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BRIEF COMMUNICATION

Nitric oxide donor SNP regulates the ascorbate and glutathione metabolism in *Agropyron cristatum* leaves through MEK1/2

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Abstract

This study investigated the role of the protein kinase MEK1/2 in the regulation of ascorbate and glutathione metabolism by nitric oxide in *Agropyron cristatum* leaves. The results showed that NO donor, sodium nitroprusside (SNP), induced the increased activities of ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase, L-galactono-1,4-lactone dehydrogenase, and γ-glutamylcysteine synthetase (γ-ECS), the transcription of respective genes, and the content of reduced ascorbate, reduced glutathione, total ascorbate, and total glutathione. All the above increases, except the activity of γ-ECS, were suppressed by pre-treatment with MEK1/2 inhibitors 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one and 1,4-diamino-2,3-dicyano-1,4-*bis*(*o*-aminophenyl-mercapto) butadiene. The results of Western blot showed that SNP induced the increase in the phosphorylation of MEK1/2. Our results suggested that SNP induced the phosphorylation of MEK1/2, which, in turn, up-regulated the ascorbate and glutathione metabolism in *A. cristatum* leaves.

Addtional key words: ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase, monodehydroascorbate reductase, protein kinase.

Ascorbate (AsA) and glutathione (GSH) have important roles in maintaining the redox equilibrium in cell and in defending against oxidative stress (Höller *et al.* 2015, Shan *et al.* 2015). Nitric oxide (NO) is an important signal molecule, which has diverse physiological functions in plants. It could affect seed germination (Fan *et al.* 2013), growth and development (Alemayehu *et al.* 2015), and play important roles in reproductive processes (Zhou *et al.* 2012), respiration (Zottini *et al.* 2002), and responses to stresses (Yildiztugay *et al.* 2014, Tian *et al.* 2015). Increasing evidence shows an important role of NO in the regulation of ascorbate and glutathione metabolism in plants (Ai *et al.* 2008, Shan *et al.* 2012, 2015, Shan and Yang 2017). The mitogen-activated protein kinase (MAPK) cascade is a major pathway by which extracellular stimuli are transduced into intracellular responses in all eukaryotic cells (Zhao *et al.* 2015). Mitogen-activated protein kinase kinases (MAPKKs) are essential components of the MAPK cascade that regulates a variety of fundamental cellular processes in response to environmental stimuli (Jonak *et al.* 2002). MEK1/2 belongs to the MAPKK family and the phospho-MEK1/2 is its activated form. So, the activation of MEK1/2 can facilitate the phosphorylation of downstream MAPKs and then initiate MAPK pathway. Our previous study showed that MEK1/2 participated in

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Abbreviations: APX - ascorbate peroxidase; AsA - reduced ascorbate; DHAR - dehydroascorbate reductase; γ-ECS - γ-glutamylcysteine synthetase; GalLDH - L-galactono-1,4-lactone dehydrogenase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; MAPK - mitogen-activated protein kinase; MDHAR - monodehydroascorbate reductase; PD98059 - 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; SNP - sodium nitroprusside; U0126 - 1,4-diamino-2,3-dicyano-1,4-*bis*(*o*-amino-phenylmercapto) butadiene.
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the signal transduction of jasmonic acid (JA) in regulating ascorbate and glutathione metabolism (Shan *et al.* 2011). However, whether MEK1/2 participates in the signal transduction of NO in regulating the ascorbate and glutathione metabolism remains unknown. Therefore, we investigated the transcription and activities of enzymes involved in AsA and GSH, the content of AsA and GSH, and the phosphorylation level of MEK1/2 in the leaves of *Agropyron cristatum* exposed to NO donor sodium nitroprusside (SNP).

The seeds of *Agropyron cristatum* (L.) Gaertn. were sown in plastic trays filled with sand + *Vermiculite* moistened with half-strength Hoagland's solution and grown in a greenhouse under a temperature of 25 - 30 °C, a 12-h photoperiod, and an irradiance of 500 μmol m^{-2} s^{-1} (photosynthetically active radiation). When the fifth leaf was fully expanded, plants were pre-treated with 50 μM 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) or 10 μM 1,4-diamino-2,3-dicyano-1,4-*bis* (*o*-aminophenyl-mercapto) butadiene (U0126) for 8 h (U0126 and PD98059 can bind to the inactive forms of MEK1/2 and prevent its activation; Favata *et al.* 1998). Then the plants were exposed to 100 μM SNP solution at a temperature of 25 °C and a continuous irradiance of 200 μmol m^{-2} s^{-1} for 12 or 24 h. The fifth leaf was collected, frozen in liquid nitrogen, and then kept at -80 °C until analyses.

The activities of ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and L-galactono-1,4-lactone dehydrogenase (GalLDH) were determined according to Shan and Liang (2010) and the activity of γ-glutamylcysteine synthetase (γ-ECS) was determined according to Rüeggseger and Brunold (1992).The activity units were defined in the previous paper (Shan and Yang 2017). Protein content was measured according to Bradford (1976) and AsA and DHA content according to Hodges *et al.* (1996). Total ascorbate content was measured after reduction of dehydroascorbate to AsA by dithiothreitol. Content of total glutathione, oxidized glutathione (GSSG), and GSH were measured according to Griffith (1980). Total glutathione was determined by an enzymatic cycling assay method. GSSG was measured by removing GSH by 2-vinylpyridine derivatization. GSH content was then estimated from the difference between total glutathione and GSSG.

Transcriptions of *APX*, *GR*, *DHAR*, *MDHAR*, *GalLDH*, and *γ-ECS* genes were detected by reverse transcription (RT)-PCR according to the method of Shan and Liang (2010). Total RNA was isolated from leaves by using *TRNzol* reagent (*Tiangen*, China). Approximately 3 μg of total RNA were reverse transcribed using oligo(dT) primer and *TIANScript* RT kit. The cDNA was amplified with the *GeneAmp PCR System 9700* (*Applied Biosystems*, USA) using primers designed according to the gene fragment of these enzymes in *A. cristatum*. To standardize the results, the relative abundance of *β-actin* was used as the internal standard. The relative expressions of target genes were analyzed by *GeneTools* from *SynGene*. The phosphorylation status of MEK1/2 was done by Western blot according to Shan *et al.* (2011). To standardize the results, the expression of *β-actin* was used as the internal standard. The relative amount of phospho-MEK1/2 was analyzed by *Gel-pro* analyzer from *Media Cybernetics* (Bethesda, USA).

The presented results are the means of five replicates. Means were compared by one-way *ANOVA* and Duncan's multiple range test at the 5 % level of significance.

The effects of 0, 50, 100, 150, and 200 μM SNP on the content of AsA, total ascorbate, GSH and total glutathione were investigated and 100 μM SNP was a the most suitable concentration for this study. Compared with the control, 100 μM SNP increased the amount of the phospho-MEK1/2 (Table 2), the transcription and activities of APX, GR, DHAR, MDHAR, γ-ECS, and GalLDH (Tables 2 and 3), and the content of AsA, GSH, total ascorbate, and total glutathione (Table 3) in

Table 1. Effects of different SNP concentrations on the content [μmol g^{-1}(f.m.)] of AsA, total ascorbate, GSH, and total glutathione. The plants were exposed to SNP for 24 h. Means ± SEs, $n = 5$; different letters indicate significant differences at $P \leq 0.05$.

SNP [μM]	AsA	Total ascorbate	GSH	Total glutathione
0	4.05±0.36d	4.25±0.50d	0.22±0.02d	0.24±0.03d
50	6.10±0.69b	6.44±0.48b	0.35±0.03b	0.38±0.03b
100	7.89±0.71a	8.35±0.99a	0.43±0.04a	0.46±0.05a
150	6.20±0.55b	7.70±0.62b	0.34±0.04b	0.36±0.04b
200	4.88±0.61c	5.68±0.68c	0.28±0.03c	0.32±0.04c

Table 2. Effect of 100 μM sodium nitropruside (SNP) and pre-treatment with MEK1/2 kinase inhibitors 50 μM PD98059 or 10 μM U0126 on the relative expressions of *APX*, *GR*, *DHAR*, *MDHAR*, *γ-ECS*, and *GalLDH* genes. *β-actin* was used as internal standard. The plants were pre-treated with PD98059 and U0126 for 8 h, and then exposed to SNP for 24 h. Means ± SEs, $n = 5$; different letters indicate significant differences at $P \leq 0.05$.

Genes	Control	SNP	PD98059 + SNP	U0126 + SNP
APX	0.17±0.03b	0.68±0.08a	0.07±0.01c	0.09±0.02c
GR	0.25±0.04b	0.63±0.07a	0.28±0.03b	0.19±0.03c
DHAR	0.02±0.00b	0.25±0.04a	0.03±0.01b	0.02±0.00b
MDHAR	0.02±0.00b	0.30±0.05a	0.05±0.01b	0.04±0.01b
γ-ECS	0.20±0.04b	0.72±0.09a	0.10±0.02c	0.12±0.02c
GalLDH	0.24±0.04b	0.43±0.06a	0.40±0.05a	0.45±0.08a

A. cristatum leaves. Pre-treatment with PD98059 and U0126 significantly prevented the increases in the transcriptions and activities of APX, GR, DHAR, MDHAR and GalLDH, the transcription of γ-ECS, and the content of AsA, GSH, total ascorbate, and total glutathione induced by SNP. The pre-treatment with PD98059 and U0126 did not reduce the activity of γ-ECS induced by SNP.

Many studies have reported that NO has an important role in the regulation of ascorbate and glutathione metabolism in plants under stress (Ai *et al.* 2008, Shan *et al.* 2012, 2015). Zhang *et al.* (2007) reported that NO increased the transcription and activity of APX and GR in maize leaves. Hermes *et al.* (2013) reported that NO could increase the transcription of γ-ECS and APX in maize leaves. Hasanuzzaman *et al.* (2011) showed that NO could regulate the ascorbate and glutathione metabolism through changes in GR, DHAR, and MDHAR activities in wheat seedlings. Our previous study reported that endogenous NO could increase the activities of APX, GR, DHAR, MDHAR, GalLDH and γ-ECS, and the content of AsA, GSH, total ascorbate and total glutathione (Shan *et al.* 2012, 2015). In this study, our results also showed that NO donor SNP had the same effects. It has been documented that the content of NO increases under various stresses, such as cadmium stress and water stress (Shan *et al.* 2012, Alemayehu *et al.* 2015). Therefore, NO has an important role in responses to stresses through the regulation of the ascorbate and glutathione metabolism.

The MAPK cascade plays an important role in plants (Moon *et al.* 2003, Xiong and Yang 2003). Our results showed that MEK1/2 was probably involved in the increases in the content of AsA, GSH, total ascorbate, and total glutathione induced by NO. MEK1/2 could be a signal for AsA synthesis through the activation of GalLDH as well as AsA regeneration through the activation of DHAR and MDHAR. Besides, our results showed that MEK1/2 could affect GSH regeneration through the activation of GR. For GSH synthesis, MEK1/2 was involved in the regulation of the transcription of *γ-ECS*, but not the γ-ECS activity.

It has been documented that H_2O_2, NO, and MEK1/2 are involved in the signal transduction of JA in regulating the ascorbate and glutathione metabolism (Shan *et al.* 2011, 2015, Dai *et al.* 2015, Shan and Yang 2017). Our previous study showed that JA could induce the activation of MEK1/2 by increasing its phosphorylation, which, in turn, resulted in the up-regulation of the content of ascorbate and glutathione through the increases in the transcriptions and activities of APX, GR, MDHAR, DHAR, and GalLDH (Shan *et al.* 2011). Dai *et al* (2015)

Table 3. Effects of 100 μM SNP and pretreatment with MEK1/2 kinase inhibitors 50 μM PD98059 or 10 μM U0126 on the the relative amount of phospho-MEK1/2, activities of enzymes involved in ascorbate and glutathione metabolism, and the content of AsA, GSH, total ascorbate, and total glutathione. The plants were pretreated with PD98059 or U0126 for 8 h and then exposed to SNP for 24 h. Means ± SEs, $n = 5$; different letters indicate significant differences at $P \leq 0.05$.

Parameters	Time [h]	Control	SNP	PD98059 + SNP	U0126 + SNP
phospho-MEK1/2	12	0.22±0.03c	0.28±0.04b	-	-
	24	-	0.36±0.04a	-	-
APX [μmol mg^{-1}(protein) min^{-1}]	12	1.90±0.23c	4.42±0.38a	2.42±0.25b	2.22±0.20b
	24	1.67±0.15c	4.63±0.46a	2.00±0.18b	2.00±0.23b
GR [μmol mg^{-1}(protein) min^{-1}]	12	1.00±0.13c	3.00±0.33a	1.62±0.18b	1.62±0.17b
	24	1.25±0.11c	2.51±0.21a	1.35±0.12b	1.33±0.13b
DHAR [μmol mg^{-1}(protein) min^{-1}]	12	2.95±0.25b	4.90±0.53a	2.81±0.31b	2.52±0.21b
	24	2.66±0.28b	4.37±0.38a	2.33±0.19b	2.32±0.26b
MDHAR [μmol mg^{-1}(protein) min^{-1}]	12	1.65±0.17c	3.56±0.29a	2.28±0.20b	2.20±0.21b
	24	1.53±0.13c	3.84±0.41a	2.20±0.23b	2.30±0.18b
GalLDH [nmol mg^{-1}(protein) min^{-1}]	12	8.05±0.90c	15.00±1.38a	11.39±1.25b	11.90±1.28b
	24	10.00±0.93c	16. 15±1.85a	12.70±1.31b	12.22±1.52b
γ-ECS [nmol mg^{-1}(protein) min^{-1}]	12	2.73±0.27b	4.00±0.37a	4.34±0.40a	3.95±0.45a
	24	2.85±0.34b	4.35±0.44a	3.96±0.35a	3.80±0.34a
AsA [μmol g^{-1}(f.m.)]	12	3.02±0.27c	6.55±0.75a	5.00±0.42b	4.35±0.38b
	24	2.81±0.33c	6.02±0.61a	4.26±0.48b	4.00±0.33b
GSH [μmol g^{-1}(f.m.)]	12	0.18±0.02c	0.42±0.05a	0.29±0.04b	0.26±0.03b
	24	0.16±0.02c	0.38±0.04a	0.26±0.03b	0.24±0.03b
Total ascorbate [μmol g^{-1}(f.m.)]	12	3.15±0.37c	7.45±0.81a	5.49±0.57b	5.00±0.42b
	24	2.90±0.26c	7.80±0.93a	4.65±0.51b	4.42±0.47b
Total glutathione [μmol g^{-1}(f.m.)]	12	0.21±0.03c	0.46±0.04a	0.32±0.04b	0.29±0.03b
	24	0.18±0.02c	0.41±0.05a	0.29±0.03b	0.28±0.03b

reported that H_2O_2 could activate MEK1/2 in the signal transduction of JA in regulating the ascorbate and glutathione metabolism. In this study, we confirmed that NO could activate MEK1/2 and so regulate the ascorbate and glutathione metabolism. Besides, it has been documented that H_2O_2 and NO are involved in the signal transduction of abscisic acid (ABA) in regulating the ascorbate and glutathione metabolism (Zhang *et al.* 2007). However, whether MEK1/2 is involved in the signal transduction of ABA is still unknown.

In conclusion, our results suggested that MEK1/2 participated in the signal transduction of NO in regulating the ascorbate and glutathione metabolism. This knowledge contributes to elucidation of the regulation of the metabolism of antioxidants in plants by NO.

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