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Involvement of nitric oxide in 5-aminolevulinic acid-induced antioxidant defense in roots of *Elymus nutans* exposed to cold stress

J.J. FU, X.T. CHU, Y.F. SUN, Y.F. XU*, and T.M. HU*

Department of Grassland Science, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi, 712100, P.R. China

Abstract

Nitric oxide (NO) and 5-aminolevulinic acid (5ALA) play fundamental roles in plant responses to environmental stresses, but their cross-talk in antioxidant defense in cold-stressed *Elymus nutans* Griseb. have not been investigated. We herein report that 5ALA and NO donor, sodium nitroprusside (SNP), alleviated cold stress-induced plant growth inhibition and lipid peroxidation in roots of two *E. nutans* ecotypes (Damxung, DX and Zhengdao, ZD). However, application of an NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (PTIO) differentially blocked these protective effects indicating that an inhibition of NO accumulation reduced 5ALA-enhanced cold resistance. Application of exogenous 5ALA or NO markedly up-regulated activities of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase, enhanced reduced glutathione accumulation and reduced glutathione to oxidized glutathione ratio, activated plasma membrane (PM) H^+-ATPase, and reduced Na^+/K^+ ratio in roots of the two *E. nutans* ecotypes. Moreover, in the presence of 5ALA, nitric oxide synthase (NOS) activity and NO release in cold-resistant DX were higher than those in cold-sensitive ZD. Conversely, both NO treatment and inhibition of endogenous NO accumulation by PTIO or NOS inhibitor N^{ω}-nitro-L-arginine did not induce 5ALA production. These results suggest that NO might be acting as a downstream signal to mediate 5ALA-induced cold resistance by activating antioxidant defense and PM H^+-ATPase and maintaining Na^+ and K^+ homeostasis.

Additional key words: lipid peroxidation, Na^+/K^+ homeostasis, plasma membrane H^+-ATPase activity, PTIO.

Introduction

Cold stress is one of the major abiotic factors that limit plant growth, development, survival, and productivity (Yu *et al.* 2003). Exposure to a low temperature may disrupt a balance between antioxidants and reactive oxygen species (ROS) resulting in oxidative stress. To survive such a stress, plants have evolved various antioxidant defense mechanisms responding to cold stress (Mittler 2002). These antioxidants include enzymes, such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR); water-soluble compounds, such as reduced ascorbate (AsA), redued glutathione (GSH), and flavonoids; and lipid soluble compounds, such as carotenoids and tocopherols (Kang and Saltveit 2002, Erdal and Dumlupinar 2011). It has been proved that cold stress may cause many physiological, biochemical, and structural disorders like a decline in chlorophyll content, photosynthetic rate, production of

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Abbreviations: 5ALA - 5-aminolevulinic acid; APX - ascorbate peroxidase; AsA - reduced ascorbate; CAT - catalase; CK - control; DHA - dehydroascorbate; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; H_2O_2 - hydrogen peroxide; L-NNA - N^{ω}-nitro-L-arginine; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; NADPH - nicotinamide adenine dinucleotide phosphate; NO - nitric oxide; NR - nitrate reductase; NOS - nitric oxide synthase; PGR - plant growth regulator; PM - plasma membrane; PTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; ROS - reactive oxygen species; RNS - reactive nitrogen species; SNP - sodium nitroprusside; SOD - superoxide dismutase; TCA - trichloroacetic acid; Tu - tungstate.

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* Corresponding author; fax: (+86) 29 87092355, e-mail: xuyuefei1980@163.com; hutianming@126.com

biomass, and uptake of essential elements, directly or indirectly, through oxidative damage (Wang *et al.* 2004, Mishra *et al.* 2011).

Nowadays, the use of plant growth regulators (PGRs) has become a promising approach to increase plant tolerance against abiotic stresses (Ali *et al.* 2013, 2014). Five-aminolevulinic acid (5ALA) is an important PGR involved in regulation of growth, development, and various physiological responses (Akram and Ashraf 2013). Accumulating evidences indicate that 5ALA plays critical roles in inducing plant resistance to various abiotic and biotic stresses including cold stress (Zhang *et al.* 2008, Sun *et al.* 2009, Balestrasse *et al.* 2010, Korkmaz *et al.* 2010, Naeem *et al.* 2011, Zhang *et al.* 2012, Ali *et al.* 2013). Application of 5ALA has been shown to increase the resistance to cold stress in diverse plant species such as rice (Hotta *et al.* 1998), melon (Wang *et al.* 2004), watermelon (Sun *et al.* 2009), soybean (Balestrasse *et al.* 2010), and pepper (Korkmaz *et al.* 2010). Recently, it has also been observed that 5ALA ameliorates mineral uptake in *Brassica napus* under salinity and lead excess (Naeem *et al.* 2010, Ali *et al.* 2014). A significant decrease in malondialdehyde (MDA) content and an increase in activities of antioxidant enzymes have also been reported in 5ALA-treated oilseed rape under Pb contamination (Ali *et al.* 2014). However, mechanisms underlying 5ALA-enhanced stress resistance of plants remain to be determined.

Nitric oxide (NO), a reactive nitrogen species (RNS), is a gaseous signal molecule involved in various physiological processes in plants, including plant growth, development, and responses to abiotic stresses such as UV-B, salinity, drought, high irradiance, extreme temperature, and heavy metals (Arasimowicz-Jelonek *et al.* 2009, Santa-Cruz *et al.* 2010, Xu *et al.* 2010, Khan *et al.* 2012, Kong *et al.* 2012, Ma *et al.* 2012, Gill *et al.* 2013). Nitric oxide may protect plants against oxidative stress by acting as antioxidant directly scavenging ROS generated under stress conditions or operating as a signal molecule in the cascade of events leading to gene expression (Wendehenne *et al.* 2001). Accumulating evidence has shown that NO serves as signal in developmental, hormonal, and environmental responses in plants (Zhang *et al.* 2007, 2011). However, whether NO is involved in 5ALA-induced antioxidant defense is still poorly understood.

In the present investigation, experiments were conducted to elucidate the roles of NO in 5ALA-induced oxidative stress resistance in *Elymus nutans* roots. First of all, the effects of 5ALA on oxidative stress resistance were investigated. Subsequently, pretreatment with an NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassium salt (PTIO) and an nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NNA) in the roots of *E. nutans* exposed to a cold stress helped us to determine whether NO is involved in 5ALA-induced cold resistance.

Materials and methods

Plants, growth conditions, and treatments: *Elymus nutans* Griseb. seeds were obtained from two sources: the seeds of Damxung (DX) were collected in September 2012 from wild plants growing in Damxung County (30° 28′ N, 91° 6′ E, and an altitude of 4 678 m), located in the middle of Tibet, China. The Zhengdao (ZD) seeds were obtained in September 2012, from *Beijing Rytway Ecotechnology Co*., located in Changping District (40° 6′ N, 116° 24′ E, and an altitude of 550 m), Beijing, China. The seeds were cleaned and stored at 4 °C in paper bags until the start of the experiments. In the previous study, ZD was found to be more susceptible to cold stress than DX (Fu *et al.* 2014).

The seeds were surface sterilized in 0.1 % (m/v) sodium hypochlorite, rinsed several times in distilled water, and germinated on moistened filter paper for 7 d. The morphologically uniform seedlings were cultured in silica sand irrigated with Hoagland's nutrient solution once every 5 d. The plants were germinated and grown in a growth chamber at day/night temperatures of 25/20 °C, a relative humidity of 70 %, a 12-h photoperiod, and a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1} provided by a fluorescent lamp (*Philips Electronics N.V. Holland,* Nanjing, China). After four weeks, the plants were treated for 12 h with the nutrient solution (control) or the nutrient solution with 100 μM sodium nitroprusside (SNP), 200 μM PTIO, 150 μM L-NNA, 100 μM tungstate (Tu), and different concentrations of 5ALA (0, 0.5, 1, 5, 10, 25 mg dm^{-3}) according to the doses described by Xu *et al.* (2010) and Fu *et al.* (2014). Afterwards, the plants were subjected to a cold stress at 5 °C for 5 d under the same conditions as indicated above (Aroca *et al.* 2001). The plants not exposed to the cold stress and grown in the growth chamber at 25 °C were used as control. Each treatment was replicated three times. After 5 d of the cold treatment, root samples for morphological, biochemical, and physiological studies were taken according to procedure described below.

For measurement of plant biomass, five plants per treatment were weighed immediately after being harvested and then placed into an oven at 80 °C until it exhibited a constant mass. Root surface area, root volume, root diameter, and number of root tips of selected plants were determined using a root automatic scan apparatus (*Perfection V700 Photo*, Epson, Japan), equipped with the *WinRHIZO* software offered by *Regent Instruments* (Quebec, Canada). Average values of five plants were considered as one replicate.

Determination of electrolyte leakage, lipid peroxidation, and ROS accumulation: Electrolyte leakage was determined according to the procedure reported by Song *et al.* (2006) with some modifications. Fresh roots (0.5 g) were washed with deionized water and placed in Petri dishes containing 5 cm^3 of deionized water at 25 °C for 2 h. After the incubation, electrical conductivity (EC_1) was measured using a conductivity meter (*INESA Scientific Instruments*, Shanghai, China). Then the samples were boiled for 20 min and electrical conductivity was read again (EC_2). The electrolyte leakage was calculated as EC_1/EC_2.

Membrane lipid peroxidation was measured as content of MDA produced using 10 % (m/v) trichloroacetic acid (TCA) according to the procedure reported by Dhindsa *et al.* (1981). The absorbances of the supernatant were measured at 450, 532, and 600 nm by a spectrophotometer *V1000* (*AOE Instruments,* Shanghai, China).

Content of H_2O_2 was determined by monitoring the absorbance of a titanium-peroxide complex at 415 nm according to the method described by Shi *et al.* (2013). Superoxide radical production rate was determined with a plant $O_2^{\bullet-}$ ELISA kit (*DG*, Beijing, China) based on an antibody-antigen-enzyme-antibody complex following the manufacturer's instructions (Shi *et al.* 2013).

Measurements of antioxidants: Reduced glutathione and oxidized glutathione (GSSG) content were determined according to the method reported by Law *et al.* (1983) with some modifications. Roots (0.3 g) were homogenized with 5 cm^3 of 10 % (m/v) TCA and the homogenate was centrifuged at 15 000 *g* for 15 min. To assay total glutathione, 0.15 cm^3 of the supernatant was added to 0.1 cm^3 of 6 mM 5,5′-dithio-2-nitrobenzoic acid, 0.05 cm^3 of GR, and 0.7 cm^3 of 0.3 mM nicotinamide adenine dinucleotide phosphate (NADPH). To measure GSSG, 0.12 cm^3 of the supernatant was added to 0.01 cm^3 of 2-vinylpyridine followed by 0.02 cm^3 of 50 % (v/v) triethanolamine. The solution was vortex-mixed for 30 s and incubated at 25 °C for 25 min. A calibration curve was developed by using GSSG samples treated exactly as above and GSH content was determined by subtracting GSSG from the total glutathione content.

After 0.2 g of roots was suspended in 3 cm^3 of 6 % TCA and centrifuged at 4 °C and 15 000 *g* for 20 min, content of AsA and total ascorbate were determined at 525 nm (Kampfenkel *et al.* 1995). The difference between the content of total ascorbate and AsA was used for estimating the extent of ascorbate oxidation.

Roots (0.5 g) were homogenized with a mortar and pestle at 4 °C in 5 cm^3 of a 50 mM phosphate buffer (pH 7.8) containing 1 mM Na_2EDTA and 2 % (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12 000 *g* and 4 ° C for 20 min and the supernatant was used for enzyme activity assays. Protein content in the supernatant was determined according to the method described by Bradford (1976) with bovine serum albumin as standard.

Activity of APX was measured following the method of Nakano and Asada (1981) by recording the rate of ascorbate oxidation at 290 nm. One unit of APX was defined as 1 μmol(ascorbate oxidized) cm^{-3} min^{-1}. Activity of CAT was measured by following the consumption of H_2O_2 at 240 nm according to the procedure reported by Cakmak and Marschner (1992). One unit of CAT activity was defined as 1 μmol(H_2O_2 decomposed) cm^{-3} min^{-1}. Activity of GR was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation as described by Shaedle and Bassham (1977). One unit of GR was defined as 1 μmol(GSSG reduced) cm^{-3} min^{-1}. Activity of SOD was determined according to the method described by Beauchamp and Fridovich (1971) by following the photoreduction of nitroblue tetrazolium (NBT) at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause a 50 % inhibition of NBT reduction.

Determination of plasma membrane H^+-ATPase activity and Na^+ and K^+ content: Plasma membrane (PM) vesicles were isolated from fresh roots by phase partitioning according to the procedure described by Palmgren *et al.* (1990). Plasma membrane H^+-ATPase activity was measured according to the procedure reported by Ahn *et al.* (1999).

A root sample (1 g) was dry-ashed, extracted with 1:1 HNO_3:$HClO_4$ and finally filtered. Content of Na^+ and K^+ were determined by flame atomic absorption spectrometry (*Model 410*, Sherwood, England).

Measurement of NO production, NOS activity, and 5ALA content: Endogenous NO in plant leaves was quantified using hemoglobin assay by determining the conversion of oxyhemoglobin to methemoglobin spectrophotometrically as previously described by Shi *et al.* (2012). Activity of NOS was determined according to the method reported by Murphy and Noack (1994).

Roots (0.1 g) were homogenized in 2 cm^3 of a 1 M sodium acetate buffer (pH 4.6) and centrifuged at 12 000 *g* for 10 min. The assay mixture consisted of 0.1 cm^3 of the supernatant, 0.4 cm^3 of distilled water, and 0.025 cm^3 of acetylacetone. The absorbance of the extract was measured at 555 nm and 5ALA content was determined from a standard curve of 5ALA (Harel and Klein 1972).

Statistical anaysis: Each experiment was repeated at least three times. All values were expressed as means ± SDs. Statistics was performed by analysis of variance (*ANOVA*) using the *SPSS-17* statistical software (*SPSS Inc*., Chicago, IL, USA). The means were separated using Duncan's least significance difference test at $\alpha = 0.05$.

Results

The cold stress resulted in a considerable inhibition of biomass and root growth of the two ecotypes of *E. nutans* (Table 1). The growth of the DX and ZD seedlings was significantly improved after 5 d of the 5ALA treatment as compared to the cold-stressed seedlings. Dry matter accumulation in roots was notably greater than in shoots under the 1 mg dm^{-3} 5ALA pretreatment, resulting in 15 and 12 % increases of root-to-shoot ratio in DX and ZD roots compared to non-treated roots under the cold stress (Table 1). Root length, root surface area, root diameter, root volume, and root tips of the DX seedlings were enhanced ($P < 0.05$) after application of 5ALA under the cold stress as compared to the seedlings treated with the cold stress only. Roots of the ZD seedlings showed much smaller changes. A similar alleviation of plant growth was also observed in SNP-treated roots, whereas the effects were completely blocked by application of the NO specific scavenger PTIO (Table 1). As expected, the pretreatment with the NO scavenger PTIO blocked the 5ALA-induced increases in plant growth of *E. nutans*. Moreover, the 5ALA-induced enhancement in biomass and root growth occurred in a dose-dependent manner in the concentration range of 0.5 - 25 mg dm^{-3} 5ALA (data not shown), with 1 mg dm^{-3} 5ALA being the most effective. Thus, 1 mg dm^{-3} 5ALA was used in the further experiments.

Table 1. Effects of 5ALA and NO on dry matter of roots and shoots [g $plant^{-1}$], length [cm $plant^{-1}$], surface area [cm^2 $plant^{-1}$], diameter [mm], volume [cm^3 $plant^{-1}$], and tips [tips $plant^{-1}$] of roots of *Elymus nutans*. CK - control, 25 °C, C - cold, 5 °C, C+A - cold + 1 mg dm^{-3} 5ALA, C+S - cold + 100 μM SNP, C+A+P - cold + 1 mg dm^{-3} 5ALA + 200 μM PTIO). Data are *m*eans ± SDs ($n = 5$). Different letters in rows indicate significant differences ($P < 0.05$) between treatments according to Duncan's multiple range test.

Ecotypes	Treatments	CK	C	C+A	C+S	C+P	C+A+P
DX	root	0.0079^{a}	0.0061^{b}	0.0076^{a}	0.0072^{a}	0.0047^{c}	0.0052^{c}
	shoot	0.0155^{a}	0.0138^{b}	0.0150^{a}	0.0147^{ab}	0.0102^{d}	0.0122^{c}
	root:shoot	0.5097^{a}	0.4420^{c}	0.5067^{a}	0.4898^{ab}	0.4608^{b}	0.4812^{b}
	length	458.0132^{a}	427.2155^{b}	448.3024^{a}	446.2137^{a}	375.9243^{c}	415.2365^{b}
	surface area	126.0021^{a}	103.1152^{c}	123.4582^{a}	122.6654^{a}	92.1268^{d}	115.6324^{b}
	diameter	0.8036^{a}	0.6547^{c}	0.8326^{a}	0.8259^{a}	0.5423^{d}	0.7235^{b}
	volume	2.5675^{a}	2.0124^{b}	2.4684^{a}	2.3896^{a}	1.6743^{c}	2.1453^{b}
	tips	745^{a}	684^{c}	752^{a}	749^{a}	615^{d}	723^{b}
ZD	root	0.0103^{a}	0.0076^{c}	0.0093^{ab}	0.0087^{b}	0.0052^{e}	0.0068^{d}
	shoot	0.0214^{a}	0.0178^{c}	0.0195^{b}	0.0201^{ab}	0.0153^{d}	0.0182^{c}
	root:shoot	0.4813^{a}	0.4270^{b}	0.4769^{a}	0.4328^{b}	0.3398^{c}	0.3956^{c}
	length	518.5345^{a}	447.1564^{c}	491.3358^{b}	458.4217^{c}	406.2266^{e}	424.5421^{d}
	surface area	145.2623^{a}	106.6136^{b}	140.8896^{a}	118.2143^{b}	90.0123^{c}	95.8954^{c}
	diameter	1.0358^{a}	0.7245^{b}	1.0312^{a}	0.7756^{b}	0.6002^{c}	0.6523^{c}
	volume	2.8654^{a}	2.0026^{b}	2.7065^{a}	2.1689^{b}	1.7542^{c}	1.689^{d}
	tips	842^{a}	713^{b}	834^{a}	739^{b}	643^{d}	668^{c}

The cold stress led to enhancements in electrolyte leakage, MDA content, H_2O_2 and $O_2^{\cdot-}$ accumulation in roots of DX and ZD (Fig. 1). Exogenous 5ALA or SNP significantly alleviated cellular membrane damage induced by the cold stress in roots of both the *E. nutans* ecotypes. However, the 5ALA-induced reductions of lipid peroxidation and ROS content were inhibited when PTIO was added. Following the application of 200 μM PTIO, lipid peroxidation and ROS accumulation markedly increased in roots of both the cold-treated *E. nutans* ecotypes but had no effect on membrane lipid peroxidation and ROS accumulation in control roots (data not shown).

Exogenous 5ALA and SNP triggered ($P < 0.05$) increases in activities of antioxidant enzymes including SOD, CAT, APX, and GR, whereas the PTIO treatment decreased the enhancements of these enzymes (Fig. 2). Similar enhancements of GSH, total glutathione content, and GSH/GSSG ratio were also observed in 5ALA or SNP-treated roots of DX (Fig. 3). However, the application of 5ALA or SNP had little effect on AsA content and AsA/dehydroascorbate (DHA) ratio in roots of both the ecotypes (data not shown). The combination of the 5ALA and PTIO treatments displayed no changes in activities of these antioxidant enzymes in comparison with control roots (data not shown).

Activity of PM H^+-ATPase increased ($P < 0.05$) in roots of the two cold stress-treated *E. nutans* ecotypes compared to control roots, especially in DX roots (Fig. 4*A*). The addition of exogenous 5ALA or NO further improved the cold-induced increase in activity of H^+-ATPase. However, the 5ALA-induced increase in H^+-ATPase activity in cold-stressed roots could be abated by the NO scavenger PTIO. The pretreatment with PTIO

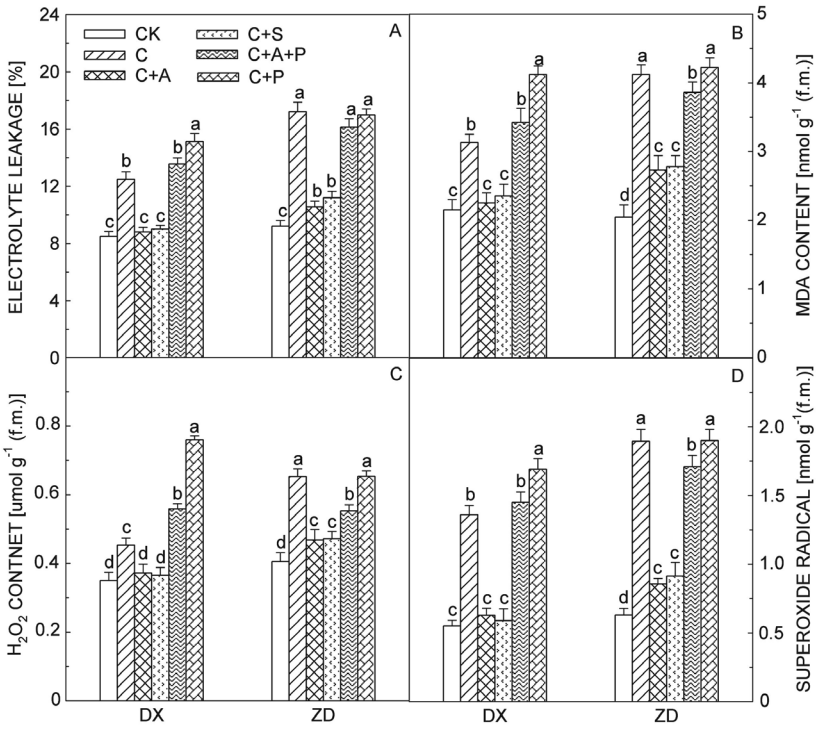

Fig. 1. Effects of 5ALA, NO, and PTIO on electrolyte leakage (*A*), MDA content (*B*), H_2O_2 content (*C*), and superoxide radical accumulation (*D*) in roots of DX and ZD ecotypes of *Elymus nutans* exposed to a cold stress of 5 ºC. CK - control, 25 °C, C - cold, 5 °C, C+A - cold + 1 mg dm^{-3} 5ALA, C+S - cold + 100 μM SNP, C+A+P - cold + 1 mg dm^{-3} 5ALA + 200 μM PTIO, C+P - cold + 200 μM PTIO. Means ± SDs, $n = 3$. Different letters indicate significant differences at the 0.05 level according to Duncan's multiple range test.

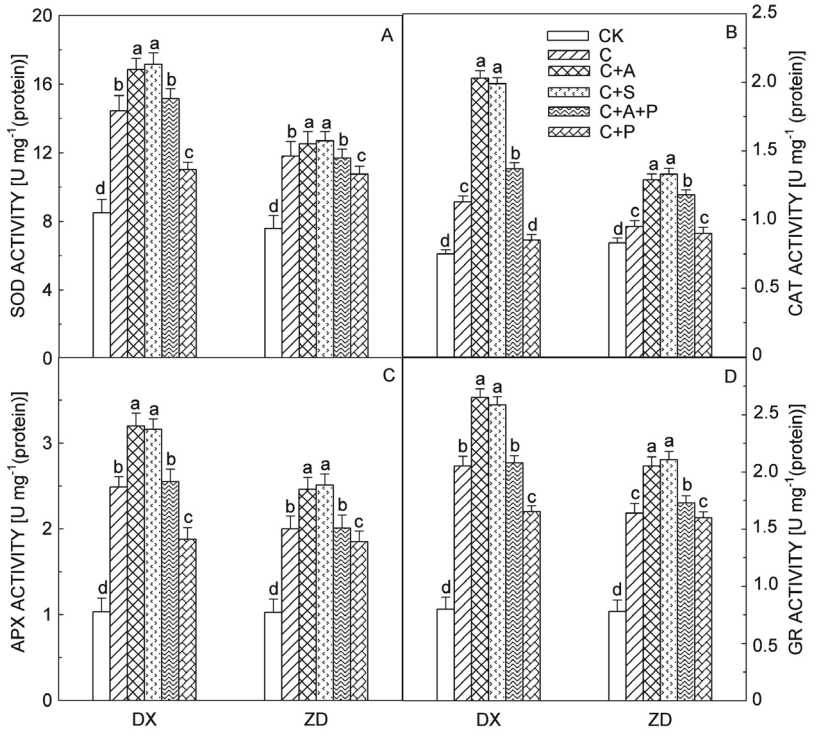

Fig. 2. Effects of 5ALA, NO, and PTIO on activities of antioxidant enzymes SOD (*A*), CAT (*B*), APX (*C*), and GR (*D*) in roots of DX and ZD ecotypes of *Elymus nutans* exposed to a cold stress of 5 ºC. For treatment abbreviations see Fig. 1. Means ± SDs, $n = 3$. Different letters indicate significant differences at the 0.05 level according to Duncan's multiple range test.

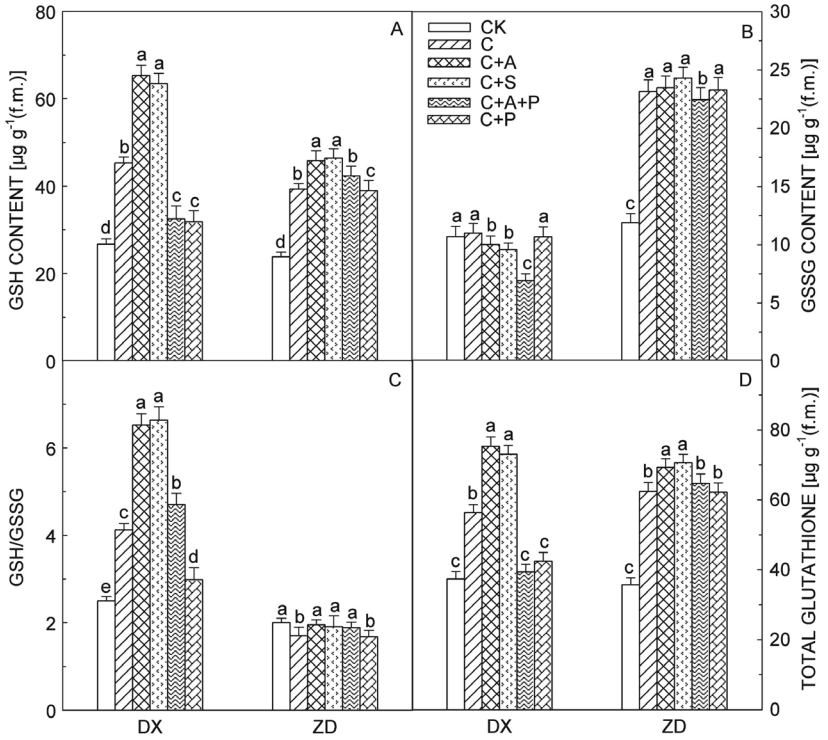

Fig. 3. Effects of 5ALA, NO, and PTIO on GSH content (*A*), GSSG content (*B*), ratio of GSH/GSSG (*C*), and total glutathione content (*D*) in roots of DX and ZD ecotypes of *Elymus nutans* exposed to a cold stress of 5 ºC. For treatment abbreviations see Fig. 1. Means ± SDs, $n = 3$. Different letters indicate significant differences at the 0.05 level according to Duncan's multiple range test.

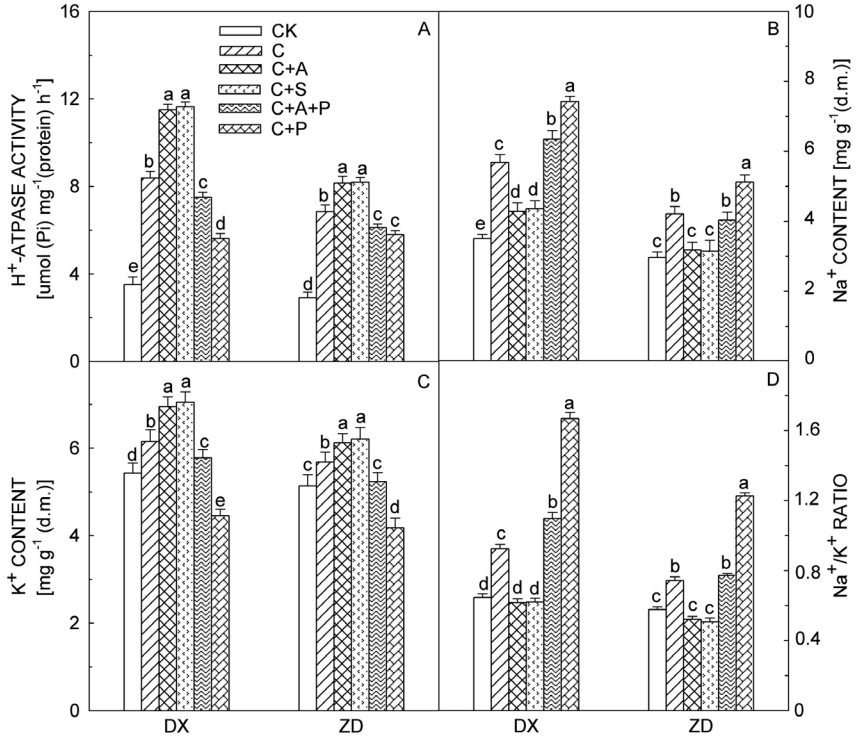

Fig. 4. Effects of 5ALA, NO, and PTIO on activity of H^+-ATPase (*A*), Na^+ content (*B*), K^+ content (*C*), and Na^+/K^+ ratio (*D*) in roots of DX and ZD ecotypes of *Elymus nutans* exposed to a cold stress of 5 ºC. For treatment abbreviations see Fig. 1. Means ± SDs, $n = 3$. Different letters indicate significant differences at the 0.05 level according to Duncan's multiple range test.

alone did not affect PM H^+-ATPase activity in both the ecotypes under the control conditions (data not shown).

The cold increased Na^+ uptake and Na^+/K^+ ratio in roots of DX and ZD (Fig. 4*B-D*). The application of 5ALA or SNP substantially reduced Na^+ uptake and Na^+/K^+ ratio and enhanced K^+ uptake in roots of both the ecotypes, especially in DX roots. The pretreatment with PTIO blocked the 5ALA-induced reduction of Na^+ uptake and Na^+/K^+ ratio in cold-stressed roots, whereas PTIO alone had little effect on Na^+ and K^+ accumulation under the control conditions (data not shown).

Endogenous NO generation and NOS activity were enhanced ($P < 0.05$) in cold-treated DX roots, but had no significant change in ZD roots (Fig. 5*A,B*). The pretreatment with the NO scavenger PTIO or NOS enzyme inhibitor L-NNA inhibited accumulation of NO during the cold stress. In contrast, NO generation during the cold stress was insensitive to the nitrate reductase (NR) enzyme inhibitor Tu and thus, NO accumulation was significantly greater. The exogenous 5ALA pretreatment significantly increased endogenous 5ALA content in roots, and both the 1 mg dm^{-3} 5ALA pretreatment and the 100 μM SNP pretreatment significantly increased NO content. The 200 μM PTIO or NOS inhibitor L-NNA but not the NR inhibitor Tu significantly reduced NO content and also significantly lowered 5ALA-induced NO production. Interestingly, the SNP treatment did not increase 5ALA content in roots of DX and ZD. The pretreatments with PTIO, L-NNA, and Tu in the presence of 5ALA also failed to affect 5ALA production in roots of the two *E. nutans* ecotypes (Fig. 5*C*).

Discussion

Previous studies have shown that cold stress-induced oxidative stress lead to lipid peroxidation, cellular membrane disruption, and growth inhibition (Mittler 2002, Tartoura and Youssef 2011). In this study, we found that the cold stress resulted in a more severe oxidative damage and growth suppression in ZD than in DX indicating that the DX ecotype is relatively more cold-resistant in comparison with ZD (Table 1, Fig. 1).

The regulatory roles of exogenous 5ALA and NO in plant development and environmental stress response has been studied extensively (Vital *et al.* 2008, Zhao *et al.* 2008, Zhang *et al.* 2009a,b, Akram and Ashraf 2013). In this study, we found that the ameliorating effects of 5ALA and NO on the plants suffering from the cold stress have been attributed to the up-regulated activities of APX, CAT, GR, and SOD; and to accumulation of AsA and GSH, thus resulting in the protection of the plants against oxidative damage (Figs. 1 - 3). These responses are in agreement with the findings of Korkmaz *et al.* (2010) and Bai *et al.* (2012). These results suggest that exogenous 5ALA and NO can elevate cold resistance in roots of *E. nutans* by enhancing the antioxidant defense system resulting in a reduced ROS accumulation alleviating oxidative injury.

Cold-induced NO accumulation alleviating cold injury by enhancing the antioxidant defense systems in the loquat fruit has been demonstrated (Xu *et al.* 2012). Similarly, an increased NO production was observed in roots of DX and ZD (Fig. 5*A*). In plants, NO synthesis from L-arginine by an NOS-like enzyme or from nitrite by NR has been reported previously (He *et al.* 2004). Here, we found that the pretreatment with the NOS inhibitor L-NNA, in contrast to the NR inhibitor Tu, reduced cold-induced NO accumulation in roots of DX, which indicates that NOS probably contributed to cold-induced NO synthesis (Fig. 5). Similar results have been reported in pea plants in response to a low temperature stress (Corpas *et al.* 2008). In contrast, Ryter *et al.* (2002) detected NR-activity-dependent NO synthesis in *Arabidopsis* under a low temperature. The discrepancy regarding the NO synthesis between these two studies could be attributed to the difference in the experimental materials used. Based on these results, we propose that NOS-dependent NO production is mechanism for protection of *E. nutans* from damage induced by cold stress. These findings agree with a previous study in reed and tall fescue demonstrating that an up-regulation of NOS associated with exogenous NO application results in an increased resistance to heat stress and high irradiance (Song *et al.* 2008, Xu *et al.* 2016).

A reduction of Na^+/K^+ ratio has been demonstrated to be involved in an enhanced cold resistance in wheat seedlings (Mishra *et al.* 2011). Cytosolic Na^+/K^+ ratio depends on the ability of plants to extrude Na^+ either outside the cell or into the vacuole by Na^+/H^+ antiport. The potassium cation plays a major role in maintaining pressure potential within the cell (Ma *et al.* 2012). The present result shows that the cold stress induced a higher Na^+ accumulation and Na^+/K^+ ratio in ZD than in DX. The pretreatment with 5ALA and SNP decreased Na^+ accumulation and Na^+/K^+ ratio, and this effect was reversed by PTIO (Fig. 4), which supports the findings of several previous studies in which 5ALA treatments reduce Na^+ uptake and Na^+/K^+ ratio in oilseed rape exposed to salt and lead stresses (Naeem *et al.* 2010, 2012, Ali *et al.* 2014). Similarly, Crawford (2006) showed that the effects of NO on salinity resistance are also related to regulation of PM H^+-ATPase and Na^+/K^+ ratio. Nitric oxide stimulates H^+-ATPase, thereby produces a H^+ gradient and offers the force for Na^+/H^+ exchange. Such an increase of Na^+/H^+ exchange may contribute to K^+ and Na^+ homeostasis (Zhang *et al.* 2006). These observations identify the essential role of PM H^+-ATPase and Na^+/K^+ ratio in enhancing cold

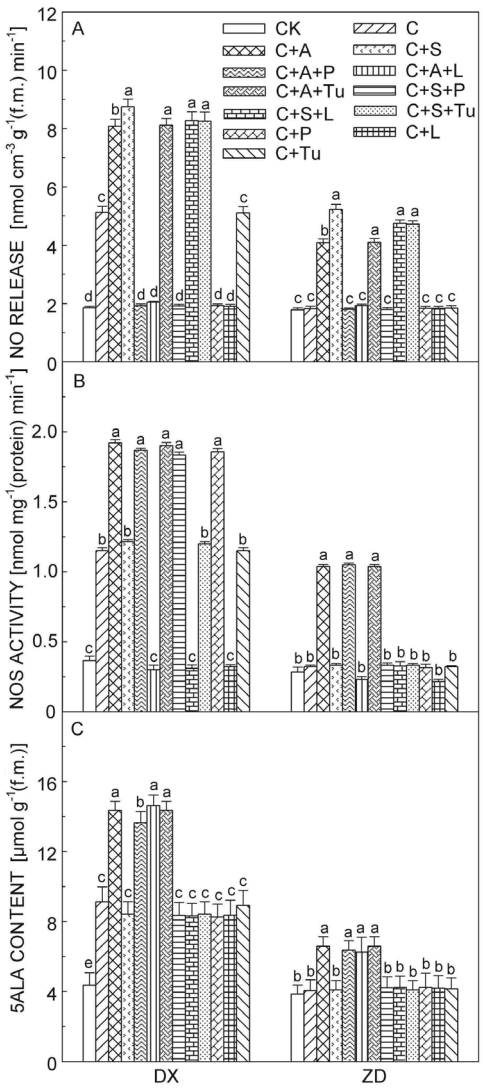

Fig. 5. NO production (*A*), NOS activity (*B*), and endogenous 5ALA content (*C*) in roots of DX and ZD ecotypes of *Elymus nutans* exposed to a cold stress of 5 ºC. CK - control, 25 °C, C - cold, 5 °C, C+A - cold + 1 mg dm^{-3} 5ALA, C+S - cold + 100 μM SNP, C+A+P - cold + 1 mg dm^{-3} 5ALA + 200 μM PTIO, C+A+L - cold + 1 mg dm^{-3} 5ALA + 150 μM L-NNA, C+A+Tu - cold + 1 mg dm^{-3} 5ALA + 100 μM Tu, C+S+P - cold + 100 μM SNP + 200 μM PTIO, C+S+L - cold + 100 μM SNP + 150 μM L-NNA, C+S+Tu - cold + 100 μM SNP + 100 μM Tu, C+P - cold + 200 μM PTIO, C+L - cold +150 μM L-NNA, C+Tu - cold + 100 μM Tu. Means ± SDs, n = 3. Different letters indicate significant differences at the 0.05 level according to Duncan's multiple range test.

resistance.

Although the roles of 5ALA and NO have been investigated extensively with respect to plant physiological processes under stress conditions, the cross-talk between 5ALA and NO has remained unexplored. In this study, several important findings related to 5ALA and cold resistance we are observed. First, endogenous 5ALA content in *E. nutans* roots was significantly induced in response to the cold stress and, accordingly, the 5ALA application significantly improved cold resistance of both the *E. nutans* ecotypes. Secondly, the 5ALA application rapidly enhanced NO content and NOS activity in roots, whereas the NO donor, NO scavenger, as well as NOS inhibitor applications had no significant effects on 5ALA content (Fig. 5). Thirdly, prevention of NO accumulation by PTIO or L-NNA could eliminate the protective effect of exogenous 5ALA against cold injury in both the ecotypes. These results illustrate that both 5ALA and NO were involved in acquisition of cold resistance in roots of the two *E. nutans* ecotypes, and NO might be a downstream signal in 5ALA-induced cold resistance similar to the previous finding that NO acts downstream of CO to regulate cold stress (Bai *et al.* 2012). This protective mechanism in the cold-resistant DX was found to be more efficient than that in the cold-sensitive ZD.

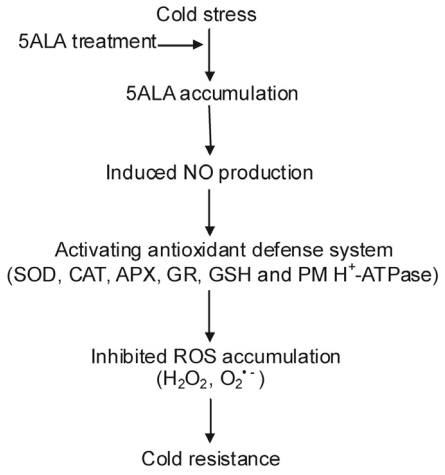

Fig. 6. A model illustrating the hypothetical function of NO in 5ALA-induced protection by modulating antioxidant defense and ROS ontent in roots of *Elymus nutans* exposed to a cold stress of 5 ºC.

Based on evidence provided herein, a novel model for 5ALA and NO-mediated cold resistance in roots of *E. nutans* is proposed (Fig. 6). Cold treatment induces accumulation of 5ALA, which has a stimulatory effect on NO accumulation, which is mechanism for protection of *E. nutans* from oxidative damage induced by cold stress. Exogenous 5ALA further increases NO accumulation to activate the antioxidant defense system and PM H^+-ATPase. The enhanced antioxidant defense system can scavenge the excess ROS accumulation induced by cold stress to alleviate oxidative damage in the roots of both the *E. nutans* ecotypes.

In conclusion, the present study revealed that the enhancement of 5ALA-induced cold resistance in *E. nutans* roots was associated with an up-regulation in the antioxidant defense system, Na^+ and K^+ homeostasis, and PM H^+-ATPase activity, thereby depressing

overproduction of ROS to alleviate cold-induced oxidative damage. Nitric oxide synthase-dependent NO production was mechanism of 5ALA for protection of *E. nutans* roots from damage induced by the cold stress, and the cross-talk between NO and 5ALA played a pivotal role in cold resistance of *E. nutans* roots. These findings help us in gaining further insights into the 5ALA and NO signal transduction pathway in plants under cold stress.

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