

Interactions between abscisic acid and cytokinins during water stress and subsequent rehydration

J. POSPÍŠILOVÁ*, M. VÁGNER**, J. MALBECK**, A. TRÁVNÍČKOVÁ** and P. BAŤKOVÁ***

*Institute of Experimental Botany, Academy of Sciences of the Czech Republic,
Na Karlovce 1a, CZ-16000 Praha 6*, Rozvojová 135, CZ-16500 Praha 6**, Czech Republic
Charles University of Prague, Faculty of Science, Viničná 5, CZ-12844 Praha 2, Czech Republic****

Abstract

With the aim to contribute to elucidation of the role of phytohormones in plant responses to stresses the endogenous contents of abscisic acid (ABA) and cytokinins (CK) were followed in French bean, maize, sugar beet, and tobacco during water stress and subsequent rehydration. The effects of pre-treatments with exogenous ABA or benzyladenine (BA) before imposition of water stress were also evaluated. The content of ABA increased by water stress, and with the exception of bean plants increased content of ABA remained also after rehydration. In all plant species the ABA content was further increased by ABA pre-treatment, but in bean and maize it decreased by BA pre-treatment. The highest total content of CK was observed in bean and the lowest in maize during water stress. In their spectrum, the storage CK were dominant in bean, and inactive CK in tobacco while in sugar beet and maize all groups were present in comparable amounts. In all plant species, the contents of CK increased during water stress and with exception of bean they decreased back after rehydration. ABA pre-treatment further increased contents of CK in water-stressed bean and tobacco. BA pre-treatment increased contents of CK in sugar beet and tobacco after rehydration.

Additional key words: benzyladenine, isopentenyladenine, *Phaseolus vulgaris*, relative water content, zeatin, zeatin riboside.

Introduction

Hormonal regulation of plant growth and metabolism is complex and interactions among phytohormones are widespread. The interactions include both positive and negative reciprocal effects on the phytohormone synthesis, and different relations between signalling pathways (e.g. LeNoble *et al.* 2004).

Water stress affects many metabolic pathways, mineral uptake, membrane structure, *etc.* Therefore it is not surprising that hormone contents can be also changed by water stress. This is very important because plant hormones are considered as main signals in root-to-shoot communication and *vice versa* (for review see, e.g., Davies and Zhang 1991, Tardieu and Davies 1993, Davies 1995, Naqvi 1995). In consequence, the change in hormonal balance might play the key role in the sequence

of events induced by stress (Itai 1999). Despite of this, the precise mechanism of induction of a hormonal shift by an environmental change is not known.

Abscisic acid (ABA) has been implicated as a key component in water deficit-induced responses including those triggered by drought, salinity, and low temperature. Although well-watered plants contain some ABA, water stress stimulates ABA biosynthesis in both roots and leaves. The first committed step of ABA biosynthesis is the oxidative cleavage of the carotenoids 9'-*cis*-violaxanthin or 9'-*cis*-neoxanthin to xanthoxin by plastid enzymes 9'-*cis*-epoxycarotenoid diogenases. In the second step, xanthoxin is converted to abscisic aldehyde by xanthoxin oxidase. In the last step, abscisic aldehyde oxidase catalyses conversion of abscisic aldehyde to

Received 4 April 2005, accepted 17 May 2005.

Abbreviations: ABA - abscisic acid; AC - biologically active CK; BA - benzyladenine; C - control plants; CK - cytokinin(s); cZ - cis-derivatives of zeatin; IC - inactive CK; RWC - relative water content; SC - storage CK; Z - zeatin; ZR - zeatin riboside.

Acknowledgements: The authors acknowledge critical reading of manuscript by Dr. J. Čatský and Dr. Z. Šesták, skilful technical assistance of Mrs. L. Hávková and B. Čermáková, and the financial support of the Grant Agency of the Czech Republic (grant Nos. 522/02/1099 and 522/04/0710).

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

ABA (for recent reviews see Taylor *et al.* 2000, Schwartz *et al.* 2003). During water stress, activities of the above mentioned enzymes as well as their mRNA transcript abundance increases in both leaves and roots. In the roots, where xanthophyll contents are relatively low, zeaxanthin epoxidation to violaxanthin might be a further regulatory step of water stress-induced ABA biosynthesis.

Decreased leaf CK content in response to drought stress has been observed (for reviews see Naqvi 1994) although it is difficult to predict the actual change of any given CK species. For example, dehydration of wheat seedlings decreased shoot content of zeatin (Z) nucleotide and zeatin 9-N-glucoside, but the total content of Z derivatives as well as the content of free base of Z remained almost constant (Mustafina *et al.* 1997/98). Mild water deficit had no effect on sunflower xylem zeatin riboside (ZR) concentration, but decreased ZR flux to the shoot. More severe water deficit decreased both concentration and flux of ZR (Shashidhar *et al.* 1996). However, increased content of CK was observed in water-stressed lychee by Stern *et al.* 2003. The increased CK contents were also observed after partial root excision in wheat seedlings (Vysotskaya *et al.* 2003).

In the previous experiments we focused on interactions between ABA and CK in regulation of stomatal opening, transpiration rate and net photosynthetic rate. In bean plants sufficiently supplied with water, benzyladenine (BA) sprayed on leaves or

added to substrate simultaneously with ABA partially alleviated the effect of ABA (Pospíšilová 2003b). Similarly in sugar beet, all gas exchange parameters were higher after ABA+BA application to the substrate than when ABA was applied alone. However, in maize the combination ABA+BA decreased gas exchange parameters similarly to application of ABA (Pospíšilová and Baťková 2004). When ABA and BA were applied simultaneously, BA partially reversed the effect of ABA on stomatal closure in broad bean (Tichá 2004). The species specific effect of BA application was also found under water stress when content of endogenous ABA increased (Pospíšilová 2003b, Pospíšilová and Baťková 2004).

It is possible that the interactions between both phytohormones in regulation of stomatal opening are not only at the level of guard cell functioning but also at the level of their synthesis. Therefore the aims of the following experiments were 1) to compare changes in contents of ABA and CK during water stress and subsequent rehydration in leaves of plant species with different sensitivity to water stress, 2) to determine if water stress induced accumulation of ABA can be modified by CK application, and 3) to determine if further increase in ABA content by exogenous application can affect content and composition of endogenous CK.

Materials and methods

Seedlings of the C₃ species French bean (*Phaseolus vulgaris* L. cv. Jantar), sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* Döll, cv. Elán) and tobacco (*Nicotiana tabacum* L. cv. SR-1), and the C₄ species maize (*Zea mays* L. cv. Anjou 245) were grown in pots with coarse sand or fine *Perlite* sufficiently moistened with Hewitt nutrient solution. Plant were cultivated in growth chambers at 16-h photoperiod, irradiance of 250 $\mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ (400 - 700 nm), day/night temperature of 25/20 °C, and relative humidity of about 50 %. Air temperature and humidity were measured with the *JUMO Humitherm TDAc-70* (M.K. Juchheim, Fulda, Germany). Irradiance was measured with the *LI 185B* radiometer with a quantum sensor (*Li-COR*, Lincoln, USA). Water stress was induced by cessation of watering. Before imposition of water stress, the plants were pre-treated with 50 cm³ of water (control), 100 μM ABA, or 10 μM BA. During water stress, when visible wilting occurred, and 2 d after rehydration samples (1 or 2 g) of leaf tissue were cut (mixture samples from different leaves of 3 plants) and deeply frozen for further determinations of ABA and CK contents. In addition, samples were taken also under mild water stress in French bean and tobacco. To evaluate water stress,

relative water content (RWC) was measured gravimetrically in leaf discs (0.5 cm²) water-saturated by immersing into holes of fully moistened polyurethane foam under dark according to Čatský (1960).

For ABA analyses, 1 g of fresh matter of plant sample was frozen in liquid nitrogen and extracted in methanol. Tritiated (\pm)-ABA was used as internal standard to quantify the loss during purification process. Samples were twice purified by ether partitioning followed by column chromatography on *Polyclar AT* (Prewin *et al.* 2004). Evaporated samples were derivatized with diazomethane. Content of ABA was measured by gas chromatography (*Hewlett Packard 5890*) equipped with electron capture detector and *OV-1* chromatographic column. Nitrogen served as carrier gas, and oven, injector, and detector temperatures were set to 210, 250, and 300 °C, respectively (Vágner *et al.* 1998).

For the CK analysis, the frozen tissue was ground under liquid nitrogen and extracted overnight at -20 °C with Bielecki (1964) solvent. Deuterium-labelled CK were added as internal standards. After clarifying by centrifugation, the extracts were passed through two *Sep-Pak C₁₈* cartridges (*Waters Corporation*, Milford, USA) connected in series, and the extract evaporated to

water phase. After correcting the pH to 6.5, this extract was passed through a *DEAE Sephadex* (Serva) column (3 cm³, HCO₃⁻) and a *Sep-Pak C₁₈* cartridge connected in series. CK bases, ribosides, and glucosides were eluted from the cartridge with 5 cm³ 80 % methanol after two washes with 5 cm³ of de-ionized water and 3 cm³ of 10 % methanol. The eluted fraction was evaporated to dryness in a *Speed Vac* concentrator. *DEAE Sephadex* columns containing CK phosphates (CK nucleotides) were eluted with 10 cm³ 1 M NH₄HCO₃. The sample was neutralized and fractions were passed through a *Sep-Pak C₁₈* cartridge. Then CK phosphates were eluted with 8 cm³ 80 % methanol, evaporated to water phase, 0.1 M Tris (pH 9.6) was added, and the sample was treated with alkaline phosphatase (30 min at 37 °C). After neutralization, the solution was passed through a *Sep-Pak C₁₈* cartridge. After washing with 5 cm³ of de-ionized water and 3 cm³ of 10 % methanol, CK nucleotides were eluted

with 5 cm³ 80 % methanol and evaporated to dryness. Samples were stored at -20 °C until used for further analysis.

CK fractions were separated and quantified by HPLC (*FLUX Rheos 2000* quaternary pump and *CTC Analytics HTS PAL* autosampler with *CSI 6200 Series HPLC Oven*) linked to a mass spectrometer (*Finnigan LCQ*) equipped with an *ESI* source. Samples (0.01 cm³) were injected onto a *C₁₈* column (*Phenomenex, AQUA*, 2 × 250 mm, 5 μm) and eluted with 0.001 % acetic acid (A) and acetonitrile (B). The following gradient profile was used: 5 min, 10 % B, then to 17 % in 10 min, then to 46 % in 10 min, at a flow rate of 0.2 cm³ min⁻¹. The column temperature was 30 °C. The effluent was introduced into the *ESI* source [220 °C, +4.5 kV capillary, sheath gas (N₂) 90 units; auxiliary gas (N₂) 8 units]. Data acquisition was performed at MS/MS full scan, two microscans at maximum ion time of 100 ms.

Results and discussion

C₄ plants such as maize are better adapted to water deficit because of lower stomatal conductance, better water use efficiency, and ability to carry on photosynthesis under low CO₂ concentration than C₃ plants (for review see Matsuoka *et al.* 2001). Maize had better osmotic adjustment and higher accumulation of proline as a possible osmolyte during water stress and faster recovery after rehydration than wheat (Nayyar 2003). To ensure a similar rate of water stress development in all species studied we used maize plants with larger leaf area per the same volume of substrate than in other species. In all plant species, opening of stomata decreased very soon after imposition of water stress. In consequence, stomatal conductance, transpiration rate, and net photosynthetic rate decreased while RWC remained high during mild water stress (for detail see Pospíšilová and Bařková 2004). These very small changes in RWC observed in French bean (Fig. 1) and tobacco (Fig. 2) was the reason why we did not measure contents of endogenous phytohormones under mild stress also in sugar beet and maize. Visible wilting was observed 5 - 7 d after cessation of watering (severe water stress). In control plants RWC decreased to 48 % in French bean, 55 % in maize, 66 % in sugar beet, and 68 % in tobacco (Figs. 1 - 4). In the same time stomatal conductance, transpiration rate, and net photosynthetic rate decreased to very low values (Pospíšilová and Bařková 2004). Pre-treatment with ABA slightly increased RWC in French bean under mild stress (Fig. 1) and in tobacco under mild and severe stress (Fig. 2). Pre-treatment with BA delayed development of water stress only in French bean.

The accumulation of endogenous ABA in leaf tissue during water stress was observed in bean, tobacco, and maize (Figs. 1,2,4). The content of ABA under water

stress was highest in bean and lowest in tobacco. In tobacco and maize, increased content of ABA remained also after 2 d rehydration. In bean plants, ABA content slightly decreased after rehydration but after 2 d it was still higher than in control plants. On the contrary, ABA accumulation in sugar beet was slow and the highest ABA content was observed after rehydration (Fig. 3).

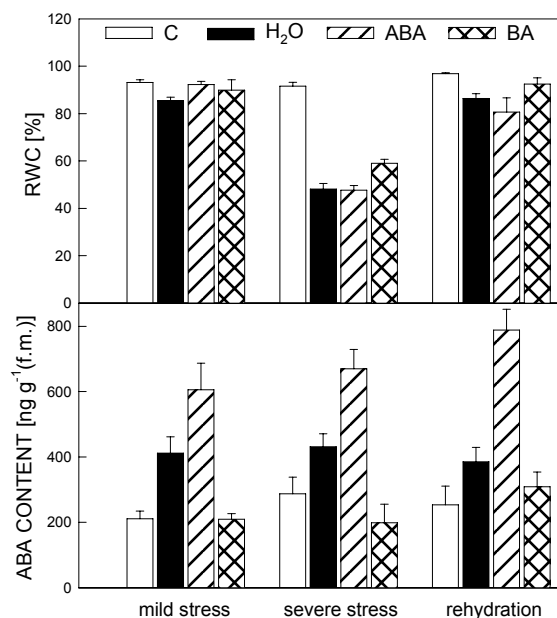


Fig. 1. Relative water content (RWC) and ABA content during development of water stress and subsequent rehydration of French bean plants pre-treated with water (H₂O), 100 μM abscisic acid (ABA) or 10 μM benzyladenine (BA). Control plants (C) were sufficiently supplied with water.

Opposite results were obtained with *Acacia*, *Leucaena*, and *Manihot*, where high ABA content under water deficit was completely reversed to control level 1 d after rewatering (Liang and Zhang 1999, Alves and Setter 2000). However, for the regulation of stomatal opening, ABA content in the vicinity of guard cells is of vital importance and this content might be different from bulk leaf ABA content (for review see, *e.g.*, Pospíšilová 2003a).

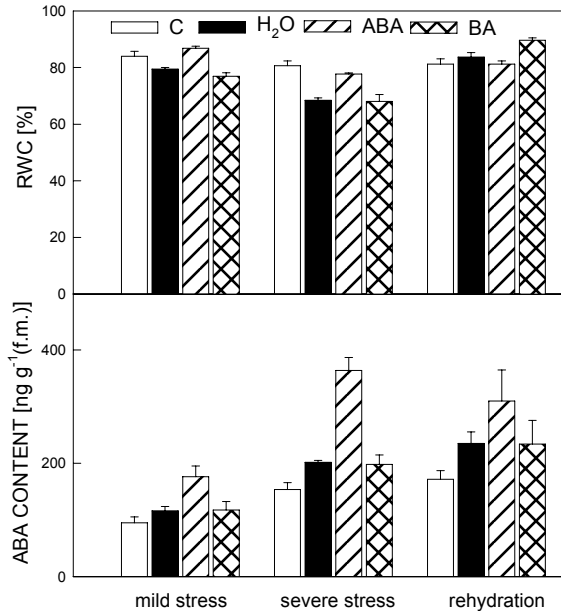


Fig. 2. Relative water content (RWC) and ABA content during development of water stress and subsequent rehydration of tobacco plants pre-treated with water (H₂O), 100 μM abscisic acid (ABA) or 10 μM benzyladenine (BA). Control plants (C) were sufficiently supplied with water.

ABA-deficient mutants help in elucidation of the role of ABA in response of plants to water stress; nevertheless, their disadvantage is an aberrant growth also under non-stressed conditions (Thompson *et al.* 2004). Another approach is to increase the endogenous ABA content more than it is normally under water stress by ABA pre-treatment.

Content of endogenous ABA was markedly increased after ABA pre-treatment in all plant species (Figs. 1-4). These results proved that applied ABA was rapidly absorbed by roots and transported to leaves. It was in agreement with previous results showing that ABA can move freely through the plant in both the xylem and the phloem (Sauer and Hartung 2000, Sauer *et al.* 2002). In all plant species studied, exogenous ABA decreased stomatal conductance already 1 h after application (Pospíšilová and Bařková 2004). However, the values of stomatal conductance, transpiration rate, and net photosynthetic rate remained higher during water stress in plants pre-treated with ABA than in plants pre-treated with water. ABA pre-treatment also stimulated recovery

of gas exchange parameters after rehydration (Pospíšilová and Bařková 2004). In wheat plants, ABA application ameliorated negative effect of PEG-induced water stress

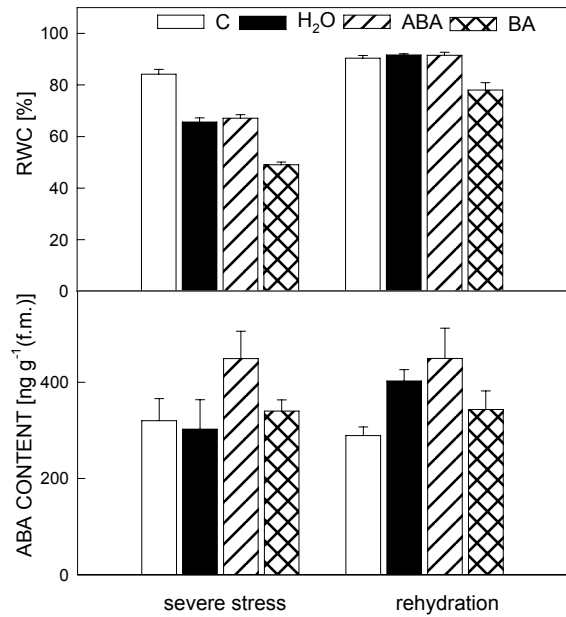


Fig. 3. Relative water content (RWC) and ABA content during development of water stress and subsequent rehydration of sugar beet plants pre-treated with water (H₂O), 100 μM abscisic acid (ABA) or 10 μM benzyladenine (BA). Control plants (C) were sufficiently supplied with water.

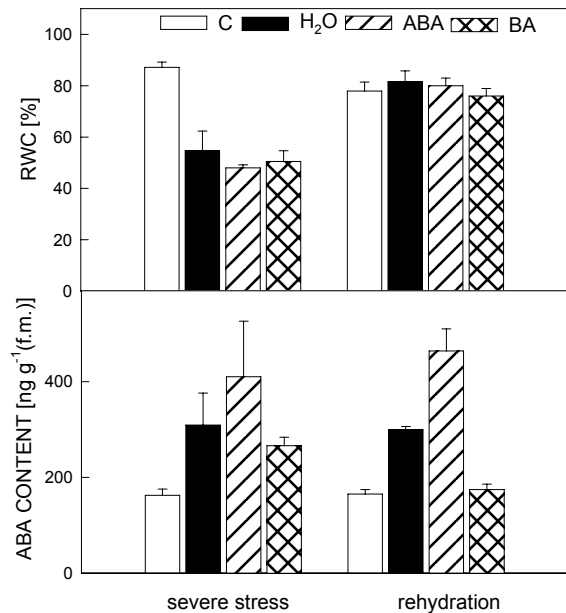


Fig. 4. Relative water content (RWC) and ABA content during development of water stress and subsequent rehydration of maize plants pre-treated with water (H₂O), 100 μM abscisic acid (ABA) or 10 μM benzyladenine (BA). Control plants (C) were sufficiently supplied with water.

on stomatal conductance and photosynthetic rate (Nayyar and Kaushal 2002).

In tobacco plants, water stress-induced increase in content of endogenous ABA was not affected by application of BA (Fig. 2). In contrast, water stress-induced ABA accumulation was inhibited by BA application in bean (Fig. 1) and maize (Fig. 4) plants. In bean, sugar beet (Fig. 3), and maize plants pre-treated with BA the content of ABA after rehydration was lower than in plants irrigated with water. To the best of my knowledge, the effect of CK on ABA accumulation has

been studied only in senna leaves, where content of ABA was also lower in leaves sprayed with BA than in non-treated leaves (Singh *et al.* 2000).

Pre-treatment with BA ameliorated the effects of water stress on gas exchange parameters in senna, bean and cotton (Singh *et al.* 2001, Pospíšilová 2003b, Pandey *et al.* 2003/4, Pospíšilová and Bačková 2004) and the effect of mild water stress in tobacco (unpublished results). In contrast, stomatal conductance, transpiration rate, and net photosynthetic rate in sugar beet and maize decreased more during water stress in plants pre-treated

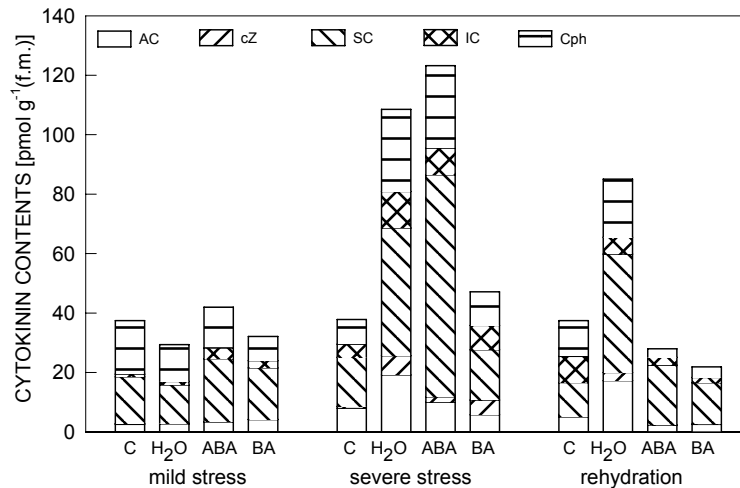


Fig. 5. Contents of endogenous cytokinins (CK) during development of water stress and subsequent rehydration in French bean plants pre-treated with water (H₂O), 100 μ M abscisic acid (ABA) or 10 μ M benzyladenine (BA). Control plants (C) were sufficiently supplied with water. AC - biologically active CK (*trans*-zeatin, dihydrozeatin, *trans*-zeatin-9-riboside, dihydrozeatin-9-riboside, isopentenyladenine, isopentenyladenine-9-riboside), cZ - *cis*-derivatives of zeatin (*cis*-zeatin, *cis*-zeatin-9-riboside), SC - storage CK (*trans*-zeatin-O-glucoside, *trans*-zeatin-9-riboside-O-glucoside, dihydrozeatin-9-riboside-O-glucoside), IC - inactive CK (*trans*-zeatin-7-glucoside, dihydrozeatin-7-glucoside, *trans*-zeatin-9-glucoside, dihydrozeatin-9-glucoside, isopentenyladenine-7-glucoside, isopentenyladenine-9-glucoside), Cph - cytokinin phosphates.

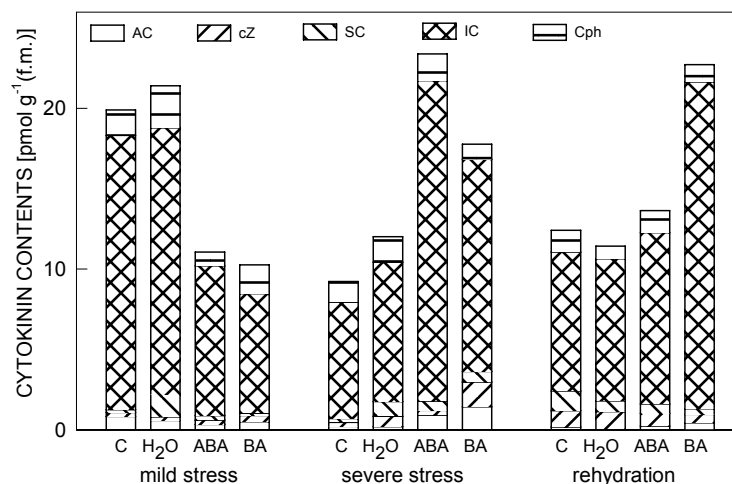


Fig. 6. Contents of endogenous cytokinins (CK) during development of water stress and subsequent rehydration of tobacco plants pre-treated with water (H₂O), 100 μ M abscisic acid (ABA) or 10 μ M benzyladenine (BA). Control plants (C) were sufficiently supplied with water. AC - biologically active CK, cZ - *cis*-derivatives of zeatin, SC - storage CK, IC - inactive CK, Cph - cytokinin phosphates.

with BA than in those pre-treated with water or ABA (Pospíšilová and Bařková 2004).

Contents and composition of endogenous CK in plants sufficiently supplied with water as well as during water stress and subsequent rehydration are different in different plant species (Badenoch-Jones *et al.* 1996, Pospíšilová 2003, Pospíšilová and Dodd 2005). To simplify the interpretation, CK were divided to five functionally different groups (Figs. 5-8) similarly as in the recent paper of Ananieva *et al.* (2004): 1) biologically active CK (*trans*-zeatin, dihydrozeatin, *trans*-zeatin-9-riboside, dihydrozeatin-9-riboside, isopentenyladenine, isopentenyladenine-9-riboside), 2) storage CK (*trans*-zeatin-O-glucoside, *trans*-zeatin-9-riboside-O-glucoside, dihydrozeatin-9-riboside-O-glucoside), 3) *cis*-derivatives of zeatin, 4) inactive CK (*trans*-zeatin-7-glucoside, dihydrozeatin-7-glucoside, *trans*-zeatin-9-glucoside, dihydrozeatin-9-glucoside, isopentenyl-adenin-7-glucoside, isopentenyl-adenin-9-glucoside), and 5) CK phosphates.

From the species studied, the highest total content of CK was observed in French bean (Fig. 5). In its spectrum the storage CK followed by CK phosphates were dominant. During severe water stress not only the total CK content but also the contents of biologically active CK increased. Further, the CK contents increased as a result of ABA pre-treatment. CK contents after BA pre-treatment were similar as in control plants, but the increased CK contents induced by mild water stress were not observed.

In tobacco plants (Fig. 6), the total content of CK was lower than in bean plants and in its spectrum inactive CK

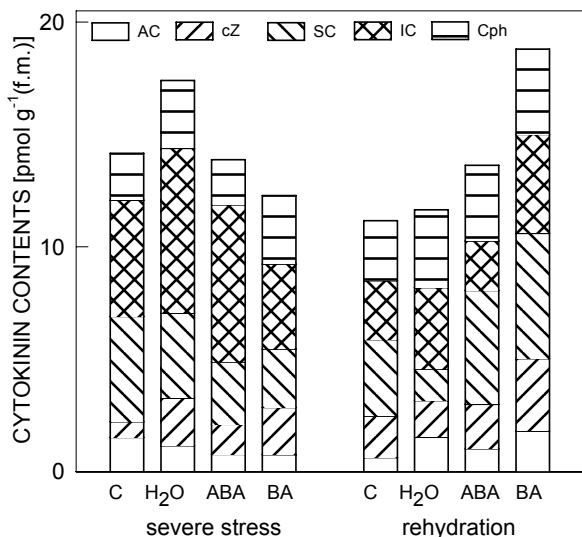


Fig. 7. Contents of endogenous cytokinins (CK) during water stress and subsequent rehydration of sugar beet plants pre-treated with water (H₂O), 100 μ M abscisic acid (ABA) or 10 μ M benzyladenine (BA). Control plants (C) were sufficiently supplied with water. AC - biologically active CK, cZ - *cis*-derivatives of zeatin, SC - storage CK, IC - inactive CK, Cph - cytokinin phosphates.

were dominant. Water stress induced slight accumulation of CK. Contents of CK decreased after ABA or BA pre-treatment during mild water stress but increased during severe water stress. After rehydration the highest CK contents were observed in plants pre-treated with BA.

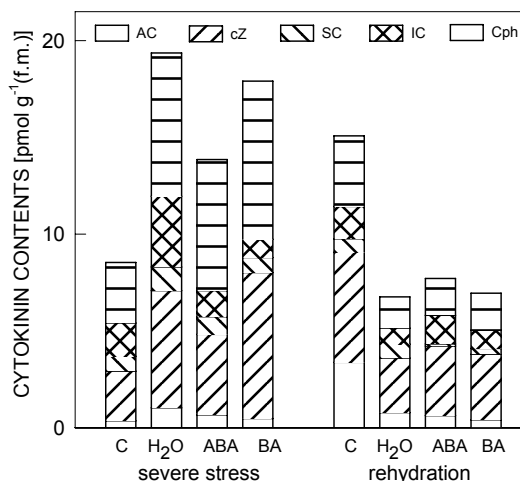


Fig. 8. Contents of endogenous cytokinins (CK) during water stress and subsequent rehydration of maize plants pre-treated with water (H₂O), 100 μ M abscisic acid (ABA) or 10 μ M benzyladenine (BA). Control plants (C) were sufficiently supplied with water. AC - biologically active CK, cZ - *cis*-derivatives of zeatin, SC - storage CK, IC - inactive CK, Cph - cytokinin phosphates.

The low contents of CK were also found in sugar beet (Fig. 7) and especially in maize (Fig. 8). It was the reason why changes in their contents were studied only under severe stress and after rehydration. In their CK spectrum all types were present including *cis*-derivatives of zeatin. In both species the highest CK contents during water stress were found in plants irrigated with water. In maize, CK contents were increased during water stress also in plants pre-treated with ABA or BA but less than in plants pre-treated with water. On the other hand, in sugar beet plants pre-treated with ABA and especially BA, CK contents did not increase during water stress but increased after rehydration (Fig. 7). In maize the CK content was surprisingly increased in the time of rehydration in regularly irrigated control plants (Fig. 8).

Increase in xylem-sap contents of *trans*-zeatin-riboside and dihydrozeatin-riboside with decrease in stem water potential was observed in lychee (Stern *et al.* 2003) or under severe water stress in *Craterostigma wilmsii* (Vicr  *et al.* 2004). In contrast, contents of zeatin, zeatin-riboside, isopentenyl-adenine and isopentenyladenosine in xylem sap of rice decreased during root drying and increased again after rewatering (Bano *et al.* 1993). CK content in alfalfa and grapevine also decreased due to water stress (Goicoechea *et al.* 1995, Stoll *et al.* 2000, Nikolaou *et al.* 2003) but remained unchanged in maize (Setter *et al.* 2001). Diurnal variations of xylem sap CK

and ABA concentrations were observed in desert-grown almond (Fusseder *et al.* 1992). The maximum CK concentration was found in the morning, but maximum ABA concentration in the afternoon. ABA and CK interactions also depended on CK type, *e.g.* in mesocarp of ripening avocado application of isopentenyladenine increased the content of ABA while application of zeatin decreased it (Cowan *et al.* 1999). These authors suggested that CK may suppress xanthin dehydrogenase activity or enhance conversion of ABA to phaseic acid.

These results also proved the statements of Hare *et al.* (1997) and Kamínek *et al.* (1997) that the composition and contents of endogenous phytohormones in the site of action might be different from the site of application.

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