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Effect of abiotic stresses on the activity of antioxidative enzymes and contents of phytohormones in wild type and AtCKX2 transgenic tobacco plants

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Abstract

The responses of antioxidant enzymes (AOE) ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) in soluble protein extracts from leaves and roots of tobacco (*Nicotiana tabacum* L. cv. Samsun NN) plants to the drought stress, salinity and enhanced zinc concentration were investigated. The studied tobacco included wild-type (WT) and transgenic plants (AtCKX2) harbouring the cytokinin oxidase/dehydrogenase gene under control of 35S promoter from *Arabidopsis thaliana* (*AtCKX2*). The transgenic plants exhibited highly enhanced CKX activity and decreased contents of cytokinins and abscisic acid in both leaves and roots, altered phenotype, retarded growth, and postponed senescence onset. Under control conditions, the AtCKX2 plants exhibited noticeably higher activity of GR in leaves and APX and SOD in roots. CAT activity in leaves always decreased upon stresses in WT while increased in AtCKX2 plants. On the contrary, the SOD activity was enhanced in WT but declined in AtCKX2 leaves. In roots, the APX activity prevailingly increased in WT while mainly decreased in AtCKX2 in response to the stresses. Both WT and AtCKX2 leaves as well as roots exhibited elevated abscisic acid content and increased CKX activity under all stresses while endogenous CKs and IAA contents were not much affected by stress treatments in either WT or transgenic plants.

Additional key words: abscisic acid, cytokinin, cytokinin oxidase/dehydrogenase, drought, salinity, zinc.

Introduction

Abiotic stresses lead to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al.* 2001). Oxidative stress is characterised by an increase in concentration of reactive oxygen species (ROS) such as singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radical, that cause an injury to the cell compartments and macromolecules. Plants are equipped with an enzymatic and non-enzymatic antioxidative protective systems to maintain the ROS at the harmless level. A more potent antioxidant defence can thus increase plant tolerance to different stresses (Wang *et al.* 2003). The most important antioxidant enzymes (AOE) are superoxide dismutase (EC 1.15.1.1; SOD), ascorbate peroxidase (EC 1.11.1.11; APX), glutathione reductase (EC 1.6.4.2; GR) and catalase (EC 1.11.1.6; CAT) (Mittler 2002).

It is difficult to generalise how the activities of AOE are affected by stresses. In most cases, the activities of AOE increased during enhanced concentration of NaCl (Hernandéz *et al.* 1999, Arbona *et al.* 2003). However, there were some exceptions to this generalization, *e.g.*, the activity of SOD was found to decrease in salinity stressed plants (Dionisio-Sese and Tobita 1998, Savouré *et al.* 1999, Rios-Gondalez *et al.* 2002, Zhu *et al.* 2004). In so far reported cases of zinc-induced stress the

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Abbreviations: ABA - abscisic acid; AtCKX2 - transgenic tobacco harbouring cytokinin oxidase/dehydrogenase gene from *Arabidopsis thaliana*; AOE - antioxidative enzymes; APX - ascorbate peroxidase; β-car - β-carotene; CAT - catalase; Chl - chlorophyll; CK - cytokinin; CKX - cytokinin oxidase/dehydrogenase; DEPS - deepoxidation state; GR - glutathione reductase; SOD - superoxide dismutase; WT - wild type.

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activities of AOE increased (Bonnet *et al.* 2000, Tripathi and Gaur 2004). Responses of AOE activities in plants subjected to drought-induced stress are the most differing. Moran *et al.* (1994) observed that activities of APX, GR and CAT were reduced whereas activity of SOD increased in pea plants under drought. While Ünyayar *et al.* (2005) reported that the activity of APX decreased and GR, CAT and SOD activities were enhanced in tomatoes in the similar stress condition. It is obvious that the response of individual AOE activities depend on plant species, level of stress, and level of tolerance to stress. It is expectable that more tolerant plants will respond to a stress by enhancing of AOE activities. Bor *et al.* (2003) compared the effects of salt stress on AOE activities of two beet species, the sugar beet (*Beta vulgaris L.*) and its wild salt tolerant relative *Beta maritima.* Already under control conditions the activities of AOE were higher in wild beet and during stress they increased even more.

Abscisic acid (ABA) is considered a plant "stress hormone" (Zeevaart and Creelman 1988). Plants respond to unfavourable environmental conditions by changes in ABA availability, either *via* re-distribution (Slovik *et al.* 1995, Wilkinson and Davies 1997), by increased biosynthesis (Zeevaart and Creelman 1988) or by modifying the sensitivity to the hormone signal. ABA is, however, also an endogenous signal required for proper plant development. ABA signalling comprises various cellular events including pressure potential regulation and differential gene expression in response to stresses (for review see, *e.g.*, Christmann *et al.* 2003). Also cytokinins (CKs) play an important role in controlling plant responses to adverse environmental conditions (Hare *et al.* 1997). In contrast to ABA, CK contents tend to decrease under environmental constraints. A general view has emerged that during the stress, a reduction of CKs supply from the root alters gene expression in the shoot and thereby elicits appropriate responses to ameliorate the effects of stress (Hare *et al.* 1997). It was also reported that CKs could be assumed as non-enzymatic antioxidants. The CK zeatin riboside was found to act as a scavenger of superoxide anions that might help to maintain seed viability by detoxifying ROS (Gidrol *et al.* 1994). CKs themselves may also be directly affected by salt stress. Roots are the first tissues exposed to salinity stress, and also the site of cytokinin synthesis. Drought and salt stress could also affect the transport of CKs from the root to the leaves (Singh *et al.* 1988), thereby influencing CK-induced gene expression (Abdelghani *et al.* 1991).

Content of cytokinins with prenyl side chain in plant tissues is controlled by irreversible degradation by cytokinin oxidase/dehydrogenase (CKX). Transgenic tobacco plants overexpressing *Arabidopsis CKX* (*AtCKX*) gene were engineered having a reduced content of CKs, altered appearance, altered root system, retarded growth, and postponed senescence onset (Werner *et al.* 2001). In this work, we studied activities of AOE during abiotic stresses (salinity, drought and enhanced concentration of zinc) in the leaves and roots of AtCKX2 transgenic tobacco. In accordance with the data by Werner *et al.* (2001) we have recently reported that these CK-deficient transgenic plants exhibit a later onset of senescence (Mýtinová *et al.* 2006). Moreover, we have found that they have a better antioxidant protection even in young leaves, based on analyses of a content of β-carotene, pigments of xanthophyll cycle, and activities of GR and SOD (Mýtinová *et al.* 2006, Mýtinová unpublished results). The above mentioned stresses, similarly to ageing, are characterised as forms of oxidative stress. Therefore, on the basis of our previous results we hypothesize that the CK-deficient transgenic plants could be, due to their better photoprotection and antioxidant defence, also more tolerant to abiotic stresses. In this paper, we attempted to verify such assumption.

Materials and methods

Wild type and transgenic (clone AtCKX2-38) leaves and roots of tobacco (*Nicotiana tabacum* L. cv. Samsun NN) were used in this study. The seeds of transformed plants were kindly gifted by Prof. T. Schmülling (Freie Universität Berlin, Germany). Construction of transgenic plants overexpressing the *AtCKX2* gene from *Arabidopsis thaliana* positioned under the control of a constitutive 35S promoter as well as growth conditions for their selection were described elsewhere (Werner *et al.* 2001). Following *in vitro* pre-cultivation (MS medium, day/night temperature 25/20 °C, 16-h photoperiod, irradiance of 130 μmol m^{-2} s^{-1}) the 42-d-old plants (both WT and AtCKX2) were replanted into *Perlite* saturated with Knop solution and cultivated in a growth chamber (*SANYO MLR 350H,* Osaka, Japan) at a 16-h photoperiod (irradiance of 130 μmol m^{-2} s^{-1}), day/night temperature 22/18 ºC and relative humidity *ca.* 80 %. During cultivation, the plants were watered twice (days 4 and 7 after replanting) with Knop solution.

After 10 d, the control plants were watered regularly, while the others were subjected to a water stress (not watered), or salinity (100 mM NaCl) or increased zinc concentration (5 mM $ZnSO_4$). The stress conditions were chosen on the basis of our previous experience obtained with tobacco as well as other plant species (Gaudinová *et al.* 2004, Mýtinová *et al.* 2005). Following 4 d of stress, all leaves and roots of plants were collected and frozen in liquid nitrogen and stored at -70 ºC until analyses.

Soluble protein extracts were prepared by leaf or root homogenisation in 0.1 M Tris-HCl extraction buffer containing 1 mM dithiotreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 % (m/v) *Triton X-100* and 5 mM ascorbic acid (pH 7.8) in ratio 5 cm^3 g^{-1}(f.m.). The extracts immersed into ice bath were incubated for

1 min in ultrasound (*Tesla,* Czechoslovakia), left for 30 min in dark and ice, and centrifuged (20 000 *g*, 10 min, 4 °C; *Heraeus Biofuge 28RS*, Osterode, Germany). One quarter of each filtered supernatant was passed through *Sephadex G-25* and desalted for SOD measurement. Samples were frozen in liquid nitrogen and stored at -70 °C.

Activities of antioxidant enzymes (APX, GR and SOD) were measured spectrophotometrically (*Hitachi U 3300*, Tokyo, Japan) at 25 °C. Activity of APX was determined in 0.1 M HEPES-NaOH (pH 7.0) containing 1 mM EDTA, 0.5 mM ascorbate and 0.88 mM H_2O_2 as a decrease of reduced ascorbate at 290 nm (Nakano and Asada 1981). The GR activity was assayed according to Goldberg and Spooner (1983) as a decrease of NADPH concentration at 340 nm in the reaction mixture consisting of 0.1 M Tris-HCl (pH 7.8), 1 mM EDTA, 0.13 mM oxidised glutathione (GSSG) and 0.1 mM NADPH. The SOD activity was measured at 470 nm. Production of superoxide was provided by conversion of xanthine catalysed by xanthine oxidase (Ukeda *et al.* 1997) in the reagent solution containing 50 mM Na_2CO_3 (pH 10.2), 0.1 mM EDTA, 0.1 mM xanthine, 0.025 mM sodium 3,3´-{-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) and 9 mU cm^{-3} xanthine oxidase. One unit of the SOD activity was defined as amount of the enzyme required for 50 % inhibition of reaction rate of XTT as a detection molecule reduced by superoxide. Activity of CAT was measured polarographically using the oxygen electrode (*Hansatech Instruments*, King´s Lynn, UK). Reaction cell contained 0.1 M sodium phosphate buffer (pH 7.0) and 10 mM H_2O_2 (Thomas *et al.* 1998).

For determination of chlorophyll (Chl) and carotenoid contents in leaves, 3 - 6 leaf discs (0.5 cm^2) were extracted with acetone and analyzed using HPLC (*ECOM,* Prague, Czech Republic) using a reversed-phase column *Watrex Nucleosil* 120-5-C18, 5 µm particle size, 125 × 4 mm (*ECOM*, Prague, Czech Republic). The solvent system was acetonitrile:methanol:water (80:12:6, v/v/v) followed by 100 % methanol, the gradient run was 25 min, flow rate 1 cm^3 min^{-1}, the detection wavelength 445 nm (Mýtinová *et al.* 2006). Data were captured and calculated by PC-software *Clarity* (*DataApex*, Prague, Czech Republic). The deepoxidation state (DEPS) was calculated as the ratio $(Z + 0.5A)/(V + A + Z)$, where Z, V and A represent zeaxanthin, violaxanthin and antheraxanthin contents, respectively (all expressed in µg cm^{-2}).

Phytohormones were extracted in methanol: water:formic acid (15:4:1, v/v/v) mixture and purified using dual-mode solid phase extraction method according to Dobrev and Kamínek (2002). Detection and quantification were carried out using HPLC/MS (*Finnigan*, San Jose, CA, USA) operated in the positive ion full-scan MS/MS mode using a multilevel calibration graph with [^{2}H]-labelled cytokinins as internal standards. Detection limits of different cytokinins were between 0.5 and 1.0 pmol. IAA and ABA were determined by two-dimensional HPLC as described by Dobrev *et al.* (2005).

The CKX was extracted and partially purified according to Motyka *et al.* (2003) and the activity determined by *in vitro* assays based on conversion of [2-^{3}H]-iP (7.4 TBq mol^{-1}, prepared by Dr. Jan Hanuš, Isotope Laboratory, Institute of Experimental Botany AS CR, Prague, Czech Republic) to [^{3}H]-adenine in the reaction mixture (0.05 cm^3 final volume) including 100 mM TAPS-NaOH buffer (pH 8.5) and 75 µM 2,6-dichloroindophenol. Separation of the substrate from the product was achieved by HPLC as described elsewhere (Gaudinová *et al.* 2005). Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

All analyses of pigment contents and AOE activities were done in two different sets and their average values are presented. The values from each set are the mean from four measurements. Analysis of variance was performed using the *NCSS 6.0 Jr.* programme (*NCSS*, Kayswille, USA), statistical significance of differences was evaluated by post-hoc Scheffé's test at $P < 0.05$. For determinations of phytohormone contents and CKX activity, each experiment was repeated twice. As both experiments differed in their absolute values but showed similar tendencies, the results of one experiment representing the means of two independent biological samples and two HPLC injections (phytohormones analyses) or duplicated assays (CKX analyses) for each sample are shown.

Results

Effects of selected abiotic stresses (water deficit, salinity or increased zinc concentration) on activities of AOE in WT and AtCKX2 tobacco plants were studied. As a result of overexpression of *AtCKX2* gene, the transgenic plants exhibited highly enhanced (up to 3500-times) activity of CKX (Fig. 1) and, consequently, reduced concentrations of endogenous CKs in leaves and roots (data not shown).

The WT plants responded more sensitively to drought compared to other stresses, they were lower in comparison with non-stressed plants and displayed pronounced signs of wilting with yellowish bottom leaves. The effect of drought on AtCKX2 tobacco was less marked and visible. On the other hand, the most harmful stress for AtCKX2 was treatment with zinc. These plants were as high as their non-stressed controls, but their bottom leaves were yellow. Resembling but less pronounced effect was found in WT. The AtCKX2 and WT plants showed similar responses to salt stress with their bottom leaves only a little lighter compared to their unstressed controls (data not shown).

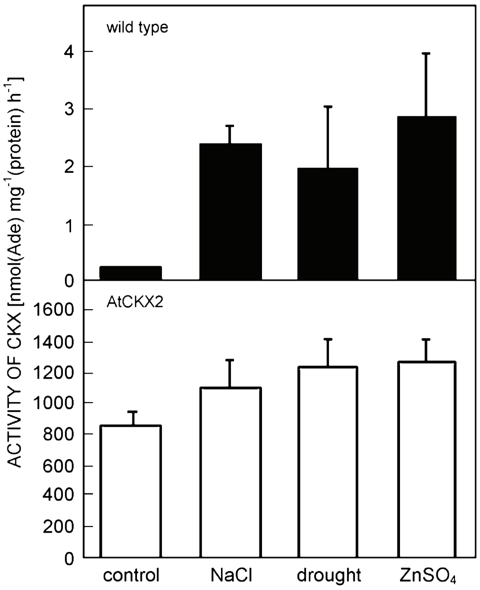

Fig. 1. The activity of cytokinin oxidase/dehydrogenase (CKX) in leaves of wild type and AtCKX2 transgenic plants grown in control (non-stressed) conditions, and under salt and drought stress and high $ZnSO_4$ concentration. *Vertical bars* represent the SE for two independent biological samples each assayed in duplicate.

Changes in total Chl content were used as a marker of stress impact on tobacco leaves. Content of Chl in AtCKX2 leaves was higher compared to WT even in non-stressed conditions (Fig. 2*A*). Upon all stresses, the Chl content decreased in both WT and AtCKX2 plants. The lowest Chl content was observed in salt stressed WT leaves. The only case when Chl amount in AtCKX2 leaves was lower than in WT was observed after zinc treatment. The Chl content was equal in both tobacco types following drought stress.

The content of β-carotene was also higher in AtCKX2 leaves in non-stressed conditions and stress responses in both tobacco groups differed (Fig. 2*B*). In WT, the β-carotene content increased in response to stresses except for salt stress when it declined. On the other hand, β-carotene content declined in transgenic plants with the highest difference between WT and AtCKX2 observed after drought stress. The de-epoxidation state (DEPS) of xantophyll cycle pigments was also higher in AtCKX2 in control conditions compared to WT (Fig. 2*C*). In response to stresses, DEPS increased in all cases, but the extent differed in both plant types. Following salt stress, the DEPS was higher in WT while after zinc treatment it was higher in AtCKX2.

The activities of APX, GR, SOD and CAT were estimated separately in leaves and roots of both WT and AtCKX2 tobacco during control and stress conditions. Under all conditions, the activities of APX were significantly higher and activities of CAT markedly lower in both WT and AtCKX2 roots (Figs. 3*B*, 6*B*) compared to their respective leaves (Figs. 3*A*, 6*A*).

In control (non-stressed) plants, the activities of APX and SOD in roots (Figs. 3*B*, 5*B*) and GR in leaves (Fig. 4*A*) of transgenic plants were significantly higher compared to WT. On the other hand, the activity of CAT in non-stressed leaves (Fig. 6*A*) was lower in AtCKX2 than in WT.

During the salt stress, the activities of only SOD increased in WT leaves (Fig. 5*A*) and the APX activity increased in WT roots (Fig. 3*B*). On the other hand, the activity of CAT decreased in leaves of WT (Fig. 6*A*). Concerning transgenic AtCKX2 tobacco, the activities of SOD decreased and CAT increased in leaves of salt stressed plants. In roots of these transgenic plants also the activity of APX (Fig. 3*B*) and SOD (Fig. 5*B*) were reduced in response to salinity.

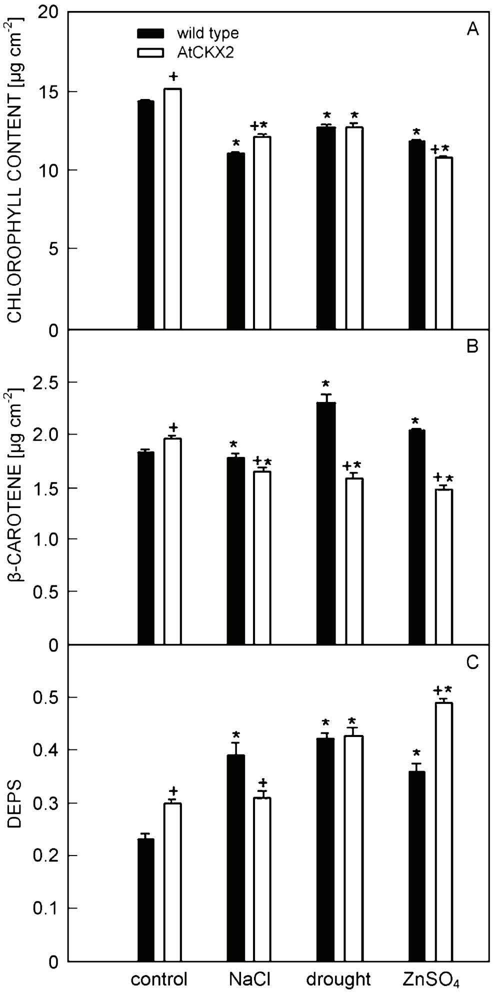

Fig. 2. Content of chlorophyll *a*+*b* (Chl) (*A*), β-carotene (*B*), and deepoxidation state of xanthophyll cycle pigments (DEPS) calculated as (Z + 0.5A)/(V + A + Z) (*C*) in leaves of wild type and AtCKX2 transgenic plants grown in control (non-stressed) conditions, and under salt and drought stress and high $ZnSO_4$ concentration. Z, V and A in the formula above represent zeaxanthin, violaxanthin and antheraxanthin contents, respectively. *Asterisks* indicate significant differences of particular stress from control conditions. *Crosses* indicate significant differences of transgenic plants from wild type. Significance of difference was tested at $P < 0.05$.

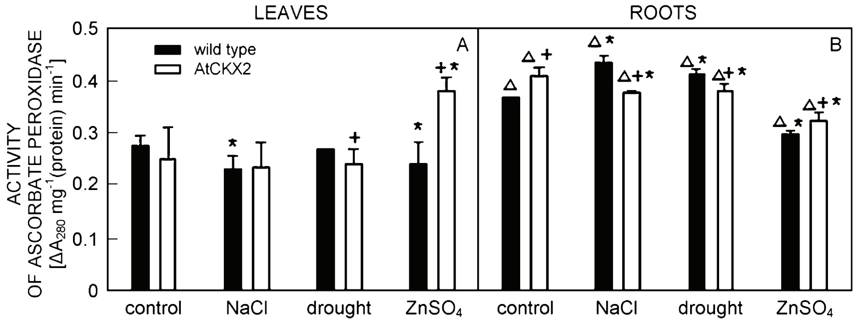

Fig. 3. The activity of ascorbate peroxidase (APX) in wild type and AtCKX2 transgenic plant leaves (*A*) and roots (*B*) grown in control conditions, and under salt and drought stress and high $ZnSO_4$ concentration. *Asterisks* indicate significant differences of particular stress from control conditions. *Crosses* indicate significant differences of transgenic plants from wild type. Triangles indicate significant differences of roots from leaves within the same plant and condition. Significance of difference was tested at $P < 0.05$.

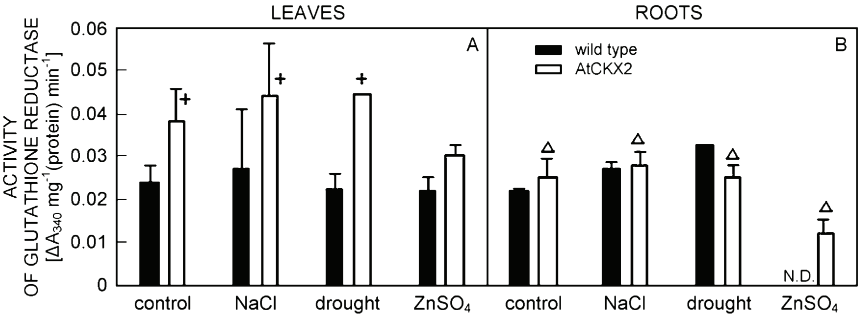

Fig. 4. The activity of glutathione reductase (GR) in wild type and AtCKX2 transgenic plant leaves (*A*) and roots (*B*) grown in control conditions, and under salt and drought stress and high $ZnSO_4$ concentration. N.D. - not detected. For other details see Fig. 3.

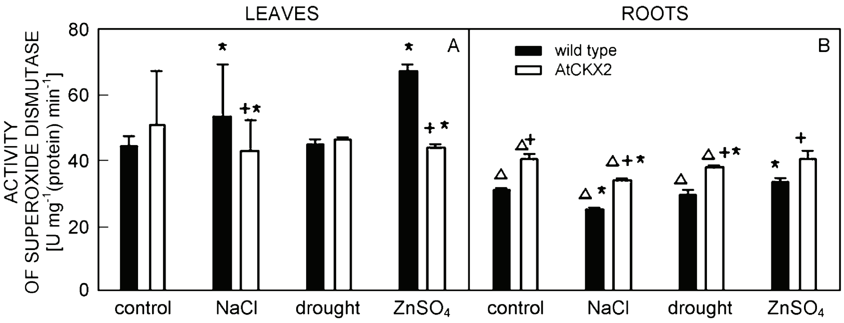

Fig. 5. The activity of superoxide dismutase (SOD) in wild type and AtCKX2 transgenic plant leaves (*A*) and roots (*B*) grown in control conditions, and under salt and drought stress and high $ZnSO_4$ concentration. For other details see Fig. 3.

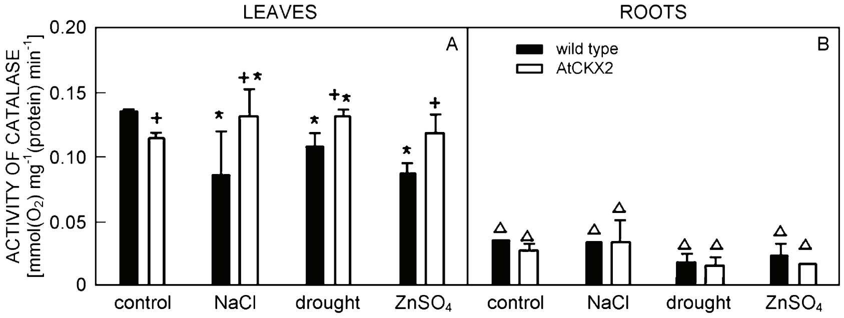

Fig. 6. The activity of catalase (CAT) in wild type and AtCKX2 transgenic plant leaves (*A*) and roots (*B*) grown in control conditions, and under salt and drought stress and high $ZnSO_4$ concentration. For other details see Fig. 3.

The activities of all studied AOE were unchanged during drought stress in leaves of WT except for CAT decline (Fig. 6*A*). In WT roots the activities of APX (Fig. 3*B*) and GR (Fig. 4*B*) increased due to the lack of water. In AtCKX2 drought stressed leaves, only the activity of CAT (Fig. 6*A*) differed from controls being elevated, while in AtCKX2 roots the activities of APX (Fig. 3*B*) and SOD (Fig. 5*B*) were depressed upon drought stress.

Zinc application to WT plants led to a decrease of APX and CAT activities in leaves (Figs. 3*A*, 6*A*). The SOD activity increased in both leaves (Fig. 5*A*) and roots (Fig. 5*B*) of WT in these conditions. In AtCKX2 plants zinc caused a high increase in the activity of APX (Fig. 3*A*) in leaves. On the other hand, the decrease was observed in activity of SOD in leaves (Fig. 5*A*) and in activities of APX (Fig. 3*B*) and GR (Fig. 4*B*) in roots following zinc treatment.

The activity of CKX was highly increased and concentrations of endogenous CKs reduced in AtCKX2 leaves and roots (see above). While the endogenous CK levels were not much affected by stress treatments in either WT or AtCKX2, the CKX activity was increased considerably under all stress conditions in both leaves and roots of WT and, in lesser extent, of transgenic tobacco (Fig. 1 and data not shown). Markedly reduced concentration of ABA compared to WT was found in AtCKX2 plants, both in leaves and roots (*ca*. 2- to 4.5-times). ABA content was elevated in leaves of both WT and AtCKX2 tobacco upon all stress treatments (Fig. 7) and, with the exception of zinc application, also in roots (data not shown). On the other hand, IAA content was only elevated in roots of WT plants subjected to drought stress or treated by zinc, in all other cases IAA levels were not much influenced by stress treatments (data not shown).

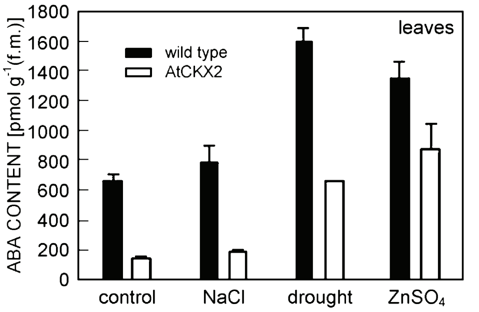

Fig. 7. Concentration of abscisic acid (ABA) in leaves of wild type and AtCKX2 transgenic plants grown in control conditions, and under salt and drought stress and high $ZnSO_4$ concentration. *Vertical bars* represent the SE for two independent biological samples each applied in two HPLC injections.

Discussion

Salinity, drought and treatment with Zn^{2+} have been considered to induce oxidative stress and thus reduce growth parameters of plants. Antioxidant enzymes prevent harmful accumulation of ROS generated during oxidative stress. Plants differ in their antioxidant defence that could be the basis of their different tolerance to stress (Sumithra *et al.* 2006). In this work, we tested the tolerance to abiotic stresses of transgenic tobacco overexpressing the *Arabidopsis* CKX (*AtCKX2*) gene. As a result of overexpression, the AtCKX2 transgenic plants exhibited highly enhanced CKX activity (Fig. 1) and hence a diminished contents of cytokinins. These cytokinin-deficient transgenic plants display retardation in their development and thus later onset of senescence (Werner *et al.* 2001, Mýtinová *et al.* 2006). Interestingly, also the contents of ABA as well as IAA were found significantly reduced in AtCKX2 leaves and roots compared to WT plants in our experiments (Fig. 7 for ABA and data not shown for IAA). For IAA, similar results were reported by Werner *et al.* (2003) in AtCKX1 and AtCKX2 tobacco seedlings. Their findings as well as our data, however, do not necessarily indicate a direct regulation of auxin or ABA metabolism by cytokinins; it is possible that, *e.g.,* the different tissue composition in transgenic plants might result in a lower proportion of auxin- or ABA-producing tissues. In this work, a special attention was given to the study of stress effects on the activities of AOE in AtCKX2 transgenics where altered antioxidant capacity was previously found during leaf ageing (Mýtinová, unpublished data).

The higher antioxidant capacity of a plant is assumed to be associated with an improved tolerance to evironmental stresses. In our previous study (Mýtinová *et al.* 2006) we reported better photoprotection, namely due to enhanced xanthophyll cycle activity and β-carotene content in leaves at different developmental stages of 12-week-old AtCKX2 tobacco. In this work, APX and SOD activities were found higher in roots of 8-week-old AtCKX2 plants compared to WT under control non-stressed conditions. On the other hand, only activity of GR was markedly higher in leaves of AtCKX2 transgenics under non-stressed conditions in comparison with WT while activity of CAT was even lower. It is apparent, that mainly in roots, but almost not at all in leaves, the antioxidant enzyme defence of non-stressed plants was better for AtCKX2 than WT.

The WT and AtCKX2 tobacco plants differed in their responses to particular stresses. In this work, we have not tested consequences of an oxidative injury on the level of cellular components. According to their appearance, the WT plants were quite sensitive to drought stress and in lesser extent also to other stresses. On the other hand, the appearance of AtCKX2 plants was changed only after zinc-induced stress, but just rather slightly by mild yellowing of the oldest leaves.

As concerns AOE, no unambiguous and uniform increase in activities of tested enzymes due to stress treatments was observed in either WT or AtCKX2 in this experiment. It is therefore not possible to discriminate from these results whether WT or AtCKX2 plants are more tolerant to unfavorable conditions.

The salt stress had marked impact on WT as apparent from pronounced decline of Chl content in comparison with non-stressed conditions. Also activities of APX and CAT decreased in leaves of WT tobacco. On the other hand, CAT activity was elevated in salt stressed AtCKX2 plants. Enhancement in the AOE activities after salt treatment has been reported previously in a number of other plant species such as pea (Hernandéz *et al.* 1999), beet (Bor *et al.* 2003), cucumber (Zhu *et al.* 2004) and tomato (Ünyayar *et al.* 2005) and was associated with salinity resistance.

It is evident from literature data that different plant species differ considerably in their AOE activities in response to water deficit (Moran *et al.* 1994, Zhang and Kirkham 1996, Ünyayar *et al.* 2005). In this work we observed that activities of AOE in WT leaves were almost not affected during water deficit in contrast to roots where APX and GR activities increased upon stress. However, the activity of CAT increased in leaves and the SOD and APX activities decreased in roots of AtCKX2. In spite of the fact that the WT plants as well as their leaves were highly reduced in size upon drought stress compared to AtCKX2, the content of Chl was equal in leaves of both tobacco types.

Zinc plays a dual role in a regulation of plant growth and development. At low concentrations it is indispensable for normal plant growth as a cofactor of more than 300 enzymes and proteins involved in cell division, nucleic acid metabolism, and protein synthesis (Hacisalihoglu *et al.* 2003). Zinc added to a soil improves plant performance as it causes increases of plant biomass, stomatal conductance and quantum yield of photosystem 2 (Wang *et al.* 2009). Zinc might also participate in oxidative stress-induced expression of genes encoding antioxidative defence enzymes such as APX and GR (Cakmak 2000). On the other hand, when present at higher concentrations zinc plays a negative toxic role in plants by inactivating the cellular antioxidant pool, disrupting the metabolic balance, eventually enhancing the amount of ROS (Triphati and Gaur 2004). However, Zn^{2+} effects does not depend only on zinc concentration but also on tolerance of particular plant species. In this work, based on our previous experiments (Mýtinová *et al.* 2005) 5 mM concentration of Zn^{2+} was used that caused oxidative stress with visible symptoms of yellowing of the bottom leaves of both WT and transgenic plants. Based on the decline in Chl content it is evident that AtCKX2 plants were heavily impaired compared to WT. The AOE activities were affected by zinc in different way in WT and AtCKX2. While a decrease in activity of APX and CAT appeared after zinc treatment in WT leaves, a decrease in GR and SOD activities in AtCKX2 leaves was found. In transgenic AtCKX2 roots the decline in APX and GR activities were observed. This finding is in contrast to increased activities of AOE reported in other plant species (Bonnet *et al.* 2000, Tripathi and Gaur 2004). In our work, the SOD activity in leaves and roots of WT plants increased. It might be connected with higher zinc supply, which is essential in Cu,Zn-SOD as the most abundant enzyme contributing to the total SOD activity in higher plants (Alscher *et al.* 1997).

In this aspect the opposite effect was observed in leaves of AtCKX2 tobacco. Here the same zinc concentration seemed to be more harmful. Zinc application was the most devastating for AtCKX2 plants among all stresses and simultaneously also SOD activity declined in AtCKX2 leaves.

The differences in responses between roots and leaves of a particular plant as well as between WT and AtCKX2 to zinc treatment could be attributed to different time course of stress response (Clijsters *et al.* 1999, Cuypers *et al.* 2001). Moreover, the root system of AtCKX2 transgenic plants was highly dispersed and spreading within a soil compared to WT (data not shown), which could hence promote higher intake of the metal by transgenics.

There are only few reports comparing differences in the antioxidant protection between leaves and roots. It is obvious that the roots are directly and primarily exposed to stresses originating from the soil as drought, salinity as well as metal application. Interestingly, Cuypers *et al.* (2002) found only limited impact of zinc on roots of *Phaseolus* compared to its primary leaves, which was substantiated by the fact that zinc was readily transported to the plant shoot. Dixit *et al.* (2001) reported different antioxidant responses to cadmium treatment in roots and leaves of pea and Liu and Huang (2000) distinct activities of AOE during heat stress in creeping bentgrass. In our experiments, the stress-induced responses of AOE activities often differed between leaves and roots, both in WT and transgenic plants. In general, the APX activity was higher and CAT activity lower in roots compared to leaves in both plant types in control and even in stress conditions. The SOD activity was lower in WT roots under control conditions and upon salt stress, however, in all other stresses the activities of SOD in roots and leaves were comparable. Interestingly, activity of GR was enhanced in roots of WT plants compared to leaves in response to salt and drought stress.

ABA is generally known to play a crucial role in plants compensating the consequences of adverse environmental conditions and its intracellular levels were repeatedly reported to be significantly enhanced by various stress factors (for review see, *e.g.*, Zeevaart and Creelman 1988). Correspondingly, elevated ABA levels were found here in leaves of both WT and AtCKX2 tobacco upon all stress treatments (Fig. 7). Similar, but less pronounced increases of ABA contents were also found in response to all applied stresses in WT and AtCKX2 roots. The IAA levels were generally not much affected by stress treatments in either WT and transgenic plants in our experiments. Cytokinins are commonly considered to exhibit antagonistic effects to ABA in plant stress responses. This antagonism might result from metabolic interaction between both phytohormones, particularly as cytokinins share, at least in part, a common biosynthetic origin with ABA (Cowan *et al.* 1999). In contrast to ABA, cytokinin levels generally tend to decrease under adverse environmental conditions (*e.g.* Hare *et al.* 1997). In our experiments, however, endogenous CK concentrations were not much influenced by stress treatments in either WT or AtCKX2. On the other hand, the CKX activity was markedly increased under all stress conditions in both leaves and roots of WT and transgenic tobacco (Fig. 1 and data not shown). These findings suggest that degradation of cytokinins catalysed by the enhanced CKX activity upon stress treatments of both WT and AtCKX2 plants might be counteracted by other regulatory mechanism(s) preventing the decline of cytokinin levels and contributing to establishment or maintenance of cytokinin homeostasis. An important role of CKX in the abiotic stress response has been recently supported by a strong increase of *Ckx1* gene expression in heat stressed maize kernels (Brugière *et al.* 2003). As these authors reported also a stimulatory effect of ABA on the *Ckx1* gene expression it is possible that the enhancement of CKX activity during abiotic stresses might be mediated *via* ABA.

To summarize, the responses of AOE activities to individual stresses differed in leaves and roots of both WT and AtCKX2 tobacco plants. Interestingly, responses of CAT activity in leaves were opposite: it always decreased in WT plants but increased (except for zinc treatment) in AtCKX2 tobacco. On the other hand, the SOD activity increased in WT but decreased after all stresses except for drought in AtCKX2 plants. Similarly, the APX activity prevailingly increased in roots of WT tobacco while mostly decreased in roots of AtCKX2 plants.

The higher tolerance of AtCKX2 plants could be attributed to noticeably higher activity of GR in leaves and APX, SOD and CAT in roots displayed already under control conditions. The higher activity of GR in leaves of AtCKX2 observed under non-stressed conditions increased even more in response to two of three stresses. It is known that GR provides satisfactory level of reduced glutathione, leading to favourable redox state of this compound. The importance of GR was already reported in resistance, *e.g.*, to drought stress (Contour-Ansel *et al.* 2006). Although transgenic tobacco plants possess, at least partially, better antioxidant protection in non-stressed conditions, they were not confirmed to be more tolerant to abiotic stresses. Their sensitivity and response to stresses was probably modified compared to WT, which need not to be directly associated with transformation. Analogously with this supposition, Vaillant *et al.* (2005) reported different sensitivity in four different members of *Datura* species to zinc stress. The difference between transgenic and WT plants in their stress responses could be associated with divergent sensitivity to imposed stress in consequence of protracted development of AtCKX2. The WT and AtCKX2 tobacco may thus differ in their general stress response. On the other hand, effects of all applied abiotic stresses on phytohormone levels and CKX activity were more or less uniform and the observed tendencies did not considerably differ between WT and AtCKX2 leaves and roots.

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