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BRIEF COMMUNICATION

Nitric oxide is involved in the regulation of ascorbate and glutathione metabolism in *Agropyron cristatum* leaves under water stress

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Abstract

This study investigated the regulation of ascorbate and glutathione metabolism by nitric oxide in *Agropyron cristatum* leaves under water stress. The activities of ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), L-galactono-1,4-lactone dehydrogenase (GalLDH) and γ-glutamylcysteine synthetase (γ-ECS), and the contents of NO, reduced ascorbic acid (AsA), reduced glutathione (GSH), total ascorbate and total glutathione increased under water stress. These increases were suppressed by pretreatments with NO synthesis inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and 4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). However, application of L-NAME and cPTIO to plants sufficiently supplied with water did not affect the activities of above mentioned enzymes and the contents of NO and above mentioned antioxidants. Pretreatments with L-NAME and cPTIO increased the malondialdehyde (MDA) content and electrolyte leakage of plants under water stress. Our results suggested that water stress-induced NO is a signal that leads to the up-regulation of ascorbate and glutathione metabolism and has important role for acquisition of water stress tolerance.

Additional key words: ascorbate peroxidase, cPTIO, electrolyte leakage, glutathione reductase, L-NAME, malondialdehyde.

Water stress usually causes oxidative damage to plants by inducing the accumulation of reactive oxygen species (ROS) (Apel and Hirt 2004). Plants could protect themselves against oxidative stress by enhancing activities of antioxidative enzymes and contents of nonenzymatic antioxidants (*e.g.* Mýtinová *et al.* 2010, Vuletić *et al.* 2010). Ascorbate and glutathione are two crucial nonenzymatic antioxidants and plants can modulate their regeneration and biosynthesis. The main biosynthetic pathway of ascorbate in plants is L-galactose pathway and L-galactono-1,4-lactone dehydrogenase (GalLDH) is the last enzyme in this way (Wheeler *et al.* 1998). The first enzyme for glutathione biosynthesis is γ-glutamylcysteine synthetase (γ-ECS; Dringen 2000). Ascorbate-glutathione cycle is the recycling pathway with the key enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer 1998).

Nitric oxide is a signal molecule in plants involved in plant growth and development (Pagnussat *et al.* 2004, Hu *et al.* 2005), maturation and senescence (Guo and

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Abbreviations: APX - ascorbate peroxidase; AsA - reduced ascorbic acid; DHAR - dehydroascorbate reductase; γ-ECS - γ-glutamylcysteine synthetase; GalLDH - L-galactono-1,4-lactone dehydrogenase; GR - glutathione reductase; GSH - reduced glutathione; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; L-NAME-N^G-nitro-L-arginine methyl ester; cPTIO - 4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.
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Crawford 2005, Wang *et al.* 2010) and respiratory metabolism (Zottini *et al.* 2002). Recently, many experiments have shown that NO also plays important role in the responses to biotic and abiotic stresses (Zeier *et al.* 2004, Neill *et al.* 2008, Song *et al.* 2008). It has been documented that NO is involved in the signal transduction in regulating the regeneration of glutathione through GR under salt and low temperature stresses (Ruan *et al.* 2005, Wu *et al.* 2009), and the regeneration of ascorbate through APX, DHAR and MDHAR under low temperature stress (Wu *et al.* 2009). Under water stress, Zhang *et al.* (2007) reported that NO regulated the regeneration of ascorbate and glutathione through APX and GR. However, whether NO regulates the regeneration of ascorbate through DHAR and MDHAR, and the biosynthesis of ascorbate and glutathione under water stress remains unknown.

Agropyron cristatum (L.) Gaertn. is a native from the semiarid area of northwestern China. In our previous observation, *A. cristatum* showed strong antioxidant ability under water stress (data not shown). However, the regulation mechanism of antioxidant metabolism of *A. cristatum* is still unknown. Therefore, aim of this study was to elucidate the role of NO in the regulation of ascorbate and glutathione metabolism of *A. cristatum*, and provide new theoretical knowledge to antioxidant metabolism in plants under water stress.

Seeds of *Agropyron cristatum* were sown in plastic trays filled with sand and *Vermiculite* (2:1, v/v) and grown in a greenhouse under 25 - 30 °C, 500 μmol m^{-2} s^{-1} photosynthetic active radiation and a 12-h photoperiod. The seedlings were watered daily with half-strength Hoagland's solution. When the fifth leaf was fully expanded, the seedlings of uniform height were taken out of trays. Roots were washed softly and thoroughly and placed in distilled water for 12 h. Plant roots were then placed in glass beakers wrapped with aluminum foil containing 100 cm^3 10 % (m/v) PEG solution for 24 h at 25 °C and a continuous irradiance of 200 μmol m^{-2} s^{-1}. To study the effects of NO inhibitors, a group of plants were pretreated with 200 μM N^G-nitro-L-arginine methyl ester (L-NAME) and 100 μM 4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) for 8 h and then exposed to PEG solution or distilled water for 24 h. Control plants were treated with distilled water alone. After treatment of 0, 4, 8, 12 and 24 h, the fifth leaves were collected and frozen in liquid N_2, and kept at -80 °C until the assays.

APX, GR, DHAR and MDHAR were extracted by grounding each sample (0.5 g) into a fine powder in liquid N_2 and then homogenizing fine powder in 6 cm^3 50 mM KH_2PO_4 (pH 7.5) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.3 % (v/v) *Triton X-100* and 1 % (m/v) polyvinylpolypyrrolidone, with addition of 1 mM AsA for APX assay (Grace and Logan 1996). The extract was centrifuged at 13 000 *g* for 15 min at 2 °C. The supernatant was used for assays. The activities of APX, GR, MDHAR and DHAR were assayed according to Nakano and Asada (1981), Grace and Logan (1996), Miyake and Asada (1992), and Dalton *et al.* (1986), respectively.

GalLDH was extracted by grounding each sample (0.1 g) into a fine powder in liquid N_2 and then homogenizing fine powder in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.4 M sucrose. The extract was passed through 2 layers of *Miracloth* and centrifuged at 300 *g* for 10 min at 2 °C. The supernatant was centrifuged at 10 000 *g* for 20 min at 2 °C. The sediment was suspended in 0.5 cm^3 of above buffer and GalLDH activity was assayed according to Shan and Liang (2010). γ-ECS was extracted by grounding each sample (0.2 g) into a fine powder in liquid N_2 and then homogenizing fine powder in 0.1 M Tris-HCl containing 5 mM EDTA (pH 8.0). The extract was centrifuged for 10 min at 30 000 *g* at 4 °C. A supernatant was desalted using *Sephadex G-25* equilibrated with extraction buffer. The eluate was used for assays done according to Rüeggseger and Brunold (1992). AsA and total ascorbate were measured according to Hodges *et al.* (1996). GSH and total glutathione were measured according to Griffith (1980).

NO content was measured according to Song *et al.* (2008) with some modifications. Fresh leaves (0.5 g) were incubated with 100 units catalase and 100 units superoxide dismutase for 5 min to remove ROS before addition of 3 cm^3 5 mM oxyhaemoglobin (HbO_2). After further 2 min incubation, NO content was estimated by following the conversion of HbO_2 to methaemoglobin (metHb) at 577 and 591 nm. MDA content and electrolyte leakage were measured according to Hodges *et al.* (1999) and Zhao *et al.* (2004), respectively. Protein content was assayed according to Bradford (1976).

The results presented were the means of five independent samples. Means were compared by one-way *ANOVA* and Duncan's test at 5 % of significance.

Water stress led to an increase in NO content within 24 h of treatment. After 4 h of treatment, NO content increased 2.16-fold, compared to the control (Table 1). Pretreatments with L-NAME and cPTIO markedly inhibited the accumulation of NO in leaves under water stress. However, pretreatments with L-NAME and cPTIO did not affect NO content in non-stressed leaves (Table 1).

The activities of APX, GR, DHAR, MDHAR, γ-ECS and GalLDH increased under water stress. Pretreatments with L-NAME and cPTIO significantly reduced the activities of above enzymes in stressed leaves, while did not affect the activities of these enzymes in non-stressed leaves (Table 2). To further investigate whether the ascorbate and glutathione metabolism were related to the accumulation of NO in *A. cristatum* leaves, the effects of pretreatments with L-NAME and cPTIO on the contents of AsA, total ascorbate, GSH, and total glutathione under water stress were studied. The results showed that pretreatments with L-NAME and cPTIO significantly

reduced the contents of AsA, GSH, total ascorbate and total glutathione induced by water stress. Pretreatments with L-NAME and cPTIO did not affect the contents of above antioxidants in non-stressed leaves (Table 2).

To further investigate whether NO has important role for water stress tolerance in *A. cristatum,* the effects of pretreatments with L-NAME and cPTIO on MDA content and electrolyte leakage under water stress were studied. The results showed that pretreatments with L-NAME and cPTIO significantly increased MDA content and electrolyte leakage of stressed leaves. However, pretreatments with L-NAME and cPTIO alone did not affect MDA content and electrolyte leakage in non-stressed leaves (Table 2).

AsA is an important compound of antioxidant system and a major redox compound in plants. It has been documented that APX activity increased in pepper leaves under drought stress (Hu *et al.* 2010) and NO could increase APX activity under water stress (Zhang *et al.* 2007). Wu *et al.* (2009) reported that NO increased the activities of APX, DHAR and MDHAR, and AsA content in loquat leaves under low temperature stress, which was consistent with our results. Besides, our study also indicated that NO increased GalLDH activity and total

Table 1. Effects of water stress (W; 10 % PEG), 200 μM L-NAME and 100 μM cPTIO on NO content [μmol mg^{-1}(protein)] in *A. cristatum* leaves. The plants were pretreated with L-NAME and cPTIO for 8 h, and then exposed to water stress or distilled water for 0, 4, 8, 12 and 24 h. Means ± SE, n = 5. Different letters within the same row indicate statistically significant differences according to Duncan's test ($P < 0.05$).

Treatment [h]	Control	L-NAME+W	cPTIO+W	W	L-NAME	cPTIO
0	2.0±0.16a	2.2±0.18a	1.8±0.18a	2.0±0.17a	2.0±0.19a	2.2±0.20a
4	2.3±0.15b	2.6±0.22b	2.4±0.19b	5.0±0.41a	2.3±0.16b	2.1±0.16b
8	2.3±0.17b	2.7±0.21b	2.4±0.15b	4.3±0.35a	2.2±0.15b	2.4±0.18b
12	2.2±0.14b	2.2±0.16b	2.0±0.13b	3.5±0.27a	2.2±0.17b	2.3±0.16b
24	2.0±0.12b	2.5±0.13b	2.3±0.13b	3.2±0.25a	2.0±0.14b	2.0±0.13b

Table 2. Effects of water stress (W; 10 % PEG), 200 μM L-NAME and 100 μM cPTIO on the activities of enzymes involved in ascorbate and glutathione metabolism, the contents of AsA, total ascorbate, GSH, total glutathione and MDA and electrolyte leakage. For other details see Table 1.

Parameters	[h]	Control	L-NAME+W	cPTIO+W	W	L-NAME	cPTIO
APX [U mg^{-1}(protein)]	4	1.30±0.09b	1.40±0.09b	1.20±0.07b	2.80±0.16a	1.40±0.09b	1.30±0.08b
	8	1.30±0.08b	1.30±0.07b	1.20±0.08b	3.10±0.16a	1.50±0.10b	1.30±0.06b
GR [U mg^{-1}(protein)]	4	1.50±0.11b	1.40±0.09b	1.30±0.09b	2.50±0.14a	1.40±0.08b	1.50±0.09b
	8	1.60±0.09b	1.60±0.10b	1.40±0.09b	2.30±0.13a	1.40±0.07b	1.60±0.08b
DHAR [U mg^{-1}(protein)]	4	1.60±0.07b	1.80±0.10b	1.80±0.11b	3.20±0.20a	1.60±0.08b	1.80±0.10b
	8	1.40±0.08b	1.70±0.09b	1.60±0.07b	3.60±0.18a	1.70±0.11b	1.60±0.07b
MDHAR [U mg^{-1}(protein)]	4	1.00±0.06b	1.20±.05b	1.10±0.06b	2.20±0.13a	1.30±0.06b	1.10±0.05b
	8	1.20±0.05b	1.30±0.06b	1.20±0.06b	2.50±0.15a	1.20±0.06b	1.30±0.06b
γ-ECS [nmol min^{-1} mg^{-1}(protein)]	4	2.00±0.16b	2.30±0.20b	2.00±0.14b	3.70±0.19a	2.30±0.12b	2.30±0.12b
	8	2.30±0.14b	2.50±0.19b	2.20±0.15b	4.00±0.22a	2.40±0.14b	2.20±0.15b
GalLDH [U g^{-1}(f.m.)]	4	1.20±0.05b	1.40±0.07b	1.20±0.06b	2.60±0.17a	1.50±0.07b	1.30±0.06b
	8	1.50±0.09b	1.50±0.06b	1.30±0.07b	3.00±0.18a	1.60±0.09b	1.50±0.09b
AsA [μmol g^{-1}(f.m.)]	4	3.06±0.19b	3.15±0.23b	2.76±0.17b	5.46±0.45a	3.35±0.21b	3.24±0.21b
	8	3.34±0.18b	3.12±0.22b	2.91±0.16b	5.67±0.42a	3.51±0.15b	3.26±0.14b
Total ascorbate [μmol g^{-1}(f.m.)]	4	3.31±0.17b	3.52±0.23b	3.23±0.20b	6.02±0.47a	3.61±0.25b	3.51±0.19b
	8	3.63±0.24b	3.51±0.25b	3.42±0.21b	6.30±0.51a	3.80±0.17b	3.52±0.20b
GSH [μmol g^{-1}(f.m.)]	4	0.21±0.03b	0.21±0.02b	0.19±0.02b	0.41±0.04a	0.24±0.03b	0.22±0.02b
	8	0.24±0.02b	0.23±0.03b	0.20±0.02b	0.37±0.04a	0.22±0.02b	0.23±0.03b
Total glutathione [μmol g^{-1}(f.m.)]	4	0.22±0.03b	0.23±0.03b	0.21±0.02b	0.44±0.05a	0.25±0.03b	0.23±0.02b
	8	0.25±0.02b	0.26±0.03b	0.22±0.02b	0.40±0.04a	0.23±0.02b	0.24±0.03b
MDA content [nmol g^{-1}(f.m.)]	8	4.00±0.29c	11.0±0.75a	10.5±0.61a	8.00±0.51b	4.50±0.38c	3.90±0.30c
Electrolyte leakage [%]	8	6.50±0.43c	21.0±1.41a	20.0±1.22a	14.0±0.87b	6.50±0.46c	7.00±0.55c

ascorbate content under water stress.

GSH is another important compound of plant antioxidant system. The cellular content of GSH can be determined by γ-ECS and GR activity. It has been reported that NO induced the accumulation of GSH and the increase in GR activity under low temperature and salt stresses (Wu *et al.* 2009, Ruan *et al.* 2005). Our results also showed that NO increased GR activity and GSH content under water stress. Zhang *et al.* (2007) reported that NO increased GR activity in maize leaves under water stress, which was consistent with our experimental results. Besides, our study also indicated that the accumulation of NO increased γ-ECS activity and total glutathione content under water stress.

It has been documented that plant hormones abscisic acid (ABA) and jasmonic acid (JA) increased as a result of water stress and have important roles in defense against oxidative stress in plant cells (Li *et al.* 1998, Jiang and Zhang 2002, Lan *et al*. 2004). Jiang and Zhang (2002) reported that NO was involved in the regulation of ascorbate-glutathione cycle in ABA signaling pathway under water stress. However, whether NO is involved in the regulation of ascorbate and glutathione biosynthesis induced by ABA remains unknown. It was also reported that JA had an important role in the regulation of ascorbate and glutathione metabolism under water stress (Shan and Liang 2010).

In conclusion, our results clearly suggest that NO participates in the regulation of ascorbate and glutathione metabolism, which, in turn, enhances the antioxidant ability and protects *A. cristatum* against oxidative stress induced by water stress. These results provide new theoretical knowledge to the antioxidant metabolism in plants under water stress.

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