Changes in Growth and Activity of Enzymes Involved in Nitrate Reduction and Ammonium Assimilation in Tomato Seedlings in Response to NaCl Stress

MOHAMED DEBOUBA1, HOUDA MAÂROUI-DGHIMI1, AKIRA SUZUKI2,*, MOHAMED HABIB GHORBEL1 and HOUDA GOUIA1

1Unité de Recherche Nutrition et Métabolisme Azotés et Protéines de Stress 99/UR/C 09-20, Département des Sciences Biologiques, Faculté des Sciences de Tunis, 1060 Tunisie and 2Unité de Nutrition Azotée des Plantes, INRA Route de Saint-Cyr, 78026 Versailles cedex, France

Received: 22 November 2006 Returned for revision: 5 January 2007 Accepted: 7 February 2007 Published electronically: 20 April 2007

INTRODUCTION

Salinity is considered to be one of the major factors that limit crop productivity in arid and semi-arid countries. Almost 1000 million ha of cultivated lands are affected by high salinity (Szabolcs, 1994). Soil salinity is becoming more problematic due to the increase in irrigation around the world. Salt water in the root zone induces osmotic changes and interferes with nutrient uptake (Cornillon and Halperin, 1999). Physiological and molecular responses of plants to salt stress have been extensively studied, while the underlying mechanisms are still not well understood (Hasegawa et al., 2001). In order to increase tolerance of plants to salt, much effort is directed to identifying physiological, biochemical and molecular processes that are affected by salt stress (Apse and Blumwald, 2002). Previous works have reported effects of salt stress on nitrogen metabolism (Abd-ElBaki et al., 2000; Flores et al., 2000; Carillo et al., 2005). Nitrate, taken up by NO3 transporters, is reduced to ammonium by the sequential reaction of nitrate reductase (NR, EC 1.6.6.1) in the cytosol and nitrite reductase (NIR, EC 1.6.6.4) in the plastids/chloroplasts. Ammonium derived from the primary nitrate reduction as well as other metabolic pathways, including root uptake, photorespiration and amino acid catabolism, is converted first to glutamine by glutamine synthetase (GS, EC 6.3.1.2) then to glutamate by glutamate synthase (Fd-GOGAT, EC 1.4.7.1 and NADH-GOGAT, EC 1.4.1.14) (Ireland and Lea, 1999). Ammonium can be directly incorporated into glutamate by the aminating reaction of glutamate dehydrogenase (NADH-GDH, EC 1.4.1.2). Since GDH reversibly deaminates glutamate to NH4 and 2-oxoglutarate, the physiological role of GDH in vivo remains controversial. The ammonium assimilation into glutamine and glutamate is vital for plant growth as these two amino acids serve as the precursors for the synthesis of the other amino acids as well as almost all nitrogenous compounds. Salt stress inhibits the ammonium assimilation (Chandra et al., 2001; Khadri et al., 2001) and induces changes in the pool of amino acids (Lacerda et al., 2001; Ashraf and Bashir, 2003). Nitrate reduction and ammonium assimilation seem to be highly implicated in the regulation of plant growth in response to salt stress.

In previous work, it was shown that increasing NaCl stress decreased plant growth and inhibited the nitrate reduction and ammonium assimilation after 10 d of treatment (Debouba et al., 2006a). However, there is a great need to monitor nitrogen metabolism behaviour upon onset of salt treatment. In fact, plant response to salt stress takes place within hours (Su et al., 2001, Kreps et al., 2002), days (Yamada et al., 1995; Viegas et al., 1999) and development stages (Debez et al., 2004). In the present study, changes in growth, nitrate reduction and...
ammonium assimilation were assessed in the leaves and roots of tomato seedlings subjected to 100 mM NaCl, in order to determine whether transient and/or permanent changes may occur during treatment.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of tomato (Solanum lycopersicum, Mill. 'Chibli F1') were sterilized in 10% H2O2 for 20 min. Seeds were then germinated on moistened filter papers at 25°C in the dark. The seedlings thus obtained were transferred to pots (seven plants per 6 L) containing 3 mM KNO3, 1 mM Ca(NO3)2, 2 mM KH2PO4, 0.5 mM MgSO4, 32.9 μM Fe-K-EDTA and the micronutrients 30 μM H2BO4, 5 μM MnSO4, 1 μM CuSO4, 1 μM ZnSO4 and 1 μM (NH4)6Mo7O24. The nutrient solutions were continuously aerated and renewed every 3 d to maintain pH (5.6–6) and nutrient composition. Plants were grown in a growth chamber: 26°C/70% relative humidity during the day and 20°C/90% relative humidity during the night; photoperiod is 16 h daily with a light irradiance of 150 μmol m−2 s−1 at the level of the plant canopy. Plants were grown for 10 d in control medium, and then for 10 d in the medium containing 100 mM NaCl. The NaCl concentration was increased to 50 mM on the first day and then to 100 mM on the second day to avoid osmotic shock. Plants were harvested 6 h after the beginning of the light phase, and immediately separated into leaves and roots. The roots were washed three times with cold distilled water and blotted with filter paper. The fresh weight was immediately determined, and the dry weight was measured after 48 h of desiccation in an oven at 60°C.

Ion analysis

Inorganic ions were extracted from dry materials with 0.5 N H2SO4 at room temperature for 48 h (Gouia et al., 1994). Sodium was analysed by flame emission using a spectrophotometer (Eppendorf, Netheler-Hinz, GmbH Hamburg Germany). Chloride was quantified by a colorimetric method using a Digital Chloridometer (HaaKeBuchler, Buchler Instruments Inc., NJ, USA). Nitrate was colorimetrically determined with an automatic analyser (Dual Tubingpump, Instrumenten B.V, Breda The Netherlands) following diazotation of the nitrite obtained by reduction of NO3 on a cadmium column. Ammonium was extracted at 4°C with 0.3 mM H2SO4 and 0.5% (w/v) polyclar AT. The ammonium concentration was determined according to the reaction of Berthelot modified by Weatherburn (1967).

Soluble proteins

Soluble protein concentrations were determined using Coomassie brilliant blue (Bradford, 1976) with bovine serum albumin as a protein standard.

Enzyme assays

Nitrate reductase. Frozen plant material was homogenized in a chilled mortar and pestle with 100 mM potassium phosphate buffer (pH 7.4), containing 7.5 mM cysteine, 1 mM EDTA and 1.5% (w/v) casein. The homogenate was centrifuged at 30 000 g for 15 min at 4°C. Nitrate reductase activity (NRA) was determined according to the method of Robin (1979). The extract was incubated in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.4), 10 mM EDTA, 0.15 mM NADH and 0.1 mM KNO3 at 30°C for 30 min. The reaction was stopped by 100 μL of 1 m zinc acetate. Absorbance of the supernatant was determined at 540 nm after diazotation of nitrite ions with 5.8 mM sulfanilamide and 0.8 mM N-(1-naphthyl)-ethylenediamine-dihydrochloride (NNEDD).

Nitrite reductase. Enzyme extracts were prepared as described above for nitrate reductase. Nitrite reductase activity (NiRA) was assayed by the method of Losada and Paneque (1971). The extract was incubated in a solution containing 100 mM potassium phosphate buffer (pH 7.4), 15 mM sodium nitrite, 5 mM methyl viologen, 86.2 mM sodium dithionite in 190 mM NaHCO3. The reaction was stopped by violent agitation on a vortex mixer. Nitrite ion concentrations were measured at 540 nm after diazotation with 5.8 mM sulfanilamide and 0.8 mM NNEDD.

Glutamate synthetase. Frozen samples were homogenized in a cold mortar and pestle with grinding medium containing 25 mM Tris–HCl buffer (pH 7.6), 1 mM MgCl2, 1 mM EDTA, 14 mM β-mercaptoethanol and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 25 000 g for 30 min at 4°C. GS activity was determined using hydroxylamine as substrate, and the formation of γ-glutamylhydroxamate (γ-GHM) was determined with acidified ferric chloride (Walls groove et al., 1979). The γ-GHM was quantified using commercial glutamine as a standard after reading the absorbance of the incubation medium at 540 nm.

Glutamate synthase. Fd-GOGAT and NADH-GOGAT activities were measured as described by Suzuki et al. (1994). Glutamate synthase was extracted with 25 mM sodium sulfate buffer (pH 7.5), containing 14 mM β-mercaptoethanol and 1 mM dithiothreitol (DTT). Fd-GOGAT activity was determined in a reaction mixture containing 25 mM sodium sulfate buffer (pH 7.5), 100 mM glutamine, 100 mM 2-oxoglutarate, 3.9 mM methyl viologen and 190 mM sodium dithionite in 180 mM NaHCO3. NADH-GOGAT was assayed using the same reaction mixture, except that methyl viologen and sodium dithionite were replaced by 1.4 mM NADH. Glutamate produced by both GOGAT activities was determined using high performance liquid chromatography (HPLC) (Suzuki et al., 1994).

Glutamate dehydrogenase. GDH extraction was performed according to the method described by Magalhaes and Huber (1991). Frozen samples were homogenized in a cold mortar and pestle with 100 mM Tris–HCl (pH 7.5),
14 mM β-mercaptoethanol and 1% (w/v) PVP. Extracts were centrifuged at 12,000 g for 15 min at 4°C. NADH-dependent aminating and NAD-dependent deamidating activities of GDH were determined by following the absorbance changes at 340 nm (Loyala-Vergas and De Jimenez, 1984).

Enzyme assays

The data presented in this work are the average of at least five replicates per treatment; means ± s.e. are given in the figures. Each experiment was carried out in duplicate. Comparisons with P-values ≤ 0.05 were considered significantly different according to Tukey test.

RESULTS

Seedling growth

The leaf and root dry weight increased over time in both control and NaCl-treated plants (Fig. 1A, B). During the first 3 d, the NaCl-treated plants sustained normal growth compared with the control plants. Significant NaCl effects on the leaf and root dry weight were recorded starting from 5 and 7 d of treatment, respectively. At 10 d, the dry weight decreased by about 40% in the leaves (Fig. 1A) and 20% in the roots (Fig. 1B), with respect to the control plants. Under salinity, the tomato leaves did not show any chlorosis or necrosis, but the leaf colour turned out to be brownish (data not shown).

Sodium and chloride concentrations

One day following plant exposure to salt, Na⁺ and Cl⁻ were rapidly accumulated in the leaves (Fig. 2A, C) and roots (Fig. 2B, D). At 10 d, Na⁺ ion concentration was higher in the roots than in the leaves, while Cl⁻ ions were accumulated more in the leaves than in the roots.

Nitrate and ammonium concentrations

The salt stress resulted in a decrease of NO₃⁻ concentrations in the leaves after 3 d of the NaCl treatment, representing 50 and 20% of the control at 5 and 10 d, respectively (Fig. 2E). In the roots, a rapid decrease of 25% in NO₃⁻ concentrations occurred after 1 d of the NaCl treatment (Fig. 2F). This decrease was thereafter accentuated; it reached 60% at the end of the salt treatment period.

Beyond 4 d of treatment, NH₄⁺ concentrations in the leaves of stressed plants were increased compared with controls (Fig. 2G). In roots, a significant increase in NH₄⁺ concentrations by salt stress was recorded at the end of the experimental period (Fig. 2H).

Nitrate reduction to ammonium

NRA decreased to 60% in only 24 h by the NaCl treatment (Fig. 3A). NRA in stressed plants gradually increased after 7 d, and it reached 50% of the control value at the end of treatment. In contrast, the NRA in the roots decreased in both the NaCl-treated and control plants (Fig. 3B). Unexpectedly, the NRA in the NaCl-treated roots was higher than in the control after 6 d.

After 1 week of treatment, NiRRA increased in the leaves (Fig. 3C), while it decreased in the roots irrespective of NaCl supply (Fig. 3D). The NaCl inhibited the NiRRA in leaves by 25% at 10 d. The NiRRA in the roots was not affected significantly by NaCl stress until 7 d of the treatment, and 40% of the inhibition was detected at 10 d.

Ammonium assimilation

In leaves, significant NaCl stress effects on the GS activity were observed after 4 d of treatment (Fig. 4A). GS activity was lowered by 20–25% with respect to control leaves. Conversely, GS activity in roots of NaCl-treated plants became significantly higher than in the control after 7 d (Fig. 4B).

Fd-GOGAT activity increased about 60% in the control leaves at 3 d and was then unchanged throughout the treatment (Fig. 4C). Adding NaCl inhibited 38% of the activity on the first day. Then, the initial value was recovered after a peak at 4 and 6 d (Fig. 4C). Fd-GOGAT activity in the roots

Fig. 1. Effects of adding 100 mM NaCl to the medium on the dry weight in (A) the leaves and (B) the roots of tomato. Plants were grown with (+NaCl) or without (Control) 100 mM NaCl during 10 d. Each point is the average of five plants ± s.e.; means sharing at least one letter are not significantly different, Tukey test at the 0.05 level.
declined gradually in response to NaCl stress until 6 d of treatment, where it reached 10% of the control value (Fig. 4D). Thereafter, it remained constant, and it was about 25% of control at the end of experiment.

In leaves, NADH-GOGAT decreased to a greater extent under NaCl stress (Fig. 4E). It was inhibited by 30 and 40% compared with controls at 7 and 10 d, respectively. In roots, NADH-GOGAT activity was increased by NaCl.
treatment, with a peak occurring on the first day. Then the root NADH-GOGAT activity dropped to the initial value, while it remained higher than that in the control (Fig. 4F).

Glutamate metabolism

In leaves, the deaminating activity of glutamate dehydrogenase (NAD-GDH) increased in the control but it was decreased by NaCl (Fig. 5A). The aminating activity (NADH-GDH) in the control leaves did not change, while it increased after 4 d of salt treatment (Fig. 5C).

The deaminating GDH activity in the roots decreased by 60 and 30 % in the NaCl-treated and control plants, respectively, at 10 d (Fig 5B). The aminating GDH activity in the roots decreased to a greater extent in the control than in NaCl-treated plants (Fig. 5D).

**DISCUSSION**

NaCl treatment was associated with differential effects on growth, metabolite concentrations and enzyme activities in the leaves and roots of tomato. During 3 d of salt treatment, both leaves and roots showed normal growth, and thereafter the reduction in dry weight occurred first in the leaves (Fig. 1A) and then in roots (Fig. 1B). Tomato leaves were more affected by salt than roots. It seems that tomato leaves did not compartmentalize a high concentration of salt ions (Fig. 2A, C), and chloride and sodium may be accumulated in the cytosol and apoplasm (Debouba *et al.*, 2006b). However, many authors suggest that growth reduction by NaCl under a short time scale is caused by external osmotic changes (Munns and Termaat, 1986; Munns, 1993). Osmotic effects probably display inhibitory signals in roots (Munns, 2002). After 1 d of salt stress, an inhibition of NRA was measured in the leaves (Fig. 3A), while dry weight in the leaves was not reduced (Fig. 1A). These results are consistent with the observation that nitrate reduction exceeds nitrogen demand (Abd-El Baki *et al.*, 2000). The fast NRA decrease in the leaves by the external supply of NaCl may be related to osmotic changes following NaCl addition to the medium. In fact, NRA is inhibited by osmotic effects of NaCl treatment in cashew (Viegas *et al.*, 1999). The reduction of the maximum extractable NRA in the leaves (Fig. 3A) could be due to a lower NR protein content. Fe´rrario-Méry *et al.* (1998) showed that the NRA decrease under water

---

**Fig. 3.** Changes during treatment of the (A, B) nitrate reductase activity and (C, D) nitrite reductase activity in the leaves and roots of tomato. Plants were grown in (+ NaCl) or without (Control) 100 mM NaCl during 10 d. Each point is the average of five plants ± s.e.; means sharing at least one letter are not significantly different, Tukey test at the 0.05 level.
stress was due to a loss of NR protein during 3 d, and NR mRNA during the following days. They suggested that the reduction in NR mRNA levels is related to lower levels of NO$_3^-$ and glutamine in leaves. Consistently, the NR depression could be related to the low NO$_3^-$ availability in the salt-treated leaves during the subsequent days (Fig. 2E). Indeed, NO$_3^-$ regulates NR transcription, translation and activation in higher plants (Férrario-Méry et al., 1998; Wang et al., 2004). It has been reported that NaCl reduced NO$_3^-$ fluxes from roots to leaves and impaired the NRA in leaves (Gouia et al., 1994; Foyer et al., 1998). NRA was less affected in the roots than in the leaves despite the large decrease in NO$_3^-$ concentrations due to NaCl in both organs (Figs 2F and 3B). These results suggest that NR is regulated differently by NO$_3^-$ availability in the leaves and roots under NaCl stress. The decrease in NO$_3^-$ concentrations by NaCl treatment may result from a disruption of root membrane integrity (Carvajal et al., 1999), an inhibition of NO$_3^-$ uptake (Bourgeais-Chaillou et al., 1992; Parida and Das, 2004) and low NO$_3^-$ loading into root xylem (Abd-El Baki et al., 2000). As Cl$^-$ ions inhibit NO$_3^-$ uptake, the decrease in NO$_3^-$ concentrations in both roots and leaves can be attributed to competition between Cl$^-$ and NO$_3^-$ for uptake by NO$_3^-$ transporters (Deane-Drummond, 1986), and/or an inactivation of NO$_3^-$ transporters by toxic effects of salt ions (Lin et al., 1997). Accordingly, the greater NO$_3^-$ concentration decline in the leaves (Fig. 2E) was associated with the higher Cl$^-$ accumulation relative to that in the roots (Fig. 2C).

**Fig. 4.** Changes during treatment of the (A, B) glutamine synthetase activity, (C, D) Fd-GOGAT activity and (E, F) NADH-GOGAT activity measured in the leaves and roots of tomato. Plants were grown with (+NaCl) or without (Control) 100 mM NaCl during 10 d. Each point is the average of five plants ± s.e.; means sharing at least one letter are not significantly different, Tukey test at the 0.05 level.
A lesser salt stress effect was observed for NiR (Fig. 3C) and GS (Fig. 4A) relative to NR in leaves (Fig. 3A). Salt ions are in large part accumulated in the cytosol when tomato seedlings are subjected to high salinity (Debouba et al., 2006b), thus NR is directly exposed to sodium and chloride effects. GS was only detected in chloroplasts (GS2) in tomato leaves (Debouba et al., 2006c). NiR and GS2, both localized in the chloroplasts, can therefore partially avoid the toxic effects of salt ions. However, it was found that plastidial Fd-GOGAT and NADH-GOGAT in roots (Fig. 4D, F) and mitochondrial NAD-GDH in leaves (Fig. 5A) were rapidly inhibited by salinity. These results provide evidence that NaCl induced an enzyme-specific effect irrespective of subcellular localization. According to the changes in the enzyme activity under NaCl stress, two types of response were distinguished: (a) early responding enzymes, which changed as early as 24 h after adding NaCl, i.e. NR and deaminating GDH in leaves, and Fd-GOGAT in the leaves and roots; and (b) late responding enzymes, which were affected at least 4 d after the NaCl treatment, i.e. NAD-GOGAT in leaves, NAD-GDH in roots, and NiR, GS and aminating GDH in both leaves and roots.

At the end of treatment, NH$_4^+$ accumulated in the leaves of NaCl-treated plants (Fig. 2G). It is reported that NaCl stress results in an NH$_4^+$ accumulation released by increased proteolysis (Santos and Caldeira, 1999; Hoai et al., 2003; Debouba et al., 2006a). Moreover, the reduced activity of GS (Fig. 4A), Fd-GOGAT and NADH-GOGAT (Fig. 4C, E) due to NaCl stress in the leaves can contribute to the observed increase in NH$_4^+$ concentration.

In control plants, NR (Fig. 3A, B), GS (Fig. 4A, B), Fd-GOGAT (Fig. 4C, D) and GDH (Fig. 5A–D) activities decreased in the roots during ageing, whereas these activities were constant or increased in the leaves. These findings indicate that during early stages of development (before the two fully expanded leaf stage), tomato roots may contribute to nitrate reduction and ammonium assimilation. Thereafter, these processes were predominant in the leaves, and GS and GOGAT activities in the roots may be involved in amino acid translocation to shoots as GS1 and NADH-GOGAT are located in vascular tissue (Yamaya et al., 1992; Hayakawa et al., 1999).

CONCLUSIONS

The present work showed that NaCl addition to the culture medium of tomato induced (a) transient effects on stimulation of NADH-GOGAT activity in roots after 1 d of salt supply; and (b) permanents effects starting from the first
day and lasting throughout the experiment period. This includes (a) the decrease of NO3\(^-\) concentrations in roots and inhibition of the early responding enzymes; and (b) the following effects appearing after more than 4 d of treatment: a decrease in growth; an increase of NH4\(^+\) concentrations in leaves and roots; a decrease of NO3\(^-\) concentrations in leaves; and changes in the late responding enzyme activities, i.e. enhancement of NR and GS activities in roots and NADH-GDH in leaves and roots, and inhibition of NiR activity in both tissues. However, it is believed that a longer period of salt treatment of tomato will give further insight into nitrogen nutrition and metabolism in response to NaCl stress.

LITERATURE CITED


