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# Molecular markers of delayed senescence in transgenic tobacco with enhanced cytokinin level

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**ABSTRACT**

Cytokinins are phytohormones that promote plant growth and development. They stimulate cell division and upregulate expression of photosynthetic-associated genes. However, the main reason for cytokinin research is their anti-senescing action used to delay the onset of plant senescence, improve productivity and postharvest storage. Exogenously applied cytokinins, like 6-benzylaminopurine, are commonly use to slow down or even inhibit the leaf yellowing and prolong lifespan of economically important plants. Similar effects, the regreening of mature fully-developed basal leaves, can be obtained by removal the top of the plant redirecting cytokinins flow from roots back to the ageing structures. Nevertheless, the real breakthrough was the engineering of transgenic tobacco plants with an inserted gene of cytokinins biosynthetic pathway (*ipt*) fused with a senescence-specific promoter ($P_{SAG12}$). Enhanced hormones synthesis results in a leaf ageing retardation and its negative auto-regulated activity prevents developmental abnormalities. We studied the capacity of elevated hormone level in the transgenic tobacco to reduce chlorophyll, soluble proteins and Rubisco degradation in plants exposed to nine days of light deprivation. We also investigated possible correlation between the enhanced cytokinin synthesis and antioxidant properties of $P_{SAG12}$-*IPT* plants analyzing three isoforms of superoxide dismutase. This paper is an attempt to determine molecular and physiological basis of cytokinin role in an inhibition of leaf ageing.

***Keywords*:** tobacco, delayed senescence, cytokinin, *ipt*, SAG12, chlorophyll, Rubisco LSU, superoxide dismutase

## 1. INTRODUCTION

Cytokinins are group of plant hormones discovered for the first time by Haberlandt in the early twentieth century. For next decades cytokinins studies were limited till late fifties when Miller and Skoog proposed the auxin-cytokinin model to explain the regulation of plant morphogenesis (Zažímalová et al., 1999). First plant cytokinin was isolated by Miller in 1961 from immature kernels of corn (*Zea mays* L.) and called zeatin. In recent years numerous cytokinin derivatives were obtained with different substituents attached to nitrogen $N^6$. In most cases $N^6$ is substituted with isopentenyl chain, rather benzyl or hydroxybenzyl group (Czerpak and Piotrowska, 2003).

Role of cytokinins in the functioning of plant meristems has been illustrated in the hormone-deficient mutants characterized by a decrease amount of meristematic cells and reduce ability to form new organs (Werner and Schmülling, 2009). Cytokinins affect also plant ageing and the formation of photosynthetically active chloroplasts from immature plastids. Their influence on photosynthesis is connected with ability to increase activity of Rubisco and other Calvin cycle enzymes (Pilarska et al., 2015). Cytokinins stimulate synthesis of chlorophylls, carotenoids and formation of chlorophyll-binding protein complexes (Czerpak and Piotrowska, 2003). They also modify chloroplast ultrastructure (Zubo et al., 2008) and regulate chloroplast-associated genes expression both in nuclear and chloroplast DNA (chlDNA).

The most feasible mechanism of signal transduction involves cytokinin-binding protein located in the chloroplasts (Choquet and Wollman, 2002; Herrin and Nickelsen, 2004). The way the plant cells response to cytokinins correlates with their age and current metabolic activity. Data obtained by Baumgartner et al. (1989) confirmed the age-related correlation of hormones influence. The youngest cells, situated in the basal part of the leaf, as well as the oldest ones from the edge were almost unaffected by hormone treatment. Cytokinins are mostly active in fully developed but still young and genetically active cells from a middle part of the leaf blade. Hence, anti-senescing action of cytokinins also limits the decline in transcription activity, allowing to delay the onset of senescence and prolong lifespan. Majority of upregulated genes are associated with photosynthesis like photosystem II proteins (*psbA* and *psbD*), large subunit of Rubisco (*rbcL*) and ATP-synthase (*atpB*). Common feature of cytokinin-depended gene expression is simultaneous light exposure. Previous study has shown that separated factors were insufficient to modify the transcription activity (Zubo et al., 2008). Proposed mechanism, explaining cooperation of these two, physically different factors, assumes that light exposure induces expression of cytokinin-binding protein in chloroplast (Kulaeva et al., 2002) or sigma factor mediating promoter recognition by plastid-encoded RNA polymerase (PEP) (Lysenko, 2007).

Plant senescence refers to two different processes. The first is responsible for a physiological ageing in the subsequent growth phases. However, because plants are sessile organisms and they are exposed to adverse and harmful factors able to induce physiological stress or even death, the ageing is often a pathological process. The leaf senescence, which is the last stage in its development, is an important process managed by genes. Controlled dismantling allows to recover most of nutrients (predominantly nitrogen) accumulated in leaves (source), and redistribute them to young developing organs and structures (sink) (Clausen and Apel, 1991). Despite obvious benefits, age-related premature relocation reduces crop yield and the quality of plant destination products like tobacco leaves. Molecular

analysis has allowed to distinguish two major groups of genes influenced by senescence in opposite manner. The first, called SDG, includes mainly photosynthetic-associated genes like *cab* and *ssu*, down-regulated during progression of senescence. The latter - SAG, assembles two classes of genes, expressed either strictly during onset of senescence (e.g. *sag12*, *sag13*) or at early growth stages (Gan and Amasino, 1997).

The influence of light availability on metabolism of plants growing in high density is well documented (Hensel et al., 1993; King et al., 1995). Lower leaves, shaded by the upper structures, are receiving substantially less light, which disturbs balance between production and utilization of assimilates. Proximity of plant canopies changes light quality modifying a red to far-red ratio. Spectrum of light, filtered through leaves, is shifted toward longer wavelength region. Significance of proper light intensity and quality in maintaining high metabolic rate of plant cells is successfully used in the method called DIS (dark-induced senescence). It allows to induce artificial death of plant structures like leaves under controlled conditions during 3-5 days of light deprivation. Despite several differences between the natural senescence and this induced by leaf shading, the rate of chlorophyll decline (Spundová et al., 2003) and gene expression pattern appear to be similar in both (Balazadeh et al., 2014).

Senescence is also regulated by phytohormones like abscisic acid, ethylene and cytokinins. As mentioned before, cytokinins have an ability to delay the onset of senescence and exogenously applied can be used to prolong lifespan of plant organs. At the same time researches have shown that during physiological ageing endogenous concentration of cytokinins is consistently decreasing. Hence, they have developed strategies to obtain genetically modified tobacco plants capable to overproduce endogenous cytokinins. The gene encodes isopentenyl transferase, the enzyme catalyzes the rate limiting reaction in cytokinin biosynthesis, was fused to senescence-specific SAG12 promoter. The *ipt*, gene originated from *Agrobacterium tumefaciens*, is activated by the onset of senescence stimulating SAG12 promoter. As a result endogenous level of cytokinins is increasing which in turn inhibits senescence. Delayed senescence in a stay-green plant attenuates the activity of SAG12, and creates kind of auto-regulated biochemical loop, preventing overexpression of *ipt* gene and side effects of enhanced cytokinins level (Gan and Amasino, 1995).

The aim of the study was to evaluate the influence of enhanced cytokinin biosynthesis on the onset of the leaf senescence. Tobacco plants, with the inserted gene of cytokinin biosynthesis expressed in senescence-related manner, were used to assessed physiological and molecular markers of stay-green phenotype.

## 2. METHODS

### 2. 1. Plant material

Three different genotypes of tobacco (*Nicotiana tabacum* L.) were used: 1) wild type WT (*N. tabacum* cv. Wisconsin) and two transgenic plants: 2) $P_{SAG12}$-*IPT* with inserted isopentenyl transferase gene (*ipt*) fused with senescence-associated gene promoter ($P_{SAG12}$) and 3) $P_{SAG12}$-*GUS*, with analogue genetic construct, but with *ipt* gene replaced by reporter gene *uidA* of β-D-glucuronidase (GUS system) (Ori et al., 1999). The latter one has been used as the internal control to distinguish between hypothetical effects of construct insertion and direct influence of increased cytokinin level on molecular markers during leaf ageing.

Three weeks after sowing plant seedlings were transferred to P9 containers filled with tobacco substrate (white peat, black peat, perlite and NPK 9:5:10). Plants were grown in greenhouse at average light intensity of 400 µmol $m^{-2}$ $s^{-1}$, supplemented with HPS lamps for three/four hours to sustain photoperiod of 16/8 h per day. Relative humidity was 60-70%, with temperature varied in the range 25-30 °C/18 °C day/night. Six weeks after sowing, the plants were transferred to 3.5 litter containers filled with the same substrate and growing in exact conditions. Plants were fertilized once a week with NPK (9:5:10) fertilizer. Eleven weeks after sowing, plants of each genotype were divided into two groups – control with retained previous light regime (16/8h) and second darkened for 9 days (DIS), to induce the leaf senescence. Next fragments from internervial parts of the leaf blade were collected, immediately frozen at –80 °C and kept for further biochemical analysis. Frozen leaf fragments of each group were grinded in liquid nitrogen to a fine powder with mortar and pestle, weighted and split for approximately 100 mg per tube.

**2. 2. Chlorophyll content**

Chlorophyll *a* + *b* content was determined spectrophotometrically according to method proposed by McCabe et al. (2001), with slight modifications. Pigments were extracted from leaf powder (100 mg) with 1.5 mL of buffered aqueous 80% acetone containing 2.5 mM sodium phosphate (pH 7.8) to reduce chlorophylls conversion to phaeophytins. Samples were vortexed for 30 s, covered and incubated for 30 min with inversion every 10 min to improve extraction. Homogenized sample mixture was centrifuged for 15 min at 10 000 g, 4 °C. The supernatant was carefully collected avoiding to not disturb pellet, transferred to new tube and mixed for 15 sec. 100 µl of the upper phase of each sample were mixed with 900 µl of buffered 80% acetone. Concentration of chlorophyll was determined at 663.6, 646.6 and 750 nm, according to optimized method by Porra et al. (1989).

**2. 3. Quantification of total soluble protein**

Soluble protein were extracted and determined as described by McCabe et al. (2001) with modifications. Frozen leaf powder (100 mg) were mixed with 1 mL of pre-cooled phosphate extraction buffer (pH 7.5) containing 2 mM dithiothreitol (DTT), 15% glycerol, 2% poly(vinylpolypyrrolidone) (PVPP), 2 mM EDTA, 10 mM $MgCl_2$ and protease inhibitor cocktail (ProteoBlock™; Fermentas, Waltham, MA). Samples were thoroughly mixed for 30 s and centrifuged for 5 min at 10 000 g, 4 °C. The collected supernatant was transferred to new tubes and quickly mixed. Spare tubes were immediately frozen at -80°C for Rubisco and SOD assessment. Total soluble protein were quantified spectrophotometrically according to Bradford (1976) with modifications using Roti®-Nanoquant (Carl Roth, Germany). The standard curve for the Bradford assay, was obtained with BSA dilutions.

**2. 4. Rubisco LSU abundance**

Previously obtained protein extracts were used for Rubisco LSU assay. Samples were standardized to 10 µg of protein per well and mixed (4:1) with sample buffer containing 350 mM Tris-HCl, pH 6.8, 55.6% glycerol (v/v), 5% LDS, 0.06% bromophenol blue, 55.5 mM DTT and incubated at 95°C for 5 min. Each sample, cooled to RT, were loaded onto SDS-PAGE gel. The gels were run at RT with constant current 40 mA for 90 min. Rubisco content was assessed by densitometric analysis of its large subunit bands visualized on gel

with PageBlue™ Protein Staining Solution (Thermo Fisher Scientific, USA) with ImageJ software (National Institute of Health, USA).

### 2. 5. SOD activity

Extracted proteins, frozen in –80 °C, were quickly transferred on an ice and mixed with a cold loading buffer (5:1), containing 25 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 0.025% bromphenol blue and vortexed for 15 sec. The individual samples were loaded onto Native-PAGE gel after the previous unification of protein content to 5 μg per well. The electrophoresis was run for 120 min at 4 °C. Gels with separated proteins were gently placed in the containers with staining solution in dark room, covered and incubated at RT for 20 min with slowly shaking. Staining solution was containing: 25 mL of 50 mM phosphate buffer (pH 7.5), 10 mM EDTA, 1.25 mM methionine, 75 μM nitroblue tetrazolium (NBT), 2 μM riboflavin. Next gels were irradiated under the fluorescent light tubes of 40 μmol $m^{-2}$ $s^{-1}$ for 10 min. SOD isoforms activity was assessed with densitometric analysis (ImageJ) based on the SOD-inhibited NBT reduction. The bright bands, corresponding to active form of SOD, has been visualized on the dark background.

### 2. 6. Statistical analysis

Analysis of variance (ANOVA) and post hoc test analysis were used after testing data to meet conditions of normality. Tukey's multiple range test was used to test differences among more than two means at the 0.05 significance level using STATISTICA (12.0, StatSoft, Poland).

## 3. RESULTS

In the study we analyzed influence of enhanced cytokinin level on senescence process in transgenic tobacco plants. Two control groups: the wild type (WT) and the control of senescence-activated promoter ($P_{SAG12}$-*GUS*), were compared with the $P_{SAG12}$-*IPT* genotype with ageing-regulated biosynthesis of cytokinins. Leaves at the same age were selected and darkened for 9-days to induce senescence. Method allows to change the profile of molecular markers in similar way to those observed for the typical physiological ageing (Spundová et al., 2003). To assess the impact of DIS the following parameters have been analyzed: 1) concentration of chlorophyll *a* and *b*, 2) total concentration of soluble protein (TSP), 3) content of the large subunit of Rubisco (LSU) and 4) activity of superoxide dismutase isoforms.

### 3. 1. Chlorophyll *a* + *b* content

The highest content of the total chlorophyll was indicated in $P_{SAG12}$-*IPT* tobacco, both in control (2.4 mg) and DIS group (1.5 mg). Measured decrease in chlorophyll content after DIS was 29.4% (WT), 37.5% ($P_{SAG12}$-*IPT*) and 38.9% ($P_{SAG12}$-*GUS*), respectively. Obtained results are opposite to previous data, that has showed important role of cytokinins in preservation of photosynthetic pigments during senescence (Table 1).

**Table 1.** Groups used in the experiments for each dataset (n ± SD).

| Groups | **WT** | **WT DIS** | **IPT** | **IPT DIS** | **GUS** | **GUS DIS** |
|---|---|---|---|---|---|---|
| **Rubisco LSU** [relative content] | - | 0.7±0.06 | - | 0.8±0.03a* | - | 0.7±0.07a |
| **Rubisco LSU abundance** [AU $\mu g^{-1}$ TSP] | 3.5±0.20a | 2.5±0.20b | 3.8±0.20 | 3.1±0.11 | 3.4±0.22a | 2.3±0.23b |
| **Mn-SOD activity** [AU] | 3.9±0.20a | 1.6±0.15b | 4.7±0.14 | 2.2±0.10 | 4.0±0.23a | 1.3±0.13b |
| **Fe-SOD activity** [AU] | 7.0±0.17 | 2.6±0.17 | 7.4±0.28 | 3.6±0.17 | 6.5±0.30 | 2.1±0.13 |
| **Cu/Zn-SOD activity** [AU] | 5.6±0.19 | 2.4±0.15a | 6.0±0.18 | 2.4±0.20a | 3.4±0.21 | 1.7±0.16 |
| **chlorophyll *a+b*** [mg $g^{-1}$ FW] | 1.7±0.12a | 1.2±0.12b | 2.4±0.18 | 1.5±0.15a | 1.8±0.16a | 1.1±0.08b |
| **Total soluble protein** [mg $g^{-1}$ FW] | 17.9±1.48a | 4.8±0.46 | 17.4±1.42ab | 2.8±0.27c | 17.0±0.95ab | 2.8±0.25c |

* The same letters in the same line denote not statistically significant differences between the means ($p < 0.05$).

### 3. 2. Total soluble protein

Average content of soluble protein documented in the study was in the range of 17-18 mg per gram of FW (fresh weight). After DIS treatment we noticed significant drop in the protein concentration. Estimated relative decrease was: 73.2%, 83.9% and 83.5% for WT, $P_{SAG12}$-*IPT*, $P_{SAG12}$-*GUS*, respectively (Tab. 1).

### 3. 3 Rubisco LSU abundance

Changes in Rubisco level was determined with densitometric analysis of 55 kDa LSU bands visualized on SDS-PAGE gel (Fig. 1). Table 1 presents the abundance of Rubisco LSU [AU $\mu g^{-1}$ TSP] and its relative content in DIS group.

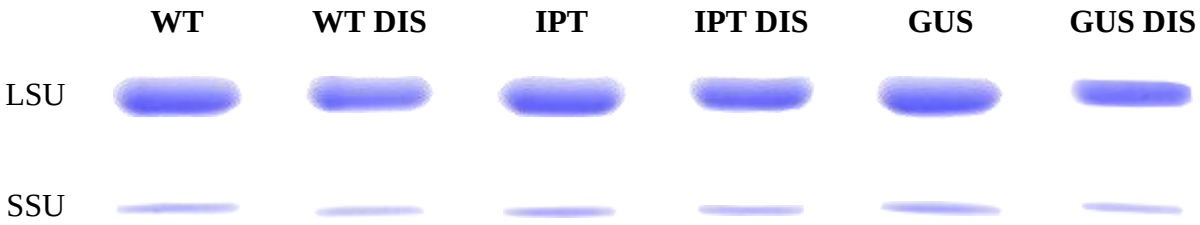
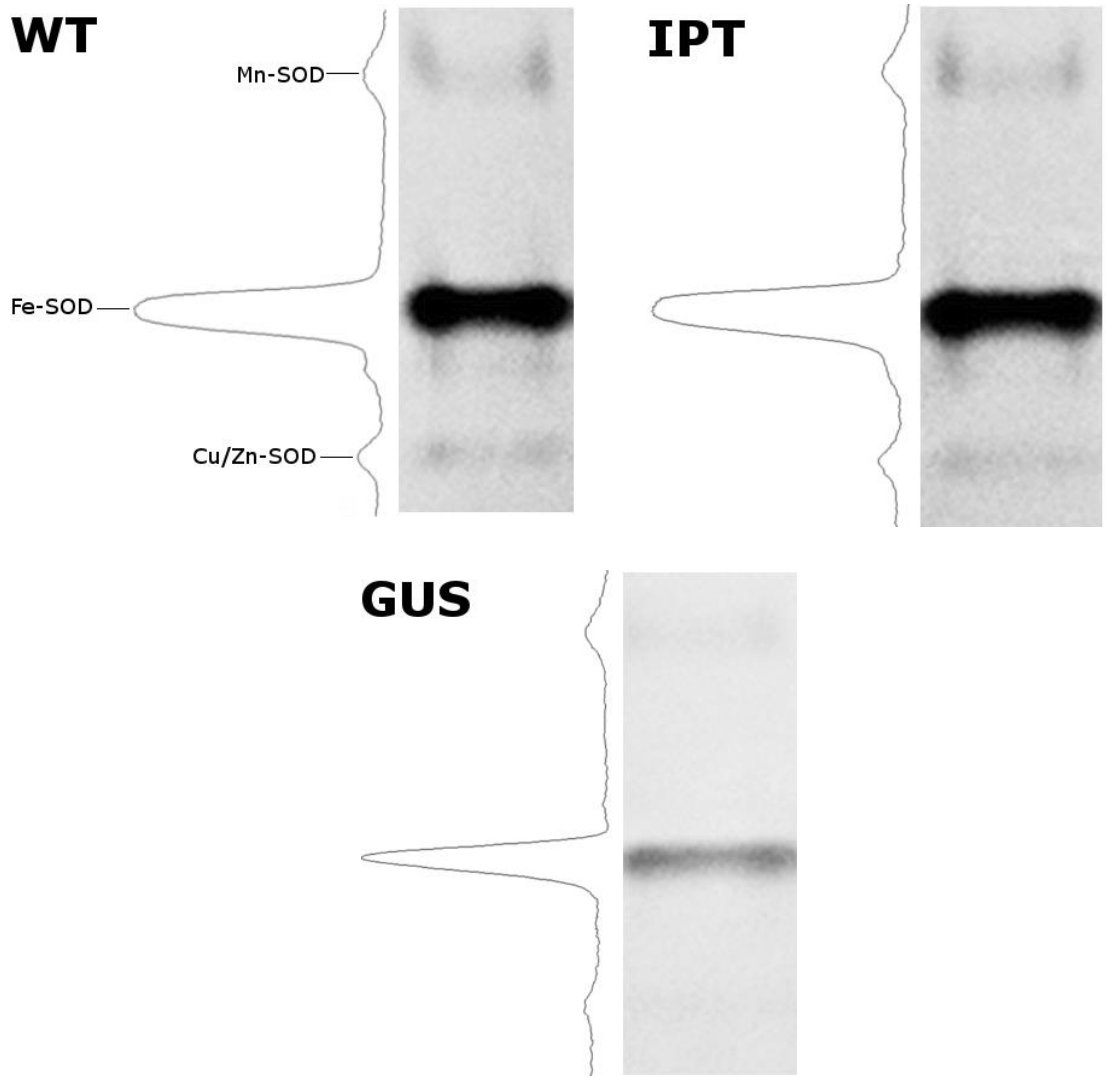



**Figure 1.** SDS-PAGE gel with Rubisco large (LSU) and small (SSU) subunits in leaf total soluble protein extracts of *Nicotiana tabacum* L.: WT, $P_{SAG12}$-*IPT* and $P_{SAG12}$-*GUS*. Plants were grown in normal or limited light conditions (DIS).

### 3. 4. Superoxide dismutase activity



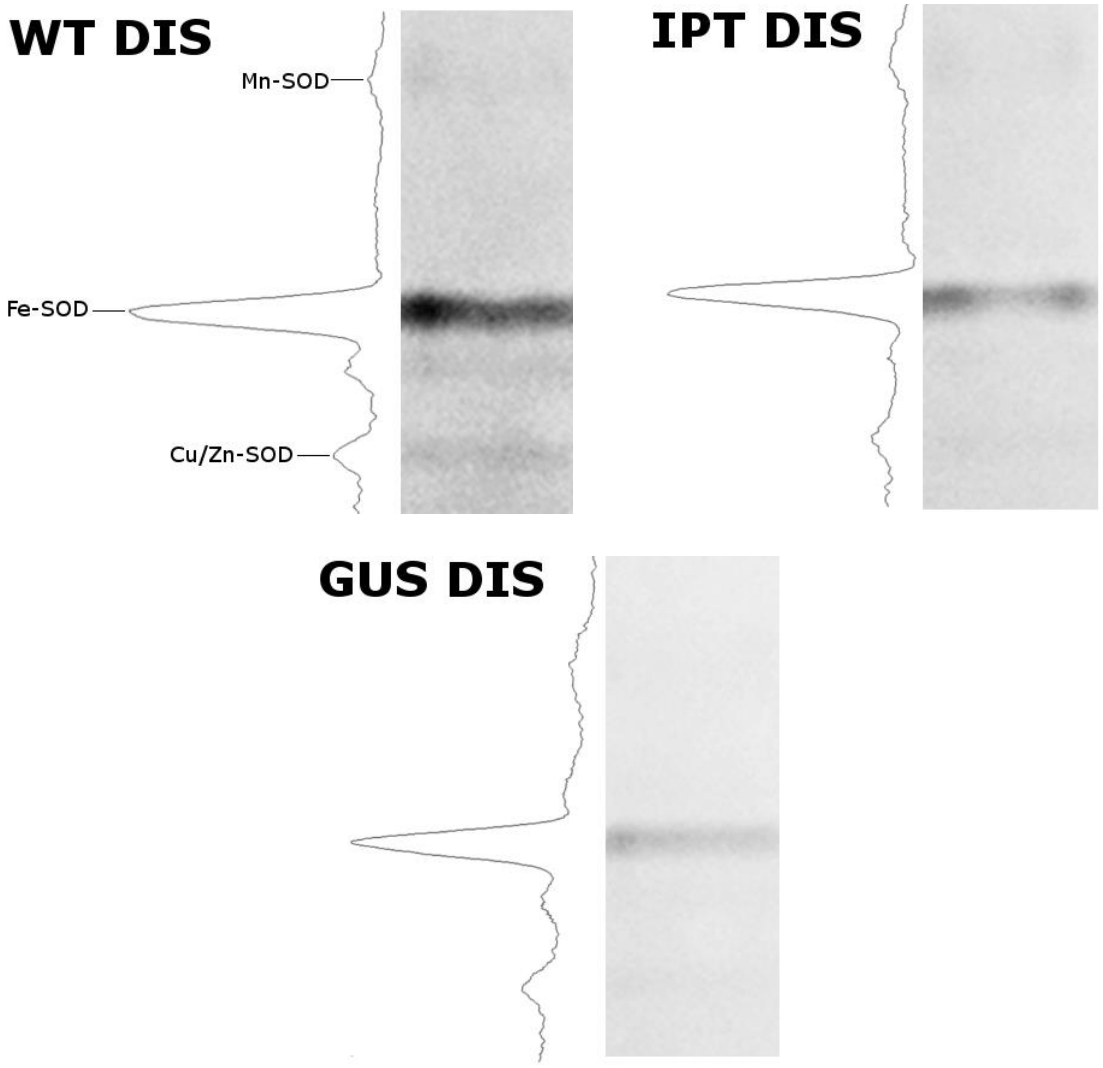



**Figure 2.** Identification of different SOD isoforms of the three genotypes of *Nicotiana tabacum* L.: WT, $P_{SAG12}$-*IPT* and $P_{SAG12}$-*GUS* on Native-PAGE gel (negative photo), and the corresponding densitograms. Plants were grown in normal or limited light conditions (DIS).

A number of studies have shown that the ageing process worked as a signal initiating changes of antioxidant enzymes activity, such as superoxide dismutase (Casano et al., 1994; del Río et al., 2003). Hence, it is useful marker to evaluate the level of oxidative stress in ageing plants. We analyzed the activity of three isoforms of superoxide dismutase, that catalyze superoxide anion scavenging in different cell compartments. Furthermore, based on previous data we assumed that activity of SOD may be modified by DIS and elevated cytokinin level. SOD isoforms were identified by Native-PAGE gel separation (Fig. 2) based on their different migration rate. Molecular mass of defined SOD isoforms were determined as 34, 48 and 82 kDa for Cu/Zn-, Fe-, Mn-SOD, respectively (Miszalski et al., 1998). Results of the densitometric analysis of singular SOD bands are presented in Table 1.

## 4. DISCUSSION

Leaf senescence has an important role in plant nitrogen metabolism and redistribution of limited mineral nutrients. The main source of remobilized nitrogen are chloroplasts, which accumulate most of plant proteins such as Rubisco, consider to be the most globally abundant protein in land biota (Raven, 2013). Proteins, tightly attached to inner membranes, as thylakoid protein complexes are utilized in later stages (Thomas et al., 2002). Concentration of total soluble protein assessed in control groups was approximately 17-18 mg $g^{-1}$ FW. However, significant drop in the protein abundance was noted in senescent leaves of all groups in the range of 73% to 84% for WT and $P_{SAG12}$-*IPT*, respectively. The higher overall concentration of the soluble proteins and more pronounced differences between tobacco genotypes (WT, $P_{SAG12}$-*IPT*) have been presented by Jordi et al. (2000). Possible explanation of this discrepancy is different mechanism used to induce the leaf ageing. Light deprivation, besides its ability to accelerate the onset of senescence, has also direct impact on gene expression. Signal, perceived by light receptor (phytochrome), activates photosynthetic- and chlorophyll-associated genes. Additionally, increased concentration of monosaccharides, may be also a potential factor inhibiting the expression of various proteins genes and attenuating the effect exerted by cytokinins (Wingler et al., 1998).

Molecular analysis has revealed that despite the leaf yellowing is obvious marker of advancing senescence, chlorophyll degradation is preceded by soluble protein disintegration. Mobilization of photosynthetic pigments is strictly related to the stability of thylakoid membrane complexes like LHCII (Thomas et al., 2002). Data obtained in this study showed that leaves of $P_{SAG12}$-*IPT* tobacco contained at averaged 2.4 mg of chlorophyll *a* and *b*. The lowest content has been found in WT. Nevertheless, after DIS treatment we noted significant decrease in pigments concentration especially in both transgenic genotypes of tobacco. The results are opposite to data obtained by Jordi et al. (2000), pointed that wild type is characterized by the highest amount of chlorophyll, which dropped very rapidly after the onset of the leaf ageing. Despite lower concentration of chlorophyll in $P_{SAG12}$-*IPT*, its level was almost unaffected by ageing. According to Wingler et. al. (2005) plants with stay-green phenotype caused by cytokinin anti-senescing action do not preserve photosynthetic activity, which starts to decline. Study stated that observed pigmentation is an effect of increased level of free chlorophyll fraction, released from the protein complexes. Mechanism of delayed senescence in response to enhanced cytokinin level may be therefore considered more as preventing chlorophyll degradation than sustaining the photosynthetic activity. Light absorbed by unbound chlorophyll molecules induces pigment excitation which is the main source of reactive oxygen species. Such action may cause premature cell death visualized by necrotic lesions on a leaf blade.

Ribulose-1,5-bisphosphate carboxylase/oxygenase, commonly known as Rubisco, is a key enzyme catalyzing process of $CO_2$ assimilation during Calvin-Benson cycle. The highest content of Rubisco LSU has been documented in $P_{SAG12}$-*IPT* control, the lowest in $P_{SAG12}$-*GUS* control. Concurrent results were obtained by Jordi et al. (2000) and Wingler et al. (1998). Interestingly, we have not found any correlation between reported increased level of LSU in *ipt* tobacco and total soluble protein concentration. Mechanism of artificial senescence induction, used in our study, is quick and reproducible method to mimic natural process both at physiological and molecular level. Nevertheless, light deprivation limits the formation of reactive oxygen species and reduces the risk of stress-related cell damages. According to

Sedigheh et al. (2011) oxidative stress, which occurs during the leaf ageing, is a key factor accelerating protein disintegration. It is postulated that ROS affect Rubisco by modifying its structure and facilitating degradation by proteases.

It is not quite clear if the formation of reactive oxygen species is a necessary condition to trigger the onset of the leaf ageing. Data obtained by Wingler et al. (2005) confirmed that antioxidant properties of senescent tissues start to decline, as a result of decreased activity of antioxidant enzymes like SOD. In this paper we documented negative influence of DIS on SOD isoforms activity in all tested tobacco groups. Activity of manganese and iron isoforms were reduced in all genotypes and only in $P_{SAG}$-*IPT* tobacco it was maintained at approximately 50% of initial value, which may be result of cytokinin-stimulated expression of SOD genes or reduce degradation of its active forms (Stoparić and Maksimović, 2008). The copper/zinc isoform retained the highest activity in $P_{SAG12}$-*GUS*. Similar results were obtained by Casano et al. (1994) of the barley leaves study. Comparison of relative activity of tested isoforms showed that the most active is Fe-SOD, localized in chloroplasts. It may be explained by higher, than in other cell compartments, rate of ROS formation. Moreover, as showed in Miszalski et al. (1998), chloroplasts are more sensitive to oxidative stress than mitochondria.

## 5. CONCLUSION

Study confirmed that auto-regulated synthesis of phytohormones in ageing-depended manner prevents abnormalities and prolongs lifespan of plants. Based on our results and data obtained in previous researches we postulate that protective action of cytokinins can be explained by their ability to alleviate the effect of reactive oxygen species by both: direct antioxidant properties of cytokinins and as a result of increased antioxidant potential of plant cells. Additionally, cytokinins reduce the rate of Rubisco degradation and inhibit early remobilization of nutrients from senescent leaves. Increased biosynthesis of cytokinins was however insufficient to inhibit chlorophyll and protein degradation in the way that has been reported in similar studies. It may be connected with the light deprivation as a method selected to induce senescence. Previous data showed that cytokinin ability to delay ageing is directly connected with light exposure, which is a potent regulator of photosyntethic-asscociated gene expression.

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