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Exogenous nitric oxide limits salt-induced oxidative damage in maize by altering superoxide dismutase activity

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ABSTRACT

Salinity stress causes accumulation of reactive oxygen species (ROS) to levels that are toxic to plants. Inefficient scavenging of ROS by antioxidant enzymes like superoxide dismutase (SOD; EC 1.15.1.1) results in cell death and inhibition of growth, which ultimately leads to reduced crop productivity. Recent studies suggest that nitric oxide (NO) can enhance plant tolerance to salinity stress but the molecular mechanism for the NO-mediated salinity stress tolerance is only partially understood. This study thus evaluated the effect of exogenously applied NO on maize leaf superoxide accumulation and SOD enzymatic activity in the presence and absence of salinity stress to delineate SOD isoforms that contribute to NO-mediated salinity tolerance. Salinity stress caused elevation of superoxide generation and lipid peroxidation in maize leaves, along with elevated activity of a number of SOD isoforms. Exogenous application of 2,2′-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO) to salinity-treated maize reduced the salinity-induced superoxide accumulation and lowered the salinity-induced lipid peroxidation in maize leaves and corresponded with an amplification of the increase in the activity of some SOD isoforms. Based on this analysis, the study suggests that exogenously applied NO reduces salinity-induced oxidative stress by up-regulating the enzymatic activity of some SOD isoforms, thus increasing the scavenging of excessive superoxide radicals to limit oxidative stress.

1. Introduction

Salinity stress is one of the most important environmental factors that cause reduction in growth, development and productivity of maize worldwide. Salinity stress changes the morphological, physiological and biochemical responses of plants (Agarwal and Shaheen, 2007; Siringam et al., 2011; Klein et al., 2013). There is evidence that high salt concentrations cause an imbalance in cellular ions, resulting in ion toxicity and osmotic stress; which leads to the generation of ROS known to damage DNA, lipids and proteins (Yasar et al., 2006). The widespread nature of this problem highlights the importance of finding a way to protect plants from accumulation of ROS and subsequent oxidative damage to cellular macromolecules (Noctor and Foyer, 1998). Exceedingly high levels of ROS must be detoxified by various cellular responses, if the plant is to survive and grow (Gratão et al., 2005). ROS scavenging depends on the detoxification mechanism, which may occur as a result of sequential and simultaneous action of a number of antioxidant enzymes, including SOD. ROS that accumulate to excessive levels as a result of salinity include O_2^- and H_2O_2 (Zilli et al., 2009). Despite their toxic effects, ROS at homeostatically favored levels are required for cellular signaling that regulates proper plant growth and development (Rodriguez et al., 2002; Foreman et al., 2003; Sagi and Fluhr, 2006). Recently, it has been shown that accumulation of O_2^- triggers enhanced SOD enzymatic activity, which acts to detoxify O_2^- by converting it to H_2O_2 and is thus one of the antioxidant enzymes that protect maize roots against oxidative damage (Keyster et al., 2012; Klein et al., 2013).

The characterization of the responses of *Atriplex centralasiatica* seedlings to salinity stress consolidated the role of NO in mediating salinity stress tolerance in plants by modulating the accumulation of ROS, the activity of antioxidant enzymes and restriction of oxidative damage (Xu et al., 2011). This reactive nitrogen species is believed to act as a signal molecule mediating responses to both biotic and abiotic stresses in plants (Crawford and Guo, 2005; Delledonne, 2005). NO induces seed germination (Beligni and Lamattina, 2000), affects growth and development in plants (Durner and Klessig, 1999; Keyster et al., 2012), regulates iron homeostasis (Murgia et al., 2002) and controls plant maturation and senescence. Several lines of investigations into the role of NO have established that exogenously applied NO enhances plant tolerance against salinity stress (Zhao et al., 2004; Zhang et al., 2006; Keyster

Abbreviations: ANOVA, analysis of variance; DETA/NO, 2,2′-(hydroxynitrosohydrazono)bis-ethanimine; MDA, malondialdehyde; NBT, nitrotetrazolium blue chloride; ROS, reactive oxygen species; RWC, relative water content; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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et al., 2012). Furthermore, it appears that the enhancement of plant tolerance against salinity stress may be mediated in part by antioxidant enzymes that act to prevent oxidative stress (Shi et al., 2007; Tanou et al., 2009; Zheng et al., 2009; Molassiotis et al., 2010; Wu et al., 2011; Keyster et al., 2012).

To understand the molecular mechanisms by which NO regulates salt stress tolerance in maize plants; given the role of NO as an antioxidant involved in the scavenging of ROS, coupled with the knowledge that salinity alters ROS production in plants; this study investigates if NO (exogenously supplied as the NO donor DETA/NO) participates in the regulation of O_2^- accumulation, lipid peroxidation and the modulation of SOD activity during salinity stress (imposed as 150 mM NaCl) in maize.

2. Materials and methods

2.1. Plant growth

Maize (*Zea mays* L. cv. Silverking) seeds (donated by Capstone Seeds Pty Ltd., Howick, South Africa) were surface-sterilized in 0.35% sodium hypochlorite for 10 min, followed by four washes with sterile distilled water. Seeds were imbibed in sterile distilled water for 30 min and sown in 2 l of filtered silica sand (98% SiO_2, Rolfes® Silica, Brits, South Africa) in 20 cm diameter plastic pots. The sand was kept moist by watering only with distilled water during germination. Germinated seedlings (thinned to one plant per pot) were grown on a temperature cycle so that the day temperature is 25 °C and the night temperature is 19 °C. The day cycle had an average photosynthetic photon flux density of 300 μmol photons·m^{-2}·s^{-1} over a period of 16 h and the dark (night) period was 8 h. Once the plants reached the V1 stage of vegetative growth, they were supplied with nutrient solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM KNO_3, 10 mM NH_4NO_3, 1 mM K_2HPO_4 buffer at pH 6.4, 5 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 2 μM Na_2MoO_4, 1 μM $CoSO_4$, 100 μM Fe-NaEDTA and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 6.4].

2.2. Treatment of plants

One week after the plants had reached the V1 stage, control plants were supplied with nutrient solution every third day. For treatments, the nutrient solution was supplemented with the following final concentrations: 150 mM NaCl, 10 μM DETA/NO (a nitric oxide donor), 10 μM diethylenetriamine (DETA, which lacks the NO moiety and thus serves as a control for NO treatments), a combination of 150 mM NaCl with 10 μM DETA/NO or a combination of 150 mM NaCl with 10 μM DETA. Treatments or nutrient solution (200 ml per pot) was applied every three days to each plant (directly to the sand at the base of the stem).

2.3. Measurement of superoxide content

For detection of O_2^- in maize leaf tissue, a modified procedure described by Schneider and Schlegel (1981) was used. For each treatment (three plants per treatment), 100 mg (per plant) of freshly harvested leaf tissue was homogenized in 500 μl of 65 mM phosphate buffer (pH 7.8). The homogenate was centrifuged at 5000 ×*g* for 10 min to obtain the O_2^- extract. An aliquot (100 μl) of the extract was mixed with 90 μl phosphate buffer and 10 μl of 10 mM hydroxylammonium chloride, followed by incubation at 25 °C for 20 min. After incubation, 100 μl of the reaction mixture was mixed with 100 μl of 18 mM 4-aminobenzene sulfonic acid and 100 μl of 7 mM 1-naphthylamine, followed by mixing with 300 μl of ethyl ether. The pink aqueous phase (200 μl) was used to measure O_2^- generation by monitoring the absorbance of the aqueous phase at 530 nm (A_{530}), based on a standard curve constructed with NO_2^- since chemical reaction of O_2^- with hydroxylammonium chloride produces NO_2^-.

2.4. Measurement of lipid peroxidation

The level of lipid peroxidation was estimated on the basis of the amount of malondialdehyde (MDA) measured in plant tissue. The MDA content was measured using the method described by Heath and Packer (1968). Fresh leaves (200 mg) were homogenized in 800 μl of 5% (w/v) trichloroacetic acid (TCA) buffer. The extracts were then centrifuged at 4000 ×*g* for 10 min. The supernatant (200 μl) was mixed with 800 μl of 0.5% (w/v) thiobarbituric acid (TBA, prepared in 20% TCA), followed by boiling at 100 °C for 20 min. The reaction mixture was cooled immediately on ice for 5 min and the absorbance was read at both 532 nm and 600 nm. The MDA concentration was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

2.5. Determination of superoxide dismutase activity

To determine total SOD activity in maize leaf tissue, total soluble protein extracts were obtained by homogenizing 100 mg of leaf tissue with 500 μl of extraction buffer [40 mM K_2HPO_4, pH 7.4, 1 mM EDTA, 5% (w/v) polyvinylpyrrolidone (PVP, molecular weight = 40,000)]. The resulting homogenates were centrifuged at 12,000 ×*g* for 20 min at 4 °C and the supernatants were used for a spectrophotometric SOD assay as described by Keyster et al. (2012), which is a modification of the method described by Beyer and Fridovich (1987). Briefly, 10 μl of the supernatant (protein extract) was added to an assay mixture containing 50 mM K_2HPO_4, pH 7.8, 0.1 mM EDTA, 0.025% (w/v) Triton X-100, 0.1 mM xanthine, 6.25 nM xanthine oxidase, 0.1 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1). This assay mixture was incubated for 20 min at 37 °C and absorbance readings were recorded at 450 nm. SOD activity was calculated based on the amount of enzyme that was required to cause 50% decrease in the reduction of WST-1. Protein concentrations in the extracts (supernatants) were determined as described by Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.6. Detection and analysis of superoxide dismutase isoforms

For the detection of SOD isoforms in maize leaf tissue, total soluble protein extracts were obtained by homogenizing 100 mg of leaf tissue with 500 μl of extraction buffer (as described for total SOD activity). The extracted proteins were separated from cell debris by centrifugation at 12,000 ×*g* for 20 min at 4 °C and used for the detection of SOD isoforms. Protein concentrations of the extracts were determined as described by Bradford (1976) using bovine serum albumin (BSA) as a standard.

Protein extracts from various treatments (containing 80 μg protein per sample) were separated on a 10% native polyacrylamide gel at 4 °C. SOD activity was detected by photochemical staining with riboflavin and nitrotetrazolium blue chloride (NBT) as described by Beauchamp and Fridovich (1971). SOD isoforms were identified by incubating gels in 6 mM H_2O_2 to inhibit Cu/ZnSOD and FeSOD, or in 5 mM KCN to inhibit only Cu/ZnSOD, with MnSOD activity assigned on the basis of its resistance to both H_2O_2 and KCN (Archibald and Fridovich, 1982). Densitometry analysis, indicative of the level of SOD activity, was performed using the Spot Denso tool (AlphaEase FC Imaging software, Alpha Innotech Corporation). SOD activity of each isoform was scored as described by Klein et al. (2013).

2.7. Statistical analysis

GraphPad Prism 5.03 software was used for the statistical analyses. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance of the collected data, with the Turkey-Kramer test set at 5% level to evaluate the statistical significance. All results are the mean of three independent experiments with three biological replicates.