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Nitric oxide affects salt-induced changes in free amino acid levels in maize

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Abstract:
It was assumed that salt-induced redox changes affect amino acid metabolism in maize (*Zea mays* L.), and this influence may be modified by NO. The applied NaCl treatment reduced the fresh weight of shoots and roots. This decrease was smaller after the combined application of NaCl and an NO-donor ((Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate, *ETA/NO*) in the shoots, while it was greater after simultaneous treatment with NaCl and nitro-l-arginine (l-NNA, inhibitor of NO synthesis) in the roots. The quantum yield efficiency of photosystem II was not influenced by the treatments. NaCl had a significant effect on the redox environment in the leaves as it was shown by the increase in the amount of glutathione disulphide and in the redox potential of the glutathione/glutathione disulphide redox pair. This influence of NaCl was modified by DETA/NO and l-NNA. Pharmacological modification of NO levels affected salt-induced changes in both the total free amino acid content and in the free amino acid composition. NaCl alone increased the concentration of almost all amino acids which effect was strengthened by DETA/NO in the case of Pro. l-NNA treatment resulted in a significant increase in the Ala, Val, Gly and Tyr contents. The Ile, Lys and Val concentrations rose considerably after the combined application of NaCl and DETA/NO compared to NaCl treatment alone in the recovery phase. NaCl also increased the expression of several genes related to the amino acid and antioxidant metabolism, and this effect was modified by DETA/NO. In conclusion, modification of NO levels affected salt-induced, glutathione-dependent redox changes and simultaneously the free amino acid composition and the level of several free amino acids. The observed much higher Pro content in plants treated with both NaCl and DETA/NO during recovery may contribute to the protective effect of NO against salt stress.

Introduction

Adverse environmental conditions result in substantial reductions in crop yields. Yield losses caused by drought can be mitigated by irrigation, but this may lead to the deposition of salt from the river water or groundwater applied. High salt

concentrations induce oxidative stress due to an increase in the amount of reactive oxygen species, so antioxidants are activated as a part of the defence system (Foyer and Noctor, 2011). The accumulation of amino acids, especially Pro and Arg, contributes to the osmoprotection of plants (Rai, 2002). Increase in Arg content may lead to the accumulation of polyamines which protect as polycations the vital negatively charged, macromolecules in the cells (Alcázar et al., 2006). In addition, reactive oxygen species, antioxidants, amino acids and polyamines are all involved in signalling pathways which may activate further protective mechanisms. During salt stress there is cross-talk between various signalling pathways involving reactive oxygen, nitrogen species and plant growth regulators (Moreau et al., 2010; Gémes et al., 2011).

The involvement of antioxidants in the response to salt stress was shown in maize, where NaCl treatment increased both the catalase and superoxide dismutase (SOD) transcript levels and the catalase activity (Menezes-Benavente et al., 2004). Transcriptome analysis revealed a rapid induction of genes encoding antioxidants by salt in a tolerant poplar species, while their induction occurred only after longer exposure to salt in the sensitive one (Ding et al., 2010). Salt stress increased the activity of SOD, ascorbate peroxidase (APX) and catalase in chickpea leaves (Sheokand et al., 2008). Further evidence for the protective role of antioxidants was observed in rice, since a salt-tolerant genotype was found to have greater ascorbate and glutathione (GSH) contents, GSH/glutathione-disulphide (GSSG) and ascorbate/dehydroascorbate ratios and antioxidant enzyme activity than a sensitive one (Vaidyanathan et al., 2003; El-Shabrawi et al., 2010). Similarly, the salt-tolerant species *Plantago maritima* exhibited better tolerance to salt stress than the salt-sensitive *Plantago media* due to its greater antioxidant activities (APX, glutathione reductase (GR), SOD, catalase) (Sekmen et al., 2007). The salinity tolerance of *Medicago trunculata* was found to be related to the induction and sustained expression of highly regulated antioxidant mechanisms in the roots and leaves (Mhadhbi et al., 2011). In the induction of antioxidants the osmotic effect of salt may be important, since osmotic stress induced by the water deficit also affected antioxidant levels (Varga et al., 2012). Besides antioxidants, amino acids are also important in the response to salt stress. In broad bean the amino acid content was decreased by increasing salinity and a marked increase was only observed in the proline content (Abd El-Samad et al., 2011). Although Wang et al. (2003) found no alteration in the overall free amino acid concentration in maize, the amino acid composition was changed by salt stress, with an increase in the proline and asparagine contents. Salt-induced changes in amino acid levels affect polyamine concentrations as it was shown in wheat (Simon-Sarkadi et al., 2007). All these findings indicate that the level of antioxidants, amino acids and polyamines are adjusted to salt stress conditions, which may improve salt tolerance.

NO was found to serve as a signal inducing salt tolerance in reed (Zhao et al., 2004). NO enhanced salt tolerance in maize due to the activation of proton pumps and the Na^+/H^+ antiport, as indicated by improved growth, increased dry matter accumulation and greater chlorophyll content (Zhang et al., 2006). The protective

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effect of NO against salt stress in maize can also be linked to the enhanced activity of antioxidant enzymes (Bai et al., 2011; Keyster et al., 2012). The induction of various antioxidant enzymes (catalase, APX, SOD) by NO was also observed under salt stress in chickpea (Sheokand et al., 2008), wheat (Zheng et al., 2009) and rice (Uchida et al., 2002). The possible effect of NO on amino acids during salt stress was only investigated in the case of Pro in cucumber where the NO-induced increase in Pro was assumed to be responsible for an improved salt tolerance (Fan et al., 2012).

According to our hypothesis salt-induced redox changes may affect amino acid metabolism in maize and in this process NO may be involved, too. To test this assumption, free amino acid concentrations were measured in plants subjected to salt stress without and with simultaneous modification of NO levels and possible redox changes were monitored by determination of GSH and GSSG concentrations.

Materials and methods

Plant material and growth conditions

Maize (*Zea mays* L. cv. Silverking) seeds were imbibed in sterile distilled water for 30 min and sown in 1 l of pre-soaked (distilled water) filtered silica sand in 15 cm diameter plastic pots. The seedlings were grown in a PGR-15 growth chamber (Conviron, Canada) at 22 °C, 400 µmol m^{-2} s^{-1} and 12 h illumination, and irrigated with modified Hoagland solution (Kellő s et al., 2008) twice a week. The following treatments were applied as supplementation of the nutrient solution at the 3-leaf stage of the plants: (1) control (addition of no chemical); (2) 150 mM NaCl; (3) 5 µM (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (*DETA/NO*); (4) 5 µM *DETA/NO* + 150 mM NaCl; (5) 5 µM diethylenetriamine (DETA); (6) 5 µM DETA + 150 mM NaCl; (7) 5 µM nitro-l-arginine (l-NNA); (8) 5 µM l-NNA + 150 mM NaCl; (9) 150 mM KNO_2. DETA/NO is a nitric oxide donor. DETA, which is produced by the dissociation of DETA/NO, was included among the treatments to act as a control for the NO treatments. Interestingly, it was found to affect the As-induced oxidative stress in maize (Stoeva et al., 2005). l-NNA is an inhibitor of NO synthesis, while KNO_2 is a possible endogenous source of NO. The treatments were followed by a 1-week recovery phase when no chemicals were added to the basic nutrient solution. Sampling and the determination of fresh weight were done before the addition of the various compounds, after 3 and 11 days treatment and after 1 week recovery. Three independent experiments were performed with three–three parallels.

Measurement of the quantum yield efficiency of photosystem II

The maximum potential quantum efficiency of photosystem II was characterized by the F_v/F_m (variable to maximum fluorescence) ratio which was measured using a pulse amplitude-modulated fluorometer (PAM 2000, Walz, Germany).

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Analysis of thiols

The qualitative and quantitative identification of the thiols was performed using reverse-phase HPLC (Waters, Milford, MA, USA) connected to a fluorescence detector (W474 scanning fluorescence detector, Waters) as previously described (Kranner and Grill, 1996; Kocsy et al., 2001). The half-cell reduction potential of the thiol/thiol disulphide redox couples was calculated according to Schafer and Buettner (2001).

Determination of free amino acid content

Shoot samples of 300–600 mg fresh weight were crushed in liquid nitrogen and extracted with 2 ml cold 10% trichloroacetic acid for 1 h with gentle agitation on a shaker (C. Gerhardt GmbH & Co. KG, Germany) at room temperature. Each sample was filtered through a 0.2 µm pore membrane filter (Sartorius AG, Germany). The biochemical analysis was carried out on an automatic amino acid analyser (Ingos Ltd., Czech Republic) equipped with an Ionex Ostion LCP5020 cation-exchange column (22 cm × 0.37 cm). The free amino acids were separated by stepwise gradient elution using a Li^{+}-citric buffer system (Ingos Ltd., Czech Republic). Colorimetric detection was accomplished at 570 nm and 440 nm (for Pro) after post-column derivatization with ninhydrin reagent.

Gene expression studies

For the real-time PCR analysis of the genes encoding enzymes involved in the amino acid and polyamine metabolism or in the antioxidant defence and death processes, the DNase treatment of RNA and first-strand cDNA synthesis were carried out according to Altpeter et al. (2005). The PCR amplification mixture (20 µl) contained 0.5 µl cDNA, 10 µl QuantiTect SYBR Green PCR Master mix (Qiagen, Hilden, Germany) and 0.8 µl forward and reverse primer (10 µM). The primers are listed in Supplementary Table S1. The amplification of DNA and normalization against glyceraldehyde-3-phosphate dehydrogenase (Kocsy et al., 2010) were performed as described earlier (Altpeter et al., 2005).

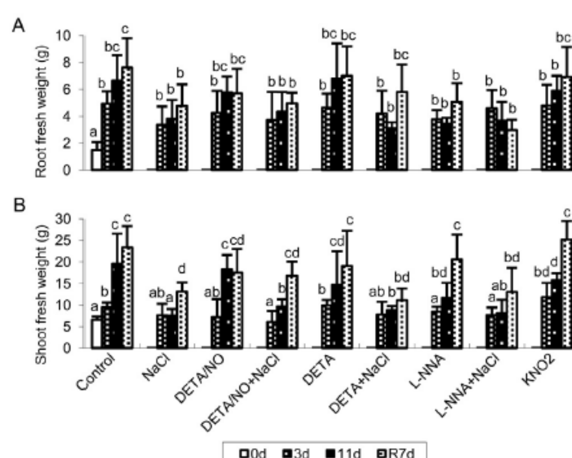

Fig. 1. Effect of NO on salt-induced changes in fresh weight. The fresh weight of roots (A; significant difference, SD: 0.46) and shoots (B; SD: 0.69) was measured after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 µmol; DETA: 5 µmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level.

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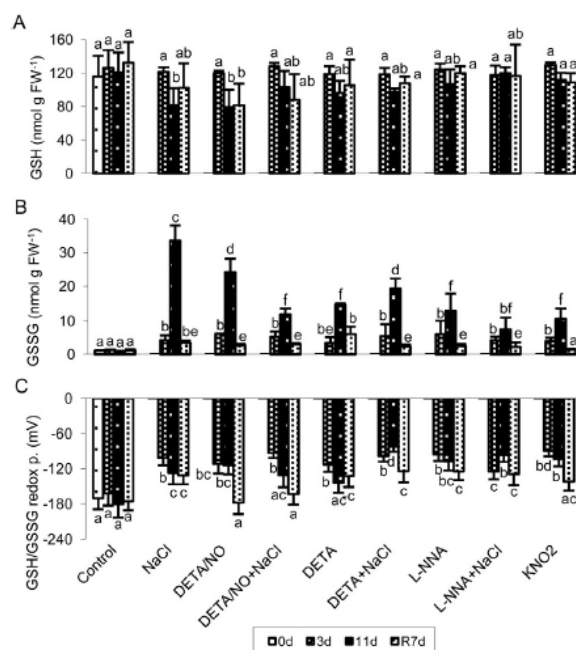

Fig. 2. Effect of NO on salt-induced changes in glutathione and glutathione disulphide content and in their redox potential. The amounts of glutathione (GSH, A; SD: 13.1) and glutathione disulphide (GSSG, B; SD: 1.2) and the redox potential of the GSH/GSSG redox couple (C; SD: 19.3) were measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 μmol; DETA: 5 μmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level.

Statistics

The statistical analysis was done using two-component (treatments, samplings) analysis of variance. Significant differences (SD) were calculated with the *t*-test at the $p \leq 0.05$ level.

Results

Fresh weight

NaCl reduced the fresh weight of both roots (to 63%) and shoots (to 38%) compared to the control plants, but the difference was greater for the shoots after 11 days (Fig. 1). The inhibition of NO synthesis in salt-stressed maize resulted in an additional reduction in root growth to 58% during the recovery phase compared to plants treated only with NaCl (Fig. 1A). DETA/NO, added simultaneously with NaCl was able to restore to 74% of control the salt-induced growth inhibition of the shoots (Fig. 1B). Like the NO donor DETA/NO, the addition of KNO_2, a possible source of NO in plants, did not affect the growth of the plants.

Quantum yield efficiency of photosystem II

The quantum yield efficiency of photosystem II decreased slightly in the course of the experiment but the various compounds did not induce significant changes in this parameter (Supplementary Fig. S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.02.006.

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Thiols, thiol disulphides and their redox potentials

With the exception of NaCl and l-NNA + NaCl all the compounds induced a great reduction (to 26–40%) in the cysteine content (precursor of GSH) during the recovery phase, and some of the treatments (DETA + NaCl, l-NNA + NaCl, KNO_2) increased the cystine content (to 316%, 202% and 267%) after 11 days salt stress

(Supplementary Figs. S2A and S2B). The treatments increased the redox potential of the cysteine/cystine couple (by 24–48%, end of recovery), except for l-NNA + NaCl (Supplementary Fig. S2C).

Similarly to cysteine, the greatest changes in the -y-glutamylcysteine (-yEC) content (intermediary product of GSH synthesis) occurred during the recovery phase (Supplementary Fig. S3A), when its level was greatly reduced by DETA/NO (to 44%), DETA + NaCl (to 42%), l-NNA (to 33%) and KNO_2 (to 28%). While the -y-glutamylcystine (ESSE) content was not affected by NaCl alone, its concentration was increased by the other treatments (to 127–248%, end of recovery) with the exception of DETA/NO + NaCl (Supplementary Fig. S3B). The redox potential of the EC/ESSE redox pair was not affected by salt, but was increased by the addition of DETA/NO (by 25%), l-NNA (by 30%) and KNO_2 (by 36%) during the recovery (Supplementary Fig. S3C).

The amount of GSH exhibited only slight changes after the addition of the various compounds for 11 days except for its decrease after NaCl (to 67%) and DETA/NO (to 65%) treatment (Fig. 2A), but all the treatments resulted in a great increase (8–37-fold) in the GSSG concentration after 11 days (Fig. 2B). The greatest changes were induced by NaCl (37-fold) and DETA/NO (27-fold). Both DETA/NO and l-NNA greatly reduced (to 35% and 22%) the NaCl-induced increase in GSSG content. The redox potential of the GSH/GSSG couple increased (by 50–55%, 11 days) after the treatments, and it remained at high level during the recovery phase except for DETA/NO and DETA/NO + NaCl treatments (Fig. 2C).

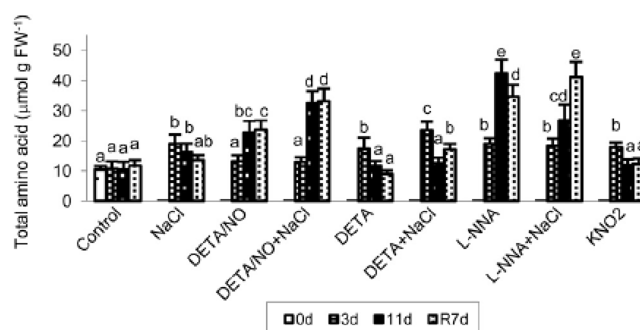

Fig. 3. Effect of NO on total free amino acid content. The free amino acid concentrations were measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 μmol; DETA: 5 μmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level. SD: 3.4.

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Free amino acids

All treatments increased the total free amino content at least in one sampling point (Fig. 3). However, the greatest increase was observed after the addition of DETA/NO + NaCl (to 218%), l-NNA (to 408%) and l-NNA + NaCl (to 257%) for 11 days. Not only the total free amino acid content, but the amino acid composition was affected by the treatments, too (Fig. 4). The ratio of the amino acids belonging to the aspartate family decreased (by 17% and 15%) and that of the amino acids of glutamate and alanine family increased (by 7% and 5%, by 10% and 7%) after 3 days treatment with DETA/NO and DETA/NO + NaCl. After 11 days DETA/NO partly neutralized the effect of NaCl, and the ratios after the application of DETA/NO + NaCl became more similar (1–7% difference) to control than after the addition of NaCl (12–15% difference) alone.

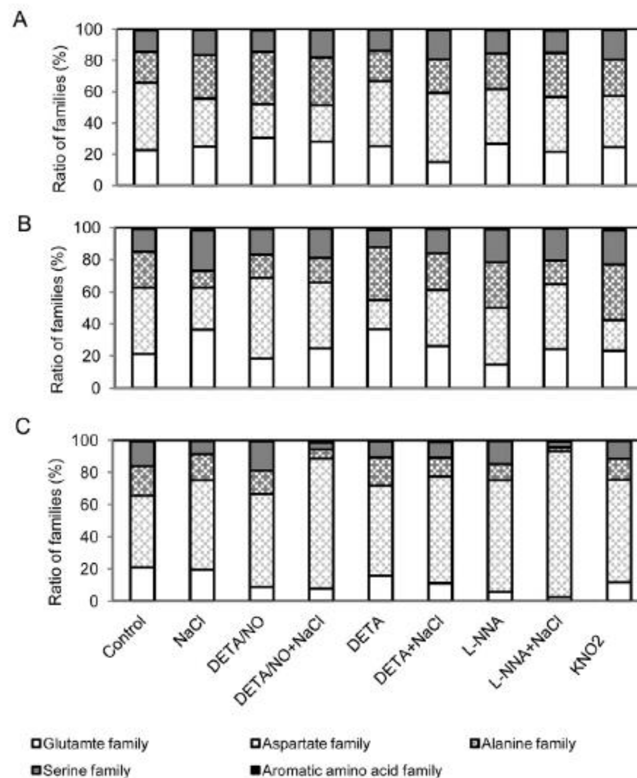

Fig. 4. Effect of NO on the ratio of amino acids belonging to the different amino acid families. The ratio was determined in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 µmol; DETA: 5 µmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The ratio of amino acids belonging to the aromatic amino acid family was between 0.4 and 1.6. SD: 2.3.

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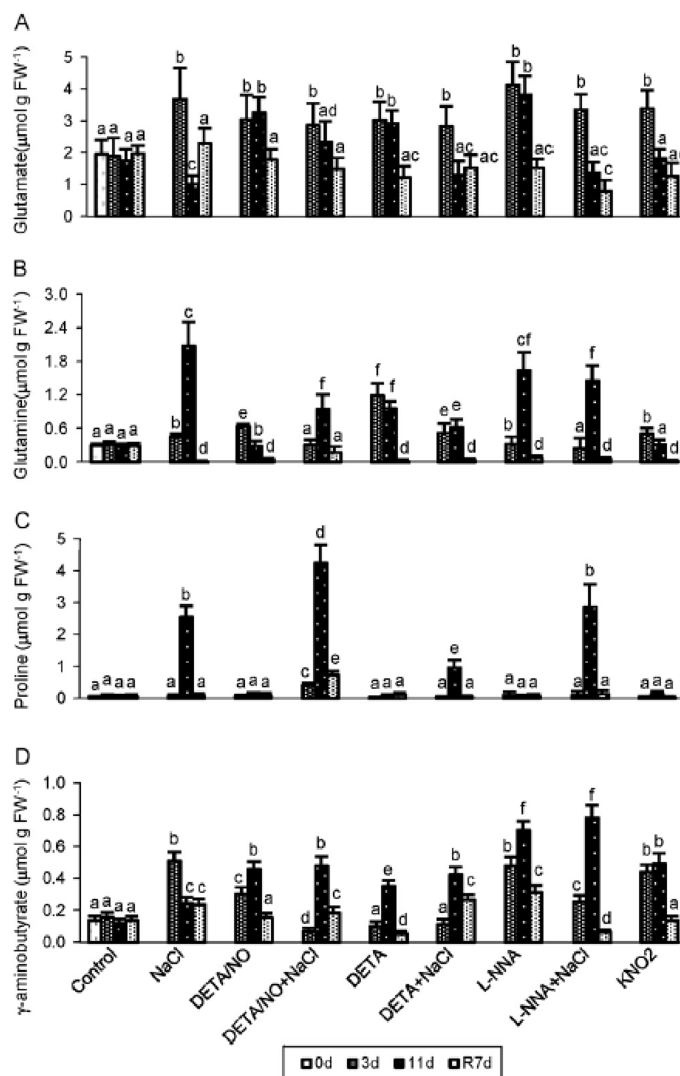

Fig. 5. Effect of NO on salt-induced changes in the concentration of amino acids belonging to the glutamate family. The amounts of glutamate (A; SD: 0.25), glutamine (B; SD: 0.063), proline (C; SD: 0.92) and γ-aminobutyrate (D; SD: 0.021) were measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 µmol; DETA: 5 µmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level.

Interestingly, at the end of the 7 days recovery phase the ratio of the amino acids of aspartate family increased (DETA/NO: by 13%; l-NNA: by 26%) and that of the amino acids of glutamate family decreased (DETA/NO: by 12%, l-NNA: 15%) after the modification of NO levels, and this effect was strengthened (additional 23% and 21% increase) by the simultaneous addition of NaCl in the case of aspartate family.

The quantity of amino acids increased in the treatments, but generally decreased to the control level during the recovery phase (Figs. 5–9). Four amino acids of the glutamate family were present in detectable quantities (Fig. 5). The amount of glutamate increased (to 151–217%) in all treatments after 3 days, but subsequently (11 days treatment) decreased to the control or lower levels in the case of NaCl (to 59%), DETA + NaCl (to 75%), l-NNA + NaCl (to 80%) and KNO_2 (to 105%) (Fig. 5A). The glutamine concentration exhibited a great increase after 11-day NaCl (7.6-fold), l-NNA (6- fold) and l-NNA + NaCl (5.3-fold) treatments

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(Fig. 5B). The greatest increase was observed for proline when the plants were treated with NaCl alone (35-fold) or in combination with other compounds (NaCl + DETA/NO: 58-fold, NACl + DETA: 13-fold, NaCl + l-NNA: 40-fold) for 11 days (Fig. 5C).

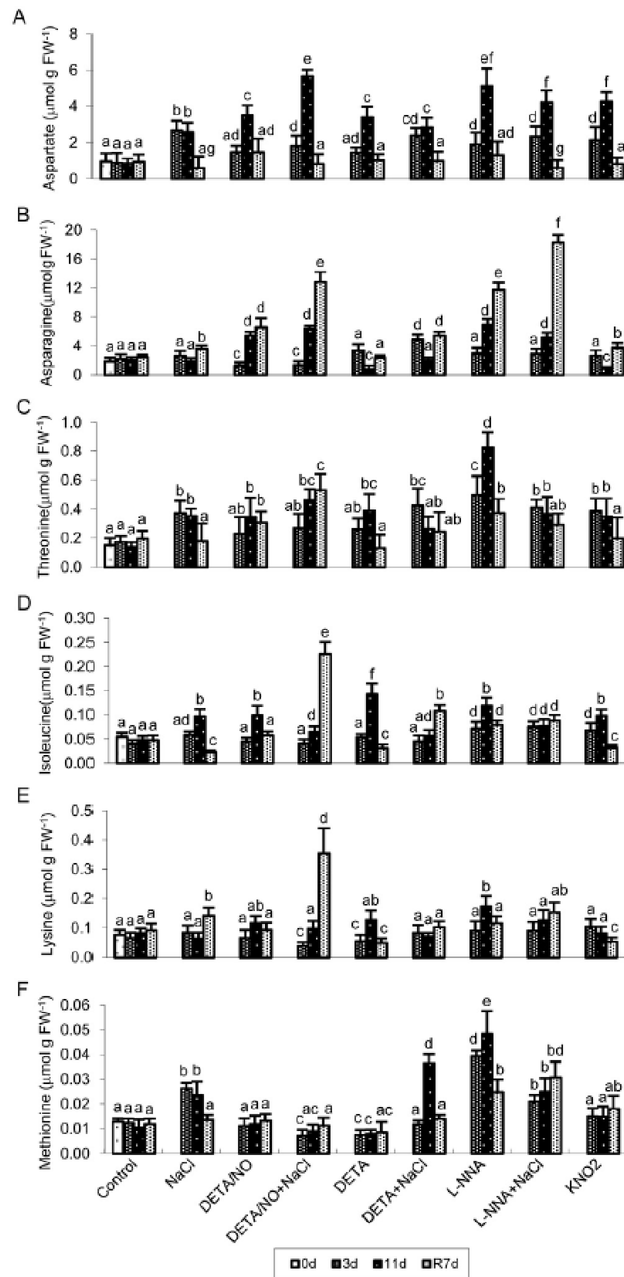

Fig. 6. Effect of NO on salt-induced changes in the concentration of amino acids belonging to the aspartate family. The amounts of aspartate (A; SD: 0.17), asparagine (B; SD: 0.28), threonine (C; SD: 0.017), isoleucine (D; SD: 0.012), lysine (E; SD: 0.015) and methionine (F; SD: 0.002) were measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 μmol; DETA: 5 μmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level.

At the end of the recovery phase, as in the case of Gln, its level was much greater (5.5–15-fold) after the addition of DETA/NO + NaCl than in the other treatments. The -y-aminobutyrate (GABA) concentration was increased (2.6–4-fold) after 11 days by all the treatments, but decreased (by 60–92%) during the recovery phase except for NaCl (Fig. 5D). The greatest increase was detected when l-NNA was applied alone (5.7-fold) or with NaCl (6.4-fold). Interestingly, arginine could be

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detected only in very low concentrations (0.05–0.1 µmol g FW^{-1}) after 3 days in plants treated with NaCl alone or combined with other compounds (data not shown) which can be explained by its low basic level and by the greatly increased use of its precursor, Glu for Pro and Gln synthesis.

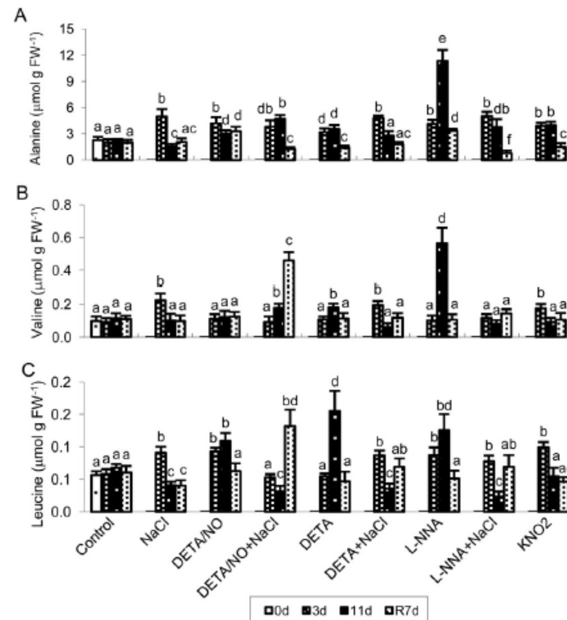

Fig. 7. Effect of NO on salt-induced changes in the concentration of amino acids belonging to the pyruvate family. The amounts of alanine (A; SD: 0.38), valine (B; SD: 0.015) and leucine (C; SD: 0.013) were measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 µmol; DETA: 5 µmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level.

Six amino acids of the aspartate family could be detected. All the chemicals induced a large increase (3–6.7-fold) in the aspartate content after 11 days, the greatest effect being detected after treatment with DETA/NO + NaCl (6.7-fold) and l-NNA (6-fold) (Fig. 6A). In contrast to Asp, the asparagine content further increased during recovery (Fig. 6B). Its highest concentration was observed after the addition of DETA/NO + NaCl, l-NNA and l-NNA + NaCl (5.2-, 4.8- and 7.6-fold greater) compared to the control. The threonine content was increased (2.5–5.9-fold) by all the compounds, and this increase was especially large (5.9-fold) at the end of the l-NNA treatment (Fig. 6C). Although the isoleucine and lysine contents were increased by almost all the compounds, their levels were at least 2 times greater after DETA/NO + NaCl addition than in the other treatments at the end of the recovery (Fig. 6D and E). The inhibition of NO synthesis resulted in a very large increase (4.6-fold) in the methionine concentration after 11 days (Fig. 6F).

The investigation of the pyruvate family revealed that both the alanine and valine contents were increased 5-fold by l-NNA by the end of the 11-day treatment, while the other treatments had no or much weaker effect (Ala: max. 2.2-fold, Val: max. 1.6-fold increase) (Fig. 7A and B). Much higher Val (4.2-fold) and Leu levels (2.2-fold) were found after addition of DETA/NO + NaCl compared to the other treatments at the end of the one-week recovery phase (Fig. 7B and C).

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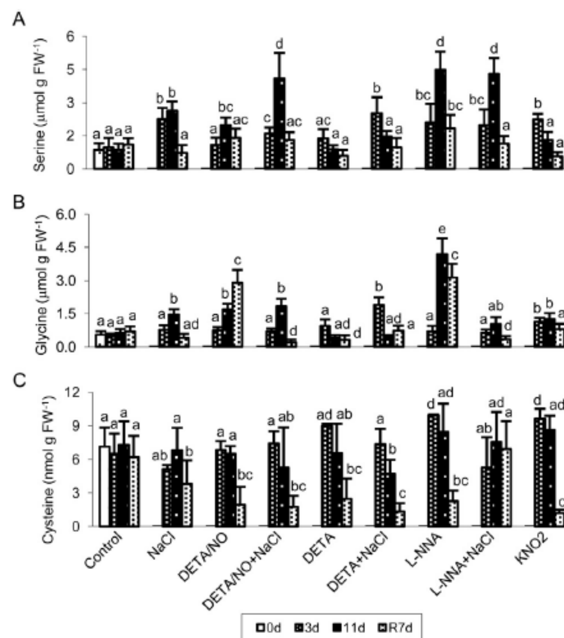

Fig. 8. Effect of NO on salt-induced changes in the concentration of amino acids belonging to the serine family. The amounts of serine (A; SD: 0.11), glycine (B; SD: 0.09) and cysteine (C; SD: 0.61) were measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 µmol; DETA: 5 µmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level.

In the serine family, the serine and glycine concentrations were increased (1.5–5.2-fold and 1.7–6.8-fold) and the cysteine content decreased (by 28–36%) by most of the treatments (Fig. 8). The accumulation of Ser (5.2-fold) and Gly (6.8-fold) was induced by 11- day l-NNA treatment (Fig. 8A and B). In addition, a great increase in Ser content was detected after DETA/NO + NaCl (4.8-fold) and l-NNA + NaCl treatments (5-fold) and the Gly concentration was elevated (4.5-fold) in the DETA/NO-treated plants during the recovery.

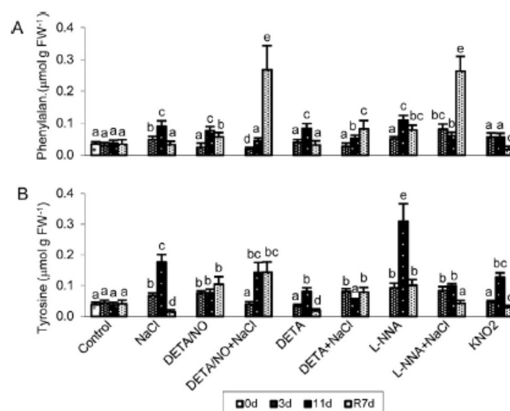

Fig. 9. Effect of NO on salt-induced changes in the concentration of amino acids belonging to the aromatic amino acid family. The amount of phenylalanine (A; SD: 0.012) and tyrosine (B; SD: 0.015) were measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). NaCl: 150 mM; DETA/NO (NO-donor): 5 µmol; DETA: 5 µmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM. The values indicated by different letters are significantly different at $p \leq 0.05$ level.

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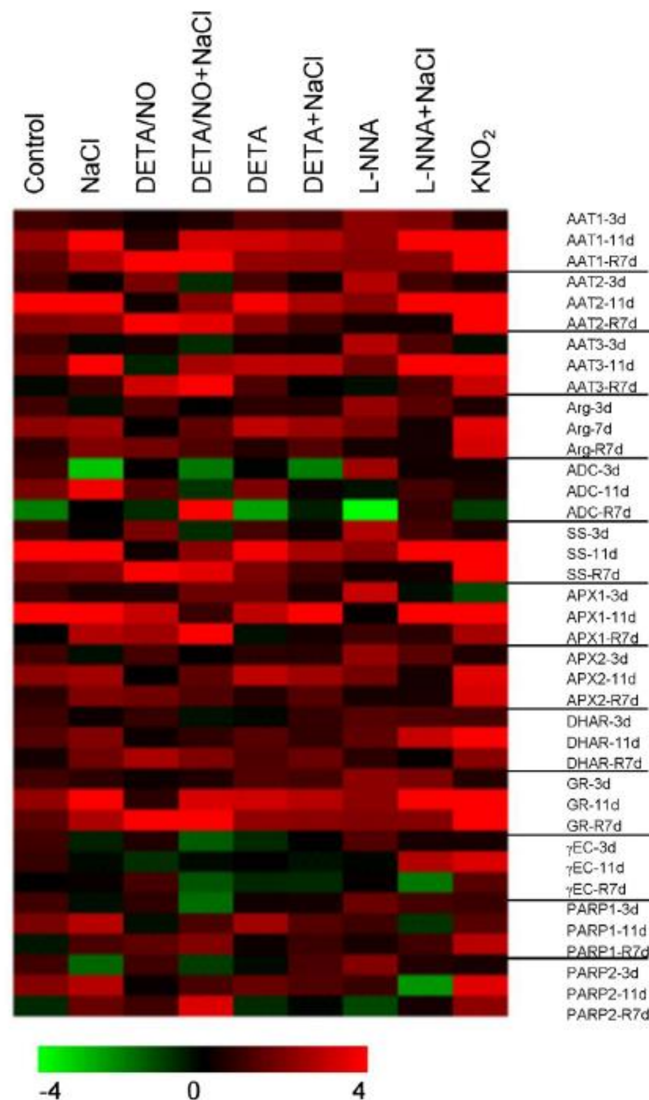

Fig. 10. Effect of NO on salt-induced changes in the expression of genes related to the amino acid metabolism, antioxidants and death processes. Expression was measured in the youngest fully developed leaves after 0, 3 and 11 days of treatment with various compounds and after 7 days of recovery (without these compounds). All the values are given relative to the transcript levels measured before the treatments. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene. NaCl: 150 mM; DETA/NO (NO-donor): 5 µmol; DETA: 5 µmol; L-NNA (inhibitor of NO-synthesis): 1 mM; KNO_2 (precursor of NO): 150 mM.

The Cys content was much lower (by 39–81%) in the recovery phase after the treatments than in the control, except for l-NNA + NaCl addition (no change) (Fig. 8C).

Among the members of the aromatic amino acid family, a 7.7- fold increase in the phenylalanine content was shown in plants treated with DETA/NO + NaCl and l-NNA + NaCl at the end of the one-week recovery period (Fig. 9A). l-NNA induced a huge increase (8.3-fold) in the tyrosine content after 11 days, but this effect was much smaller (2.7-fold increase) when it was combined with NaCl (Fig. 9B). The other treatments increased (1.4–4.7-fold) the Tyr content, too.

Gene expression studies

Gene expression was investigated for several genes related to the amino acid, polyamine and antioxidant metabolism and to death processes. The transcript

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level of the three aspartate aminotransferase and arginase genes was increased (1.5–7.5-fold) by most of the treatments, except for DETA/NO after 11 days NaCl treatment (Fig. 10). The expression of arginase was induced by NaCl (2.5-fold), DETA (2.9-fold) and KNO_2 (3.5-fold) after 11 days, and that of spermine synthase by DETA/NO (4.1-fold), l-NNA (5.3-fold) and KNO_2 (7.5-fold) during recovery. The gene encoding APX1 was greatly induced (3–6-fold) by the various treatments after 11 days except DETA/NO + NaCl and l-NNA. The transcription of the GR gene was increased (2.2–7.5-fold, except for DETA/NO) and that of)'EC synthetase was not changed (except for l-NAA + NaCl – 2.7-fold increase and KNO_2 – 3.4-fold increase) by most of the treatments. A great increase (2.8-fold) in transcript level of poly(ADP-ribose) polymerase1 (PARP1) and PARP2 was observed after NaCl treatment, while no change was detected after the DETA/NO + NaCl treatment.

Discussion

DETA/NO was successfully used to elevate the endogenous NO level and reduce salt-induced damage in maize (Keyster et al., 2012). This effect could be reversed by NO metabolic scavengers and inhibitors (Bai et al., 2011). In the present study, the protective role of NO against salt stress was confirmed in maize using DETA/NO and by inhibiting its synthesis with l-NNA. The improvement in salt tolerance was indicated not only by the growth data, but it was also shown at the gene expression level since the transcript level of PARP1 and PARP2, enzymes involved in the cell death process, was only increased by NaCl but not by DETA/NO + NaCl treatment in maize leaves. The reduction of salt-induced injuries by NO was confirmed by the simultaneous decrease in cell death and caspase-like activity in maize roots, too (Keyster et al., 2012).

Change in the GSH- and GSSG-dependent redox potential was suggested to be a marker of the stress-induced damages (Kranner et al., 2006). NaCl had a great effect on the redox environment in the maize leaves as it was shown by the increase in the amount of GSSG and in the redox potential of the GSH/GSSG redox pair. This influence of NaCl was modified by DETA/NO and l-NNA. The effect of NO on GSH synthesis was shown in *Medicago trunculata*, too (Innocenti et al., 2007). The protective effect of NO on tolerance to osmotic stress was mediated by GSH in *Agropyron cristatum* (Shan et al., 2012). NO-dependent changes in the amount and redox potential of glutathione may protect plants from stress-induced injuries directly by the removal of the excess of reactive oxygen species and indirectly by activation of defence mechanisms and adjustment of metabolism to the altered environmental conditions (Noctor et al., 2012). In addition, the role of the other components of redox system in the mediation of the effect of NO on the response to salt stress is indicated by the increased expression of GR and APX in the leaves of maize treated with DETA/NO + NaCl in the present study. Similarly, the combined application of DETA/NO and NaCl increased the activity of these two enzymes, and also that of GPX and DHAR in the roots of maize (Keyster et al., 2012). These results were confirmed in salt-stressed rice, where NO pre-treatment increased the activity of APX, GR, catalase and SOD and also the expression of stress-related genes (Uchida et al., 2002). The activation of antioxidants by NO during salt stress reduces

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injuries as it was shown in chickpea and soybean (Sheokand et al., 2008; Simaei et al., 2011).

Salt-induced changes in the amino acid metabolism were affected by NO in maize as shown by the alterations in the amino acid composition and in the amount of several amino acids. The effect of NO_2^- as an NO donor in plants was also tested (Crawford and Guo, 2005), and it was found to have an effect very similar to that of DETA/NO on the time-course of changes in the concentrations of several amino acids (Gln, Pro, GABA, Ile, Val, Cys).

Although NO affected the salt-induced accumulation of almost all the amino acids, its greatest effect was observed in the case of Pro, which effectively reduces NaCl-induced damage as an osmoprotectant (Simon-Sarkadi et al., 2007). The Pro content in DETA/NO-treated plants was several times higher than that in the other treatments both after 3 days stress and during recovery. Similarly to the present findings, NO affected Pro concentration during salt stress in cabbage (López-Carrion et al., 2008). The NO-induced Pro accumulation was a result of enhanced Pro synthesis and decreased degradation in maize (Yang and Gong, 2009). Although a relationship between the NO and the Pro precursor Glu was found in tobacco cells, where the involvement of a Glu-receptor in NO production and plant defence signalling was observed (Vatsa et al., 2011), the pattern of salt- and NO-dependent changes for Pro and Glu was not similar in maize in the present experiment. This difference can be explained by the possible simultaneous use of Glu for Pro, GSH and/or polyamine synthesis. Both GSH and polyamines have important role in the reduction of injuries occurring during abiotic stress (Simon-Sarkadi et al., 2007; Kelló˝ s et al., 2008). The increased need for Glu because of greater Pro, GSH and polyamine synthesis may be ensured from the enhanced catabolism of Lys described in stressed plants (Galili et al., 2001). However, NO did not activate Lys catabolism in the present experimental system, since greater accumulation of Lys was observed in maize seedlings treated with DETA/NO + NaCl compared to NaCl-treated ones during the recovery.

Besides Pro, the branched-chain amino acids, Ile, Val and Leu, accumulated to a much greater extent during the recovery phase after the addition of DETA/NO + NaCl than after the other treatments, indicating their involvement in the NO-mediated response to salt stress. In the case of Ile this change may be the result of a simultaneous increase in the level of its precursor Thr after the DETA/NO + NaCl treatment. A recent study emphasized the importance of branched-chain amino acids as osmolytes which function might contribute to the improved salt tolerance in the present experiment (Joshi et al., 2010).

The inhibition of NO synthesis increased the Gln and GABA levels, but this effect was similar in both the absence and presence of NaCl, therefore cannot be related to a protective mechanism in maize. However, the results obtained in tobacco (Akc¸ ay et al., 2012) suggest that the increase in the GABA content induced by inhibition of NO synthesis using l-NNA may have a protective role during salt stress. NO and GABA regulate mutually the concentration of each other, since GABA also affected

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NO synthesis in potato (Floryszak-Wieczorek et al., 2012). Similarly to maize, the effect of NO on Gln could be shown in root nodules of *Medicago trunculata*, where a Gln synthase was found to be a target of NO (Melo et al., 2011). In contrast to Gln and GABA, l-NNA only increased the concentrations of Thr, Ala and Tyr to very high levels compared to the other treatments in the absence of NaCl, indicating that NO has a negative regulatory role on the concentration of these three amino acids. Interestingly, the Gly concentrations reached high levels after both the addition of NO donor and the inhibition of NO synthesis. This effect disappeared in the presence of NaCl. Compared to Gly the opposite changes were observed for Phe, the level of which was high after the combined application of NaCl and DETA/NO or l-NNA, but low if these compounds were added without salt. Thus, the Phe level was only affected by NO during salt stress. Interestingly, higher and lower NO levels had a similar effect on the Gly and Phe metabolism which contradiction may be explained by possible induction of their synthesis by high NO levels and by possible inhibition of their degradation at low NO concentration.

In conclusion, modification of NO levels affected salt-induced, glutathione-dependent redox changes and simultaneously the level of several amino acids. These could be independent effects, but it cannot be excluded that the influence of NO on free amino acid concentration is mediated by redox signalling. The observed much higher Pro content in plants treated with both NaCl and DETA/NO during recovery, may contribute to the protective effect of NO against salt stress.

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