Crosstalk of nitric oxide with calcium induced tolerance of tall fescue leaves to high irradiance

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Abstract

Calcium ion (Ca^{2+}) is essential secondary messenger in plant signaling networks. In this study, the effect of Ca^{2+} on oxidative damage caused by a high irradiance (HI) was investigated in the leaves of two cultivars of tall fescue (Arid3 and Houndog5). Pretreatment of the tall fescue leaves with a $CaCl_2$ solution significantly increased Ca^{2+} content and intrinsic HI tolerance due to a decreased ion leakage and content of malondialdehyde, hydrogen peroxide, and superoxide radicals. Moreover, the activities of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase increased in both the cultivars in the presence of Ca^{2+} under the HI stress. In contrast, treatments with a Ca^{2+} chelator ethylene glycol-*bis*(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) or a plasma membrane Ca^{2+} channel blocker $LaCl_3$ reversed these effects. On the other hand, a pronounced increase in nitric oxide synthase-like activity and NO release by exogenous Ca^{2+} treatment was observed in the tolerant Arid3 plants after exposure to the HI, whereas only a small increase was observed in more sensitive Houndog5. Moreover, the inhibition of NO production by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide or N^{ω}-nitro-L-arginine blocked the protective effect of exogenous Ca^{2+}, whereas the inhibition of Ca^{2+} by EGTA or $LaCl_3$ had no influence on the protective effect of NO. The results indicate that NO might be involved in the Ca^{2+}-induced activities of antioxidant enzymes further protecting against HI-induced oxidative damage. This protective mechanism was found to be more efficient in Arid3 than in Houndog5.

Additional key words: antioxidant enzymes, *Festuca arundinacea,* hydrogen peroxide, malondialdehyde.

Introduction

In the natural environment, sun is the ultimate source of energy for growth and development of plants. However, an excessive radiation induces formation and accumulation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{·-}$), hydroxyl radical ($HO^{•}$), and singlet oxygen (O_2^{1}) (Asada 2006). If these ROS are not scavenged immediately, they can directly damage lipids, proteins, pigments, and nucleic acids. To avoid ROS-induced cellular injury, plants utilize various antioxidative enzymes and non-enzymatic antioxidants (Apel and Hirt 2004).

Nitric oxide (NO), a key signaling molecule produced in response to various stimuli, is involved in many plant physiological processes such as seed germination, de-etiolation, cell senescence, and programmed cell death (Beligni and Lamattina 2000, Neill *et al*. 2003, Bright *et al*. 2006). Moreover, NO was also found to mediate plant responses to abiotic stresses caused by drought, salinity, UV-B radiation, and heavy metals (Laspina *et al*. 2005, Zhao *et al*. 2008, Corpas *et al*. 2011, Nasir Khan *et al*. 2012, Tossi *et al*. 2012, Bai *et al*. 2015). Additionally, recent studies have indicated that NO

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; CAT - catalase; EGTA - ethylene glycol-*bis*(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; GR - glutathione reductase; HI - high irradiance; LI - low irradiance; LNNA - N^{ω}-nitro-L-arginine; NO - nitric oxide; NOS - nitric oxide synthase; PPFD - photosynthetic photon flux density; PTIO - 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SNP - sodium nitroprusside; SOD - superoxide dismutase.
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activates antioxidant defenses during UV irradiation, heat, and salt stresses. The studies also indicate a link between NO and Ca^{2+} (Gong *et al*. 1997, Jiang and Huang 2001, Bhattacharjee 2008, Al-Whaibi *et al*. 2012, Tossi *et al*. 2012).

Calcium is one of the important, ubiquitous intracellular secondary messengers involved in regulation of plant adaptive responses to various environmental stresses and diverse physiological and developmental processes (Gong *et al*. 1998, Shi *et al*. 2014). Signal molecules, such as NO, H_2O_2, and Ca^{2+}, are involved in abscisic acid (ABA)-triggered stomatal closure. In this signaling event, Ca^{2+} induces NO production by inducing a nitric oxide synthase (NOS)-like activity (Garcia-Mata and Lamattina 2007). Further, Ca^{2+} is involved in NO- and auxin-induced lateral root formation (Chen and Kao 2012). Our earlier study has shown that NO is involved in the ABA-induced activities of antioxidant enzymes, further protecting against injuries caused by a high irradiance (HI) (Xu *et al*. 2010, 2013). However, the mechanism how antioxidant enzymes are induced by NO and Ca^{2+} has not been elucidated in tall fescue leaves.

Tall fescue (*Festuca arundinacea*) is widely used cold-season turf grass in China. It is often grown at fluctuating photosynthetic photon flux densities and improving HI tolerance is very important for turf grass engineering. In a preliminary experiment conducted on two cultivars Arid3 and Houndog5, it was found that Houndog5 is more susceptible to an HI stress than Arid3. Our earlier study also demonstrated that ABA protects tall fescue against HI-induced oxidative damage by promoting NO release by inducing NOS-like activity (Xu *et al*. 2013). Thus, NO may act as signaling molecule triggering enhanced activities of antioxidant enzymes further protecting against injuries caused by an HI (Xu *et al*. 2010). The objective of this study was to elucidate the role of Ca^{2+} in alleviating HI-induced oxidative damage in leaves of two tall fescue cultivars, and also how NO and Ca^{2+} interact in these processes.

Materials and methods

The seeds of tall fescue [*F. arundinacea* (Schreb.) cvs. Arid3 and Houndog5] were obtained from the *Clover Seed & Turf Company*, Beijing, China. The seeds were surface sterilized in 0.1 % (m/v) sodium hypochlorite, rinsed several times in distilled water, and germinated on moistened filter paper at room temperature for 7 d. The healthy seedlings were selected and placed into 5-dm^3 plastic containers containing 4 dm^3 of a nutrient solution. Each plastic container contained six plants. The seedlings were cultured hydroponically in a continuously aerated nutrient solution (Xu *et al*. 2010). The plants were grown at day/night temperatures of 25/20 °C, a relative humidity of 70 %, a 14-h photoperiod, and a photosynthetic photon flux density (PPFD) at the plant height of 100 μmol m^{-2} s^{-1} [the lighting system was 16 cool white fluorescent lamps (17 W) and two incandescent lamps (40 W)].

The first experiment was conducted to elucidate the role of Ca^{2+} in alleviating HI-induced oxidative damage in leaves. After 21 d of pre-culture, the plants were grown under HI (500 μmol m^{-2} s^{-1} PPFD), whereas control plants were grown under low irradiance (100 μmol m^{-2} s^{-1}; LI). A 20 mM Ca^{2+} (by adding $CaCl_2$), or a 2 mM calcium chelator ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), or a 1 mM calcium channel blocker ($LaCl_3$) were applied into a nutrient solution of the HI-treated plants (An *et al*. 2005, Laspina *et al*. 2005). After 7 d of treatments, the plants were harvested and frozen in liquid nitrogen and then stored at -80 °C for further analyses.

The second experiment was designed to investigate the role of Ca^{2+} in NO induced tolerance in tall fescue under the HI stress. Stress treatments were carried out after 21 d of pre-culture. The plants were grown under the HI or LI. A 20 mM $CaCl_2$, 1 mM $LaCl_3$, 2 mM EGTA, 100 μM NO donor sodium nitroprusside (SNP), 200 μM NOS inhibitor N^{ω}-nitro-L-arginine (LNNA), or 150 μM NO scavenger 2-(4-carboxy-2-phenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide (PTIO) were applied into nutrient solutions under the HI treatments. After 7 d of treatments, plant height was immediately measured and after taking fresh mass, the samples were placed in an oven and maintained at a temperature of 60 °C for 48 h to record dry mass.

Relative ion leakage was determined according to Sairam and Srivastava (2002). Fresh leaves (0.5 g) were harvested and cut into 20 mm pieces. They were washed in deionized water and placed in Petri dishes with 5 cm^3 of deionized water at 25 °C for 2 h. After the incubation, conductivity was measured (C1). Then, the samples were boiled for 20 min, and conductivity was read again (C2). Relative ion leakage [%] = C1/C2 × 100.

Content of H_2O_2 was measured according to Veljovic-Jovanovic *et al*. (2002). Leaves (0.5 g) were ground in liquid nitrogen and the powder was extracted in 2 cm^3 of 1 M $HClO_4$ in the presence of 5 % (m/v) polyvinyl-pyrrolidone (PVP). The homogenate was centrifuged at 12 000 *g* for 10 min, and the supernatant was neutralized with 5 M K_2CO_3 (pH 5.6) in the presence of 0.1 cm^3 of 0.3 M phosphate buffer (pH 5.6). The solution was centrifuged at 12 000 *g* for 1 min, and the sample was incubated for 10 min with 1 U of ascorbate oxidase to oxidize ascorbate prior to assay. A reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 3.3 mM 3-(dimethylamino) benzoic acid, 0.07 mM 3-methyl-

2-benzothiazoline hydrazone, 0.3 U of guaiacol peroxidase, and 0.2 cm^3 of the supernatant. Changes in absorbance at 590 nm were monitored at 25 °C.

Superoxide radical content was determined by the modified method of Elstner and Heupel (1976). Leaves (1.0 g) were homogenized in 3 cm^3 of 50 mM potassium phosphate buffer (pH 7.8) and centrifuged at 12 000 *g* for 20 min. A reaction mixture contained 1 cm^3 of the supernatant, 1 cm^3 of 50 mM potassium phosphate buffer (pH 7.8), and 1 cm^3 of 1 mM hydroxylaminonium chloride and was incubated at 25 °C for 20 min. The mixture was subsequently incubated with 2 cm^3 of 17 mM sulphanilic acid and 2 cm^3 of 7 mM α-naphthyl amine at 25 °C for 20 min. The final solution was mixed with an equal volume of ethyl ether, and absorbance was read at 530 nm.

Membrane lipid peroxidation was estimated according to malondialdehyde production using a slightly modified thiobarbituric acid method of Buege and Aust (1978). Leaves (0.5 g) were homogenized with a mortar and pestle in 10 % (m/v) trichloroacetic acid, and then the homogenate was centrifuged at 4 000 *g* for 30 min. A 2 cm^3 aliquot of the supernatant was mixed with 2 cm^3 of 10 % trichloroacetic acid containing 0.5 % (m/v) thiobarbituric acid. The mixture was heated at 100 °C for 30 min. The absorbance of the supernatant was measured at 532 nm with a reading at 600 nm subtracted from it to account for a non-specific turbidity.

Leaves (1.0 g) were homogenized with a mortar and pestle at 4 °C in 5 cm^3 of 50 mM phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid disodium salt and 2 % (m/v) PVP. The homogenate was centrifuged at 12 000 *g* and 4 °C for 20 min and the supernatant was used for the following enzyme activity assays. Protein content in the supernatant was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the nitroblue tetrazolium method of Beauchamp and Fridovich (1971). One unit of SOD was defined as the amount of the enzyme required to cause a 50 % inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm. Catalase (CAT, EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 at 240 nm (the coefficient of absorbance ε = 39.4 mM^{-1} cm^{-1}) by the method of Aebi (1984). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to the method of Foyer and Halliwell (1976); it depends on the rate of decrease in the absorbance of NADPH at 340 nm (ε = 6.2 mM^{-1} cm^{-1}).

Nitric oxide production was performed according to the method of Murphy and Noack (1994). The fresh leaves (0.5 g) were incubated with 100 U of catalase and 100 U of superoxide dismutase for 5 min to remove endogenous ROS before addition of 5 cm^3 of oxyhaemoglobin (5 mM). After 2 min incubation, NO content was estimated by following the conversion of oxyhaemoglobin to methaemoglobin at 577 and 591 nm.

Activity of NOS was measured according to the method of Chandok *et al.* (2003). Leaves (1.0 g) were homogenized in 2 cm^3 of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.5 mM ethylenediaminetetraacetic acid, 1 mM leupeptin, 1 mM pepstatin, 7 mM glutathione, and 0.2 mM phenylmethylsulphonyl fluoride. After centrifugation at 10 000 *g* for 20 min (4 °C), the supernatant was collected and recentrifuged at 100 000 *g* for 45 min. The supernatant was used for NOS determination. A reaction mixture (a total volume of 1 cm^3) in 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.0) contained 0.2 cm^3 of the enzyme extract, 1 mM arginine, 1 mM magnesium diacetate, 1 mM $CaCl_2$, 1 µM calmodulin, and 4 µM flavin adenine dinucleotide. The mixture was incubated at 37 °C for 30 min. The reaction was stopped by heat inactivation at 50 °C for 10 min. Activity of NOS was determined by subtracting the amount of methaemoglobin (metHb) produced in the presence of this inhibitor from the amount generated in its absence. The reaction was started by addition of NADPH (a final concentration of 100 µM) and tetrahydrobiopterin (a final concentration of 10 µM). A change in absorbance was recorded at 401 nm.

Concentration of Ca^{2+} in the cell sap was analyzed on a 1:100 (v/v) dilution using an inductively coupled plasma spectrophotometer (*Fisons Accuris*, *Fisons Insruments*, Beverly, USA). For analysis of Ca^{2+} concentration, leaves were frozen and pressed with a hydraulic press to collect cell sap. The cell sap was stored at -80 °C before analysis (Jiang and Huang 2001).

These experiments were in a completely randomized design. Each experiment was repeated three times. All values were expressed as means ± SDs (n = 3). Statistical analyses were performed by analysis of variance (*ANOVA*) using the *SPSS v. 17* statistical software (*SPSS Inc*., Chicago, IL, USA). The means were separated using Duncan's least significance difference test at $P \leq 0.05$.

Results

After 7 d under the HI, the plant height was remarkably influenced in both the cultivars. The plant height decreased by 11.28 and 25.84 % in Arid3 and Houndog5, respectively. The Ca^{2+} addition before the HI stress significantly increased the plant heights of Arid3 and Houndog5. To elucidate the role of Ca^{2+} in HI response, the calcium channel blocker $LaCl_3$ or the calcium chelator EGTA was applied alone, which had no effect on Arid3 and Houndog5 under the control conditions (data not shown). Under the HI, $LaCl_3$ or EGTA decreased the plant height of Arid3 more than the HI alone, but slightly affected Houndog5 (Fig. 1*A*). Similarly, the HI decreased the dry masses of Arid3 and Houndog5 by 34.8 and 61.27 %, respectively, compared to those of the control plants. The application of exogenous Ca^{2+} alleviated the adverse effects of the 7-d HI stress and enhanced the dry masses of the stressed plants, whereas the addition of $LaCl_3$ or EGTA decreased the dry masses, particularly in Arid3 (Fig. 1*B*).

Malondialdehyde content is usually used to measure

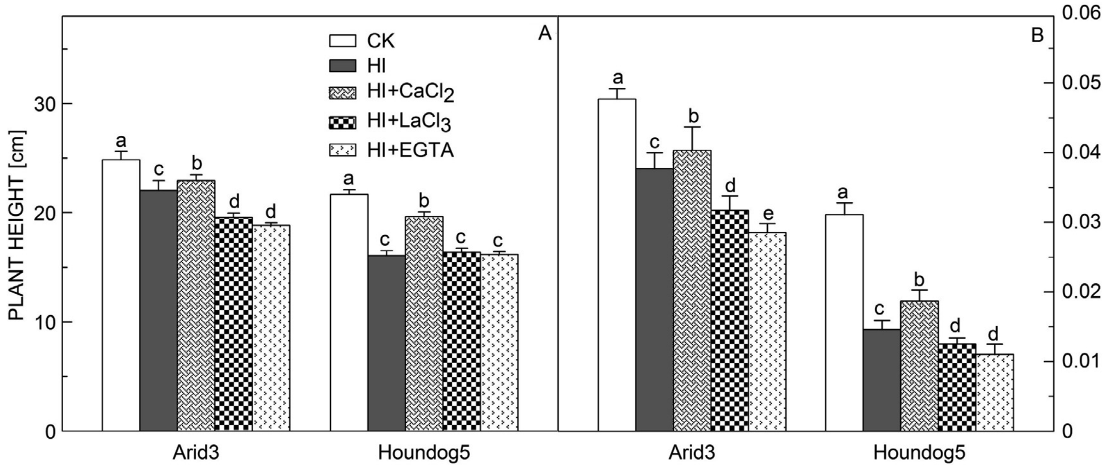

Fig. 1. The effects of calcium on plant height (*A*) and dry mass (*B*) in Arid3 and Houndog5 under a high irradiance (500 μmol m^{-2} s^{-1}) stress. The plants were grown under PPFD of 100 μmol m^{-2} s^{-1} (LI; CK) or 500 μmol m^{-2} s^{-1} (HI) and with the addition of 20 mM $CaCl_2$, 1 mM $LaCl_3$, or 2 mM EGTA. Means ± SDs, *n* = 3; bars with different letters are significantly different at 5 % level.

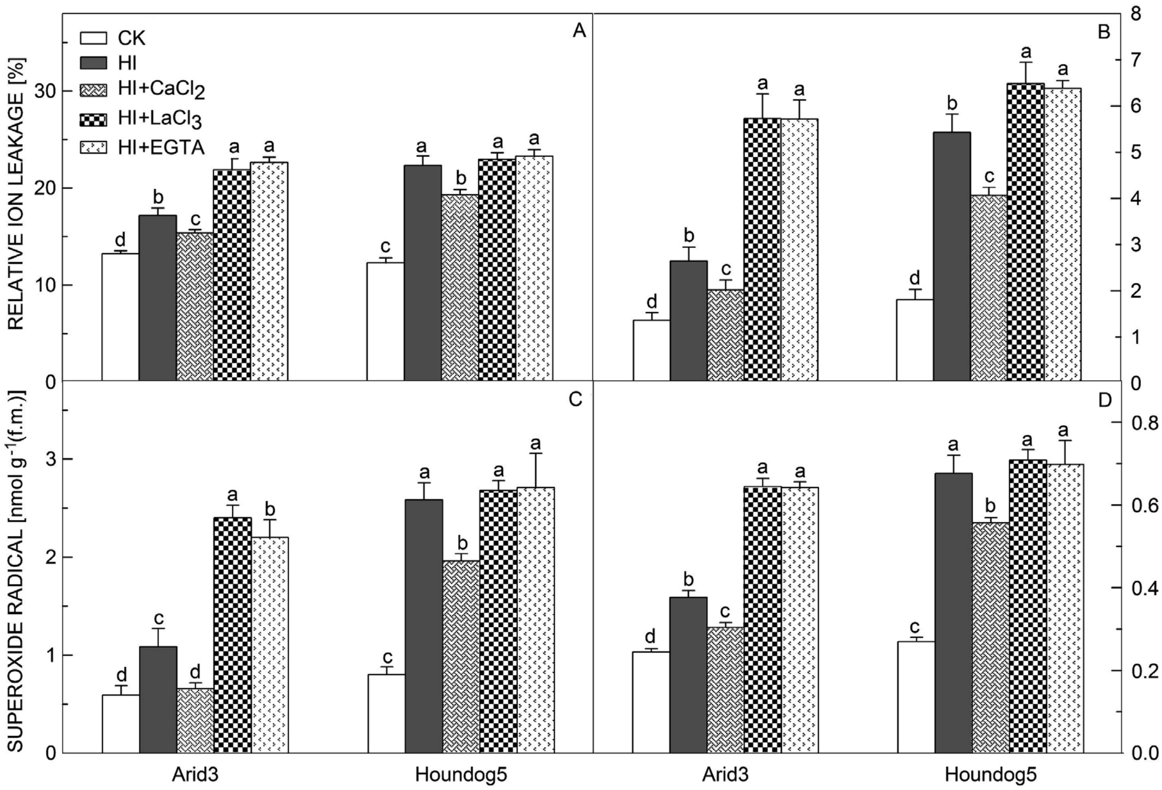

Fig. 2. The effects of calcium on ion leakage (*A*) and content of malondialdehyde (*B*), superoxide radical (*C*), and hydrogen peroxide (*D*) in leaves of Arid3 and Houndog5 under a high irradiance (500 μmol m^{-2} s^{-1}) stress. Other details as in Fig. 1.

the extent of lipid peroxidation resulting from oxidative stress, and ion leakage is indicator of disintegration of cell membranes. The HI stress significantly increased the MDA content along with an increase in the ion leakage of Arid3 and Houndog5 (Fig. 2*A*). The pretreatment of plant leaves with exogenous Ca^{2+} significantly decreased the MDA content and ion leakage compared to the HI alone in the leaves of Arid3 and Houndog5, whereas the supplementation with $LaCl_3$ or EGTA led to a significant increase in MDA content and ion leakage in the leaves of Arid3, but had a slight effect on Houndog5 (Fig. 2*B*). The HI stress also significantly increased the content of H_2O_2 and $O_2^{\cdot-}$ compared to the control plants. The exogenous Ca^{2+} reduced the production of H_2O_2 and $O_2^{\cdot-}$, whereas $LaCl_3$ or EGTA enhanced the H_2O_2 and $O_2^{\cdot-}$ content, particularly in the Arid3 leaves (Fig. 2*C*,*D*).

After 7 d of the HI treatment, the activities of SOD, CAT, APX, and GR increased significantly ($P \leq 0.05$) in Arid3, whereas only slightly in Houndog5. The pretreatment of the seedlings with the $CaCl_2$ solution further increased the SOD, CAT, APX, and GR activities compared to the HI alone, particularly in Houndog5. However, $LaCl_3$ or EGTA decreased the SOD, CAT, APX, and GR activities under the HI stress (Fig. 3).

After 7 d of the HI treatment, the NOS-like activity and NO generation increased significantly ($P \leq 0.05$) in Arid3, but slightly in Houndog5 (Fig. 4). The pretreatment of the seedlings with the $CaCl_2$ solution further enhanced the NOS-like activity and NO generation, whereas the use of $LaCl_3$ or EGTA had the opposite effect. However, in our preliminary experiment, $CaCl_2$ was used alone, which had no effect on NO production and NOS-like activity of tall fescue under the control conditions (data not shown). To further reveal the effect of NO in HI stress response, we used LNNA under the control conditions, and it had no effect on both the tall fescue cultivars (data not shown). The combination of SNP with LNNA, $LaCl_3$, or EGTA significantly decreased the NOS-like activity despite an increase in NO production from the NO donor (SNP) in the Arid3 leaves compared to those under the HI treatment alone. The PTIO or Ca + PTIO treatments enhanced the NOS-like activity, but reduced the NO production in the Arid3 leaves compared to that under the HI alone. The application of the NO scavenger PTIO only reduced the NO release, whereas the application of LNNA reduced the NOS-like activity and NO production. This indicates that the NOS-like activity was essential for NO synthesis, and NO release was inhibited when NOS-like activity was suppressed. This also suggests that NO might act as intermediate molecule mediating Ca^{2+}-induced tolerance to the HI stress.

To elucidate the key role of Ca^{2+} in response of Arid3 and Houndog5 to the HI stress, Ca^{2+} concentration in the cell sap was measured. After the HI treatment, the Ca^{2+} concentration significantly increased in Arid3, but only

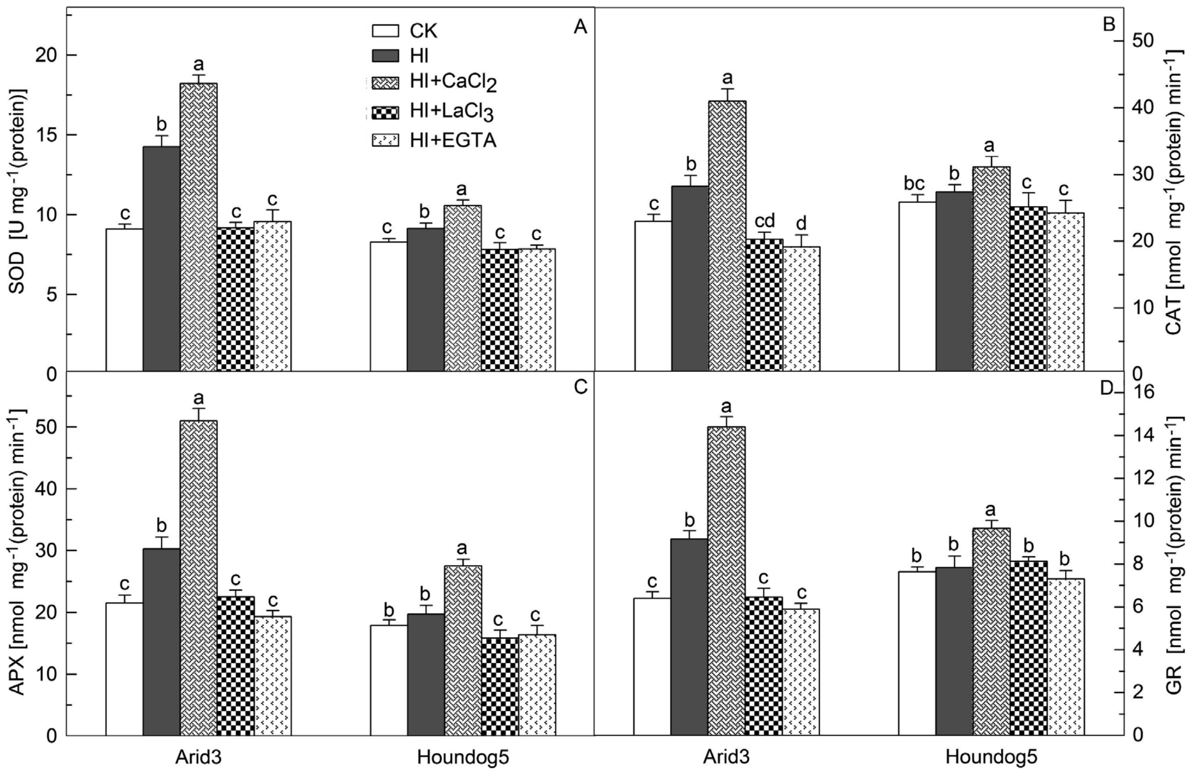

Fig. 3. The effects of calcium on activities of antioxidant enzymes in leaves of Arid3 and Houndog5 under a high irradiance (500 µmol m^{-2} s^{-1}) stress. SOD (*A*), CAT (*B*), APX (*C*), and GR (*D*). Other details as in Fig. 1.

slightly in Houndog5. The endogenous Ca^{2+} concentration in the Arid3 leaves was 3.95 times higher than in the Houndog5 leaves under the HI stress. The exogenous Ca^{2+} treatment increased the endogenous Ca^{2+} concentration in the cell sap in both the cultivars. Moreover, the combination of Ca^{2+} with either PTIO or LNNA remarkably increased the endogenous Ca^{2+} concentration, whereas the pretreatment with $LaCl_3$, EGTA, $LaCl_3$ + SNP, or EGTA + SNP significantly decreased the endogenous Ca^{2+} concentration (more pronounced in Arid3, Fig. 5).

Discussion

In the natural environment, plant chloroplasts often absorb more sun radiation than can be consumed by photosynthetic processes resulting in an increased ROS production (Asada 2006). This can cause lipid peroxidation and damage to cell membranes, thus ultimately inhibiting plant growth. In this study, the 7-d HI treatment led to an increased MDA content and ion leakage and resulted in a decreased plant height and dry mass in both the Arid3 and Houndog5 cultivars (Fig. 1). The exogenous Ca^{2+} application alleviated the HI. Our results are consistent with previous reports on different tall fescue cultivars (Jiang and Huang 2001). Additionally, the HI stress remarkably increased the endogenous Ca^{2+} content in the cell sap of Arid3, but had no significant effect on Houndog5 (Fig. 4*A*). Similar studies were also conducted on *Zea mays*, *Amaranthus lividus*, and *Triticum aestivum* (Gong *et al*. 1997, Bhattacharjee 2008, Al-Whaibi *et al*. 2012).

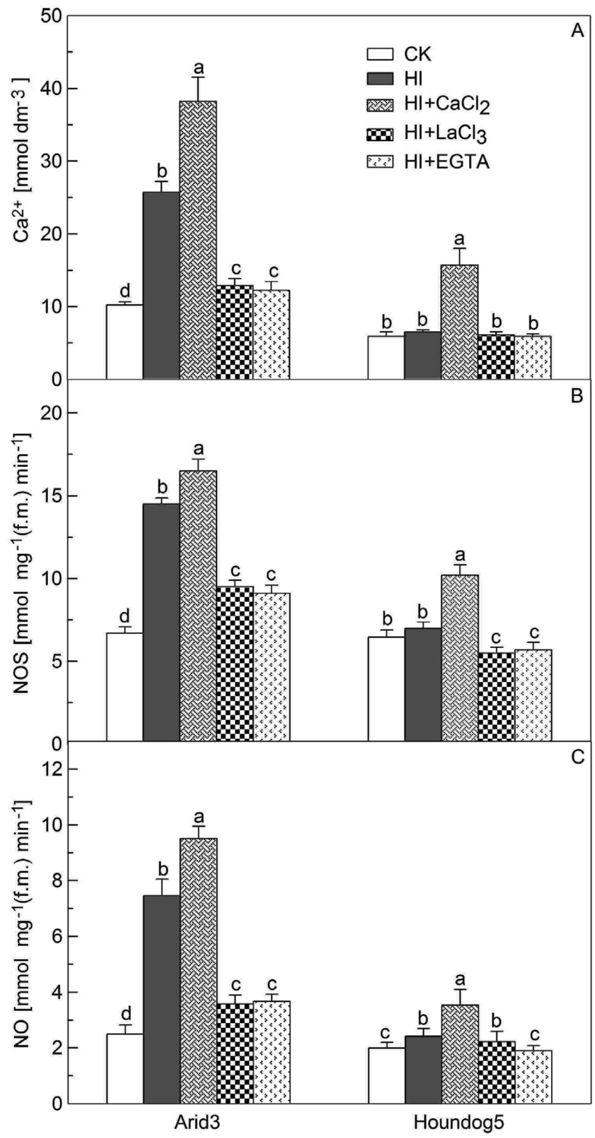

Fig. 4. The effects of calcium on cell sap Ca^{2+} concentration (*A*), NOS-like activity (*B*), and NO production (*C*) in leaves of Arid3 and Houndog5 under a high irradiance (500 μmol m^{-2} s^{-1}) stress. Other details as in Fig. 1.

Increased activities of antioxidant enzymes belong to important mechanisms of plant tolerance to an HI stress (Burritt and Mackenzie 2003, Ali *et al*. 2005). In this study, the two cultivars of tall fescue exhibited different increases in activities of antioxidant enzymes: higher increases in the activities of SOD, CAT, APX, and GR and a lower content of ROS were observed in Arid3 than in Houndog5 indicating that Arid3 alleviated oxidative injuries by increasing the antioxidant enzyme activities to counter ROS accumulation. These two cultivars also exhibited a distinct photoacclimation and ABA protected them against an HI-induced oxidative stress by promoting NO release by inducing NOS-like activity (Xu *et al*. 2013). Thus, NO may act as signaling molecule triggering the enhanced activities of antioxidant enzymes and further protecting against injuries caused by an HI (Xu *et al*. 2010).

A previous study have shown that the NO production pathways in plants include nonenzymatic (Neill *et al*. 2003) as well as enzymatic sources such as NOS (Guo *et al*. 2003), and nitrate reductase (Bright *et al*. 2006). In this study, the exogenous Ca^{2+}-induced antioxidant enzyme activity and Ca^{2+}-triggered NO generation were reversed in the presence of LNNA indicating that NOS-like activity might play a key role in the NO-mediated Ca^{2+}-induced antioxidant enzyme activity. This result is in agreement with a previous observation (Garcia-Mata and Lamattina 2007). The results indicate that Ca^{2+} induced NO production by activating a LNNA-sensitive NOS-like enzyme. These findings have been discussed in our earlier work in relation to the NOS-like enzyme in plants (Xu *et al.* 2010).

Calcium is one of the universal intracellular secondary messengers (Courtois *et al.* 2008). Notably, an increase in cytosolic Ca^{2+} concentration has been detected in response to heat and cold stresses (Jiang and Huang 2001, Shi *et al.* 2014). In this study, the Ca^{2+} concentration increased by 151.96 and 10.77 % in the cell sap of the Arid3 and Houndog5 leaves, respectively, under the HI stress compared to the control plants (Fig. 4*A*). Recent studies have demonstrated that Ca^{2+} and NO can counteract oxidative damage and display a protective effect against salt and UV-B stresses (Nasir Khan *et al.* 2012, Tossi *et al.* 2012). In this study, exogenous Ca^{2+} and SNP (NO donor) remarkably improved activities of SOD, CAT, APX, and GR, reduced H_2O_2 and $O_2^{\cdot-}$ accumulation, and inhibited lipid peroxidation and membrane damage similarly as in previous study (Xu *et al.* 2010). However, these effects were reversed when the Ca^{2+} channel blocker $LaCl_3$, Ca^{2+} chelator EGTA, or NO scavenger PTIO were applied (Fig. 5). This is in agreement with the postulated roles of Ca^{2+} and NO as signaling molecules involved in inducing an increase in antioxidant enzyme activities in enhancing tolerance to salt stress (Nasir Khan *et al.* 2012).

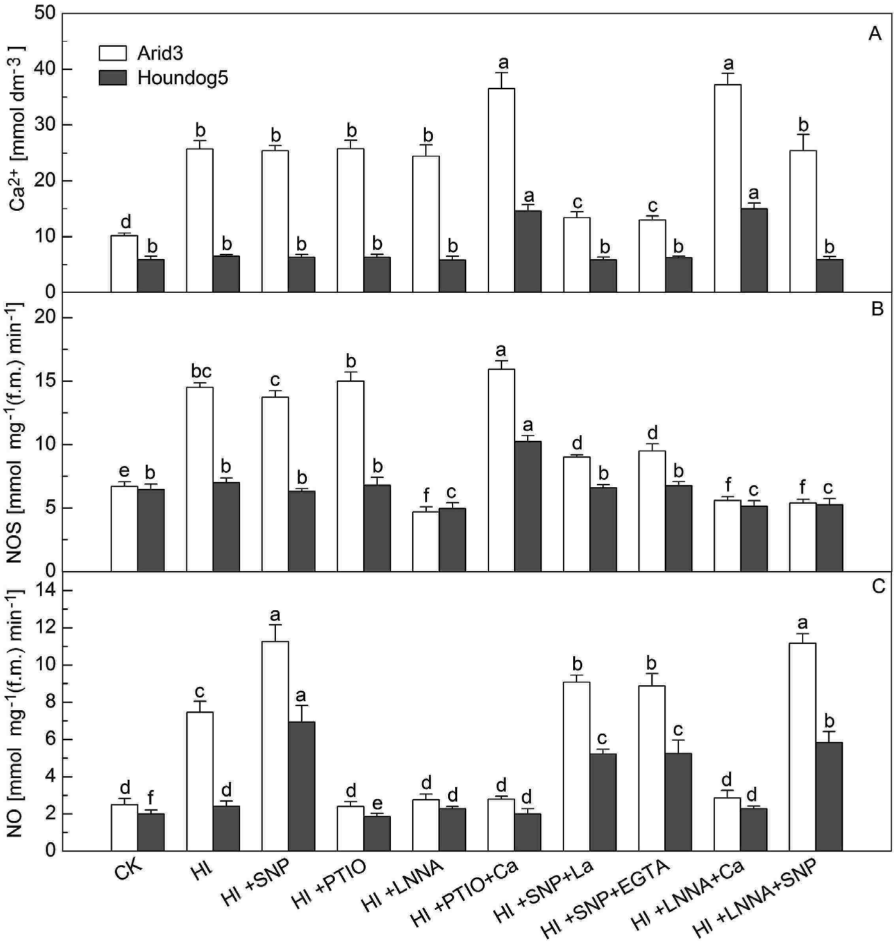

Fig. 5. Cell sap Ca^{2+} concentration (*A*), NOS-like activity (*B*), and NO production (*C*) in leaves of Arid3 and Houndog5 under a high irradiance stress. Plants were grown under PPFD of 100 µmol m^{-2} s^{-1} (CK) or 500 µmol m^{-2} s^{-1} (HI) and were treated with 100 µM SNP, 200 µM PTIO, 150 µM LNNA, 200 µM PTIO + 20 mM $CaCl_2$, 100 µM SNP + 1 mM $LaCl_3$, 100 µM SNP + 2 mM EGTA, 150 µM LNNA + 20 mM $CaCl_2$, and 150 µM LNNA + 100 µM SNP. Means ± SDs, *n* = 3; bars with different letters are significantly different at 5 % level.

Both Ca^{2+} and NO have been reported to regulate many physiological processes during plant growth and development, including regulation of stomatal movement (Courtois *et al.* 2008) and activities of antioxidative enzymes under abiotic stresses (Nasir Khan *et al.* 2012). In this study, PTIO or LNNA could reverse the protective effect of exogenous Ca^{2+} (Fig. 5). However, $LaCl_3$ and EGTA could not eliminate the protective effect of SNP indicating that the protective effect of Ca^{2+} under the HI stress might be mediated by NO. Interestingly, this study as well as one previous study (Garcia-Mata and Lamattina 2007) has shown that the pretreatment with exogenous Ca^{2+} significantly induced NOS-like activity and increased NO production. As an NOS-like enzyme has been implicated as possible source of ABA-induced

NO production in tall fescue under an HI (Xu *et al.* 2013), the dependence of NO production on the Ca^{2+} signal is consistent with the requirement of Ca^{2+} signal for NOS-like enzyme activation (Wu and Wu 2008). In this study, $LaCl_3$ and EGTA were used to reduce the NOS-like activity and NO release in the Arid3 leaves under the HI stress. Moreover, neither the exogenous SNP nor PTIO or LNNA treatments had any effect on Ca^{2+} concentration in the cell sap (Fig. 4), indicating Ca^{2+}-induced HI tolerance in Arid3 by promoting NO release due to inducing NOS-like activity. Moreover, the exogenous Ca^{2+} treatment significantly increased the NOS-like activity and NO generation in the Houndog5 leaves, whereas the SNP treatment had no effect on Ca^{2+} concentration. This suggests that NO might operate downstream of Ca^{2+} and mediate the protective effect of Ca^{2+} under the HI stress. In other words, Ca^{2+} might act as signal in induction of endogenous NO synthesis under the HI stress. Our result is in agreement with a previous study (Garcia-Mata and Lamattina 2007), but there is also evidence that Ca^{2+} can act upstream of NO production in plants (Lanteri *et al.* 2006).

Based on the results and above discussion, crosstalk between NO and Ca^{2+} forms an intricate network and participates in the regulation of diverse biotic and abiotic stresses in plant cells. Similarly, other signal molecules such as ABA, salicylic acid, and protein kinases activate plant responses to environment stresses (Jiang and Zhang 2003, Wu and Wu 2008). However, many questions remain unanswered including an interrelationship among them and signaling pathways under an HI stress. Thus, further research on crosstalk among all signaling molecules in response to an HI stress in plants will continue in the future.

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