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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.

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

absorbance at 390 nm was recorded and the content of hydrogen peroxide was calculated using the standard curve.

Superoxide anion was measured according to Elstner and Heupel (1976) with some modifications. The mixture including 1 mL supernatant and 0.25 mL hydroxylamine hydrochloride (10 mM) was incubated at 25 °C for 20 min. Then 1 mL sulfanilic acid (17 mM) and 1 mL α-naphthylamine (7 mM) were added to the mixture, allowing for reaction at 30 °C for 30 min. The absorbance was measured at 530 nm and the content of superoxide anion was calculated using a standard curve.

2.6. Glutathione and ascorbate

The method of glutathione and ascorbate determination was according to Knörzer et al. (1996) with some modifications. Leaf was homogenized to a fine powder in liquid nitrogen and 50–100 mg samples were extracted with 0.5 mL 5% (w/v) pre-cooled metaphosphoric acid. Centrifugation was carried out at 4 °C, 15 000 × g for 30 min and the supernatant was used for measurement.

For total ascorbate (T-AsA), 200 μL sample extraction, 48 μL triethanolamine (1.84 M), 802 μL phosphate buffer (pH 7.5, 50 mM, 2.5 mM EDTA), and 100 μL dithiothreitol (DTT, 10 mM) were mixed and maintained at 25 °C for 10 min. After all the dehydroascorbic acid was reduced, 100 μL *N*-ethylmaleimide (0.5%, w/v) was added to remove excessive DTT. For the mixtures, 400 μL trichloroacetic acid (10%, w/v), 400 μL phosphoric acid (44%, v/v), 400 μL 2,2-dipyridyl (4% [w/v] in 70% ethanol), and 200 μL $FeCl_3$ (3%, w/v) were added and incubated at 37 °C for 1 h. For the determination of reduced ascorbate (AsA), the DTT and *N*-ethylmaleimide were replaced by 300 μL PBS (pH 7.5, 50 mM, 2.5 mM EDTA). The absorbance at 525 nm was recorded and the content of total AsA and reduced AsA were calculated using a standard curve. The concentration of oxidized ascorbate (DHA) was calculated by subtracting AsA from T-AsA.

For oxidized glutathione (GSSG), 100 μL sample extraction, 100 μL sulfosalicylic acid (5%, w/v), 48 μL triethanolamine (1.84 M), and 100 μL 2-vinylpyridine (10% [w/v] in 70% ethanol) were mixed and incubated at 25 °C for 1 h to mask GSH from derivatization. Then 1412 μL PBS (pH 7.5, 50 mM, 2.5 mM EDTA), 40 μL NADPH (10 mM), and 160 μL 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, 12.5 mM) were added to the mixture and maintained at 25 °C for 10 min. Thereafter, 40 μL glutathione reductase (50 U/mL) was added to start the reaction and the absorbance at 412 nm was monitored. For total glutathione (T-GSH), the 2-vinylpyridine was replaced by 70% ethanol. The calculation of GSSG and T-GSH was based on the standard curve. The concentration of reduced glutathione (GSH) was calculated by deducting GSSG from T-GSH.

2.7. Antioxidant enzyme

For antioxidant enzyme activity, leaf sample (0.1 g) was homogenized in 1.2 mL pre-cooling extraction buffer including 50 mM KH_2PO_4-KOH (pH 7.5), 0.1 mM EDTA, 20% (v/v) glycerine and 2% (w/v) polyvinyl pyrrolidone. The homogenate was incubated at 4 °C for 10 min and centrifuged at 4 °C, 12 000 × g for 15 min, and the supernatant was used for subsequent analysis.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by monitoring the decrease in absorbance at 290 nm according to Hossain and Asada (1984). The reaction system included 50 mM Hepes-KOH (pH 7.6), 0.5 mM AsA, and 1 mM H_2O_2 and 50 μL crude enzyme. H_2O_2 was added to start the reaction at 25 °C.

Monodehydroascorbate reductase (MDHAR, EC, 1.6.5.4) activity was measured at 340 nm according to Miyake and Asada (1992). The reaction system included 0.1 mM NADH, 0.25 mM AsA, 0.3 units of ascorbate oxidase (AO), and 100-μL crude enzyme. The reaction was started by AO at 25 °C.

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured at 265 nm according to Nakano and Asada (1980) and reaction solution included 100 mM Hepes-KOH (pH 7.0), 2.5 mM GSH, 0.2 mM dehydroascorbate (DHA), and 100 μL crude enzyme extraction. The reaction was initiated by DHA at 25 °C.

Glutathione reductase (GR, EC 1.6.4.2) activity was measured at 340 nm using 3 mL reaction solution including 100 mM Tris-HCl (pH 8.0), 1 mM GSSG, 0.2 mM NADPH, and 0.1 mL enzyme extraction by the method of Grace and Logan (1996). The reaction was initiated by the addition of NADPH.

The activity of glutathione peroxidase (GPX, EC 1.11.1.9) and glutathione transferase (GST, EC 2.5.1.18) were tested according to Nagalakshmi and Prasad (2001). The measurement of GPX was conducted in 3 mL reaction solution including 100 mM phosphate buffer (pH 7.0), 2 mM EDTA, 200 mM NaCl, 2 mM GSH, 0.4 mM NADPH, 0.5 mM H_2O_2, 1 unit of GR, and 50 μL enzyme extraction. The reaction mixture was initiated by GR. The measurement of GST activity was performed in 3 mL reaction solution including 100 mM phosphate buffer (pH 6.5), 1 mM GSH, and 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB), and 0.1 mL enzyme extraction. The reaction was started by CDNB, and the absorbance change at 340 nm was recorded for calculation of GPX and GST activity.

2.8. Total RNA extraction and gene expression analysis

Frozen leaf sample (50–100 mg) was ground into powder under liquid nitrogen and total RNA was extracted using TRIzol Reagent (Thermo Fisher, USA). Then, 1 μg total RNA of each sample was reverse transcribed according to manufacturer instructions (HiScript II Q RT SuperMix for qPCR, Vazyme, China). The gene-specific primers were designed by Primer Premier 5.0 and are shown in Table 1; qRT-PCR was performed in 20 μL volume according to manufacturer instructions (AceQ qPCR SYBR Green Master Mix, Vazyme, China). Two-step PCR method was used and the PCR conditions are as follows: pre-denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 15 s and 60.5 °C for 30 s. For the normalization of gene expression, *Actin* (gene ID: AB181991) was an internal reference.

2.9. Statistical analysis

The experiment was conducted using completely randomized design. ANOVA and Duncan's multiple range test ($P \leq 0.05$) were performed using the statistical program SPSS 19.0 (IBM, USA). Data was plotted using SigmaPlot 12.5 (Systat, USA).

3. Results

3.1. Physiological responses in melatonin-treated seedlings under drought stress

Melatonin is a pleiotropic signaling molecule that provides protection against environmental stress. Here, we observed the alleviation effect of melatonin in drought stress. Higher photosynthetic rate and maximum efficiency of photosystem II (Fv/Fm) was observed in melatonin-treated wheat seedlings compared with control group (Fig. 1A and B). Melatonin treatment obviously relieved oxidative damage induced by drought stress. Thicker lamina, more intact chloroplast grana lamella and leaf structure were observed in melatonin-treated seedlings under drought stress (Fig. 2A and B; Fig. 3C, E). Specifically, we observed lesser membrane damage and low level of ROS, as demonstrated by low content of MDA, hydrogen peroxide, superoxide anion, and lower

Table 1
The specific primers of genes related to GSH-AsA cycle.

Gene Name	Gene ID	Sense primer	Anti-sense primer
Actin	AB181991	ATGCTATCCTTCGTTTGGACCTT	CGTAAGCGAGCTTCTCCTTTATGT
MDHAR 4	JX034702.1	AAAGCAAGAAAGACGTGCCTGAC	CCTGCCGTACCAATAACCGAAT
APX	EF555121.1	TTCAATTAGATACGAGGAAGAGTACACCC	CGCTTTAATAGGCTCAAGGAGATCAATA
DHAR	AY074784	AAGTGAACCCGTAAGCCCTCC	TCGCTCGCATTATTCCAACCTA
GST2	JX051004	CCACGGCAGGAGGCAACTAA	CGCAAGAGCAACGGAACCAG
GR	AK332288.1	GATGGTTCAAAGCAAAGGCACA	AAGAATCACAGCACGTTTCGGTAG
GSHS	AJ579382	ATATCATATCATTTAGTCGGGACCAAGA	TTTCAAGAAACCTTTCAAGCACGT
GPX1	AF475124	GTTTGCCTGCACTCGCTTCA	GGCATAGCGGTCCACAACG

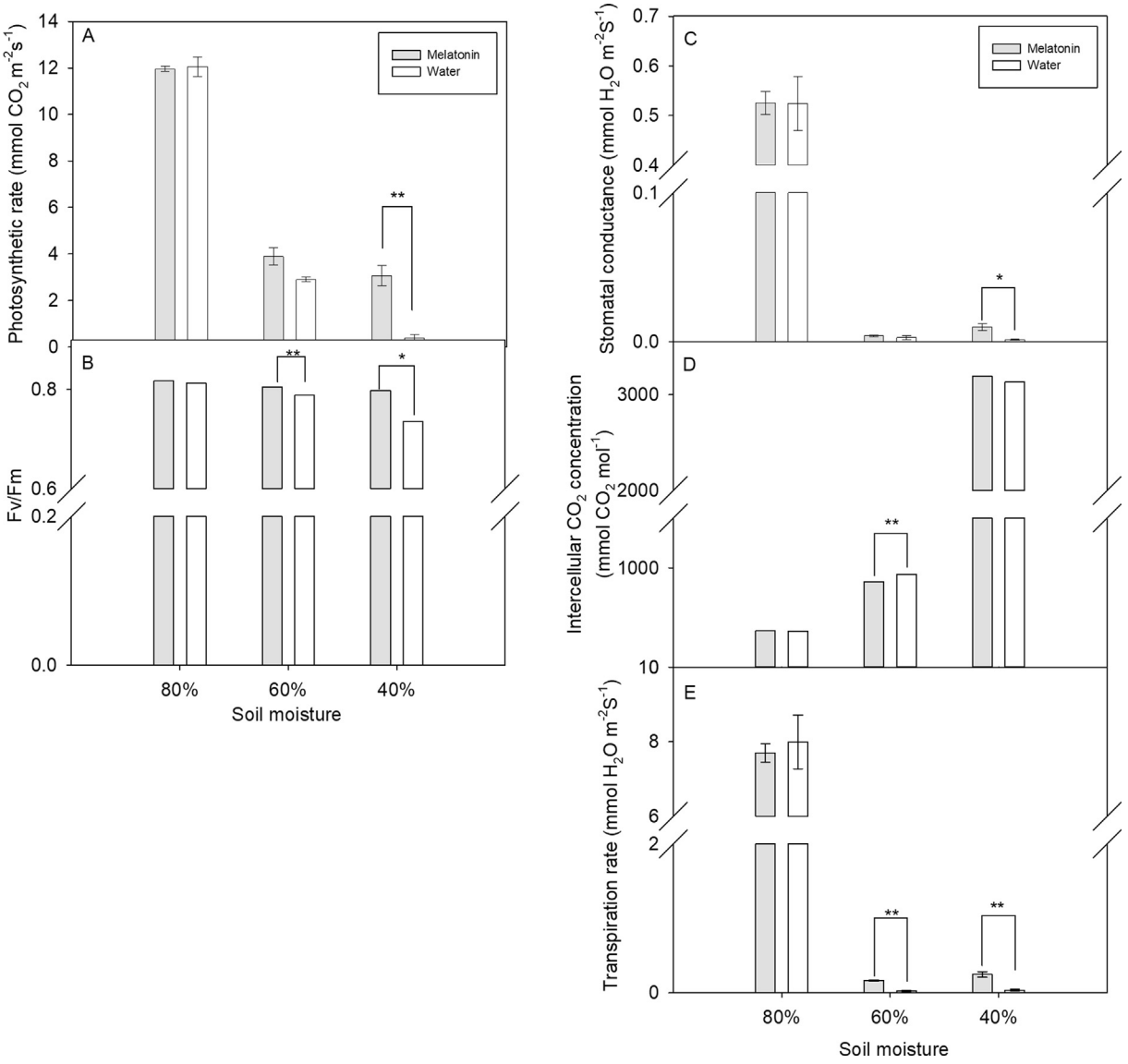

Fig. 1. Photosynthetic rate (A), maximum potential efficiency of photosystem II (B), stomatal conductance (C), intercellular CO_2 concentration (D), and transpiration rate (E). Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the parameters were measured at 80%, 60%, and 40% of field capacity. ** and * means the difference is significant at the level of $P < 0.01$ and 0.05.

relative conductivity in melatonin-treated seedlings (Fig. 4).

3.2. Effect of melatonin on GSH and AsA content in wheat seedlings response to drought

Amounts of GSH and AsA produced were analyzed in control and melatonin-treated samples. The content of total GSH and AsA, GSH and AsA in wheat leaf were significantly increased by exogenous melatonin at 60% and 40% FC of soil water compared with the control seedlings (Figs. 5 and 6). Meanwhile, a higher rate of AsA/(AsA + DHA) and GSH/(GSH + GSSG) was observed in melatonin-treated samples under drought stress (Fig. 5D; Fig. 6D). In addition, the GSSG content was increased by melatonin at 40% FC and almost unchanged at 60% FC (Fig. 5C) while the content of DHA was

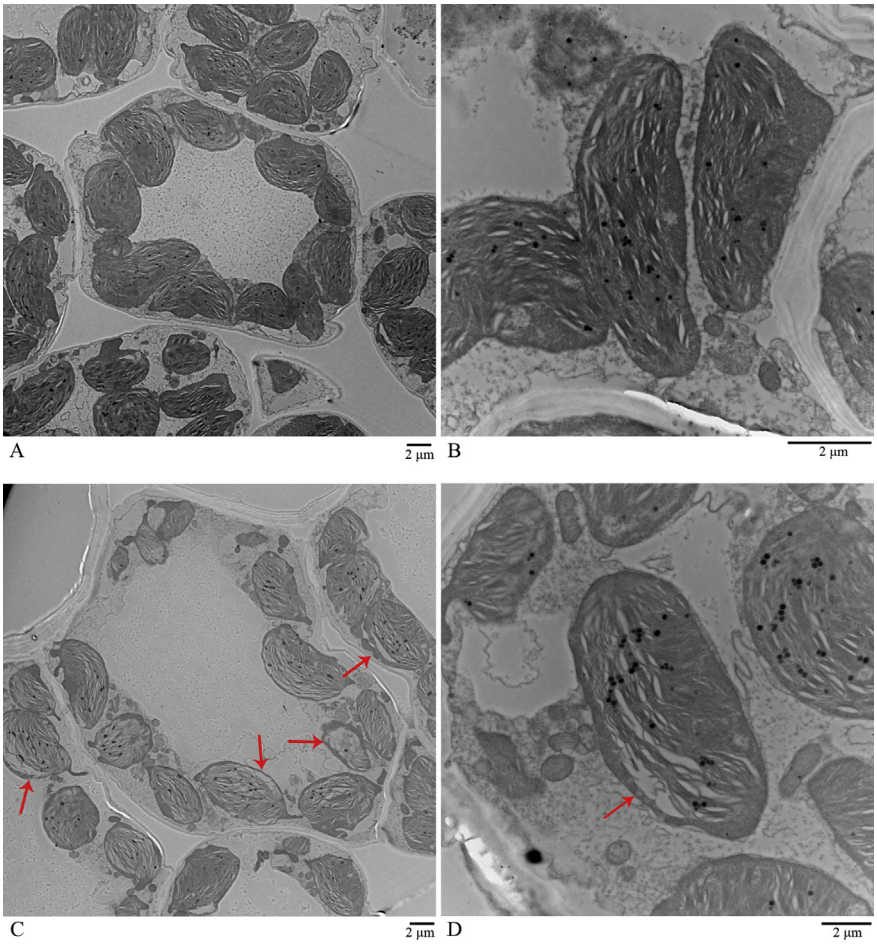

Fig. 2. Ultrastructure of leaf chloroplast by transmission electron microscope. Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the seedling leaves were fixed at 40% of field capacity. A and B: 500 μM melatonin-treated seedlings, C and D: water-treated seedlings. Arrows indicate degradation of chloroplast grana lamella.

decreased by melatonin at 60% FC, but almost unchanged at 40% FC (Fig. 6C).

3.3. Effect of melatonin on enzymatic activity of GSH-AsA cycle in wheat seedlings response to drought

The activity of enzymes, including APX, DHAR, MDHAR, GR, and GPX, which are related to GSH-AsA cycle was largely increased under drought stress (Fig. 7). Melatonin treatment significantly improved the activity of enzymes including APX, DHAR, and MDHAR at different soil moisture levels (Fig. 7A, B, C), which were key enzymes of AsA renovation. The enzymes responsible for GSH production, including GR and GPX, were commonly increased by exogenous melatonin treatment even at different soil drought, except that GR was decreased in melatonin-treated samples compared with the control at 40% FC (Fig. 7D and E). In addition, GST, the enzyme responsible for GSH-coupled detoxification under stress was significantly increased by melatonin treatment (Fig. 7F).

3.4. Effect of melatonin on the gene expression of GSH-AsA cycle in wheat seedlings response to drought

Through quantitative real-time PCR, the transcript levels of seven GSH-AsA cycle genes (*APX, DHAR, MDHAR4, GPX, GPX1, GR,* and *GST2*) in wheat seedling leaves were found to be significantly affected by soil drought and melatonin treatment (Fig. 8). The expression of *APX* and *MDHAR4*, was markedly increased by melatonin treatment compared to the control under different soil moisture conditions (Fig. 8A, C). The expression level of *DHAR* exhibited no difference under melatonin treatment at 60% of FC, but was largely upregulated by melatonin at 40% of FC (Fig. 8B). Clear upregulated expression of *GPX1* and *GPX* was observed in melatonin-treated seedlings under drought stress, except *GPX* was downregulated at 40% FC (Fig. 8D and E). In addition, the transcripts of *GR* and *GST2* were also significantly increased by melatonin at 60% FC, but no obvious change was observed at 40% FC between melatonin-treated and control samples (Fig. 8F and G).

4. Discussions

It has been widely known that abiotic stress can result in the accumulation of ROS. Although moderate ROS level is important for activation of stress-response and defense pathway, high levels of ROS will harm biomembrane, increasing the biomembrane permeability, and eventually resulting in the degradation of organelle and cell death (Apel and Hirt, 2004). In the present study, water deficit caused cell shrinking and ROS accumulation (Fig. 3E and F; Fig. 4B, C), resulting in membrane lipid peroxidation, higher membrane permeability, damaged grana lamella of chloroplast (Fig. 4A, D; Fig. 2C, D), which may be responsible for the decreased photosynthetic rate (Fig. 1A). Notably, the negative effects of drought were reversed by exogenous melatonin treatment. The

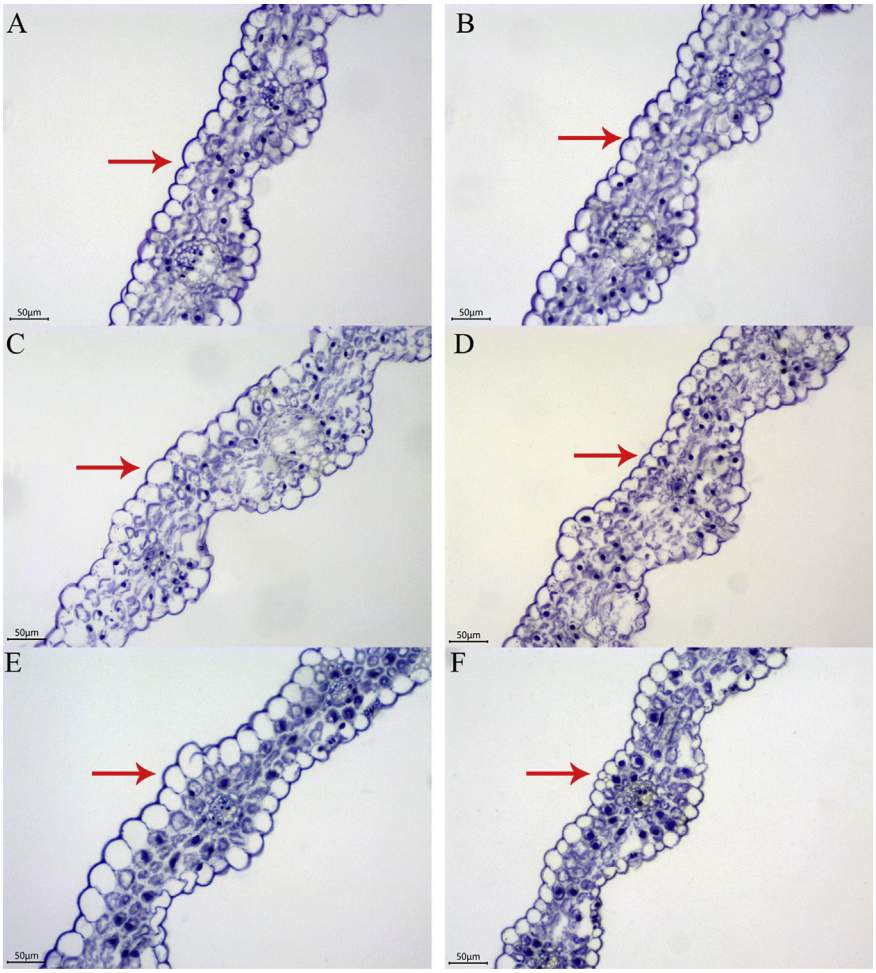

Fig. 3. Leaf structure by paraffin section. Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the seedlings were fixed at 80%, 60%, and 40% of field capacity. A, C, and E: 500 μM melatonin-treated seedlings at 80%, 60%, and 40% of field capacity; B, D, and F: water-treated seedlings at 80%, 60%, and 40% of field capacity. Arrows indicate epidermis cells of leaf.

wheat seedlings treated with exogenous melatonin had higher levels of antioxidants, lower ROS level, higher photosynthetic rate and Fv/Fm (Fig. 1; Fig. 4), in accordance with previous observations in wheat and maize (Fleta-Soriano et al., 2017; Ye et al., 2015). Apart from mitigating chloroplast damage, thicker leaves were maintained even under severe drought when supplemented with melatonin treatment (Fig. 2C and D; Fig. 3C, E), indicating the possible role of melatonin in drought tolerance of wheat. Previous studies reported that stomatal conductance increased with melatonin treatment and exogenous melatonin could cause stomata to reopen in cucumber and *Malus* (Li et al., 2015; Zhang et al., 2013). In this study, enhanced stomatal conductance was observed in melatonin-treated wheat seedlings (Fig. 1C), suggesting a role for melatonin in stomatal regulation in wheat. Enhanced stomatal conductance can improve the transportation of H_2O, CO_2, and O_2, to maintain a steady state of photosynthesis even under drought stress, as seen in the increased photosynthesis and transpiration rates in melatonin-treated seedlings (Fig. 1A, E). All these results suggest the effect of melatonin on photosynthesis and water use by influencing stomatal activity. In addition, the water content of leaf was influenced by exogenous melatonin and was found to be decreased at 60% FC but no obvious decrease occurred at 40% FC (Fig. 4F), which was in accordance with a previous report in soybean (Wei et al., 2015). This may be attributed to the stomatal opening induced by melatonin. Melatonin-treated seedlings had thicker leaf tissue and the mesophyll cells maintained a relatively high turgor pressure compared with the drought control (Fig. 3C, D, E, F). This was also observed in maize seedlings with higher stomatal conductance and turgor pressure (Ye et al., 2016). The accumulation of osmotic substances and thicker epidermal cells may be responsible for the relatively high turgor pressure of mesophyll cells and water holding capacity in melatonin-treated wheat seedlings at 40% of FC based on previous reports (Antoniou et al., 2017; Turk et al., 2014) and the results from this study. These findings indicate that melatonin regulates water balance and maintains cell turgor in wheat seedlings in response to drought stress. In addition, we highlight a novel role for melatonin in epidermal cell growth, which can be helpful in reducing water loss. Overall, from this study and other reports, comparing the response of different plants to drought stress, it is evident that melatonin enhanced the drought tolerance of plants by improving their antioxidant capacity, protecting the photosynthetic apparatus, decreasing the osmotic potential, and increasing the water-holding capacity (Antoniou et al., 2017; Fleta-Soriano et al., 2017; Wei et al., 2015; Ye et al., 2015, 2016). Taken together, the application of exogenous melatonin significantly decreased the accumulation of ROS, protected photosynthetic apparatus, maintained higher cell turgor and water holding capacity compared with the control under drought stress, resulting in improved drought tolerance of melatonin-treated wheat seedlings.

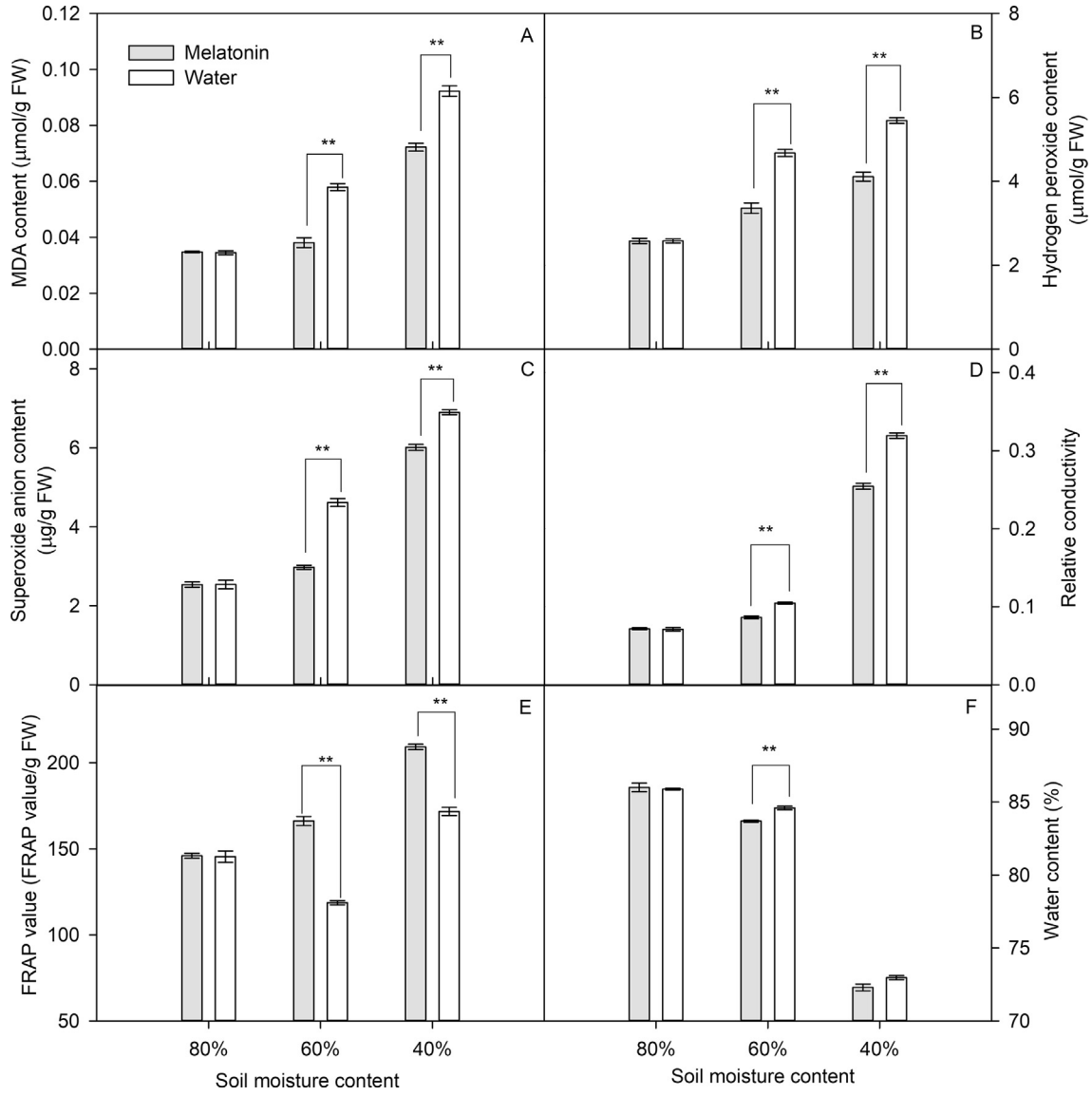

Fig. 4. Content of malondialdehyde (MDA), hydrogen peroxide, superoxide anion, relative conductivity, total antioxidant capacity (FRAP value), and water content. Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the seedlings were harvested at 80%, 60%, and 40% of field capacity.** means the significant level is $P < 0.01$.

GSH-AsA cycle, an important antioxidative system in plant, can stabilize ROS level in chloroplasts (Kuźniak and Skłodowska, 2001). It has been reported that the production of GSH and AsA is induced by melatonin under drought stress and is associated with low H_2O_2 content in wheat and other plants (Liu et al., 2015; Wang et al., 2013; Ye et al., 2015). Here, we report increased total AsA and GSH content in melatonin-treated seedlings (Fig. 5A; Fig. 6A), which suggests that melatonin plays an important role in the biosynthesis of GSH and AsA. Low levels of H_2O_2 and superoxide anion also correlated to with higher GSH and AsA content as well as melatonin treatment, highlighting the role of GSH-AsA and melatonin in ROS balance. Glutamylcysteine synthetase (GCS) is the rate-limiting enzyme of GSH synthesis (Hell and Bergmann, 1990) and the increased total GSH content in melatonin-treated seedlings suggests the contributions of melatonin to GCS activity. However, no direct evidence is available at this time to unambiguously prove that melatonin directly increases the activity of GCS, and therefore, further research is needed.

Relatively higher levels of GSH/GSSG and AsA/DHA is fundamental to many physiological functions of the cell (Meister and Anderson, 1983). APX, MDHAR, DHAR and GR are four key enzymes in the GSH-AsA cycle (Fig. 9), that serve as scavengers for H_2O_2 and superoxide radicals in the cellular compartments, particularly in the chloroplast (Logan et al., 2006). In this study, these enzymatic activities and gene expression were commonly upregulated by exogenous melatonin compared to the control (Fig. 7; Fig. 8). Upregulation of genes of GSG-GSH and AsA-DHA might be responsible for the high rates of GSH/GSSG and AsA/DHA. GR plays a key role in the reduction of GSSG to GSH and is beneficial for AsA homeostasis in chloroplast (Foyer and Halliwell, 1976). A previous study revealed that melatonin caused down-regulation of GR gene expression and enzymatic activity in apple (Wang et al., 2012). Down regulation of GR gene and enzymatic activity was also observed at 40% FC in melatonin-treated seedlings in this study and corresponds to the increased GSSG content at 40% FC (Fig. 5C). It also indicates that GR might not be a key factor for

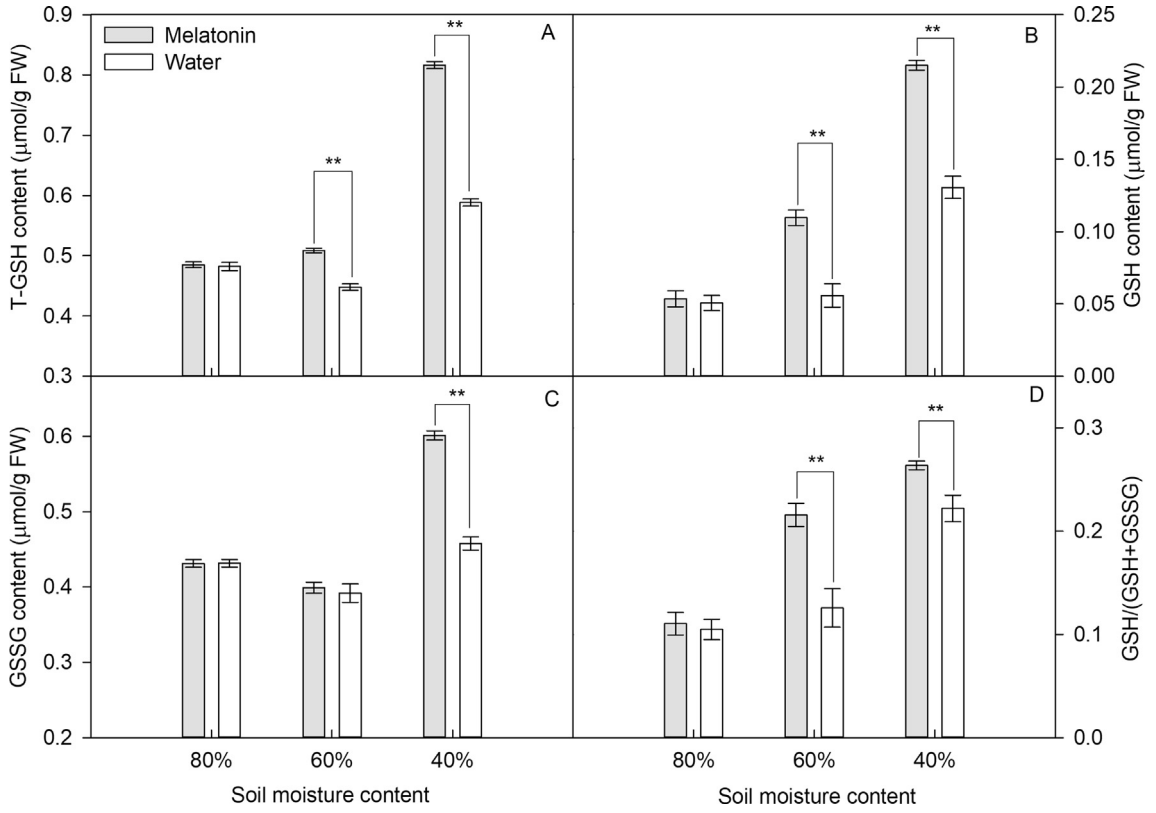

Fig. 5. Total glutathione (T-GSH), reduced glutathione (GSH), oxidative glutathione (GSSG), and the rate of GSH/(GSH + GSSG). Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the seedlings were harvested at 80%, 60%, and 40% of field capacity. ** means the difference is significant at the level of $P < 0.01$.

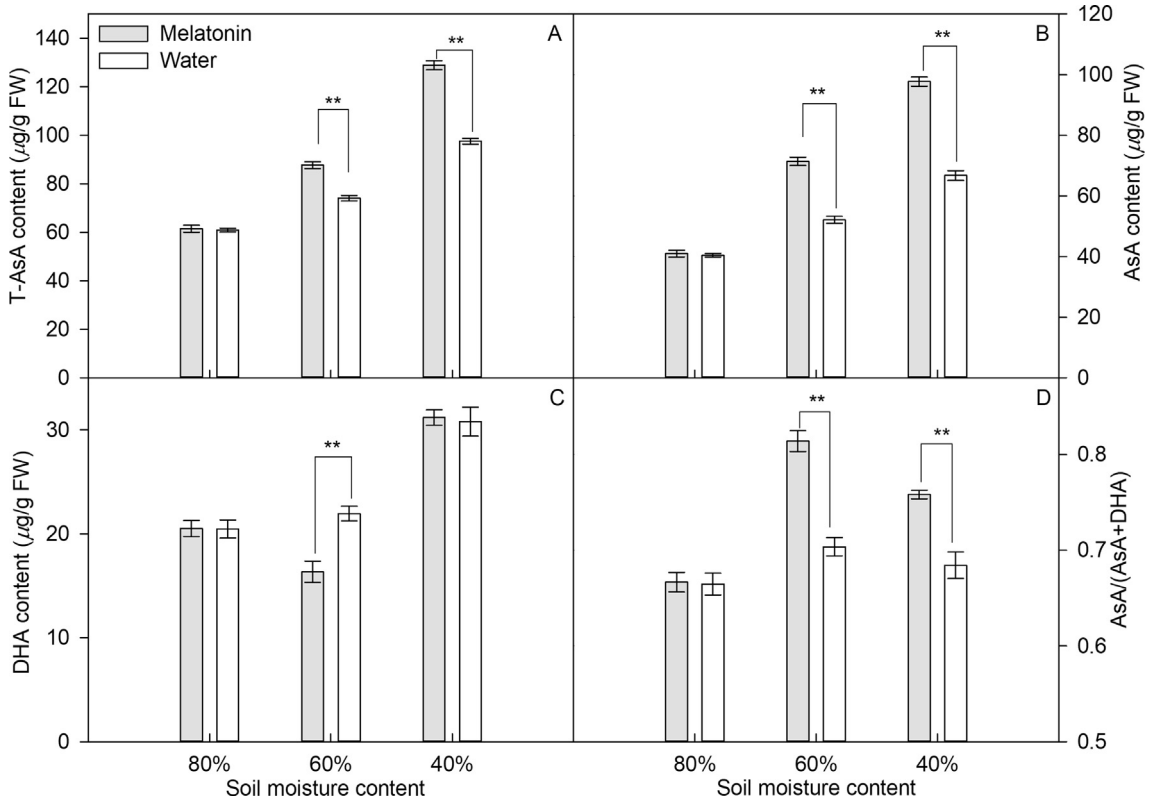

Fig. 6. Total ascorbate (T-AsA), reduced ascorbate (AsA), oxidative AsA (DHA), and the rate of AsA/(AsA + DHA). Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the seedlings were harvested at 80%, 60%, and 40% of field capacity. ** means the difference is significant at the level of $P < 0.01$.

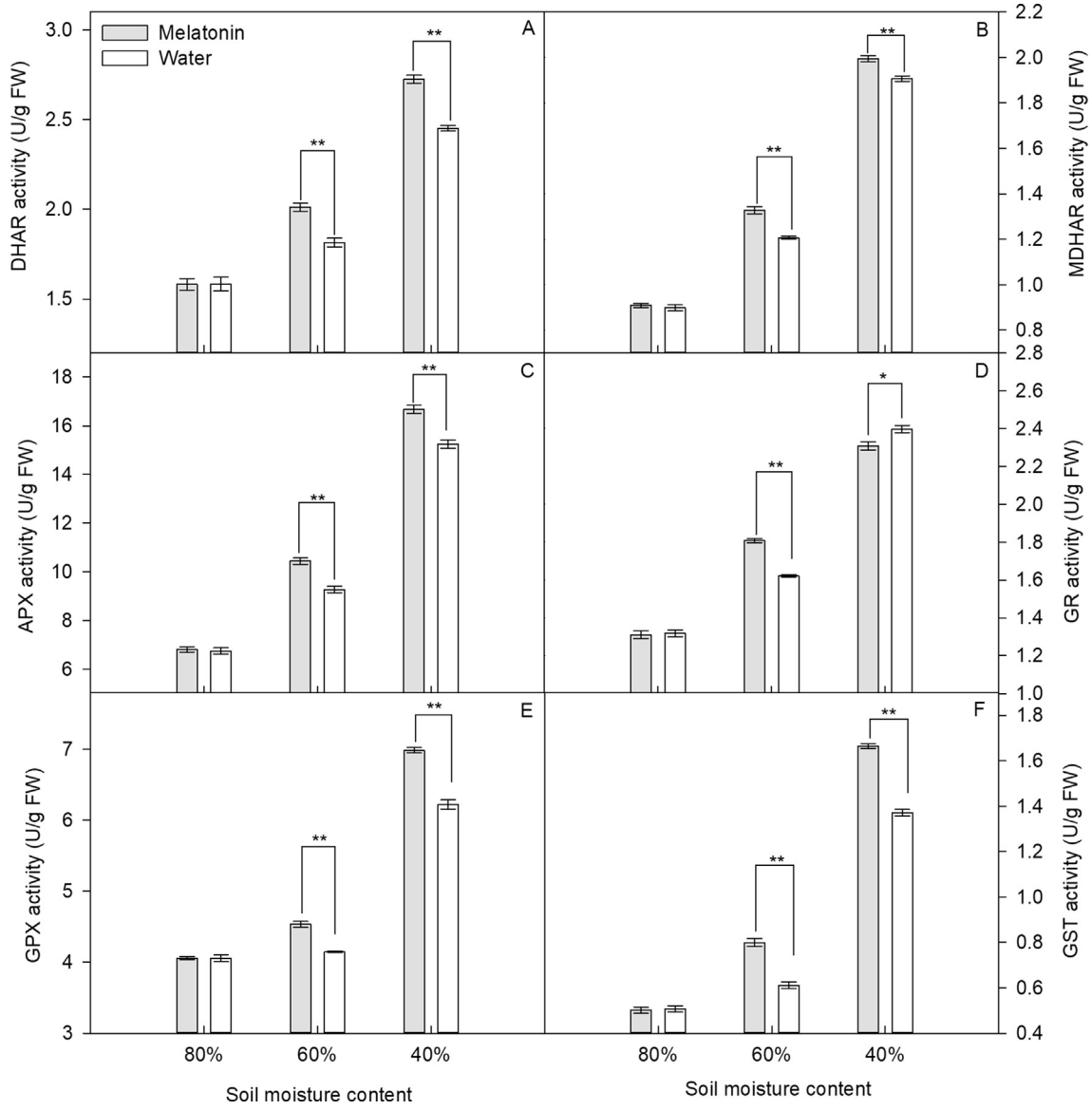

Fig. 7. Activity of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione transferase (GST). Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the seedlings were harvested at 80%, 60%, and 40% of field capacity. ** and * means the difference is significant at the level of $P < 0.01$ and 0.05.

melatonin-induced antioxidant capacity. All these results indicate that melatonin can maintain a relatively higher AsA/DHA and GSH/GSSG levels by accelerating the conversion of DHA to AsA and GSSG to GSH, as well as the biosynthesis of GSH and AsA under moderate drought conditions. In severe drought conditions, higher GSH/GSSG levels might be attributed to the biosynthesis of excessive GSH more than the threshold conversion rate from GSH to GSSG.

GPXs is a peroxidase containing sulfhydryl and can efficiently eliminate H_2O_2, hydroperoxides and lipid peroxide (Bela et al., 2015). In this study, the activity of GPX significantly increased with melatonin treatment under drought stress (Fig. 7E). The transcripts levels of *GPX* and *GPX1* in melatonin-treated group was 2-fold higher than the control at 60% of FC and *GPX1* was upregulated by melatonin even at 40% of FC (Fig. 8D, E). In contrast, the expression of *GPX* was down regulated by melatonin treatment at 40% of FC (Fig. 8D), indicating incomplete synchronization of homologous gene expression. GSTs catalyze the electrophilic substitution reaction between glutathione and a variety of electrophilic compounds including cytotoxic drugs, carcinogens, mutagens, etc., playing an important role in cell detoxification (Banerjee and Goswami, 2010). As GST activity and its gene expression are upregulated by melatonin (Fig. 7F; Fig. 8G), it will contribute to the alleviation of toxicity or lethal effects induced by drought stress, highlighting the important role of melatonin in detoxification.

5. Conclusion

In this study, 500 μM melatonin was used for testing the role of melatonin in drought tolerance of wheat. Our results demonstrate that melatonin treatment remarkably increased drought tolerance of wheat seedlings, as evidenced by increased antioxidant capacity, decreased endogenous ROS level, decreased membrane damage, thicker epidermal cell, intact grana lamella of chloroplast and leaf structure, higher photosynthetic rate and maximum efficiency of photosystem II (Fv/Fm) in melatonin-treated seedlings. Moreover, the biosynthesis of GSH and AsA was induced by melatonin, and the

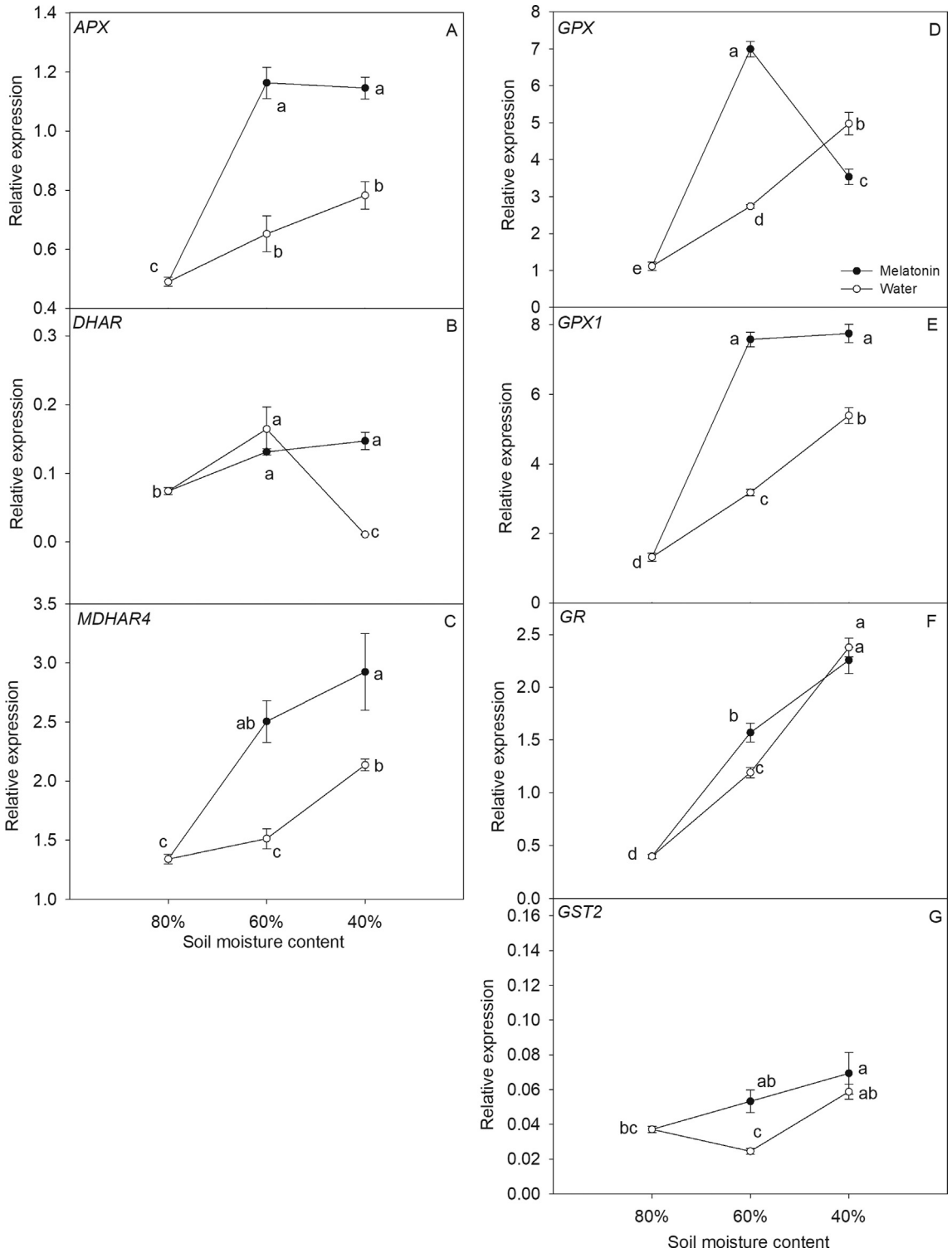

Fig. 8. Relative expressions of ascorbate peroxidase gene (*APX*), dehydroascorbate reductase gene (*DHAR*), monodehydroascorbate reductase gene (*MDHAR4*), glutathione peroxidase gene (*GPX*, *GPX1*), glutathione reductase gene (*GR*), and glutathione S-transferase gene (*GST2*). Different letters means the difference is significant at $P < 0.05$ (Duncan's multiple comparison test, $n = 5$). Wheat seedlings (21 days old) were treated by 500 μM melatonin or water until the soil moisture was down to 40% of field capacity and the seedlings were harvested at 80%, 60%, and 40% of field capacity.

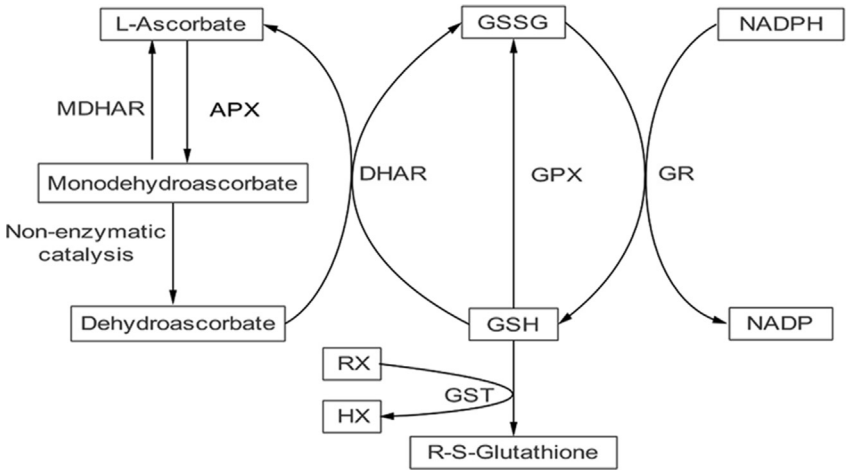

Fig. 9. GSH-AsA cycle, MDHAR: monodehydroascorbate reductase, APX: ascorbate peroxidase, DHAR: dehydroascorbate reductase, GSSG: oxidative glutathione, GSH: reduced glutathione, GPX: glutathione peroxidase, GR: glutathione reductase, NADPH: reduced nicotinamide adenine dinucleotide phosphate, NADP: nicotinamide adenine dinucleotide phosphate, RX: organic halide, GST: glutathione S-transferase, HX: hydrogen halide. R may be an aliphatic, aromatic, or heterocyclic group; X may be a sulfate, nitrile, or halide group.

formation of GSH-GSSG and AsA-DHA was significantly enhanced in melatonin-treated seedlings. The increased enzyme activity and genes expression of APX, DHAR, and MDHAR were responsible for increased GSH/(GSH + GSSG) and AsA/(AsA + DHA).

Contributions

Y. Xi designed the experiment and drafted the manuscript. G. Cui finished the experiment and prepared the manuscript. X. Zhao contributed to the previous draft version of the manuscript. S. Liu was the supervisor of the experiment and contributed to the early design. F. Sun contributed by editing the manuscript drafts. C. Zhang provided assistance in measurement of photosynthesis and fluorescence parameters.

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Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.plaphy.2017.06.014.

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