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Research article

Beneficial effects of melatonin in overcoming drought stress in wheat seedlings

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ABSTRACT

Melatonin plays an important role in abiotic stress in plant, but its role in wheat drought tolerance is less known. To verify its role, wheat seedlings (*Triticum aestivum* L. 'Yan 995') at 60% and 40% of field capacity were treated with 500 μM melatonin in this study. Melatonin treatment significantly enhanced the drought tolerance of wheat seedlings, as demonstrated by decreased membrane damage, more intact grana lamella of chloroplast, higher photosynthetic rate, and maximum efficiency of photosystem II, as well as higher cell turgor and water holding capacity in melatonin-treated seedlings. Besides, melatonin markedly decreased the content of hydrogen peroxide and superoxide anion in melatonin-treated seedlings, which is attributed to the increased total antioxidant capacity, GSH and AsA contents, as well as enzyme activity including ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), and glutathione transferase (GST). The GSH-AsA related genes including *APX*, *MDHAR*, and *DHAR* were commonly upregulated by melatonin and correlated to the antioxidant enzyme activity as well as the content of GSH and AsA, indicating that the increase of GSH and AsA was attributed to the expression of these genes. Our result confirmed the mitigation potential of melatonin in drought stress and certain mechanisms of melatonin-induced GSH and AsA accumulation, which could deepen our understanding of melatonin-induced drought tolerance in wheat.

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1. Introduction

Wheat is one of the most important food crops worldwide and its production is affected by drought stress (Kosová et al., 2016). A common effect of drought stress is the excessive accumulation of reactive oxygen species (ROS) (Smirnoff, 1998). Since photosynthesis and respiration generate ROS, excessive accumulation of ROS causes oxidative damage to proteins, DNA, RNA, and enzyme activity (Mittler, 2002). To eliminate excessive ROS in cell, enzymatic antioxidants, including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), non-enzymatic antioxidants, including glutathione (GSH) and ascorbate (AsA), as well as vitamins, polyphenols, carotenoids have evolved in plants (Apel and Hirt, 2004). Especially, glutathione-ascorbate (GSH-AsA) cycle, including GSH and AsA and related antioxidant enzymes (APX, DHAR, MDHAR, and GR) which participate in GSH and AsA renovation, play an important role in ROS homeostasis and stress tolerance including drought stress (Apel and Hirt, 2004).

Many studies reported that melatonin increases the drought tolerance and plays multiple roles in plant including delaying leaf senescence, regulating water balance, promoting lateral root formation and seed germination, maintaining the integrity of leaf and chloroplast, modulating nitro-oxidative homeostasis and proline metabolism (Antoniou et al., 2017; Li et al., 2015; Meng et al., 2014; Wang et al., 2013; Wei et al., 2015). Specifically, the increased antioxidant capacity and high ROS homeostasis were related to melatonin content in drought-stressed plants, including apple (Li et al., 2015; Wang et al., 2013), cucumber (Zhang et al., 2013), grape (Meng et al., 2014), *Arabidopsis* (Zuo et al., 2014), tomato (Liu et al., 2015), Bermuda grass (Shi et al., 2015), soybean (Wei et al., 2015), rice (Li et al., 2016) and *Medicago sativa* (Antoniou et al., 2017). Tolerance to drought stress was initially attributed to the antioxidant capacity of melatonin, as evidenced by its direct interaction with ROS (Allegra et al., 2003). Secondly, melatonin can modulate the activity of antioxidant enzymes and antioxidants in

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response to excessive ROS (Rodriguez et al., 2004). GSH and AsA were also largely increased by melatonin under drought stress in various plants (Liu et al., 2015; Meng et al., 2014; Shi et al., 2015; Wang et al., 2012). Although Turk et al. (2014) and Ye et al. (2015) reported that melatonin can improve resistance to cold stress in wheat seedlings and polyethylene glycol (PEG) stress, the role of melatonin in wheat response to stress is still less well known. Furthermore, regulation of the GSH-AsA cycle influenced by melatonin has not been reported in wheat response to drought stress.

The objective of this study was to investigate the effects of melatonin in improving drought tolerance and to analyze the mechanism of increased drought tolerance induced by melatonin. Here, we were able to show the photosynthetic performance, the microstructure of leaf and chloroplast, the ROS level, and the membrane damage of wheat seedlings under drought stress. Specifically, we analyzed the content of GSH and AsA, the enzyme activity of GSH-AsA cycle, and the genes expression of related enzyme in GSH-AsA cycle to highlight the exact mechanism of melatonin-induced antioxidant properties. All of these observations will be beneficial for further understanding the biological function of melatonin in wheat.

2. Material and methods

2.1. Plant material and experimental treatments

Seeds of wheat (*Triticum aestivum* L. 'Yan 995') induced to germinate were planted in black plastic pots (15 cm × 20 cm) containing 1.7 kg mixture of farmland topsoil/sand/grass peat (1:1:2, v:v:v) (pH, 7.65; organic matter, 43.97 g/kg; available N, P, and K, 55.22, 31.67, 73.87 g/kg; maximum field capacity, FC, 29.3%). These pots were placed in a phytotron with a light/dark cycle (20/15 °C, 14/10 h) under a relative humidity of 70± 5% at Northwest A&F University, Yangling, China. The soil moisture in those plots were maintained at 80% of FC for 21 days by adding lost water and fifteen plants with same growth vigor were maintained for treatment. Thereafter, the watering was adjusted by adding 20 mL melatonin solution (500 μM, treatment group) or distilled water (control group) after dark every other day until soil moisture was down to 40% of FC. The concentration of melatonin was calculated in a simple experiment. When the soil moisture is at 80%, 60% and 40% of FC, the top third leaf of seedlings were harvested 5 h after dark because of the high photosensitivity of melatonin (Boccalandro et al., 2011), quickly frozen with liquid nitrogen, and stored at −80 °C. To ensure the accuracy of determination, fifteen leaves were pooled for one biological repeat and five biological repeats were used.

2.2. Photosynthetic gas exchange and chlorophyll fluorescence

For measuring photosynthetic gas exchange parameters, the third leaf from top was prepared and experiment started at 9:00 h. The photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate were measured using LI-6400XT (LI-COR, USA) with light 500 μmol m^{-2} s^{-1}. For measuring fluorescence parameters, the third leaf from top was placed in dark for 30 min. The maximum potential efficiency of photosystem II (Fv/Fm) was measured using Dual-PAM-100 (Walz, Germany).

2.3. Leaf structure and chloroplast damage

Damage to chloroplast was observed using a transmission electron microscope (Hitachi, Japan). Fresh leaf sample was fixed at 4% glutaraldehyde (in 0.2 M phosphate buffer [pH 6.8]) for 6 h, followed by rinsing with phosphate buffer and fixation with 0.1 M osmic acid, and rinsing again and dehydrating tissue samples with ethyl alcohol. This was followed by permeation, embedding and slicing ultra-thin sections according to Meng et al. (2014).

The leaf structure was observed using a paraffin section according to Kothar and Varshney (1998). About 0.5 mm × 0.5 mm section of the middle part of seedling leaf was fixed in FAA fixative for 24 h and was dehydrated through a series of ethanol washes (30%, 50%, 70%, 85%, 95%, and 100%). After dehydration, the leaf sample hyalinized in xylene gradient (50% xylene in ethanol, pure xylene, and pure xylene) for 1 h. Next, the sample was immersed overnight in 50% liquid paraffin in xylene at 37 °C. Next day the sample was transferred to an incubator at 59 °C and the paraffin wax was changed three times. Finally, leaf tissue was embedded in paraffin and 15 μm tissue sections were used for general microscopy.

2.4. Water content and antioxidant capacity

The fresh weight (*FW*) was measured immediately after harvesting, and then was dried in an oven at 105 °C for 15 min. Thereafter the sample was dried at 80 °C until weight remained constant (*DW*). Leaf water content (*WC*) was calculated using the equation:

$$WC = \frac{(FW - DW)}{FW} \times 100\%.$$

The ferric reducing ability of plasma (FRAP) method was used for determining antioxidant capacity according to Benzie and Strain (1996). Nearly 0.1 g leaf sample were ground in 2 mL deionized water and centrifuged at 15 000 × g, 4 °C for 10 min. The supernatant was used for determining antioxidant capacity. The FRAP reagent contained 0.3 M NaAc-HAc buffer (pH 3.6), 10 mM tripyridyltriazine (TPTZ, in 40 mM HCl), and 20 mM $FeCl_3$ (10/1/1, v/v/v). Reaction solution including 2.4 mL FRAP reagent and 100 μL supernatant was mixed and incubated at 37 °C for 10 min. The absorbance at 593 nm was recorded and the result was standardized based on the absorbance of 1.0 mM $FeSO_4$.

2.5. Electrolyte leakage, malondialdehyde (MDA), hydrogen peroxide, and superoxide anion levels

Relative electrolyte leakage was measured according to Dionisio-Sese and Tobita (1998). Five fresh leaves were washed for 3 times with ultrapure water and 50 mL test tubes were prepared. The electrical conductivity of ultrapure water was analyzed using an electrical conductivity analyzer (Thermo Fisher, USA) before immersing the leaf section, and after immersing at 32 °C for 2 h, and after boiling it for 20 min, respectively as EC_0, EC_1, and EC_2. The relative electrolyte leakage (*REL*) was calculated using the formula: $REL = (EC_1 - EC_0)/(EC_2 - EC_0)$.

For MDA, hydrogen peroxide, and superoxide anion, leaf samples (0.3 g) were ground in 5 mL 100 mM pre-cooling phosphate buffer (pH 7.0) and the homogenate was centrifuged at 12 000 g, 4 °C for 15 min. The supernatant was used for subsequent analysis.

For malondialdehyde (MDA), the mixture including 1 mL supernatant and 2 mL thiobarbituric acid (0.6%, w/v) were boiled for 30 min, cooled and centrifuged at 3000 × g for 15 min. The supernatant was used for absorbance measurement at 450, 532, and 600 nm according to Dionisio-Sese and Tobita (1998).

The method of Velikova et al. (2000) was used to quantify hydrogen peroxide. The mixture including 1 mL supernatant and 1 mL KI (1 M) was incubated at 25 °C for 1 h under dark. The

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