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Cadmium stress tolerance in wheat seedlings induced by ascorbic acid was mediated by NO signaling pathways

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ABSTRACT

Ascorbic acid (AsA) and nitric oxide (NO) are well known and widespread antioxidants and gaseous molecules that regulate plant tolerance to several stresses. However, the relationship between them in plant response to stress, especially heavy stress, is largely unclear. This study demonstrated that both AsA and NO could enhance the tolerance of wheat seedlings to cadmium stress evidenced by root length change, which resulted from their roles in maintaining the balance in reactive oxygen species (ROS) and reducing the absorption of Cd. Furthermore, exogenous AsA led to a significant increase of NO content and endogenous AsA content in wheat roots, which could be weakened by the NO scavenger c-PTIO. In addition, c-PTIO also inhibits the NO-induced production of endogenous AsA. Although the AsA synthesis inhibitor lycorine significantly inhibited the inductive effect of exogenous AsA on endogenous AsA production, it has little effect on NO content. In addition, we found that the protective effects of NO and AsA on Cd stress were removed by c-PTIO and lycorine. These results indicated that NO accumulation could be necessary for exogenous AsA-induced cadmium tolerance and endogenous AsA production, and the exogenous AsA-induced endogenous AsA production was likely mediated by NO signaling pathways and together they induced the tolerance of wheat to cadmium stress.

1. Introduction

A high cadmium content in soils can affect the growth of plants and the yield of certain crops in different regions. Thus, it is a frequent constraint to agriculture in several countries (Clemens, 2006). The accumulation of cadmium in plants can cause the occurrence of oxidative stress and damage, including the inhibition of root and shoot growth and the reduction of chlorophyll content, which may result from the excessive accumulation of reactive oxygen species (ROS) (Wang et al., 2007; Sharma and Dietz, 2009; Zhang et al., 2009; Feng et al., 2010; Shi et al., 2010; Song et al., 2011). Certain antioxidant enzymes, such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and catalase (CAT), as well as several antioxidants (ascorbic acid, AsA and reduced glutathione, GSH), are used by plants to cope with oxidative stress (Amudha and Balasubramani, 2010; Foyer and Noctor, 2011). It has been shown that some stresses, such as chilling, drought, salt and UV–B stress, up-regulated or down-regulated antioxidant enzymes and decreased the contents of AsA and GSH (Chao et al., 2010; Kostopoulou et al., 2015; Liu et al., 2014; Sun et al., 2014; Shukla et al., 2015). In addition, the exogenous addition of AsA enhances the resistance to certain stresses including drought, high salt and ozone in different plants (Guo et al., 2005; Shalata et al., 2001). More detailed and accurate evidences were presented using *Arabidopsis* AsA-deficient (*vtc1*) mutants. Mutant *vtc1* was hypersensitive to ozone, UV radiation and salt stresses and so on (Filkowski et al., 2004; Gao et al., 2008; Huang et al., 2005). Although the roles of AsA in protecting against oxidative stress under different stress conditions were extensively studied, there are no reports about the signal node of AsA in protecting certain plants against stress injury.

Nitric oxide (NO) acts as a signal molecule in higher plants, it mediates a large number of physiological processes such as growth and development (Arasimowicz and Floryszak-Wieczorek, 2007). In addition, the responses and tolerance of plants to certain abiotic stresses were also reported to be associated with NO production, which depends on two key enzymes (NR and NOS) in plants. Several stress factor treatments and hormones induced an endogenous NO increase, which might be essential for stress sensing, signal transduction, and activation of adaptive stress response

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(Zhao et al., 2008; Song et al., 2006; Lopez-Carrion et al., 2008; Singh et al., 2008). Numerous reports in plants demonstrated that NO might serve as a key secondary messenger in stress response, and it could act as a downstream target of other signal molecules or activate downstream signal transduction. Likewise, certain interactions between NO and some molecules, including SA (Zottini et al., 2007), ABA (Guo et al., 2003), H_2O_2 (Bright et al., 2006) and auxin (Kolbert et al., 2008), were reported in several studies. However, whether NO can act as the downstream target of AsA in plant stress response is still unclear. Application of SNP (NO donor) could alleviate some adverse effects caused by several stresses (Tossi et al., 2011, 2012; Zhao et al., 2004, 2007, 2009) by improving the activities of SOD, CAT, APX and POD and subsequently decrease the ROS accumulation. However, whether NO can enhance the tolerance of wheat to Cd stress is not yet well known. In the present work, we address the possible relationships between AsA and NO and how exogenous AsA affects the tolerance of wheat under Cd stress, thereby providing evidence of the involvement of AsA in alleviating the oxidative stress during the growth of wheat seedlings.

2. Materials and methods

2.1. Plant material and chemical treatments

Wheat (*Triticum aestivum*, cv Longchun 27) seeds were purchased from Gansu Agricultural Academy. The seeds were surface-sterilized with 0.1% (w/v) $HgCl_2$ for 10 min and germinated for 24 h at 25 °C under dark conditions. Then, the germinated seeds were cultivated in the 1/4 Hoagland solution (pH 6.0) at 23 ± 1 °C with a 16/8 h light/dark photoperiod, and the light density was 200 μmol m^{-2} s^{-1}. The Hoagland solution was updated every two days. The relative humidity was approximately 70%. The 3-DAYS-old seedlings were treated with different concentrations of $CdCl_2$ in the nutrient solution for Cd stress experiments. To examine the role of AsA and NO in wheat tolerance to Cd stress, the seedlings of wheat were pre-treated with the AsA (1 mM, Sigma), NO scavenger 2-(4-carboxyphenyl) − 4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 100 μM, Sigma), nitrate reductase (NR) inhibitor tungstate (100 μM, Sigma), nitric oxide synthase (NOS) inhibitor L-NAME (100 μM, Sigma) and an NO donor sodium nitroprusside (SNP, 200 μM, Sigma) for 6 h before exposure to 100 μM $CdCl_2$ for the indicated time under the same conditions as described earlier. Each experiment was repeated at least three times.

2.2. Lipid peroxidation assay

The lipid peroxidation was measured following the method of Hodges et al. (1999) with some modifications. Approximately 0.5 g of sample was homogenized in 10% trichloroacetic acid (TCA) at 4 °C. After centrifugation at 3000 g for 15 min, the supernatant was collected and incubated with an equal volume of 0.5% thiobarbituric acid (TBA) 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 non-specific turbidity. The amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.3. Ascorbate acid content determination

The ascorbate acid content was determined according to the method described by Foyer et al. (1983). In detail, 0.5 g wheat roots were ground in 2 mL 1 M $HClO_4$, and then centrifuged at 12,000 g at 4 °C for 15 min, and the supernatant was neutralized to pH 5.6 with saturated K_2CO_3; the assay mixture contained 100 mM sodium-phosphate buffer, pH 5.6, 1 unit of ascorbate oxidase and 100 μL above saturated supernatant. The absorbance changes at 265 nm were recorded and used to assay the ascorbate acid content.

2.4. Determination of H_2O_2 content

The H_2O_2 content was determined according to the method of Veljovic-Jovanovic et al. (2002). Briefly, 0.5 g wheat roots were ground in 2 mL $HClO_4$ including 5% PVP, and then centrifuged at 12,000 g for 10 min. The supernatant was neutralized with 5 M K_2CO_3 to pH 5.6 and centrifuged again for 1 min. The obtained sample was incubated with 1 U ascorbate oxidase and, subsequently, assayed for the H_2O_2 content. The reaction mixture consisted of 0.1 M pH 5.6 phosphate buffer, 3.3 mM DMAB, 0.07 mM MBTH, 0.3 U POX and 200 μL sample. The absorbance changes at 590 nm were recorded. The H_2O_2 content was calculated from a standard curve.

2.5. Growth and chlorophyll content analysis

The growth of the plants was determined by measuring the shoot length and root length. After treatment, the length of the shoot and root were measured with a ruler using 15–20 plants per treatment at the indicated time. The chlorophyll was extracted and measured according to the method of Porra et al. (1989). Approximately 0.5 g of fresh roots were ground to powder by adding a spot of quartz sand and then placed in 5 mL acetone (80%) and incubated at 4 °C until the samples turned white. The samples were transferred into a 25 mL container, at the same time washing the mortar and the draffy repeatedly. Finally, the volume of the samples was fixed using 25 mL of 80% acetone and filtrated. The absorbance was measured under the wavelengths of 663, 645, 652 nm.

2.6. Determination of the Cd content

The sampled plants were washed with deionized water, dried at 80 °C in an oven to a constant weight, and then weighed. The dried plant tissues were then prepared for the Cd concentration measurement. Approximately 0.1 g of dry sample was mixed with HNO_3 and digested in a microwave digestion instrument. The solution was used to measure the Cd concentration with ICP-MS.

2.7. Determination of NO by hemoglobin

The NO content was assayed by monitoring the conversion of oxyhaemoglobin (HbO_2) to methaemoglobin (MetHb) as described by Murphy and Noack (1994). The method is based on the direct reaction between NO and HbO_2, which yields MetHb. Briefly, 0.5 g of wheat roots were incubated with 100 units of CAT and 100 units of SOD for 5 min to remove endogenous ROS before adding 10 mL of 5 mM HbO_2. After incubation for 5 min, NO was calculated by measuring the conversion of HbO_2 to MetHb spectrophotometrically at 401 and 421 nm, using an extinction coefficient of 77 mM^{-1} cm^{-1}.

2.8. Determination of NR and NOS activity

The activity of NR and NOS was determined as described by Tian et al. (2007) and Lin et al. (2012), respectively. Briefly, 0.2 g of wheat roots were ground in 2 mL of extraction buffer (50 mM Hepes-KOH, pH 7.5, 5% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine and 10 μM FAD), and then centrifuged at 15,000 g for 20 min. The reactions started by the addition of