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Content of carotenoids during ageing and senescence of tobacco leaves with genetically modulated life-span

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Abstract

We exploited leaves of tobacco (*Nicotiana tabacum* L., cv. Wisconsin 38) with introduced chimeric construct consisting of *SAG*12 promoter fused with *ipt* gene for cytokinin synthesis and therefore prolonged life-span. As a control we used its wild type. In 12-week-old plants, the first leaves of control plants showed senescence symptoms at the time of sampling. Carotenoid content decreased with increasing leaf age both in control and in transgenic plants. On the other hand, the first leaves of transgenic plants demonstrated better antioxidant capacity represented by carotenoids compared to the leaves of control plants of the same age. They stayed still green at this age.

Additional key words: cytokinin, reactive oxygen species, xanthophylls.

Introduction

Leaf senescence is a phase of development during which cells undergo distinct metabolic and structural changes leading to cell death (Noodén 1988). It is an active process, during which catabolic activities such as macromolecular breakdown of nucleic acids, proteins and lipids are intensified through the induction of a number of hydrolytic enzymes. The main purpose of the cellular activities of leaf senescence is to convert accumulated material into exportable nutrients that can then be transported and supplied to other actively growing tissues or developing seeds (Kim *et al*. 2007).

Leaf senescence is often associated with increased oxidative damage to cellular macromolecules by reactive oxygen species (ROS) such as singlet oxygen, superoxide radicals, hydrogen peroxide and hydroxyl radicals. Plants possess – in addition to enzymatic antioxidants – a group of non-enzymatic antioxidants, which can keep senescence under control to its late phases by overcoming oxygen toxicity and preventing the deleterious effects of ROS.

One of them is a group of lipophilic carotenoids, which comprises xanthophylls (with one or more oxygen molecules) and carotenes (the parent hydrocarbons) (Halliwell and Gutteridge 1989). Xanthophylls are involved in the radiationless dissipation of excess energy in the pigment bed by the conversion from violaxanthin with two epoxide groups to antheraxanthin with only one epoxide group and to zeaxanthin with no epoxide group in the violaxanthin cycle (Demmig-Adams *et al*. 1994). Similarly a photoprotective function has the lutein epoxide cycle, which is based on de-epoxidation of lutein-5,6-epoxide (Garcia-Plazaola *et al*. 2007). This cycle has been indentified in holoparasitic *Cuscuta reflexa* (Bungard *et al*. 1999), hemiparasitic plants (Matsubara *et al*. 2001; 2003), *Quercus* species (Garcia-Plazaola *et al*. 2002) or avocado (Förster *et al*. 2009). In addition, β-carotene can quench $^1O_2$ produced from interaction of ^{3}P680 and O_2 in the photosystem II (PS II) reaction centre (Telfer *et al*. 1994).

The improved understanding of senescence is important for the increase of crop yield and for prolongation of a product shelf life-span. The development of transgenic plants with modulated life-span enables a new access into senescence studies. One of these transgenic plants are tobacco plants with *SAG*12 promoter fused with *ipt* gene which encodes isopenthenyl

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Abbreviations: C plants – control plants; Chl – chlorophyll; F_M – maximum fluorescence in the dark-adapted state; F_V – variable fluorescence in the dark-adapted state; F_V/F_M – maximum quantum efficiency of PSII photochemistry; F_V/F_0 – maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PSII; F_0 – minimum fluorescence in the dark-adapted state; F_0/F_M – basal quantum yield of non-photochemical processes in PSII; NPQ – non-photochemical quenching; PS II – photosystem II; ROS – reactive oxygen species; SAG – transgenic plants with increased levels of cytokinins; v – volume.

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transferase, an enzyme which catalyses the rate-limiting step in cytokinin synthesis. Life-span of these transgenic plants is significantly longer compared to the wild type plants. At the onset of leaf senescence the promoter is activated and cytokinin level is increased due to a higher isopenthenyl transferase expression (Gan and Amasino 1995). The deep investigation of these plants is highly important because they seem to be more resistant against abiobic stresses: *e.g.* they had minimal yield loss under drought stress (Rivero *et al.* 2007).

In order to elucidate the role of carotenoid antioxidants during ageing and senescence, their content was determined in leaves of above mentioned transgenic tobacco with prolonged life-span and compared to its wild type.

Materials and methods

Plants: We used tobacco (*Nicotiana tabacum* L., cv. Wisconsin) transformed with a construct consisting of *SAG*12 promoter fused with *ipt* gene for cytokinin synthesis (SAG plants). As a control we used its wild type (C plants). Plants were grown after *in vitro* pre-cultivation in pots with soil substrate in a greenhouse under day/night temperature of 25/18 ºC day/night and 60 % relative humidity. Natural irradiance was prolonged to 16 h by additional illumination (*ca*. 200 μmol m^{-2} s^{-1}). Plants were irrigated by tap water, two times a week by Hewitt nutrient solution (Procházková *et al.* 2008).

For experiments, we used the first seven leaves at the plant age of 12 weeks when the lowermost (oldest) leaves of C plants turned yellow. The leaves were numbered from the bottom: the oldest leaves as the 1st, the youngest as the 7th. Leaf sample withdrawal took place 4 h after the beginning of the light period.

Pigment contents were established by HPLC (*ECOM, s.r.o.*, Prague, Czech Republic) from acetone extracts 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 ml min^{-1}, the detection wavelength was 445 nm (Procházková and Wilhelmová 2007a). Pigment content was calculated according to Lichtenthaler (1987).

Chlorophyll (Chl) fluorescence parameters from slow kinetics were measured after a 15 min dark period with the *PAM* Chl 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} (Wilhelmová *et al.* 2005). The *DA 100* data acquisition system (*Walz*, Effeltrich, Germany) was used for sampling and calculation. All parameters, *i.e.* maximum ratio of quantum yield of photochemical and concurrent non-photochemical processes in PS II (F_V/F_0), basal quantum yield of non-photochemical processes in PS II (F_0/F_M), and non-photochemical quenching (NPQ) of maximum Chl fluorescence were calculated according to Roháček (2002).

Statistical evaluation: The results presented are the means of three independent experiments. Each sample was assayed in triplicate. Sample variability is given as the standard deviation of the mean. Statistical significance of differences was evaluated with analysis of variance (*ANOVA*, *Duncan's* multiple range test) using *NCC 6.0 jr.* programme (*NCSS*, Kaysville, USA).

Results

C plants had 7 fully developed leaves at the time of sampling. The first leaves from the bottom were yellow. SAG plants were slightly shorter compared to C plants, however the number and size of leaves were the same. All their leaves were green.

The total Chl content decreased with increasing leaf age both in C and in SAG plants (Fig. 1*A*). The decrease of Chl content in the 1st leaves was significant in both genotypes. This content was significantly higher in the 1st leaves of SAG plants compared to the C leaves of the same position. F_V/F_0 ratio was the highest in the 7th leaves in C plants and henceforward it decreased with leaf age (Fig. 1*B*). This ratio was significantly lower in the 1st leaves of C plants compared to the 7th ones. In SAG plants, F_V/F_0 ratio decreased in the 5th leaves and then sustained with increasing age almost at the same level. F_V/F_0 ratio was significantly higher in the 1st leaves of SAG plants compared to the leaves of C plants of the same position. On the contrary, F_0/F_M ratio significantly increased in the 1st leaves of C plants (Fig. 1*C*). In SAG plants, there was no significant difference between the 1st and 7th leaves. This ratio was significantly higher in the 1st leaves of C plants compared to the 1st leaves of SAG plants. The maximum NPQ was found in young leaves in both genotypes (Fig. 1*D*) without any significant difference between the oldest leaves of C and SAG plants.

Except zeaxanthin content in the 6th and 7th leaves and β-carotene in 3rd leaves, the level of all carotenoids was higher in SAG leaves of all ages compared to control ones. The content of β-carotene increased from the 7th to the 5th leaves in both genotypes (Fig. 2*A*). Its content decreased afterwards with increasing leaf age in C plants with a significant difference between the 7th and the

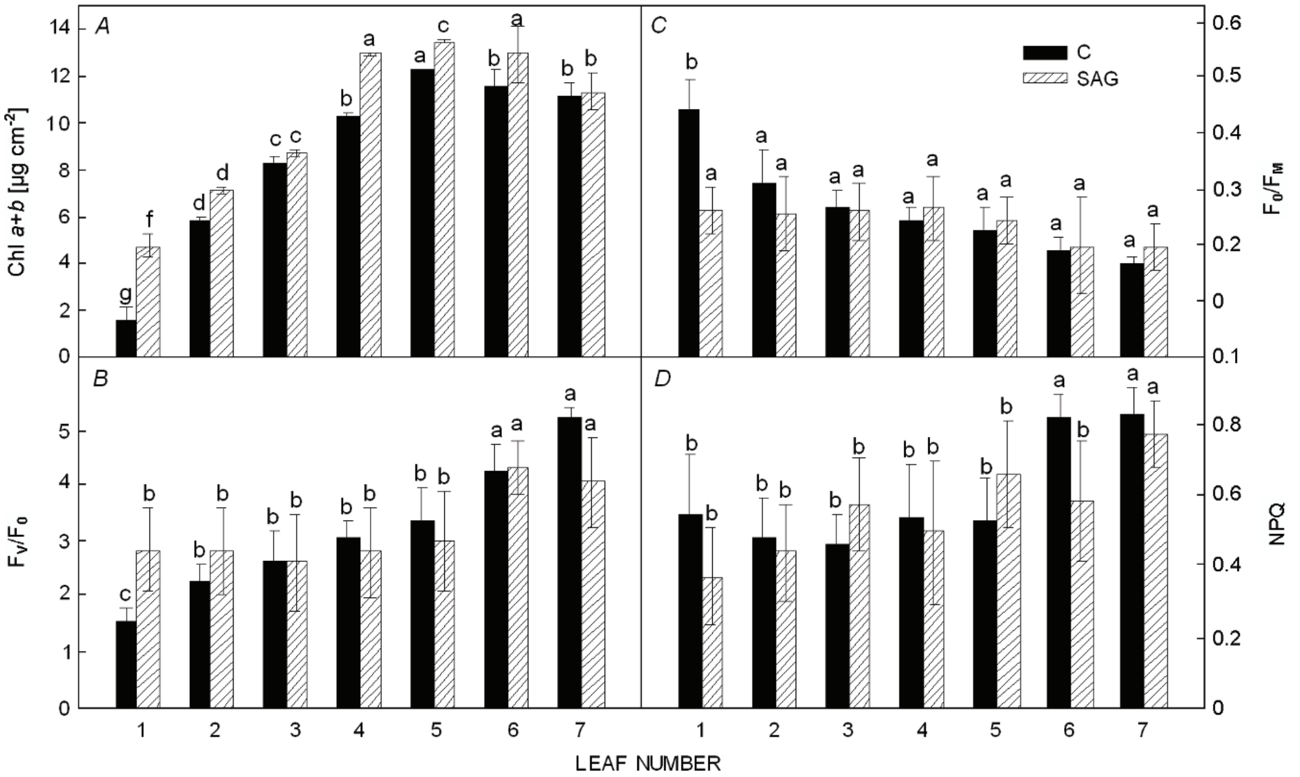

Fig. 1. *A*: Content of total chlorophyll (Chl), *B*: maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in photosystem II (PSII) (F_V/F_0), *C*: basal quantum yield of non-photochemical processes in PSII (F_0/F_M) and *D*: non-photochemical quenching (NPQ) of variable Chl fluorescence in leaves of wild tobacco type (C) and transgenic type (SAG) during increasing leaf age. Leaves are numbered from the bottom. Means ± SD are given as vertical bars (n = 9). Means followed by the same letters are not significantly different at p=0.05 (*Duncan´*s multiple range test).

1st leaves. The decrease in SAG leaves was very sharp between the 4th and the 3rd leaves but the content did not change afterwards. There was a significant difference between the 7th and the 1st leaves of SAG plants and the content was significantly higher in the 1st SAG leaves compared to the 1st leaves of C plants. Violaxanthin content decreased from the 3rd leaves in C plants with a significant difference between the 7th and the 1st leaves (Fig. 2*B*). The content of violaxanthin was lower in C plants compared to SAG ones in leaves of all ages. In SAG plants, there was a significant difference in the content of violaxanthin between the 7th and the 1st leaves and the content in the 1st leaves was more than twice higher compared to C plants leaves of the same age. Antheraxanthin content decreased with leaf age in C plants with significant difference between the 7th and the 1st leaves tested (Fig. 2*C*). Its content was lower compared to SAG leaves of all ages. In SAG plants, antheraxanthin content was decreasing slightly from the 7th up to the 2nd leaves with a significant decrease in the 1st leaves. Zeaxanthin content decreased with age in leaves of C plants with significant difference between the 7th and the 1st leaves (Fig. 2*D*). Zeaxanthin content practically did not change up to the 4th leaves in SAG plants, then decreased with increasing age. Lutein content decreased with leaf age in both genotypes being higher in SAG plants with a significant difference between the 7th and the 1st leaves in both genotypes (Fig. 2*E*). Lutein content was significantly higher in the 1st leaves of SAG plants compared to the same leaves of C plants. Neoxanthin content was almost the same in a range of 7th – 3th leaves in C plants then it rapidly decreased with significant difference between the 7th and the 1st leaves of C plants (Fig. 2*F*). In SAG plants, there was also a significant difference in neoxanthin level between the 7th and the 1st SAG leaves and its content was significantly higher in the 1st and 2nd leaves of SAG plants compared to the leaves of the same age in C plants.

Discussion

In the present study, we investigated changes in levels of several carotenoids with antioxidant function in the course of ageing and senescence in leaves of wild type tobacco (C plants) and in tobacco with elevated levels of cytokinins in the old leaves and therefore prolonged their life-span (SAG plants) (Gan and Amasino 1995).

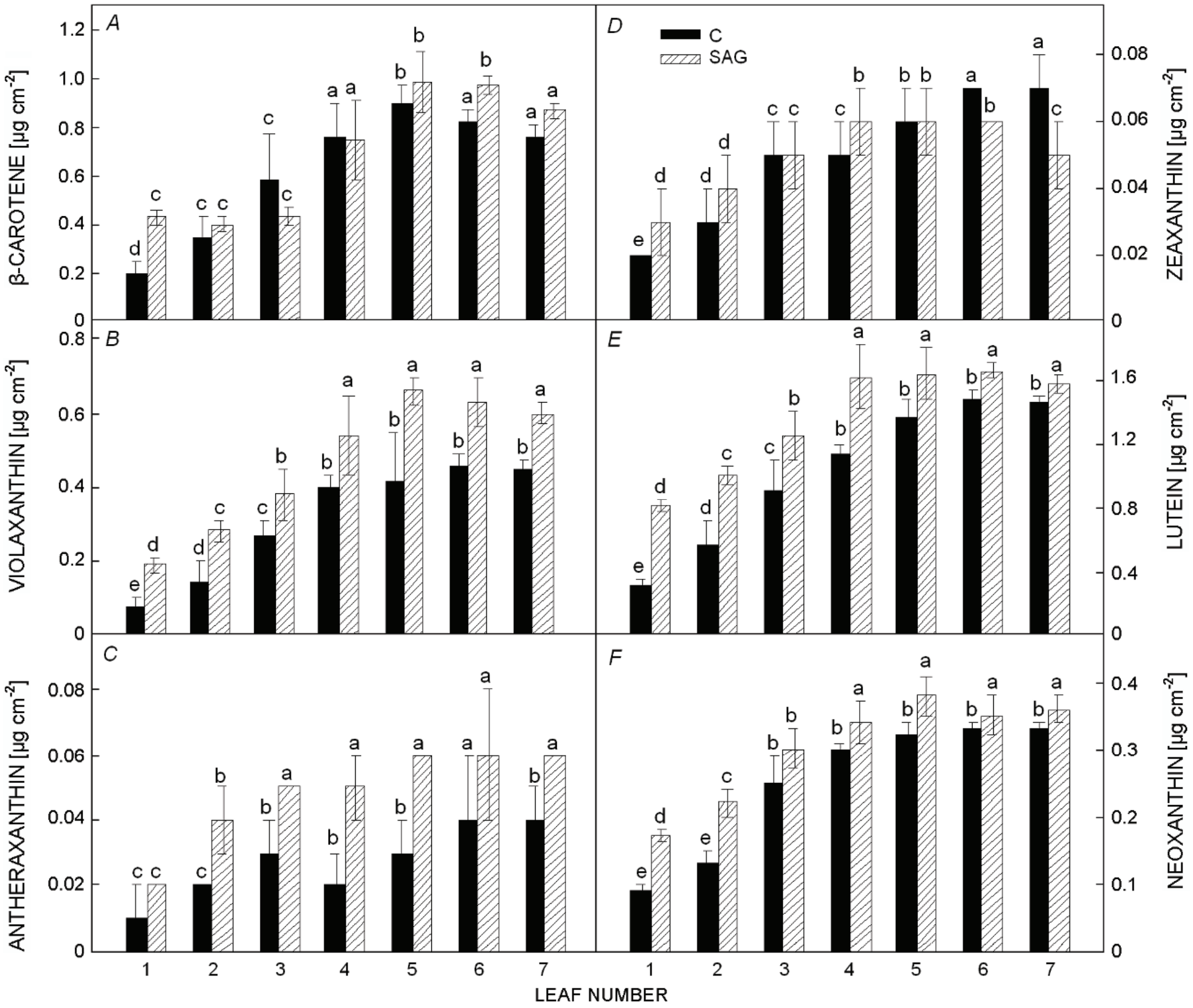

Fig. 2. Contents of β-carotene (*A*), violaxanthin (*B*), antheraxanthin (*C*), zeaxanthin (*D*), lutein (*E*) and neoxanthin (*F)* in leaves of wild tobacco type (C) and transgenic type (SAG) during increasing leaf age. Leaves are numbered from the bottom. Means ± SD are given as vertical bars ($n = 9$). Where bars are not shown, they are too small to be visible in the selected graph scale. Means followed by the same letters are not significantly different at p=0.05 (*Duncan*´s multiple range test).

In our previous studies (Procházková *et al.* 2008) we reported that the maximum photochemical efficiency (F_V/F_M) fell to 0.6 in the 1st leaves of C plants. On the other hand, the F_V/F_M ratio was practically constant in SAG leaves of different age but is was under the optimum value in all measured leaves.

Chl content has been widely used as a benchmark of leaf senescence (Matile 1992). In C plants, Chl content started to decrease in 5th leaves. Hence, we can aver that senescence was in the progress in 1st C leaves however we are not able to determine the senescence onset. Chl fluorescence parameters are the reliable indices for the assessment of physiological status of plants (Roháček and Barták 1999). The decrease of F_V/F_0 – an indicator of changes in the maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS II related to dark-adapted state and/or potential photosynthetic activity of healthy as well as stressed plants (Roháček 2002) – demonstrated that senescence in C plants was in progress. The same fact was supported by the increase of F_0/F_M ratio – an indicator of basal quantum yield of non-photochemical processes in PS II, which increases in stressed or damaged plants (Bilger *et al.* 1987). We found that NPQ was high in young leaves, in 5th leaves it decreased and henceforth sustained on the same level. This indicates the need to dissipate excess radiant energy by non-photochemical mechanisms with age progression.

The higher Chl content, higher F_V/F_0 ratio and lower levels of F_0/F_M ratio and NPQ of SAG plants indicate the better physiological status of their 1st leaves compared to C plants, which is in accordance with results of Dertinger *et al*. (2003). On the other hand, although the 1st leaves of SAG plants remained green, their physiological parameters did not achieve the level of the 7th leaves. We presume that SAG plants were not able to achieve a complete rejuvenation and to return to the physiological status of the young ones. Our results signify that these transgenic plants were able only to slow down the process of senescence but not to reverse it completely. This is in contrast to the results by Jordi *et al*. (2000). Similar conclusions are also apparent from the contents of tested lipophilic antioxidants. Their level declined in both genotypes, in SAG tobacco less markedly. However, we found out that these contents did not reach those of the young leaves.

Total antioxidant protection declines usually at the later stages of leaf senescence (Procházková and Wilhelmová 2007a,b,c) however it is not a general rule. In tobacco genotypes studied here, activities of most of the antioxidant enzymes decreased in whole leaf extracts of C plants (Dertinger *et al*. 2003) but superoxide dismutase increased in the oldest leaves of SAG plants both in whole leaves extracts and in chloroplasts. Similarly ascorbate peroxidase activity significantly increased in chloroplasts of the oldest C plant leaves (Procházková *et al*. 2008). It is impossible to define the explicit response of lipophilic antioxidants to ageing as well. Dertinger *et al*. (2003) as well as our laboratory (unpublished data) reported that concentration of α-tocopherol increased continuously with age both in C and SAG plants. On the other hand, α-tocopherol decreased to undetectable level in senescing leaves of *Pistacia lentiscus* (Munné-Bosch and Peñuellas 2003).

In our experiments, contents of monitored lipophilic antioxidants decreased continuously with leaf age both in C and in SAG plants being higher in old leaves of SAG plants compared to C plant leaves of the same age.

We suppose that senescence process in old leaves of C plants was influenced by the substantial decrease of dissipation of excess excitation energy in the violaxanthin cycle as levels of all these pigments declined. The decrease of zeaxanthin content might be also important, as zeaxanthin is thought – in addition to its main function, *i.e.* thermal dissipation of excess energy – to influence thylakoid membrane fluidity (Gruszecki and Strzalka 1991, Munné-Bosch and Peñuellas 2003).

In contrast to C plants, there was no sharp decline in β-carotene content in the oldest three leaves of SAG plants. Photosystem reaction centres are rich in β-carotene (Siefermann-Harms 1985, Markgraf and Oelmüller 1991) and its high level might help to sustain higher photosynthetic parameters in old SAG leaves compared to C plant leaves of the same age.

In animals, lutein is thought to have important antioxidant functions (Mares-Perlman *et al*. 2002) however our knowledge of lutein as a leaf antioxidant is very limited. Recent works have shown that the xantophyll cycle involving lutein operates together with violaxanthin cycle (Bungard *et al*. 1999, Matsubara *et al*. 2001, Garcia-Plazaola *et al*. 2007, Kruk and Szymańska 2008). Its content decreased with increasing leaf age in *Pistacia lentiscus* (Munné-Bosch and Peñuellas 2003) and in tobacco (Mýtinová *et al*. 2006). Our results showed the similarity between the time course of lutein content and the time course of other lipophilic antioxidants in both genotypes. Nevertheless it is not a proof of existence of lutein cycle in tobacco and this question deserves further studies.

The decrease of neoxanthin content with increasing age was observed both in C and SAG plants. Besides the fact that neoxanthin is regarded as a precursor of abscisic acid (Parry *et al*. 1991, Bouvier *et al*. 2000), which contributes to the initiation of plant senescence (Noodén 1988), it is also an internal structural component of the antenna complexes (Llorens *et al*. 2002). Its expressive decrease in C plants might reflect the worse stability of photosynthetic apparatus in old leaves of C plants.

The functionality of selected lipophilic antioxidants was maintained higher in old leaves of SAG plants compared to C plant leaves of the same age. It was similar to the course of enzymatic antioxidants activities (Dertinger *et al*. 2003, Procházková *et al*. 2008) suggesting that both antioxidant systems work in coordination. Probably one mechanism alone is not enough to protect leaves from uncontrolled oxidative stress during senescence, but the complete set of antioxidant defence, including both enzymatic and low-molecular antioxidants, is necessary to provide antioxidant protection during ageing and senescence.

In experiments presented, we demonstrated (*1*) the close relationship between the decrease of photosynthetic parameters and the decrease of lipophilic antioxidants with leaf ageing and (*2*) incapability of transgenic plants to completely reverse the senescence process, although they have prolonged life-span.

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