The role of antioxidants in short-term acclimation of leaves to the changing light environment

Summary of PhD thesis

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INTRODUCTION

Plants have high adaptive potential to acclimate to different light regimes, that involves the protection against the potential hazardous effects of high photosynthetically active radiation (PAR) and ultraviolet (UV) radiation reaching the Earth's surface (290-400 nm). Although these sunlight components are essential or beneficial for the development and life of plants as energy (PAR) or information source (PAR and UV), these can manifest as oxidative stressors. Acclimation processes are aiming at avoiding these harmful effects mainly by maintaining the prooxidant-antioxidant balance.

Plants adapt to fluctuating environmental conditions primarily by preserving their photosynthetic capacity, since light energy can be damaging when absorbed but not used in photochemistry. High intensities of PAR can lead to the light-induced inhibition of photosynthesis, photoinhibition, a process characterized by the increase in the concentrations of reactive oxygen species (ROS) in plant cells due to various mechanisms. ROS are highly reactive and potentially harmful molecules derived from molecular oxygen. During photoinhibition, from the components of the photosynthetic electron transport chain in the thylakoid membrane of chloroplasts, electrons can be transferred to molecular oxygen resulting in the formnation of superoxide radical ($O_2^{\bullet-}$), that can be further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). In another way, by transferring energy from the triplet excited state of chlorophyll to oxygen, singlet oxygen ($^1O_2$) can be formed.

ROS can generate further reactive radical or non-radical species (prooxidants) in a chain reaction, leading to the degradation of the phosystems, proteins and cell components, and can lead to lipid peroxidation, and by provoking oxidative stress, finally to cell death.

Plants are equipped with efficient mechanisms for preventing elevated ROS formation and oxidative stress caused by changing light environment. or for scavenging these species. The former, preventive defense is mainly achieved by dissipating excess energy through alternative pathways. The scavenging of ROS is realized through the action of the antioxidant system, comprising of a co-operative, multilevel system of enzymes and non-enzymatic compounds. While levels of $O_2^{\bullet-}$ and H_2O_2 is primarily regulated by enzymes (superoxide dismutases and peroxidases), most ROS are not aimed by specialized enzymes and therefore these are neutralized by a set of antioxidant compounds. The most significant of these compounds are glutathion, ascorbate, carotenoids, tocopherols (E-vitamins), B6-vitamins and the wide group of phenolic compounds, including flavonoids.

Indeed the balance between prooxidants and antioxidants form the redox homeostasis of cells, that regulates the metabolic and developmental processes and is the key factor in acclimatory response to environmental cues. Stressors directly or indirectly, or through triggering enhanced ROS production can induce antioxidative defense responses, thereby promoting the installation of a new balance leading to acclimation to the changing (light) environment.

The other component of sunlight, UV-radiation has an influence on photosynthesis, morphology and metabolism of plants as well. High doses (and mainly without PAR background) of UV-B (290-315 nm) can lead to direct ROS production ($^{\bullet}OH$, $O_2^{\bullet-}$) and oxidative stress. However, lower UV-doses supplementing visible light can participate in the induction of acclimatory responses through inducing the antioxidant system or by signal transduction processes possibly mediated by low ROS concentrations.

When plants are exposed to serious stress effects, their pro- and antioxidant relations are clearly disrupted, and the process of oxidative stress, i.e. the toxic overproduction of free radicals can be followed by detecting the oxidized reaction products or by detecting the primarily induced ROS forms directly. In case of acclimation, i.e. when the increase in antioxidant concentrations or activities keep pace with ROS production, monitoring the antioxidants can serve as a clue on the state of the system.

In our experiments we were aiming at elucidating the role of this antioxidant system in the acclimation of plants to changing light environment in artificially adjusted greenhouse or naturally occurring sunlight conditions. The antioxidants were characterized by total antioxidant capacities (TAC), like Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP) and total phenolics content (TPC). ROS-specific antioxidant capacities (SAC) were analysed as well, to gain a more comprehensive insight of changes in the antioxidant system. To achieve this, we employed the widely used test for superoxide scavenging activity, in which the radical is created in an artificial system in a chemical reaction, and the radical neutralizing activity of the added extract is determined by its inhibition effect on the ROS-induced bleaching of a dye. In analogy with this method, we applied antioxidant capacity tests for hydroxyl radical and singlet oxygen scavenging adapted to plant extracts in our laboratory. Further, we analysed the role of the compounds referred to as „UV-B absorbing pigments" in the literature and carotenoids in the acclimation processes as well.

AIMS

Our experiments were aimed at dissecting the antioxidant content of plants grown under artificially controlled greenhouse light conditions or natural sunlight to analyse its role in acclimation to different light conditions. Acclimation to low, close to ambient (and PAR supplemented) UV-radiation was studied in relation to leaf antioxidant contents. Experiments were carried out with different plant material (grapevine, tobacco, linden) and under various light conditions (greenhouse and natural sunlight) to gain a more comprehensive insight on the complex relations between antioxidant content changes and acclimation to the light environment.

The main goal of our work was to study how antioxidant contents described by total and ROS-specific antioxidant parameters eflect the plant's light acclimation potential. To achieve this, we aimed to answer the following questions:

1. How sensitive are the different TAC and SAC parameters to the antioxidant changes accompanying different physiologial states of leaves?
2. What correlations describe the relations between TAC and SAC parameters and physiological changes attending naturally (senescence, age) or artificially (high light, UV-radiation) induced changes in plant physiological states?

   We were elucidating this question in two approaches:

   a) by analysing whether the responses of greenhouse grown plants differ for supplemental UV-radiation due to age-related differences in leaf antioxidants,

   b) and by investigating whether the UV-acclimation potential can be improved by changing antioxidant content of leaves (with high light pretreatment) prior to the UV-treatment.
3. What is the role of flavonoids, a group of UV-absorbing pigments, and ROS-scavenging in acclimation to natural sunlight conditions?

METHODS

Greenhouse grown tobacco (*Nicotiana tabacum* L. cv. Petite Havana) and grapevine (*Vitis vinifera* L. cv. Chardonnay) were used as plant material. Senescence related effects were studied on nonstressed tobacco plants. During UV-treatments biologically effective daily UV-doses corresponded to 8,95 kJ m^{-2}, centered in the UV-B region (8,04 kJ m^{-2}); this was applied for 4 days in case of grapevine and 6 days in case of tobacco. For high light pretreatment tobacco were kept for 6 days under 5-fold higher light intensities than growth light. To study acclimation to natural sunlight sun and shade leaves of naturally grown (Szeged) linden tree (*Tilia platyphyllos* Scop.) were used.

Light acclimation of plants in physiological terms was characterized by their photosynthetic performance, measuring CO_2-fixation (LI-6400 gas analyser). Photosynthetic electron transport and energy dissipation pathways were studied by measuring variable chlorophyll fluorescence (Imaging-PAM).

Pigments were determined by spectrophotometric methods. Carotenoids were extracted in acetone and UV-B absorbing pigments were determined by measuring sum absorption between 280-315 nm after extraction in acidified methanol.

Leaf antioxidant capacities were characterized by total and ROS-specific antioxidant capacities. TAC assays were carried out from water extracts of leaves. Total phenolics content was determined by the Folin-Ciocalteu reagent. Trolox equivalent antioxidant capacities described the extracts ability to neutralize an artificial radical (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), $ABTS^{\bullet+}$). Ferric reducing antioxidant power was measured by the ability of the extract to reduce Fe(II)-TPTZ (2,4,6-tripyridyl-s-triazine) complex.

Specific antioxidant capacities were carried out from water (grapevine, tobacco) and methanolic (linden) extracts of leaves. Superoxide scavenging ability was based on measuring the antioxidant ability of the extracts to prevent the $O_2^{\bullet-}$-induced bleaching of a dye (nitro blue tetrazolium). $O_2^{\bullet-}$ was generated enzymatically from xanthin by xanthine oxidase. Hydroxyl radical scavenging capacity test measured the ability of leaf extract antioxidants to prevent 2-hydroterephtalete formation from the reaction between $^{\bullet}OH$ and terephtalic acid. $^{\bullet}OH$ was generated by Fenton reaction. For testing singlet oxygen antioxidant capacity $^1O_2$ was generated in a photosenzitized reaction by illuminating methylene blue dye. The inhibition of $^1O_2$ generation was followed by the color change of

an indicator compound (*p*-nitroso-dimethylaniline or 1,3-diphenylisobenzofuran). The specific ROS-scavenging ability of leaf extracts was followed optometrically, by the inhibitoion of the above dye-bleaching or fluorescence-inducing reactions.

Absorption spectra of linden leaf (water-methanol) extracts (was determined between 280 and 500 nm. Pure flavonoid compounds (quercetin, kaempferol, myricetin, quercetin-3-glucoside and kaempferol-3-glucoside) were dissolved in 50% methanol and absorption spectra were determined photometrically.

Flavonoid content of linden leaves was determined by HPLC-DAD-ESI-MS/MS method.

RESULTS

1. **Our results showed that TAC and SAC parameters are sensitive enough to follow the physiological changes deriving from natural processes, like senescence or leaf age, or occurring in response to acclimatory effects** (**Majer et al. 2010**). Although in the literature TEAC, FRAP and TPC are used as measures of the same parameter, our studies proved that based on the nature of the effect (senescence, oxidative stress, light acclimation), these parameters do not describe antioxidant status uniformly. One has to be aware of the interpretation of TAC results as well: regarding the fine-tuned redox balance, low antioxidant capacities can either be a sign of oxidative stress (insufficient antioxidant defense) or a new balance set up in an acclimation process. Therefore these assays should always be complemented with physiological (photosynthetic) measurements to gain a picture of the plant's status. Although several attempts were made to estabilish a standard method for characterize antioxidant content of samples, our results show that instead of a simple, one-dimensional method, more TAC parameters are to be used, supplemented with the ROS-specific antioxidant testd.

2. **Monitoring the UV-acclimation of grapevine leaves of different age lead us to the conclusion that younger leaves have a greater ability to adapt to UV and that this is based on the induction of antioxidants in these leaves in response to UV (Majer and Hideg 2012a). In contrary, old leaves were not protected from the damaging effects of UV by their originally higher capacities**. Besides TAC, responses of SAC were found to be different as well, for example the process of senescence was not characterized by

specific changes in these parameters, but with the help of these tests we could gain evidences on the role of $O_2^{\bullet-}$, $^{\bullet}OH$, and $^1O_2$ antioxidants in UV-acclimation.

3. **We stated that a few-days long antioxidant-stimulating high light pretreatment of tobacco aided the acclimation efficiency of plants to the subsequent UV-treatment (Majer and Hideg 2012b).** This result indicated the existence of potential cross-tolerance pathways between high visible and UV-light induced acclimatory responses. This assumption was further supported by our observation, that the components of the group generally referred to as UV-B absorbing pigments are induced by high light as well. Therefore we directed our attention to the dissection of the role of flavonoids, the most outstanding group of UV-B absorbing phenolics, in acclimation to light.

4. **We observed that in tobacco leaves acclimated to sunlight, the increase in $^1O_2$ specific antioxidants is significant, besides the operation of preventive energy dissipating pathways (Hideg and Majer 2010).** Since we found a major role for $^1O_2$ svavenging even in short-term adaptation to sunlight, the flavonoid contents of natural sunlight grown sun and shade linden leaves were studied in connection to this parameter.

5. **We stated that the significant differences in the flavonoid contents of sun and shade linden leaves were mainly derived from the increse in quercetin:kaempferol ratio and in myricetin concentrations in sun leaves. The *in vitro* analysis of the $^1O_2$ antioxidant capacity and the absorption spectra of flavonoids together lead to the conclusion that their role in light acclimation is rather based on their antioxidant activity, than their screening efficiency as a UV-B absorbing compound (Majer et al. *in prep*.).** These conclusions show that the role of the compounds referred to as „UV-B absorbing pigments" are more manifold than suspected and suggests the possibility, that UV-inducible reaction pathways may aid protection against oxidative stress caused by the visible (PAR) component of sunlight.

PUBLICATIONS

Peer-reviewed publications related to the PhD theses:

Majer P, Stoyanova S., Hideg É (2010) Do leaf total antioxidant capacities (TAC) reflect specific antioxidant potentials? - A comparison of TAC and reactive oxygen scavenging in tobacco leaf extracts. Journal of Photochemistry and Photobiology B: Biology 100(1):38-43.
IF: 2,116

Hideg É, Majer P (2010) Factors contributing to the high light tolerance of leaves in vivo – Involvement of photo-protective energy dissipation and singlet oxygen scavenging. Acta Biologica Hungarica 61.(Suppl.): 49-60.
IF: 0,793

Majer P, Hideg É (2012a) Developmental stage is an important factor that determines the antioxidant responses of young and old grapevine leaves under UV irradiation in a green-house. Plant Physiology and Biochemistry 50:15-23.
IF: 2,402

Majer P, Hideg É (2012b) Existing antioxidant levels are more important in acclimation to supplemental UV-B irradiation than inducible ones: Studies with high light pretreated tobacco leaves. Emirates Journal of Food and Agriculture 24(6): 598-606.

Majer P, Neugart S, Krumbein A, Schreiner M, Hideg É (*in prep.*) Singlet oxygen scavenging by leaf flavonoids contributes to sunlight acclimation in *Tilia platyphyllos*.

Other peer-reviewed publications:

Fehér-Juhász E.*, Majer P*, Sass L, Csiszár J, Turóczy Z, Mihály R, Mai A, Horváth VG, Vass I, Dudits D, Pauk J. Phenotyping shows improved physiological traits of wheat plants expressing the alfalfa aldo-keto reductase under drought. *kézirat*

Nagy B, Majer P, Mihály R, Dudits D, Horváth VG (2011) Transient and transgenic approaches for functional testing of candidate genes in barley. Acta Biologica Szegediensis. 55(1): 129-133.

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IF: 1,243

Conference abstracts:

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Hideg É, Majer P, Czégény Gy, Sándor Gy, Poage M, Dix PJ (2012) Consequences of enhanced chloroplast SOD activity on the acclimation of leaves to supplemental UV-B. Abstracts of the Plant Biology Congress, 29 July-3 August 2012, Freiburg, Germany, pp. 337.

Majer P, Hideg É (2012) Preceding high light acclimation promotes toleration of supplemental UV-B radiation via enhancing base antioxidant levels. Abstracts of the COST Action FA0906 WG3 mini-conference „Plant responses to ultraviolet radiation – roles of antioxidants and pro-oxidants" 2-3 February 2012, Copenhagen, Denmark, pp. 12.

Majer P, Neugart S, Krumbein A, Schreiner M, Hideg É (2011) Living under the sun: singlet oxygen neutralizing by flavonoids in sun and shade linden leaves. Abstracts of the COST Action UV4growth mini-conference „MetabolUV - Interactive effects of UV-B radiation with abiotic and biotic factors" 23-24 November 2011, Cork, Ireland

Majer P, Neugart S, Krumbein A, Schreiner M, Hideg É (2011) Singlet oxygen scavenging in linden leaves. (International Training Course Alumni Conference „Multidisciplinary Approaches to Biological Problems", 1-3 September 2011, Szeged, Hungary

Majer P, Hideg É (2011) Age related differences in antioxidant responses of grapevine (*Vitis vinifera* L.) leaves to supplemental UV-B radiation. Abstracts of the 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants, 5-8 July 2011, Budapest, Hungary, pp. 115.

Majer P, Hideg É (2011) Are young ones more vigorous? A comparative study of photosynthesis and antioxidant responses of younger and older Vitis leaves under UV-B radiation in a green-house experiment. Abstracts of the 1st Annual Meeting of COST Action FA0906 UV4growth, 7-9 February 2011, Szeged, Hungary, ISBN 978-963-508-606-1, pp. 21.

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Jancsó M, Majer P, Lantos C, Simon-Kiss I, Dudits D, Pauk J (2007) Diagnostic system for detection of drought tolerant rice genotypes. Proceedings of the 4th International Temperate Rice Conference, June 25-28, 2007, Novara, Italy, pp. 330-331.

Book chapters:

Sass L, Majer P, Hideg É (2012) Leaf hue measurements: a high-throughput screening of chlorophyll content. Methods in Molecular Biology vol. 918:61-69. High-Throughput Phenotyping in Plants: Methods and Protocols. Ed: J. Normanly. Humana Press. ISBN 978-1-61779-994-5