BIOLOGIA PLANTARUM 45 (3): 417-421, 2002

Accumulation of microsomal polypeptides in barley roots during aluminium stress

I. MISTRÍK, L. TAMÁS, J. HUTTOVÁ and V. GEJDOŠOVÁ

Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 14, SK-84223 Bratislava, Slovakia

Abstract

Accumulation of two peripheral membrane polypeptides (20 and 28 kDa) in roots of Al-sensitive (cv. Alfor) and Al-resistant (cv. Bavaria) barley cultivars were analysed during Al stress. Both cultivars were subjected to Al concentration ranging from 0 to 150 μM for 24, 48, 72 and 96 h. Accumulation of both polypeptides was determined 24 h after exposure of plants to Al and content of both polypeptides showed only small dependence upon Al concentration and duration of Al treatment. Although, based on root growth test, Bavaria showed significantly greater resistance to Al than Alfor, analysis of 20 and 28 kDa polypeptide pattern has not revealed significant difference between the two cultivars. However, accumulation of 20 and 28 kDa polypeptides in Alfor was selectively induced by Al treatment because different pH of the root media (pH 3.5 to 6.5) or application of other metals (Cu, Co, or Cd) failed to induce these two bands. On the other hand, accumulation of these polypeptides in Bavaria was induced not only by Al, but also by Cd and in a lesser extent by Co treatment.

Additional key words: aluminium, *Hordeum vulgare*, peripheral membrane proteins, polypeptide pattern, roots, stress.

Introduction

Aluminium toxicity became one of the major factors limiting plant distribution and yield of crops on acid soils. In spite of the fact that several plants species accumulate Al in leaves (*e.g. Camelia sinensis*) or in sepals (*e.g. Hydrangea macrophylla*) without apparently suffering from Al toxicity, majority of plant species and crop plants are sensitive even to micromolar concentrations of the soluble forms of Al.

Application of molecular methods revealed that Al activates several genes with known function (Richards and Gardner 1994, Hamel *et al.* 1998) as well as genes and proteins with unknown functions (Ownby and Hruschka 1991, Basu *et al.* 1994a). In wheat Al treatment induced genes (*wali 1-7*) whose transcript encode plant metallothionein-like protein, proteinase inhibitors, phenylalanine-ammonia lyase and proteins with sequence similarity to the putative proteins encoded by maize wound-induced genes (Snowden *et al.* 1995). Aluminium-induced peroxidase, cysteine proteinase, phenylalanine-ammonia lyase, and oxalate oxidase genes (*War* genes) were isolated by Hamel *et al.* (1998) from wheat. Due to the fact that most of the Al-induced genes were induced in both Al-resistant and Al-sensitive genotypes indicates that they are not involved in Al tolerance, and probably represent a part of a suite of stress genes induced not only by Al treatment but also by other biotic and abiotic stresses (Cruz-Ortega and Ownby 1993, Didierjean *et al.* 1996).

Specific 51 kDa Al-induced polypeptide related to Al tolerance was described by Basu *et al.* (1994a) in root apex of an Al-resistant wheat cultivar and one 18.6 kDa membrane protein by Ownby and Hruschka (1991). The same two proteins were not found in the root apex of an Al-sensitive cultivar. Two cDNA clones, pAL111, and pAL142 from cultured tobacco cells grown under Al stress were isolated by Ezaki *et al.* (1995). Their amino

Received 16 March 2001, *accepted* 19 June 2001.

Abbreviations: BHT - butylated hydroxytoluene; BSA - bovine serum albumine; DTT - dithiothreitol; EGTA - ethyleneglycol-bis-(β-amino-ethyl ether)N,N′-tetraacetic acid; PVP - polyvinylpyrrolidone; SHAM - salicylhydroxamic acid.

Acknovledgements: The authors thank Mrs. A. Grycová for the excellent technical assistance. This work was partially supported by Grant Agency for Science VEGA, project No. 2/1066/01.

Fax: (+421) 7 54771948, e-mail: botumist@savba.sk

acid sequencing result indicated that pAL111 is identical to the *parA* gene of *Nicotiana tabacum* L. and pAL142 is highly homologous to the tobacco *parB* gene (Takahashi and Nagata 1992) which has a glutathione S-transferase (GST) activity. It has been reported that Al ions enhance the peroxidation of membrane lipids (Cacmak and Horst 1991) therefore GST encoded by pAL142 probably prevent the peroxidation process caused by Al (Ezaki *et al.* 1995). Another new clone (pAL201) whose expression is induced by Al treatment or phosphate starvation encodes a moderately anionic peroxidase (Ezaki *et al.* 1996). The authors suppose that Al ion stress can control the activity of some peroxidase isozymes supporting the view of Richards *et al.* (1998) that there are some common mechanisms for gene induction between Al toxicity and oxidative stress.

In our previous work dealing with the analyses of protein profiles of barley plants (Tamás *et al.* 1999) and maize (Mistrík *et al.* 2000) exposed to Al stress we described qualitative changes in protein composition of cell wall, cytoplasmic and membrane proteins. Especially in the fraction of peripheral membrane proteins we identified two polypeptides with apparent molecular mass of 20 and 28 kDa amount of which was significantly enhanced by Al treatment. The present work was undertaken to characterise the time course of the accumulation of these two polypeptides in roots of Al-sensitive and Al-resistant barley cultivars exposed to different concentrations of Al. Al-induced changes in accumulation of 20 and 28 kDa polypeptides are compared with the changes induced by different pH of root medium (pH 3.5 - 6.5), and by application of 10 μM concentration of other toxic metals (Cu, Cd, and Co) into the root medium.

Materials and methods

Seeds of barley (*Hordeum vulgare* L.) cultivars, the Al-sensitive cv. Alfor and Al-resistant cv. Bavaria, were surface sterilised with 12 % H_2O_2 and germinated at 25 °C, relative humidity of 98 % in darkness. Three-day-old seedlings were transferred into 3 dm^3 glass jars containing 1 mM $CaCl_2$ solution, pH 4.5, and cultivated in a growth chamber (*E 8 Conviron*, Winnipeg, Canada) at light/dark temperature of 20/16 °C and irradiance 70 μmol m^{-2} s^{-1} (400-700 nm) for 16-h photoperiod. After 4 d of cultivation the control plants were transferred into a fresh 1 mM $CaCl_2$ solution with pH 4.5. To analyse the effect of different pH on the accumulation of 20 and 28 kD polypeptides, plants were transferred into a fresh 1 mM $CaCl_2$ solutions with pH 3.5, 4.5, 5.5 or 6.5. Experiments with other metal ion application were performed on plants transferred into a 1 mM $CaCl_2$ solution, pH 4.5, containing 10 μM $AlCl_3$, $CuCl_2$, $CdCl_2$, or $CoCl_2$. In the case of Al concentration impact on the accumulation of 20 and 28 kDa polypeptides 1, 5, 10, 50, 100, and 150 μM $AlCl_3 . 6 H_2O$ solutions were added into 1 mM $CaCl_2$ solution, pH 4.5. After 4 d of Al (or other metals) treatments, whole roots were harvested and immediately frozen in liquid nitrogen. Samples were ground to a fine powder in a cold mortar in liquid nitrogen and the resulting powder was re-homogenized in extraction buffer (100 mM Tris buffer, pH 8.0, 0.3 mM sucrose, 1 mM EGTA, 2.5 mM potassium metabisulfite, 2 mM SHAM, 1 mM DTT, and 3 % PVP) with homogenizator (*DIAX 900 Heidolph GmbH,* Keilheim, Germany). After filtration the homogenate was centrifuged, first at 1500 *g* for 5 min, then the supernatant was re-centrifuged at 12 000 *g* for 15 min, then again at 150 000 *g* for 30 min (*L8-M Beckman Instruments*, Palo Alto, USA). The resulting pellet represented the microsomal membrane fraction. The microsomal membrane fraction was washed with 10 mM Tris-maleate buffer, pH 7.3 containing 0.15 M NaCl, 1 mM EDTA and 10 μg cm^{-3} BHT and re-centrifuged at 150 000 *g* for 30 min to obtained peripheral membrane proteins. Proteins were precipitated with ice cold acetone than solubilized and separated under denaturing condition on 12 - 18 % gradient polyacrylamide slab gels using the discontinuous buffer system (Laemmli 1970) and silver stained (Heukesloven and Dernick 1985). Protein concentrations were determined by the slightly modified method of Lowry *et al.* (1951) with bovine serum albumin as the standard. The apparent molecular masses of polypeptides were calculated based on the mobilities of protein standards obtained from *Bio Rad Laboratories* (Hercules, USA) with gel documentation system (*UVP 5000*, Cambridge, England).

Results

Accumulation of 20 and 28 kDa polypeptides was determined 24 h after exposure of plants to Al stress (Fig. 1). Al at 1 μM concentration was sufficient to start the accumulation of both polypeptides in roots of the Al-sensitive cv. Alfor. In Al-resistant cv. Bavaria accumulation of 20 kDa polypeptide started at the same threshold concentration, but accumulation of 28 kDa polypeptide started at 5 μM Al. The amount of 20 and 28 kDa

polypeptides continuously increased with raising Al concentration in cv. Bavaria. In Al-sensitive Alfor the amount of both polypeptides reached the maximum level at 50 μM and than remained unchanged up to 150 μM Al.

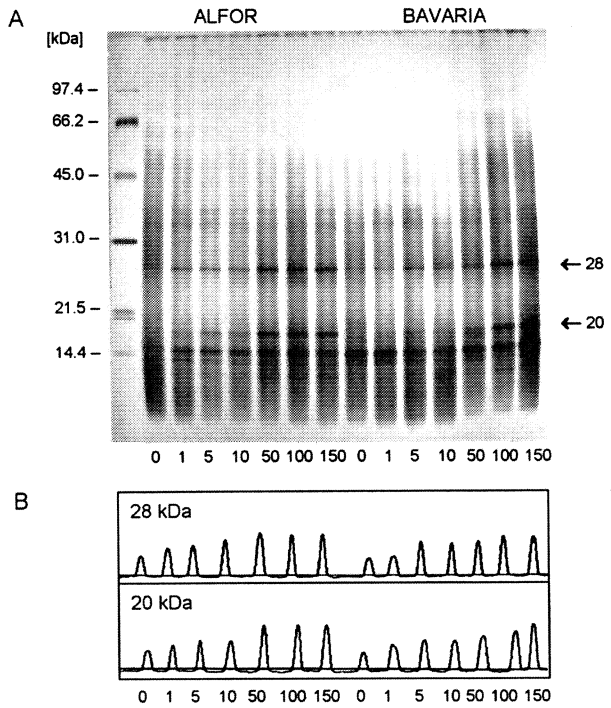

Fig. 1. SDS-PAGE analysis of peripheral membrane proteins isolated from roots of Al-sensitive (Alfor) and Al-resistant (Bavaria) barley cultivars grown for four days in the presence of different concentrations of Al (0, 1, 5, 10, 50, 100 and 150 μM) (*A*). Densitometric scans correspond to 20 and 28 kDa polypeptides of the gel (*B*).

In order to characterise the time course of the accumulation of 20 and 28 kDa polypeptides in roots of both cultivars we analysed their content 0, 1, 2, 3, 4 d after treatment (Fig. 2). Almost identical quantitative changes were found in the content of 28 kDa polypeptide in both barley cultivars, while the content of 20 kDa polypeptide in roots of Bavaria slightly declined with time.

To find out whether different pH of root media (3.5 - 6.5) or some other fytotoxic metals (Cu, Co, Cd) influence the polypeptide composition of peripheral membrane proteins in the same manner as Al does, we determined the content of 20 and 28 kDa polypeptides in roots exposed 24 h to different pH (Fig. 3) or 10 μM concentration of Cu, Co, or Cd (Fig. 4). pH of root media in the range from 3.5 to 6.5 does not significantly influence the content of both polypeptides. Surprisingly, no changes were found in polypeptide composition of peripheral membrane proteins isolated from the roots of cv. Alfor exposed to Cu, Cd or Co treatment (Fig. 4). In contrast, Cd or Co treated roots of cv. Bavaria accumulated both 20 and 28 kDa polypeptides in very similar extent as Al did.

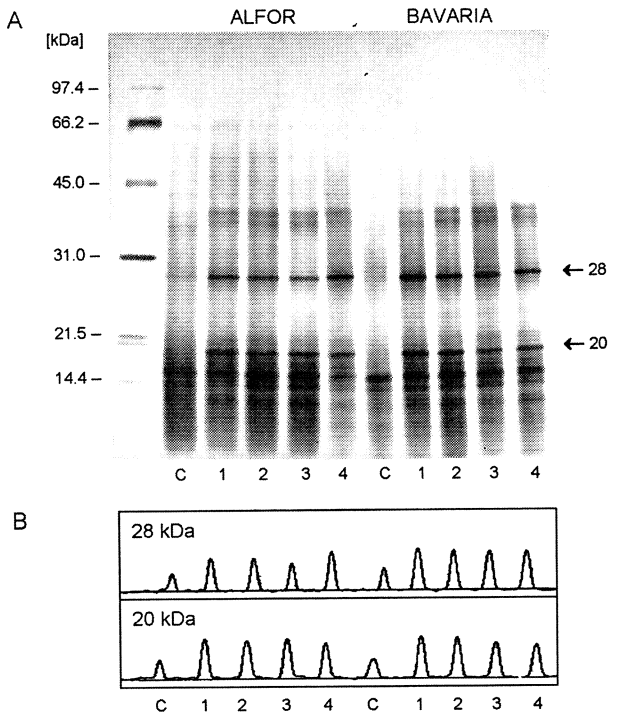

Fig. 2. SDS-PAGE analysis of peripheral membrane proteins isolated from roots of Al-sensitive (Alfor) and Al-resistant (Bavaria) barley cultivars grown 0 (C-control), 1, 2, 3 or 4 d in the presence of 10 μM Al (*A*). Densitometric scans correspond to 20 and 28 kDa polypeptides of the gel (*B*).

Discussion

Induction of extracellular and intracellular proteins by Al-treatment is well documented phenomenon in the literature. Basu *et al.* (1994b) showed that Al stress induces exudation of a number of different polypeptides (12, 23, 43.5 kDa) from the roots of Al-sensitive and Al-resistant wheat cultivars. One of them a 23 kDa polypeptide showed significant Al-binding capacity. Over-expression of other genes, *e.g.*, *wali* or *par* genes (Snowden and Gardner 1993, Ezaki *et al.* 1995) induced by Al did not show close relationship to Al tolerance and was also induced by various other stresses (P_i starvation, wounding, heavy metal and oxidative stress, *etc.*) suggesting their protective role not only in Al stress but also in oxidative stresses and heat shock stress (Ezaki *et al.* 1998).

Our results on Al-induced accumulation of two

peripheral membrane proteins in both Al-resistant and Al-sensitive barley cultivars suggests some linkage of these polypeptides to Al stress. Accumulation of both polypeptides was found in root tissues 24 h after exposure of plants to Al and only slow quantitative changes were observed with prolonged exposure of plants to Al.

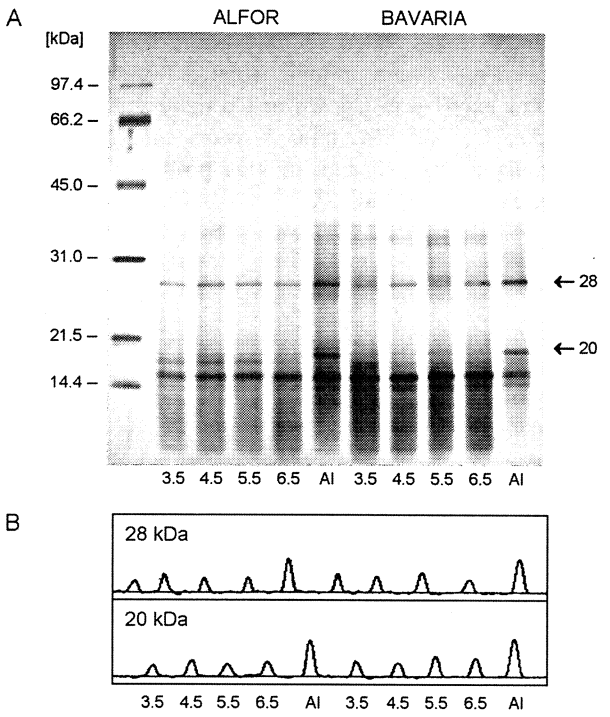

Fig. 3. SDS-PAGE analysis of peripheral membrane proteins isolated from roots of Al-sensitive (Alfor) and Al-resistant (Bavaria) barley cultivars grown for four days in the presence of 10 μM Al (Al), or in the root media with different pH values (1 mM $CaCl_2$, pH 3.5, 4.5, 5.5 or 6.5) (*A*). Densitometric scans correspond to 20 and 28 kDa polypeptides of the gel (*B*).

Because acid soils or low pH of root media alone affects gene expression (Espino *et al.* 1998) we followed the amount of 20 and 28 kDa polypeptides during acid stress (pH 3.5, 4.5). During this experiment no significant changes were observed in the accumulation of 20 and 28 kDa polypeptides as well as in root growth of both barley cultivars.

In order to determine the relationship of these two polypeptides to Al toxicity we exposed the barley plants to several heavy metals. The root growth test showed that cv. Bavaria posses increased resistance not only to Al but also to Cd and Co (root growth inhibition by 10 μM of metals increased in the following manner: Al 15 % < Co 25 % < Cd 28 % << Cu 45 %). In cv. Alfor root growth inhibition was higher in comparison to Bavaria and followed the order: Co 30 % < Al 35 % < Cu 42 % = Cd 43 %. Almost identical inhibition of root growth by Co and Al, and even higher by Cu and Cd in cv. Alfor did not cause accumulation of 20 and 28 kDa polypeptides as did Al and polypeptide pattern of peripheral membrane proteins remained unchanged even after 4 d exposure of plants to Cu, Co, or Cd. Surprisingly, in roots of cv. Bavaria 10 μM Cd or Co treatment induced almost identical accumulation of 20 a 28 kDa polypeptides as we

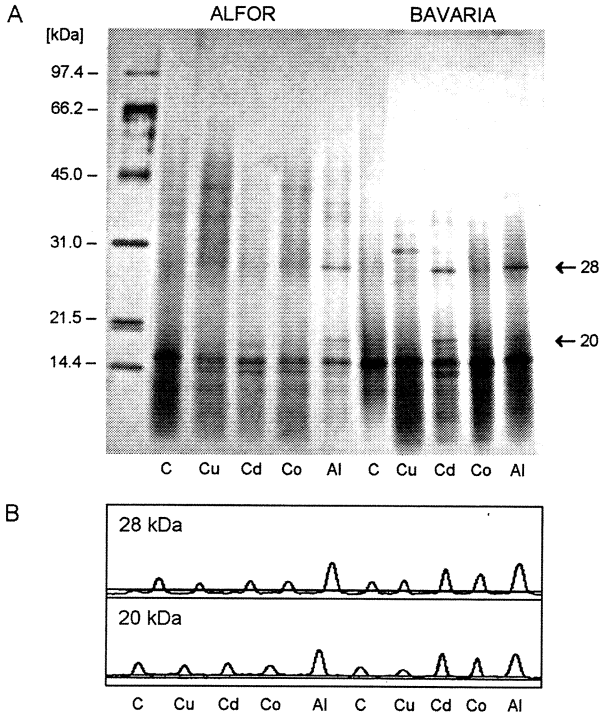

Fig. 4. SDS-PAGE analysis of peripheral membrane proteins isolated from roots of Al-sensitive (Alfor) and Al-resistant (Bavaria) barley cultivars grown for four days in the presence of Al, Cu, Cd and Co (10 μM), or in 1 mM $CaCl_2$ - control plants, C (*A*). Densitometric scans correspond to 20 and 28 kDa polypeptides of the gel (*B*).

found after Al treatment. In spite of almost 50 % inhibition of root growth by 10 μM Cu, polypeptide pattern of peripheral membrane proteins isolated from the roots of Bavaria was unaffected by Cu treatment. Similar results were described by Snowden *et al.* (1995), who found that Al-induced *wali* genes (*wali1, wali3, wali4,* and *wali5*) are also induced by Cd, Cu, Zn, Ga, and by wounding. These results and results on non-specific induction of same proteins by different biotic or abiotic stresses (*e.g.*, toxic metals, wounding, UV-radiation) described by Cruz-Ortega and Ownby (1993) and Didierjean *et al.* (1996) support the hypothesis that transcripts of most of the Al-induced genes belong to the general stress-related polypeptides. Poor linkage of 20 and 28 kDa polypeptides of barley roots to Al resistance was confirmed by Al-induced identical quantitative changes in the content of these polypeptides in both cultivars which does not correspond with their different

resistance to Al. Especially during longer exposure (four and more days) of barley plants to the low concentration of Al (5 and 10 μM) root growth increment of cv. Bavaria was 2 - 3 times higher than of cv. Alfor. No correlation between Al-resistance and callose synthesis in these two cultivars, as well as almost identical amount of Al accumulation in their roots and similar Al-induced root cell damages (Budíková and Mistrík 1999) suggest that increased Al-resistance in Bavaria is not associated with Al exclusion mechanisms but rather by mechanisms conferring the ability to tolerate Al in the plant symplasm. The question whether the Al-induced accumulation of 20 and 28 kDa peripheral membrane polypeptides might play a role in Al tolerance needs a better characterisation of their function, especially their Al-binding capacity and involvement in oxidative stress which are the subjects of ongoing experiments.

References

Basu, A., Basu, U., Taylor, G.J.: Induction of microsomal membrane proteins in roots of an aluminium-resistant cultivar of *Triticum aestivum* L. under conditions of aluminium stress. - Plant Physiol. **104**: 1007-1013, 1994a.

Basu, U., Basu, A., Taylor, G.J.: Different exudation of polypeptides by roots of aluminum-resistant and aluminum-sensitive cultivars of *Triticum aestivum* L. in response to aluminum stress. - Plant Physiol. **106**: 151-158, 1994b.

Budíková, S., Mistrík, I.: Cultivar characterisation of aluminium tolerance of barley seedlings by root growth, aluminium and callose distribution. - Biologia (Bratislava) **54**: 447-451, 1999.

Cacmak, I., Horst, W.J.: Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). - Physiol. Plant. **83**: 463-468, 1991.

Cruz-Ortega, R., Ownby, J.D.: A protein similar to PR (pathogenesis-related) proteins is elicited by metal toxicity in wheat roots. - Physiol. Plant. **89**: 211-219, 1993.

Didierjean, L., Frendo, P., Nasser, W., Genot, G., Marivet, J., Burkard, G.: Heavy-metal-responsive genes in maize: identification and comparison of their expression upon various forms of abiotic stress. - Planta **199**: 1-8, 1996.

Espino, F.J., González-Jean, M.T., Ibánez, J., Sendino, A.M., Vázquez, A.M.: Aluminum and low pH effects on translatable RNA population from bean calli. - Protoplasma **201**: 85-91, 1998.

Ezaki, B., Gardner, C.R., Ezaki, Y., Kondo, H., Matsumoto, H.: Protective roles of two aluminum (Al)-induced genes, *HSP150* and *SEDI* of *Saccharomyces cerevisiae,* in Al and oxidative stresses. - FEMS Microbiol. Lett. **159**: 99-105, 1998.

Ezaki, B., Tsugita, S., Matsumoto, H.: Expression of moderately anionic peroxidase is induced by aluminum treatment in tobacco cells: Possible involvement of peroxidase isozymes in aluminum ion stress. - Physiol. Plant. **96**: 21-28, 1996.

Ezaki, B., Yamamoto, Y., Matsumoto, H.: Cloning and sequencing of the cDNA induced by aluminium treatment and P_i starvation in cultured tobacco cells. - Physiol. Plant. **93**: 11-18, 1995.

Hamel, F., Breton, C., Houde, M.: Isolation and characterization of wheat aluminum-regulated genes: possible involvement of aluminum as a pathogenesis response elicitor. - Planta **205**: 531-538, 1998.

Heukesloven, J., Dernick, R.: Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. - Electrophoresis **6**: 103-112, 1985.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. - Nature **277**: 680-685, 1970.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Mistrík, I., Tamás, L., Huttová, J.: Quantitative changes in maize membrane proteins induced by aluminium. - Biol. Plant. **43**: 85-91, 2000.

Ownby, J.D., Hruschka, W.R.: Quantitative changes in cytoplasmic and microsomal proteins associated with aluminium toxicity in two cultivars of winter wheat. - Plant Cell Environ. **14**: 303-309, 1991.

Richards, K.D., Gardner, R.C.: The effect of aluminium treatment on wheat roots: expression of heat shock, histone and SHH genes. - Plant Sci. **98**: 37-45, 1994.

Richards, K.D., Schott, J., Sharma, Y.K., Davis, K.R., Gardner, R.C.: Aluminum induces oxidative stress genes in *Arabidopsis thaliana.* - Plant Physiol. **116**: 409-418, 1998.

Snowden, K.C., Gardner, R.C.: Five genes induced by aluminum in wheat (*Triticum aestivum* L.) roots. - Plant Physiol. **103**: 855-861, 1993.

Snowden, K.C., Richards, K.D., Gardner, R.C.: Aluminium-induced genes. Induction by toxic metals, low calcium, and wounding and pattern of expression in root tips. - Plant Physiol. **107**: 341-348, 1995.

Takahashi, Y., Nagata, T.: *parB*: An auxin-regulated genome encoding glutathione S-transferase. - Proc. nat. Acad. Sci. USA **89**: 56-59, 1992.

Tamás, L., Mistrík, I., Huttová, J.: Protein profiles in root of aluminium sensitive and resistant barley cultivars after aluminium treatment. - Biologia (Bratislava) **54**: 459-465, 1999.

PHOTOSYNTHESIS AND CROP PRODUCTION

International Conference to be held in Kyiv, Ukraine
7 - 11 October 2002

ORGANIZED BY

Ministry of Education and Science of Ukraine
Ukrainian Society of Plant Physiology
Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine

CONFERENCE TOPICS

1. Structure and function of photosynthetic membranes.
2. Electron transport and ATP synthesis regulation in photosynthetic systems.
3. Photosynthetic carbon metabolism regulation.
4. Source-sink regulation in photosynthesis.
5. Effect of biotic and abiotic factors on the photosynthetic apparatus.
6. Photosynthesis and mineral nutrition.
7. Effect of plant growth regulators on photosynthesis.
8. Photosynthesis in gene-modificated plants.
9. Photosynthesis during leaf ontogeny.
10. Photosynthesis in canopy and remote sensing of crop production.
11. Photosynthesis in the condition of global climate change.

The main objective of the Conference is to present comprehensive and up-to date reviews of recent achievements in the field of Photosynthesis and Crop Production. The Conference is the opportunity to establish and strength the international working links between Ukrainian and international researchers in photosynthesis and to make the quaintance of Ukrainian plants and environment. The special attention will be concentrated on the molecular mechanisms of photosynthesis regulation, effects of the stresses in different combination on the photosynthesis and source-sink regulation, using of transgenic plants in the studing of photosynthesis and solution of food question, applying of the remote sensing methods in the sustainable agriculture. The program will include lectures by prominent scientists in these fields of study and will be completed with lectures selected on the basis of abstracts sent in advance and poster sessions. The official language of the Conference is English.

For further details please contact:
Tamara M. Shadchina
Department of Physiology and Ecology of Photosynthesis, Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine,03022, Kyiv-22, Vasylkivska 31/17, Ukraine
PhoneTel: (+38) 044.2633108; fax: (+38) 044 2635150; e-mail: shad@i.kiev.ua
http://www.photosynthesis-kyiv.iptelecom.net.ua