The role of carotenoids in higher plants ontogenesis and adaptation to stress conditions

Summary of Ph.D. Thesis

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1. Introduction

Carotenoids are the most important group of natural pigments in the plant and animal kingdoms. Plant carotenoids are red, orange, and yellow lipid-soluble pigments embedded in the membranes of chloroplasts and chromoplasts. Their colour is masked by chlorophyll in photosynthetic tissues, but in late stages of plant ontogeny these pigments contribute to the bright colours of many leaves, flowers, fruits and roots. The greatest production of carotenoids occurs in the photosynthetic tissues. β-carotene was firstly isolated in 19th century from plant material, and after short time many other photosynthetic carotenoids were identified.

Carotenoids are generally C₄₀ terpenoid compounds formed by the condensation of eight isoprene units. At the centre of the molecule, the linkage order is reversed, so the molecule as a whole is symetric. A set of conjugated double bonds is responsible for the absorption of light in the visible region of the spectrum. The ends of molecules are often cyclised to ionone rings with functional substituents, for example hydroxy-, epoxy-, keto- and carboxy- groups. In plants, two groups of carotenoids are differentiated – carotenes without substituents in ionone rings and xanthophylls with hydroxy- or epoxy- groups.

Plant carotenoids play fundamental roles as light-harvesting pigments for photosynthesis, protectors against photoinhibition, are structural determinants in plastid pigment-protein complexes, and precursors of abscisic acid.

Light-harvesting function is necessary for the absorption of light in the blue region of the spectrum. The absorbed energy can be transferred to the chlorophylls. In higher plants, mostly lutein and neoxanthin fulfil this function.

Protective function of carotenoids is in two different levels. In plants and algae, the most important function of β-carotene in the core complexes of the reaction center is the protection of photosystems against photooxidation by quenching the excitation energy of the chlorophyll in triplet state or singlet oxygen. The second protective mechanism in higher plants is xanthophyll cycle, important process in thylakoids membranes. During stress conditions, deepoxidation of light-harvesting carotenoid violaxanthin to zeaxanthin via antheraxanthin is activated. These enzymatic reactions are connected with thermal dissipation of excess light energy within light harvesting antenna proteins usually measured as non-photochemical quenching of chlorophyll fluorescence.

Structural function in pigment-protein complexes is typical for all carotenoids presented in higher plants and it consists in stabilisation of photosynthetic components. Pigments induce conformation changes on pigment-protein complexes of photosynthetic apparatus and without carotenoids they cannot function properly. Every carotenoid has its accurate placement with specific functions, for example lutein and neoxanthin in light-harvesting complexes or β-carotene in reaction core of photosystems.
**Abscisic acid biosynthesis.** The carotenoids neoxanthin and violaxanthin are precursors of abscisic acid, the plant hormone that participates in the control of water relations in plants and in many other physiological processes.

In spite of huge amount of literature concerning carotenoids, which appeared from time of their discovery till now, many unsolved questions still remain. In the presented Ph.D thesis I tried to throw light on a few of them.
2. Aims

The goal of this Ph.D. thesis was to elucidate the roles of carotenoids during ontogeny of plants in different environmental conditions and in response to stresses. The research was aimed to answer the following questions:

1) How do carotenoid contents and functions change during in vitro cultivation and after ex vitro transfer?
2) Is the presence of sucrose in medium necessary for sufficient carotenoids biosynthesis?
3) How do in vitro cultivation conditions (irradiance, sucrose and CO₂ concentration) affect ability of plants to ex vitro acclimation?
4) Can carotenoids sufficiently protect plants against photoinhibition during transfer from low irradiance in vitro to considerably higher irradiance ex vitro?
5) Can carotenoids sufficiently protect plants against photoinhibition during water stress?
6) Can be contents and function of carotenoids affected by phytohormones such as abscisic acid and cytokinins?
7) Can increased production of cytokinins in transgenic plants affect contents and function of carotenoids?
3. Methods

Plant material

Tobacco (*Nicotiana tabacum* L.) was a model plant species used in the most of experiments. Obtained results were proved on sugar beet and French bean (*C₃* plants) and maize (*C₄* plant). Cultivation details are presented in individual papers and briefly mentioned in the survey of results.

HPLC analysis of photosynthetic pigments

Photosynthetic pigments were extracted twice from leaf discs (6 cm²) with acetone and after centrifugation both clear supernatant were mixed. After drying under gas nitrogen, samples were resolved in 0.3 cm³ of acetone and analyzed by HPLC (firstly by old and later by new configuration).

**Old HPLC** was from *Spectra-Physics* (San Jose, USA) and contained absorption detector *Spectra 100*, gradient pump *SP8800* and integrator *ChromJet*. Reversed-phase column (*Sepharon SGX C18*, 5 μm particle size, 150 mm × 3 mm, Tessek, Prague, Czech Republic) were used for analysis. The solvent system was acetonitrile/methanol/water (80:12:6) followed by 100 % methanol, and the gradient was run from 8 to 12 min. The flow rate was 1 cm³ min⁻¹, the detection wavelength 445 nm. Total time of chromatography was 30 min.

**New HPLC** is from *ECOM* (Prague, Czech Republic) and contains absorption detector *Sapphire*, gradient pump *Beta10* and autosampler *HTA300*. Reversed-phase column (*Watrex Nucleosil 120-5-C18*, 5 μm particle size, 125 mm × 4 mm, *ECOM*, Prague, Czech Republic) was used for analysis. The solvent system was acetonitrile/methanol/water (80:12:10) followed by methanol/ethylacetate (95:5), and the gradient was run from 2 to 6 min. The flow rate was 1 cm³ min⁻¹, the detection wavelength 445 nm. Total time of chromatography was 25 min.

By both methods we obtained following data:

contents of chlorophyll *a* and *b*, carotenoids β-carotene, antheraxanthin, lutein, neoxanthin, violaxanthin and zeaxanthin, and degree of xanthophyll cycle pigments de-epoxidation
4. Results

4.1. Carotenoids content during plant *in vitro* growth and after *ex vitro* transfer

The influence of irradiance and presence of sucrose in medium

*Nicotiana tabacum* L. cv. Samsun plantlets were grown *in vitro* photoautotrophically (0% sucrose) and photomixotrophically (3% sucrose) at two different irradiances (LL; 60 \(\mu\)mol m\(^{-2}\)s\(^{-1}\) and HL; 200 \(\mu\)mol m\(^{-2}\)s\(^{-1}\)) to investigate the effect of these cultivation conditions on photosynthetic performance and growth. Plants were cultivated *in vitro* for 42 days.

The content of \(\beta\)-carotene decreased during ontogeny (from day 14 to day 42) more markedly in plantlets grown under HL. The influence of sucrose was not significant. The content of light-harvesting carotenoid lutein slightly increased up to 36 d of cultivation and further decreased. The content of second light-harvesting carotenoid neoxanthin increased during the whole *in vitro* cultivation in HL plantlets grown on medium with sucrose, but decreased at the end of cultivation in variants without sucrose. The content of xanthophyll cycle pigments (antheraxanthin, violaxanthin and zeaxanthin) was almost constant in plantlets grown under LL, only at the end of cultivation slightly decreased. Content of xanthophyll cycle pigments was markedly higher in plantlets grown under HL than under LL but decreased from day 14. The de-epoxidation state of xanthophyll cycle pigments (DEPS, \([(\text{zeaxanthin} + 0.5 \text{ antheraxanthin})/\text{zeaxanthin} + \text{antheraxanthin} + \text{violaxanthin}]\), which is usually connected with thermal dissipation of the excess of light energy, was markedly higher in plantlets grown under HL than under LL and mostly decreased during ontogeny in all variants. These results proved protective function of carotenoids under higher irradiance. The presence of sucrose in medium was not prerequisite for sufficient carotenoid biosynthesis.

Acclimation of *in vitro* grown plants to *ex vitro* conditions

Plantlets grown *in vitro* for 35 d under above mentioned conditions (HL, LL, with or without sucrose) were transferred into pots with soil and grown for 7 d under two different irradiances (200 and 700 \(\mu\)mol m\(^{-2}\)s\(^{-1}\)).

After *ex vitro* transfer, the content of \(\beta\)-carotene did not change considerably in plantlets grown on medium with sucrose, while it increased in plantlets grown without sucrose. Contents of lutein and neoxanthin decreased first day and then increased up to day 7 in plantlets grown on medium with sucrose and only increased in those grown on medium without sucrose. The content of xanthophyll cycle pigments increased during 7th d *ex vitro*
cultivation in plantlets grown under LL more in those grown *ex vitro* under irradiance 700 \( \mu \text{mol m}^{-2}\text{s}^{-1} \) than 200 \( \mu \text{mol m}^{-2}\text{s}^{-1} \). On the other hand, content of xanthophyll cycle pigments in plantlets grown under HL and with sucrose did not change at irradiance 200 \( \mu \text{mol m}^{-2}\text{s}^{-1} \) and only slightly increased at 700 \( \mu \text{mol m}^{-2}\text{s}^{-1} \), while dramatically increased in those grown without sucrose.

These results showed the influence of previous *in vitro* conditions on pigments content during *ex vitro* acclimation in addition to direct impact of *ex vitro* conditions.

### The influence of CO₂ on carotenoids content

Contents and functioning of photosynthetic pigments in *Nicotiana tabacum* leaves were also studied in plantlets cultivated under different CO₂ supply. The plantlets were grown *in vitro* for six weeks either in glass vessels tightly closed with aluminium foil (G-plants) or in polycarbonate Magenta GA-7 vessels covered with microporous vents (M-plants).

Photosynthetic pigment contents were higher (Chl \( a \) by about 36 \%, Chl \( b \) 30 \%, \( \beta \)-carotene 33 \%) in leaves of M-plants that in leaves of G-plants. The differences might be caused by better CO₂ supply combined with slightly higher irradiance of M-plants. This corresponded also with the higher Chl \( a/b \) ratio in M-plants, which meant that smaller light-harvesting complexes containing Chl \( b \) were needed for energy capture. The contents of lutein and neoxanthin were not significantly different, but contents of xanthophyll cycle pigments were lower by 30 \% in M-plants than in G-plants. Even if also DEPS was slightly higher in G-plants than in M-plants its value did not indicate any photoinhibition.

### The influence of CO₂ and abscisic acid application on acclimation plants to *ex vitro* conditions

In first set of experiments, *Nicotiana tabacum* L. plants grown *in vitro* were transferred to *ex vitro* conditions and grown for 28 d in a greenhouse under normal CO₂ concentration (CA, 650 mg m\(^{-3}\)) or elevated CO₂ concentration (CE, 1200 mg m\(^{-3}\)). During *ex vitro* acclimation, contents of carotenoids increased only in CA plants. Consequently, CE plants had lower contents of \( \beta \)-carotene, lutein, neoxanthin and xanthophyll cycle pigments, but higher DEPS.

In further experiments we used plants precultivated *in vitro* under low (G-plants) and normal (M-plants) concentration of CO₂ for 6 weeks. Then the plantlets were transferred into pot with sand moistened either with water or 5 \( \mu \text{M} \) solution of abscisic acid (ABA). Plants were grown *ex vitro* under normal (CA) or elevated (CE) CO₂ concentration. Higher contents of Chl \( a \) and Chl \( b \) were found in leaves of M-plants than in leaves of G-plants also after *ex*
vitro transfer, while higher total carotenoid content in M-plants was observed only in plants treated by ABA. During further ex vitro growth the differences between M- and G-plants disappeared. The content of xanthophyll cycle pigments was higher in G-plants than in M-plants grown in vitro and similar differences were found also after ex vitro cultivation. Their content was always lower under CE than under CA and lowest in M-plants treated with ABA and grown under CE. On the other hand, DEPS was similar in G- and M-plants, and higher under CE than under CA.

4.2. Carotenoids during water stress and consequent rehydration

The influence of abscisic acid and benzyladenine on photosynthetic pigments during water stress and rehydration

During water stress, carotenoids play protective function as well as serve as abscisic acid precursors. Within the study of the role of phytohormones in responses of plants to water stress, we studied the influence of ABA and benzyladenine (BA) on photosynthetic pigments in four plant species (Phaseolus vulgaris, Nicotiana tabacum, Beta vulgaris and Zea mays) during water stress and consequent rehydration. 100 μM ABA or 10 μM BA was added to substrate before development of water stress, control plants were treated only with water. The total carotenoid content was the highest in Zea mays and the lowest in Phaseolus vulgaris. In control plants, the content of carotenoids decreased during water stress and consequent rehydration. On the other hand, in ABA and BA treated plants content of carotenoids increased during water stress and decreased after rehydration to the level before stress. The content of lutein and neoxanthin in control plants decreased during water stress and this trend continued for lutein after rehydration, while content of neoxanthin increased after rehydration. The content of neoxanthin decreased in plants treated with ABA and BA with exception of BA-treated tobacco plants.

Contents of xanthophyll cycle pigments increased during water stress in plants pre-treated with ABA or BA more than in those pre-treated with water. This might be important for their protection against photoinhibition under conditions of more severe water stress or higher irradiance. The highest increase was observed in ABA pre-treated maize. DEPS increased during water stress in all plant species in plants pre-treated with water and the highest increase was observed in maize. Increase of DEPS in plants pre-treated with ABA or BA was observed in bean, beet and tobacco. After rehydration, DEPS remained high in all plants pre-treated with water, but decreased in BA pre-treated bean and ABA or BA pre-treated sugar beet and tobacco.

The results obtained confirmed our hypothesis about positive effect of ABA and BA pre-treatment on response of plants to water stress and protection of photosynthetic apparatus.
The influence of trans-zeatin-\textit{O}-glukosyltransferase gene expression on carotenoid contents during water stress of transgenic plants

For further study of cytokinins and ABA interactions we used plants with increased endogenous level of both of these hormones. Endogenous content of cytokinins was reached in transgenic tobacco plants with trans-zeatin-\textit{O}-glukosyltransferase gene under constitutive (35S:ZOG1) or senescence-inducible promoter (SAG12:ZOG1). The level of endogenous ABA was increased by water stress. We compared leaves of different insertion level (upper, middle and lower) before water stress, 7 days lasting stress and 7 days after rehydration of both transgenics and wild type (WT) plants.

The total carotenoid content exhibited gradual increase during the stress progression in all tobacco plants and gradual decrease was found upon rehydration. In \textit{SAG}:ZOG and even more in 35S:ZOG plants, increase in carotenoid content was postponed and lower in comparison with WT. Also carotenoid content decline after rehydration was slower. The changes in \(\beta\)-carotene content were similar to those of total carotenoids with the exception of its more rapid degradation in lower leaves of transgenics than in lower leaves of WT. In WT, slight increase of lutein and neoxanthin contents was found at mild stress. During stress progression, an increase of lutein content occurred in upper and middle leaves, while in lower leaves a decrease of lutein content was found from the third day of stress. Upon rehydration both pigments decreased in WT middle leaves. In \textit{SAG}:ZOG as well as in 35S:ZOG plants increase of lutein content was delayed. Neoxanthin content was higher in both transgenics than in WT. Upon rehydration decrease of lutein and neoxanthin contents were slower in both transgenics than in WT. In WT the content of xanthophyll cycle pigments gradually increased during stress development and decreased after rehydration. In both transgenic plants the changes were less expressive with exception of marked elevation of xanthophyll cycle pigments in 35S:ZOG plants after 7-d dehydration. DEPS increased markedly 3 d and 7 d after cessation of watering and slightly decreased after rehydration in all tobacco plants. The highest values were found in lower leaves of \textit{SAG}:ZOG plants (3-d stress) and middle leaves of 35S:ZOG (7-d stress). With exception of lower leaves, DEPSC at moderate stress was lower in both transgenics than in WT and in middle leaves also after rehydration. The results obtained showed that both transgenics coped better with water stress.
5. Conclusions

On the basis of results obtained and presented in this Ph.D. thesis, we can answer the question setting in “Aims“:

1. During ontogeny of plants grown in vitro, the content and function of carotenoids changed in dependence on cultivation conditions (irradiance, presence of sucrose in medium, CO₂ concentration). These cultivation conditions affected carotenoid pattern also after ex vitro transfer.

2. Protective function of carotenoids was positively affected by sucrose in medium under higher irradiance but not under low irradiance.

3. Higher irradiance in combination with sucrose in cultivation medium had positive effect on carotenoid contents in beginning of ex vitro acclimation.

4. Changes in carotenoids content and function after ex vitro transfer were dependent on previous in vitro cultivation as well as on conditions during ex vitro growth. Very important were irradiance and CO₂ concentration. The shift of function of carotenoids from light-harvesting to protective enabled that photoinhibition was not observed after ex vitro transfer.

5. The content of xanthophyll cycle pigments and their de-epoxidation state increased during water stress. Also in these experiments carotenoids protected plants against photoinhibition damage.

6. Biosynthesis of carotenoids including xanthophyll cycle pigments during water stress was stimulated by abscisic acid and benzyladenine application and thereby protect of plants against photoinhibition.

7. Higher content of cytokinins in transgenic plants influence the content and composition of carotenoids and their changes during ontogeny.
6. List of original publications, on which this Ph.D. thesis is based

Poster 1


Publication 1


Publication 2


Publication 3


Publication 4


Publication 5


Publication 6


Publication 7


Publication 8

7. List of original publications of the author


