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Antioxidant protection during ageing and senescence in transgenic tobacco with enhanced activity of cytokinin oxidase/dehydrogenase

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Abstract

We studied changes in physiological parameters of whole leaves and in antioxidant protection of chloroplasts during ageing and senescence of tobacco (*Nicotiana tabacum* L. cv. Samsun NN) leaves with enhanced cytokinin oxidase/dehydrogenase activity (CKX) or without it (WT). Old leaves of CKX plants maintained higher pigment content and photosystem 2 activity compared to WT leaves of the same age. Chloroplasts of old CKX plants showed better antioxidant capacity represented by higher superoxide dismutase, dehydroascorbate reductase and glutathione reductase activities.

Additional key words: oxidative stress; reactive oxygen species, transgenic plants.

Introduction

Leaf senescence has been defined as endogenously controlled disintegrative processes, culminating in the death of a leaf, during which plants recover as many nutrients as possible from the senescing tissues for further use in other plant organs (Noodén 1988). This is characterized by intensive losses of chlorophyll, cessation of photosynthesis, disintegration of organelle structures and increases in oxidation of cellular components such as proteins and nucleic acids, and by peroxidation of lipids and increase in membrane leakiness (Buchanan-Wollaston 1997). These changes are mainly due to a strong enhancement in the generation of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical ($O_2^{\bullet -}$) and its more toxic derivatives, hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$). Relatively low contents of ROS can operate as intermediate signalling molecules to regulate the expression of genes associated with antioxidant defence mechanisms (Vranová *et al*. 2002, Neill *et al*. 2002, Navabpour *et al*. 2003). The production of ROS increases with plant age or in stress conditions and can contribute to the initiation of senescence.

Plants defend against oxidative stress by removing ROS (Scandalios 1997). This response includes the production of both non-enzymatic antioxidants such as ascorbate, glutathione, tocopherols, β-carotene, pigments of xanthophyll cycle (violaxanthin, antheraxanthin and zeaxanthin) and flavonoids (Del Río *et al*. 1998) and enzymatic antioxidants such as superoxide dismutase (SOD, EC 1.15.1.1) (Bowler *et al*. 1994, Halliwell and Gutteridge 1998), catalase (EC 1.11.1.6) (Kunce *et al*. 1988) and enzymes of ascorbate-glutathione cycle: ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.8.1.7) (Foyer and Halliwell 1976). Antioxidant protection decreases usually during senescence when determined in whole leaf extracts (Procházková and Wilhelmová 2007a). On the other hand, prolongation of life span is often combined with prolongation of period with higher antioxidant enzyme activities. For example, Dertinger *et al*. (2003) reported higher SOD, APX and GR activities in old leaves of tobacco with enhanced cytokinin production

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbate; BSA - bovine serum albumin; Car - carotenoids; Chl - chlorophyll; CKX - transgenic tobacco with enhanced cytokinin oxidase/dehydrogenase activity; CKs - cytokinins; DHAR - dehydroascorbate reductase; DTNB - 5,5-dithiobis-2-nitrobenzoic acid; DTT - dithiothreitol; F_M - maximum fluorescence; F_V - variable fluorescence; GR - glutathione reductase; MDHAR - monodehydroascorbate reductase; PS - photosystem; ROS - reactive oxygen species; SOD - superoxide dismutase; WT - wild type plants; XTT - 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt.

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compared to old leaves of wild type tobacco. Bean cotyledons with prolonged life span by excision of the whole shoots above them showed prolonged period of high activity of antioxidant enzymes as well (Procházková and Wilhelmová 2007b).

Changes in antioxidant protection on sub-cellular level seem to be more complicated. For example, Mn-SOD activity of leaf peroxisomes increased significantly in senescent leaves and two new CuZn-SODs were induced (Pastori and Del Río 1994). On the other hand, dark-induced leaf senescence was characterized by a decrease of SOD, APX, MDHAR, DHAR and GR mitochondrial activities in pea leaves as well as by a reduction in the ascorbate and glutathione pools of these organelles (Jimenéz *et al*. 1998).

Cytokinins (CKs) play roles in many aspects of plant growth and development, including leaf senescence. They also appear to mediate a number of light-regulated processes, such as de-etiolation and chloroplast differentiation (Mok 1994). The decrease of CKs with increasing leaf age is often regarded as one of the main causes for the beginning of senescence and an external application of CKs often delays senescence (Pell and Dann 1991).

The decline in CK contents appears to be mainly due to the increased activity of cytokinin oxidase/dehydrogenase (EC 1.5.99.12), which catalyzes the irreversible degradation of cytokinins and in many plant species is responsible for the majority of metabolic cytokinin inactivation (Mok and Mok 2001). Werner *et al*. (2001) reported the cloning of four putative CKX genes from *Arabidopsis thaliana*. In contrast to the expectation, leaf senescence of these transgenic plants did not occur earlier and their leaves stayed green longer compared to the wild type tobacco.

It was found out that photoprotection was higher in whole old leaves of transgenic tobacco plants with increased cytokinin oxidase/dehydrogenase activity compared to their wild type (Mýtinová *et al*. 2006). Our knowledge of antioxidant protection on sub-cellular level during ageing and senescence is still limited. Chloroplasts, where ROS production is relatively high, are especially at risk of oxidative stress. It was of special interest to determine whether antioxidant capacity will be higher also on the chloroplast level.

Materials and methods

We used leaves and chloroplasts of tobacco (*Nicotiana tabacum* L., cv. Samsun NN) with inserted gene *AtCKX2* from *Arabidopsis thaliana* positioned under the control of a constitutive 35S promoter (CKX plants). As a control we used its wild type (WT plants). Plants were grown after *in vitro* pre-cultivation in pots with soil in a greenhouse under the following conditions: 16-h photoperiod, irradiance of *ca*. 200 μmol m^{-2} s^{-1}, day/night temperature of 25/18 ºC and relative humidity about 60 %. Plants were irrigated by tap water and twice a week by Hewitt nutrient solution. For experiments we used the first five leaves at the plant age of 12 weeks when the oldest leaves of WT plants turned to yellow. The leaves were numbered from the 1st one (the first leaf from the bottom) to the 5th one, *i.e*. the oldest leaves were indicated as 1st and the youngest ones as 5th.

Pigment content was established spectrophotometrically (*Hitachi UV 3300*, Tokyo, Japan) from dimethylformamide extracts (Porra *et al*. 1989).

Chlorophyll fluorescence parameters were measured after a 15 min dark period with the *PAM* chlorophyll fluorometer (*Walz*, Effeltrich, Germany) on adaxial side of fresh leaves at room temperature. Measuring irradiance was 0.35 μmol m^{-2} s^{-1}, actinic irradiance 200 μmol m^{-2} s^{-1}, 700 ms saturated flash of 2 500 μmol m^{-2} s^{-1} (for detail see Wilhelmová *et al*. 2005). The *DA 100* data acquisition system (*Walz*, Effeltrich, Germany) was used for sampling and calculation. The quantum efficiency of open photosystem 2 (PS 2) centres (F_V/F_M) was calculated using formulas of Van Kooten and Snel (1990). Effective quantum yield of PS 2 was determined according to Genty *et al*. (1989).

Organelles were separated from each leaf type by the modified method of Šindelářová *et al*. (2000). Fresh leaves without the main veins were cut into pieces and homogenized in an ice cold isolation medium containing 0.4 M sucrose, 165 mM Tricine, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA Na_2, 1 mM dithiothreitol (DTT), 25 mM ascorbate (AsA), 0.5 % bovine serum albumin (BSA), pH 7.5. The homogenates were filtered through four layers of cheesecloth and centrifuged at 100 *g* for 1 min at 4 ºC (*Jouan GR 2220*, Saint-Herblain, France). The super-natants were centrifuged at 500 *g* for 2 min at 4 ºC. The pellets were resuspended in medium, containing 0.4 M sucrose, 10 mM Tricine, 1 mM EDTA Na_2, 1 mM DTT, 25 mM AsA, pH 7.2 and re-centrifuged at 2 000 *g* for 2 min at 4 ºC. The pellets were resuspended again in resuspending medium and centrifuged in 40 % *Percoll* at 2 000 g for 3 min at 4 ºC. The pellets were resuspended in a very small volume of resuspending medium and the intactness of chloroplasts was monitored by the ferricyanide method (Walker 1987) using a liquid-phase oxygen electrode (*Hansatech*, King´s Lynn, UK). The chloroplast intactness exceeded 75 % in the case of senescent WT leaves and 80 % in the case of the others. The chloroplast fraction was without detectable activity of catalase and fumarase as specific markers for the presence of peroxisomes and mitochondria, respectively. After the detection of chloroplast intactness their fraction was homogenized with a mortar and pestle with 50 mM K phosphate buffer (pH 7.8) containing 1mM EDTA Na_2, 10 mM $MgCl_2$, 0.1 % *Triton X-100*, 1 mM DTT in the case of MDHAR and DHAR assays and with 0.1 M Tris buffer (pH 7.8) containing 1 mM DTT, 1 mM EDTA Na_2

0.5 % *Triton X-100*, 5 mM AsA in the case of SOD, APOD and GR. The chloroplast homogenate was then processed for 2 min by ultrasound (*Tesla UC006 DM1*, Prague, Czech Republic) in an ice bath and finally incubated in ice for 30 min in the dark. After centrifugation at 20 000 *g* for 30 min at 4º C (*Sorvall*, *Discovery 90SE*, Asheville, USA) fresh supernatant was used immediately in the case of APX assay. For SOD assay the extract was desalted on a column of *Sephadex G-25*, using the extraction buffer for the preparation of the column, then frozen in liquid nitrogen and stored at -70º C. For MDHAR, DHAR and GR assays the extracts were immediately frozen in liquid nitrogen and stored at -70º C.

Activities of all enzymes were assayed spectrophotometrically (*Hitachi UV 3300*, Tokyo, Japan). SOD activity was measured at 470 nm using the method by Ukeda *et al*. (1997) in 50 mM Na_2CO_3, pH 10.2, containing 0.1 mM EDTA Na_2, 0.1 mM xanthin, 0.75 mM 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetra-zolium-5-carboxanilide sodium salt (XTT), 11.2 mU xanthin oxidase (from bovine milk, *Sigma*) and 10 mm^3 enzyme extract (total volume 3 cm^3). One unit of enzyme activity represents 50 % inhibition of activity without extract. APX activity was assayed as the decrease in absorbance at 290 nm due to AsA oxidation by the method of Nakano and Asada (1981) in 0.1 M Hepes, pH 7.0, 0.5 mM Na-ascorbate, 0.88 mM H_2O_2 and 0.1 cm^3 enzyme extract (total volume 3 cm^3). DHAR activity was assayed as the increase in absorbance at 265 nm due to AsA formation according to Hossain and Asada (1984) in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM dehydroascorbate, 5 mM reduced glutathione and 0.1 cm^3 enzyme extract (total volume 3 cm^3). MDHAR activity was assayed as the decrease in absorbance at 340 nm due to NADH oxidation by the method of Hossain *et al*. (1984) in 50 mM Tris buffer, pH 7.5, containing 0.2 mM NADH reduced, 2.5 mM ascorbate, 0.15 U ascorbate oxidase (from *Cucurbita* species, *Sigma*) and 0.1 cm^3 enzyme extract (total volume 3 cm^3). GR activity was assayed as the increase in absorbance at 412 nm due to the formation of a coloured complex by reduced glutathione with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according to the method of Smith *et al.* (1988) in 50 mM KH_2PO_4 buffer, pH 7.8, containing 0,5 mM DTNB, 0.2 mM NADPH, 0.2 mM oxidized glutathione and 0.1 cm^3 enzyme extract (total volume 3 cm^3). Protein content was determined by the method of Bradford (1976) with standard curves prepared using BSA.

The results presented are the means of three independent experiments. Each sample was assayed in triplicate. Sample variability is given as the standard error of the mean. Statistical significance of difference was evaluated with analysis of variance (*ANOVA*) using *NCC 6.0 jr.* programme (*NCSS*, Kaysville, USA).

Results

Wild type plants were 40 - 50 cm high with ten fully developed leaves at the time of sampling. Their first leaves from the bottom were yellow. CKX plants were dwarfed (15 - 20 cm) with bushy appearance. They had eight fully developed leaves. All their leaves were green.

The total chlorophyll content significantly decreased in the oldest leaves in both WT plants and CKX plants with significant difference between the oldest WT and CKX leaves (Fig. 1*A*). The content in the oldest WT leaves represented only 8 % of the content in the young WT leaves but it was still 47 % in the case of CKX plants. Chl *a*+*b*/Car ratio decreased with age in WT plants (41 % of the young leaves) but remained almost unchanged in CKX plants (Fig. 1*B*). The optimum value of F_V/F_M is in the range of 0.85 - 0.75. In the case of WT plants this ratio dropped below the optimum value in the 3rd leaves (Fig. 1*C*). This ratio henceforth decreased and in the oldest WT leaves reached 43 % of the young ones. On the other hand, this ratio remained on high level in CKX plants and decreased only slightly below the optimum value in the oldest leaves (0.743). The effective quantum yield of PS 2 decreased with age in the case of WT plants (Fig. 1*D*). In the oldest leaves, the effective quantum yield reached 44 % of the young leaves in the case of WT plants. This level was 114 % in the case of old CKX leaves.

Car content significantly decreased in old leaves both in WT plants and in CKX plants (22 and 55 % of the young leaves, respectively) (Fig. 2*A*). SOD activity in chloroplasts of WT plants was the highest in 3rd leaves without any difference between 1st and 5th leaves (Fig. 2*B*). In 3rd to 5th leaves, the activity of chloroplast SOD was higher in WT plants, while in the oldest leaves this activity was higher in CKX plants. SOD activity in chloroplasts of CKX plants reached its maximum in 2nd leaves whereas the activity in 1st leaves reached 153 % of the activity in 5th leaves.

APX activity was the highest in chloroplasts of 2nd WT plant leaves (Fig. 2*C*), however in chloroplasts of the oldest leaves the activity was almost 170 % of the 5th leaves. APX activity uniformly increased in chloroplasts of CKX plants with age and this activity in the oldest leaves reached 390 % of the 5th leaves. The activity was always lower compared to WT but significantly only in 2nd and 5th leaves.

Chloroplast MDHAR activity increased from 5th leaves of WT plants to 3rd leaves and after a slight decrease in 2nd leaves the activity increased again in the oldest leaves (Fig. 2*D*). The activity decreased in chloroplasts of the oldest CKX leaves (81 % of the young). DHAR activity was the highest in the 3rd leaves in WT plants, without significant differences between remaining leaves (Fig. 2*E*). In CKX plants, the activity was the highest in 4th leaves. In the oldest leaves the decrease occurred

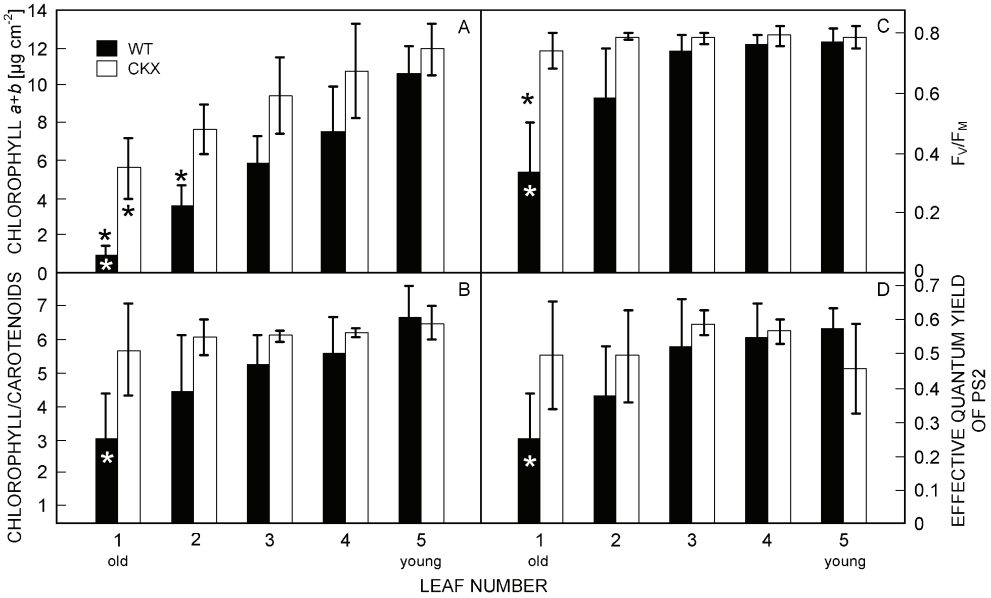

Fig. 1. Content of chlorophyll *a*+*b* (*A*), Chl/Car ratio (*B*), maximum efficiency of PS 2 (F_V/F_M) (*C*) and effective quantum yield of PS 2 (*D*) in leaves of different insertion in wild tobacco (WT - *black columns*) and transgenic plants (CKX - *white columns*). Means ± SE (*vertical bars*) are given. *Asterisks* above columns indicate significant difference between CKX and WT of the same age. *Asterisks* inside columns indicate significant difference between old and young leaves.

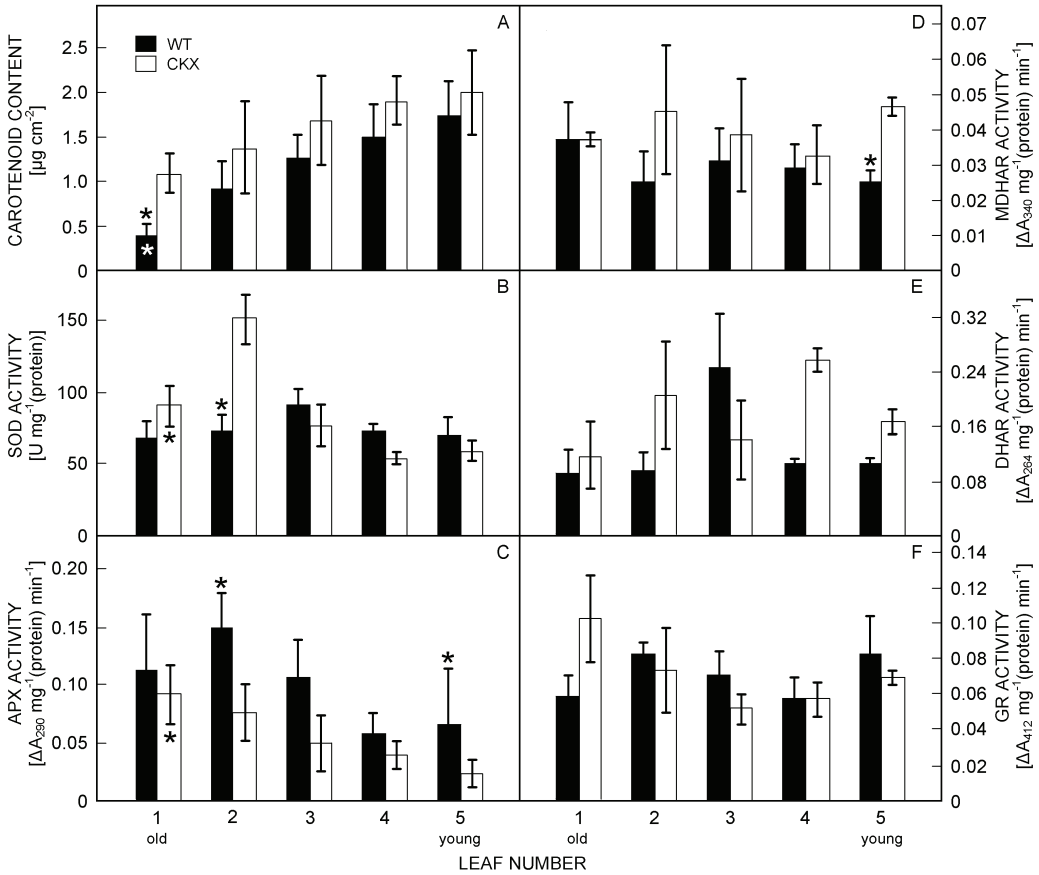

Fig. 2 Carotenoid content (*A*) and activities of SOD (*B*), APX (*C*), MDHAR (*D*), DHAR (*E*) and GR (*F*) in chloroplasts of leaves of different insertion in wild tobacco (WT - *black columns*) and transgenic plants (CKX - *white columns*). Means ± SE (*vertical bars*) are given. *Asterisks* above columns indicate significant difference between CKX and WT of the same age. *Asterisks* inside columns indicate significant difference between old and young leaves..

(70 % of the young).

Nonsignificant decrease in chloroplast GR activity occurred in chloroplasts of the oldest leaves of WT plants (71 % of the young leaves) (Fig. 2*F*). This activity increased in the oldest leaves of CKX plants being 147 % of the young ones.

Discussion

In the present study we investigated the changes in antioxidant capacity of chloroplasts in leaves of control tobacco plants (WT plants) and in leaves of transgenic plants with enhanced activity of cytokinin oxidase/ dehydrogenase (CKX plants) during ageing.

One of the markers commonly used for the specification of the ageing and senescence of plants is the loss of chlorophyll and the associated yellowing of the leaves (Dertinger *et al*. 2003). As an indicator of the photoinhibition or other kind of injury caused to the PS 2 complex as well as ageing fluorescence parameter F_V/F_M has been often used (Roháček 2002). The ratio typically ranging between 0.75 - 0.85 is proportional to the effectiveness of light energy utilization under standard conditions of CO_2 fixation and to the quantum yield of photochemical processes (Demming and Björkman 1987, Čaňová *et al*. 2008). From the total chlorophyll content as well as from the F_V/F_M ratio and effective quantum yield we can assume that the onset of senescence process was initiated in the oldest leaves of WT plants. Chlorophyll content in leaves of CKX plants decreased with leaf age but it was higher compared to WT and the decline was relatively lower. On the other hand, their F_V/F_M ratio decreased only slightly under optimum value even in their oldest leaves. Hence we concluded that senescence was retarded in CKX plants. It was reported before that leaves stayed green longer in CKX plants compared to WT plants due to their retarded development (Werner *et al*. 2003). Cytokinins regulate a number of growth and developmental processes in plants such as stimulating cell division and prolongation (Gan and Amasino 1995) therefore their decreased level led to a significant reduction of the leaf area and to a dwarfed appearance of CKX plants.

Although chloroplasts are the organelles where the first symptoms of senescence are visible, there was no significant decrease in chloroplast antioxidant protection of the oldest leaves of WT plants compared to the 5th leaves. The exception was GR activity, which decreased in the chloroplasts of the oldest WT leaves. It is in agreement with our results concerning chloroplasts of tobacco cv. Wisconsin, in which part of their antioxidant protection worked fully even when senescence was in progress (Procházková *et al*. 2008). We presume that the maintaining of relatively high antioxidant protection even when senescence was in progress bears witness to the hypothesis that decrease of antioxidants is a consequence, but not the cause, for the start of the senescence programme (Dertinger *et al*. 2003).

The decrease in F_V/F_M and effective quantum yield in the 2nd leaves was attended by the increase in activities of chloroplast SOD and GR in the 2nd leaves and MDHAR and DHAR in the 3rd leaves. Senescence may be reversed during the phase of early mobilization of nutrients by suitable changes in environmental conditions (Peñarrubia and Moreno 1995). Maybe the increase in antioxidant activity in the 2nd and 3rd leaves guarantees the maintaining ROS on harmless level for the sake of necessity to reverse senescence process.

The physiological status (F_V/F_M, effective quantum yield, chlorophyll content) of CKX plants was better compared to WT plants. In chloroplasts of old leaves of CKX plants, the activities of antioxidant enzymes were higher (except APX) compared to WT plants. Also antioxidant and photo-protection in whole leaves of these transgenic plants was higher in the oldest leaves compared to the WT leaves of the same age (Mýtinová *et al*. 2006, 2010). It is probable that CKX plants were under severe stress during their whole life span due to the cytokinin deficiency. Hence, we assume that higher antioxidant enzyme activities, including the higher APX and GR activities on chloroplast level, could be partially associated with the postponed senescence onset.

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