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Amelioration of cold-induced oxidative stress by exogenous 24-epibrassinolide treatment in grapevine seedlings: Toward regulating the ascorbate–glutathione cycle

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ABSTRACT

Ascorbate-glutathione (AsA-GSH) cycle plays a key role in tolerance of plant under biotic or abiotic stress. However, little is known regarding the role of brassinolide in AsA-GSH cycle in grape under chilling stress. In this study, a wine grape cultivar, Cabernet Sauvignon (*Vitis vinifera*), was subjected to chilling stress for different time points after treated by 24-Epibrassinolide (EBR). The result showed EBR plays a positive role in amelioration of leaf morphology and root growth under chilling stress. In terms of physiological aspect, EBR treatment reduce the O_2^- and H_2O_2 contents than that in the control. This is primarily related to the improved antioxidant system and the regulation of EBR to AsA-GSH cycle. There was an obvious increase in superoxide dismutase (SOD) activities in EBR treatment compared with in the control, and similar result also was observed in level of AsA, and AsA/DHA. Alleviation of EBR to chilling-stressed injury was also reflected in glutathione (GSH) and GSH/GSSG, as high levels of these parameters were observed in EBR treatment. Furthermore, ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbic acid reductase (DHAR) and monodehydroascorbate reductase (MDHAR) also showed elevated activities in the EBR treatment. Interestingly, the regulation of EBR to these components in AsA-GSH cycle showed a difference in time course, suggesting that the cycle might be affected by EBR at earlier and middle term of chilling stress. Our work will provide the information for understanding the role of EBR in AsA-GSH cycle.

1. Introduction

Abiotic or biotic stress are often unfavorable for growth and development of plants (Zhu, 2016). For example, under severe chilling stress, plant will suffer undesirable morphological, physiological, biochemical and molecular damages as they are sessile and incapable of long distance migration (Pedmale et al., 2016). Generally, the stress can cause the excessive accumulation of reactive oxygen species (ROS) thus breaking the steady-state of ROS in normal conditions (Foyer and Noctor, 2016). ROS often are considered to be unfavorable byproducts that are highly reactive with membrane lipids, protein, and DNA (Choudhury et al., 2017). In order to mitigate the chilling damage, plants have developed several protective mechanisms, including ascorbate-glutathione (AsA-GSH) cycle, which improves tolerance of plant to oxidative stress (Mittler et al., 2004).

The AsA-GSH cycle is an antioxidant system of great importance that can regulate the oxidative and reductive environment by modulating glutathione/glutathione disulfide (GSH/GSSG) and ascorbate/dehydroascorbate (AsA/DHA) interconversion in chloroplasts, mitochondria, peroxisomes, and the cytoplasm (Ogawa, 2005; Palma et al., 2006). In this cycle, AsA is oxidized to a monodehydroascorbate (MDHA) by ascorbate peroxidase (APX) to scavenge hydrogen peroxide (H_2O_2). However, MDHA is not stable that will turn into AsA by monodehydroascorbateaccelerator reductase (MDHAR) or dehydroascorbate (DHA) spontaneously. By dehydroascorbate reductase (DHAR), DHA uses reduced glutathione (GSH) as substrate to produce oxidized glutathione (GSSG) that itself turns back into AsA. Also, GSH could be recycle from GSSG by glutathione reductase (GR) (Ma et al., 2008; Wang et al., 2012a). Thus, ROS resulting in damage to macromolecules are usually minimized by the regulation of AsA-GSH cycle

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; AsA-GSH, ascorbate-glutathione; BRs, brassinosteroids; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EBR, 24-epibrassinolide; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NADH, nicotinamide adenine dinucleotide; O_2^-, superoxide radical; ROS, reactive oxygen species; SOD, superoxide dismutase

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(Singh et al., 2015), and above-mentioned enzymes in this cycle are of importance in determining plant tolerance to oxidative stress (Eltayeb et al., 2006).

Grape (*Vitis vinifera* L.) is one of the most cultivated fruit trees and grown widely throughout the world (Algarra Alarcon et al., 2015). In China, however, because of the continental climate, the grape planting in northwest China often faces the threatens to winter cold and spring frost. Since plant hormones were reported to play an important role in response to chilling stress, hormone treatment is one of the effective ways to alleviate the chilling damage (Wang and Li, 2006). Brassinosteroids (BRs) are a type of steroidal compounds, which occur in free form and conjugated to sugars and fatty acids. BR presents in almost all organisms of the plant (fruits, seeds, leaves, etc.) and is considered a novel plant hormone, which plays a vital role in the growth and development of plants (Bajguz and Hayat, 2009). Previous study have reported that BR can mediate tolerance of plant to biotic and abiotic stress (Anjum et al., 2011; Bajguz and Hayat, 2009; Kim et al., 2013; Li et al., 2012; Xi et al., 2013). Exogenous BRs could elevate redox state of AsA and GSH under oxidative stress, which improves the tolerance of plants to damaged effects and leads to enhancement of plant defense (Talaat et al., 2015). This indicated that there was a close relationship between BRs and the AsA-GSH cycle. However, little is known regarding the role of BRs in plant defense affected by AsA-GSH cycle in grape under chilling stress. Therefore, regulatory mechanism of BRs to the cycle is important to enhance the tolerance of the grapevines to chilling stress.

Our previous study observed that exogenous EBR improved the tolerance of young grapevine plants to short-term chilling stress with an increase of the antioxidation defense system and osmoregulation substances in the leaves after the treatments (Xi et al., 2013). In this study, the role of BRs in the response of AsA-GHS cycle in grape seedlings under chilling stress is investigated. Our Object is to study the effect of BRs on AsA-GSH cycle in grape. This work will provide an insight in the mechanism by which BRs regulate AsA-GSH process in grape and provide theoretical basis for promoting cold resistance of grape.

2. Materials and methods

2.1. Plant materials and treatments

One-year-old cuttings of wine grape cultivar, Cabernet Sauvignon (*Vitis vinifera*), were collected from a vineyard at the Northwest A&F University, Shaanxi, China. The rhizogenic cuttings were gown in pots containing a mixture of 50% sand and 50% perlite for 12–15 weeks. Young grape seedlings with 7 ~ 10 functional leaves were chosen and divided into two groups. One group was sprayed by 0.1 mg / L EBR-ethanol solution with 1% Tween-80 for 6 days, while the other group as the control was treated by water for 6 days. Later, the two groups were transferred to an illumination incubator with 4 °C low temperature, 75% relative humidity and 500 μmol m^{-1} s^{-1} and a 12 h day/night cycle for 6 h, 12 h, 24 h, 48 h and 72 h.

2.2. Root morphology measurement

Root was sampled and washed off the dust on root surface with double distilled water, while root morphology including root length, root diameter and root volume were measured using root scanner (v39, EPSON, Japan).

2.3. Measurement of contributed parameters

The 2nd to 8th leaves of grape seedlings were sampled to determine these contributed parameters including H_2O_2 level, O_2^- producing rate, activities of antioxidant enzymes, antioxidant content.

2.3.1. Measurement of H_2O_2 level and O_2^- producing rate

The contents of H_2O_2 was determined using the kit of Cominbio company (Cominbio, Jiangsu, China).

The O_2^- producing rate was measured based on the method described by Wang and Luo (1990) with a slight modification. Leaf (0.2 *g*) was homogenized in 2 mL potassium phosphate buffer (50 mmol/L, pH 7.8) and centrifuged at 10,000 rpm for 10 min at 4 °C. The 0.2 mL supernatant was taken and mixed with incubation solution (0.2 mL potassium phosphate buffer (50 mmol/L, pH 7.8), 0.2 mL hydroxylamine hydrochloride (10 mmol/L)). After incubated at 25 °C for 1 h, the mixture was mixed with 0.2 mL sulphanilic acid (17 mmol/L) and 0.2 mL α-naphthylamine (7 mmol/L) for 20 min. Then the absorbance of the reaction solution was measured at 530 nm A standard curve with $NaNO_2$ was used to calculate the production rate of O_2^- (μg g^{-1}).

2.3.2. Measurement of activity of antioxidant enzymes

For activity of antioxidant enzymes, leaves (0.5 *g*) was homogenized in 5 mL phosphate buffer (5 mM, pH7.0) (1% polyvinylpyrrolidone, 1 mM ethylenediaminetetraacetic acid and 5 mM AsA). The homogenate was centrifuged at 10,000 rpm for 25 min, and the supernatant was used as the source of enzymes for measurement. The experiment was carried out at 4 °C.

The activity of SOD was determined by the method of Beauchamp and Fridovich (1971) with a slight modification. The reaction mixture (50 mM phosphate buffer (pH7.8), 130 mM methionine, 750 μM nitroblue tetrazolium, 20 μM riboflavin, 100 μM Ethylene Diamine Tetraacetic Acid and 25 μL enzyme extraction. The dark reaction was conducted in the black circumstance after riboflavin was added to the reaction mixture, while the color reaction was carried out under a 4000 lx fluorescent at 30 °C for 20 min. The absorbance was measured at 560 nm and one unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of nitroblue tetrazolium

The APX activity was measured by the method of Nakano and Asada (1981) with a slight modification. The reaction mixture contained 50 mM potassium phosphate buffer (pH7.0), 0.1 mM Ethylene Diamine Tetraacetic Acid, 0.2 mM H_2O_2, 15 mM ascorbic acid (AsA) and 0.2 mL enzyme extraction. The reaction was carried out when 25 μL AsA (15 mM) was added. The result was calculated using the change of the absorbance at 290 nm in 0 ~ 5 min.

The activities of MDHAR and GR were determined by the method of Krivosheeva et al. (1996), while method of DHAR activity was determined from description of Nakano and Asada, (1981). MDHAR activity was assayed at 340 nm in 1 mL reaction mixture (2 mmol L^{-1} ascorbate, 2 mmol L^{-1} nicotinamide adenine dinucleotide (NADH), ascorbate oxidase (2 units) and 100 μL enzyme extraction). The reaction was carried out by adding AsA oxidase.

DHAR activity was determined by monitoring the increase in absorbance at 265 nm owing to AsA formation. 1.5 mL reaction solution contained 50 mM phosphate buffer (pH 7.0), 2.5 mM GSH, 0.1 mM Ethylene Diamine Tetraacetic Acid, 0.2 mM DHA, and 50 μL enzyme extract. The reaction was initiated by adding DHA.

GR activity was assayed by monitoring the decrease in absorbance at 340 nm owing to reduced NADH oxidation. The 1 mL reaction mixture contained 50 mM phosphate buffer, pH 7.6, 0.4 mM Ethylene Diamine Tetraacetic Acid, 0.2 mM NADH, 0.5 mM GSSG and 75 μL enzyme extract. The reaction was initiated by adding NADH.

2.3.3. Extraction of antioxidant metabolites

The leaves (0.5 *g*) was ground with a mortar and pestle in precooling 5% sulfosalicylic acid and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was used for assays of total AsA and reductive-form AsA contents. The total AsA content was measured according to the method of Kampfenkel et al. (1995) with some slight modification. The reaction mixture containing (300 μL extracted sample solution, 1.84 M triethanolamine, 50 mM, pH7.5 phosphate buffer with 2.5 mM Ethylene