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Nitric oxide influences glycine betaine content and ascorbate peroxidase activity in maize

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ABSTRACT

Compatible solutes, such as glycine betaine (GB), are involved in improving plant tolerance to abiotic stress. In this study, we investigated the effects of exogenously applied nitric oxide (NO) donor 2,2′-(hydroxynitrosohydrazono) bis-ethanimine and nitric oxide synthase (NOS) inhibitor N_{ω}-Nitro-L-Arginine methyl ester (L-NAME) on GB content and its influence on ascorbate peroxidase (APX) enzymatic activity in roots and leaves of maize seedlings. Application of L-NAME (2 mM L-NAME or in combination with 200 μM DETA) significantly increased cell death, H_2O_2 content, and lipid peroxidation but reduced GB content and APX activity. The effects of L-NAME treatment on maize were reversed by application of the NO donor 2, 2′-(hydroxynitrosohydrazono) bis-ethanimine (DETA/NO). Application of the NO donor to plants treated with L-NAME reversed the effects of L-NAME on GB content and APX activity, which were increased to levels higher than those in plants treated with L-NAME alone. These results show that exogenous application of the NOS inhibitor reduces APX activity and GB accumulation. Our data suggest that NOS activity plays a role in regulating the antioxidant defense mechanism and osmoprotection in plants.

1. Introduction

Accumulation of glycine betaine (GB) in the cytosol of plants is a response which aims at combating and acclimation to osmotic stress. This is a cellular approach employed by certain plant species during abiotic stress (Mulder and Breure, 2003). GB is an amphoteric quaternary amine which plays a vital role as a compatible solute in plants during abiotic stress (Gadallah, 1999; Ma et al., 2006; Zhao et al., 2007; Chen and Murata, 2011). It is synthesized in the cell to protect against osmotic stress and is dependent on water status, crop growth stage, and cultivar of the plant (Ashraaf and Foolad, 2007; Zhang et al., 2009).

Apart from its established role as an osmolyte (Gorham, 1995), this low-molecular-weight water-soluble compound is also involved in the scavenging of reactive oxygen species (ROS) (Cruz et al., 2013; Fariduddin et al., 2013). In plants, the biosynthesis of GB is a two-step oxidation of choline that involves an intermediate betaine aldehyde. The first oxidation step is catalyzed by choline monooxygenase (CMO, EC 1.14.15.7), and the second oxidation step is catalyzed by betaine aldehyde dehydrogenase (BADH, EC 1.2.1.81) in a process that occurs in chloroplasts (Sakamoto and Murata, 2002; Sithtisarn et al., 2009). Different plants accumulate varying levels of GB. In fact, accumulation of high levels of GB has been correlated with the extent of increased plant tolerance to abiotic stress (Chen et al., 2000; Joseph et al., 2013).

There are different types of compatible solutes and their accumulation varies in different plant species (Rhodes and Hanson, 1993; Bohnert and Jensen, 1996). Unlike other GB accumulators, maize lacks the ability to synthesize GB in high amounts (Zwart et al., 2003). As such, exogenous application of GB has most recently become an effective way of inducing tolerance in maize plants under water (Ali and Ashraaf, 2011), chilling (Farooq et al., 2008), drought (Anjum et al., 2012), and salt stress (Nazia et al., 2014). In many regions of South Africa, maize (*Zea mays*) is one of the most important staple foods and cash crops available.

During abiotic stress, plants employ signaling molecules, such as NO, which help to mitigate the toxic effects resulting from the accumulation of ROS. Apart from its role in maintaining normal physiological processes in plants (Delldonne et al., 1998; Beligni and Lamattina, 2000; Mishina et al., 2007), several studies have shown the induction of antioxidant enzyme activity by NO during long-term drought (Farooq et al.,

Abbreviations: APX, Ascorbate peroxidase; BADH, Betaine aldehyde dehydrogenase; CMO, Choline monooxygenase; DETA, Diethylenetriamine; DETA/NO, 2,2(hydroxynitrosohydrazono) bis-ethanimine; GB, Glycine betaine; L-NAME, N_{ω}-Nitro-L-arginine methyl ester; L-NNA, N_{ω}-Nitro-L-arginine; MDA, Malondialdehyde; NBT, Nitrotetrazolium Blue chloride; NO•, Nitric oxide; NOS, Nitric oxide synthase; ROS, Reactive oxygen species.

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2009; Cechin et al., 2015) and salt stress (Egbichi et al., 2014; Sheokand et al., 2010). Among these enzymes is ascorbate peroxidase (APX, EC 1.11.1.11), which plays a vital role in defense against oxidative stress.

APX utilizes ascorbate (AsA) as its specific electron donor to reduce hydrogen peroxide (H_2O_2) to H_2O, with the concomitant generation of monodehydroascorbate/dehydroascorbate (MDHA/DHA) (Dalton et al., 1986; Asada, 1994; Iturbe et al., 2001). In analogy to animals, plants have NOS enzymatic activity, which catalyzes the conversion of L-arginine to L-citrulline, with a simultaneous release of NO (Wendehenne et al., 2001).

Although the identity of NOS in plants has not been resolved (Bates et al., 1995; Barroso et al., 1999; Corpas et al., 2001; Crawford and Guo, 2005; Zamojtel et al., 2006), the L-arginine-dependent NO production provides a convenient tool to investigate a possible similar NO production pathway in plants. In fact, several studies have used compounds such as N_ω-Nitro-L-Arginine methyl ester (L-NAME) (Corpas et al., 2009; Leach et al., 2010) and N_G-monomethyl-L-arginine acetate (LNMMA) (Zhang et al., 2003). These analogues of L-arginine, which function as competitive inhibitors of animal NOS-mediated NO synthesis on plants, result in decreased NO content.

There are several studies on the individual role of GB and NO in mediating plant tolerance against various abiotic stresses. However, to our knowledge, no investigations have been done on the effects of endogenous NO on GB content and its effect thereof on the APX enzymatic activity. In view of this fact, we investigated the effect of inhibition of NOS activity with L-NAME on GB accumulation and the resulting effect on APX activity in maize leaves and roots. We also investigated the effect of L-NAME on cell viability, lipid peroxidation and H_2O_2 level in maize seedlings when supplemented with an NO donor (DETA/NO).

2. Materials and methods

2.1. Plant growth

Maize (*Zea mays* L. cv Silverking) seeds (donated by Capstone Seeds Pty Ltd) were surface-sterilized in 0.35% sodium hypochlorite for 10 min and then rinsed four times with sterile distilled water. The seeds were imbibed in sterile distilled water for 20 min and sown in 2 L of pre-soaked (distilled water) filtered silica sand (98% SiO2, Rolfes® Silica, Brits), in 20 cm diameter plastic pots. The sand was kept moist by watering with distilled water during germination. Germinated seedlings (one plant per pot) were grown on a regulated condition of 25/19 °C day/night temperature cycle under a 16/8 h light/dark cycle, at a photosynthetic photon flux density of 300 μmol photons.m^{-2}.s^{-1} during the day phase. Plants 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 7.2, 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 7.2] at the V1 stage (when the collar of the first true leaf is visible). Plants at the V1 stage which were of similar height were selected for all experiments.

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: 2 mM L-NAME, a combination 2 mM L-NAME with 200 μM DETA/NO, and finally a combination of 2 mM L-NAME with 200 μM Diethylenetriamine (DETA). DETA lacks the NO moiety and serves as a control for NO treatments. Treatments or nutrient solution (200 ml per pot) were applied to each plant directly to the sand at the base of the stem of the plant in the pot every three days. After 10 days of treatment, plants were carefully removed from the sand, then used immediately for cell viability or snap-frozen (in liquid nitrogen) for use in all other experiments.

2.3. Determination of protein concentration

Protein concentrations for all assays were measured in the extracts as instructed for the RC DC™ Protein Assay Kit II (Bio-Rad Laboratories).

2.4. Determination of glycine betaine content

Estimation of endogenous glycine betaine content in *Zea mays* was carried out by modifying a method previously described by Sairam et al. (2002). Plant root and leaf tissue (250 mg) were ground to a fine powder in liquid nitrogen. The tissue was incubated in tubes containing 20 ml of de-ionized water for 24 h at 25 °C. The samples were filtered and mixed with 2 N H_2SO_4. An aliquot (0.25 ml) was transferred into a test tube and cooled in ice water for 1 h. Cold potassium iodide-iodine reagent (0.1 ml) was added, vortexed, and then centrifuged at 1000 × *g* for 30 min at 4 °C. The sample was incubated for 24 h at 4 °C. The formed periodite crystals were dissolved in 14 ml of 1,2-dichloroethane and shaken at room temperature for 48 h. The absorbance was then read at a wavelength of 365 nm using a FLUOstar Omega UV-visible spectrophotometer (BMG LabTech GmbH, Ortenberg, Germany).

2.5. Estimation of H_2O_2 content

In order to determine if the inhibition of NOS activity affects ROS accumulation, we measured H_2O_2 content in the maize treatments. The H_2O_2 content was determined in the maize root and leaf extracts by modifying a method previously described by Velikova et al. (2000). *Zea mays* tissue (100 mg) was ground to a fine powder in liquid nitrogen and homogenized in 400 μl of cold 6% (w/v) TCA. The extracts were centrifuged at 12,000 × *g* for 30 min at 4 °C and 50 μl of the supernatant was used to initiate the reaction in a mixture (total volume of 200 μl) containing 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. The reaction was incubated at 25 °C for 20 min and absorbance readings were recorded at 390 nm. H_2O_2 content was calculated using a standard curve based on the absorbance (A390 nm) of H_2O_2 standards.

2.6. Measurement of lipid peroxidation

Lipid peroxidation was determined in *Zea mays* root and leaf tissue by measuring malondialdehyde (MDA) formation, using the thiobarbituric acid (TBA) method as previously described by Buege and Aust (1978). Plant tissue (100 mg) was ground into a fine powder in liquid nitrogen and homogenized in 400 μl of cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 x *g* for 30 min at 4 °C. Aliquots (100 μl) of the supernatant were mixed with 400 μl of 0.5% TBA (prepared in 20% TCA). The mixture was incubated at 95 °C for 30 min and the reaction was stopped by placing the mixture on ice for 5 2min. The mixture was further centrifuged at 12,000 x *g* for 5 min at 4 °C. The absorbance of the supernatant was measured at 532 and 600 nm. After subtracting the non-specific absorbance (A600 nm), the MDA concentration was determined by its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} of fresh weight.

2.7. Evaluation of cell viability in Zea mays roots and leaves

In order to establish if application of NO (200 mμ DETA/NO) could maintain *Zea mays* cell viability after the inhibition of NOS, evaluation of root and leaf cell viability was carried out. This cell viability assay was estimated by modifying a method previously described by Sanevas et al. (2007). The tissues (100 mg per treatment) were harvested and stained with 0.25% (w/v) Evans Blue for 15 min at room temperature. The roots and leaves were then washed for 30 min in distilled water, followed by extraction of the Evans Blue stain (taken up by