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Effects of abscisic acid or benzyladenine on pigment contents, chlorophyll fluorescence, and chloroplast ultrastructure during water stress and after rehydration

D. HAISEL, J. POSPÍŠILOVÁ*, H. SYNKOVÁ, R. SCHNABLOVÁ, and P. BAŤKOVÁ

Institute of Experimental Botany, Academy of Sciences of the Czech Republic,
Na Karlovce 1a, CZ-160 00 Praha 6, Czech Republic

Abstract

With the aim to contribute to the elucidation of the role of phytohormones in response of plants to adverse environmental conditions, seedlings of *Phaseolus vulgaris*, *Nicotiana tabacum*, *Beta vulgaris*, and *Zea mays* were supplied with water, 100 μM abscisic acid (ABA), or 10 μM N^6-benzyladenine (BA) immediately before imposition of water stress (WS). In all four species, contents of chlorophylls (Chls) and carotenoids were markedly decreased during WS and after rehydration only in plants pre-treated with water but not in those pre-treated with ABA or BA. Contents of pigments of xanthophyll cycle increased during WS more in plants pre-treated with ABA or BA than in those pre-treated with water, but the degree of their de-epoxidation was highest in the later. Similarly, the efficiency of photosystem 2, determined as variable to maximal Chl fluorescence ratio, was not markedly decreased in bean plants pre-treated with ABA or BA in contrast to those pre-treated with water. The imposed WS was not severe enough to damage chloroplast ultrastructure. However, different changes in a size of starch inclusions were observed. In bean plants, the amount of starch increased considerably in plants pre-treated with water, while it decreased in BA pre-treated plants and no change was found in ABA pre-treated ones. The starch content declined under WS in sugar beet and tobacco plants but only moderate changes were found in ABA or BA pre-treated plants. Thus the application of BA and especially of ABA reduced the negative effects of subsequent WS.

Additional key words: *Beta vulgaris*; carotenoids; *Nicotiana tabacum; Phaseolus vulgaris*; starch content; xanthophylls; *Zea mays.*

Introduction

During water stress (WS), most plants avoid shoot desiccation by closing their stomata to decrease transpiration. Decreased stomatal conductance is accompanied by a reduction in internal CO_2 concentration and decreased diffusion of CO_2 *via* mesophyll cell walls, membranes, cytoplasm, and chloroplast envelope leading to decreased chloroplast CO_2 concentration which decreases CO_2 fixation rate. However, many other processes such as contents of photosynthetic pigments, activities of enzymes, photochemical efficiency of photosystems, and structure and ultrastructure of chloroplasts can be also influenced by WS, especially by the severe one. The effects of WS depend not only on its severity but also on dynamics of dehydration. The recovery of plants after rehydration is not less important. Both processes are species specific and depend on plant age, growing conditions, and contemporary stresses (for recent reviews see Athar and Ashraf 2005, Dubey 2005).

Photosynthetic apparatus can be seriously damaged when leaves are exposed to high irradiance during drought. To avoid photoinhibition, enhanced formation of antheraxanthin and zeaxanthin during summer drought was observed, *e.g.* in European beech seedlings (García-Plazaola and Becerril 2000) and lavender (Munné-Bosch and Alegre 2000), or during dehydration in pea (Iturbe-Ormaetxe *et al.* 1998), maize (Saccardy *et al.* 1998), and wheat (Tambussi *et al.* 2002).

Abscisic acid (ABA) has been implicated as a key

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[*] Corresponding author; fax: (+420) 224310113, e-mail: pospisilova@ueb.cas.cz

Abbreviations: ABA – abscisic acid; BA – benzyladenine; C – control plants; Car – carotenoids; Chl – chlorophyll; CK – cytokinin(s); DEPS – degreee of XCP de-epoxidation; DEPSC – de-epoxidised XCP per Chl unit; PS – photosystem; RWC – relative water content; WS – water stress; WUE – water use efficiency; XCP – xanthophyll cycle pigments.

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component in water deficit-induced responses. Its biosynthesis starts with the carotenoids 9′-*cis*-violaxanthin or 9′-*cis*-neoxanthin, from which xanthoxin is formed by the plastidic enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED). Subsequently, xanthoxin is converted by xanthoxin oxidase to abscisic aldehyde, and this one by abscisic aldehyde oxidase to ABA (for recent reviews see Hose *et al*. 2002, Xiong and Zhu 2003).

Involvement of ABA in the regulation of stomatal opening is generally accepted. However, non-stomatal effects of ABA on photosynthesis (*e.g*. on the activity or regeneration of ribulose-1,5-bisphosphate carboxylase or on chloroplast ultrastructure) are not sufficiently confirmed and explained. ABA affected expression of many genes including those encoding small and large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbsS* and *rbsL*), *cab* genes encoding proteins of light-harvesting pigment-protein complexes, or *psbA* gene encoding D1 protein (Giraudat *et al*. 1994, Bray 2002). The application of ABA reduced degradation of chlorophyll (Chl) and carotenoids (Car) in barley and wheat under moderate WS (Mizrahi *et al*. 1974, Agarwal *et al*. 2005). Application of ABA to barley and maize resulted in partial protection of the photosystem 2 (PS2) photochemistry against photoinhibition. This was accompanied with higher photochemical and non-photochemical quenching in ABA-treated leaves, considerable increase in the amount of total Car and xanthophylls and activity of xanthophyll cycle (Ivanov *et al.* 1995, Jia and Lu 2003). The ABA effect on photosynthesis can be associated also with changes in chloroplast ultrastructure (Lopez-Carbonell *et al*. 1994a,b, Pastor *et al*. 1999).

There is only scarce information about stress-induced changes in contents of cytokinins (CKs) and their possible effects on stomata and photosynthesis (for recent reviews see Pospíšilová *et al*. 2000, Pospíšilová 2003a, Pospíšilová and Dodd 2005). Interaction between CKs and ABA were found not only in the site of their action (*e.g.* on stomatal closure) but also during their synthesis (Pospíšilová and Baťková 2004, Pospíšilová *et al*. 2005). Non-stomatal effects of CKs have been reported (for recent review see Nyitrai 2005) including alleviation of the negative effects of WS on Chl and Car contents, photochemical activities of PS1 and PS2, and content and activity of ribulose-1,5-bisphosphate carboxylase or phospho*enol*pyruvate carboxylase by applied CKs (Metwally *et al.* 1997, Chernyad′ev and Monakhova 1998, 2003, Pandey *et al.* 2000, Singh *et al.* 2001). Further, CKs delays leaf senescence (*e.g.* Čatský *et al.* 1996, Naqvi 1999, Ananieva *et al*. 2004, Xie *et al*. 2004).

The aim of our research was to contribute to elucidation of still not sufficiently known role of ABA and CKs in plant responses to WS. In previous experiments we concentrated on their effects on plants sufficiently supplied with water (Pospíšilová 2003b), on stomatal regulation of gas exchange (Pospíšilová and Baťková 2004), and on changes in endogenous contents of these phytohormones (Pospíšilová *et al.* 2005) during water stress and after rehydration. This paper is focused on non-stomatal limitations to photosynthesis. The aim of this research was to reduce negative effects of WS on leaf pigments, Chl fluorescence, and chloroplast ultrastructure by pre-treatment of plants with ABA or CK benzyladenine (BA). The goal of ABA application was to confirm or reject the new hypothesis that exogenous ABA which is readily absorbed by plants can substitute endogenous ABA synthesized from neoxanthin or violaxanthin during WS and thus a higher pool of xanthophyll cycle pigments remains for dissipation of excess energy absorbed in thylakoids. BA was used with the aim to delay stress-induced leaf senescence or to promote Chl synthesis.

Materials and methods

Plants and treatments: Similarly as in previous experiments (Pospíšilová and Baťková 2004, Pospíšilová *et al*. 2005), seedlings of French bean (*Phaseolus vulgaris* L. cv. Jantar), tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR-1), sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Döll cv. Elán), and maize (*Zea mays* L. cv. Anjou 245) grown in pots with coarse sand or fine *Perlite* sufficiently moistened with Hewitt nutrient solution in growth chambers at 16-h photoperiod, irradiance of 250 μmol(photon) m^{-2} s^{-1} (400–700 nm), day/night temperature of 25/20 °C, and relative humidity of about 50 % were used. WS was induced by cessation of watering. Before imposition of WS, the plants were pretreated with 50 cm^3 of water, 100 μM ABA, or 10 μM BA. These concentrations were chosen according to previous experiments (*e.g*. Pospíšilová *et al*. 2001). Control plants (C) were watered regularly. Relative water content (RWC) was measured gravimetrically in leaf discs (0.5 cm^2) water-saturated by immersing into holes of fully moistened polyurethane foam under dark according to Čatský (1960).

Contents of photosynthetic pigments were determined during WS (when visible wilting occurred, for detail see Results and discussion), and 2 d after rehydration in acetone extracts of leaf discs by HPLC (*Spectra-Physics*, San Jose, USA) using a reversed phase column (*Sepharon SGX C18*, 5 μm particle size, 150×3 mm, *Tessek*, Prague, Czech Republic). The solvent system was acetonitrile : methanol : water (80 : 12 : 6) followed by 100 % methanol, and the gradient was run from 8 to 12 min. The flow rate was 1 cm^3 min^{-1}, the detection wavelength was 445 nm. Data were captured and calculated by PC-software *Clarity* (*DataApex*, Prague, Czech Republic). Means and standard errors of means were calculated from 3 samples [each contained 3–5 discs (Ø 1 cm) from blades of different leaves]. Experiments were repeated twice with similar results.

Chl *a* fluorescence characteristics of attached bean leaves were measured after a 15-min dark period with a *PAM Chlorophyll Fluorometer* (*Walz,* Effeltrich, Germany). Actinic radiation (650 nm; 100 µmol m^{-2} s^{-1}) was provided by *102L LED* lamp. Saturating pulses of "white light" (duration 700 ms, 2 500 µmol m^{-2} s^{-1}) were applied in 100-s intervals. Data sampling, control, and calculation were served by the *DA 100 Data Acquisition System* (*Walz,* Effeltrich, Germany).

Chloroplast ultrastructure was observed in C and WS bean, sugar beet, and tobacco leaves. As it is not possible to suppose rapid changes in chloroplast utrastructure, we did not measure plants under mild WS and 2 d after rehydration. Samples were taken from mature leaves of 5 plants (four pieces from a leaf) in each experimental variant and experiments were repeated twice. Ultra-thin sections of Spurr´s embedded leaf samples were contrasted with uranyl acetate followed by lead citrate treatment and examined using a transmission electron microscope *JEM 1010* (*Jeol,* Kyoto, Japan) (for detail see Schnablová *et al.* 2006). Volume density of starch inclusions was counted on pictures taken by CCD camera using the program *LUCIA 4.61*.

Results and discussion

As shown in previous study (Pospíšilová and Baťková 2004), visible wilting was usually observed 5 d after cessation of watering. RWC decreased rather slowly: from 85–92 % in the first day to 80–85 % after 2–3 d, and to 65–75 % after 5 d. RWC increased quickly after rehydration and reached the original values after 2 d. In contrast, stomatal conductance, transpiration rate, and net photosynthetic rate decreased rapidly to very low values and did not recover completely after rehydration.

In the actual experiments, in C plants (sufficiently supplied with water) Chl content per leaf area unit was highest in maize and lowest in French bean and tobacco, but these differences almost disappeared in the WS plants and after rehydration.

Also Car content was highest in maize and in this case also under WS and after rehydration. In plants pre-treated with water, contents of Chl and Car markedly decreased during WS. This decrease continued also after rehydration (Figs. 1 and 2). The 2-d-period was probably too short for recovery of pigment synthesis. In agreement with this, in two populations of beech seedlings recovery of pigment contents after rehydration was observed after

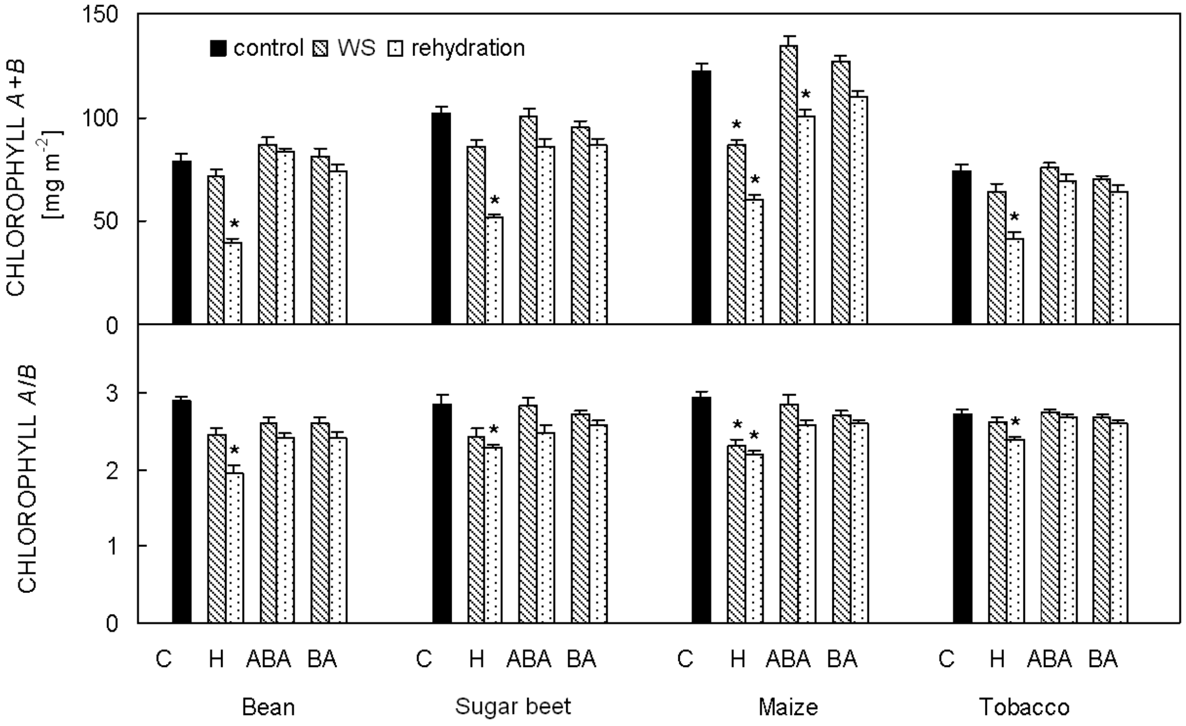

Fig. 1. Changes in chlorophyll *a*+*b* content and chlorophyll *a*/*b* ratio during water stress (WS) and subsequent rehydration. Before imposition of WS, plants were pre-treated with water (H), abscisic acid (ABA), or benzyladenine (BA). Control plants (C) were irrigated regularly. Means ± SE, $n = 3$, statistically significant differences at $p=0.05$ are marked by *.

Table 1. The contents of neoxanthin and lutein during WS(WS) and subsequent rehydration. Before imposition of WS, plants were pre-treated with water (H_2O), abscisic acid (ABA), or benzyladenine (BA). Control plants were irrigated regularly. Statistically significant differences at p=0.05 are marked by *.

Treatment		Neoxanthin [mg m^{-2}]				Lutein [mg m^{-2}]			
		sugar beet	French bean	tobacco	maize	sugar beet	French bean	tobacco	maize
Control		4.4	3.9	4.2	5.0	14.0	11.0	15.4	11.6
H_2O	WS	3.4*	2.7*	4.0	3.5*	12.2	8.1	10.7	11.1
	rehydration	4.6	3.3	4.1	4.1	9.0*	5.7*	6.0*	9.5*
ABA	WS	5.5*	4.7*	5.4*	8.6*	14.1	10.9	14.1	13.3
	rehydration	4.3	3.4	4.8	6.8*	12.8	10.2	10.3*	12.0
BA	WS	5.0	4.5	4.1	8.0*	14.1	10.7	15.4	11.3
	rehydration	4.2	4.0	3.6	6.3	11.0	9.0	11.9	9.8

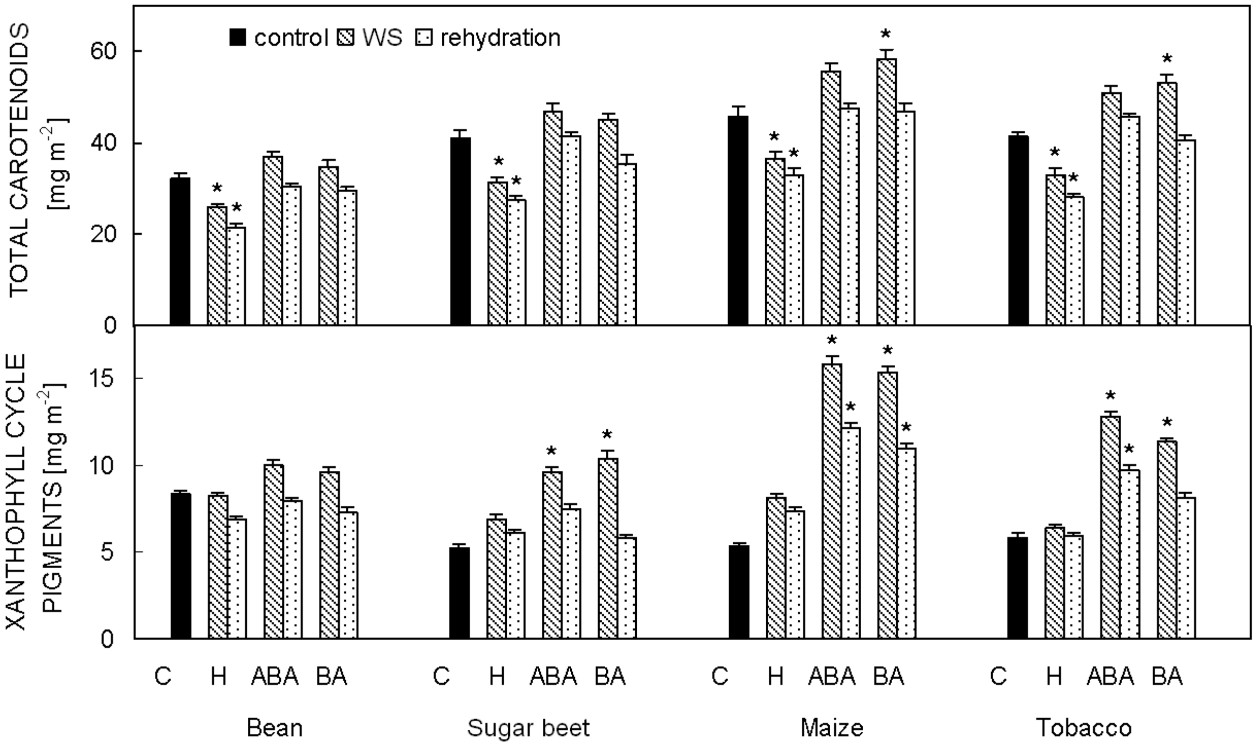

Fig. 2. Changes in total carotenoids and xanthophyll cycle pigment (zeaxanthin + antheraxanthin + violaxanthin) contents during water stress (WS) and subsequent rehydration. Before imposition of WS, plants were pre-treated with water (H), abscisic acid (ABA), or benzyladenine (BA). Control plants (C) were irrigated regularly. Means ± SE, n = 3, statistically significant differences at p=0.05 are marked by *.

5 and 7 d, respectively (Tognetti *et al.* 1995).

In all tested species, pre-treatment with ABA or BA protected photosynthetic pigments or stimulated their biosynthesis in the ensuing WS period: Chl and Car contents in ABA or BA pre-treated leaves were not markedly different from those in C plants during WS and subsequent rehydration. The positive effect of ABA application on Chl and Car retention during moderate WS has been observed also in wheat and barley (Mizrahi *et al.* 1974, Agarwal *et al.* 2005). Similarly, application of BA reduced the decrease in Chl and Car contents in WS wheat (Chernyad′ev and Monakhova 2003) or in Chl content in *Cassia* (Singh *et al.* 2001). However, in WS cotton Chl *a* content increased due to BA pre-treatment but decreased due to ABA pre-treatment, and contents of Chl *b* and Car decreased as a result of both pre-treatments (Pandey *et al.* 2003/4). In our previous experiments, BA applied during re-watering did influence pigment contents in bean and sugar beet plants (Rulcová and Pospíšilová 2001, Vomáčka and Pospíšilová 2003) but this was probably due to the fact that WS applied was not severe enough to influence markedly pigment contents also in non-treated plants.

Contents of light-harvesting pigments lutein and neoxanthin (Table 1) decreased under WS in plants pre-treated with water. The content of lutein continued in decreasing after rehydration while the content of neoxanthin increased. The decrease in lutein content was also observed in *Arbutus unedo* but only under severe WS (Munné-Bosch and Peñuelas 2004). In plants pre-treated with ABA and BA the contents of neoxanthin during WS were mostly higher than those of plants pre-treated with water with the exception of tobacco plants pre-treated with BA. The contents of lutein and neoxanthin in plants pre-treated with ABA or BA decreased after rehydration (Table 1).

Contents of xanthophyll cycle pigments (XCP = zeaxanthin + antheraxanthin + violaxanthin) increased during WS in plants pre-treated with ABA or BA more than in those pre-treated with water (Fig. 2). This might be important for their protection against photoinhibition under more severe WS or higher irradiance. The highest increase was observed in ABA pre-treated maize in agreement with increased XCP content in long-term ABA-treated maize plants as reported by Jia and Lu (2003). The degree of XCP de-epoxidation [DEPS = (zeaxanthin + 0.5 antheraxanthin)/(zeaxanthin + antheraxanthin + violaxanthin)] increased during WS in all plant species in plants pre-treated with water and the highest increase was observed in maize (Fig. 3). This increase in DEPS during WS was in agreement with the results of Saccardy *et al.* (1998), Medrano *et al.* (2002), and Tambussi *et al.* (2002). Increase of DEPS in plants pre-treated with ABA or BA was observed in bean, sugar beet, and tobacco. After rehydration, DEPS remained high in all plants pre-treated with water, but decreased in BA pre-treated bean and ABA or BA pre-treated sugar beet and tobacco. The ratio of de-epoxidised XCP per Chl [DEPSC = (zeaxanthin + 0.5 antheraxanthin)/(Chl *a*+*b*)] rapidly increased during WS in all plant species and all pre-treatments and mostly decreased after rehydration in plants pre-treated with ABA or BA. In plants pre-treated with water, DEPSC continued in increasing also after rehydration (Fig. 3).

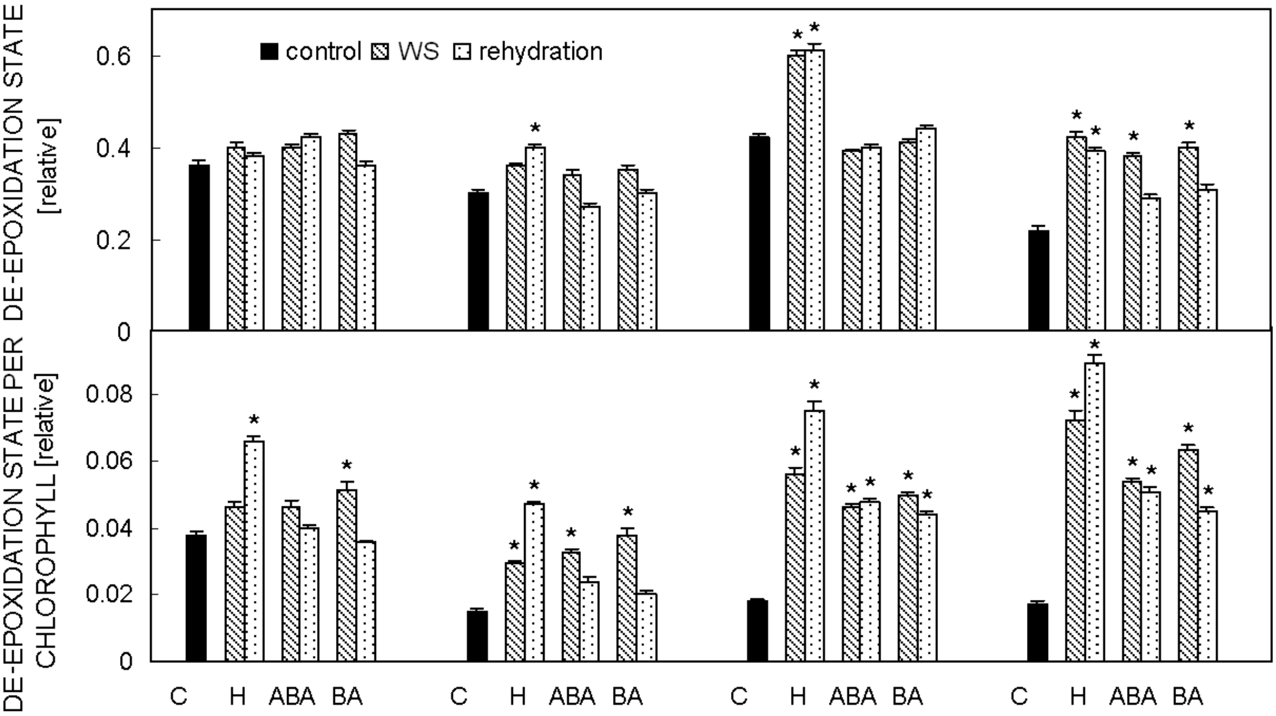

Fig. 3. Changes in deepoxidation state of XCP [DEPS = (zeaxanthin + 0.5 antheraxanthin)/(zeaxanthin + antheraxanthin + violaxanthin)] and deepoxidation state per chlorophyll [DEPSC = (zeaxanthin + 0.5 antheraxanthin)/(chlorophyll a+b)] during water stress (WS) and subsequent rehydration. Before imposition of WS, plants were pre-treated with water (H), abscisic acid (ABA), or benzyladenine (BA). Control plants (C) were irrigated regularly. Means ± SE, $n = 3$, statistically significant differences at p=0.05 are marked by *

To prove the ability of ABA or BA to reverse the negative effect of WS on photosynthetic apparatus we measured in bean plants parameters of Chl *a* fluorescence induction kinetics which are often used as non-destructive tool for the determination of photochemical and non-photochemical processes in thylakoid membranes of chloroplasts (for review see Roháček 2002). The maximum fluorescence, maximum photochemical efficiency of PS2, determined as variable to maximum Chl fluorescence ratio (F_v/F_m), photochemical quenching (q_P), and ratio of fluorescence decrease (Rfd) decreased in plants pre-treated with water but mostly not in those pre-treated with ABA or BA (Fig. 4). This further confirmed the ABA and BA induced protection of photosynthetic apparatus. Our results are in agreement with the relative stability of Chl *a* fluorescence parameters in drought-resistant maize line with high content of endogenous ABA during soil drying (Ristic and Cass 1993). The Chl fluorescence parameters are usually not changed during mild WS as we observed earlier (Rulcová and Pospíšilová 2001, Vomáčka and Pospíšilová 2003). Similarly, Chl fluorescence parameters were not decreased in kidney beans until water was withheld for 7 d (Miyashita *et al.* 2005). Therefore in the present experiments we examined parameters of Chl *a* fluorescence in bean plants under more severe WS than in the previous ones.

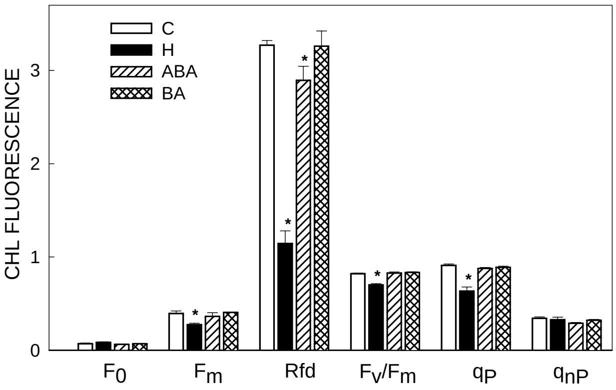

Fig. 4. Effect of pre-treatment with H_2O, 100 μM ABA, or 10 μM BA on chlorophyll *a* fluorescence parameters (F_0 – initial fluorescence, F_m – maximum fluorescence, Rfd – ratio of fluorescence decrease, F_v/F_m – variable to maximum fluorescence ratio, q_P – photochemical quenching, q_{nP} – non-photochemical quenching) in French bean under water stress (RWC decreased to about 70 %). Control plants (C) were sufficiently supplied with water. Means ± SE, *n* = 5, statistically significant differences at p=0.05 are marked by *.

Nevertheless, the imposed WS was not severe enough to damage chloroplast ultrastructure. The only visible changes found were in a frequency and size of starch grains. The amount of starch changed more in non-pre-treated WS plants compared to ABA or BAP pre-treated plants. The changes were species specific but mostly not statistically significant (Table 2). Nevertheless, the tendency to increase the starch content was found in French bean, whereas the decrease was observed in tobacco and sugar beet. Usually ABA pre-treated stressed plants showed the similar structure as controls, while BA pre-treated stressed plants exhibited the similar starch contents as water pre-treated stressed plants (Fig. 5). In tobacco, water pre-treated plants showed dilatation of thylakoids under stress. These plants usually contained also larger plastoglobuli, but the differences were not statistically significant. The alterations in chloroplast ultrastructure caused by WS share many common features with the development of senescence manifested as disappearance of starch inclusion, appearance of large plastoglobuli, and thylakoid swelling and dilatation (Kolodziejek *et al.* 2003, 2007). Changes in water supply seem to affect the structural characteristics of chloroplasts differently in various plant species or cultivars (Ristic and Cass 1993). Although increase in chloroplast starch accumulation has been observed in some plants (Ackerson and Hebert 1981, Utrillas and Alegre 1997), the disappearance of starch inclusions was more often reported under WS (Giles *et al.* 1976, Pastor *et al.* 1999). WS affects significantly the activity of key enzymes involved in sucrose and starch metabolism. Thus, water deficit induces an increase in leaf sucrose, glucose, or fructose contents that is frequently associated with starch depletion (Pelleschi *et al.* 1997). The severity of that stress also determines the final effect (Chang and Ryan 1987). This could be also the reason for differences in starch content that we found among plant species in our experiments.

In all species studied, stomata sensitively regulated gas exchange during WS (Pospíšilová *et al.* 2004). Due to markedly decreased *E*, water content in the leaf during WS remained sufficiently high to prevent real damage of photosynthetic apparatus. In addition, the irradiance in the growth chamber was relatively low, therefore we suppose that the observed changes in measured parameters were mostly caused by WS and not by photoinhibition. Nevertheless, excess of absorbed energy cannot be excluded even under rather low irradiance when stomata are closed.

Endogenous ABA content increases during WS in many plant species. In our previous experiments ABA content in bean, maize, and tobacco also increased considerably during WS, and further increased by ABA pre-treatment (Pospíšilová *et al.* 2005). Probably after ABA pre-treatment sufficient amount of ABA is absorbed from the substrate, Car being not necessary for ABA synthesis and therefore XCP pool increases during water stress in plants pre-treated with ABA (Fig. 2). Our results support the hypothesis that exogenous ABA might substitute for endogenous ABA synthesized from violaxanthin and thus increase the available XCP pool. It might be important because restriction of CO_2 transport by low stomatal conductance leads to decreased sink of reducing power in

Table 2. Starch content as a percentage of the chloroplast volume in sugar beet, French bean, and tobacco plants. The values were calculated from TEM images of chloroplasts from control plants and water-stressed plants pre-treated with water, ABA, or BA. Statistically significant differences at p=0.05 are marked by *.

Treatment	Sugar beet	French bean	Tobacco
Control	2.40	3.08	7.07
H_2O	1.27	5.23	0.00^*
ABA	0.67^*	2.99	5.01
BA	1.54	1.18	0.72*

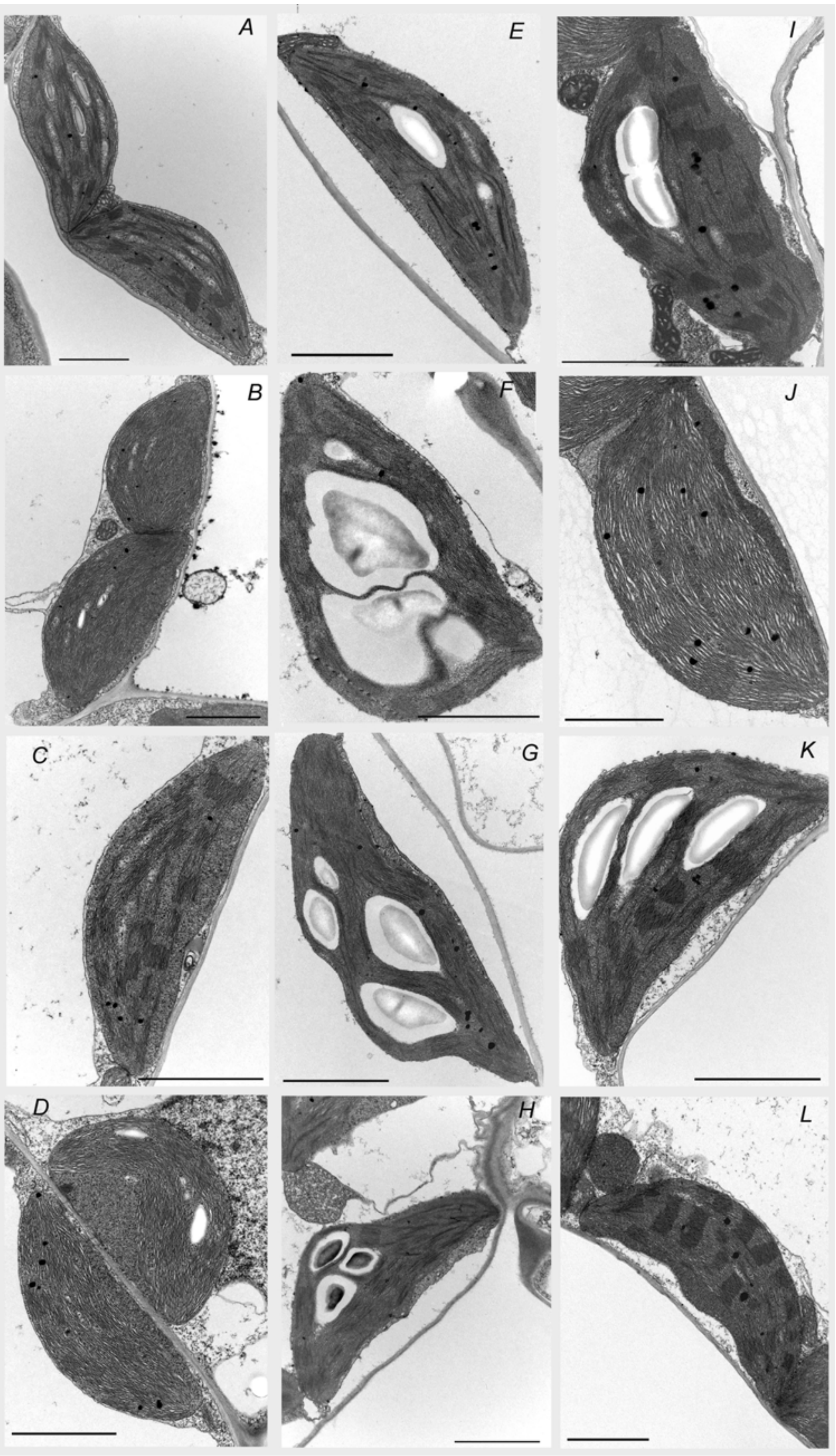

Fig. 5. The ultrastructure of chloroplasts from control plants (*A*, *E*, *I*) or water stressed plants pre-treated with water (*B*, *F*, *J*), ABA (C, G, K), or BA (*D*, *H*, *L*) of sugar beet (*A*–*D*), French bean (*E*–*H*), and tobacco (*I*–*L*). *Scale bars* = 2 µm.

CO_2 fixation which may result in an over-reduction of the primary quinone electron acceptor. One of the main strategies which prevent photodamage of PS2 is just a thermal dissipation of excess energy which is connected with the function of xanthophyll pigment cycle. Thus, the better protection of photosynthetic apparatus may alleviate the negative effects of WS on Chl content, Chl *a* fluorescence parameters, and chloroplast ultrastructure observed after ABA pre-treatment (Figs. 1, 4, and 5).

Alleviation of negative effect of WS was observed also after pre-treatment with BA but it was usually less substantial than after the ABA pre-treatment. Cytokinins are able to delay leaf senescence (*e.g.* Pospíšilová and Dodd 2005). Hence our results support the hypothesis that the above mentioned alleviation might be in connection with delayed WS induced leaf senescence after BA application.

In conclusion, the pre-treatment with BA and especially with ABA can protect the photosynthetic apparatus during subsequent WS. These results confirm importance of these two phytohormones in plant responses to drought.

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