthetic rate (P_N), maximal quantum yield of PSII photochemistry (F_v/F_m), and effective quantum yield of PSII photochemistry (Φ_{PSII}) decreased with increasing water deficit. In contrast, the nonphotochemical quenching of Chl fluorescence (NPQ) significantly increased, as well as the amounts of violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z). In the whole levels, the photosynthetic pigment composition did not display significant changes in WS seedlings. However, the de-epoxidation state of xanthophyll cycle pigments [(Z+0.5A)/VAZ] generally exhibited higher values in WS seedlings. The significant inhibition of de-epoxidation by dithiothreitol (DTT) and negligible changes of epoxidation of Z by glucosamine (Gla) were both observed; the slight but stably upregulated transcript level of violaxanthin de-epoxidase (VDE) and downregulated zeaxanthin epoxidase (ZEP) expression profile were found during WS period, indicating that they were regulated on post-transcript levels. VDE activity, *via* the accumulation of Z and A, which confers a greater capacity of photoprotection, appears to contribute to the survival of severely stressed plants.

Additional key words: *Cerasus humilis* Bunge; energy dissipation; recovery; violaxanthin; violaxanthin de-epoxidase; water stress; xanthophyll cycle; zeaxanthin; zeaxanthin epoxidase.

Introduction

Photosynthesis is one of the most important physiological processes inhibited by water deficit (Kaiser 1987). Drought stress can reduce photosynthesis in three ways: limiting the entrance of CO_2 into the leaf (stomatal limitation), decreasing the CO_2 diffusion within the mesophyll (mesophyll limitation) or inhibiting the photochemical and metabolic processes associated with photosynthesis (photochemical and enzymatic limitations) (Flexas *et al*. 2002, Flexas and Medrano 2002). In order to attain maximal photosynthetic efficiency and minimal photodamage, plants have developed different mechanisms that enable optimization of light use and protection under rapidly changing environmental conditions. These acclimation mechanisms involve four main groups of processes: (*1*) regulation of excitation energy reaching the reaction centers; (*2*) energy dissipation as heat in the antenna, which is estimated by the rate of NPQ; (*3*) scavenging of oxidative molecules; and (*4*) repair of oxidative damage (Fernández-Marín *et al*. 2009).

Among these protective mechanisms, carotenoids play a pivotal role in avoidance of the harmful effects of light excess. Some carotenoids are involved in the xanthophyll cycle (also called VAZ cycle), which is especially relevant because of its relationship to thermal

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Abbreviations: A − antheraxanthin; Chl − chlorophyll; DEPS − the de-epoxidation state of xanthophyll cycle pigments; E – transpiration rate; F_v/F_m – maximal quantum yield of PSII photochemistry; g_s – stomatal conductance; HPLC − high-performance liquid chromatography; LRWC – leaf relative water content; NPQ − nonphotochemical quenching; P_N − net photosynthetic rate; PSII − photosystem II; RCs − reaction centers; V − violaxanthin; VAZ − total amount of xanthophyll cycle components; Z – zeaxanthin; Φ_{PSII} − effective quantum yield of PSII photochemistry.

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energy dissipation (Demmig-Adams and Adams 1996). The cycle implies interconversions between three carotenoids in the thylakoid membrane: V, A, and Z, and is ubiquitous in all land plants and green algae. VDE catalyzes the light-induced de-epoxidation of V to Z *via* A, and ZEP catalyzes the epoxidation of Z back to V upon weak light. The combined activity of the two enzymes generates a light-dependent daily cycle.

Mature individuals of Chinese dwarf cherry (*Cerasus humilis* Bunge), a species of Cerasus family that originates in Northwest China, have the characteristics of being drought-, saline-, alkali-, cold- and sterile resistant, all of which make it a wild fruit tree species with considerably strong adaptabilities. On the other hand, *C. humilis* fruits contain a variety of mineral elements beneficial to human health, especially higher calcium contents of fruit than the average. In recent years, there is an urgent need to enlarge cultivated area to meet the growing demand. However, *C. humilis* is typically exposed to WS during the spring and summer in northwest China. Besides, drought has been found to be responsible for diverse effects in seedlings of at least 20 studied species (Moles and Westoby 2004). More specifically, photosynthetic resistance to WS differs between seedlings and mature individuals in many woody perennials (Bond 2000). In the early seedling periods, for example, *C. humilis* not only grows slowly, but is vulnerable to the environmental changes. In our previous studies, it was proven that foliar sprays of photosynthetic bacteria could effectively improve the growth and antioxidative capability on *C. humilis* seedlings (Yin *et al.* 2011). Apart from this, at the moment there are no studies addressing the effects of drought stress on photochemical efficiency, photosynthesis and carotenoid composition of *C. humilis* seedlings. Also, little is known about the response to subsequent recovery after the removal of the WS.

The main purpose of this study was (*1*) to investigate the adaptive strategies of *C. humilis* seedlings to cope with extreme WS induced by withholding water for 21 d and recovery of 7 d, and (*2*) to determine the relationship between xanthophyll cycle activity and thermal dissipation of excitation energy, consequently elucidating the mechanisms that might confer protection from photoinhibition. The physiological responses were monitored by measuring gas exchange, Chl fluorescence, and photosynthetic pigments, with an emphasis on the role of the xanthophyll cycle in the dissipation of excess photon energy. Specifically, we tested the changes of xanthophyll-cycle pigment composition when we treated the seedlings with exogenous inhibitors of VDE and ZEP as well as its mRNA transcript levels of VDE and ZEP in the whole treatment period.

Materials and methods

Plants and experimental design: Cuttings of Chinese dwarf cherry (cv. Lamagoumen) were obtained from the forest bureau of Langxiang, Heilongjiang Province, China. Each cutting was transplanted into a container (35 × 35 × 25 cm) filled with organic soil, irrigated regularly by half-strength Enshi nutrient solution (Yu and Matsui 1997) under a 12-h photoperiod at temperatures ranging from about 17–25°C, photosynthetic photon flux density (PPFD) of 600 μmol(photon) m^{-2} s^{-1} and the relative humidity of 70–75% in the greenhouse. Seedlings at the 35–40-leaf stage were randomly allocated to one of two treatments: control plants (control) were watered daily to field capacity, whilst water was withheld from water-stressed plants (drought). The drought treatment lasted for 21 d. Recovery was measured 7 d after plants of drought treatment were rewatered on a daily basis with the water amount used for control plants. All measurements of physiological parameters were carried out on the youngest fully expanded leaves, with at least 30 plants per treatment.

Plant water status was evaluated by measuring LRWC which was calculated as LRWC = (FM – DM)/FM, where FM refers to fresh mass and DM refers to dry mass, respectively.

Photosynthesis and chlorophyll fluorescence: Measurements of net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) were performed on a fully expanded leaf of *C. humilis* seedlings using an open system (*Ciras-2, PP-Systems*, UK). The light-saturating photosynthetic rate was made at a CO_2 concentration of 360 μmol $mol^{-1}$and at a temperature of 25°C with a relative humidity of 80% and saturating light of 1,200 μmol(photon) m^{-2} s^{-1}. Chl fluorescence was measured simultaneously with a pulse-modulated fluorometer (*FMS2, PP-Systems*, UK). Prior to each measurement, a clip was placed on the leaf for 30 min for dark adaptation. The weak modulated irradiance, “actinic light”, and saturating pulse (typically less than 1 s) were 0.05, 200, and 5,000 μmol(photon) m^{-2} s^{-1}, respectively. Using both light- and dark fluorescence parameters, we calculated: (*1*) $F_v/F_m = (F_m - F_o)/F_m$, (*2*) $NPQ = (F_m/F_m') - 1$ (van Kooten and Snel 1990), and (*3*) $\Phi_{PSII} = (F_m' - F_s)/F_m'$ (Genty *et al.* 1989).

Photosynthetic pigments: Samples were taken from the same leaves in which Chl fluorescence was measured. Leaf pigments were later extracted with acetone in the presence of sodium ascorbate and stored as described previously (Abadía and Abadía 1993). Pigment extracts were thawed on ice, filtered through a 0.45 µm filter and analysed by an isocratic HPLC method (Larbi *et al.* 2004). All chemicals used were of HPLC quality. The method is able to quantify a specific group of xanthophylls (oxygen-containing carotenoids), involved in the so-called xanthophyll cycle. Z is formed by de-epoxidation of V *via* A. The de-epoxidation state was defined as (Z+0.5A)/VAZ. This index is an estimation of the actual number of molecules within the xanthophyll cycle partially or totally de-epoxidized over the maximum possible. A low (Z+0.5A)/VAZ ratio would indicate a displacement towards V, whereas a large one would indicate that Z and/or A account for a large part of the pool of the xanthophyll cycle pigments (Abadía *et al.* 1999, Gilmore and Yamamoto 1993).

Treatments with metabolic inhibitors: In order to prevent xanthophyll cycle de-epoxidation or epoxidation during drought treatment, some *C. humilis* seedlings after 21 d of WS treatment were sprayed with 10 mL (one plant) of an aqueous solution of 3 mM DTT (*Sigma*, USA), an inhibitor of VDE activity (Yamamoto and Komite 1972), and 10 mL (one plant) of a concentration of 0.5% (v/v) Gla (*Sigma*, USA), an inhibitor of ZEP activity (Latowski *et al.* 2007), respectively. Several WS plants ($n \geq 5$) were sprayed by 10 mL of distilled water as control. After 24-h spraying with inhibitors or distilled water, (Z+0.5A)/VAZ was measured by the above mentioned methods.

Assay of qRT-PCR: For qRT-PCR, duplicate samples were analysed in a Quantitative PCR instrument (Bole, USA). Total RNA was prepared using CTAB, then RNA was treated with RNase-free DNase I. 5 µg of total RNA was performed in reverse transcription with RevertAid Reverse Transcriptase (Fermentas) and Oligo d(T)primers (TaKaRa). PCR amplification was performed with 40 cycles as follows: 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min. The relative expression levels of genes were presented by $2^{-\Delta\Delta CT}$ (fold change). The cycle threshold (CT) is defined as the number of cycles required for the fluorescent signal to cross the threshold (*i.e.* exceeding the background level). CT levels are inversely proportional to the amount of target nucleic acid in the sample. ΔCT is the difference of CT between the control gene products and the target gene products, $\Delta\Delta CT = (\Delta CT_{CK} - \Delta CT_{WS})$.

PCR reactions employed the following primers, actin - F (5′-GTGAAGGCTGGGTTTGCT-3′), actin - R (5′-CCCATCCCAACCATAACA-3′), VDE - F (5′-GTGGGGAATTTGTCTTGGAG-3′), VDE - R (5′-AGGCTGATTTGGGTCTTGC-3′), ZEP - F (5′-GGAAGAACTTGTTTGGAT-3′), and ZEP - R (5′-GGAATTAATGTCAGCAGG-3′), respectively.

Statistics: Data of physiological variables were analysed by *ANOVA* using *PC SAS* version *8.2* (*SAS Institute*, Cary, NC, USA). When significant differences were noted, LSD multiple range test was used to determine where differences existed. The statistical significance was set at the $P<0.05$ confidence level. An *F*-test assessing the null hypothesis for one curve fitting was used in order to compare the best fit lines of NPQ *vs.* (Z+0.5A)/VAZ (*SPSS 11.5*, IL, USA).

Results

LRWC in WS seedlings decreased by 61% gradually throughout 21-d drought period in comparison with control plants until the 21th day. After 7 d of recovery, a slight decrease by 25% was observed (Fig. 1*A*).

After 7 d of water shortage, P_N of WS seedlings began to decrease, reaching a minimum value of about 6 µmol m^{-2} s^{-1} by the end of the drought treatment (21 d), then recovered to a value close to the control after 7 d of recovery under clement conditions. On the contrary, P_N remained almost stable in control seedlings in the whole experimental period (Fig. 1*A*). Such a gradual decrease of P_N in WS seedlings was mostly due to the stomatal closure induced by water shortage, as the g_s began to drop in WS seedlings since 7th day (Fig. 1*B*), together with P_N, where no limitation was observed in control plants. Similarly, *E* followed the same change pattern as g_s (Fig. 1*B*) .

In the stressed seedlings, F_v/F_m exhibited a drop starting from 7th day of the experiment; values around 0.75 were reached by the end of 21st day of WS, representing about 88% of the control value (~0.85) that were typical of healthy nonphotoinhibited leaves. However, this parameter fully recovered after rewatering (Fig. 1*C*). The Φ_{PSII} followed similar change patterns (Fig. 1*C*). Φ_{PSII} declined slightly from 7th day, which was attributed to a decrease in the photochemical quenching of Chl fluorescence (q_P) and F_v'/F_m' (data not shown), then gradually recovered when returned to normal conditions. In comparison, WS caused a rapid increase in NPQ with increasing time, with a 45% increase compared with control seedlings by the end of 21st day. After 7 d of recovery, NPQ was still higher than the control level, although it decreased with the recovery time (Fig. 1*D*).

Chl *a*, Chl *b*, Chl *a*/*b*, N, L, β-Car, and VAZ were quantified in both WS and control seedlings of *C. humilis*. Our results indicated that WS did not modify the photosynthetic pigment composition of *C. humilis* seedlings in the whole levels, excepting Chl *a*, which decreased from control values by 20% at the end of 21st day of WS (Table 1). However, the values of VAZ from WS seedlings were significantly ($P<0.05$) increased compared with control plants after 21 d of drought (Table 1). The de-epoxidation state of xanthophyll cycle pigments [DEPS = (Z+0.5A)/VAZ] generally exhibited higher values in WS seedlings compared with controls (Fig. 2*A*). Values of 0.4 were reached at 21st day in treated seedlings and indicated that almost half of the V was de-epoxidized to Z. After recovery on 7th day, the DEPS of WS seedlings was still higher than that of control plants (Fig. 2*A*).

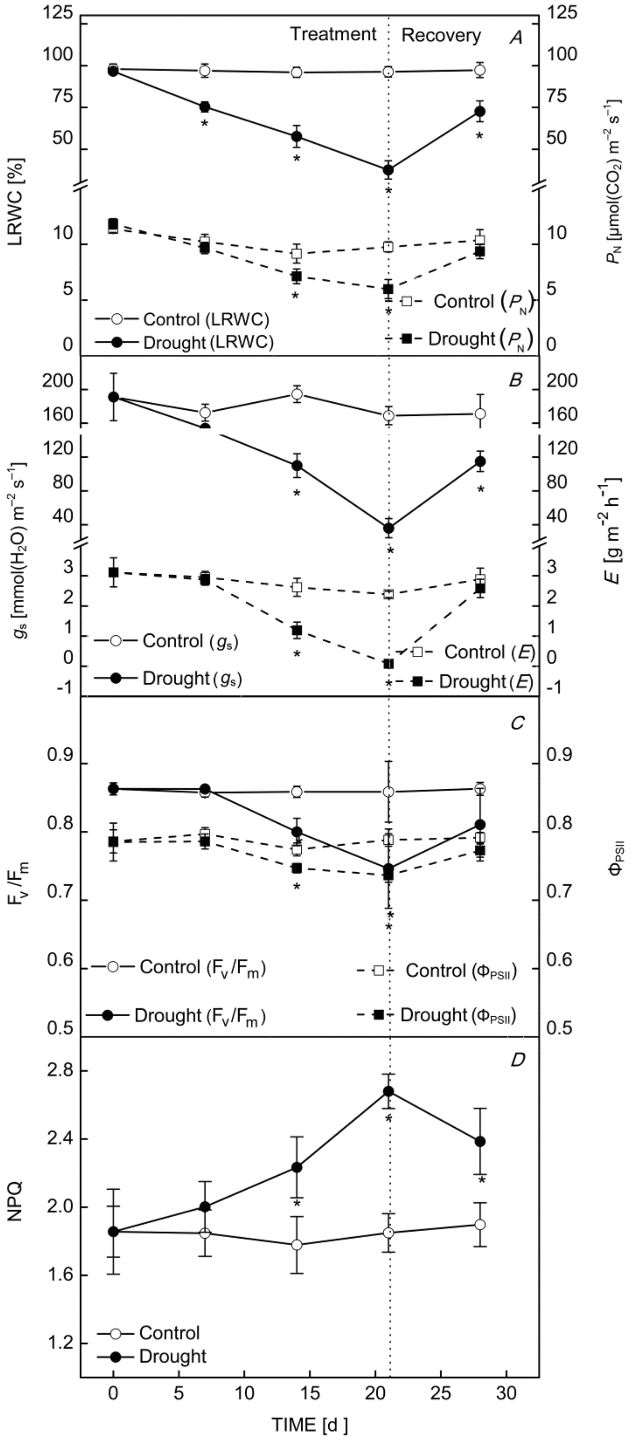

Fig. 1. Effects of drought stress on leaf relative water content (LRWC) and net photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) and transpiration rate (*E*) (*B*), maximal quantum yield of PSII photochemistry (F_v/F_m) and effective quantum yield of PSII photochemistry (Φ_{PSII}) (*C*), and nonphotochemical quenching (NPQ) (*D*). The vertical *dashed line* indicates the transfer of plants back to the control conditions. *Open symbols* indicate control plants, *solid symbols* indicate drought-stressed plants. In *A, B* and *C*, the *solid lines* indicate the data of left axis, *dash lines* indicate the data of right axis, * – statistically significant differences ($P<0.05$) between the control- and drought treatments. Data are the means of 5 replicates with SE shown by *vertical bars*.

Changes in PSII over the course of the experiment closely tracked the parallel pattern of xanthophyll (Z+0.5A)/VAZ. The increase in NPQ was linearly related to the increase in the de-epoxidized fraction of xanthophylls for WS (Fig. 2*B*), and the relationship between xanthophyll (Z+0.5A)/VAZ and PSII was similar, irrespective of the source of variation (drought alone in midday measurements, irradiance, or a combination of irradiance and drought in midday values, data not shown).

Next, we used DTT and Gla, inhibitors of the VDE and ZEP respectively, to study the mechanism of Z formation exposed to WS in *C. humilis* seedlings. As shown in Fig. 3, DTT significantly inhibited xanthophyll de-epoxidation induced by drought stress. In contrast, Gla had no effect on the epoxidation of Z (Fig. 3). Furthermore, to examine the response of VDE and ZEP expressions on Z formation in *C. humilis* leaves during WS and subsequent recovery, transcript levels of VDE and ZEP genes were analysed by qRT-PCR (Fig. 4) in the same samples as used in the photosynthetic analysis. A slight but gradual increase in transcript level of VDE was observed during the WS period. Up to the recovery on 7th day, however, there were no changes in VDE expression between treated and control seedlings (Fig. 4*A*). In contrast, for ZEP expression, a significant decrease was found even in the early period of drought stress (7 d). Thereafter, ZEP transcript abundance was sustained in lower level plants with time treated. Only 1/5 of the transcript level of ZEP was detected by the end of 21st day of WS compared with control seedling. After the 7-d recovery, it still could not recover to the control level (Fig. 4*B*).

Discussion

Water deficit, generally associated with high solar irradiance and high temperatures during the summer, is the main limiting factor for plants growing in North China. The inhibitory effect of drought on photosynthetic activity has been widely described and is mainly associated with stomatal and metabolic limitations (Chaves *et al.* 2009; Lawlor and Tezara 2009). The decrease in LRWC (Fig. 1*A*) confirmed the deterioration of leaf water status in WS plants. In the present experiment, gas exchange decreased during WS, but recovered rapidly following rewatering (Fig. 1*A*), which indicates that decreased gas exchange was temporary and *C. humilis* possessed rapid recovery ability after WS was removed. The ability of this species to recover swiftly from reduced photo-

Table 1. Temporal evolution of photosynthetic pigment composition in the leaf during the water stress period. Chl – chlorophyll; Car – caroten; N – neoxanthin; L – lutein; VAZ – total amount of xanthophyll cycle components; * – statistically significant differences ($P<0.05$) among the different dates. Data are the means of 5 replicates with SE by *ANOVA*.

Day	Chlorophylls Chl *a* [mg g^{-1}(FM)]		Chl *b* [mg g^{-1}(FM)]		Chl *a*/*b*		Carotenoids N [mg g^{-1}(FM)]		L [mg g^{-1}(FM)]		β-Car [mg g^{-1}(FM)]		VAZ [mg g^{-1}(FM)]	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
0	3.76±0.10	3.95±0.08	0.66±0.13	0.70±0.03	5.86±1.5	5.68±0.04	0.65±0.05	0.64±0.05	3.71±0.09	3.31±0.36	0.63±0.06	0.61±0.09	1.25±0.09	1.40±0.04
7	3.47±0.09	3.86±0.03	0.69±0.08	0.67±0.07	5.08±0.73	5.82±0.82	0.59±0.12	0.72±0.12	3.66±0.16	3.45±0.11	0.65±0.04	0.67±0.07	1.23±0.10	1.45±0.05
14	3.26±0.24	3.50±0.19	0.66±0.07	0.64±0.06	4.96±0.81	5.54±0.99	0.69±0.12	0.76±0.12	3.47±0.13	3.65±0.11	0.71±0.11	0.73±0.08	1.10±0.12	1.53±0.10
21	3.45±0.12	2.78±0.06*	0.71±0.03	0.58±0.02	4.84±0.14	4.80±0.80	0.61±0.06	0.74±0.05	3.38±0.11	3.54±0.12	0.61±0.05	0.73±0.06	1.23±0.11	1.69±0.06*
28	3.34±0.30	3.86±0.18	0.65±0.11	0.66±0.05	5.23±1.09	5.85±0.64	0.65±0.04	0.70±0.06	3.41±0.15	3.65±0.10	0.60±0.03	0.68±0.09	1.30±0.09	1.54±0.09

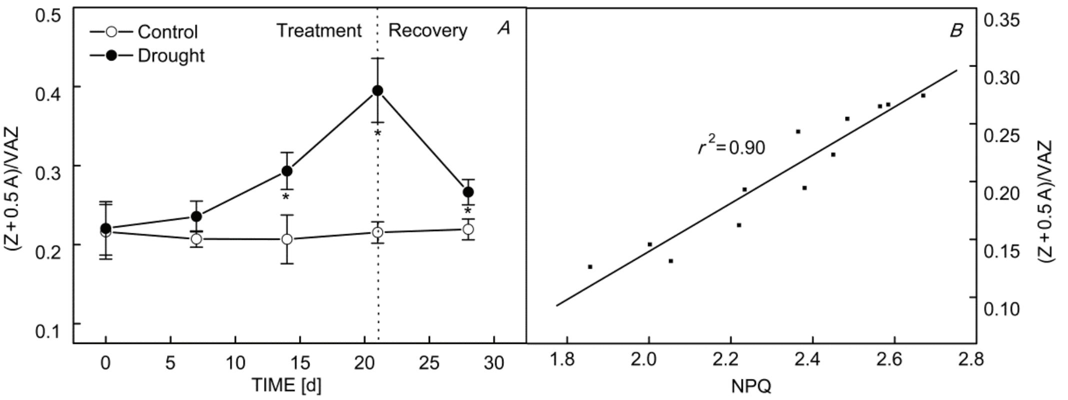

Fig. 2. Change of (Z+0.5A)/VAZ (*A*) and the correlation between NPQ and (Z+0.5A)/VAZ (*B*) as influenced by drought stress. The vertical *dashed line* indicates the transfer of plants back to the control conditions (*A*). * – statistically significant differences ($P<0.05$) between the control- and drought treatments. Data are the means of 5 replicates with SE shown by *vertical bars*.

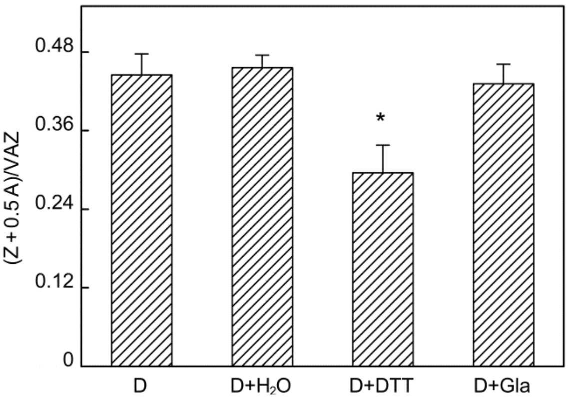

Fig. 3. Change of (Z+0.5A)/VAZ induced by inhibitors after 21 days of WS treatment. D – drought stress; D+H_2O – drought stress + distilled water; D+DTT – drought stress + dithiothreitol (DTT); D+Glu – drought stress + glucosamine (Gla); * – statistically significant differences ($P<0.05$) among the different treaments. Data are the means of 5 replicates with SE shown by *vertical bars*.

synthetic activity may enable it to survive and to grow in periodically drought areas such as Northwest and Northeast China.

F_v/F_m was reduced under WS (Fig. 1*C*). But the reduction was not sustained after rewatering and up to control levels by the end of 7-d recovery. An analogous transient drop during water deficit and recovery was detected in seedlings of *Phlomis fruticosa* (Petsas and Grammatikopoulos 2009). We can assume that it was probably due to higher drought resistance of these species. This is also clearly seen in the pigment composition, which remained fairly unchanged during the whole drought cycle (Table 1). The preservation of an intact photosynthetic pigment machinery at the end of the drought cycle could contribute to the rapid recovery of *C. humilis* after a long drought-stress period, explaining the rapid recovery in CO_2 net assimilation and stomatal conductance after irrigation of intense WS plants (Peguero-Pina *et al*. 2008).

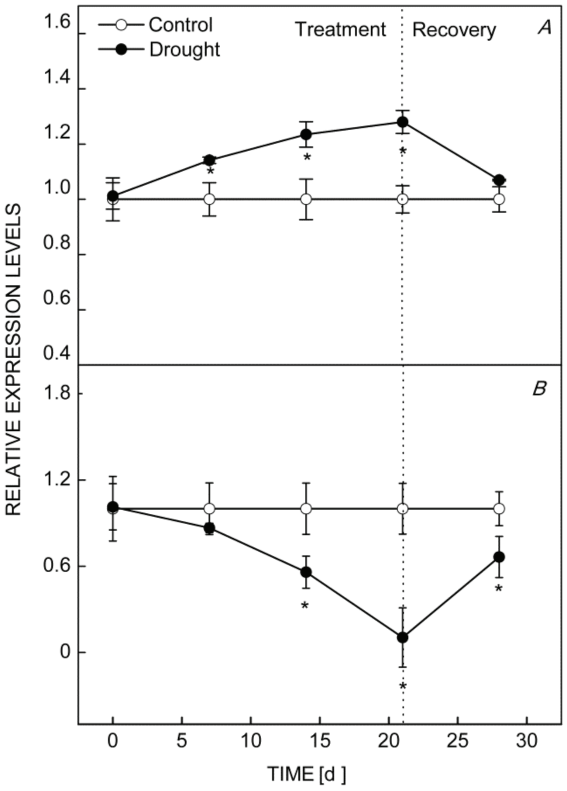

Fig. 4. Effects of WS on expression pattern of VDE (*A*) and ZEP (*B*) by qRT-PCR. The vertical *dashed line* indicates the transfer of plants back to the control conditions. Relative expression levels = $2^{-\Delta\Delta CT}$, The relative expression values are scaled so that CK expression is equal to 1. * – statistically significant differences ($P<0.05$) between the control- and drought treatments. Data are the means of 5 replicates with SE shown by *vertical bars*.

In our measurements, Φ_{PSII}, called the efficiency of excitation energy capture by open PSII reaction centers (RCs), exhibited negative responses to WS (Fig. 1*C*), suggesting that WS decreased the efficiency of excitation energy capture by open PSII RCs. Similar results were also observed in *Pisum sativum* responding to WS (Havaux *et al*. 1991). It is worth mentioning that variables related to PSII efficiency (*i.e.* F_v/F_m and Φ_{PSII}) still showed a high degree of resistance, with slight or moderate decreases (Fig. 1*C*).

As regards NPQ, which reflects the influences of nonphotochemical processes on Chl fluorescence emission during the transition of sample from dark-adapted to light-adapted state (Roháček and Barták 1999), it was higher throughout WS period (Fig. 1*D*), and showed more dissipation of excitation energy in the PSII antennae. It is the photoprotective mechanism under stress (Bigras 2005). Demmig-Adams *et al*. (1996) suggested that one of the major photoprotective responses of plants to avoid photoinhibition under WS is harmless thermal dissipation of excess excitation energy through the xanthophyll cycle function usually estimated by NPQ measurement. The xanthophyll cycle pigments Z and A are formed from V under conditions of excess excitation energy and are both thought to be involved in the photoprotective dissipation process (Demmig-Adams and Adams 2006). Our results showed that drought stress induced a greater increase in both NPQ and de-epoxidation status of the xanthophyll cycle in WS plants (Figs. 1*D*, 2*A*), which probably contributed to the dissipation of excess energy in thylakoids as heat. WS also largely altered the proportions of Z and V, resulting in a considerable retention of Z and A as the drought stress became more intense (Fig. 2*A*). This retention was previously reported both during the summer in *N. oleander*, *Z. mays*, *R. officinalis*, and *Yucca* species (Demmig *et al*. 1988, Saccardy *et al*. 1998, Munné-Bosch and Alegre 2000, Barker *et al*. 2002) and during the winter in several conifers, broad-leaved evergreen species, shrubs, and herbs (Adams and Demmig-Adams 1995, Barker *et al*. 1998, Logan *et al*. 1998, Garcia-Plazaola *et al*. 1999, Verhoeven *et al*. 1999, Barker *et al*. 2002) as a response to different environmental types of stress. Since we found a strong positive correlation (r^2 = 0.90) between NPQ and the amount of de-epoxidized xanthophyll-cycle component (Fig. 2*B*), we conclude that there was a retention of Z+A in a state primed for energy dissipation, as suggested by several authors (Adams and Demmig-Adams 1995, Garcia-Plazaola *et al*. 1999, Verhoeven *et al*. 1999, Levizou *et al*. 2004). A similar relationship has been reported for two *Yucca* species (Barker *et al*. 2002). Thus, the results in this study suggest that the xanthophyll cycle plays an important role in dissipating excess excitations in order to avoid possible photodamage to PSII when WS was imposed under a long term.

In this study, we used for the first time, to our knowledge, inhibitors and qRT-PCR in parallel to assess the extent to which enzyme, VDE or ZEP, contributes to Z formation after drought stress. The inhibition of de-epoxidation by DTT (Fig. 3) suggests that VDE is the enzyme responsible for the conversion of V into Z during WS, in accordance with the data reported by North *et al*. (2005), who analyzed the contributions of VDE in *Arabidopsis* to drought-stress resistance. But negligible changes of epoxidation of Z by Gla (Fig. 3) indicated that inhibition of ZEP did not, at least partly, account for de-epoxidation status of the xanthophyll cycle. Compared with the changes of VDE and ZEP by inhibitors, the slight but stable upregulated transcript level of VDE and down-regulated ZEP expression profile were observed during WS period (Fig. 4), indicating that they were regulated on post-transcript levels (Jahns *et al*. 2009). Furthermore, we may safely come to the conclusion that NPQ-VAZ regulation under drought is mostly – if not fully – regulated by transcript regulation of ZEP, and not VDE. The retention of Z was mainly resulted from de-epoxidation by VDE. Increased VDE activities might be important for accumulation of Z and more effective protection to the photosynthetic apparatus (North *et al*. 2005).

In summary, the ability of *C. humilis* seedlings to maintain the functionality of the xanthophyll cycle during the long-term WS and subsequent recovery appears as an efficient mechanism to protect the photosynthetic machinery from irreversible damages. In addition, the VDE activity could mainly contribute to the accumulation of Z and A, which confers a greater capacity of photoprotection and thus contributes to the survival of severely stressed plants.

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