Regulation of aldehyde oxidase and nitrate reductase in roots of barley (Hordeum vulgare L.) by nitrogen source and salinity

Rustem T. Omarov¹, Moshe Sagi and S. Herman Lips

Biostress Research Laboratory (J. Blaustein Institute for Desert Research) and Department of Life Sciences, Ben-Gurion University of the Negev, Sede Boqer 84990, Israel

Received 8 September 1997; Accepted 18 December 1997

Abstract

The molybdenum cofactor (MoCo) is a component of aldehyde oxidase (AO EC 1.2.3.1), xanthine dehydrogenase (XDH EC 1.2.1.37) and nitrate reductase (NR, EC 1.6.6.1). The activity of AO, which catalyses the last step of the synthesis of abscisic acid (ABA), was studied in leaves and roots of barley (Hordeum vulgare L.) plants grown on nitrate or ammonia with or without salinity. The activity of AO in roots was enhanced in plants grown with ammonium while nitrate-grown plants exhibited only traces. Root AO in barley was enhanced by salinity in the presence of nitrate or ammonia in the nutrient medium while leaf AO was not significantly affected by the nitrogen source or salinity of the medium.

Salinity and ammonium decreased NR activity in roots while increasing the overall MoCo content of the tissue. The highest level of AO in barley roots was observed in plants grown with ammonium and NaCl, treatments that had only a marginal effect on leaf AO. ABA concentration in leaves of plants increased with salinity and ammonium.

Key words: ABA, aldehyde oxidase, ammonium, nitrate, salinity.

Introduction

Information on AO regulation in plants is scant and nothing is known about its sensitivity to environmental factors. Most of the interest in AO stems from its involvement in the biosynthesis of ABA (Walker-Simmons et al., 1989) and indole acetic acid (IAA) (Koshiba et al., 1996). The barley (Hordeum vulgare) mutant Az34 which exhibited a low basal level of ABA also had a reduced capacity to produce abscisic acid during water stress (Walker-Simmons et al., 1989). The mutation is located in one of the genes controlling the expression of MoCo (Mendel, 1997), a constitutive component of Mo-enzymes such as NR, XDH and AO. This mutant was found to lack aldehyde oxidase activity in the presence of several substrates such as abscisic aldehyde and heptaldehyde. Wilting mutants of tomato (Lycopersicon esculentum), sitiens and flacca, which do not produce ABA have been shown to lack the capacity to oxidize abscisic aldehyde (Taylor et al., 1988; Sindhu et al., 1990). Recently it has been found that the flc mutant of tomato lacks aldehyde oxidase and xanthine dehydrogenase but not nitrate reductase (Marin and Marion-Poll, 1997). The aba3 mutant of Arabidopsis (A. thaliana) seems to have lost the capacity to introduce S into MoCo as required by the hydroxylases AO and XDH (Schwartz et al., 1997).

ABA is a plant hormone important in seed development and dormancy and in plant response to environmental stress (Marin et al., 1996; Kende and Zeevaart, 1997). The endogenous ABA level in leaves and roots of Ricinus communis increased in response to ammonium nutrition and salinity while nitrate had the reverse effect. The flow of ABA in both xylem and phloem was also increased by ammonium compared to nitrate (Peuke et al., 1994). Salinity and drought brought about an increase of ABA and a decrease of cytokinins (Kende, 1965; Downton and Loveys, 1981; Bano et al., 1993). ABA concentration in the xylem sap was enhanced when the soil became dry, saline, nitrogen deficient or compacted (Munns and Cramer, 1996).

Nitrogen is a mineral nutrient required in large amounts...
by plants. The oxidation stage of nitrogen entering the root, the organ in which it is assimilated and the forms in which it is transported to the shoot, may be linked to the transmission of growth-regulating signals from root to shoot (Lips, 1997). The two major forms of nitrogen taken up by plants are NO$_3^-$ and NH$_4^+$ ions, each of which differently influences the growth and development of plants (Lips et al., 1990; Pilbeam and Kirkby, 1992). While nitrate is transported and assimilated in the shoots by practically all annual crops characterized by fast growth rates, ammonium must be assimilated in the roots and the assimilation products transported to the leaves (Lips et al., 1990).

Fast growth correlates with the transport of nitrate and cytokinins from roots to shoots (Lips, 1997) and this transport was inhibited by salinity (Cramer et al., 1995).

Since nitrate transport through the xylem seems to be linked to that of cytokinin, ammonium nutrition may be related to ABA levels in the root (Peuke et al., 1994). This rationale led us to study the effect of nitrogen sources (nitrate and ammonium) and salinity on the molybdo-enzymes aldehyde oxidase, nitrate reductase and xanthin dehydrogenase. In the present paper, the effects of nitrogen source and salinity on the activities of aldehyde oxidase, xanthine dehydrogenase and nitrate reductase in roots of barley plants are described. The levels of ABA concentration in leaves and roots of barley grown on nitrate or ammonium and their response to mild salinity were determined.

Materials and methods

Plant material

Barley (Hordeum vulgare L.) cv. Steptoe seedlings were grown in hydroponics (201 containers) or in vermiculite (51 pots) as previously described (Savidov et al., 1997a). The seeds were germinated in 0.2 mM CaSO$_4$ in the dark at room temperature over 3 d. Seedlings were allowed to develop under non-saline conditions during the first 3 d after germination. Uniform plants, 23 pot$^{-1}$, were grown in 4–8 replications in a completely randomized block design for each treatment. The experiments were conducted in a greenhouse, in which average day temperatures fluctuated during the growth period between 20 and 25 °C, and night temperature between 8 and 12 °C. Midday PPFD was 900–1000 μmol m$^{-2}$ s$^{-1}$.

Each of the nitrogen sources, NH$_4$Cl and NaNO$_3$, was supplied at a concentration of 4 mM. Salinity treatments consisted of NaCl (50 mM) in half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1938) which resulted in an overall electroconductivity (EC) of 11.2 dS m$^{-1}$. Half-strength Hoagland nutrient solution (EC 2 dS m$^{-1}$) served as the control treatment. The pH of the nitrogen solutions was adjusted to 6.0–6.5 with H$_2$SO$_4$. Nitrapyrin (N-serve 24E; Dow Chemical Co., King's Lynn, England), 4 μl l$^{-1}$, and dicyandiamide (Sigma Chemicals, St Louis, MO, USA) 7.5 mg l$^{-1}$, were added to the nutrient solutions to prevent ammonium nitrification. The plants grown in vermiculite were irrigated twice daily with identical volumes of nutrient solution to prevent an increase of more than 10% EC in the leachate as compared with that of the input solution.

Tissue extraction

Part of the barley roots were frozen in liquid nitrogen immediately after their harvest and ground in an ice-cold mortar with acid-washed sand with ice-cold extraction medium containing 250 mM TRIS-HCl (pH 8.5), 1 mM EDTA, 1 mM dithiothreitol, 3 mM reduced glutathione, and 3% (w/v) polyvinylpyrrolidone. Samples of 4 g fresh weight of tissue were extracted in 8 ml buffer (1:2 w/v) for AO determination. A ratio of 1 g tissue to 3 ml buffer (1:3, w/v) was used for NR assays. The homogenized plant material was centrifuged at 27 000 g in a Centrikon T-124 refrigerated centrifuge at 4 °C for 15 min. The resulting supernatant was used for subsequent assays.

Aldehyde oxidase determination

Ammonium sulphate was added to the supernatant to 60% saturation. The resulting mixture was stirred for 30 min and then centrifuged at 15 000 g for 15 min. The precipitated proteins were dissolved in a small volume of 50 mM K-phosphate buffer (pH 7.8) and desalted on Sephadex G-25 (Pharmacia) columns equilibrated with the same buffer. AO activity was assayed by monitoring the decrease of absorbance at 600 nm in a Genesys-2 (Milton Roy, USA) spectrophotometer using DCIP as an electron donor (Courtright, 1967). The reaction mixture (1 ml) contained 100 μl of the enzyme extract, 50 mM K-phosphate buffer (pH 7.4), 0.002% 2,6-dichloroindophenol (DCIP), 0.1 mM phenazine methosulfate, and 1.5 mM heptaldehyde or 2 mM indole-3-aldehyde. AO activity was expressed as nmol$^{-1}$ DCIP mg$^{-1}$ protein min$^{-1}$.

AO activity was also detected in polyacrylamide gels by staining after native electrophoresis. Native gels were prepared with 7.5% acrylamide gel (Laemmli, 1970) in the absence of SDS at 4 °C. The gel was immersed after electrophoresis in 0.2 M phosphate buffer, pH 8.0, for 10 min followed by gentle shaking of the gel at room temperature in a reaction mixture containing 0.1 M TRIS-HCl, pH 8.0, 1.5 mM heptaldehyde or 1 mM indole-3-aldehyde, 0.1 mM phenazine methosulfate, and 1 mM MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazole-bromide).

NADH-nitrite reductase assay

NR was assayed in a reaction mixture containing 15 mM K-phosphate buffer pH 7.5, 12.5 mM KNO$_3$ and 0.4 mM NADH (Cramer et al., 1996). The reaction was started with the addition of 50 μl of the enzyme extract. The total volume of the assay solution consisted of 500 μl and the enzyme reaction took place at 30 °C for 15 min. The assay was terminated by addition of a 0.1 ml mixture of 0.15 mM phenazine methosulfate and 0.5 M Zn acetate, and mixed vigorously to remove residual NADH. Nitrite was determined using 1 ml of a 1:1 (w/v) mixture of 1% (w/v) sulfanilamide in 3.0 M HCl and 0.02% (w/v) N-1-naphthyl-(1)-dihydrochloride. Absorbance at 540 nm was measured after 20 min. NR activity was expressed as μmol NO$_2^-$ mg$^{-1}$ protein min$^{-1}$ or μmol NO$_2^-$ g$^{-1}$ fresh weight h$^{-1}$.

Xanthine dehydrogenase assay

XDH activity was detected after native gel-electrophoresis using xanthine or hypoxanthine as substrates (Mendel and Müller, 1976).
Molybdenum cofactor activity in plant tissue was estimated using a pleiotropic nit-1 mutant of Neurospora crassa according to Nason et al. (1970). The original procedure (Mendel et al., 1985) was carried out essentially as recently modified and described (Sagi et al., 1997; Savidov et al., 1997b).

**MoCo determination**

The determination of MoCo activity in barley roots was performed using a pleiotropic nit-1 mutant of Neurospora crassa according to Nason et al. (1970). The original procedure (Mendel et al., 1985) was carried out essentially as recently modified and described (Sagi et al., 1997; Savidov et al., 1997b).

**Abscisic acid determination**

ABA was analysed immunologically by ELISA procedure with monoclonal antibodies as described (Mertens et al., 1985). The original procedure (Mendel et al., 1985) was carried out essentially as recently modified and described (Sagi et al., 1997; Savidov et al., 1997b).

**Protein determination**

Soluble proteins were estimated by the Bio-Rad micro assay modification of the Bradford procedure (1976) using crystalline bovine serum albumin as a reference.

**Statistics**

Each treatment was analysed with at least three replicate tissue samples bulked from at least five plants. All treatments were repeated at least three times.

**Results**

**Aldehyde oxidase activity**

Significant activity of AO in roots of barley was observed only in plants grown with ammonium as their nitrogen source. Plants grown with nitrate exhibited very low levels of AO activity in their roots (Fig. 1). Salinity significantly increased root AO activity in plants grown in either \( \text{NO}_3^- \) or \( \text{NH}_4^+ \). The highest level of AO activity, more than 5-fold higher than in nitrate-grown plants, was observed in roots of \( \text{NH}_4^+ \)-fed plants under saline conditions. AO activity in leaves of the same plants was not significantly affected by the same treatments, yielding rates ranging between 32 and 37 nmol DCPIP mg\(^{-1}\) min\(^{-1}\).

**NADH-NR assay**

NR activity in roots of \( \text{NO}_3^- \)-fed plants decreased with salinity, when calculated either on a protein or on a fresh weight basis (Fig. 2). The decline was more marked when calculations were performed on a protein basis. A low level of NR activity, essentially the constitutive enzyme level, was detected in \( \text{NH}_4^+ \)-fed plants under both saline and non-saline conditions.

**Xanthine dehydrogenase activity**

XDH activity, another plant MoCo enzyme with a considerable part of its amino acid sequence homologous to that of AO (Ori et al., 1997), was observed in roots of barley plants grown in all the treatments applied. Salinity and N source, contrary to their effect on root AO, did not significantly affect XDH activity (not shown) in barley. Aldehyde oxidase and xanthine dehydrogenase in barley roots differed in molecular weight and in their substrate specificity as evidenced by their staining after exposure of native PAGE gels to hypoxanthine and heptaldehyde, yielding two distinctive bands in the gel (Fig. 3).

**MoCo assay**

The combination of salinity and ammonium brought about a considerable increase of the overall MoCo activity detected in the root (Fig. 4). The activity of MoCo did not change significantly in roots of plants grown on the other treatments tested. The MoCo pool size in barley roots was not directly related to changes in the allocation of this cofactor to Mo-enzymes in the root.
Fig. 3. Staining of AO and XDH in native gels using heptaldehyde and hypoxanthine as substrates. Extracts were obtained from roots of ammonium-grown plants.

Fig. 4. MoCo activity in barley roots as affected by salinity (50 mM NaCl) and N source (4 mM NaNO₃ or 4 mM NH₄Cl). Bars indicate standard error of the mean.

Abscisic acid content in barley roots and shoots

ABA concentration in leaves of nitrate-fed plants was between 130 and 140 pmol g⁻¹ FW and increased with salinity and ammonium. Addition of NaCl to the ammonium containing growth medium increased slightly the ABA content of barley leaves (Fig. 5) in spite of the fact that very little changes were observed in the leaf AO activity. Root ABA levels were low (data not shown) and differences between treatments were not significant. ABA in the xylem sap was not measured in these experiments.

Discussion

Aldehyde oxidase is a Mo-enzyme which oxidizes abscisic aldehyde (ABAld) to abscisic acid (ABA) in higher plants (Walker-Simmons et al., 1989; Sindhhu et al., 1990; Leydecker et al., 1995). The response of a given plant to salinity and other abiotic stresses depends on its physiological and genetic characteristics. The changes in the hormonal balance triggered by these stresses are among the factors determining the plant tolerance or susceptibility to the stresses (Fedina et al., 1994).

ABA is a plant hormone related to stress regulation (Bano et al., 1993; Schill et al., 1996). Exposure of barley and cotton plants to NaCl reduced transpiration and increased ABA levels in leaves, roots and xylem sap (Kefu et al., 1991). ABA levels in plant tissues were increased by ammonium nutrition and salinity while nitrate had the reverse effect (Peuke et al., 1994). Slow seed germination can be accelerated by hormonal treatments or by nitrate (Vincent and Roberts, 1977; Khan, 1997).

Relatively little information has been published concerning biochemical characterization of plant AO. Maize AO has been found to have biochemical and molecular features very similar to animal AO (Koshiha et al., 1996; Sekimoto et al., 1997). The tomato AO protein has a considerable extent of sequence homology with aldehyde oxidase and xanthine dehydrogenase from various organisms (Ori et al., 1997). Both animal and plant AO exhibited affinity for a number of different aldehydes (Koshiha et al., 1996). Maize AO activity in the coleoptile apex, a known producer of auxin, oxidized indole-3-acetaldehyde into IAA (Koshiha et al., 1996). Since AO seemed to be capable of oxidizing abscisic aldehyde as well as indole acetaldehyde, one may speculate that salinity and N source affected the root enzyme involved mainly, but not only, in ABA biosynthesis, while having an insignificant effect on leaf AO which does not produce
much ABA. ABA and IAA may interact to signal the need for the changes in plant functions required for adaptation to stress conditions (Dunlap and Robacker, 1990). Increasing concentrations of NaCl reduced the endogenous levels of IAA in roots, although this decrease in root IAA occurred independently with the increase in root ABA (Dunlap and Binzel, 1996).

In view of the low effect of salinity on leaf AO, the increase of ABA level observed in leaves may be the result of enhanced ABA transport via the xylem following the induction/activation of AO in the roots rather than of an increased local production of the hormone. The main site of ABA synthesis is the root from where it is transported to the shoot, especially under stress conditions (Wolf et al., 1990; Baño et al., 1993). ABA synthesis in roots was enhanced under salinity conditions (Schnapp et al., 1990).

Root AO activity of barley plants grown with ammonium in the nutrient medium was higher than in nitrate-fed plants (Fig. 1). Salinity and drought (data not shown) significantly enhanced the activity of AO in roots of both NH$_4^+$ and NO$_3^-$-fed plants. Contrary to the changes observed in root AO, leaf AO was not significantly affected by nitrogen source and/or salinity. The increased activity of AO in roots of plants exposed to salinity may be the result of enhanced expression of the gene coding for the AO apoprotein or of a post-translational activation of existing enzyme molecules. The lack of effect of the AO enhancing treatments on XDHN in the barley plants may rule out regulation of the gene expressing the protein responsible for the sulphur addition to hydroxylase MoCo (Schwartz et al., 1997). According to the results of this study, ABA concentrations in leaves of barley were higher in ammonium- than nitrate-grown plants and were enhanced by salinity in all cases (Fig. 5).

ABA content increased significantly in leaves of Ricinus communis L. only in ammonium-fed plants and salinity slightly enhanced ABA content in leaves of NH$_4^+$-fed plants, but had no effect on nitrate-fed plants (Peuke et al., 1994). However, salt stress considerably increased ABA concentration in xylem exudates of nitrate-fed plants, but not in leaves. Hormone concentration in a given plant is determined by the balance between synthesis and degradation along with other factors such as compartmentalization and transport (Hetherington and Quatrano, 1991).

The MoCo pool in roots of ammonium-fed plants increased under saline conditions over that in nitrate-grown plants (Fig. 4). Salinity and N concentration increased MoCo content in ryegrass (Lolium multiflorum) roots and shoots (Sagi et al., 1997). The need for the increased MoCo level under saline conditions is not clear since the level of this cofactor in the tissue is vastly greater than that found in the three major plant Mo-enzymes known. The increase of AO in ammonium-grown plants under saline conditions is accompanied by a considerably larger increase of MoCo in the roots (Figs 1, 4). The apparent excess of MoCo in the tissue may be the result of considerable amounts of inactive Mo-enzymes as extensively described for NR (Moorhead et al., 1996). Roots of barley grown in nitrate did not increase their MoCo level under saline conditions (Fig. 4).

The increase of aldehyde oxidase under saline and drought (data not shown) conditions may be responsible for the increase of the ABA levels in roots and its subsequent rise in the xylem sap and may constitute one of the early events of plant adaptation to stress. The response of AO to inorganic nitrogen ions may bring us closer to understanding the effects of these ions in the control of dormancy and seed germination. The way in which nitrate, ammonium and salinity affect the activity or synthesis of AO and the resulting fluctuations of ABA levels in different plant organs may involve the regulation of gene expression required for MoCo biosynthesis (Mendel, 1997) or of the genes responsible for the synthesis of the apoproteins of the Mo-hydroxylases. The extent of post-translational regulation of AO and XDHN is unknown at the moment.

Acknowledgements

This work has been possible thanks to the financial support of AID/CDR to project CA15–124 and to the Fohs Foundation (USA). Our gratitude also to Genia Shichman and Fu Xiao Ping for their excellent technical assistance.

References


Hoagland DR, Arnon DI. 1938. The water culture method for growing plants without soil. California Agricultural Experimental Station Circular 347, 1–32.


Sindhu RK, Griffin DH, Walton DC. 1990. Abscisic aldehyde is an intermediate in the enzymatic conversion of xanthoxin to abscisic acid in Phaseolus vulgaris L. leaves Plant Physiology 93, 689–94.


