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BRIEF COMMUNICATION

Nitric oxide acts downstream of hydrogen peroxide in the regulation of ascorbate and glutathione metabolism by jasmonic acid in *Agropyron cristatum* leaves

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Abstract

The relationship between hydrogen peroxide (H_2O_2) and nitric oxide (NO) in the regulation of ascorbate and glutathione metabolism by jasmonic acid (JA) in *Agropyron cristatum* leaves were studied. Results showed that JA increased the production of H_2O_2 and NO, 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), as well as transcription of the respective genes and also the content of reduced ascorbate (AsA) and reduced glutathione (GSH). Above increases were suppressed by pre-treatments with H_2O_2 synthesis inhibitor diphenylene iodonium (DPI), H_2O_2 scavenger dimethylthiourea (DMTU), NO synthesis inhibitor N^G-nitro-L-Arg methyl ester (L-NAME), and NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Pre-treatments with DPI and DMTU reduced H_2O_2 and NO production. Pre-treatments with L-NAME and cPTIO reduced NO production, but did not reduce the H_2O_2 production induced by JA. Our results suggested that NO acted downstream of H_2O_2 in JA signalling in the up-regulation of ascorbate and glutathione metabolism in *A. cristatum* leaves.

Additional key words: ascorbate peroxidase, dehydroascorbate reductase, γ-glutamylcysteine synthetase, glutathione reductase, monodehydroascorbate reductase.

Ascorbate and glutathione are two crucial nonenzymatic compounds of defence system against oxidative stress (Shan *et al.* 2015). Plants can adjust ascorbate and glutathione content by modulating their regeneration and biosynthesis. In plants, L-galactono-1,4-lactone dehydrogenase (GalLDH) is the last enzyme in the main biosynthetic pathway of ascorbate (Wheeler *et al*. 1998) and γ-glutamylcysteine synthetase (γ-ECS) is the first enzyme for glutathione biosynthesis (Dringen 2000). Ascorbate-glutathione cycle is the regeneration pathway for ascorbate (AsA) and glutathione (GSH). Ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), are enzymes involved in this cycle (Noctor and Foyer 1998).

H_2O_2 and NO are two key signal molecules in plants and play important roles in plant growth, development and many other physiological processes (Zhao *et al.* 2012, Chen *et al.* 2015, Serrano *et al.* 2015) including responses to biotic and abiotic stresses (Abramowski

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; AsA - ascorbate; cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DHAR - dehydroascorbate reductase; DMTU - dimethylthiourea; DPI - diphenylene iodonium; GalLDH - L-galactono-1,4-lactone dehydrogenase; γ-ECS - γ-glutamylcysteine synthetase; GR - glutathione reductase; GSH - reduced glutathione; JA - jasmonic acid;; L-NAME - N^G-nitro-L-Arg methyl ester; MDHAR - monodehydroascorbate reductase; SA - salicylic acid.
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et al. 2015, Cheng *et al.* 2015, Mostofa *et al.* 2015). They also participate in the signalling pathways of salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) (Gonzalez *et al.* 2012, Furlan *et al.* 2013, Mur *et al.* 2013, Dai *et al.* 2015, Mostofa *et al.* 2015).

Jasmonic acid plays important roles in regulating stress responses, plant growth, and development (Qiu *et al.* 2014, Matschi *et al.* 2015). Increasing evidence showed that JA also has an important role in the regulation of AsA and GSH metabolism in plants (Ai *et al.* 2008, Shan *et al.* 2011, 2015). It has also been documented that H_2O_2 and NO together with JA are involved in the regulation of the AsA and GSH metabolism (Zhang *et al.* 2007, Mur *et al.* 2013, Dai *et al.* 2015).

Agropyron cristatum is native in the semiarid area of northwestern China. Our previous studies have showed that JA regulated ascorbate and glutathione metabolism in *A. cristatum* leaves under water stress and JA alone could also have the same effects (Shan and Liang 2010, Shan *et al.* 2011). However, whether NO and H_2O_2 participate in the signal transduction of JA in regulating AsA and GSH metabolism in *A. cristatum* leaves remains unknown. So, it is very interesting to study the relationship between H_2O_2 and NO in JA signaling in the regulation of ascorbate and glutathione metabolism, in order to elucidate antioxidant mechanism of *A. cristatum* regulated by JA.

The seeds of *Agropyron cristatum* (L.) Gaertn. were sown in plastic trays filled with sand + *Vermiculite* mix (2:1, v/v) and grown in a greenhouse under a temperature of 25 - 30 °C, an irradiance of 500 μmol m^{-2} s^{-1} (photo-synthetically active radiation), and a 12-h photoperiod. The seedlings were watered with a half-strength Hoagland's solution every day. When the fifth leaf was fully expanded, the seedlings with uniform height and growth status were selected for all experiments. The roots of plants were washed softly and thoroughly. After rinsing in a half-strength Hoagland's solution for 12 h, the roots were placed in beakers containing 100 cm^3 1 μM JA and wrapped with aluminium foil for 24 h at 25 °C with a continuous radiation of 200 μmol m^{-2} s^{-1}. To study the effects of inhibitors or scavengers, plants were pre-treated with 100 μM H_2O_2 synthesis inhibitor diphenylene iodonium (DPI), 5 mM H_2O_2 scavenger dimethylthiourea (DMTU), 200 μM NO synthesis inhibitor N^G-nitro-L-Arg methyl ester (L-NAME), and 100 μM NO scavenger 2-(4-carbo-xyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). The roots were soaked in these compounds added to half-strength Hoagland's solution for 4 h and then exposed to 1 μM JA for 12 and 24 h. Control plants were treated with a half-strength Hoagland's solution alone. After the treatments, the fifth leaves were collected, frozen in liquid nitrogen, and then kept at -80 °C until further analyses.

Enzymes were extracted according to Shan and Liang (2010). The activities of APX (EC 1.11.1.11), GR (EC 1.6.4.2), MDHAR (EC 1.6.5.4), and DHAR (EC 1.8.5.1) were measured by the methods of Nakano and Asada (1981), Grace and Logan (1996), Miyake and Asada (1992), and Dalton *et al.* (1986), respectively. Further, GalLDH (EC 1.3.2.3) and γ-ECS (EC 6.3.2.2) were extracted and measured by the method of Shan and Liang (2010) and Rüeggseger and Brunold (1992), respectively. One unit of APX was defined as the amount of APX catalyzing the oxidation of 1 μmol of AsA per minute. One unit of GR activity was defined as the reduction of 1 μmol of NADPH per minute. One unit of DHAR activity was defined as the amount of enzyme that produces 1 μmol of AsA per minute. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 μmol of NADH per minute. One unit of GalLDH activity was defined as the amount of extract required to oxidize 1 nmol of L-Gal per minute. One unit of γ-ECS activity was defined as 1 μmol of cysteine-dependently generated PO_4^{3-} per minute. Protein content was measured by the method of Bradford (1976). The specific enzyme activity was expressed as U mg^{-1}(protein). The isolation of total RNA and semi-quantitative reverse transcription (RT)-PCR of above enzyme genes were done according to method described previously (Shan *et al.* 2011). To standardize the results, the relative abundance of *β-actin* was also determined and used as the internal standard. The relative expression of target genes were analyzed by *GeneTools* from *SynGene*. The content of AsA, GSH, H_2O_2, and NO were measured according to the method of Hodges *et al.* (1996), Griffith (1980), Jiang and Zhang (2003), and Song *et al.* (2008), respectively. The whole experiment was repeated five times with six seedlings each time. The results presented are means of five replicates. The means were compared by one-way analysis of variance and Duncan's multiple range test at the 5 % level of significance.

After 12 and 24 h of treatment, JA application increased H_2O_2 content (Table 1). Pre-treatments with DPI and DMTU markedly inhibited the production of H_2O_2 induced by JA. Meanwhile, JA increased the transcriptions and activities of APX, GR, DHAR, MDHAR, γ-ECS, and GalLDH and content of AsA and GSH after 12 and 24 h. Dai *et al.* (2015) reported that JA increases the transcriptions and activities of APX, GR, MDHAR, DHAR, GalLDH and γ-ECS, and the content of AsA and GSH in the leaves of wheat, which is consistent with our results. Pre-treatments with DPI and DMTU significantly reduced the transcriptions and activities of APX, GR, DHAR, MDHAR, γ-ECS, and GalLDH as well as the content of AsA and GSH induced by JA. These results suggested that H_2O_2 was involved in the signal transduction of JA in regulating the AsA and GSH metabolism. Chou *et al.* (2012) reported that H_2O_2 can induce the expression of APX and GR in rice leaves. Dai *et al.* (2015) reported that JA-induced H_2O_2 increases

the transcriptions and activities of APX, GR, DHAR, MDHAR, GalLDH and γ-ECS, and the content of AsA and GSH in wheat leaves. In our study, we found that JA-induced H_2O_2 also increased the transcriptions and activities of APX, GR, DHAR and MDHAR, and the content of AsA and GSH, which was consistent with previous studies. However, Noctor *et al.* (2002) reported that H_2O_2 does not affect the transcription of *γ-ECS*. This discrepancy may be due to the differences between plant species.

Jasmonic acid also increased NO content after 12 and 24 h of treatment (Tables 1,2). Pre-treatments with L-NAME and cPTIO markedly inhibited the production of NO induced by JA at both 12 and 24 h of treatment. Meanwhile, pre-treatments with L-NAME and cPTIO also significantly reduced the transcription and activities of APX, GR, DHAR, MDHAR, γ-ECS, and GalLDH and the content of AsA and GSH induced by JA. These results suggested that NO was involved in the signal transduction of JA in regulating AsA and GSH metabolism. Sun *et al.* (2015) reported that NO can increase the transcription and activities of APX, GR. and DHAR in roots of wheat. NO has been reported to activate the transcription of *γ-ECS* gene (Innocenti *et al.* 2007). In our previous study, we found that JA-induced NO increased the transcription and activities of APX, GR, DHAR and MDHAR, and the content of

Table 1. The activities of enzymes and the relative expressions of respective genes of the ascorbate and glutathione metabolism, and the content of AsA, GSH, NO, and H_2O_2 in *Agropyron cristatum* leaves. Control - half-strength Hoagland's solution; JA - 1 μM JA; DPI+JA - 100 μM DP I+ 1 μM JA; DMTU+JA - 5 mM DMTU + 1 μM JA;. The plants were pretreated with DPI and DMTU for 4 h, and then exposed to JA for 12 or 24 h. Means ± SDs, $n = 5$, different letters in the same row indicate significant differences at $P < 0.05$.

Time [h]	Parameters	Control	JA	DPI+JA	DMTU+JA
12	APX [U mg^{-1}(protein)]	1.8 ±0.15b	3.7 ±0.32a	2.2 ±0.20b	2.0 ±0.18b
	GR [U mg^{-1}(protein)]	1.2 ±0.11b	2.1 ±0.20a	1.4 ±0.12b	1.3 ±0.15b
	DHAR [U mg^{-1}(protein)]	3.5 ±0.31b	6.9 ±0.53a	3.4 ±0.35b	3.0 ±0.24b
	MDHAR [U mg^{-1}(protein)]	1.8 ±0.20b	3.7 ±0.40a	2.0 ±0.20b	2.1 ±0.23b
	γ-ECS [U mg^{-1}(protein)]	4.1 ±0.36b	8.0 ±0.71a	4.8 ±0.50b	4.5 ±0.43b
	GalLDH [U mg^{-1}(protein)]	3.7 ±0.34b	8.5 ±0.71a	4.9 ±0.44b	5.1 ±0.42b
	relative expression of *APX*	0.01±0.00b	0.20±0.03a	0.03±0.00b	0.02±0.00b
	relative expression of *GR*	0.08±0.01b	0.30±0.04a	0.08±0.01b	0.08±0.01b
	relative expression of *DHAR*	0.15±0.02b	0.35±0.04a	0.16±0.03b	0.11±0.01b
	relative expression of *MDHAR*	0.02±0.00b	0.20±0.02a	0.02±0.00b	0.03±0.00b
	relative expression of *γ-ECS*	0.13±0.02b	0.86±0.09a	0.18±0.03b	0.13±0.02b
	relative expression of *GalLDH*	0.07±0.01c	0.45±0.06a	0.11±0.02b	0.10±0.01b
	AsA [μmol g^{-1} (f.m.)]	3.9 ±0.34b	5.7 ±0.57a	4.3 ±0.42b	4.1 ±0.36b
	GSH [μmol g^{-1} (f.m.)]	0.21±0.01b	0.35±0.04a	0.23±0.02b	0.20±0.01b
	NO [μmol g^{-1}(f.m.)]	1.2 ±0.10c	4.8 ±0.44a	2.0 ±0.16b	2.4 ±0.21b
	H_2O_2 [μmol g^{-1}(f.m.)]	6.7 ±0.75c	12.5 ±1.11a	8.1 ±0.92b	7.9 ±0.85b
24	APX [U mg^{-1}(protein)]	1.6 ±0.16b	3.4 ±0.33a	1.7 ±0.14b	1.6 ±0.13b
	GR [U mg^{-1}(protein)]	1.0 ±0.10b	1.7 ±0.15a	1.1 ±0.11b	1.0 ±0.12b
	DHAR [U mg^{-1}(protein)]	2.9 ±0.25b	5.8 ±0.61a	3.1 ±0.25b	2.8 ±0.26b
	MDHAR [U mg^{-1}(protein)]	1.7 ±0.16b	3.4 ±0.29a	1.7 ±0.14b	1.8 ±0.15b
	γ-ECS [U mg^{-1}(protein)]	4.4 ±0.41b	7.3 ±0.67a	4.7 ±0.43b	4.2 ±0.37b
	GalLDH [U mg^{-1}(protein)]	4.1 ±0.40b	7.7 ±0.75a	4.5 ±0.39b	4.4 ±0.38b
	relative expression of *APX*	0.01±0.00b	0.18±0.02a	0.05±0.01b	0.02±0.00b
	relative expression of *GR*	0.07±0.01b	0.16±0.02a	0.05±0.01b	0.07±0.01b
	relative expression of *DHAR*	0.13±0.02b	0.30±0.03a	0.14±0.02b	0.10±0.01b
	relative expression of *MDHAR*	0.01±0.00b	0.27±0.03a	0.03±0.00b	0.01±0.00b
	relative expression of *γ-ECS*	0.11±0.02b	0.95±0.11a	0.13±0.02b	0.10±0.02b
	relative expression of *GalLDH*	0.09±0.01c	1.09±0.12a	0.16±0.02b	0.16±0.03b
	AsA [μmol g^{-1}(f.m.)]	4.5 ±0.51b	6.3 ±0.53a	4.8 ±0.44b	4.4 ±.37b
	GSH [μmol g^{-1}(f.m.)]	0.25±0.03b	0.40±0.05a	0.21±0.02b	0.22±0.03b
	NO [μmol g^{-1}(f.m.)]	1.5 ±0.13c	5.2 ±0.49a	2.2 ±0.19b	2.0 ±0.18b
	H_2O_2 [μmol g^{-1}(f.m.)]	6.1 ±0.61c	11.7 ±1.06a	8.1 ±0.79b	7.4 ±0.72b

AsA and GSH in wheat leaves (Shan *et al.* 2015). In the present study, we found that JA-induced NO increased the transcriptions and activities of APX, GR, DHAR, MDHAR, and γ-ECS and the content of AsA and GSH in *A. cristatum*, which is consistent with the results of previous studies. Besides, we also found that NO can increase the transcriptions of DHAR and MDHAR, and the transcriptions and activities of GalLDH and γ-ECS.

Pre-treatments with DPI and DMTU substantially decreased the production of NO induced by JA after 12 and 24 h (Table 1). Pre-treatments with L-NAME and cPTIO did not block the increases in the production of H_2O_2 induced by JA after 12 and 24 h (Table 2). These results suggested that H_2O_2 was required for the production of NO and NO did not induce H_2O_2 production in the signal transduction of JA in regulating AsA and GSH metabolism in *A. cristatum* leaves. It has been reported that NO induced the production of H_2O_2 in the calluses from *Populus euphratica* under salt stress (Zhang *et al*. 2007) and in NO-induced cell death in maize leaves (Wang *et al.* 2014). Lum *et al.* (2002) and Kong *et al.* (2013) reported that H_2O_2 induced a rapid production of NO in mung bean and *Arabidopsis* seedlings, respectively. In present study, our results showed that H_2O_2 mediated NO production, but NO did not induce H_2O_2 production in the signal transduction

Table 2. The activities of enzymes and the relative expressions of respective genes of the ascorbate and glutathione metabolism, and the content of AsA, GSH, NO, and H_2O_2 in *Agropyron cristatum* leaves. Control - half-strength Hoagland's solution; JA - 1 μM JA; L-NAME+JA - 200 μM L-NAME + 1 μM JA; cPTIO+JA - 100 μM cPTIO + 1 μM JA. The plants were pretreated with L-NAME and cPTIO for 4 h, and then exposed to JA for 12 or 24 h. Means ± SDs, $n = 5$, different letters in the same row indicate significant differences at $P < 0.05$.

Time [h]	Parameters	Control	JA	L-NAME+JA	cPTIO+JA
12	APX [U mg^{-1}(protein)]	2.3 ±0.23b	3.5 ±0.31a	1.5 ±0.13c	1.0 ±0.11d
	GR [U mg^{-1}(protein)]	2.5 ±0.25b	4.0 ±0.35a	1.7 ±0.21c	1.3 ±0.11c
	DHAR [U mg^{-1}(protein)]	2.3 ±0.22b	4.3 ±0.40a	1.5 ±0.15c	1.2 ±0.13c
	MDHAR [U mg^{-1}(protein)]	4.3 ±0.38b	5.8 ±0.52a	3.9 ±0.33b	1.9 ±0.16c
	γ-ECS [U mg^{-1}(protein)]	4.0 ±0.36b	7.4 ±0.68a	4.6 ±0.42b	4.5 ±0.44b
	GalLDH [U mg^{-1}(protein)]	4.2 ±0.40b	7.7 ±0.71a	4.5 ±0.38b	4.4 ±0.34b
	relative expression of *APX*	0.06±0.00b	0.26±0.03a	0.03±0.00c	0.02±0.00c
	relative expression of *GR*	0.01±0.01d	0.45±0.04a	0.10±0.01b	0.06±0.01c
	relative expression of *DHAR*	0.06±0.02c	0.38±0.04a	0.13±0.03b	0.13±0.01b
	relative expression of *MDHAR*	0.01±0.00c	1.03±0.02a	0.04±0.00b	0.06±0.00b
	relative expression of *γ-ECS*	0.10±0.02b	0.37±0.09a	0.06±0.03c	0.11±0.02b
	relative expression of *GalLDH*	0.11±0.01b	0.17±0.06a	0.11±0.02b	0.12±0.01b
	AsA [μmol g^{-1}(f.m.)]	5.0 ±0.45b	6.5 ±0.56a	4.9 ±0.44b	4.5 ±0.41b
	GSH [μmol g^{-1}(f.m.)]	0.21±0.02b	0.40±0.03a	0.22±0.02b	0.21±0.02b
	NO [μmol g^{-1}(f.m.)]	1.50±0.18c	3.96±0.35a	1.70±0.18b	1.82±0.17b
	H_2O_2 [μmol g^{-1}(f.m.)]	6.3 ±0.72b	13.0 ±1.22a	14.1 ±1.35a	13.5 ±1.35a
24	APX [U mg^{-1}(protein)]	2.1 ±0.18b	3.1 ±0.28a	1.8 ±0.16b	0.9 ±0.09c
	GR [U mg^{-1}(protein)]	2.8 ±0.31b	3.6 ±0.31a	1.9 ±0.18c	1.1 ±0.10d
	DHAR [U mg^{-1}(protein)]	2.6 ±0.29b	4.0 ±0.39a	1.8 ±0.19c	1.0 ±0.11d
	MDHAR [U mg^{-1}(protein)]	3.9 ±0.42b	6.5 ±0.60a	3.3 ±0.37b	1.6 ±0.15c
	γ-ECS [U mg^{-1}(protein)]	3.8 ±0.38b	6.9 ±0.61a	4.3 ±0.39b	4.0 ±0.37b
	GalLDH [U mg^{-1}(protein)]	4.0 ±0.30b	7.1 ±0.63a	4.0 ±0.40b	4.1 ±0.42b
	relative expression of *APX*	0.07±0.00b	0.28±0.02a	0.10±0.01b	0.02±0.00c
	relative expression of *GR*	0.01±0.01d	0.39±0.02a	0.17±0.01b	0.03±0.01c
	relative expression of *DHAR*	0.02±0.02d	0.28±0.03a	0.05±0.02c	0.15±0.01b
	relative expression of *MDHAR*	0.07±0.00b	0.99±0.03a	0.08±0.00b	0.08±0.00b
	relative expression of *γ-ECS*	0.12±0.02b	0.37±0.11a	0.05±0.02c	0.05±0.02c
	relative expression of *GalLDH*	0.14±0.01b	0.21±0.12a	0.13±0.02b	0.15±0.03b
	AsA [μmol g^{-1}(f.m.)]	4.7 ±0.42b	7.1 ±0.63a	5.2 ±0.55b	4.8 ±0.35b
	GSH [μmol g^{-1}(f.m.)]	0.24±0.03b	0.37±0.04a	0.25±0.03b	0.24±0.02b
	NO [μmol g^{-1}(f.m.)]	0.83±0.07c	2.70±0.31a	1.44±0.14b	1.25±0.14b
	H_2O_2 [μmol g^{-1}(f.m.)]	7.0 ±0.58b	14.7 ±1.31a	15.2 ±1.44a	14.0 ±1.33a

of JA in regulating the ascorbate and glutathione metabolism. Above differences suggested that there were diverse relationship between H_2O_2 and NO under different circumstances.

In conclusion, our results indicated that NO acts downstream of H_2O_2 in the regulation of ascorbate and glutathione metabolism by jasmonic acid in *Agropyron cristatum* leaves, which uncovered the antioxidant mechanism of *A. cristatum* regulated by JA.

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