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Research article

Physiological and iTRAQ-based proteomic analyses reveal that melatonin alleviates oxidative damage in maize leaves exposed to drought stress

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ABSTRACT

To explain the underlying mechanism of melatonin-mediated drought stress responses in maize, maize pretreated with or without melatonin was subjected to 20% PEG nutrient solution to induce drought stress. We found that exogenous melatonin significantly improved drought tolerance, demonstrated by improved photosynthesis, reduced ROS accumulation, enhanced activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), and increased content of glutathione (GSH). Comparative iTRAQ proteomic analyses revealed a higher abundance of differentially expressed proteins (DEPs) in melatonin-treated maize under drought stress for carbon fixation in photosynthetic organisms, photosynthesis, biosynthesis of amino acids, and biosynthesis of secondary metabolites, compared to untreated plants. Changes in the above molecular mechanisms could explain the melatonin-induced physiological effects associated with drought tolerance. In summary, this study provides a more integrated picture about the effects of melatonin on the physiological and molecular mechanisms in maize seedlings responding to drought stress.

1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine), a crucial animal hormone, play many physiological roles in life processes of animals (Tan et al., 2003). Intriguingly, research suggests that melatonin is naturally present in plants (Badria, 2002). Previous studies have shown that melatonin plays important roles in modulating plant metabolism and regulating growth and development of plants (including fruit ripening, seed germination, photoprotection, root regeneration, and flowering) (Arnao and Hernández-Ruiz, 2015).

In multiple plant species, melatonin treatment can enhance tolerance to biotic and abiotic stresses by removing reactive oxygen species (ROS) and improving the activity of antioxidant enzymes (Arnao and Hernández-Ruiz, 2014). Many studies have attempted to illustrate the mechanism by which exogenous application of melatonin mitigates damages from drought and osmotic stresses. However, fewer studies have focused on melatonin regulating downstream target genes and molecular signaling. Bioinformatics analyses have shown that melatonin can reprogram the expression of genes involved in the metabolism of major carbohydrate metabolism, abscisic acid (ABA) signaling, hormone metabolism, and secondary metabolism under abiotic stresses (Shi et al., 2015; Li et al., 2015; Cui et al., 2018). Moreover, melatonin can reduce lipid peroxidation in cellular membranes, prevent carbohydrate oxidation, and mitigate photosystem damage by regulating ROS-dependent signal transduction under abiotic stresses (Cui et al., 2018). Overall, previous studies have highlighted the role of melatonin as an antioxidant that can increase antioxidant enzyme activities and directly scavenge ROS to eliminate the over accumulation of ROS under multiple stress conditions.

Maize is a major cereal crop in China, with a very large planting area and high yield (Qin et al., 2016). Drought is an important abiotic stressor that has a strong negative affect on maize production (Ziyomo and Bernardo, 2013). A recent study showed that melatonin can accelerate plant growth, thereby improving photosynthesis of maize under drought stress (Ye et al., 2016). Although there have been many reports exploring the physiological effects of melatonin treatment in plants responding to environmental stress, the proteome-level response mediating the underlying mechanism of response to drought stress in maize is still not clear.

Therefore, we determined the effects of exogenous melatonin

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application on plant growth, the antioxidant system, and ROS accumulation, as well as photosynthesis during drought stress. Additionally, iTRAQ-based proteomic analyses were conducted to reveal melatonin-induced drought responses in maize. Results of pathway enrichment analysis showed that melatonin alleviated oxidative damage and decreased photosynthesis associated with regulating several metabolic pathways. This study provides integrated insights into the effects of melatonin on the physiological and potential molecular mechanisms in the response of maize seedlings to drought stress.

2. Materials and methods

2.1. Plant material and treatments

Seeds of the maize (*Zea mays* L.) cultivar Zheng Dan 958, which are widely cultivated in China, were surface sterilized in 2% hypochlorite for 10 min, then washed three times in distilled water. Afterwards, the seeds were germinated on moistened filter paper for 3 days in the dark. The germinated seedlings were placed in plastic pots containing half-Hoagland solution replaced every 2 days. The plants were grown in a culture room with a photoperiodic cycle (14 h light/10 h dark, relative humidity 75%, at a 28/22 °C day/night temperature) of 400 μmol $m^{-2}s^{-1}$ photon intensity active radiations. When the second leaf expanded, the solution was replaced with full-strength Hoagland solution and sprayed two times every day with different concentrations of melatonin (0 μM, 1 μM, 10 μM, 100 μM, and 1000 μM) for 4 days.

After four days, half of the plants were raised in full-strength Hoagland solution for 3 days. The other half of the plants were cultivated in 20% polyethylene glycol (PEG, 6000) dissolved in full-strength Hoagland solution and replaced every day for 3 days as a drought treatment. As previously reported, the osmotic potential was estimated at −0.5 MPa for 20% PEG solution (Yin et al., 2016b). Three days after continuous drought stress, the third piece of a leaf from the bottom in all treatments were separately collected and immediately frozen in liquid nitrogen and stored at −80 °C for assessment of physiological parameters and proteomics. Each treatment was designed with three replicates for physiological parameters and two replicates for proteomics.

2.2. Measurement of morphology parameters and chlorophyll content

The morphological parameters (plant height, relative water content, and dry weight of leaf tissues) were measured or calculated according to Wei et al. (2015). Chlorophyll concentrations of the third leaves were determined by measuring the SPAD value using a SPAD meter (SPAD-502, Konica-Minolta, Tokyo, Japan).

2.3. Measurement of endogenous melatonin

The extraction and quantification of endogenous melatonin was performed with a melatonin enzyme-linked immunosorbent assay (ELISA) kit (ml024033; Mlbio, Shanghai, China).

2.4. Measurement of hydrogen peroxide (H_2O_2) content and superoxide anion (O_2^-) production rate

The rate of O_2^- production was measured using a previously reported method with some modifications (Elstner and Heupel, 1976). Leaf tissues (fresh weight) were homogenized in 7 ml 50 mM phosphate buffer (pH 7.8), then centrifuged at 16,000 rpm for 10 min. One ml supernatant with 0.9 ml phosphate buffer (50 mM, pH 7.8) and 0.1 ml hydroxylamine were mixed. The mixture was incubated for 20 min at 25 °C, and then mixed with 0.5 ml p-aminobenzenesulfonic acid (17 mM) and 0.5 ml a-naphthylamine (7 mM) and incubated at 25 °C for 20 min. Then, the absorbance at 530 nm was recorded.

H_2O_2 was measured as described by Kong et al. (2015). H_2O_2 absorbance at 415 nm was recorded. Concentrations of H_2O_2 were calculated using a prepared standard curve.

2.5. Assay of antioxidant enzyme activity

To extract enzymes, leaf tissues were ground into a powder in liquid nitrogen, homogenized in 2 ml phosphate buffer (50 mM, pH 7.0), and then centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was used in the antioxidant enzyme assays.

Activity of superoxide dismutase (SOD) was detected by the photochemical reduction of nitro-blue tetrazolium (NBT), and the absorbance was measured at 560 nm. One unit of SOD was defined as the enzyme activity that suppressed NBT photochemical reduction by 50% for one unit of SOD activity (Beauchamp and Fridovich, 1971). The activities of peroxidase (POD), and catalase (CAT) were assayed using the guaiacol method and the ultraviolet absorption method, measured by monitoring the absorbance changes at A470 and A240, respectively (Kochba et al., 1977; Havir and Mchale, 1987).

2.6. Determination of glutathione (GSH) content and malonaldehyde (MDA) content

The content of GSH and MDA were measured using the methods of Anderson and Gronwald (1991) and Heath and Packer (1968), respectively.

2.7. Analysis of photosynthesis and chlorophyll fluorescence parameters

Photosynthesis parameters (net photosynthetic rate (Pn), intercellular CO2 concentrations (Ci), stomata conductance (Gs), and transpiration rate (Tr)) were measured using a photosynthetic apparatus (Li-Cor6400, Li-Cor, Inc, Lincoln, NE) between 10:00 and 11:00 a.m. Photosynthetic photon flux density (PPFD) was set at 1000 l mol $m^{-2}s^{-1}$. Chlorophyll fluorescence was measured with a portable Handy-PEA chl fluorometer (Handy-Plant Efficiency Analyser-2126, Hansatech Instruments, King's Lynn, UK) at room temperature between 10:00 to 11:00 a.m. Before measurements, the leaves were placed in the dark to acclimate for 30 min. The following fluorescence parameters were obtained using a built-in analysis software of the instrument (PEA Plus v1.10): maximal quantum yield of PSII photochemistry (Fv/Fm) and performance index on absorption based on the absorption of light energy (PI abs).

2.8. Protein extraction

Leaf samples were first ground in liquid nitrogen for 10 min and transferred to 5 mL centrifuge tubes and sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT) and 1% Protease Inhibitor Cocktail). The remaining debris was removed by centrifugation at 20,000 g at 4 °C for 10 min. The protein was precipitated with cold 15% trichloroacetic acid (TCA) for 4 h at −20 °C. After centrifugation at 20,000 g at 4 °C for 3 min, the supernatant was discarded. The remaining precipitate was washed three times with cold acetone. The protein was redissolved in buffer (8 M urea, 100 mM tetraethyl-ammonium bromide (TEAB), pH 8.0) (Zhou et al., 2018) and the protein concentrations in the supernatant were detected with a 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions.

2.9. Trypsin digestion and iTRAQ labeling

The protein solution was reduced with 10 mM dithiothreitol (DTT) for 1 h at 37 °C and alkylated with 20 mM iodoacetamide for 45 min at 25 °C in darkness. The protein sample was then diluted with less than 2 M urea by adding 100 mM tetraethyl-ammonium bromide (TEAB).

Finally, the protein sample was digested with trypsin at 1:50 mass ratios of trypsin-to-protein for the first digestion overnight and the digestion of second step was processed in 1:100 trypsin-to-protein for 4 h.

After trypsin digestion, the peptides of each sample were desalted using a Strata X C18 solid phase extraction (SPE) column (Phenomenex) and dried in vacuum environment. The peptides were reconstituted in 0.5 M TEAB (Zhou et al., 2018) and processed based on the manufacturer's instructions for the 8-plex iTRAQ kit. Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 μL acetonitrile (ACN). The peptide mixtures were then incubated for 2 h at 25 °C and then pooled, desalted, and dried using vacuum centrifugation. In the present experiment, 113- and 114 -tags were used for control treatment with two replicates, 115- and 116-tags for melatonin treatment with two replicates, 117- and 118-tags for drought treatment with two replicates, and 119- and 121-tags were used for drought and melatonin treatment with two replicates.

2.10. High performance liquid chromatography (HPLC) fractionation and LC-MS/MS analysis

The peptide mixtures of each sample were fractionated with high pH reverse-phase high performance liquid chromatography (HPLC) using an Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). The peptides were combined into 18 fractions and dried by vacuum centrifuging (Zhou et al., 2018). Peptides were dissolved with 0.1% formic acid (FA), and directly placed in a reversed-phase pre-column (Acclaim Pep Map 100, Thermo Scientific). Peptides were separated using a reversed-phase analytical column (15 cm analytical column, 50 μm internal diameter, 1.9 μm reprosil-pur and C18 particles) (Acclaim Pep Map RSLC, Thermo Scientific) and then subjected to nano-electrospray ionization (NSI) source followed by tandem mass spectrometry (MS/MS) in an Orbitrap Fusion TM (Thermo) Mass Spectrometer coupled online to the EASY-nLC 1000 ultrahigh Performance Liquid Chromatography (UPLC), the electrospray voltage was kept at 2.0 kV. A data-dependent procedure was performed using top speed mode that alternated between one MS scan followed by 20 MS/MS scans with the following parameters: above a threshold ion count of 1e4 for the selected precursor ions. Full MS scans were acquired for the mass range of 400–1600 Th at the resolution of 60,000. Twenty most abundant ions were fragmented by data-dependent MS/MS experiments at a normalized collision energy (NCE) of 38 for higher-energy collisional dissociation (HCD). The MS/MS scans were acquired at a resolution of 15,000 with a fixed first m/z of 100 Th. Dynamic exclusion was set to 30 s; Automatic gain control (AGC) was taken to prevent overfilling of the ion trap. The accumulation of 1e5 ions were used for generation of MS/MS spectras. (Zhou et al., 2018).

2.11. 11Database search

The MS/MS data were processed using the Mascot search engine (v.2.3.0). Tandem mass spectra were searched against the UniProt *Zea mays* database. Trypsin/P was specified as the cleavage enzyme allowing up to 2 missing cleavages. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl of cysteine was specified as a fixed modification and oxidation on Met was specified as a variable modification. For the protein quantification method, iTRAQ 8-plex was selected in Mascot. All identified proteins were at least two unique peptides, and the positive protein identification as the false discovery rate (FDR) was adjusted to $< 1\%$ and peptide ion intensity was set as ≥ 20. A significant change in a protein was defined as their average in both replicates over 1.2 or less than 0.833 relative to unity and with a coefficient of variation (CV) < 0.05 between biological replicates (Manteca et al., 2010).

2.12. Bioinformatics

Gene Ontology (GO) annotation of the proteome was conducted using the UniProt-GOA database (www. http://www.ebi.ac.uk/GOA/). First, the converted protein ID was mapped to GO IDs by UniProt ID. The interProScan software was used to annotate GO function of the protein if some proteins are not annotated in the UniProt-GOA database. Then, proteins were classified into three categories by GO annotation: biological process, cellular component, and molecular function as described in previous reports (Sangrador-Vegas et al., 2016). We used WOLF PSORT as an updated version of PSORT/PSORT II for the prediction of eukaryotic sequences to predict subcellular localization (Horton et al., 2007). In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate protein pathways.

2.13. Gene transcript assay using real-time quantitative PCR

Total RNA was isolated from seedlings using the Trizol reagent according to the manufacturer's instructions. RNA (3 μg) was reverse transcribed into cDNA using the SuperScript II reverse transcriptase (Invitrogen, USA). Specific primer sequences for real-time PCR were designed using Primer Premier 5.0 software and validated based on corresponding cDNA sequences using NCBI BLAST. The cDNA sequences were obtained from NCBI GenBank. The genes and primers are listed in Table S3. To normalize the results, the maize β-actin gene was used as an endogenous standard. The qRT-PCR program consisted of a preliminary denaturation step at 95 °C for 30 s followed by 50 cycles at 95 °C for 5 s and 60 °C for 1 min. Relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Adnan et al., 2011). This experiment was repeated at least three times.

2.14. Statistical analysis

Results of the physiological experiments were based on three biological repeats. All recorded values are presented as the mean ± standard deviation with three independent experiments. Data were analyzed statistically by one-way analysis of variance and Duncan's multiple range test by SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Significant differences were determined at $P < 0.05$.

3. Results

3.1. Effects of exogenous melatonin on morphology of maize seedlings exposed to PEG-stimulated drought-stress conditions

Under control conditions, melatonin treatment significantly influenced maize seedlings growth (plant height, plant fresh weight) and chlorophyll content of leaves (Fig. 1, Table 1). Positive effects on plant height, relative water content (RWC), and chlorophyll content of leaves were observed in maize seedlings treated with 10–100 μM melatonin, but negative effects on these indicators were observed in maize seedlings treated with 1000 μM melatonin. In addition, drought stress significantly reduced seedling growth (plant height), RWC, and leaf chlorophyll content. However, seedling growth (plant height), RWC, and leaf chlorophyll content in applications of melatonin under drought stress were reduced less than in untreated plants under drought stress (Table 1).

3.2. Effects of exogenous melatonin on physiological parameters of maize seedlings exposed to PEG-stimulated drought-stress conditions

The activities of several antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione (GSH) content as well as endogenous reactive oxygen species (ROS, H_2O_2 and O_2^-) levels and malondialdehyde (MDA) contents were compared between melatonin pretreated and untreated plants under

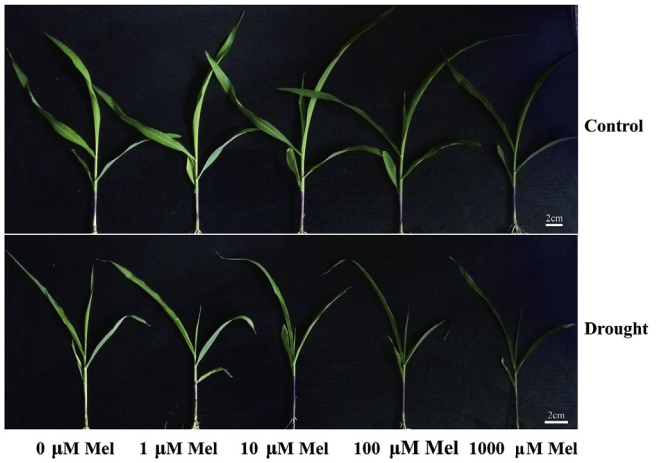

Fig. 1. Effects of different concentrations of melatonin on maize seedling growth in watered or drought stress conditions. The maize seedlings were treated with 20% PEG for 3 days after pretreatment with different concentrations of melatonin (0 μM, 1 μM, 10 μM, 100 μM, and 1000 μM) for 4 days.

drought stress conditions. Drought stress significantly increased the activities of SOD, POD, and CAT and the content of GSH in both treated and untreated maize seedlings (Fig. 2A–D). However, the activities of SOD, POD, and CAT and the content of GSH were higher in maize seedlings treated with melatonin (10–100 μM) than those untreated under drought stress. These results show that an appropriate concentration of melatonin can alleviate damage caused by drought stress in maize. However, high levels of melatonin have a negative effect on maize under drought stress.

Based on the above results, we chose the optimum concentration of melatonin (100 μM) for subsequent analysis. The endogenous concentration of melatonin was quantified (Fig. 2E). After drought stress treatment for 3 d, endogenous melatonin concentrations were increased 1.4-fold, and levels of melatonin were significantly increased 1.8-fold after melatonin pretreatment (Fig. 2E). The higher levels of endogenous melatonin under drought stress indicated that melatonin participated in the response to drought stress in maize seedlings. MDA, a widely used indicator of oxidative lipid peroxidation, H_2O_2 contents and O_2^- production rate was significantly increased under drought stress (Fig. 2 F, J, H). However, melatonin application significantly reduced ROS and MDA generation under drought stress.

3.3. Effects of exogenous melatonin on photosynthesis in maize seedlings exposed to PEG-stimulated drought-stress conditions

Under control conditions, melatonin treatment had no obvious effect on photosynthetic rate, intercellular CO_2 concentrations, stomata conductance, or transpiration rates. Drought stress significantly reduced all those parameters. The parameters included net photosynthetic rate (Pn), stomata conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) decreased by 29.8, 38.1, 45.5, and 72.7%, respectively (Table 2). However, decreases of Pn, Gs, and Ci were partly reversed by melatonin application under drought stress. Transpiration rate had no obvious variation under drought stress with or without melatonin pretreatment. As shown in Table 2, melatonin application did not influence maximal quantum yield of PSII photochemistry (Fv/Fm) or performance index on absorption based on the absorption of light energy (PI *abs*) in leaves under control conditions. Those parameters declined significantly under drought conditions. However, melatonin pretreatment could maintain higher values of the parameters (Fv/Fm and PI *abs*).

3.4. Identification of differentially expressed proteins (DEPs) of maize seedlings under drought stress with or without melatonin pretreatment

To explore the melatonin-mediated mechanism underlying response to drought stress in maize seedlings, total proteins affected by melatonin and drought treatments were extracted from the third expanded leaves, and then an integrated approach involving iTRAQ labeling and LC-MS/MS was used to quantify the dynamic changes of total proteins. In total, 5,068 identified proteins were found, among which 4,175 proteins were quantified (Table S1). The differentially expressed proteins (DEPs) are summarized in Table S2. A total of 668 DEPs were identified in Dro vs Con consisting of 431 upregulated and 237 downregulated; Mel + Dro vs Mel including 136 upregulated and 74 downregulated; Mel vs Con including 36 upregulated and 50 downregulated; and Mel + Dro vs Dro with 79 upregulated and 198 downregulated (Fig. 3A). From the overview of a hierarchical clustering analysis of DEPs in four comparison groups, the DEPs of compared groups showed different expression patterns. Mel + Dro vs Dro and Dro vs Con had 118 common DEPs; seven common DEPs were found in Mel + Dro vs Dro and Mel vs Con; and 50 common DEPs were found in Dro vs Con and Mel + Dro vs Mel (Fig. 3B). Only four DEPs overlapped in all four comparison groups. Total of 499, 125, 52, and 131 DEPs were independently expressed in Dro vs Con, Mel + Dro vs Mel, Mel vs Con, and Mel + Dro vs Dro, respectively (Fig. 3B).

3.5. GO analyses and subcellular localization of DEPs in response to melatonin and drought stress

All identified proteins were classified as cellular component, molecular function, or biological process by gene ontology (GO) annotation software. The results of the GO analyses in response to melatonin and drought stress are shown in Fig. 4A. The most common molecular functions were binding and catalytic activity, and the most common biological processes were cellular, single-organism, and metabolic process (Fig. 4A). Further analysis showed that most of these proteins are located in chloroplasts, the cytoplasm, or nuclei (Fig. 4B).

Table 1
Effect of different treatments on maize seedling height, fresh weight, leaf relative water content, and chlorophyll content. The maize seedlings were treated with 20% PEG for 3 days. After pretreatment with different concentrations of melatonin (0 μM, 1 μM, 10 μM, 100 μM, and 1000 μM) for 4 days, the plant height and the leaf relative water content and chlorophyll were measured. Data are means of three replicates (± SD). Means denoted by different letters show significant differences ($P < 0.05$).

Treatment	RWC(%)		Plant height (cm)		Plant fresh weight(g)		SPAD value	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Mel (0 μM)	93.71 ± 1.12^{ab}	74.84 ± 1.24^{c}	34.30 ± 0.95^{bc}	28.47 ± 0.78^{b}	2.78 ± 0.095^{ab}	1.35 ± 0.89^{b}	31.30 ± 0.73^{b}	27.60 ± 0.84^{cd}
Mel (1 μM)	94.71 ± 2.78^{ab}	81.89 ± 1.36^{b}	35.57 ± 1.46^{b}	29.37 ± 0.91^{b}	2.80 ± 0.46^{ab}	1.42 ± 0.17^{b}	33.80 ± 0.68^{a}	28.40 ± 1.21^{bc}
Mel (10 μM)	96.02 ± 1.68^{a}	83.11 ± 1.87^{ab}	42.53 ± 1.20^{a}	33.43 ± 1.01^{a}	3.22 ± 0.20^{a}	1.82 ± 0.23^{a}	35.40 ± 1.30^{a}	30.10 ± 1.22^{ab}
Mel (100 μM)	95.66 ± 1.24^{ab}	85.15 ± 1.15^{a}	41.53 ± 0.95^{a}	32.93 ± 1.12^{a}	3.19 ± 0.15^{a}	1.81 ± 0.19^{a}	35.20 ± 0.94^{a}	31.40 ± 1.32^{a}
Mel (1000 μM)	93.02 ± 1.44^{b}	72.82 ± 1.54^{c}	32.87 ± 1.34^{c}	28.50 ± 0.92^{b}	2.54 ± 0.34^{b}	1.24 ± 0.14^{b}	30.70 ± 1.33^{b}	25.70 ± 0.93^{d}

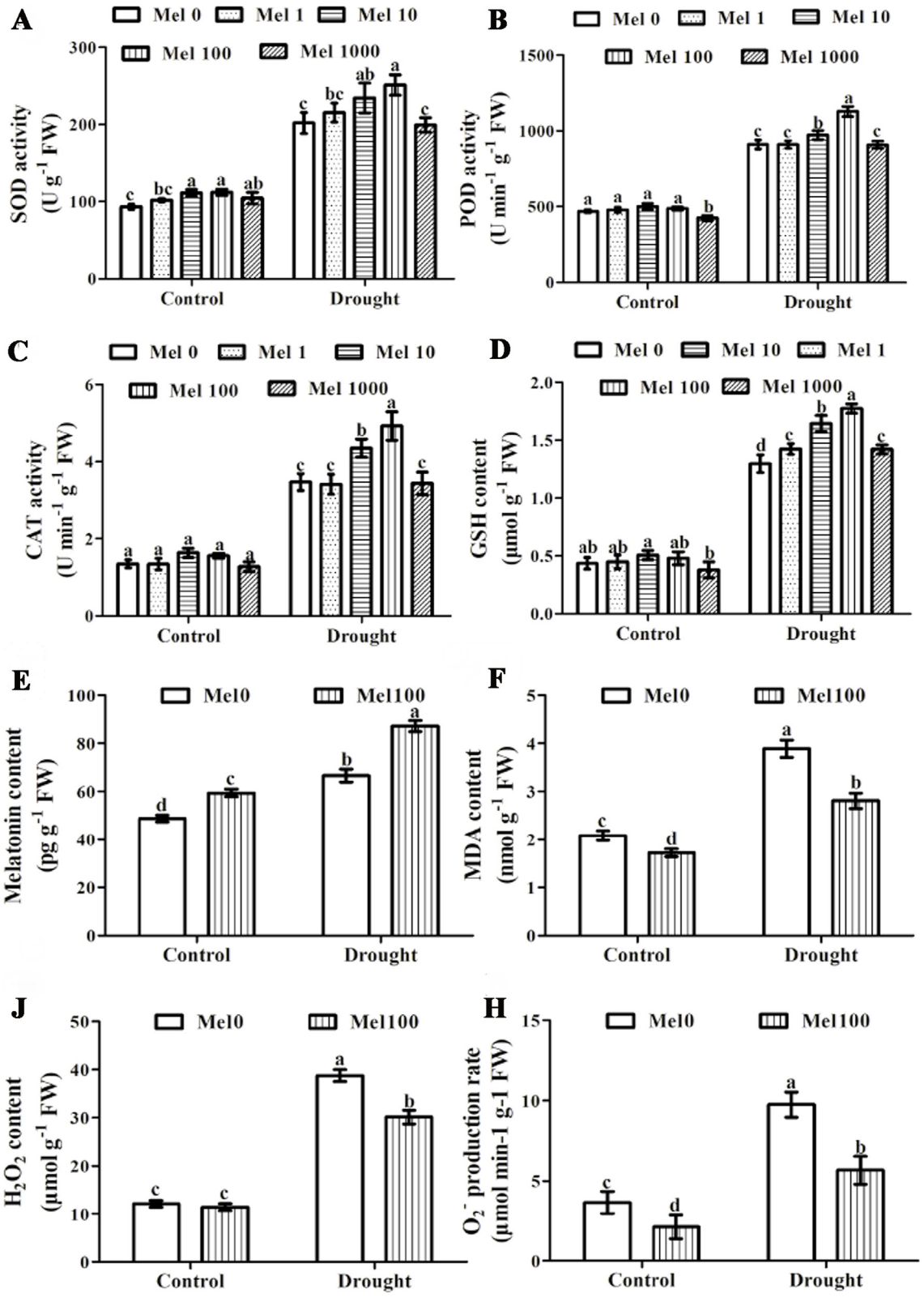

Fig. 2. Physiological effects of melatonin in maize seedlings in response to drought stress. The activities of superoxide dismutase (SOD) (A), peroxidase (POD) (B), catalase (CAT) (C), and glutathione (GSH) content (D) in the third leaves of maize plants pre-treated with different concentrations of melatonin or without melatonin in watered conditions (control) and under drought stress. Melatonin content (E), malondialdehyde (MDA) content (F), hydrogen peroxide (H_2O_2) (G) and superoxide anion rate (H) in the third leaves of maize plants pre-treated with melatonin (100 μM) or without melatonin in watered conditions (control) and under drought stress. Data are the means of three replicates (± SD). Means denoted by different letters show significant differences ($P < 0.05$).

Table 2
Photosynthesis parameters and chlorophyll fluorescence parameters in the third leaves of maize plants pre-treated with melatonin or untreated in watered conditions (control) and under drought stress. Data are means of three replicates (± SD). Means denoted by different letters show significant differences ($P < 0.05$). Pn, net photosynthetic rate; Ci, intercellular CO_2 concentration; Gs, stomata conductance; Tr, transpiration rate; Fv/Fm, maximal quantum yield of PSII photochemistry; PI (*abs*), Performance index on absorption based on the absorption of light energy. Control, control water conditions; Mel, control water conditions after 100 μM pre-treatment melatonin; Ds, drought induced by 20% PEG for 3 d; Mel + Ds drought induced by 20% PEG after 100 μM pre-treatment melatonin.

Treatment	Pn ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Gs ($mmol \cdot m^{-2} \cdot s^{-1}$)	Ci ($\mu mol \cdot mol^{-1}$)	Tr ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	*Fv/Fm*	PI abs
Control	22.85 ± 0.57^a	0.21 ± 0.006^a	237.67 ± 6.27^a	4.24 ± 0.15^a	0.78 ± 0.009^a	1.34 ± 0.034^a
Mel	24.12 ± 0.74^a	0.22 ± 0.037^a	240.94 ± 7.13^a	4.34 ± 0.42^a	0.79 ± 0.011^a	1.32 ± 0.027^a
Ds	16.03 ± 0.95^c	0.13 ± 0.041^c	129.44 ± 6.10^c	2.23 ± 0.17^b	0.74 ± 0.007^c	0.89 ± 0.042^c
Mel + Ds	19.56 ± 0.97^b	0.16 ± 0.011^b	168.52 ± 10.19^b	2.32 ± 0.26^b	0.76 ± 0.005^b	1.18 ± 0.047^b

3.6. Pathway enrichment analysis of DEPs in response to melatonin and drought stress

Pathway enrichment analysis of DEPs showed that several pathways were enriched by melatonin or drought treatments, including carbon fixation in photosynthetic organisms, carbon metabolism, biosynthesis of secondary metabolites, glutathione metabolism, and citrate cycle (TCA cycle) (Fig. 5A). Besides, we focused on 118 DEPs that were influenced and were commonly present in both comparison groups (Dro vs Con and Mel + Dro vs Dro), and also examined a number of DEPs associated with physiological parameters. Through KEGG pathway enrichment analysis, we determined the abundances of 118 DEPs involved in many pathways, such as carbon fixation in photosynthetic organisms, biosynthesis of amino acids, biosynthesis of secondary metabolites, and

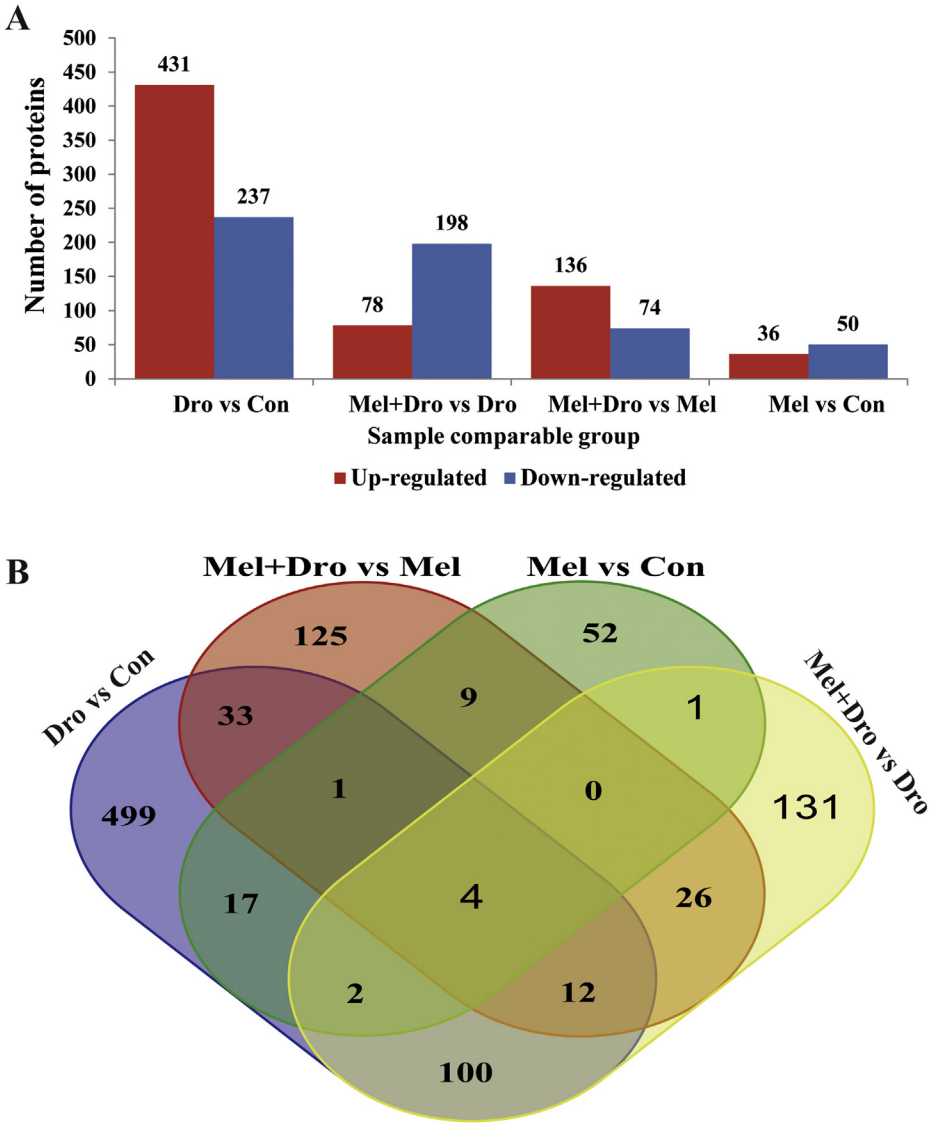

Fig. 3. Distribution of differentially expressed proteins (DEPs) following melatonin treatment and drought effects in maize seedlings. (A) The number of DEPs (fold change ≥1.2 or fold change ≤0.833 and CV value < 0.05) in maize seedlings pre-treated with melatonin or untreated in watered conditions (control) and under drought stress. (B) Venn diagram showing the number of overlapping proteins that were differentially expressed in maize seedlings pre-treated with melatonin or untreated in watered conditions (control) and under drought stress.

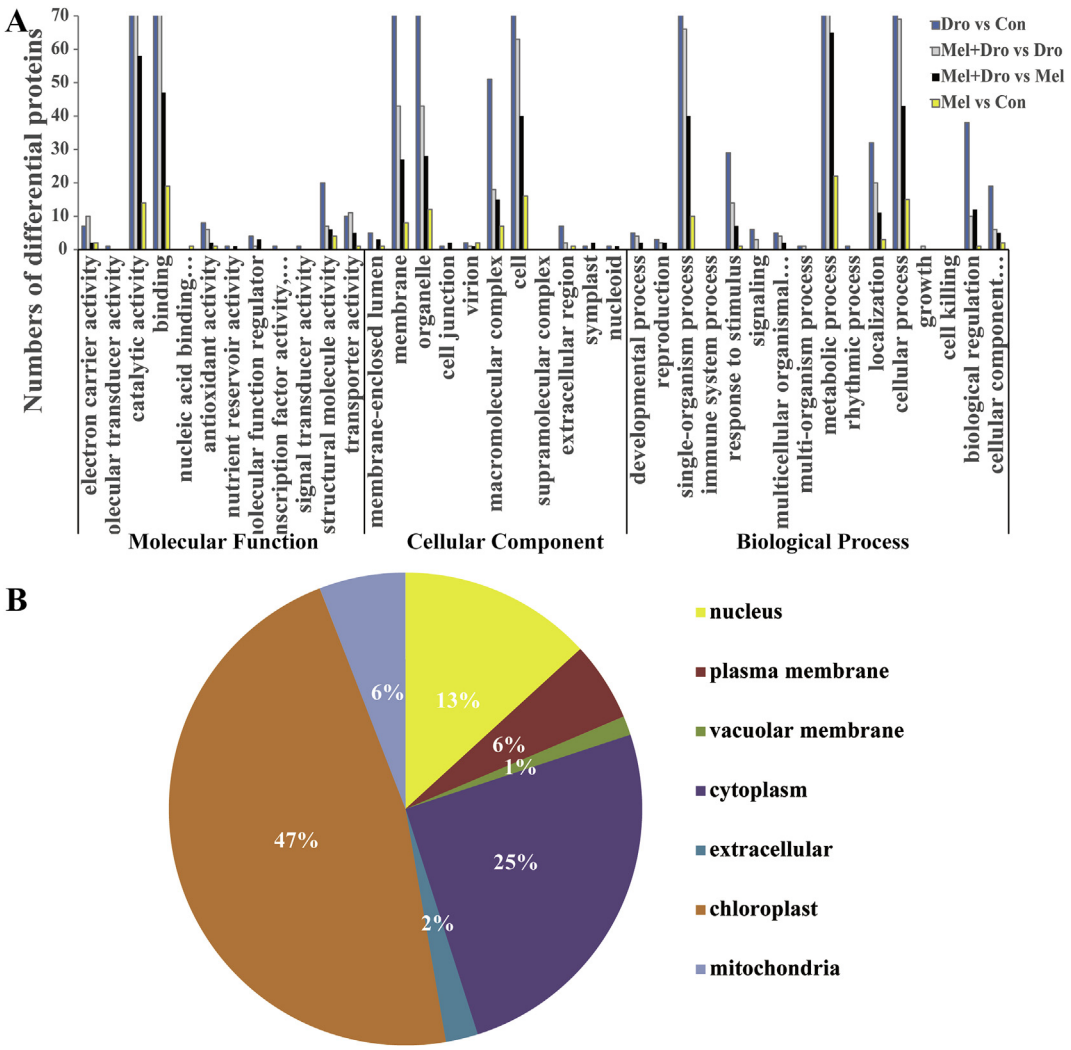

Fig. 4. Gene ontology (GO) analysis (A) and category (B) of differentially expressed proteins (DEPs) in leaves of maize plants after melatonin treatment and with drought effects. Dro vs Con: intensity ratios of different proteins in response to drought treatment compared to the control; Mel + Dro vs Mel: intensity ratios of different proteins in response to melatonin treatment in drought conditions compared to melatonin treatment in watered conditions (control); Mel + Dro vs Dro: intensity ratios of different proteins in response to melatonin treatment in drought conditions compared to untreated in drought conditions; Mel vs Con: intensity ratios of different proteins in response to melatonin treatment compared to the control.

photosynthesis associated with physiological parameters (Table 3, Fig. 5B). Results indicated that these DEPs were possibly involved in melatonin-mediated regulatory mechanism response to drought stress in maize leaves.

4. Discussion

4.1. Exogenous melatonin mitigates the inhibitory effect of PEG-induced drought stress on maize seedling growth

Drought stress critically inhibits plant growth and photosynthesis (Heath and Packer, 1968). As an abiotic anti-stressor, melatonin mediates multiple physiological processes in plants such as growth, development, and enhanced tolerance of abiotic stress (Nawaz et al., 2015). In our experiments, we also found that inhibition of maize seedling growth under drought stress was significantly reduced by melatonin treatments (Fig. 1, Table 1). In addition, when subjected to drought stress, melatonin-treated plants delayed the decline of leaf RWC compared to untreated plants, suggesting enhanced drought tolerance in maize seedlings (Table 1).

4.2. Exogenous melatonin enhances antioxidant enzyme capability and eliminates ROS over-accumulation

Drought stress triggers ROS accumulation and breaks down the balance between ROS generation and detoxification in crop plants (Miller et al., 2010). The accumulation of ROS can induce lipid peroxidation and chlorophyll degradation, and cause the loss of cell membrane integrity and photosynthetic activity (Larkindale and Huang, 2004). Plants have developed an enzymatic antioxidant system and a non-enzymatic antioxidant system to protect against ROS damage (Gill and Tuteja, 2010). Enhancing plant antioxidant ability has been considered the primary function of melatonin in plant stress tolerance (Brazão et al., 2017). In our study, melatonin application enhanced the activities of antioxidant enzymes, including SOD, CAT, and POD, increased GSH content, and decreased H_2O_2 and MDA accumulation (Fig. 2). Therefore, melatonin application enhanced plant antioxidant ability. The results of this study are in agreement with previous reports showing that melatonin application can enhance drought tolerance (Ye et al., 2016).

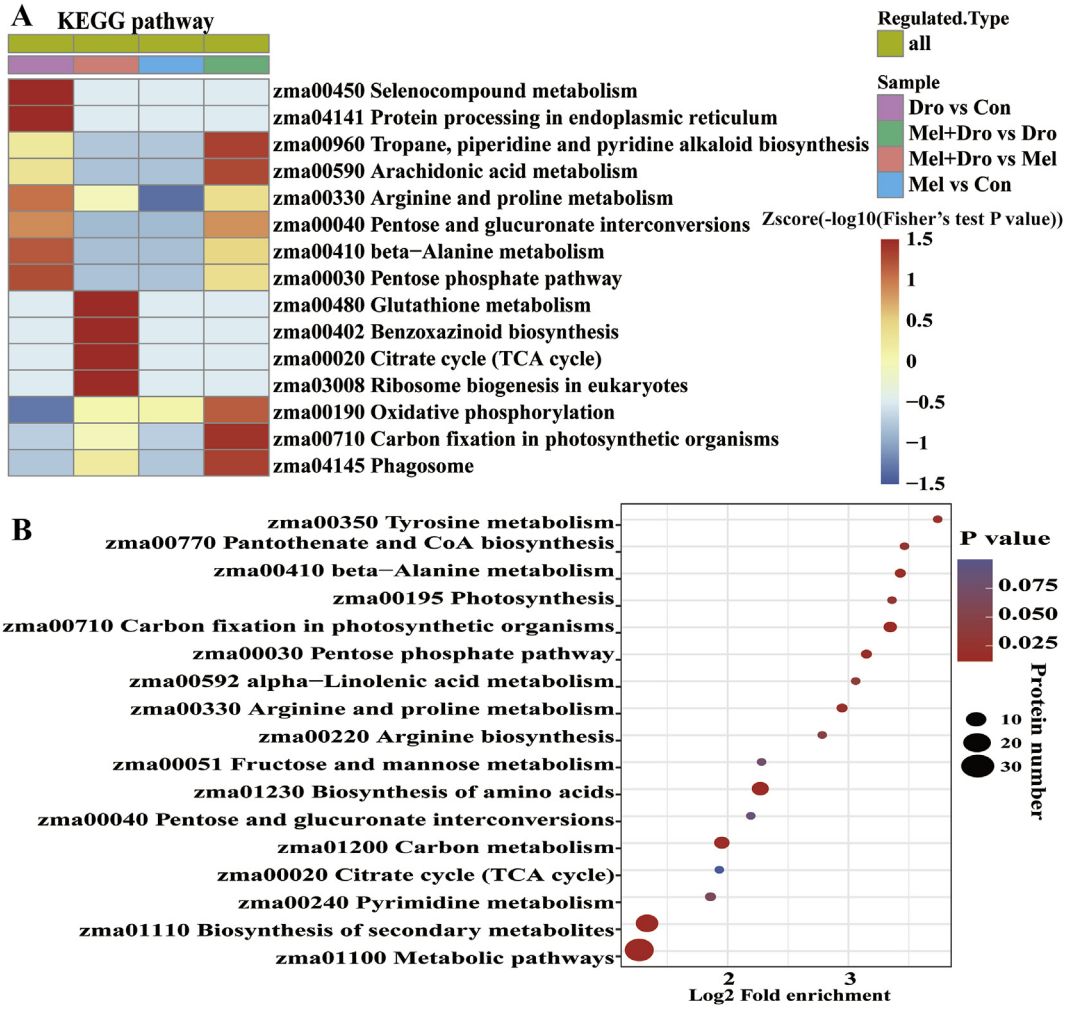

Fig. 5. Pathway enrichment analysis of differentially expressed proteins (DEPs) following melatonin treatment and drought effects in maize seedlings. (A) Pathway enrichment analysis of DEPs induced by melatonin under control or drought stress conditions in maize seedlings and (B) overlapping DEPs in both comparison groups (Dro vs Con and Mel + Dro vs Dro).

4.3. Protein profile changes in response to drought in maize seedlings treated with exogenous melatonin

In this study, we reported changes in physical parameters and a comprehensive analysis of DEPs that were influenced by melatonin in maize leave responding to drought based on iTRAQ-based quantitative proteomic and LC-MS/MS methods. In total, 5,068 identified proteins were found, and 4,175 proteins were quantified (Table S1). Among these proteins, we focused on 118 DEPs that were commonly present in both comparison groups (Dro vs Con and Mel + Dro vs Dro). The functions and pathways of these melatonin-responsive proteins are discussed in the following paragraphs.

4.4. Changes in DEPs related to photosynthesis during drought stress

In the current study, one of most remarkable changes associated with melatonin application was the up regulation of many proteins related to photosynthesis and carbohydrate metabolism in response to drought stress. Some of the typical symptoms of drought stress are decreases in Pn, Gs, Ci, Tr, and Fv/Fm, and as a consequence, carbohydrate synthesis is inhibited in plants (Farooq et al., 2009). The maintenance of positive balance in photosynthesis and carbohydrate metabolism for generating energy is an important mechanism for drought tolerance in plants (Merewitz et al., 2011). During photosynthesis, the reaction-center subunit of the photosystem serves the key site in plants that can be damaged by many kinds of stresses such as drought (Murata et al., 2007). The extent of damage depends on the balance between injury and repair.

Drought stress decreased reaction center subunits (D1, D2, CP43 reaction center, ferredoxin–NADP reductase, reaction center protein H and Cytochrome *b*6-f complex iron-sulfur subunit) in untreated plants. However, plants treated with melatonin had significant increases in photosynthetic proteins including the reaction center subunits compared to untreated plants under drought conditions (Table 3). These results implied that drought stress inhibited photosynthesis in maize seedlings, whereas melatonin could enhance energy conversion during drought stress. This may explain why melatonin-treated plants maintained higher Pn and Fv/Fm than untreated plants when exposed to the same level of leaf water deficit. Similar results can be found in the studies of Ye et al., 2016, which showed that the drought tolerance regulated by melatonin was associated with energy production through maintaining higher Pn, Gs, Ci, Tr, and Fv/Fm than in untreated plants (Table 2).

4.5. DEPs involved in carbon fixation in photosynthetic organisms during the drought response

Metabolic pathway analysis showed that DEPs involved in carbon fixation were largely enriched in maize seedlings. Carbohydrates are the most abundant metabolites in plants, and they play essential roles in

Table 3
Total of 27 key differentially expressed proteins associated with physiological parameters and metabolic pathways of 118 overlapping DEPs in both comparison groups (Dro vs Con and Mel + Dro vs Dro) by KEGG pathway enrichment analysis.

Protein accession	Protein description	Mel + Dro/Dro Ratio	CV value	Dro/Con Ratio	CV value	Mel + Dro/Mel Ratio	CV value	Mel/Con Ratio	CV value
zma00195 Photosynthesis									
P48184	Photosystem II D2 protein	1.248	0.029	0.820	0.029	0.962	0.029	1.064	0.007
P48187	Photosystem II CP43 reaction center protein	1.257	0.039	0.730	0.028	0.912	0.039	1.007	0.025
P48183	Photosystem II protein D1	1.346	0.053	0.776	0.024	1.020	0.053	1.025	0.026
B4FUM2	Ferredoxin–NADP reductase	1.721	0.145	0.656	0.008	1.232	0.145	0.915	0.015
P24993	Photosystem II reaction center protein H	1.371	0.197	0.568	0.033	0.925	0.267	0.842	0.267
B4FSD8	Cytochrome *b*6-f complex iron-sulfur subunit	1.204	0.022	0.820	0.059	0.976	0.022	1.011	0.059
zma00710 Carbon fixation in photosynthetic organisms									
B4FTI5	Fructose-bisphosphate aldolase	1.415	0.027	0.730	0.027	1.066	0.102	0.968	0.102
Q7SIC9	Transketolase, chloroplastic	1.237	0.038	0.763	0.007	0.920	0.052	1.026	0.052
C0P3W9	Phosphoenolpyruvate carboxykinase (ATP)-like protein	1.465	0.038	0.770	0.037	1.355	0.141	0.832	0.141
B4FRQ1	Ribulose-phosphate 3-epimerase	1.375	0.013	0.828	0.014	1.107	0.053	1.029	0.053
P11155	Pyruvate, phosphate dikinase 1, chloroplastic	1.367	0.009	0.787	0.009	1.057	0.031	1.018	0.031
zma01230 Biosynthesis of amino acids									
K7URE0	Aconitate hydratase	0.735	0.026	1.320	0.024	0.981	0.060	0.989	0.060
B4FRQ1	Ribulose-phosphate 3-epimerase	1.375	0.013	0.828	0.014	1.107	0.053	1.029	0.053
A0A096SG60	OSJNBb0072M01.18 protein	0.675	0.050	1.437	0.024	0.999	0.050	0.970	0.023
B4FTI5	Fructose-bisphosphate aldolase	1.415	0.027	0.730	0.027	1.066	0.102	0.968	0.102
Q7SIC9	Transketolase, chloroplastic	1.237	0.038	0.763	0.007	0.920	0.052	1.026	0.052
C4IYE1	Delta-pyrroline-5-carboxylate synthetase	0.692	0.008	2.064	0.034	1.207	0.108	1.183	0.108
K7W3B1	Delta 1-pyrroline-5-carboxylate synthetase isoform 1	0.473	0.046	3.672	0.020	1.461	0.046	1.188	0.030
B4FAW7	Putative histidinol-phosphate transaminase	1.414	0.021	0.785	0.033	1.090	0.009	1.018	0.033
zma01110 Biosynthesis of secondary metabolites									
C0HFU7	Phospholipase D	0.636	0.025	1.589	0.025	0.928	0.116	1.089	0.116
B7ZZ57	Putative polyphenol oxidase family protein	0.824	0.019	1.557	0.026	1.350	0.046	0.950	0.046
C4J3Y5	Glutamate decarboxylase	0.816	0.037	1.631	0.037	1.379	0.025	0.965	0.015
B4FTQ1	Arginase 1, mitochondrial	0.660	0.017	1.205	0.041	0.831	0.060	0.958	0.060
C4IYE1	Delta-pyrroline-5-carboxylate synthetase	0.692	0.008	2.064	0.034	1.207	0.108	1.183	0.108
C0P3W9	Phosphoenolpyruvate carboxykinase (ATP)-like protein	1.465	0.038	0.770	0.037	1.355	0.141	0.832	0.141
K7W3B1	Delta 1-pyrroline-5-carboxylate synthetase isoform 1	0.473	0.046	3.672	0.020	1.461	0.046	1.188	0.030
B4FAW7	Putative histidinol-phosphate transaminase	1.414	0.021	0.785	0.033	1.090	0.009	1.018	0.033
A1XCI7	Lipoxygenase	0.801	0.024	1.303	0.011	0.880	0.125	1.186	0.125
A5GZ73	Glucose-1-phosphate adenylyltransferase	1.298	0.030	0.732	0.030	0.925	0.009	1.027	0.009
K7URE0	Aconitate hydratase	0.735	0.026	1.320	0.024	0.981	0.060	0.989	0.060
A0A096SG60	OSJNBb0072M01.18 protein	0.675	0.050	1.437	0.024	0.999	0.050	0.970	0.023
B4FRQ1	Ribulose-phosphate 3-epimerase	1.375	0.013	0.828	0.014	1.107	0.053	1.029	0.053
B4FTI5	Fructose-bisphosphate aldolase	1.415	0.027	0.730	0.027	1.066	0.102	0.968	0.102
C0P4G2	Putative cytochrome P450 superfamily protein	0.658	0.011	1.714	0.011	1.085	0.095	1.040	0.095
Q7SIC9	Transketolase, chloroplastic	1.237	0.038	0.763	0.007	0.920	0.052	1.026	0.052
K7U7K6	9-cis-epoxycarotenoid dioxygenase 1	1.647	0.027	0.463	0.027	1.014	0.040	0.753	0.040

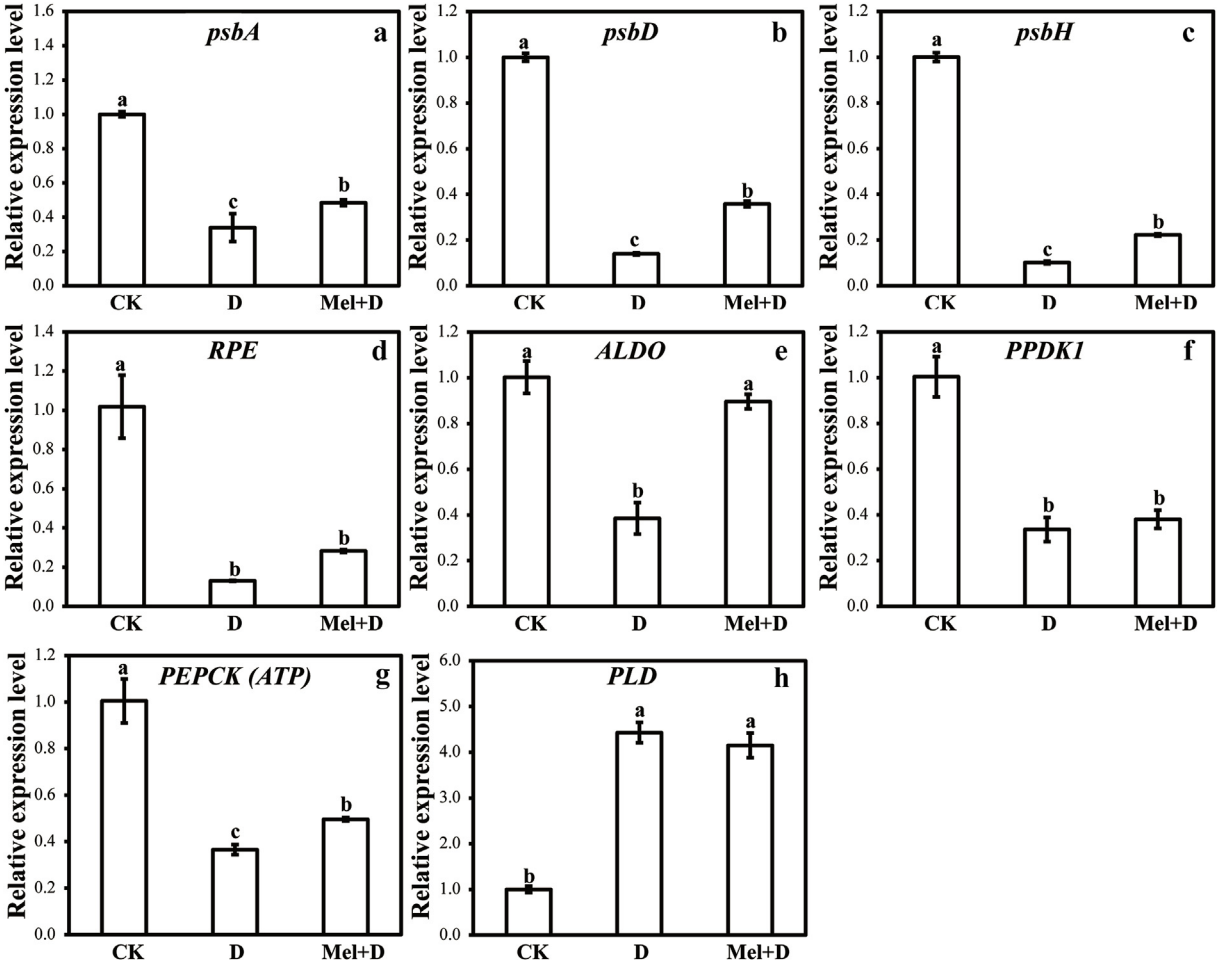

Fig. 6. Expression analyses of the genes related to photosynthesis, carbon fixation, biosynthesis of amino acids, and biosynthesis of secondary metabolites in the control and Mel 100 in maize seedlings subjected to drought stress. Relative expression levels were recorded for quantitative RT-PCR in different treatments by the means (± SD) using the $2^{-\Delta\Delta Ct}$ method. Means denoted by different letters show significant differences ($P < 0.05$). Definition of the abbreviations: (a) psbA, Photosystem II protein D1; (b) psbD, Photosystem II D2 protein; (c) psbH, Photosystem II reaction center protein H; (d) RPE, Ribulose-phosphate 3-epimerase; (e) ALDO, Fructose-bisphosphate aldolase; (f) PPDK1, Pyruvate, phosphate dikinase 1, chloroplastic; (g) PEPCK (ATP), Phosphoenolpyruvate carboxykinase (ATP)-like protein; (h) PLD, Phospholipase D.

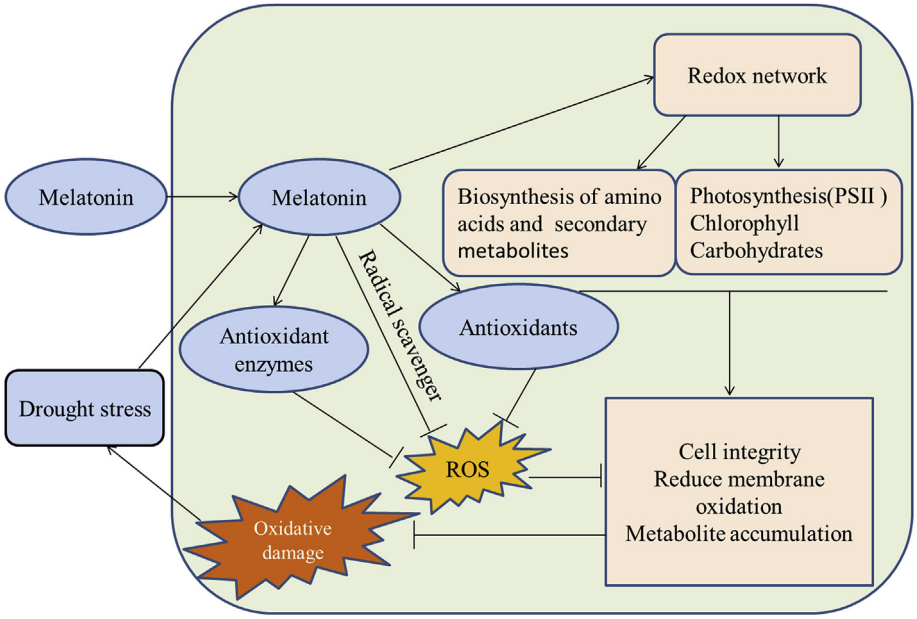

Fig. 7. Schematic illustration of a proposed model for alleviating damage to maize exposed to drought stress by melatonin treatment.

plant response to abiotic stresses, serving as energy sources, and as signaling molecules (Rolland and Baena Gonzalez, 2006). The relationship between the accumulation of carbohydrates and enhanced drought tolerance has been widely documented in different plants (Mitchell et al., 2013). Under drought conditions, carbon fixation is suppressed, but consumption of reserve carbohydrates still increases until plants die (Mcdowell et al., 2011).

Our study demonstrated that drought stress in maize induced significant decreases in the abundance of fructose-bisphosphate aldolase, transketolase, ribulose-phosphate 3-epimerase, and other DEPs related to carbon fixation in untreated plants (Table 3). However, melatonin-treated plants maintained a significantly higher abundance of these proteins compared to untreated plants under drought stress. These proteins are critical enzymes in controlling carbon fixation. Their decline might reflect the inhibition of carbon fixation in plants during abiotic stress (Doubnerová and Ryšlavá, 2011), and melatonin could enhance carbon fixation, which might also partly contribute to the regulation of drought tolerance in maize seedlings. Thus, our findings provided strong evidence in favor of the role of endogenous melatonin in the regulation of energy production and carbohydrate metabolism in response to drought stress.

4.6. DEPs involved in other biological processes and expression pattern analysis of genes

For other metabolic processes, several of the 118 most abundant DEPs were observed to be associated with biosynthesis of amino acids and biosynthesis of secondary metabolites. Amino acids play an important physiological function in resistance to stress (Rai, 2002). In this study, ribulose-phosphate 3-epimerase, fructose-bisphosphate aldolase, transketolase, and histidinol-phosphate transaminase were strongly induced by melatonin treatment in maize seedlings under drought stress. Previous reports have shown that chilling stress significantly suppressed enzyme activity of fructose-bisphosphate aldolase in tomato seedlings (Cai et al., 2017), and over-expression of ribulose-phosphate 3-epimerase promoted growth and development of *Arabidopsis thaliana* (Wang et al., 2016). In addition, the DEPs not only regulated biosynthesis of amino acids, but were also associated with biosynthesis of secondary metabolites. As reported previously, DEPs were closely related to plant drought tolerance because of their functions in directly or indirectly regulating photosynthesis and alleviating ROS damage (Cai et al., 2017; Wang et al., 2016). This result suggested that the ability of melatonin to alleviate drought stress is associated with regulating biosynthesis of amino acids and secondary metabolites.

To compare the alteration of gene transcription and protein levels, we used qRT-PCR to test relative expression levels of genes involved in several metabolic pathways from the identified overlapping DEPs and photosynthesis. As shown in Fig. 6, the levels of gene expression were clearly different between melatonin pretreated and untreated maize seedlings subjected to drought stress, and expression patterns of most these genes had similar changes with protein levels. For instance, psbA, psbD, psbH, PEPCK (ATP), and ALDO significantly increased in melatonin-pretreated maize and decreased in untreated maize under drought stress. These results indicated that changes in melatonin-induced protein levels were largely dependent on the expression level of the transcript. However, the expression profiles of the mRNA did not always have the same tendency as the proteins; trends were different for RPE, PPDK1, and PLD. We assume that the lack of correlation between mRNA and protein level in these cases indicates the existence of post-translational modifications and/or regulation, such as translational regulation and protein degradation (Pradet-Balade et al., 2001). The inconsistent expression level of transcripts and proteins were confirmed by previous reports in various plant species in response to drought stress (Yin et al., 2016a; Li et al., 2016).

5. Conclusions

In summary, pre-treatment with melatonin can effectively enhance photosynthesis, membrane stability, antioxidant enzyme activity, and decrease oxidative damage in maize seedlings under drought stress (Fig. 7). These results demonstrated that application of melatonin effectively improved drought tolerance of maize seedlings. The iTRAQ-based protein profiling provided a holistic way to reveal several major metabolic processes associated with regulation by melatonin under drought stress. These metabolic processes included photosynthesis, carbon fixation in photosynthetic organisms, biosynthesis of amino acids, and biosynthesis of secondary metabolites. In conclusion, our findings provide new evidence to support the multiple roles of melatonin under drought stress and the ability to enhance maize tolerance to drought stress through engineered modifications.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Conceived and designed the experiments: Q.Y., X.S., and L.G. Performed the experiments: X.S., X.F., Y.W., R.X., and J.G. Analyzed the data: X.S., X.F., Y.W., R.X., J.Y., and L.G. Contributed reagents/materials/analysis tools: Q.Y., L.G., X.S., and J.Y. Wrote the paper: X.S., J.Y., Q.Y., and L.G. All authors had final approval of the submitted and published versions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2019.07.012.

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