Summary of Ph.D. thesis

EFFECTS OF ENVIRONMENTAL STRESS FACTORS ON THE ANTIOXIDANT DEFENCE SYSTEMS AND MOLIBDOENZYMES IN PLANTS

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by

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INTRODUCTION

We are living in a time when the word "stress" occurs frequently in our daily life. Human, animal and plant kingdoms are all touched by this "syndrome". The present work wants to be a little contribution to the numerous, but yet not sufficient studies dealing lately with plant stress and plant stress detection.

Nowadays, a large part of plant physiology research is covered by investigations on plant stress concepts or particular aspects of plant stress. This interest is explained by the alterations happened in biosphere during the last decades: depletion of stratospheric ozone which has as a primer effect the increase of UV-B radiation reaching the earth's surface; modifications in the composition of atmosphere leading to changes in climate, materialized in the "green house effect"; presence of saline soils and continuous desertification processes, etc. And all these in conditions of increasing population. Every year, environmental stress causes considerable losses in crop quality and productivity. One of the important mechanisms by which plants are damaged during adverse environmental conditions is the excess production of active oxygen species, such as superoxide anion, hydrogen peroxide and hydroxyl radicals, giving rise to the so called "oxidative stress". Plants have evolved non-enzymatic protection mechanisms that efficiently scavenge these active oxygen species, such as ascorbic acid, glutathione, α-tocopherols, carotenoids, as well as enzymatic defence strategies including superoxide dismutase, catalase, peroxidases and glutathione reductase.

Aldehyde oxidase (AO) and xanthine dehydrogenase (XDH), two molybdenum cofactor containing enzymes, could also be involved in stress adaptation processes: through the production of abscisic acid (ABA) by AO, a known stress phytohormon implicated in the control of physiological and molecular processes involved in the response of plants to environmental stresses such as freezing, drought and salinity; XDH by producing uric acid, which is an effective scavenger of reactive oxygen species produced under stress conditions.

Due to the significant role of plants in our daily life (food for human and animals, material for certain industries etc.) it was felt that a better understanding of

plant adaptation to different environmental stress conditions and of the mechanisms involved in these processes is of great importance.

OBJECTIVES

The present study analyzed the mechanisms involved in the "stress tolerance" of plants, the ways by which plants are able to cope with unfavorable environmental conditions, represented in this case by increased UV-B irradiation and salinity.

The main aims of the present work were:

1. To investigate the effect of UV-B radiation corresponding to about 10% ozone depletion, as oxidative stress for wheat, at the physiological and biochemical levels, by answering the questions:

    - What are the stress responses to enhanced UV-B treatment in wheat?
    - Which mechanisms are involved in the protective responses?
    - Do wheat plants adapt to these circumstances?

2. To examine the effect of salinity, as environmental stress factor, on the biological clock in plants, specifically, the changes of rhythmic behavior of the antioxidant enzyme superoxide dismutase in wheat under salinity treatment.

3. To characterize the effect of salinity and nitrogen source on aldehyde oxidase, xanthine dehydrogenase and nitrate reductase activities and their distribution in adventitious or nodal roots of maize, and within the different zones of root with defined physiological functions and of progressive maturation.

MATERIAL AND METHODS

Plant material and growth conditions

Wheat seedlings (*Triticum aestivum* L. cv. Tiszatáj, Cereal Research Institute, Szeged, Hungary) were grown hydroponically in phytotrons (Conviron PGW 36). To determine the effect of UV-B treatment, in one of the phytotrons the set of tubes was supplemented with Philips sun lamps (TL 100W/01) providing 2.5 Wm^{-2} radiation of major spectral emittance between 310-315 nm (λ_{max}= 311/312 nm) (Santos et al., 1993). To determine the effect of salinity treatment on the rhythmic behavior of SOD treatments with NaCl at 0, 50, 100 and 150 mM concentrations were performed. To determine the effect of salinity on the distribution and activity of MoCo-enzymes, maize (*Zea mays* L., cv. Jubily) plants were grown in aerated hydroponic culture in a greenhouse. The nitrogen sources consisted of 4 mM $NaNO_3$ or 2 mM $(NH_4)_2SO_4$ in a half-strength modified Hoagland nutrient solution (Hoagland and Arnon, 1938). Salinity treatments consisted of 50 mM NaCl.

Analytical determinations

Enzyme assays

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring the ability of the enzyme to inhibit the photochemical reduction of Nitro Blue Tetrasolium (NBT) in the presence of riboflavin in the light (1 EU=50 % inhibition). The colour change was monitored at 560 nm. Isoenzymes were identified by using native gel electrophoresis and on the basis of their differential inhibition by H_2O_2 and KCN (H_2O_2 inhibits Cu/Zn-SOD and Fe-SOD but not Mn-SOD; KCN inhibits Cu/Zn-SOD, but not Fe-SOD or Mn-SOD) (Beauchamp and Fridovich, 1971; Dindsha et al., 1981).

Catalase (EC 1.11.1.6) activity was measured spectrophotometrically by following the decrease of H_2O_2 quantity in time at 240 nm (1 EU=mmol H_2O_2 decomposed in one minute) (Upadhyaya et al., 1985).

Guaiacol peroxidase (EC 1.11.1.7) activity was determined by monitoring the increase in absorbance at 470 nm as guaiacol was oxidised (1 EU=μmol guaiacol oxidised in one minute) (Upadhyaya et al., 1985).

Glutathione reductase (GR, EC 1.6.4.2) activity was determined by monitoring absorbance increment at 412 nm when DTNB (5,5'-dithio-*bis*(2-nitrobenzoic acid)) was reduced by GSH, generated from GSSG. Standard GR was Type III from Baker's Yeast. The rate was calculated from the linear portion of the curve and expressed as a rate/5 min (Smith et al., 1988).

Aldehyde oxidase (AO, EC 1.2.3.1) activity was detected by staining after native electrophoresis with 7.5% acrylamide gels (Laemmli, 1970). The gel was immersed after electrophoresis in 0.2 M phosphate buffer, pH 7.5, for 10 min followed by gentle shaking at room temperature in a reaction mixture containing 0.1 M Tris-HCl (pH 7.5), 0.1 mM phenazine methosulfate (PMS), 1 mM 3[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT) and 1 mM substrate (acetaldehyde, heptaldehyde, benzaldehyde or indole-3-aldehyde).

Xanthine dehydrogenase (XDH, EC 1.2.37) XDH activity was detected after native gel-electrophoresis using hypoxanthine as a substrate (Mendel and Muller, 1976).

NADH-nitrate reductase (NADH:NR, EC 1.6.6.1) was assayed in a reaction mixture containing 30 mM K-phosphate buffer (pH 7.5), 25 mM KNO_3 and 0.25 mM NADH (Sagi and Lips, 1998c). NR activity was expressed as μmol NO_2^- g^{-1} FW h^{-1} or nmol NO_2^- mg^{-1} protein min^{-1}.

Quantitation of glutathione

Chromatographic conditions: dinitro-phenyl derivates were separated and measured using a gradient HPLC (BioRad) system equipped with a BioRad Amino-5S column and UV detector (Spectra Physics) at 365 nm. Following a 100 μl injection of the centrifuged solution containing derivatized sample, the mobile phase was 75 % solution A and 25 % solution B for 10 min followed by a 30 min linear gradient to 5 % A and 95 % B. Flow rate was 1.0 ml/min. Solution A contained 80 % methanol, solution B was composed of 0.55 M sodium acetate, 12.6 % acetic acid and 64 % methanol (Fariss and Reed, 1987).

Western blot analysis for aldehyde oxidase

The AO proteins extracted from the plant material were subjected to Western blotting. After SDS-PAGE the gel with the separated proteins was electrophoretically transferred onto a nitrocellulose membrane (0.2 μm pore size; Schleicher and Schüll, Dassel, Germany). Blotting time was 1 h at 2 mA cm^{-2}. Blots were blocked for 90 min in 5% (w/v) bovine serum albumin in TBS. Immunodetection of AO was carried out with polyclonal mouse antibodies raised against the purified maize AO (Koshiba et al., 1996) after a 500-fold dilution in TBS and secondary antibodies (anti-mouse IgG, Sigma) diluted 1000-fold in TBS. The antigen/primary antibody complex was detected by binding of alkaline-phosphatase-linked goat anti-mouse IgG (Sigma, USA). Phosphatase activity was developed by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT, Sigma Fast™ tablets). Molecular weight of proteins was estimated with a mixture of protein standards: myosin (202 kD), galactosidase (109 kD), bovine serum albumin (78 kD), ovalbumin (46.7 kD). Binding of the antibodies to maize root AO proteins was detected by immunoprecipitation using protein-A-Sepharose CL-4B (Pharmacia). All of the activity (detected after native-PAGE) in the samples was removed when the antibodies and Sepharose were added, while all of the activity remained in the supernatant when control mouse serum was used.

Estimation of AO and XDH activities

Enzyme activities of the Mo-hydroxylases were estimated on the basis of MTT reduction, which resulted in the development of specific formazane bands. Quantitative analyses were made by scanning the formazane bands in the gel, with a computing laser densitometer (Molecular Dynamic) using Image Quant version 3.19.4 and NIH Image 1.6.

Fit of periodicity by Fourier transformation

For the calculation of different periodicities, Fourier analysis was carried out by a radix-2 fast Fourier transform algorithm implemented in the MATLAB 4.0 programming environment (The Mathworks Inc., Cochituate Place, 24 Prime Park Way Natick, Mass. 01760). Frequency filtering was performed by an inverse Fourier transform of the truncated frequency spectra, employing the same algorithm.

NEW RESULTS AND CONCLUSIONS

Effects of excess UV-B radiation on the antioxidant defense mechanisms in wheat seedlings

1. During UV-B irradiation, the activities of the investigated antioxidant enzymes, after reaching a transitional minimum value, caught up with the control level, i.e. acclimatization had occurred. The predominant component among them was the glutathione system, which, being one of the most powerful antioxidant in plants, brought about an intensive "detoxification" and remediation of the cellular environment.

2. Catalase, guaiacol proxidase and superoxide dismutase activities decreased, while GSSG level and glutathione reductase activity increased under UV-B irradiation.

3. The responses indicated two main phases in the adaptation process to UV-B irradiation in young wheat plants. In terms of the Selyean stress concept (Selye, 1956) these phases can be considered as parts of the general adaptation syndrome (GAS): the alarm reaction is represented by the inhibition of stem and leaf elongation, accumulation of flavonoid pigments, decrease in antioxidant enzyme activities, increased GSSG level and triggering of GR activity, while normalisation of growth, disappearance of stem pigmentation, levelling of antioxidant enzyme activities, high GR activity and decreased level of GSSG were found as parts of adaptation to UV-B irradiation.

4. Synthesis of flavonoid pigments during the alarm phase is a *syntoxic* element that contributes to the increase in tolerance, while the increase in the levels and/or activities of antioxidants can be regarded as *catatoxic* elements, which actively eliminate the oxidative component of the stressor (Leshem and Kuiper, 1996).

5. In general, moderate stress caused by the excess UV-B irradiation in the present study can be categorised as *eu-stress* (Lichtenthaler, 1996), which is a stimulating stress that activates the cell metabolism and increase resistance or acclimatisation. Moderate increase in UV-B irradiation due to the present depletion of ozone in the stratosphere, at the surface do not bring about fatal damage in plant (at least in wheat) repair mechanisms, metaoolism and growth.

Effects of salinity treatment on the changes of rhythmic behavior of superoxide dismutase in wheat seedlings

6. The activity of SOD decreased under NaCl salinity (0, 50, 100 and 150 mM) treatment in young winter wheat.

SOD activity showed a circadian rhythm when followed as the function of time, in both control and treated seedlings. Fourier analysis disclosed an increase in the amplitude of the circadian period fraction of SOD activity in the 150 mM NaCl treated samples.

7. The oscillation of SOD activity in the roots of wheat seedlings were modulated by the stressor (NaCl) suggesting a connection between environmental stress and biological rythmicity:

i) antioxidant enzyme activity (SOD) fluctuates with circadian and ultradian periodicities;
ii) salinity treatment influenced the amplitude of oscillations and left the circadian period unchanged; and
iii) ultradian fluctuations proved to be sensitive to stress treatment.

8. On the basis of these observations and literature data it can be hypothesized that the stressor may modify and desynchronize the biochemical and physiological oscillations by acting either on the input (from receptor till oscillator) or on the output (from the oscillator till the oscillating component) pathways. In these terms, *adaptation* means re-setting of the new circadian and/or ultradian

rhythms of biochemical/physiological processes and the restoration of their concerted action results in maximal resistance for the organism under the new environmental conditions.

Molibdoenzymes: effects of salinity and nitrogen ions on the level and distribution of aldehyde oxidase, xanthine dehydrogenase and nitrate reductase

9. Mo-enzymes activities in maize nodal roots were considerably higher under mild saline conditions and activities of the Mo-hydroxilases (AO and XDH) were enhanced by ammonium as nitrogen source.

10. Four bands or isoforms of AO (AO1-4) were detected after native PAGE with indole-3-aldehyde in maize roots showing the strongest activities in the AO3 and AO4 bands. Salinity and ammonium enhanced activity of AO3 and AO4 in different ways: ammonium increased the level of AO protein while salinity seemed to activate preexisting enzyme molecules. Since ABA level increased in plant tissue under the same conditions, it can be concluded the possible role of AO3 and AO4 in ABA biosynthesis in maize nodal roots.

11. Mo-enzymes were not homogeneously distributed along the longitudinal and transversal axes of the maize nodal roots, but were mainly located in the tips and in the stele of mature root zones. The location of the centers of AO activity in the tips and the stele fits two needs of the developing root: (a) The root tip seems to require ABA for sustain elongation growth (Saab et al., 1992) and (b) Synthesis of ABA in the stele of mature root zones may be related to the xylem parenchyma cells from which the hormone can be rapidly transported to the shoot via the xylem vessels.

12. Stress (salinity) and ammonium ions, affected Mo-enzymes in the different root zones. Immunoblot analysis with antibodies raised against AO protein revealed increased level of AO protein in root tips of ammonium fed plants while

salinity treatment of nitrate fed plants did not affect the enzyme protein level, in spite of the salinity-enhanced activity of these enzymes. Therefore, salinity seems to activate latent or down-regulated enzymes.

13. SDS-PAGE followed by immunoblotting, revealed besides the major 150 kD subunit of AO, two polypeptides with molecular masses of 72 and 85 kD located specifically in the cortex.

This diversity of AO, XDH and NR distribution may be tightly related with specific metabolic functions of root zones and tissues and to the involvement of Mo-enzymes in the adaptation of plants under changing environmental conditions.

These results indicate that exposure to environmental stress can stimulate plants to enhance their reactive oxygen intermediates scavenging systems, and this enhancement can apparently provide generalized stress protection.

Besides their contribution to the basic research, the present data may constitute the basis for a modern plant breeding system and commercial application for engineering new, stress tolerant plants.

The elucidation of the mechanisms involved in nitrogen assimilation and plant adaptation to saline conditions may be important for the development of environmentally friendly crops capable of producing good yields with increase use of ammonium at the expense of nitrate allowing lower inputs of fertilizers and water, as well as a better use of the saline soils.

LIST OF PUBLICATIONS

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