The impact of *trans*-zeatin *O*-glucosyltransferase gene over-expression in tobacco on pigment content and gas exchange

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Abstract

The responses of tobacco plants over-expressing *trans*-zeatin *O*-glucosyltransferase gene under constitutive or senescence-inducible promoter (*35S:ZOG1* and *SAG12:ZOG1*) and of wild type (WT) plants to water stress and subsequent rehydration were compared. In plants sufficiently supplied with water, both transgenics have higher net photosynthetic rate (P_N) in upper and middle leaves and higher stomatal conductance (g_s) in middle leaves than WT. Water use efficiency (WUE = P_N/E) was higher in both transgenics than in WT. During prolonged water stress, both P_N and E declined to a similar extent in both transgenics and WT plants. However, 7 d after rehydration P_N in *SAG:ZOG* (upper and middle leaves) and *35S:ZOG* (upper leaves) was higher than that in WT plants. Increased content of endogenous CKs in *35S:ZOG* plants did not prevent their response to ABA application and the results obtained did not support concept of CK antagonism of ABA-induced stomatal closure. The chlorophyll (Chl) *a+b* content was mostly higher in both transgenics than in WT. During water stress and subsequent rehydration it remained unchanged in upper leaves, decreased slightly in middle leaves only of WT, while rapidly in lower leaves. Total degradation of Chl, carotenoids and xanthophyll cycle pigments (XCP) was found under severe water stress in lower leaves. Carotenoid and XCP contents in middle and upper leaves mostly increased during development of water stress and decreased after rehydration. While β-carotene content was mostly higher in WT, neoxanthin content was higher in transgenics especially in *35S:ZOG* under severe stress and after rehydration. The higher content of XCP and degree of their deepoxidation were usually found in upper and middle leaves than in lower leaves with exception of *SAG:ZOG* plants during mild water stress.

Additional key words: carotenoids, chlorophylls, net photosynthetic rate, *Nicotiana tabacum*, stomatal conductance, transpiration rate, transgenic plants, xanthophyll cycle pigments.

Introduction

Almost all processes in the plant life are directly or indirectly affected by both environmental factors and phytohormones. Although much work has focused on the role of the abscisic acid (ABA) in plant response to water stress, the role of other phytohormones is still far from fully elucidated. Cytokinins (CK) are often considered abscisic acid (ABA) antagonists in many processes (for recent review see Dodd 2003, Pospíšilová 2003a, Pospíšilová and Dodd 2005). Interaction between CKs and ABA may occur not only at the site of their action (*e.g*. on stomatal aperture) but also during their synthesis, *e.g.*, application of benzyladenine (BA) decreased water stress-induced ABA accumulation in bean and maize but not in sugar beet and tobacco (Pospíšilová *et al*. 2005). However, in transgenic tobacco plants with introduced isopentenyltransferase (*ipt*) gene under control of the P_{SAG12} promoter (P_{SAG12}-*ipt*) with highly increased CK content stress-induced accumulation of ABA was similar to that in wild type tobacco plants (Cowan *et al*. 2005).

The interactions between these phytohormones can be studied in different ways: *1*) simultaneous application of both substances, *2*) application of CKs to plants with

Received 11 July 2007, *accepted* 27 September 2007.
Abbreviations: ABA - abscisic acid; BA - benzyladenine; Car - carotenoids; Chl - chlorophyll; CK - cytokinin; DEPS - degreee of XCP deepoxidation; DEPSC - deepoxidised XCP per Chl unit; PS - photosystem; RWC - relative water content; *35S:ZOG* - *25S:ZOG1*; *SAG:ZOG* - *SAG12:ZOG1*; WUE - water use effficiency; XCP - xanthophyll cycle pigments.
Acknowledgement: We are grateful to Prof. Richard Amasino (University of Wisconsin, Madison, USA) for kind donation of *SAG12* promoter and Profs. Machteld and David Mok and Dr. Ruth Martin (Oregon State University, Corvallis, USA) for kind supplementation of the seeds of *35S:ZOG1* and *SAG12:ZOG1* transformed tobacco. We express our thanks to Mrs. Ludmila Hávová for technical assistance. This work was supported by Grant Agency of the Czech Republic, project no. 522/04/0549 and by Ministry of Education, Youth and Sport CR, project no. ME868.
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increased or decreased content of endogenous ABA, *3*) response of plants to increased endogenous contents of both phytohormones, and 4) response of plants with increased content of endogenous CKs to ABA application. We gradually tried to add some results to each of them.

Firstly, application of CKs was reported to delay stomatal closure induced by exogenous ABA, *e.g*. in maize epidermal strips (Blackman and Davies 1984), detached flax leaves (Drüge and Schönbeck 1992) and leaves detached from N-deprived cotton (Radin and Hendrix 1988). Also in our previous experiments, when ABA and CK were applied simultaneously to the substrate or sprayed on French bean or sugar beet leaves of plants sufficiently supplied with water, the stomatal conductance decreased less than when ABA was applied alone (Pospíšilová 2003b, 2004). Similar results were found when ABA and BA were applied simultaneously but separately to different parts of split root system (Pospíšilová 2003b).

Further, the responses of plants to CKs application were followed in water stressed plants. Under water stress, the marked increase in content of endogenous ABA has been repeatedly described (recently *e.g*. Dodd *et al*. 2006, Verslues and Bray 2006, Kudoyarova *et al*. 2007) while only minor changes in content of endogenous CKs were usually found (*e.g.* Naqvi 1994, Pospíšilová 2003a). However, Kudoyarova *et al*. (2007) recently observed decrease in CK content in tomato leaf tissue up to 46 % during water stress but no changes in xylem sap CK content. Treatment of cucumber cotyledons with CK benzyladenine (BA; 20 μM) increased content of endogenous CKs (Li and Ma 2007). In bean plants, pre-treatment with BA ameliorated effects of water stress on gas exchange parameters (Pospíšilová and Baťková 2004) and BA application also improved recovery of drought stressed bean plants after rehydration (Rulcová and Pospíšilová 2001). However, effects of BA on gas exchange parameters were species-specific and ranged from positive to negative (Pospíšilová and Baťková 2004). Non-stomatal effects of CKs have been also reported (for recent review see Nyitrai 2005) including alleviation of the negative effects of water stress on chlorophyll (Chl) and carotenoid (Car) contents, photochemical activities of photosystem (PS) 1 and 2, and content and activity of ribulose-1,5-bisphosphate carboxylase or phosphoenol-pyruvate carboxylase (Metwally *et al.* 1997, Chernyad′ev and Monakhova 1998, 2003, Pandey *et al*. 2000, 2003/4, Singh *et al*. 2001). Similarly, BA pre-treatments ceased a degradation of Chl and Car induced by water stress, increased contents of xanthophyll cycle pigments and decreased degree of their de-epoxidation (Haisel *et al*. 2006).

Finally, transgenic plants with increased endogenous CKs contents were used and their responses to water stress (and thus increased endogenous ABA content) or to ABA application were followed. CK biosynthetic gene, which codes for isopentenyltransferase (*ipt* gene), has been introduced into plants either under its native promoter, or as chimaeric *ipt* genes driven by different promoters (*e.g*. Synková *et al*. 1997, 2006, Květoň 2006). Changes in many parameters in transgenic plants depended on the concentration of endogenous CKs reached after expression of the *ipt* gene. When *ipt*-transgenic tobacco plants had only sligthly elevated CK content stomatal and cuticular transpiration rates as well as parameters of water relations were not significantly affected (Pospíšilová *et al*. 1997/98), In transgenic tobacco plants containing *ipt* gene under the control of the promoter for the small subunit of Rubisco (*Pssu-ipt*) considerable increase in contents of endogenous CKs was associated with marked morphological changes and occurrence of water stress (Synková *et al*. 1999). Unfortunately, in these plants it was impossible to differentiate direct effects of increased CKs content and indirect effect of decreased root/shoot biomass ratio. To overcome this drawback we used in the following experiments another transgenic tobacco plants.

In these transgenic plants, over-expression of *trans*-zeatin *O*-glucosyltransferase (*ZOG1*) gene from *Phaseolus lunatus* either under constitutive (*35S*) promoter (uniform elevation of total CKs in whole plant, already before stress initiation) or under senescence-inducible (*SAG12*) promoter (CK increase related to time and location of the stress) increased the total CKs content, but especially their storage forms (*O*-glucosides), without affecting significantly the amount of the corresponding active free bases and ribosides as well as, and in consequence their morphology (Havlova *et al*. 2008). According to Badenoch-Jones *et al*. (1996) not only free bases and ribosides and but also CK *O*-glucosides may play a role in regulating stomatal opening, probably due to their fast de-glucosylation.

These transgenic tobacco plants were used as model plants for elucidation of ABA and CKs interactions. Their responses to water stress and subsequent rehydration as well as ABA application were compared with wild type plants. The plant water status was characterized by relative water content, and parameters of gas exchange and contents of photosynthetic pigments were measured in upper, middle and lower leaves. According to above mentioned previous experiments in which exogenous CKs were applied we chose parameters in which we can expect differences between wild type and transgenic plants and leaves of different insertion were measured to find possible differences between both transgenics.

Materials and methods

Plants and cultivation: Wild-type tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) and transgenic *SAG12:ZOG1* and *35S:ZOG1* tobacco plants (for detail see Martin *et al*. 2001, Havlova *et al.* 2008) were grown

in pots with *Perlite* moistened by 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 %. Water stress was induced in 2-month-old seedlings by cessation of watering and below mentioned parameters were followed during development of water stress (1, 3 and 7 d after last irrigation) and during recovery after rehydration (2 and 7 d). The response of wild type and transgenic plants (*35S:ZOG*) to abscisic acid (100 μM ABA) was also determined and individual parameters were measured 1 h and 24 h after its application to plants sufficiently supplied with water. The used ABA concentration was found suitable in previous experiments (Pospíšilová and Baťková 2004) and according to Jiang and Zhang (2002) treatment with 100 μM ABA induced similar increase in endogenous ABA content in maize leaves as water stress. In further experiment plants were pre-treated with 50 cm^3 of water (H_2O) or with 100 μM ABA before imposition of water stress and below mentioned parameters were measured during mild and severe water stress (when visible wilting occurred), and 2 d after rehydration.

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).

Gas exchange parameters: Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) were measured on upper, middle and lower attached leaves using the commercial gas exchange system *LCA-4* (*ADC Bio Scientific*, Hoddesdon, UK) with leaf chamber *LC4/PLC4BT-1/E* at a temperature of 25 °C, irradiance of 750 μmol m^{-2} s^{-1}, CO_2 concentration of 350 μmol mol^{-1}, and relative humidity of about 30 %.

Photosynthetic pigments: Contents of photosynthetic pigments were determined in acetone extracts of discs taken from upper, middle and lower leaves 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 linear gradient was run from 8 to 12 min. The flow rate was 1 cm^3 min^{-1}, the detection wavelength 445 nm. Data were captured and calculated by PC-software *Clarity* (*DataApex*, Prague, Czech Republic).

Statistics: Means and standard errors of means were calculated and significance of differences between transgenic plants and wild type was evaluated by Student *t*-test. *ANOVA* (programme *SYSTAT*, *version 7.0.1*, 1997) was further used for analysis of the effects of plant type, leaf position and treatments and their interactions. Experiments were repeated twice with similar results.

Results

Relative water content and gas exchange parameters: After cessation of watering, RWC in WT and transgenic plants decreased to about 85 % in upper leaves and 80 % in lower leaves during seven days and increased again after rehydration (Fig. 1).

In all plants stomatal conductance (g_s), transpiration rate (E) and net photosynthetic rate (P_N) were highest in the upper leaves and lowest in lower ones. All gas exchange parameters decreased rapidly during water stress to very low values. Stomata were completely closed after 7 d. On the contrary, recovery was rather slow. P_N, E and g_s increased preferentially in young leaves and recovery was complete after 7 d only in upper and middle leaves (Figs. 2, 3, 4).

When gas exchange parameters were compared in WT, *SAG:ZOG* and *35S:ZOG* plants sufficiently supplied with water, both transgenics had higher P_N in upper and middle leaves and higher g_s in middle leaves than WT (Fig. 2) while E in upper leaves of *35S:ZOG* plants was lower than that of WT plants (Fig. 2). The mean water

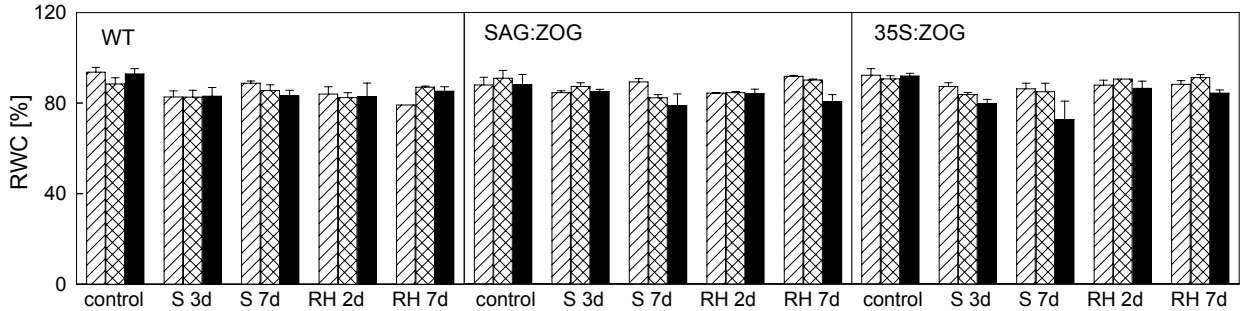

Fig. 1. Changes in relative water content (RWC) in upper (*stripped columns*), middle (*chequered columns*) and lower leaves (*full columns*) of wild type (WT) and two transgenic (SAG:ZOG and 35S:ZOG) tobacco plants during development of water stress for 3 d (S 3d) and 7 d (S 7d) after cessation of watering and during recovery 2 d (RH 2d) and 7 d (RH 7d) after re-irrigation. Means ± SE, $n = 3$.

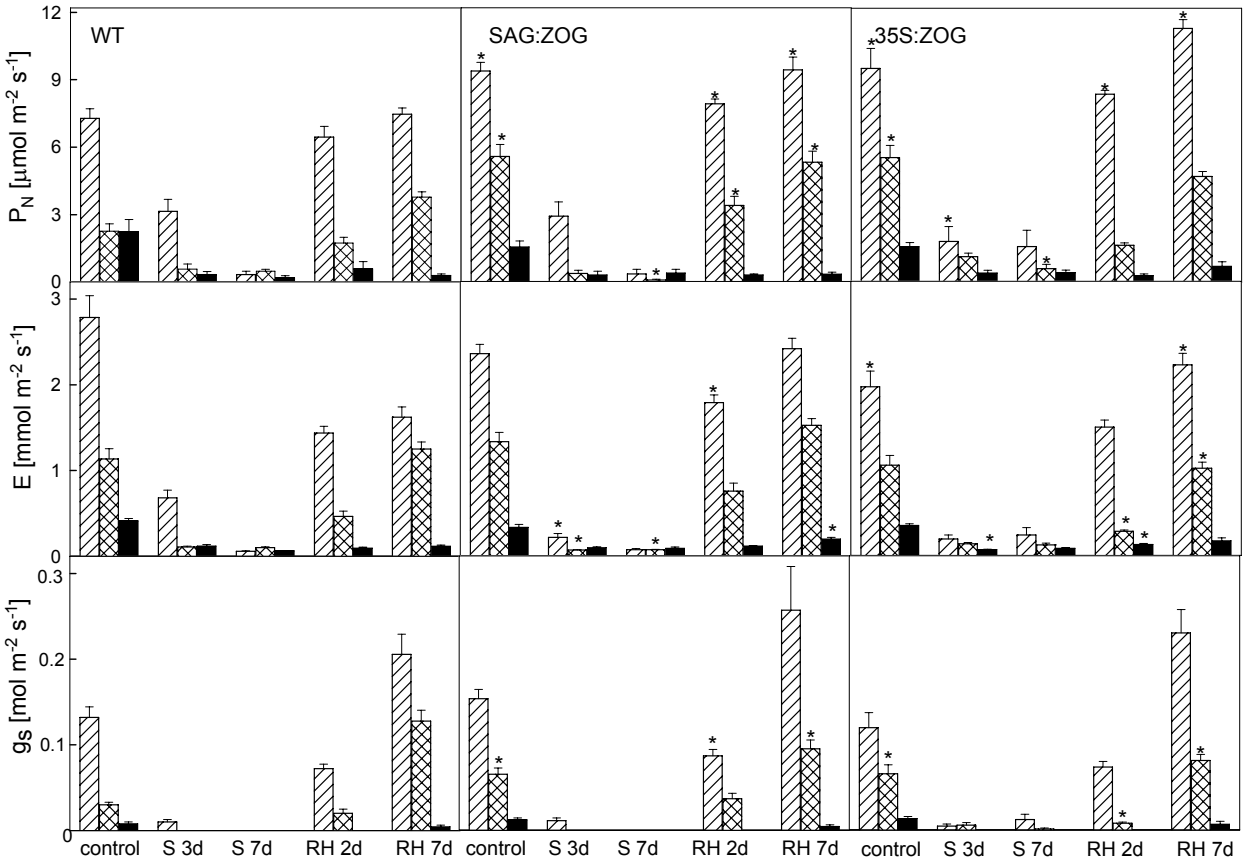

Fig. 2. Changes in net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) in upper (*stripped columns*), middle (*chequered columns*) and lower leaves (*full columns*) leaves of wild type (WT) and two transgenic (SAG:ZOG and 35S:ZOG) tobacco plants during development of water stress after cessation of watering (S 3d and S 7d) and during recovery after re-irrigation (RH 2d and RH 7d). Means ± SE, $n = 9$, * - statistically significant differences among WT and transgenics at $P = 0.05$.

use efficiency (WUE = P_N/E) was considerably higher in both transgenics (4.95 and 4.10 mmol mol^{-1} in *35S:ZOG* and *SAG:ZOG*, respectively) than in WT (2.72 mmol mol^{-1}).

During prolonged water stress, a significant decrease in gas exchange parameters was observed in both transgenics and WT plants. However, 7 d after rehydration P_N of *SAG:ZOG* (upper and middle leaves) and *35S:ZOG* (upper leaves) was higher than that in WT plants (Fig. 2). While g_s and E were slightly higher in upper leaves of *SAG:ZOG* than in WT 2 d after rehydration, g_s in middle leaves of *SAG:ZOG* was lower. In middle leaves of *35S:ZOG* g_s and E were lower than in WT 2 d and 7 d after rehydration (Fig. 2).

Table 1. Effect of application of 100 μM ABA on stomatal conductance and photosynthetic rate in WT and 35S:ZOG plants after 1 and 24 h. Means ± SE, $n = 20$. * - statistically significant differences among WT and 35S:ZOG at $P = 0.05$.

Time [h]	g_s [mol m^{-2} s^{-1}]		P_N [μmol m^{-2} s^{-1}]	
	WT	35S:ZOG	WT	35S:ZOG
0	0.088±0.003	0.083±0.005*	11.17±0.27	12.42±0.34*
1	0.068±0.005	0.032±0.005*	8.57±0.35	6.96±0.53*
24	0.034±0.004	0.012±0.003*	6.55±0.59	4.07±0.60*

Already 1 h after 100 μM ABA application g_s, E and P_N decreased and they decreased considerably after 24 h (Table 1). Increased content of endogenous CKs (predominantly of CK *O*-glucosides) in *35S:ZOG* plants did not prevent their response to ABA application. Even the effect was more pronounced than in WT plants.

ABA pre-treatment postponed development of water stress and values of P_N were higher under severe water stress and after rehydration in WT and under mild and severe water stress in *35S:ZOG* plants in comparison with H_2O pre-treated plants (data not shown).

Chlorophyll content: During development of water stress and after rehydration, both transgenics mostly had higher chlorophyll (Chl) *a* and *b* contents in comparison with WT plants. Immediately after cessation of watering, Chl content increased in WT in the upper leaves, but from the 3rd day of water stress Chl content gradually decreased, especially in the lower, and to a minor extent in the middle leaves (Fig. 3). In *SAG:ZOG* plants minor

elevation of chlorophyll content in upper leaves occurred at mild stress while in *35S:ZOG* plants this Chl increase was delayed. At prolonged stress chlorophyll loss in the lower, as well as in the middle leaves occurred in all tobacco plants. As the Chl *a* is preferentially degraded, the Chl *a*/*b* ratio in lower leaves decreased considerably 3 d after cessation of watering. After rehydration upper and middle leaves (which corresponded to the lowest survived ones) of both transgenics had almost the same Chl contents (Fig. 3) but in WT Chl content of upper leaves was higher than that of middle leaves. Chl *a*/*b* ratio in the middle leaves after rehydration was significantly higher in both transgenics than in WT.

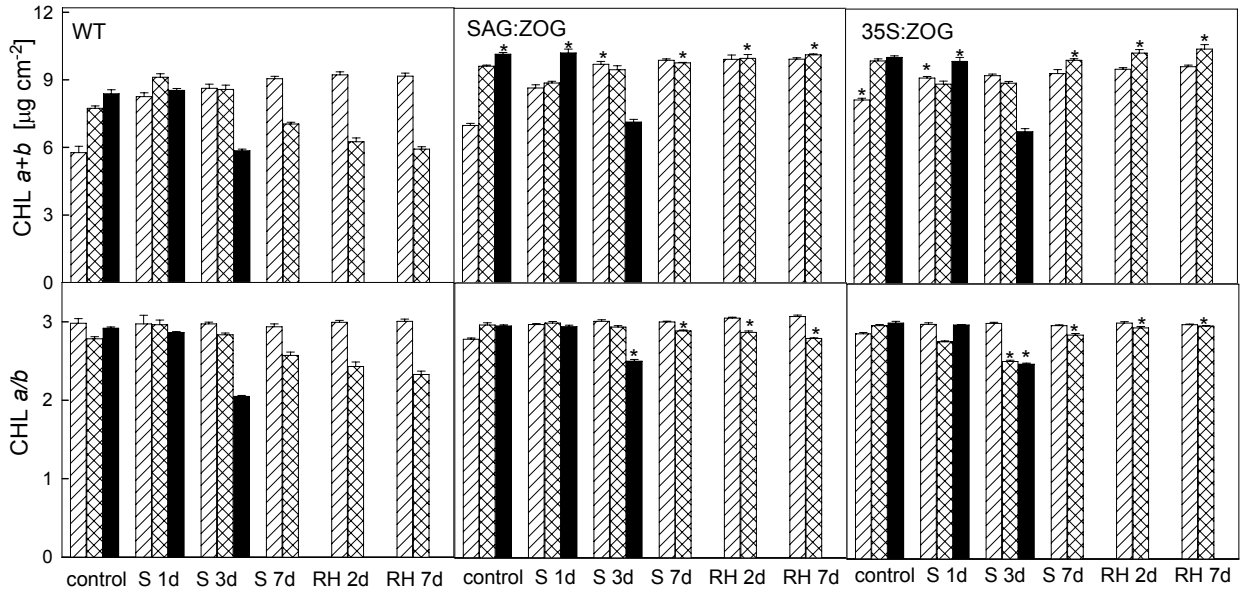

Fig. 3. Changes in chlorophyll (Chl) *a*+*b* content and Chl *a*/*b* ratio in upper (*stripped columns*), middle (*chequered columns*) and lower leaves (*full columns*) leaves of wild type (WT) and two transgenic (SAG:ZOG and 35S:ZOG) tobacco plants during development of water stress after cessation of watering and during recovery after re-irrigation. Means ± SE, n = 3, * - statistically significant differences among WT and transgenics at P = 0.05.

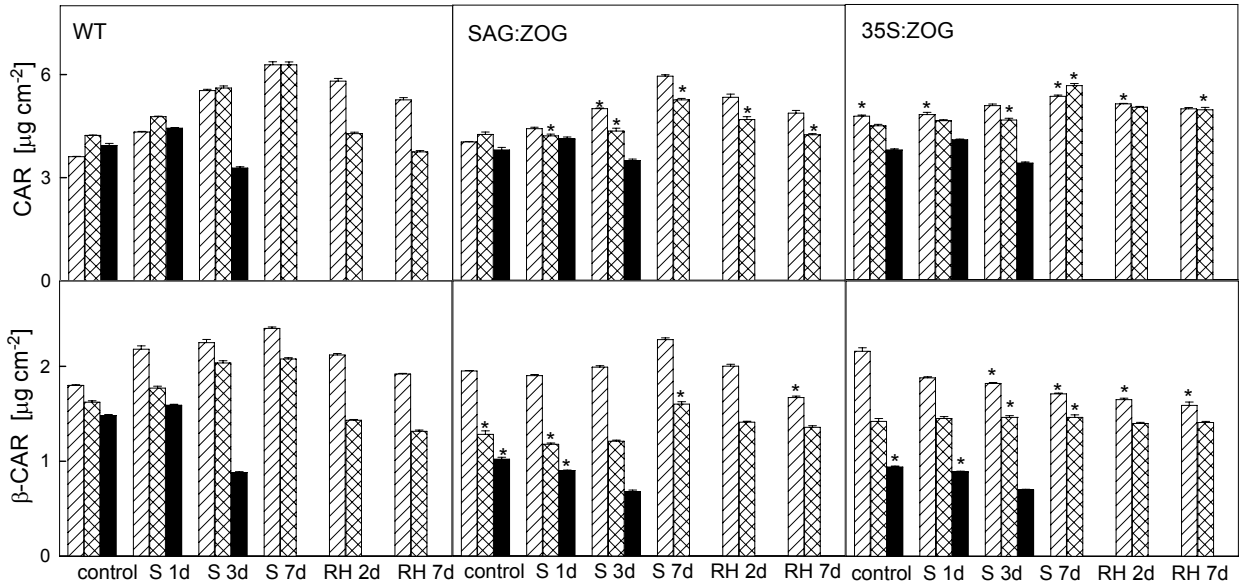

Fig. 4. Changes in total carotenoids (Car) and β-carotene (β-CAR) content in upper (*stripped columns*), middle (*chequered columns*) and lower leaves (*full columns*) leaves of wild type (WT) and two transgenic (SAG:ZOG and 35S:ZOG) tobacco plants during development of water stress after cessation of watering and during recovery after re-irrigation. Means ± SE, n = 3, * - statistically significant differences among WT and transgenics at P = 0.05.

Carotenoid contents: The total carotenoid content exhibited gradual increase during the stress progression in all tobacco plants and upon rehydration gradual decrease was found (Fig. 4). In *SAG:ZOG* and even more in *35S:ZOG* plants, increase in carotenoid content was postponed and lower in comparison with WT. Also carotenoid content decline after rehydration was slower. The changes in β-carotene content were similar to those of total carotenoids (Fig. 4) with the exception of its more rapid degradation in lower leaves of trasgenics than in lower leaves of WT.

In WT slight increase of light harvesting carotenoids lutein and neoxanthin was found at mild stress (Fig. 5). During stress progression an increase of lutein content occurred in upper and middle leaves, while in lower leaves decrease of lutein content was found from the third day of stress. Upon rehydration both pigments decreased in WT middle leaves. In *SAG:ZOG* as well as in *35S:ZOG* plants increase of lutein content was delayed. Neoxanthin content was mostly higher in both transgenics than in WT. Upon rehydration decrease of lutein and neoxanthin contents were slower in both trangenics than in WT (Fig. 5).

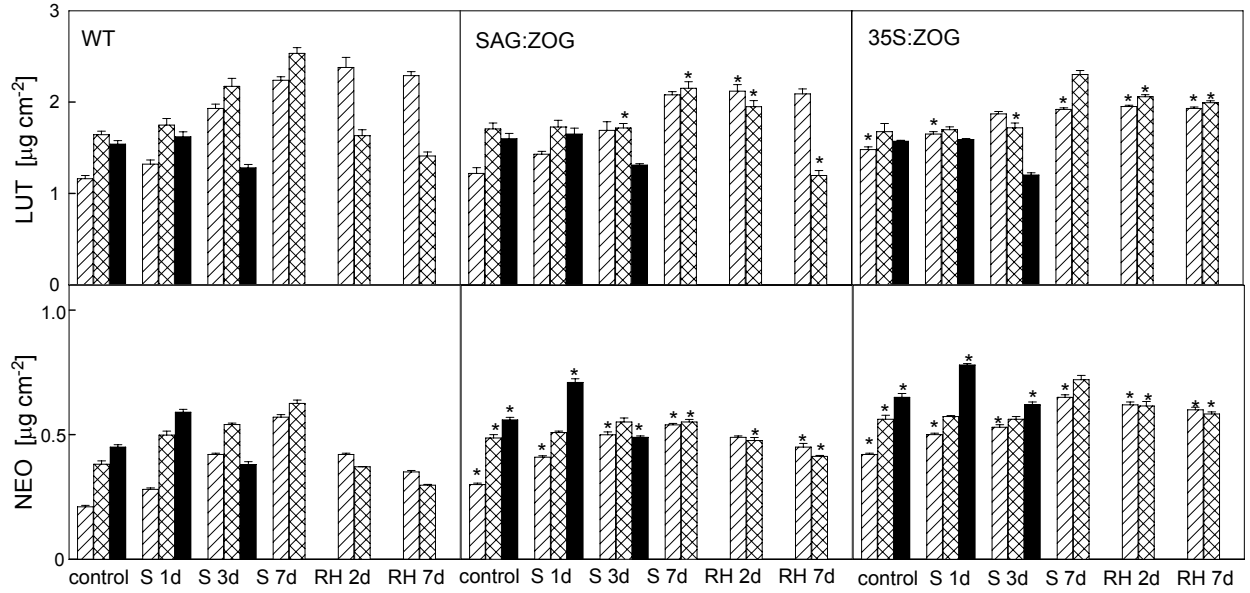

Fig. 5. Changes in lutein and neoxanthin content in upper (*stripped columns*), middle (*chequered columns*) and lower leaves (*full columns*) leaves of wild type (WT) and two transgenic (SAG:ZOG and 35S:ZOG) tobacco plants during development of water stress after cessation of watering and during recovery after re-irrigation. Means ± SE, $n = 3$, * - statistically significant differences among WT and transgenics at $P = 0.05$.

Xanthophyll cycle pigments: In WT gradual increase of XCP occurred during the whole stress progression and decreased after rehydration. In both transgenics the changes of XCP content were in a similar way but less expressive with exception of marked elevation of XCP in *35S:ZOG* plants after 7-d dehydration.

The degree of XCP deepoxidation (DEPS) increased markedly 3-d and 7-d after cessation of watering (Fig. 6) and slightly decreased after rehydration in all tobacco plants. The highest values were found in lower leaves of *SAG:ZOG* plants (3-d stress) and middle leaves of *35S:ZOG* (7-d stress). With exception of lower leaves, DEPSC at moderate stress was lower in both transgenics than in WT and in middle leaves also after rehydration (Fig. 6).

Analysis of variance: Significant differences ($P < 0.001$) were found among plant types, leaves of different position and different treatments (levels of water stress) in all measured parameters of gas exchange and pigment composition with the exception of the effect of plant type on g_s. The interaction leaf×treatment was significant for all measured parameters, the interaction plant×treatment for all parameters with the exception of g_s, and the interaction plant×leaf with the exception of g_s and E. Interaction plant×leaf×treatment was significant for all parameters with the exception of g_s. The exceptional position of g_s among measured parameters was probably due to frequent occurrence of zero values under water stress.

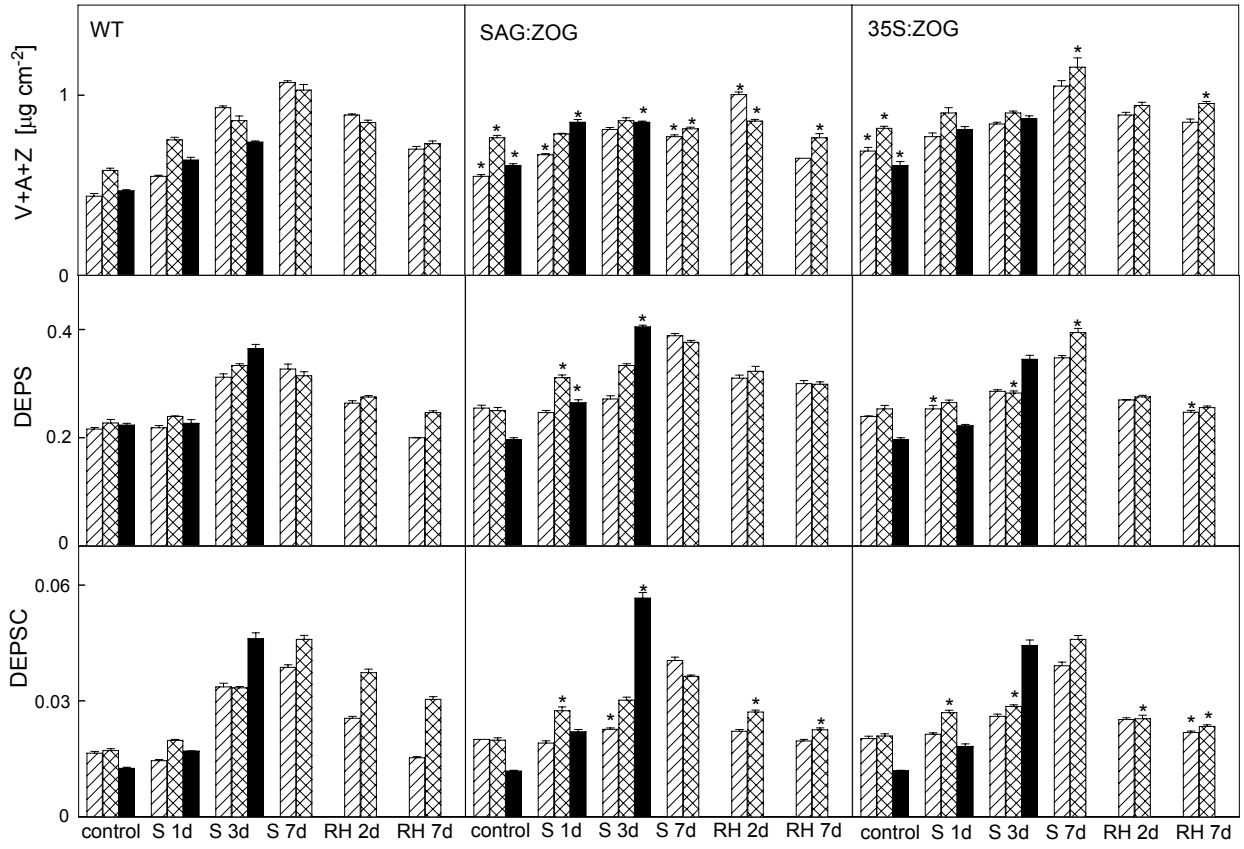

Fig. 6. Changes in xanthophyll cycle pigments (antheraxanthin + violaxanthin + zeaxanthin) content, xanthophyll cycle pigments de-epoxidation state (DEPS, (zeaxanthin + 0.5 antheraxanthin)/(antheraxanthin + violaxanthin + zeaxanthin)) and DEPS expressed per chlorophyll (DEPSC, (zeaxanthin + 0.5 antheraxanthin)/(chlorophyll *a*+*b*) in in upper (*stripped columns*), middle (*chequered columns*) and lower leaves (*full columns*) leaves of wild type (WT) and two transgenic (SAG:ZOG and 35S:ZOG) tobacco plants during development of water stress after cessation of watering and during recovery after re-irrigation. Means ± SE, n = 3, * - statistically significant differences among WT and transgenics at P = 0.05.

Discussion

In order to evaluate the impact of CKs on photosynthetic characteristics and pigment composition during water stress and subsequent recovery, we compared the behaviour of plants with CK content elevated before stress initiation (*35S:ZOG1*), plants exhibiting senescence induced CK elevation (*SAG12:ZOG1*) and the corresponding WT. Constitutive transgenics had almost double total CK content in comparison with WT (for more detail see, Havlova *et al.* 2008). This elevation was represented predominantly by CK *O*-glucosides. The increase of physiologically active CKs was negligible. As the highly increased glucosylation was not compensated by the decrease of CK degradation with cytokinin oxidase/dehydrogenase, maintenance of CK homeostasis seems to be acquired by stimulation of CK biosynthesis (or CK turn-over) in these transgenics. The difference in CK levels was only minor between juvenile, control *SAG:ZOG1* plants and WT, however, during water stress progression as well as natural senescence *SAG* promoter activity (quantified by real-time RT PCR) correlated with elevation of total CK content, which might correspond to enhanced CK turn-over.

In agreement with reported high tobacco drought resistance (*e.g.*, Riga and Vartanian 1999), stomata closed in our experiments early during development of water stress, and thus g_s decreased to very low values. Simultaneous decrease in E prevented excessive water loss which enabled plants to retain relatively high RWC for a long period under water stress (about 80 % up to 7 d). In previous experiments we also observed increase in endogenous ABA content in tobacco leaves already at mild water stress (Pospíšilová *et al*. 2005) which according to Borel *et al*. (2001) might be important for early induction of stomatal closure. Slower recovery of g_s and E than RWC after rehydration suggested that the stomatal closure was not a passive response to water deficit (Ortuño *et al*. 2005). P_N decreased more slowly than g_s and E under water stress. Therefore in tobacco plants both stomatal and non-stomatal limitations to photosynthesis were important.

During water stress, highest g_s remained in upper leaves in all plant types (Fig. 2) which corresponds to well-known preferential water supply of young leaves during stress conditions (Čatský 1962). This is also in accordance with the establishment of the gradient of physiologically active CKs in favour of the upper tobacco leaves during water deficit progression, but in contrast to the highest increase in endogenous ABA content in upper leaves, as described by Havlová *et al.* (2007). It might be explained either by lower sensitivity of stomata of young leaves to ABA or by interaction of ABA with CKs.

Our finding of higher values of P_N in upper and middle leaves of the both *ZOG* transgenics in comparison with the WT under well watered conditions is in accordance with the long-acknowledged positive effect of applied CKs on photosynthesis (for recent review see *e.g*. Dodd 2003, Pospíšilová 2003a, Pospíšilová and Dodd 2005). The higher P_N values in *ZOG* transgenics were probably connected with their consistently higher Chl content (Fig. 4), because significant differences in g_s between transgenics and WT were rather exception than the rule. Even g_s values were either higher or lower in *ZOG* transgenics than in WT. This inconsistency of response of stomata to elevated CK is in agreement with our previous experiments with exogenous application of BA where positive or negative effects on g_s were found (Pospíšilová and Baťková 2004). Higher values P_N in upper and middle leaves of the both transgenics in comparison with WT were found also after rehydration which is in agreement with the results obtained after CK application to bean plants (Rulcová and Pospíšilová 2001). However, no significant differences in gas exchange parameters were found in lower leaves in which high increase of the activity of the main CK degrading enzyme, cytokinin oxidase/dehydrogenase, as well as massive CK glucosylation were observed (Havlova *et al*. 2008). Higher WUE in both transgenics then in WT was mostly due to their higher P_N in upper and middle leaves under sufficient water supply and in the case of *35S:ZOG* also by their lower E in upper leaves.

As was mentioned above changes in many parameters observed in transgenic plants depend on the concentration of endogenous CKs: the changes induced by slightly increased CK content (10 - 30 %) were mostly positive while those induced by increase in CK content several times were negative (*e.g*. Synková *et al*. 1997, Pospíšilová *et al*. 1997/98, 2003a). Therefore no disturbance in water regime was found in both *ZOG* transgenics (Fig. 1) where content of physiologically active CKs was only slightly increased (Havlova *et al*. 2008). It was in contrast to the behaviour of above mentioned *ipt* transgenic plants. Rooted *Pssu-itp* transgenic tobacco plants suffered by water stress, RWC and g_s of both young and old leaves were considerably lower than in WT plants (Synková *et al*. 1999). However, when their water regime was improved by grafting on wild type rootstocks, g_s and P_N of young leaves were also comparable to those in WT plants while in old leaves P_N was lower (Synková *et al*. 1999). Wang *et al*. (1997) found increased g_s and E in transgenic tobacco plants with increased CK content.

The response of both *ZOG* transgenics to prolonged water stress was similar to WT plants. It was in agreement with increase in endogenous ABA content during water stress not only in WT but also in transgenics (Cowan *et al*. 2005, Havlova *et al*. 2008). While in French bean plants increase in ABA content induced by water stress was inhibited by CK application, the same was not found in tobacco (Pospíšilová *et al*. 2005). In addition, we found, that the response of *35S:ZOG* plants to applied ABA was even more pronounced than that of WT plants.

The Chl *a* and Chl *b* contents in lower leaves in all plants was completely degraded under severe water stress. On the other hand, in upper leaves Chl contents slowly increased at the beginning of water stress development, which corresponded to regular ontogenetic course in leaf Chl content, and remained on the same level during the course of experiment. In middle leaves, decrease in Chl content during water stress and subsequent rehydration was observed only in WT, but in both transgenics it remained on the same level during the experiment similarly as in young leaves. Similar changes were also observed in chlorophyll *a*/*b* ratio (Fig. 3). Thus both transgenics with elevated endogenous CKs coped with water stress more efficiently than WT plants (at least in case of the water loss lower than 20 %). This was in agreement with the protection of photosynthetic pigments by application of benzyladenine (BA) found in previous experiments (Haisel *et al*. 2006). Application of BA reduced the decrease in Chl also in water-stressed wheat (Chernyad′ev and Monakhova 2003) or in *Cassia* (Singh *et al*. 2001).

Similar profile was observed for β-carotene (Fig. 4), which serves as an antioxidant (ROS scavenger) as well as precursor of most other carotenoid biosynthesis. Almost steady level of β-carotene in upper and middle leaves of *35S:ZOG* plants might indicate that carotenoid biosynthesis and metabolism are in equilibrium. On the contrary, increased content of β-carotene in upper and middle leaves of WT plants during water stress shows its necessity to protect of reaction centre against stress or production of a carotenoid pool for XCP biosynthesis. Much lower water stress-induced increase in content of ABA precursor neoxanthin than in content of lutein might reflect its conversion to this stress hormone. In contrast, decrease in lutein and β-carotene content was observed in water-stressed peanut (Lauriano *et al*. 2006).

Xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin play very important protective role. During stress conditions, violaxanthin is de-epoxidated *via* antheraxanthin to zeaxanthin which dissipates the excess of light energy in the form of heat to avoid the damage of photosynthetic apparatus (Demmig-Adams and Adams 1996). Enhanced formation of antheraxanthin and zeaxanthin during dehydration was found, *e.g.*, in pea (Iturbe-Ormaetxe *et al*. 1998), maize (Saccardy *et al*.

1998), and wheat (Tambussi *et al,* 2002). Strongly increased content of XCP in WT plants indicated higher necessity of protection. On the contrary, *SAG:ZOG* plants only balanced the increased stress by slowly increased content of XCP and in *35S:ZOG* plants content of XCP increased only in final phase of water stress. Very similar changes of XCP and lutein contents are in accordance with participation of lutein in photoprotective function fulfilled by XCP (Kalituho *et al*. 2007). The ability of plants to defend against the stress may be also expressed by means of degree of XCP de-epoxidation (DEPS). Postponed maximum XCP content and DEPS in *35S:ZOG* plants till severe stress might indicate a delay of stress sensing in plants with constitutively increased CK levels. The increased DEPS during water stress in all plants indicated elevation of protective processes. While the effect of BA pre-treatment on Chl and carotenoid content during water stress and subsequent rehydration was in accord with the effect of increased endogenous CKs content in transgenic plants, the effect of applied BA was different in the case of XCP (Haisel *et al*. 2006) and XCP content and DEPS during water stress were higher in BA pre-treated plants than in controls. When DEPS was expressed per chlorophyll basis (as DEPSC), higher levels at middle leaves in WT plants at moderate stress and during subsequent rehydration indicated that protective mechanisms were switched off more slowly than chlorophyll degradation.

To sum up the results obtained, the transgenic plants with higher content of endogenous CKs have mostly higher P_N, WUE and Chl content when sufficiently supplied with water. From detailed comparison of changes of measured parameters during water stress and subsequent rehydration it seems that both transgenics also cope better with water stress, *e.g.*, they have higher P_N after rehydration, higher Chl content during water stress and rehydration and postponed water stress-induced increase in Car, XCP and DEPS than WT plants.

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