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Photosynthesis and protective mechanisms during ageing in transgenic tobacco leaves with over-expressed cytokinin oxidase/dehydrogenase and thus lowered cytokinin content

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Abstract

The content of cytokinins (CKs), the plant inhibitors of the final phase of plant development, senescence, is effectively controlled by irreversible degradation catalysed by cytokinin oxidase/dehydrogenase (CKX). In transgenic tobacco, denoted as AtCKX, with over-expressed CKX causing lowered CK content, we investigated changes in the time courses of chlorophyll (Chl) and xanthophyll (violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and lutein) contents. We also determined parameters of slow Chl fluorescence kinetics such as minimum Chl fluorescence yield in the dark-adapted state F_0, maximum quantum yield of PS2 photochemistry (F_v/F_m), maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in photosystem 2 (PS2), F_v/F_0, non-photochemical quenching (NPQ), and effective quantum yield of photochemical energy conversion in PS2 (Φ_2). We used three different developmental leaf stages, old, mature, and young, and compared this with time courses of these characteristics in leaves with natural CK levels. The parameters F_v/F_m, F_v/F_0, and Φ_2 were unchanged during ageing in AtCKX plants in contrast to control ones where a significant decrease in old leaves was found. In control plants F_0 increased during ageing, but in the oldest leaf a considerable decrease was observed. This could indicate progressive damage to PS2 reaction centres and then detachment and rapid degradation of Chl. This is in agreement with time course of Chl content. NPQ decreased with age and was similar in both plant types. We observed a decline of xanthophyll contents in the oldest leaves in both plant types, but the contents were enhanced in AtCKX compared to control plants, especially of neoxanthin. The higher xanthophyll contents in the transgenic plants contribute to a better photoprotection and the fluorescence parameters indicated that photosynthetic apparatus was in better condition compared to control and it consequently postponed the onset of leaf senescence.

Additional key words: chlorophyll; fluorescence; *Nicotiana tabacum*; photosynthetic efficiency; senescence; xanthophyll cycle.

Introduction

During ageing plants go through several development stages. The latest phase before death is senescence. Senescence occurs as an orderly loss of functions and structures, comprising an array of biochemical and physiological processes whose purpose is the efficient removal and retrieval of nutrients from the decomposing tissues of a plant. Reactive oxygen species (ROS), as a by-product of aerobic metabolism, contribute to the triggering of senescence (Krupinska *et al.* 2003). In chloroplasts, the Mehler reaction and the antenna pigments are the primary sources of ROS production in plants (Mittler *et al.* 2004). In addition, chloroplasts are the organelles in which the first symptoms of senescence are observed because of chlorophyll (Chl) degradation

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Abbreviations: A − antheraxanthin; ABA − abscisic acid; Chl − chlorophyll; CK − cytokinin; CKX − cytokinin oxidase/dehydrogenase; DEPS − de-epoxidation state; F_0 − minimum Chl fluorescence from dark-adapted leaves; F_m − maximum Chl fluorescence from dark-adapted leaves; F_m' − maximum Chl fluorescence from light-adapted leaves; F_s − steady state Chl fluorescence from light-adapted leaves; F_v − variable Chl fluorescence; F_v/F_m − maximum photochemical efficiency; F_v/F_0 − maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2; L − lutein; N − neoxanthin; NPQ − non-photochemical quenching; PAM − pulse amplitude modulation; PAR − photosynthetically active radiation; PS2 − photosystem 2; ROS − reactive oxygen species; V − violaxanthin; Z − zeaxanthin; Φ_2 − effective quantum yield of photochemical energy conversion in PS2.
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(Peñarrubia and Moreno 2002). In senescence, chloroplasts undergo changes as swelling, thylakoid distortion, decline of starch content, loss of ribosomes, and increase of plastoglobuli number (Hillman *et al.* 1994). Degradation of chloroplasts is evidently connected with disassembly of the photosynthetic apparatus resulting in consequent decrease in photosynthetic activity. Moreover, the decline in photosynthetic efficiency may be caused in particular by a degradation of the very important and abundant enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Chiba *et al.* 2003). Photoprotective processes minimize photooxidative damage that could potentially result from the absorption of excess radiation in chloroplasts. One such photoprotective process is the xanthophyll cycle. This cycle is based on a removal of absorbed radiation energy from the light-harvesting antennae Chl before it reaches the reaction centres thereby minimizing the over-reduction of the photosynthetic electron transport chain and protecting thus against the formation of ROS (Logan *et al.* 1999).

The xanthophyll cycle is localised in thylakoid membranes and involves de-epoxidation of violaxanthin (V) creating zeaxanthin (Z) with antheraxanthin (A) as intermediates. Along with these xanthophylls also lutein (L) and neoxanthin (N) are components of the light-harvesting complex (LHC). L has a direct role in non-photochemical quenching through thermal dissipation (Inoue 2004) and together with Z it is needed for photoprotection due to an ability of quenching ^{1}Chl and $^1O_2$. In this way they inhibit lipid peroxidation that is impaired in the absence of both Z and L (Niyogi 1999). On the other hand, N as well as V, A, and Z could be precursors for abscisic acid biosynthesis (Nambara *et al.* 2005), which accelerates leaf senescence. Among these xanthophylls, the content of L is the highest, usually between 30–60 % of total xanthophylls. N represents a smaller proportion of the total xanthophyll pool but its content is remarkably uniform across a wide range of contrasting species and growth environments (Bungard *et al.* 1999). Munné-Bosch and Peñuelas (2003) reported that the content of xanthophyll pigments (V, A, Z, L, N) decreased during senescence even if the de-epoxidation state (DEPS) increased.

In addition to excessive energy being disposed as heat it could also be re-emitted as radiation in the form of fluorescence. The studies of the xanthophyll cycle being the main part of non-photochemical quenching in the protection against photodestruction, are almost exclusively based on measurements of fluorescence kinetics (Eskling *et al.* 1997). The extent of non-photochemical as well as photochemical quenching of energy is changed during senescence. Whereas photochemical quenching is inhibited with leaf age, the non-photochemical one increases (Lu *et al*. 2001).

Senescence is regulated by phytohormones. Cytokinins (CKs) delay leaf senescence (Gan and Amasino 1995). Werner *et al*. (2001) reported the cloning of four putative CK oxidase/dehydrogenase (CKX) genes from *A. thaliana* and the results of their over-expression in tobacco. This enzyme mediates degradation of CKs with unsaturated isoprenoid side chain. The transgenic tobacco with enhanced activity of CKX and thus decreased CK content shows signs of delayed rather than accelerated senescence. This decrease in CK content could be a prerequisite for senescence, but not a signal that triggers its onset (Werner *et al*. 2003, Wilhelmová *et al.* 2004).

We used transgenic tobacco plants with enhanced activity of CKX to study the effect of decreased CK content on photosynthetic activity, pigment content, and photoprotective processes. From these results we deduced a postponed onset of senescence in the transgenic plants.

Materials and methods

Plants: We used tobacco (*Nicotiana tabacum* L. cv. Samsun NN) leaves of different age as an experimental plant. Transgenic plants had an inserted gene *AtCKX2* from *A. thaliana* positioned under the control of a constitutive 35S promoter. The tobacco seeds were a kind gift of Prof. Thomas Schmülling from Freie Universität Berlin, Germany. The transgenic plants were marked as AtCKX. Plants were grown after *in vitro* pre-cultivation in pots with soil in a glasshouse under 25/18 °C day/night and 60 % relative humidity. Day irradiance [overall integrated mid-values were *ca*. 500 μmol(quantum) m^{-2} s^{-1}] was prolonged by the additional irradiation [photon flux density *ca*. 200 μmol(quantum) m^{-2} s^{-1}] to 16 h. After 12 weeks the leaves were used for analysis. Transgenic plants developed 10 leaves while control plants 14 leaves. We exploited leaves from three positions on plant, at bottom, middle, and top position and call them old (numbered as 1 and 2 from bottom), mature (numbered 5–7 from bottom in case of control and 4–6 in case of AtCKX), and young (numbered 12–14 from bottom in case of control and 8–10 in case of AtCKX) leaves, respectively. For fluorescence parameters, leaves 1–3 were measured, and then every odd leaf.

Pigment contents: Leaves were extracted from 3–6 leaf discs (0.5 cm^2 per one disc) with acetone and analyzed by HPLC (*SpectraPhysics*, San Jose, USA) using a reversed-phase column *Sepharon SGX C18*, 5 μm particle size, 150×3 mm (*Tessek*, Praha, 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 min^{-1}, the detection wavelength 445 nm (Tichá *et al.* 1998). DEPS was calculated as the ratio (Z + 0.5A)/(V + A + Z).

Chl fluorescence parameters from slow kinetics were measured after 15 min-dark period using the *PAM* chlorophyll fluorimeter (*Walz*, Germany) on attached leaves. Measuring irradiance was 0.35 μmol m^{-2} s^{-1}, actinic irradiance 200 μmol m^{-2} s^{-1}, 700 ms saturated flashes of 2 500 μmol m^{-2} s^{-1} after 10, 250, and 265 s. The *DA 100* Data Acquisition System (*Walz*, Germany) was used for sampling, control, and calculation. The minimum Chl fluorescence yield in the dark-adapted state F_0, the Chl fluorescence ratios F_v/F_m and F_v/F_0, non-photochemical quenching $NPQ = (F_m - F_m')/F_m'$. Effective quantum yield of photochemical energy conversion in PS2 was calculated according to Roháček (2002).

Statistical evaluation: All analyses were done in three different sets and we present the representative one. The values are the means from three plants. The pigment contents were measured in two repetitions. Analysis of variance was performed using the *NCSS 6.0 Jr.* programme (*NCSS*, USA), statistical significance of differences was evaluated by post-hoc Scheffé's test at $p<0.05$.

Results

The transgenic plants had a different phenotype compared to the controls. They were lower and bushy with less smaller and thicker leaves. The control plants had about 14 leaves and the transgenic ones had about 10. We decided to compare leaves according to their age, not to their position, and the resulting three groups were classified as young, mature, and old. After 12 weeks the oldest leaves at the bottom of the control plants became yellow in contrast to transgenic plants, where they were still green. The Chl *a+b* content was higher in transgenic plants in all three observed stages (Fig. 1*A*). A reduced Chl *a+b* content was found in both control and transgenic plants. The ratio of Chl *a/b* was rather high in control plants (Fig. 1*B*). The total Chl content as well as the Chl *a/b* ratio was the highest in mature leaves of both plant types.

We observed that the total content of the xanthophyll cycle pigments was higher in transgenic plants in all leaf stages (Fig. 2*B*). In both used plants the content of V was the highest in mature leaves (Fig. 2*A*) in contrast to A (Fig. 2*C*) and Z (Fig. 2*E*) where the highest value was in the youngest leaves. DEPS was the lowest in mature leaves and the transgenic plants had a slightly lower level of de-epoxidation than the control ones (Fig. 2*D*). We observed a remarkable increase of N content in transgenic plants compared to controls (Fig. 3*B*). N as well as L was higher in mature leaves in both kinds of tobacco (Fig. 3*A*). L was also enhanced in transgenic plants. All xanthophyll pigments declined in the oldest leaves of both analysed plants.

We calculated parameters from slow Chl fluorescence kinetics that have been used to describe photochemical and non-photochemical processes in examined plants (Fig. 4*A–E*). The quantum efficiency of open photosystem 2 (PS2) centres (F_v/F_m) was almost constant in transgenic tobacco leaves of different age in contrast to controls where a considerable decline was observed in the mature leaves (Fig. 4*A*). The same course was found even in case of the effective quantum yield of photochemical energy conversion in PS2 (Φ_2) (Fig. 4*E*) and maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2, F_v/F_0 (Fig. 4*C*). The minimum fluorescence yield in the dark-adapted state (F_0) had a reverse course (Fig. 4*B*). F_0 was nearly unchanged in transgenic plants but was enhanced from the mature leaves of controls except the oldest one, where a significant decrease was observed. The non-photochemical quenching (NPQ) was similar in both transgenic and control plants and decreased with age (Fig. 4*D*).

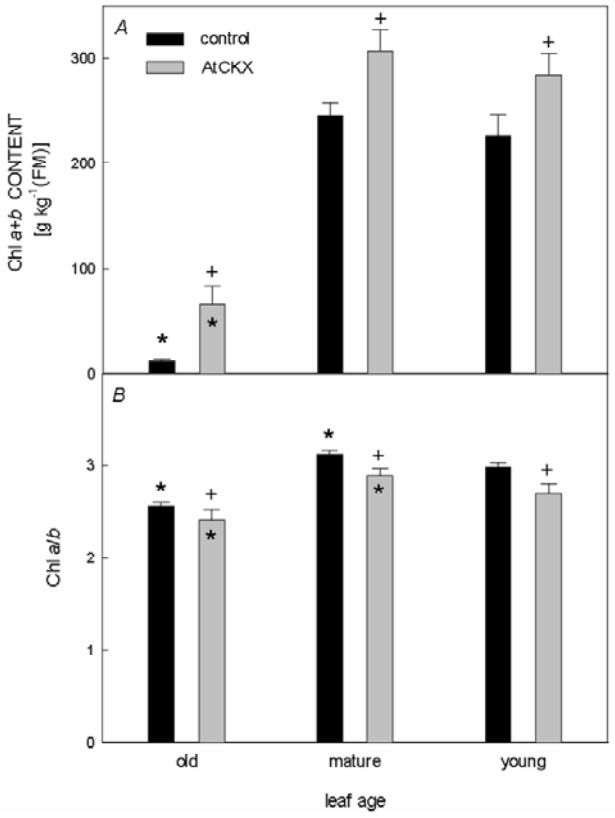

Fig. 1. Chlorophyll (Chl) contents (*A*) and Chl *a*/*b* ratio (*B*) in old, mature, and young leaves of control and transgenic tobacco AtCKX. Means ± SE (*vertical bars*). *Asterisk* indicates significant difference from the respective young leaf. *Cross* indicates significant difference of transgenic plant from control plant. Significance of difference is according to Student's *t*-test at $p<0.05$.

Discussion

In the present study, we investigated the changes in contents of Chl and xanthophyll pigments and in PS2 photochemistry in leaves of different age in control and transgenic tobacco with enhanced expression of CKX resulting in lowered CK content in all three observed stages (Jiří Malbeck, personal communication). The highest reduction of CK content was in the oldest leaves and the lowest in the youngest ones. A decline in Chl content has been widely used as a senescence marker (Jordi *et al.* 2000, Stessman *et al.* 2002, Jiao *et al.* 2003, Weng *et al.* 2005). The Chl content was higher in all leaf developmental stages (old, mature, and young) in transgenic plants. Despite the oldest leaves of the transgenic plants were still green and in the control plants they had been already yellow, we observed a decline of Chl content in both plants with leaf age. We suppose that the content of Chl in the senescence stage would still decrease in transgenic plants. We can deduce from a decline in Chl *a/b* ratio that during senescence the Chl *a* is preferentially degraded. The same results were also found in ageing bean cotyledons (Wilhelmová *et al.* 1997).

Fluorescence parameter F_v/F_m has been frequently used as an indicator of the photoinhibition or other kind of injury caused to the PS2 complex (Roháček 2002).

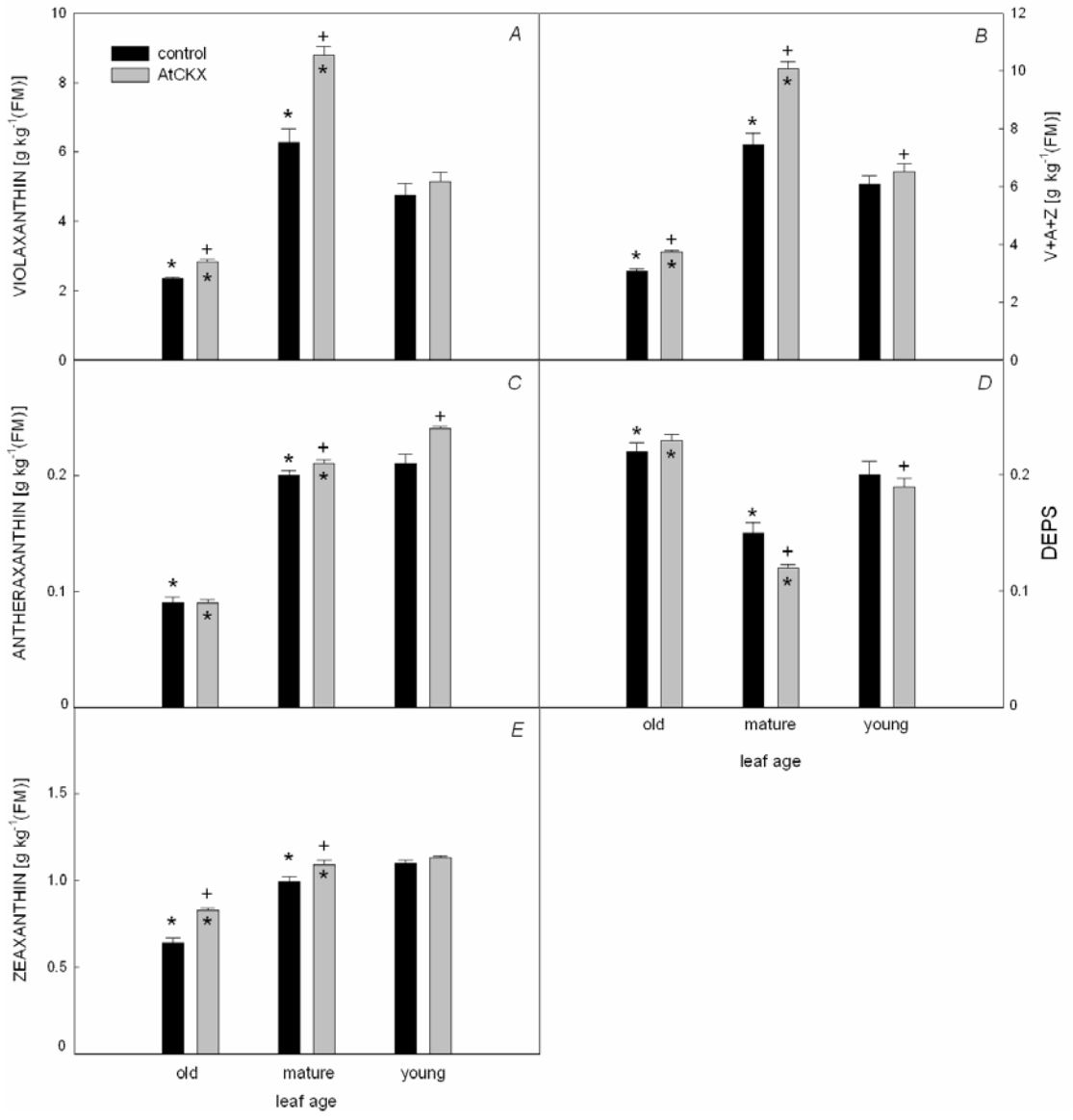

Fig. 2. Xanthophyll cycle pigment contents: violaxanthin (*A*), antheraxanthin (*C*), zeaxanthin (*E*), total content V+A+Z (*B*), and the de-epoxidation state of the xanthophyll cycle (DEPS) (*D*) in old, mature, and young leaves of control and transgenic tobacco AtCKX. Means ± SE (*vertical bars*). *Asterisk* indicates significance of the differences from the respective young leaf. *Cross* indicates significance of difference of transgenic plant from control plant. Significance of difference is according to Student's *t*-test at $p<0.05$.

Many scientists reported that this parameter decreased during senescence resulting from a decline of PS2 function (*e.g.* Lu *et al.* 2001, Procházková and Wilhelmová 2004, Weng *et al.* 2005, Wilhelmová *et al.* 2005). It is evident from our observations that the photochemical efficiency in transgenic plants was almost unchanged during all leaf developmental stages contrary to controls where a considerable decline was evident. Based on the determination of photochemical efficiency the oldest leaves of transgenic plants were not apparently senescent yet. Similar conclusions were apparent from F_v/F_0 that can be used as a sensitive indicator of the maximum efficiency of photochemical processes in PS2 and/or the potential photosynthetic activity of healthy as well as stressed plants (Roháček 2002). Later onset of senescence in old transgenic leaves was also confirmed by the fluorescence parameter Φ_2 reflecting the actual performance of PS2. The Φ_2 decreased only in control plants during ageing.

The magnitude of the F_0 is influenced by two factors (Schnettger *et al.* 1994). One is the loss of the photosynthetic pigments, resulting in a decline of F_0, and the other is damage to the PS2 reaction centres, which can contribute to an increase in F_0. Therefore we propose that the increase of F_0 observed with age in control tobacco leaves could reflect progressive damage to PS2 reaction centres, whereas the final decrease of F_0 could be a consequence of a detachment of Chl from pigment protein complexes. This was supported by the rapid degradation of Chl that we observed after Chl content determinations. Interestingly, transgenic plants did not show such time course and evidently their leaves did not undergo senescence stage.

NPQ has been widely used as an indicator of excess radiant energy dissipation to heat in the PS2 antenna complex and its increase is associated with enhancement of A and Z contents (Gilmore 1997). In our experiment NPQ decreased with age as well as xanthophyll pigments A and Z. Guiamét *et al.* (2002) reported that the decline in NPQ in senescent leaves probably depended on growth condition. Evidently, the efficiency of a plant to dispose excess energy decreases with age. This could result in increased ROS production. We detected also a decrease of anti-oxidative protection (unpublished results) with leaf age in transgenic plants with lowered CK content. However, the decrease of activities of ascorbate peroxidase and glutathione reductase was less in this tobacco type.

We found that the total content of xanthophyll cycle pigments increased in the mature leaves and decreased in the oldest leaves compared to the young ones. All time courses were similar for both tobacco types. The majority of their total amount was represented by V that indeed had the same courses. On the contrary, A and Z contents declined already in mature leaves. DEPS was significantly enhanced in old leaves. Decreasing V, A, and Z, and enhanced DEPS during ageing was also observed by other authors (Yang *et al.* 2001, Munné-Bosch and Peñuelas 2003). The same courses as V had also N and L. At first both increased during leaf development and then declined in the old senescent leaves. It is in agreement with other observations (Lu *et al.* 2001, Munné-Bosch and Peñuelas 2003). However, Yang *et al.* (2001) studied two different rice hybrids and found out opposite time-courses of N and increasing L content during leaf ageing. All measured xanthophylls were enhanced almost in all leaf stages of transgenic plants compared to controls. The higher xanthophyll contents in the transgenic plants contribute to better photoprotection *via* xanthophyll cycle. These results, *i.e.* enhancement of N and to a lesser extent of V in transgenic plants compared to controls, are in conflict with our findings of abscisic acid (ABA) contents. These pigments are precursors of ABA. ABA content is reduced in transgenic plants (Václav Motyka, personal communication), most markedly in young leaves. ABA participates in induction of leaf senescence process (Noodén 1988), its decrease thus could be another source of leaf senescence postponing in transgenic plants. Moreover, the higher xanthophyll pigments content that gives

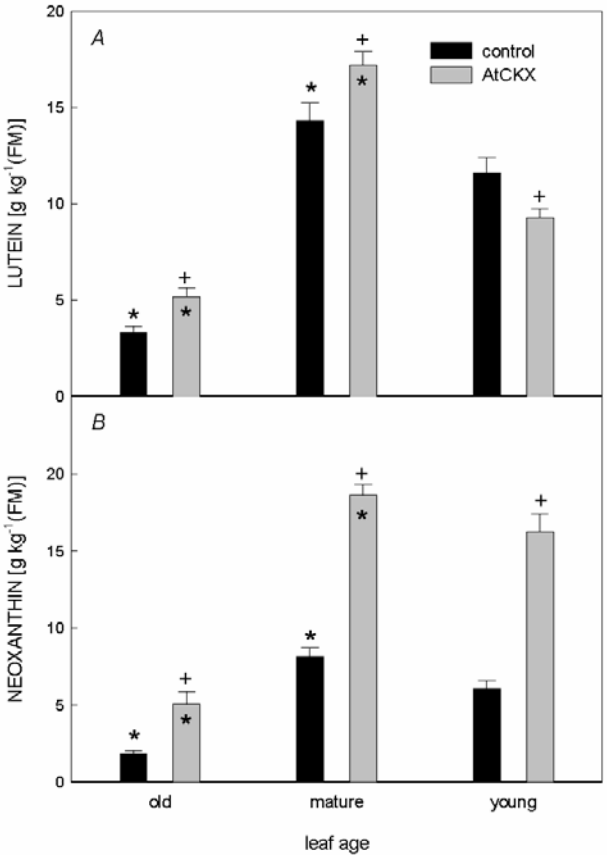

Fig. 3. Lutein (*A*) and neoxanthin (*B*) contents in old, mature, and young leaves of control and transgenic AtCKX tobacco. *Asterisk* indicates significance of the differences from the respective young leaf. *Cross* indicates significance of differences of transgenic plants from control plant. Significance of difference is according to Student's *t*-test at $p<0.05$.

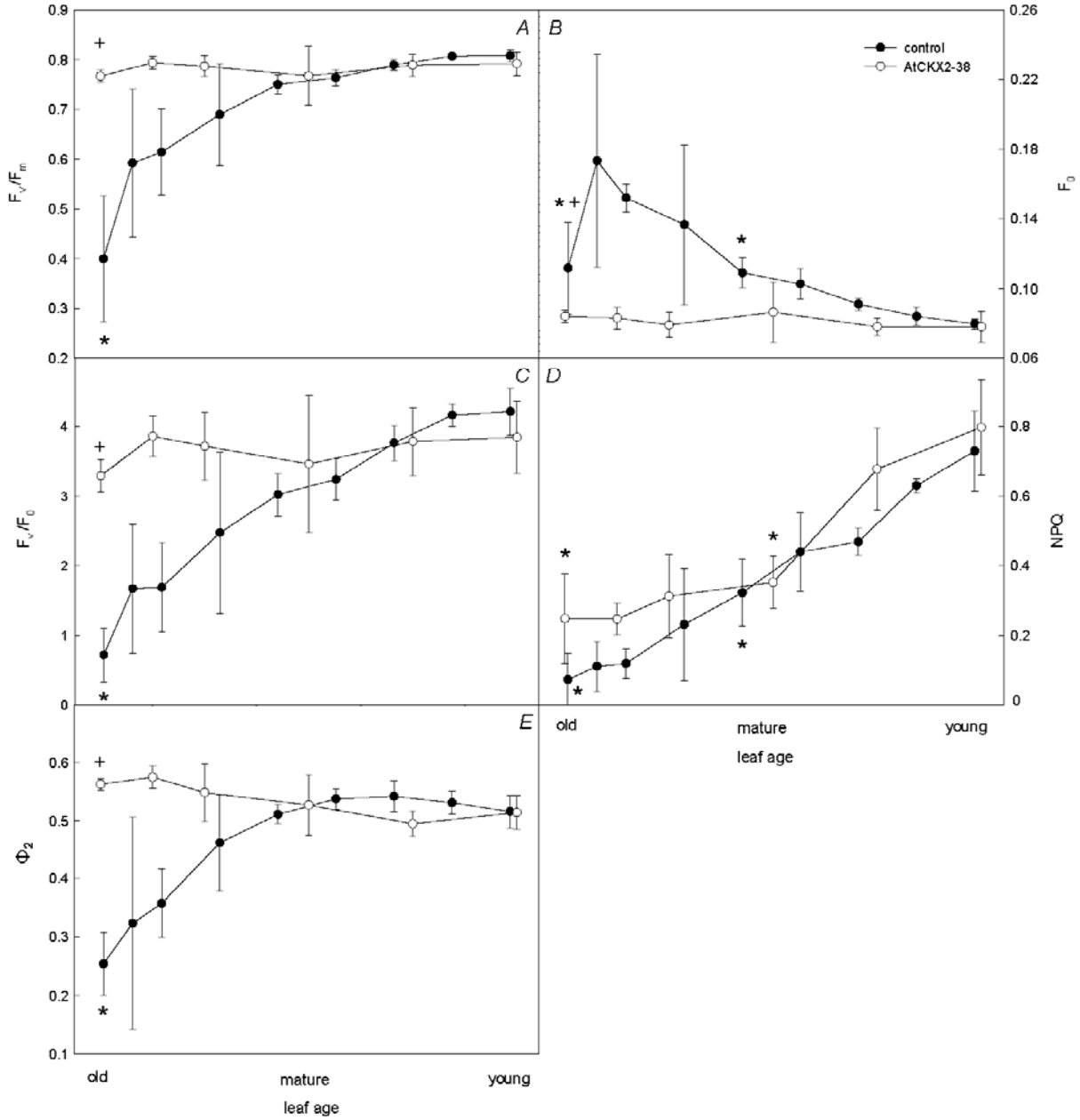

Fig. 4. Maximum photochemical efficiency, F_v/F_m (*A*), maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2, F_v/F_0 (*C*), effective quantum yield of photochemical energy conversion in PS2, Φ_2 (*E*), minimum chlorophyll *a* fluorescence yield recorded at very low irradiance (at dark-adapted state), F_0 (*B*), and non-photochemical quenching NPQ (*D*) in old, mature, and young leaves of control and transgenic tobacco AtCKX. *Asterisk* indicates significance of the differences from the respective young leaf. *Cross* indicates significance of differences of transgenic plant from control plant. Significance of difference is according to Student's *t*-test at $p<0.05$.

better photoprotection could contribute to later onset of senescence in transgenic plants.

We conclude from the presented data that the transgenic plants aged more slowly, during the experimental period they did not reach the senescence stage yet, contrary to the controls. They maintained higher Chl content and a better photoprotection based on xanthophyll cycle and also higher photosynthetic performance in the oldest leaves. On the other hand, the oldest leaves in the controls were already senescent with poor photoprotection and photosynthesis.

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