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The role of the cytokinin biosynthesis pathways in the rate of tobacco leaf senescence

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ABSTRACT

The regulation of leaf senescence depends on endogenous and exogenous factors, among them phytohormones like cytokinins (CKs). CKs are key players regulating the senescing process, as their endogenous concentration is linked to the onset and rate of senescence progression. Thus, this study aimed to identify the relationship between the activity of endogenous CKs biosynthesis pathways - the cytosolic mevalonate (MVA) and the plastid methyl-erythritol phosphate (MEP) and the rate of leaf senescence. To this end, three distinct tobacco (*Nicotiana tabacum* L.) cultivars – Xanthi, Golden Virginia and Monte Calme Yellow were analysed. The study involved treatment with exogenous CK – benzyladenine – and two different CK synthesis inhibitors: lovastatin and clomazone. The progression of senescence was induced by light deprivation and monitored with chlorophyll level (SPAD), photosynthetic activity (PAM) and changes in the Rubisco protein profile (SDS-PAGE). Analyses showed that the Xanthi cultivar was characterized by delayed onset of senescence and *stay-green* phenotype, while Golden Virginia, and particularly Monte Calme Yellow showed rapid leaf senescence. The studies provided valuable information regarding the role of MEP and MVA pathway of CK synthesis in the regulation of tobacco leaf senescence.

KEYWORDS: dark-induced leaf senescence, cytokinin biosynthesis, stay-green phenotype

Introduction

The final stage of a plant's life is senescence, which results in the ultimate death of and organ or whole organism. At the same time, however, during the ontogenesis, we observe that the rate of metabolic processes systematically decreases because of gradual aging. Environmental factors such as temperature, humidity and photoperiod influence the rate of senescence (Thomas,

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2013). Also, light, which is essential for the growth and development of plants, plays a crucial role. Prolonged reduction of light intensity or its complete deprivation are very efficient activators of leaf senescence. Therefore, dark-induced leaf senescence (DILS) assay is widely accepted as a rapid and effective method for synchronously inducing the onset of senescence (Guo *et al*., 2021).

Leaves are the most important assimilatory organs (donor-type organs) (Woo *et al*., 2013), thus their senescence is orchestrated and involves changes in the expression patterns of numerous genes as well as physiological responses. Consequently, the leaf's cellular structures and biological molecules are broken down in and ordered manner and remobilised to other, acceptor-type organs (Mayta *et al*., 2019). Moreover, the leaf senescence is related to the decrease in cytokinin (CKs) concentration and activity. Their application is effective in delaying chlorophyll breakdown and maintaining the integrity of chloroplasts (Hönig *et al*., 2018). Higher plants possess two pathways of CK biosynthesis, including the cytosolic mevalonate pathway (MVA) and the plastid-localized 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway. Clomazone and lovastatin are inhibitors of the MEP and MVA pathway, respectively.

Clomazone specifically inhibits 1-deoxy-D-xylulose-5-phosphate synthase (DXS), the enzyme catalysing the first step of the MEP pathway, while lovastatin is a specific inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), blocking the rate-limiting step of the MVA pathway (Kobayashi *et al*., 2007) (Fig. 1).

Thus, the aim of the study is to identify the relationship between the activity of endogenous CKs biosynthesis pathway of

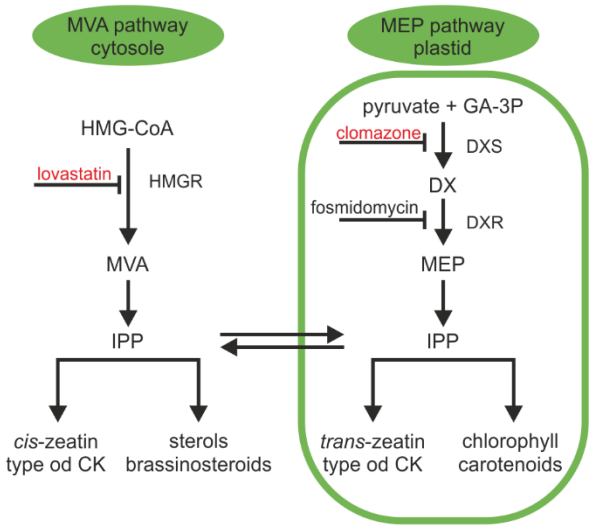

Figure 1. The alternative pathways of isoprenoid compound synthesis in plant cells are the cytosolic mevalonate pathway (MVA) and the plastidial methylerythritol phosphate pathway (MEP). The action of the applied inhibitors, lovastatin and clomazone, are indicated in red. MVA – mevalonic acid; MEP – 4-phosphate 2-methyl-D-erythritol; HMG-CoA reductase – 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMGR –3-hydroxy-3-methylglutaryl-CoA reductase; IPP – isopentenyl diphosphate; DXS – 1-deoxy-D-xylulose-5-phosphate synthase; DX – 1-deoxy-D-xylulose-5-phosphate; DXR – 1-deoxy-D-xylulose-5-phosphatereductoisomerase (according to Kobayashi *et al*., 2007; Añorga *et al*., 2020, modified).

MVA and MEP, and the rate of leaf senescence, to provide solutions to delay the onset of senescence. To this end, three distinct tobacco (*Nicotiana tabacum* L.) cultivars – Xanthi, Golden Virginia and Monte Calme Yellow were analysed. Tobacco is a plant belonging to the *Solanaceae* family, including such economically important species as tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.). In addition to tobacco industry application, tobacco is used as an important plant species in metabolic engineering and genetic manipulation research. Studies are being conducted on tobacco chloroplasts for their use in producing human proteins, while leaves are successfully utilized for the synthesis of recombinant proteins with significance in the pharmaceutical, medical, and cosmetic industries (Tsaballa *et al*., 2020). The study involved treatment with exogenous CK – benzyl-adenine – and two different CK synthesis inhibitors: lovastatin and clomazone. The progression of senescence was induced by light deprivation and monitored with chlorophyll level (SPAD), photosynthetic activity (PAM) and changes in the Rubisco protein profile (SDS-PAGE). Analyses showed that the Xanthi cultivar was characterized by delayed onset of senescence and *stay-green* phenotype, while Golden Virginia, and particularly Monte Calme Yellow showed rapid leaf senescence.

Methods

Plant material and growth conditions

The seeds of tobacco (*N. tabacum* L.) cultivars Xanthi (X), Golden Virginia (V) and Monte Calme Yellow (M) presenting different leaf pigmentation – dark (X) and pale (V and M), were sown into seedling trays with single-cell dimensions of 65 × 55 × 60 mm. Seedlings at the age of 20 DAS (*days after sowing*) were placed individually in P9 (9 × 9 × 10 cm) containers, filled with the substrate (white and black peat, perlite, and N:P:K = 9:5:10; pH 6.0–6.5), divided into groups, and transferred to environmentally controlled chambers and grown under LED RhenacM12 lamps (PXM, Podleze, Poland) delivering 200 µmol m^{-2} s^{-1} of the RGB spectrum (R:G:B; 661:633:520:434 nm) for next 25 days (Fig. 2).

Leaf senescence induction and chemical treatment

Leaf discs (10 mm in diameter) of each tobacco cultivar were cut off and analysed before treatment (day 0, "0") or incubated in Petri dishes over 72 h under the previous light regime, floating on water (negative control of senescence, "–") or without light (light deprivation) for dark-induced senescence (DILS) according to Sobieszczuk-Nowicka *et al*. (2018),

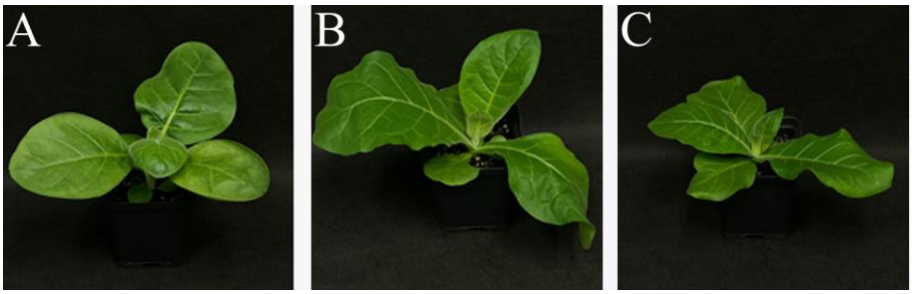

Figure 2. Morphology of 45-DAS plants of tobacco (*Nicotiana tabacum* L.) cultivars – Xanthi (A), Golden Virginia (B) and Monte Calme Yellow (C).

floating on water (positive control of senescence, "+") or with cytokinin 50 µM (benzyladenine, "B"), 50 µM lovastatin ("L") or 50 µM clomazone solution ("C").

Chlorophyll and photosynthetic activity analyses

Chlorophyll level was monitored with a non-invasive chlorophyll meter (SPAD-502, Konica Minolta, Tokyo, Japan). Photosynthetic activity was analysed with the chlorophyll a fluorescence induction curve analysis (IMAGING-PAM M-Series, Walz, Germany) and changes in the Rubisco protein profile with electrophoretic separation of proteins on a polyacrylamide gel (SDS-PAGE) stained with Coomassie Brilliant Blue R-250 Staining Solution according to Skowron and Trojak (2021) and quantified within densitometric analysis by ImageJ software (ImageJ v.1.53t., National Institutes of Health, Bethesda, USA).

Statistical analysis

Statistical analyses were performed using Statistica 13.3 software (StatSoft Inc., Oklahoma, OK, USA). The normal distribution of variables was verified using the Shapiro-Wilk test, and the equality of variances was evaluated using Levene's test. One-way ANOVA and post hoc Tukey's HSD tests were employed to analyse the differences between the investigated groups. The data are presented as mean with standard deviation (± SD). Statistical significance was determined at the 0.05 level ($p = 0.05$).

Results

Senescing leaf phenotype. Chlorophyll SPAD

It was demonstrated that the tested tobacco varieties exhibit different sensitivity to the applied compounds and rates of leaf senescence (Fig. 3).

The analysis of relative changes in chlorophyll content in leaves using the *leaf greenness index* (SPAD) for the Xanthi cultivar (X) shows SPAD value decreases after DILS compared to the control (day 0). It was noted that the

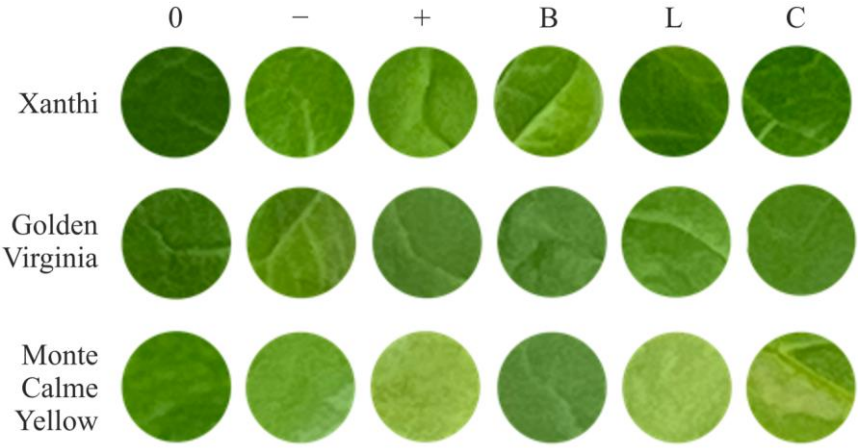

Figure 3. Phenotype observations of leaf discs cut out from 4th leaf of 45 DAS tobacco cultivars (*Nicotiana tabacum* L.): Xanthi, Golden Virginia and Monte Calme Yellow, analysed before treatment (day 0, "0") or incubated in Petri dishes over 72 h under the previous light regime (yellow area), floating on water (negative control of senescence, "−") or without light (light deprivation, grey area) for dark-induced senescence (DILS), floating on water (positive control of senescence, "+") or with cytokinin 50 µM (benzyladenine, "B"), 50 µM lovastatin ("L") or 50 µM clomazone solution ("C").

decrease in SPAD values for groups X–, X+, XB, XL, and XC amounted to 13%, 13%, 24%, 18%, and 8% respectively, relative to X0 (Fig. 4A). The SPAD analysis for the Golden Virginia cultivar (V) (Fig. 4B), like the Xanthi cultivar, indicated that chlorophyll content decreases in each treatment over the course of leaf senescence relative to day zero. However, this decrease was more pronounced, with respective reductions for V–, V+, VB, VL, and VC treatments of 35%, 27%, 22%, 31%, and 28%. The average SPAD values for groups V+, VB, VL, and VC did not exhibit statistically significant differences among them (Fig. 4B). In the case of the Monte Calme Yellow cultivar (M), the SPAD analysis (Fig. 4C) revealed that this cultivar is characterized by the lowest values at M0 compared to X0 and V0. Similar to the Xanthi and Golden Virginia cultivars, values decrease with leaf senescence in each case relative to plants at day zero. The most significant decrease (61%) was recorded for leaf disc incubation with lovastatin (Fig. 4C).

Photosynthetic activity

The effective quantum yield of photosystems II – Y(II), was measured with pulse amplitude modulation (PAM) chlorophyll a fluorometer. The analysis of the Y(II) confirmed that the most significant decrease in photosynthetic activity for Xanthi was recorded in the case of discs incubated with BA under light deprivation conditions (Fig. 5A). Statistically significant reduction in the Y(II) parameter values was noted in all groups compared to X0. For X–, X+, XL, and XC, this decrease was 13%, 25%, 39%, and 30%, respectively (Fig. 5A). In the case of Golden Virginia showed that Y(II) values of the parameter obtained in the case of the negative control V– showed 15% decrease compared to V0 (Fig. 5B). The greatest decrease in Y(II) was observed for treatment with clomazone - a decrease of 42%, and VL – a decrease of 38% compared to V0 (Fig. 5B). In the case of Y(II) in M+ plants, a decrease of 61% compared to M0 was noted. A significant decrease in Monte Calme Yellow photosynthetic activity

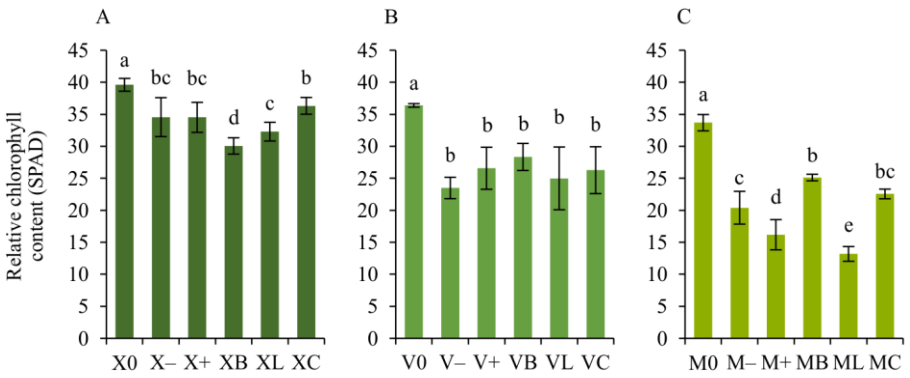

Figure 4. Analysis of relative chlorophyll content (SPAD) in leaves of tobacco (*N. tabacum* L.) cultivars – Xanthi (X) (A), Golden Virginia (V) (B) and Monte Calme Yellow (M) (C) at 45 days after sowing (DAS). 1/ "0" – material from day zero; 2/ "–" – negative senescing control, leaves kept under light in distilled water; 3/ "+" – positive senescing control, distilled water and 72 h dark incubation (DILS); 4/ "B" – 50 µM of benzyladenine (CKs) and DILS for 72 h; 5/ "L" – 50 µM lovastatin and DILS for 72 h; 6/ "C" – 50 µM clomazone and DILS for 72 h. Each bar represents the average ± SD of six independent measurements (n = 6). Different letters (a–e) indicate significant differences between treatments at p = 0.05 with a Tukey's HSD test.

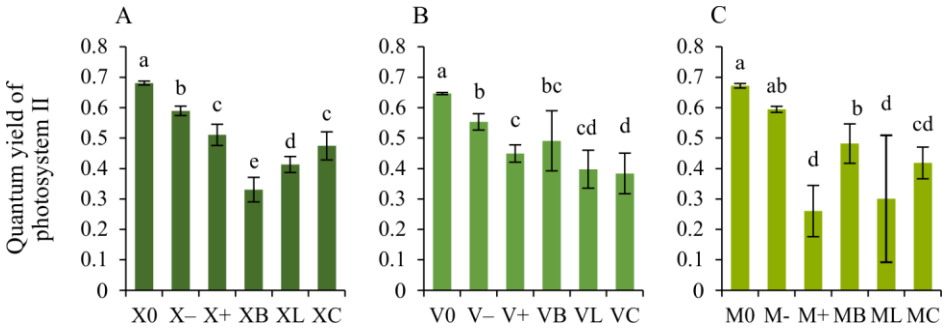

Figure 5. Analysis of photosynthetic activity with effective quantum yield of photosystem II – Y(II) in leaves of tobacco (*N. tabacum* L.) cultivars– Xanthi (X) (A), Golden Virginia (V) (B) and Monte Calme Yellow (M) (C) at 45 days after sowing (DAS). 1/ "0" – material from day zero; 2/ "–" – negative senescing control, leaves kept under light in distilled water; 3/ "+" – positive senescing control, distilled water and 72h dark incubation (DILS); 4/ "B" – 50 µM of benzyladenine (CKs) and DILS for 72h; 5/ "L" – 50 µM lovastatin and DILS for 72h; 6/ "C" – 50 µM clomazone and DILS for 72h. Each bar represents the average ± SD of six independent measurements (n = 6). Different letters (a–e) indicate significant differences between treatments at p = 0.05 with a Tukey's HSD test.

was also noted after incubation with lovastatin and clomazone. Conversely, incubation with BA exerted a protective effect on the activity of PSII despite DILS (Fig. 5C).

RuBisCO LSU and SSU

Densitometric analysis of the gel-separated protein bands (Fig. 6) revealed that the highest content of RuBisCO large (LSU) and small (SSU) subunit was noted in the control samples "0" of all cultivars tested. At the same time, leaf detachment and incubation under the previous light regime were very efficient factors in inducing senescence-related protein degradation (negative senescing control). Surprisingly, DILS incubation slowed down this process in Xanthi and Golden Virginia samples. The most effective for delaying leaf senescence was incubation with BA, which for LSU showed only a 5% lower level when compared to X0 and XB. In the case of the Golden Virginia cultivar, combined effects of light deprivation and treatment with clomazone, resulted in LSU and SSU decreases by 53% and 15%, respectively, when compared to V0 (Fig. 6). We also showed that in Monte Calme Yellow cultivar both DILS as well as clomazone treatment decrease LSU by 66%, whereas for SSU by 47% and 41%, respectively.

Discussion

The study presents preliminary results of investigation of the impact of exogenous cytokinin – benzyladenine, as well as inhibitors of CKs' alternative synthesis pathways – lovastatin and clomazone, on the leaf senescing process of *N. tabacum* L. cultivars: Xanthi, Golden Virginia, and Monte Calme Yellow induced by light deprivation (DILS). Surprisingly, the study revealed that leaf detachment and subsequent incubation under light was an even more effective factor to induce the onset of senescence than DILS itself. Similarly, Zhao *et al*. (2012) showed that mechanical leaf detachment from the plant was a sufficient factor to induce the senescing rate of wheat leaves (*Triticum aestivum* L. var. Shiluan 02-1). This phenomenon can be explained by the decrease in endogenous CK levels, which are no longer exported from the parent plant to

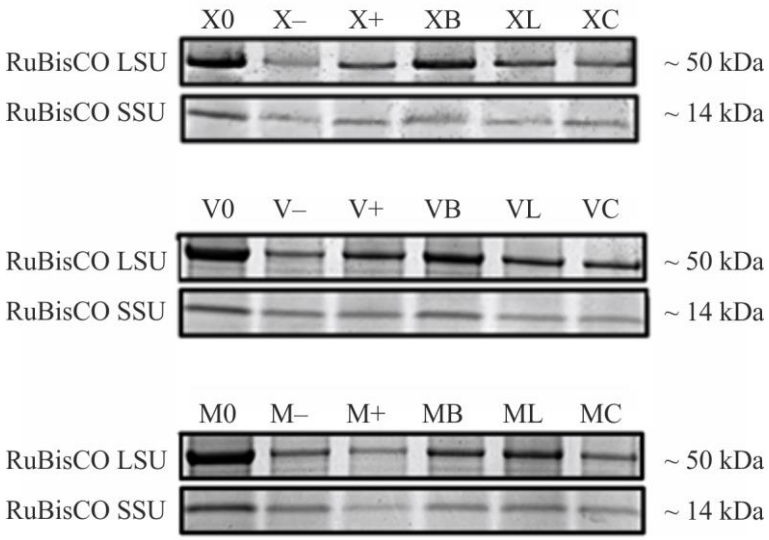

Figure 6. Analysis of total soluble proteins resolved in polyacrylamide 4–20% TGX gel stained with Coomassie Brilliant Blue reagent. The large (LSU) and small (SSU) subunits of RuBisCO isolated from tobacco (*N. tabacum* L.) leaves of Xanthi (X), Golden Virginia (V) and Monte Calme Yellow (M) at 45 days after sowing (DAS). 1/ "0" – material from day zero; 2/ "–" – negative senescing control, leaves kept under light in distilled water; 3/ "+" – positive senescing control, distilled water and 72h dark incubation (DILS); 4/ "B" – 50 μM of benzyladenine (CKs) and DILS for 72h; 5/ "L" – 50 μM lovastatin and DILS for 72h; 6/ "C" – 50 μM clomazone and DILS for 72h.

leaf tissues due to mechanical separation (Janečková *et al*., 2018).

The actual effect of clomazone on leaf senescence induced by light deprivation and/or mechanical damage, and the identification of cultivar-specific sensitivity, was realized by XC, VC, and MC comparison within corresponding X+/X–, V+/V–, and M+/M– treatments. These additional controls indicate that among the analysed cultivars Xanthi shows low sensitivity to clomazone treatment, while more clomazone sensitive is Golden Virginia and Monte Calme Yellow. Results are consistent with previous reports. Darwish *et al*. (2015) documented that the Xanthi cultivar exhibits greater tolerance to clomazone compared to the Golden Virginia type variety (Virginia vk51). In the case of the Monte Calme Yellow, although there are no direct literature analyses specific to this cultivar, studies have been conducted on the same tobacco group – Burley (Yellow Burley). Li *et al*. (2017) and Li *et al*. (2021) demonstrated that Burley group varieties, such as Monte Calme Yellow, are characterized by a light green leaf colour, resulting from a chloroplastic mutation leading to a reduction in photosynthetic pigment content – both carotenoids and chlorophyll *a* and *b*. This formed the basis for selecting this variety for analysis due to the application of selective inhibitors of MEP and MVA pathways, which, besides CK synthesis, are responsible for the synthesis of photosynthetic pigment precursors. However, results show that Monte Calme Yellow at day zero control showed a similar pigments concentration to Golden Virginia, due to enhanced nitrogen fertilization. Yet cultivar is still more

sensitive to pigment loss under the senescing progression.

Conclusions

Preliminary results of our study shows that the photosynthetic activity of the studied tobacco cultivars is not directly correlated with the leaf senescence pattern. The Xanthi exhibits the highest resistance, while Monte Calme Yellow shows the lowest resistance to factors inducing leaf senescence – mechanical detachment and light deprivation. Secondly, tobacco cultivars classified as bright types (Golden Virginia and Monte Calme Yellow) present faster progression of leaf senescence compared to cultivars with darker leaf pigmentation (Xanthi), as indicated by the SPAD analysis. All analysed tobacco cultivars exhibit different sensitivity to clomazone, lovastatin, and benzyladenine, which is significant in the context of further research. For the Xanthi, which displayed *stay-green* phenotype trait, leaf incubation with exogenous cytokinin resulted in decreased photosynthetic activity, yet this variety was insensitive to MVA and MEP pathway inhibitors. Sensitivity to clomazone was confirmed for the Golden Virginia, while a delay in leaf senescence was observed for the Monte Calme Yellow following lovastatin application.

Acknowledgements

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