Distribution of the Mo-enzymes aldehyde oxidase, xanthine dehydrogenase and nitrate reductase in maize (Zea mays L.) nodal roots as affected by nitrogen and salinity

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Abstract

The distribution of the Mo-enzymes aldehyde oxidase (AO; EC 1.2.3.1) xanthine dehydrogenase (XDH; EC 1.2.1.37) and nitrate reductase (NAD(P)H NR; EC 1.6.6.1-2) was studied along the longitudinal and transversal axes of maize (Zea mays L. cv. Jubily) nodal roots as affected by nitrogen sources and salinity. Activities of the Mo-enzymes were considerably enhanced under mild saline conditions. The activities of AO and XDH increased following addition of ammonium to the nutrient solution. Immunoblot analysis with antibodies raised against maize AO protein revealed increased levels of AO proteins in root tips of ammonium fed plants. Application of salinity to nitrate fed plants did not affect the enzyme protein level, although it enhanced the activity of the Mo-hydroxylases. The specific activities of the Mo-enzymes were the highest in root tips (0–1 cm segments) while on the transversal axis maximal activity was observed in the stele or vascular cylinder. Activity staining of AO after native PAGE of root extracts revealed four bands of AO proteins (AO1-4) capable of oxidizing a number of aliphatic and aromatic aldehydes. Increased AO activity in maize nodal roots grown with ammonium, and salinity were observed mainly at the AO3 and AO4 bands. Tips and stele contained primarily AO3 and AO4, and only traces of AO1 and AO2. SDS-PAGE of root extracts followed by Western blots revealed, besides the major 150 kD subunit of AO, two polypeptides with molecular masses of 72 and 85 kD located specifically in the cortex. Part of the polymorphism of AO in plant roots may be related to the allocation of distinct isoforms to different regions of the root, although the specific metabolic roles of the different bands have not been established. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Aldehyde oxidase; Cortex; Nitrate reductase; Maize; Nodal roots; Root tips; Stele; Xanthine dehydrogenase

1. Introduction

In higher plants the molybdenum cofactor (MoCo) constitutes the active site of at least three enzymes: aldehyde oxidase (AO, EC 1.2.3.1), xanthine dehydrogenase (XDH, EC 1.2.3.7) and nitrate reductase (NAD(P)H: NR, EC 1.6.6.1-2). AO catalyzes the final steps in the biosynthesis of two phytohormones, through the oxidation of abscisic aldehyde to abscisic acid (ABA) [1,2] and indole-3-acetaldehyde to indole-3-acetic acid (IAA) [3]. Four bands of AO proteins were reported in barley plants [4] as well as three AO isoforms in Arabidopsis [5] and ryegrass [6]. At least two maize cDNAs were cloned in maize where they are differentially expressed in a tissue specific manner, with zmAO-1 expressed at a higher level in roots [7]. In Arabidopsis seedlings,
AO1 was suggested to be responsible for IAA biosynthesis [8].

ABA controls physiological and molecular processes involved in the response of plants to environmental stresses such as freezing, drought and salinity [9]. Exposure of barley and cotton plants to NaCl reduced transpiration and increased ABA levels in the leaves and xylem sap [10]. Maize roots subjected to drought increased ABA synthesis [11]. ABA content increased significantly in leaves of *Ricinus communis* L. only in ammonium-fed plants and salinity enhanced slightly ABA content in leaves of NH4-fed plants but had no effect on nitrate-fed plants [12]. Roots of several plant species seem to be an important site of ABA synthesis under environmental stress conditions, from where the hormone was transported to the shoot [13,14]. Since ABA affects water relation parameters [15] and K⁺ transport processes [16], these physiological and transport parameters exert their influence on the K⁺-shuttle responsible for the translocation of nitrate to the shoot [17].

XDH is an oxidoreductase of importance in purine metabolism. It catalyzes the first oxidative step in the catabolism of purine and purine derivatives resulting from nucleic acid degradation. XDH takes part in ureide biosynthesis [18] in higher plants, through de novo synthesis of purines from glutamine [19].

NR is the first enzyme in the nitrate assimilation pathway, reducing nitrate to nitrite with NADH or NADPH as electron donor [20]. NR of higher plants is post-translationally regulated by a two-step mechanism that rapidly and reversibly inhibits the enzyme in response to diverse signals including light–dark transition, decrease in CO₂ levels, or an increase in cytosolic pH (for review see Refs. [21–23]).

Recent studies have described the effects of salinity and nitrogen source on Mo-enzymes activities. Salinity increased the activity of AO in barley [24] and ryegrass [6], XDH and NR in ryegrass [6,25] and NR in tomato plant roots [26].

The plant root cells divide, elongate and differentiate along its longitudinal and transversal axes. Cells differ in both developmental stage and function, showing prominent differences in their capacity for nutrient absorption, storage, translocation and assimilation [27,28]. This cell heterogeneity should be considered while interpreting biochemical and molecular data obtained with root tissue.

Zone and tissue specific gene expression in roots has been reported [29]. In-situ hybridization localized *nia* mRNA mainly in root tips and the stele of chicory roots at high nitrate concentration [30]. Post-embedding immunogold labeling revealed NR proteins in the cytoplasm of root epidermal and cortical cells, as well as in the parenchyma and pericycle cells of the stele [31]. Studies have been reported on the organ specific distribution of AO in *Arabidopsis thaliana* [8] and on AO gene expression in tomato organs [32]. AO has been observed in the apical and mature regions of maize coleoptiles [3]. Very little is known about the distribution of AO and XDH along and across the root and its main anatomical areas such as the cortex, the vascular cylinder, the root apex and the mature zones.

The aim of the present study was to characterize the effect of salinity and nitrogen sources on the distribution of Mo-enzymes in nodal roots of maize, within the different root zones with defined physiological functions and of progressive maturation.

2. Materials and methods

2.1. Plant material

Seeds of maize (*Zea mays* L., cv. Jubily) were surface sterilized with ethanol for 3 min and then germinated in 0.2 mM CaSO₄ in the dark at room temperature. The young seedlings were transferred to moist filter paper for two weeks and then to aerated nutrient solutions, wrapping a small part of the plant stem in foam rubber and inserting the plant into the plastic lids of containers with nutrient solution. The plants were grown in 20-l containers with nutrient solutions for a further 3 weeks before use as previously described [33]. Nutrient solutions were replaced once a week and the pH of the medium (6.5) was monitored and adjusted daily. Uniform maize plants, 8/pot, were grown in four replications for each treatment in a completely randomized block design. The experiments were conducted in a greenhouse, with an average day temperature of 20–25°C, and of 8–12°C during the night. Midday PPFD was 900–1000 μmol/m² per s. Nitrogen sources were 4 mM NaNO₃ or 2 mM (NH₄)₂SO₄ as part of a modified half-strength Hoagland nutrient solution [37]. Salinity consisted of 50 mM NaCl.
2.2. Excision of root segments

Maize nodal roots were selected from the first or second nodes to a length of about 12 cm, without lateral roots. A group of similar nodal roots were excised and placed on a glass plate wetted with growth medium. The roots were cut with a razor blade into three segments: 0–1, 1–2, and 2–10 cm, where the numbers represent the distance from the apex in cm. For cortex and stele separations, the first 2-cm of the root were discarded and the 2–10 cm segments stripped manually. This could be readily performed due to the limited lignification of the stele at this stage of root development. Similar root segments of 10 nodal roots constituted one of three replicates.

2.3. Tissue extraction

Root samples were extracted immediately after harvesting. Crude extracts used for the assays of AO and XDH in native gel electrophoresis were prepared by maceration with acid washed sand in a mortar with ice-cold extraction medium. The extraction medium consisted of 250 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM reduced glutathione (GSH), 5 μM FAD and 3% (w/v) polyvinylpolypyrrolidone (PVPP). Samples of 1 g root tissue were extracted in 3 ml buffer (1:3 w/v). For NR determination, the extraction buffer contained 25 mM Tris–HCl (pH 8.4), 3 mM EDTA, 10 μM FAD, 5 μM sodium molybdate, 1 mM DTT, 5 mM cystein, 10 μM antipain, 0.1 mM phenylmethyl sulfonylfluoride (PMSF), and 3% (w/v) washed PVPP (Sigma chemicals) [35]. The ratio of tissue to extraction buffer was 1:8 (w/v). The homogenized plant material was centrifuged in a Centrikon T-124 refrigerated centrifuge at 27 000 g and 4°C for 15 min. The resulting supernatant was used in subsequent assays.

2.4. Aldehyde oxidase assay

AO activity was detected in polyacrylamide gels by staining after native electrophoresis with 7.5% acrylamide gels [36] in the absence of SDS at 4°C. The gel was immersed in a reaction mixture containing 0.2 M phosphate buffer, pH 7.5, 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) for 10 min followed by gentle shaking at room temperature. Generally, AO activity was assayed on protein basis, when 80 μg of total soluble protein was loaded in each lane, or on FW basis when 100 μ/lane was used.

2.5. Xanthine dehydrogenase assay

XDH activity was detected after native gel-electrophoresis using hypoxanthine as a substrate [37]. Each lane was loaded with 80 μg of total soluble protein.

Native PAGE was carried out with a Protean II xi Cell (Bio-Rad, USA). Quantitative analyses of formazane bands in the gel, were carried out after scanning and the relative intensities of the bands estimated by NIH Image 1.6 software.

2.6. Western blot analysis

The AO proteins extracted from the plant material were subjected to Western blotting. Ground tissue (1 g FW) was extracted with 1 ml Tris–HCl buffer, pH 7.5, 3 mM DTT, 1 mM EDTA, 5 mM GSH and 50 μM leupeptin. After centrifugation, samples of the resulting supernatant were added to SDS-buffer at a ratio of 1:4 (v/v). About 40 μg total soluble protein was loaded in each lane. SDS-PAGE took place in 10% polyacrylamide gels [36]. The separated proteins were then electrophoretically transferred onto a nitrocellulose membrane (0.2 μm pore size, Schleicher and Schüll, Dassel, Germany). Blotting time was 1 h at 2 mA/cm². Blots were blocked for 90 min in 5% (w/v) bovine serum albumin in Tris-buffered saline (TBS: 10 mM Tris–HCl, pH 8.0; 150 mM sodium chloride). Immunodetection of AO with polyclonal mouse antibodies raised against the purified maize AO [3] followed a 500-fold dilution in TBS and anti-mouse IgG (Sigma) secondary antibodies 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 (66 kD), carbonic anhydrase (30 kD), lactate dehydrogenase (140 kD), myoglobin (17 kD) and ovalbumin (45 kD). KOH digestion of the gel was performed for equalizing protein content.
Fig. 1. AO (A), XDH (B) and NR (C) activities in crude extract of maize nodal roots as affected by N source and salinity (50 mM NaCl). For AO and XDH: crude protein supernatants (80 μg/lane) were subjected to native PAGE. Substrate staining of AO and XDH bands was done with indole-3-aldehyde and hypoxanthine, respectively. Controls (100%) were the enzyme activities of extracts obtained from plants grown with nitrate in the absence of salinity. Numbers above the lanes indicate percentage values obtained by densitometry and analysis by computer software (NIH Image 1.6). The zymogram represents one of at least three different experiments with similar results. For NR: enzyme activity was calculated on protein basis. Bars indicate standard error of the mean (n = 3).

kD), bovine serum albumin (78 kD), and ovalbumin (46.7 kD).

Table 1
NR, AO and XDH activities in maize nodal roots as well as AO protein level in the tips of maize nodal roots as affected by nitrogen source and salinity

<table>
<thead>
<tr>
<th>N source</th>
<th>Salinity (mM NaCl)</th>
<th>NR activity (nmol NO$_3^-$/mg per min)</th>
<th>AO activity (density as % of the control)</th>
<th>XDH activity (density as % of the control)</th>
<th>AO protein (arbitrary density units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3^-$</td>
<td>0</td>
<td>0.53</td>
<td>100 d</td>
<td>100 b</td>
<td>145 b</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.43$^b$</td>
<td>127 c</td>
<td>139 a</td>
<td>150 b</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>0</td>
<td>–</td>
<td>164 b</td>
<td>126 a</td>
<td>205 a</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>–</td>
<td>182 a</td>
<td>134 a</td>
<td>152 b</td>
</tr>
</tbody>
</table>

$^a$ AO was assayed with indole-3-aldehyde, while XDH was assayed with hypoxanthine as substrates. Different letters following the mean values in each column indicate significant differences, P < 0.05, Duncan test.

$^b$ Means significance at P < 0.001, student t-test; n = 4 different experiments.
principal contributors to total AO activity in the nodal roots of maize. XDH activity, similar to AO, was enhanced by salinity and by ammonium in the nutrient solution (Fig. 1(B) and Table 1).

NADH-NR activity in nodal roots of nitrate fed plants increased with salinity (Fig. 1(C) and Table 1). The increase was more pronounced in tips than in mature parts of the root. On the transversal root axis, NR activity increased in the stele while it decreased slightly in the cortex (Fig. 4).

3.2. Distribution of Mo-enzymes through different parts of the root

Zone and tissue specific distribution of AO, XDH and NR activities were studied in nodal roots of maize plants grown in nutrient medium containing 4 mM NH₄⁺ and 50 mM NaCl, since these conditions were previously found to enhance AO and XDH activities (Fig. 1(A,B)). The mature part (2–10 cm) of the root was further separated into cortex and stele. Crude extracts obtained from these root zones were subjected to native PAGE followed by activity staining with acetaldehyde, benzaldehyde, heptaldehyde and indole-3-aldehyde as substrates of AO, and hypoxanthine for XDH activity determinations.

AO activity was unequally distributed through the different tissues of maize nodal roots with the highest levels of enzyme activity in tips and the stele (Fig. 2). Activity staining of AO after native PAGE revealed four bands designated AO1 to AO4 (Fig. 2). Comparative analysis of AO activity in the different root zones showed that the overall AO activity in maize nodal root was given mainly by the AO3 and AO4 protein bands or isoenzymes. The tips and the vascular cylinder contained mainly AO3 and AO4 and only traces of AO1 and AO2. AO activity was expressed on a fresh weight basis with acetaldehyde as substrate (Fig. 2). Similar results were obtained when AO activity was determined on a total soluble protein basis with other aldehydes as substrates (data not shown).

NR and XDH, showed a similar pattern distribution, with their principal concentration in the first cm of the maize nodal root (tips), and on the transverse axis in the stele (Figs. 3 and 4).

3.3. Immunoblot analysis

Western blots of AO proteins from different parts of maize nodal roots, with polyclonal antibodies raised against maize AO protein [3], revealed a major band (a) of about 150 kD in extracts of root tips and stele, and two minor polypeptides (bands b and c) with molecular masses of about 72 and 85 kD in the cortex (Fig. 5). Western blots revealed enhanced levels of 150...
Fig. 4. Nitrate reductase activity in different zones of maize nodal roots as affected by salinity. Roots were grown in medium supplied with 4 mM NaNO₃ (control) and salinity consisted of 50 mM NaCl. Enzyme activity was calculated on fresh weight (A) and protein (B) basis. Bars indicate standard error of the mean (n = 3).

Fig. 5. Immunoblot analysis of AO proteins after SDS-PAGE. Crude extract samples of different parts of maize nodal roots (tips: 0–1 cm; cortex and stele from mature zone: 2–10 cm from the apex) grown with NO₃⁻ or NH₄⁺ as nitrogen source, with or without salinity (50 mM NaCl) treatment. About 40 µg total soluble protein was loaded in each lane. The relative intensities of the bands were estimated by NIH Image 1.6 software. The results represent one of at least three different experiments with similar results.

kD polypeptide in extracts of root tips of ammonium-grown plants. The 150 kD polypeptide of the cortex was observed only in plants receiving ammonium as their nitrogen source (Fig. 5). The amount of AO protein varied among different tissues and under different growth conditions. The increment of AO activity in ammonium fed plants (Fig. 1(A) and Table 1) is in agreement with the accumulation of AO protein in the root tips of ammonium fed plants (Fig. 5 and Table 1). The enhancement of AO activity of nitrate fed plants by salinity (Fig. 1(A)), was not accompanied by increased levels of AO protein in the tips (Fig. 5) and seems to be related to activation of existing enzyme molecules.

4. Discussion

AO activity in maize nodal roots increased in plants exposed to salinity and in plants grown in nutrient solutions containing ammonium (Fig. 1(A) and Table 1). Similar responses have been reported recently for barley [24], ryegrass [6] and tomato [39].
Activity staining of AO after native PAGE with indole-3-aldehyde detected four bands or isoforms in maize roots (Fig. 1(A)) showing the strongest activities in the AO3 and AO4 bands. Salinity and ammonium enhanced activity of AO (Fig. 1(A)) although in different ways: ammonium increased the level of AO protein while salinity seemed to activate pre-existing enzyme proteins. ABA level increased in plant tissues [12,4] under the same conditions. This correlation supports the possible role of AO3 and AO4 in ABA biosynthesis in maize nodal roots. Tips and central cylinders of maize roots had a higher level of total AO activity (Fig. 2). Maize root tips accumulated more ABA in response to dehydration than mature root sections [11], which is in agreement with our results showing high AO activity in the root tips. A significant contribution of the stele to the total AO activity in mature zones of the root has been observed in the present study. AO activity in the stele is probably underestimated, since the xylem vessels account for a large fraction of the tissue volume (6–9%) (data not shown) and fresh weight. It can be assumed that AO activity in the stele may be located mainly in the xylem parenchyma cells. These cells control the composition of the xylem sap by loading specific solutes onto the xylem vessels. It has been observed that under ammonium nutrition and salinity, which increase AO activity and ABA level [24] xylem loading preferences change from inorganic nitrate to organic nitrogen compounds [40]. These changes in xylem loading specificity may be related also to the increase of xylem sap pH observed during stress [41].

Activity staining of root AO of NH$_4^+$ and NaCl grown plants, revealed that the isoforms AO3 and AO4 were the main contributors to total AO activity, and that AO4 was localized mainly in the tips and the stele (Fig. 2). The difference in mobility during native PAGE of AO4 in tips and stele of roots, may be the result of modifications of the active enzyme molecule as observed in barley roots [4]. The modifications could be phosphorylation and linking of regulatory protein factors such as the 14-3-3 proteins. Tips and stele tissues contain a large number of small, protein rich, unvacuolated cells, while the cortex is made up mainly of large, vacuolated parenchyma cells.

Immunoblot analysis of AO proteins after SDS-PAGE revealed in the cortex two smaller polypeptides (band b and c) with molecular masses of about 72 and 85 kD (Fig. 5). These polypeptides, which appear in NO$_3^-$ grown plants or plants exposed to relatively high salinity, may be residues of the break up of the main AO 150 kD monomer, two of which are required for the active AO enzyme. Similar minor AO bands were detected in maize coleoptiles and roots [3], tomato [32] and following AO exposure to trypsin (Omarov and Lips, personal communication). The increased activity of AO in roots may be the result of enhanced gene expression of the AO apoprotein contributing to the increased levels of AO observed in ammonium-grown plants but not in nitrate-grown plants. Salinity may induce post-translational activation of the existing enzyme molecules as observed in tomato genotypes (Sagi et al, unpublished). Increased AO activity in ammonium fed plants (Fig. 1(A) and Table 1) corresponded to higher levels of the AO protein in the root tips (Fig. 5 and Table 1) suggesting a regulatory role of NH$_4^+$ ions on the synthesis of AO proteins in root tips and xylem parenchyma cells. On the other hand, the enhancement of AO activity in nitrate fed plants by salinity (Fig. 1(A)) was not supported by an increase of AO protein in neither tips nor stele (Fig. 5), pointing to a possible post-translational regulation. Molybdenum-containing enzymes of the hydroxylase class (AO, XDH) require a terminal sulfur atom attached to the molybdenum to hydroxylate their specific substrates. The increase of AO activity in maize nodal roots under salinity (Fig. 1(A)) could be attributed to increased activity of the Mo-hydroxylase sulfurylase (Sagi et al. unpublished results), required for the addition of sulfur which activates AO and XDH [42]. It has been shown recently that in vitro sulfurylation with Na$_2$S, reactivated pre-existing XDH and AO proteins in extracts from ABA mutant of tomato flaccus, particularly in shoots and superinduced the basal level activity in wild-type extracts [39]. In ryegrass, Western blots of AO proteins made after native-PAGE, showed higher AO band densities in root extracts of plants grown in the presence of salinity and ammonium than in nitrate fed plants [6].

XDH activity was also enhanced by salinity and ammonium (Fig. 1(B) and Table 1). The increased activity of XDH under similar conditions was previously observed in ryegrass where the enhancement correlated with a higher content of ureides in the plants [6]. It was suggested that the
role of XDH during stress might be related to the need for a more efficient use of available carbon skeletons to assimilate ammonium into organic nitrogen compounds with a much lower C:N ratio for their transport through the xylem to the shoot [6]. In pea leaves, XDH activity increased sharply together with superoxide dismutase and other oxidant dissipating enzymes during senescence [43]. XDH may be involved in the defense of plant tissues against oxidative stress induced by salinity, most probably through its product uric acid, which is an effective scavenger of active oxygen species in many organisms [44,45].

Salinity enhanced the activity of NR in maize nodal roots of nitrate fed plants, when calculated either on a fresh weight or protein basis, especially in the root tip and in the stele (Figs. 1(C) and 4). Root NR activity increased in tomato [26] and ryegrass [25] roots, while it decreased in barley [24] roots exposed to salinity. NR was found to be located mainly in the tips (0–1 cm). A similar distribution of NR was observed in root tips of carob [28], and maize seedlings [46]. NR activity was higher in the stele (Fig. 4) than in the cortex in the present study, suggesting that most of the nitrate reaching the stele did not transverse the cortex symplast inducing NR along the way, but moved through the cortex apoplast bypassing the cortex parenchyma cells.

The distribution pattern of Mo-enzymes seemed to be related to their physiological functions on the various zones of the root. The tip is the site of the root apical meristem with actively dividing cells, which are the basis of root elongation. The root tip seems to cater for its own organic nitrogen needs, independently from the main plant body, by inducing high levels of nitrogen assimilation enzymes whose products (amino acids) are used on the spot and not exported to the shoot. The apical portion of the primary maize root has been considered a sink for freshly absorbed N [27]. The second center of AO activity was the vascular cylinder and most probably the xylem parenchyma cells. The location 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 [47] 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.

Mo-enzymes were not homogeneously distributed along the longitudinal and transversal axes of the maize nodal roots, but were preferentially located in the tips and in the stele of mature root zones. Stress (salinity) and ammonium ions, affected Mo-enzymes in the different root zones. 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.

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