

## **ABSTRACT OF Ph.D. THESIS**

### ***IN VIVO* DETECTION OF LIGHT STRESS INDUCED REACTIVE OXYGEN SPECIES IN PLANTS**

Ph.D. Thesis

**Csengele Barta**

Supervisor: Dr. Éva Hideg

Biological Research Centre of the Hungarian Academy of Sciences  
Institute of Plant Biology  
Laboratory of Molecular Stress-and Photobiology

During their life cycle plants are daily exposed to adverse environmental impacts, such as high intensities of photosynthetically active radiation, increased fluxes of ultraviolet radiation, extreme temperatures, water deprivation, air pollutants or pathogen attack, or to the combination of these. When plants are exposed to various stress conditions, a number of metabolic functions are affected through the generation of reactive oxygen species (ROS). These reactive, short-lived molecules are considered in variety of roles: as primary elicitors of damage or as important mediators of cellular damage. Lately

their role as signal transduction molecules was also assessed, having important regulating role in the process of programmed cell death. ROS are produced in response to a variety of abiotic stresses, such as extreme temperature, UV irradiation, ozone exposure, osmotic stress or drought. ROS play a central role in the coordination of plant responses and are involved in acclimation responses and cross-talk as well.

Although ROS perform a wide array of roles in plant cells, there is an evident controversy in their roles: they are necessary for the survival of plants, performing signal transduction and gene-activating roles, but they can as well be lethal when they are overproduced, due to the disturbance in the balance between their production and removal. Plants are equipped with a highly effective network of enzymatic- and non-enzymatic antioxidant system, and with effective excess energy dissipating systems, which prevent ROS-induced oxidative damage under physiological and mild stress conditions. By different acclimation mechanisms plants can tolerate stress conditions up to a certain level. If oxidative damage occurs, repair processes may alleviate the damage up to a certain threshold. Since ROS are key molecules of a wide scale of biotic and abiotic stress conditions, the study of the roles of ROS in plants' life and their damaging effect is an important field of plant stress physiology.

One of the most frequent stress conditions occurring in the field is the light stress. The solar radiation reaching the

Earth's surface is divided into ultraviolet B (UV-B: 280-320 nm), ultraviolet A (UV-A: 320-400 nm) and visible (PAR: 400-700 nm) radiation, the latter one being used for photosynthesis by the vegetation. Plants may often be exposed to high irradiances of PAR (photoinhibition) and ultraviolet radiation. Due to recent increase in UV radiation on Earth, the study of the effect of increased ultraviolet radiation on biological systems had a central role in a multitude of studies in the last decades.

### **State of knowledge at the start of the project**

The oxidative nature of light stress was the objective of a wide variety of plant stress studies. In the mechanism of both acceptor side-, and donor side-induced photoinhibition various ROS were involved, but still there is a lack of consensus between the different *in vitro* (isolated membrane) and *in vivo* (leaf) studies. Photoinhibition induced protein damage was found to be accompanied by hydroxyl radical formation in the donor side-induced mechanism and by  $^1\text{O}_2$  evolution in the acceptor side-induced photoinhibition *in vitro*, by various detection methods. *In vivo*  $^1\text{O}_2$  evolution was also in the focus of several studies, and even the induction of superoxide radical production was hypothesized *in vivo*, although superoxide radicals were not detected by EPR spectroscopy as promoters of photoinhibitory damage in spinach thylakoids. Superoxide was

hypothetized to participate in photoinhibition as product of electron transport to oxygen both in functioning and in donor-side impaired PS II.

ROS induction is known to be the early plant response to UV exposure as well. High levels of UV-B radiation induce lipid peroxidation and membrane damage by the action of various reactive oxygen species. In isolated thylakoid membranes, UV-B exposure triggered hydroxyl radical generation was detected by EPR spectroscopy, but the presence of carbon centered (methyl-like), and peroxy radicals were also reported. Singlet oxygen was not found in the same preparation. UV-B induced D1 protein degradation was assigned to hydroxyl radicals. Several indirect *in vitro* and *in vivo* studies also point the oxidative nature of UV-B stress, and hypothesize the possible production of ROS other than hydroxyl and carbon centered radicals. Upon UV-B exposure, increased amounts of ascorbate radical were also detected *in vivo* (Hideg et al., 1997).

While the damaging mechanism of UV-B radiation is in the focus of several studies, less information is available on the effect of UV-A. The difference in the action site of UV-A and UV-B in photosynthesis has been intensely discussed, attributing smaller damaging effect to the longer wavelength UV-A irradiation, than to the higher photon-energy UV-B. However, ROS generating abilities of various UV energies have not been compared yet.

## **OBJECTIVES OF OUR RESEARCH**

Due to the lack of consensus between various *in vitro* and *in vivo* studies our goal was to study the nature ROS types involved in photoinhibition and stress by both UV-A and UV-B radiation *in vivo* by using a direct detection method adapted for plant stress studies.

- **We aimed to develop further a sensitive, specific method for direct *in vivo* stress-induced ROS detection.**
- **We aimed to investigate the ROS involved in *in vivo* photoinhibition.**
- **Our goal was to reveal the nature of ROS involved in UV stress in leaves, including the UV wavelength-dependency of ROS production**

## **MATERIALS AND METHODS**

The nature of stress induced ROS was studied *in vitro* in isolated thylakoid membranes as well as *in vivo* in spinach, *Arabidopsis thaliana* and in CuZnSOD deficient tobacco leaves.

The short-lived ROS were detected by fluorescent sensors.

Before being used in stress studies, various fluorescent sensors were characterized *in vitro* for specificity and stability and *in vivo* for stability, penetration and micro-localization.

The decrease induced in the ROS sensor's fluorescence was measured in leaves in spectrophotometrical, laser scanning microscopy and fluorescence imaging experiments.

Leaf and thylakoid samples were exposed to one of the following stress conditions:

- Illumination by either  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$  or  $1800 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR
- Treatment with  $27 \mu\text{mol m}^{-2} \text{s}^{-1}$  295-320 nm broadband UV-B radiation
- Treatment with  $35 \mu\text{mol m}^{-2} \text{s}^{-1}$  345-385 nm broadband UV-A radiation
- A combination of broadband UV-A and UV-B radiation
- Treatment with  $2 \times 10^{22}$  photons of quasi-monochromatic ( $\pm 8$  nm around central wavelength) UV radiation in the 280-390 nm range, corresponding to  $18\text{-}36 \mu\text{mol m}^{-2} \text{s}^{-1}$

Photoinhibition and UV irradiation induced electron transport loss in isolated thylakoids was measured as decrease in oxygen evolution.

The effect of photoinhibition and UV irradiation on the photosynthesis of various leaves was estimated from the relative decrease in their variable chlorophyll fluorescence.

## **CONCLUSIONS**

1. Although DanePy proved to be a good  $^1\text{O}_2$  sensor for *in vivo* stress induced studies, as previous studies of our group showed, there was further need to find a fluorescent ROS sensor specific to ROS types other than  $^1\text{O}_2$ . Therefore, other potential ROS sensors were introduced and characterized for *in vitro* selectivity and stability, and *in vivo* stability.

**From among the many fluorescent ROS sensor candidates for stress-induced ROS detection studies we selected two sensors, based on their stability and localization in chloroplasts: the  $^1\text{O}_2$  specific DanePy and the  $^1\text{O}_2$  and  $\text{O}_2^{\bullet-}$  reactive HO-1889NH.**

2. We applied both sensors to study photoinhibition- and UV-stress induced ROS production.

a.) We investigated the ROS-inducing effect of photoinhibition in isolated thylakoids as well as in leaves. We have shown, that **photoinhibition promoted singlet oxygen**

**evolution both *in vitro* and *in vivo*. The contribution of superoxide anion radicals was minor.**

**b.) We have studied the nature of ROS induced by both UV-A and UV-B radiation. Reactive oxygen production in leaves exposed to UV radiation was heterogenous. In spinach leaves  $O_2^{\bullet-}$  production was induced by 280 -360 nm broadband UV radiation, while  $^1O_2$  evolution was not a characteristic response.**

In order to compare the ROS triggering ability of various UV energies, specially that of UV-A and UV-B in detail, the wavelength dependency of UV-induced  $^1O_2$  and  $O_2^{\bullet-}$  production was studied in leaves, in the range of 280-390 nm.

**c.) Superoxide production was characteristic to the UV-B wavelength region but not to UV-A. We have shown the existence of a  $O_2^{\bullet-}$  yielding primary reaction occurring in the chloroplasts, possibly in the thylakoid membranes. Singlet oxygen evolution was a characteristic UV-A induced physiological response, typical for irradiation by 340-390 nm, however its source is probably not localized inside the chloroplasts.**

In summary, we have shown, that light stress is a complex oxidative stress, inducing the production of various reactive oxygen species in plants. The main product of *in vivo*



photoinhibition is singlet oxygen, while UV-B irradiation induces superoxide production. Both reactions take place in chloroplasts. On the other hand, UV-A irradiation triggered singlet oxygen production in leaves from a yet unspecified source.

## **LIST OF PUBLICATIONS**

### **I. JOURNAL ARTICLES**

**Barta**, Cs., Kálai, T., Vass, I., Hideg, É. (2002) Dansyl- and rhodamine-based fluorescent sensors for detecting singlet oxygen and superoxide production in plants *in vivo*. *Acta Biologica Szegediensis* 46, 149-150.

Hideg, É., **Barta**, Cs., Kálai, T., Vass, I., Hideg, K., Asada, K. (2002) Detection of singlet oxygen and superoxide with fluorescent sensors in leaves under stress by photoinhibition or UV-radiation. *Plant Cell Physiology* 43, 1154-1164.

**Barta**, Cs., Kálai, T., Hideg, K., Vass, I., Hideg, É. (2004) Differences in the ROS generating efficacy of various ultraviolet wavelengths in detached spinach leaves. *Functional Plant Biology* 31, 23-28.

## **II. CONFERENCE PROCEEDINGS**

Hideg, É., Asada, K., **Barta**, Cs., Bornman, J., F., Dudits, D., Hideg, K., Horváth, G. V., Kálai, T., Oberschall, A., Ogawa, K., Sár, C., Szilágyi, A., Vass, I. (2000) Detecting ROS in plants under UV-B stress (Plant and Ultraviolet-B Radiation Congress, Japan).

**Barta**, Cs., Kálai, T., Sár, C., Hideg, K., Vass, I., Hideg, É. (2001) Reaktív oxigén növényekben: újabb mérési lehetőségek (IV<sup>th</sup> Hungarian Photosynthesis Meeting, Szeged, HAS-BRC).

Hideg, É., Kálai, T., **Barta**, Cs., Ogawa, K., Vass, I., Hideg, K., Asada, K. (2001) Detecting stress induced reactive oxygen production in plants (9<sup>th</sup> Congress of the European Society for Photobiology”, Lillehammer, Norway).

**Barta**, Cs., Kálai, T., Vass, I., Hideg, K., Hideg É. (2002) Action spectrum of ROS production under different wavelength UV stress (II<sup>nd</sup> Chemistry and Biochemistry of Antioxidants Course, Wageningen, The Netherlands).

**Barta**, Cs., Kálai, T., Vass, I., Hideg, K., Hideg É. (2002) Az UV sugárzás reaktív oxigénkeltő hatásának vizsgálata spenót levelekben (III<sup>rd</sup> Hungarian Plant Physiology Conference, Biological Research Center, Szeged).

**Barta**, Cs., Ogawa, K., Asada, K., Hideg, É. (2003) The role of superoxide-dismutase in detoxifying reactive oxygen species in tobacco leaves exposed to UV-radiation (10<sup>th</sup> Congress of the European Society for Photobiology, Vienna, Austria).

**Barta, Cs., Vass, I., Hideg, É. (2003)** A szuperoxid diszmutáz szerepe a 290, illetve 360 nm-es UV besugárzásnak kitett dohánylevelekben keletkező reaktív oxigén formák semlegesítésében (V<sup>th</sup> Hungarian Photosynthesis Conference and Advanced Photosynthesis School, Noszvaj, Hungary).