Planta
https://doi.org/10.1007/s00425-018-2881-2

ORIGINAL ARTICLE

Proteomic analysis of melatonin-mediated osmotic tolerance by improving energy metabolism and autophagy in wheat (*Triticum aestivum* L.)

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Received: 1 February 2018 / Accepted: 17 March 2018

Abstract

Main conclusion **Melatonin-mediated osmotic tolerance was attributed to increased antioxidant capacity, energy metabolism, osmoregulation and autophagy in wheat (*Triticum aestivum* L.).**

Melatonin is known to play multiple roles in plant abiotic stress tolerance. However, its role in wheat has been rarely investigated. In this study, 25% polyethylene glycol 6000 (PEG 6000) was used to simulate osmotic stress, and wheat seeds and seedlings were treated with different concentrations of melatonin under PEG stress. Isobaric tag for relative and absolute quantification (iTRAQ)-based proteomic techniques were used to identify the differentially accumulated proteins from melatonin-treated and non-treated seedlings. Seeding priming with melatonin significantly increased the germination rate, coleoptile length, and primary root number of wheat under PEG stress, as well as the fresh weight, dry weight, and water content of wheat seedlings. Under PEG stress, melatonin significantly improved reactive oxygen species homeostasis, as revealed by lower H_2O_2 and $O_2^{\cdot-}$content; and the expression of antioxidant enzymes at the transcription and translation levels was increased. Melatonin maintained seedling growth by improving photosynthetic rates and instantaneous and intrinsic water use efficiencies, as well as carbon fixation and starch synthesis at the protein level. Melatonin treatment significantly affected the expression of glycolytic proteins, including fructose-1,6-bisphosphate aldolase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, and enolase, and remarkably increased the expression of the nicotinamide adenine dinucleotide transporter and nicotinamide adenine dinucleotide binding protein, thereby indirectly modulating electron transport in the respiratory chain. This indicated that melatonin improved energy production in PEG-stressed seedlings. Further, melatonin played a regulatory role in autophagy, protease expression, and ubiquitin-mediated protein degradation by significantly upregulating rab-related protein, fused signal recognition particle receptor, aspartyl protease, serine protease, ubiquitin-fold modifier 1, and ubiquitin at the mRNA or protein level. These findings suggested that melatonin might activate a metabolic cascade related to autophagy under PEG stress in wheat seedlings.

Keywords Cytophagy · Drought stress · Glycolysis · iTRAQ · *N*-Acetyl-5-methoxytryptamine · Polyethylene glycol (PEG) · Seed priming

Guibin Cui and Fengli Sun have contributed equally to this paper.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-018-2881-2) contains supplementary material, which is available to authorized users.

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Abbreviations

iTRAQ	Isobaric tag for relative and absolute quantification
DP	Differentially accumulated proteins

Introduction

Drought is one of the strongest abiotic stressors affecting plant production worldwide. Plant growth, morphogenesis, yield, pigment composition, and other traits are seriously affected by drought (Osakabe et al. 2014). Plants

Published online: 21 March 2018

have evolved various physiological mechanisms to adapt to drought, including stomatal closure to decrease water loss (Flexas and Medrano 2002; Pou et al. 2008), the production of enzymatic and non-enzymatic reactive oxygen species (ROS) antioxidants (Xu et al. 2016b), the over-accumulation of osmoprotectants (Jongdee et al. 2002), and increasing root extension for water absorption (Manschadi et al. 2008; Gowda et al. 2011).

Melatonin is an endogenic indolamine derived from tryptophan that occurs in many higher plants (Posmyk and Janas 2009; Byeon et al. 2015, 2016). It has been reported to enhance plant tolerance to environmental stressors such as chilling (Turk et al. 2014), salinity (Mukherjee et al. 2014), and osmotic stress (Zhang et al. 2013). Several studies have attempted to elucidate the mechanism by which melatonin mitigates drought and osmotic stresses. Melatonin was reported to increase the antioxidant capacity and reduce ROS damage in apple, tomato, and wheat by stimulating both enzymatic and non-enzymatic antioxidants (Wang et al. 2013a; Liu et al. 2015; Manchester et al. 2015; Reiter et al. 2016; Cui et al. 2017). It might have been the first evolutionary line of defense against oxidative stress (Tan et al. 2014; Manchester et al. 2015), can be induced by osmotic or drought stress (Smirnoff 1998) and scavenge ROS directly and efficiently (Allegra et al. 2003). Melatonin can decrease chlorophyll degradation and photosystem damage and protect the bioactivity of sugars and proteins in apple and grape under drought or osmotic stresses (Wang et al. 2013a, b; Meng et al. 2014), likely by promoting the decomposition of ROS. Melatonin establishes nitro-oxidative homeostasis by regulating the enzymes that metabolize reactive oxygen and nitrogen species (Antoniou et al. 2017). Moreover, it enhances the water absorption and retention in cucumber by increasing the root:shoot ratio in the melatonin-treated group (Zhang et al. 2013). It was also found to improve water retention in apple (Li et al. 2015). Cellular water potential was reduced by exogenous melatonin via the excessive production of osmoregulatory solutes such as free proline, amino acids, organic acids, sugars, and sugar alcohols (Wang et al. 2013a; Shi et al. 2015a; Antoniou et al. 2017). Turk and Erdal (2015) reported that melatonin increases the mineral content in maize under cold stress, and these elements might contribute to the accumulation of osmoprotectants. In addition, bermudagrass pretreated with melatonin and subjected to abiotic stress showed an overexpression of genes associated with the metabolism of nitrogen, major carbohydrates, tricarboxylic acids, and hormones, as well as the genes encoding proteins for transport, metal disposition, redox, and secondary metabolism (Shi et al. 2015a). Furthermore, melatonin also affects ROS-dependent signal transduction. ROS are key secondary messengers in plant signal transduction under stress and might cause membrane lipid peroxidation, DNA damage, protein and carbohydrate oxidation, and cell autophagy (Baxter et al. 2014; Wang et al. 2015). The calcium-dependent signal pathway in *Arabidopsis* was also reported to be influenced by melatonin (Posmyk and Janas 2009; Sarah Weeda et al. 2014). Proteins containing the WRKY and NAC domains, zinc finger transcription factors, and many defense-related genes are upregulated by melatonin (Byeon et al. 2013; Zhang et al. 2014).

Wheat is one of the most important crops worldwide. Drought seriously impedes wheat tillering, stem elongation and heading, flowering and grain formation (Brisson and Casals 2005; Jaeger et al. 2014; Hao et al. 2015). Exogenous melatonin enhances the growth and water-stress tolerance in many plants (Li et al. 2015; Liu et al. 2015; Shi et al. 2015a; Ye et al. 2016; Antoniou et al. 2017; Cui et al. 2017; Fleta-Soriano et al. 2017; Wang et al. 2017). Nevertheless, the proteome-level response mediated by exogenous melatonin in response to osmotic stress has not yet been identified in wheat. Isobaric tag for relative and absolute quantification (iTRAQ)-based quantitative proteomic analysis with tandem mass spectrometry (MS/MS) generates more accurate results than those by traditional two-dimensional gel electrophoresis, isotope-coded affinity tags, or stable isotope labeling of amino acids(Ross et al. 2004; Yang et al. 2011). In this study, wheat seeds and 14-d-old seedlings were subjected to osmotic stress simulated by 25% polyethylene glycol (PEG-6000) with or without different concentrations of melatonin. Melatonin was found to induce morphological and physiological changes and play an important role in osmotic tolerance in PEG-stressed wheat seedlings.

Materials and methods

Materials and germination tests

Wheat (*Triticum aestivum* L. Yan995) was selected for this study because a preliminary screening experiment indicated that it is highly sensitive to exogenous melatonin. The seeds of Yan 995 come from the Key Laboratory of Wheat Biology and Genetic Improvement on Northwestern China, Ministry of Agriculture, P.R. China in Yangling, Shaanxi, China. The experimental design was a completely randomized block with five replicates. Seeds were first treated with 75% (v/v) ethanol for 2 min and surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min. They were then rinsed five times with distilled water and treated with 0, 1, 10, 100, 300, 500, or 1000 μM melatonin for 12 h. The seeds were placed on Petri dishes ($d = 9$ cm) containing three layers of filter moistened either with 5 mL of distilled water or 25% PEG-6000 solution and one of the melatonin concentrations. The plates were stored in the dark at 20 °C. Germination rates, coleoptile lengths, and primary root numbers were measured on the fourth day after germination.

Seedling growth and treatments

The Yan 995 wheat seeds were surface-sterilized as described above and germinated in 9-cm Petri dishes. After 6 days, seedlings of similar size were planted in black plastic pots (9 × 9 × 12 cm) each containing 700 mL of 1/2 Hoagland's nutrient solution. The pots were maintained in a growth chamber with temperature and light/dark cycle of 20/15 °C and 14/10 h, respectively, at 70 ± 5% relative humidity. Wheat seedlings with three expanded leaves (14-days) were transferred to 25% PEG-6000 containing 0, 1, 10, 100, 300, 500, or 1000 μM melatonin for 72 h. The growth and physiological parameters of the seedlings were measured, and proteomic analyses were performed.

Determination of seedling growth

The seedlings were dried on absorbent paper, and their fresh weight (FW) was determined. The seedlings were killed by heating to 105 °C for 10 min, and their dry weight (DW) was determined by heating to a constant weight at 80 °C. The water content (WC) was calculated as follows: WC = (FW − DW)/FW (Gond et al. 1999). These measurements were repeated nine times.

Determination of physiological parameters

Five replicates were used to determine the gas exchange parameters, and four replicates were used for the determination of the other physiological parameters. Gas exchange parameters including photosynthetic rate (Pn), stomatal conductance (Cond), intercellular CO_2 concentration (Ci), and transpiration rate (Tr), were measured using a portable photosynthesis system (LI-6400XT; LI-COR Biosciences, Lincoln, NE, USA) at a light intensity of 500 μmol m^{-2} s^{-1}. The instantaneous and intrinsic water efficiencies of seedling leaves were calculated from Pn/Tr and Pn/Cond, respectively (Adam et al. 2000; Gulías et al. 2012).

Malondialdehyde (MDA) content was determined according to Kramer et al. (1991). Frozen leaves (0.1 g) was ground in 1.5 mL of 0.5% trichloroacetic acid and the homogenate was centrifuged at 4°C and 12,000*g* for 15 min. The reaction mixture consisted of 1 mL of supernatant and 2 mL of 0.6% thiobarbituric acid. The mixture was boiled for 15 min and centrifuged at 3000*g* for 15 min. The absorbance of the supernatant was measured at 450, 532, and 600 nm. The MDA content was calculated as follows:

$$\mathrm{MDA}(\mu\mathrm{mol\ g^{-1}FW}) = (6.45 \times (D532 - D600) - 0.56 \times D450) \times V/W$$

where *D*450, *D*532, and *D*600 are the supernatant absorbance values at 450, 532, and 600 nm, respectively; *V* is the supernatant volume; and *W* is the fresh weight of the leaf sample.

Potassium iodide solution was used to quantify H_2O_2 according to Velikova et al. (2000). The reaction mixture, which contained 2 mL of KI (1 M) and 1 mL of leaf extract, was incubated at 25 °C for 1 h. The absorbance at 390 nm was recorded. The H_2O_2 content was derived from a standard curve.

The superoxide radical formation was measured according to Elstner and Heupel (1976). The reaction mixture, which included 1 mL of leaf extract and 0.25 mL of hydroxylamine hydrochloride (10 mM), was incubated at 25 °C for 20 min. Next, 1 mL of sulfanilic acid (17 mM) and 1 mL of α-naphthylamine (7 mM) were added to the mixture and left to react at 30 °C for 30 min. The absorbance was measured at 530 nm, and the superoxide radical content was obtained from a standard curve.

Leaf protein extraction, trypsin digestion and iTRAQ labeling

Protein was exacted according to Chu et al. (2013). Fresh leaf powder (0.05–0.1 g) was digested with 500 μL of lysis buffer (20 mM Tris–HCl pH 7.5; 250 mM sucrose; 10 mM EGTA; 1% Triton X-100; 1 mM PI; 1 mM DTT) in an ice-bath for 20 min and centrifuged at 15,000*g* at 4 °C for 15 min. Three volumes of pre-cooling acetone were added to the supernatants, and the protein was precipitated at − 20 °C for ≥ 2 h. The precipitate was separated by centrifugation and washed three times with cold acetone. The protein concentration was determined according to the instructions provided with Bradford Reagent (B6916) (Sigma-Aldrich, St. Louis, MO, USA). The protein quality was checked using SDS-PAGE. Approximately 200 μg of protein from each sample was digested with 4 μg of 50:1 trypsin (Promega Corp., Madison, WI, USA) for ≥ 12 h at 37 °C. Digested protein was detected by mass spectrometry using a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF; 4800 Proteomics Analyzer; AB SCIEX LLC, Framingham, MA, USA) and then labeled using an iTRAQ Reagent-8plex Multiplex Kit (AB SCIEX LLC) according to the manufacturer's instructions. The labeling process is shown in Fig. 1. The control group with PEG stress (PEG) has three independent biological replicates (PEG-1, PEG-2, and PEG-3), and were labeled by the isobaric tag 115, 116, and 117, respectively. The experimental group with melatonin treatment under PEG stress (MT) also has three independent biological replicates (MT-1, MT-2, and MT-3) and were labeled by the isobaric tag 118, 119, and 121, respectively. Labeling efficiency was determined using liquid chromatography-tandem mass spectrometry (LC–MS/MS).

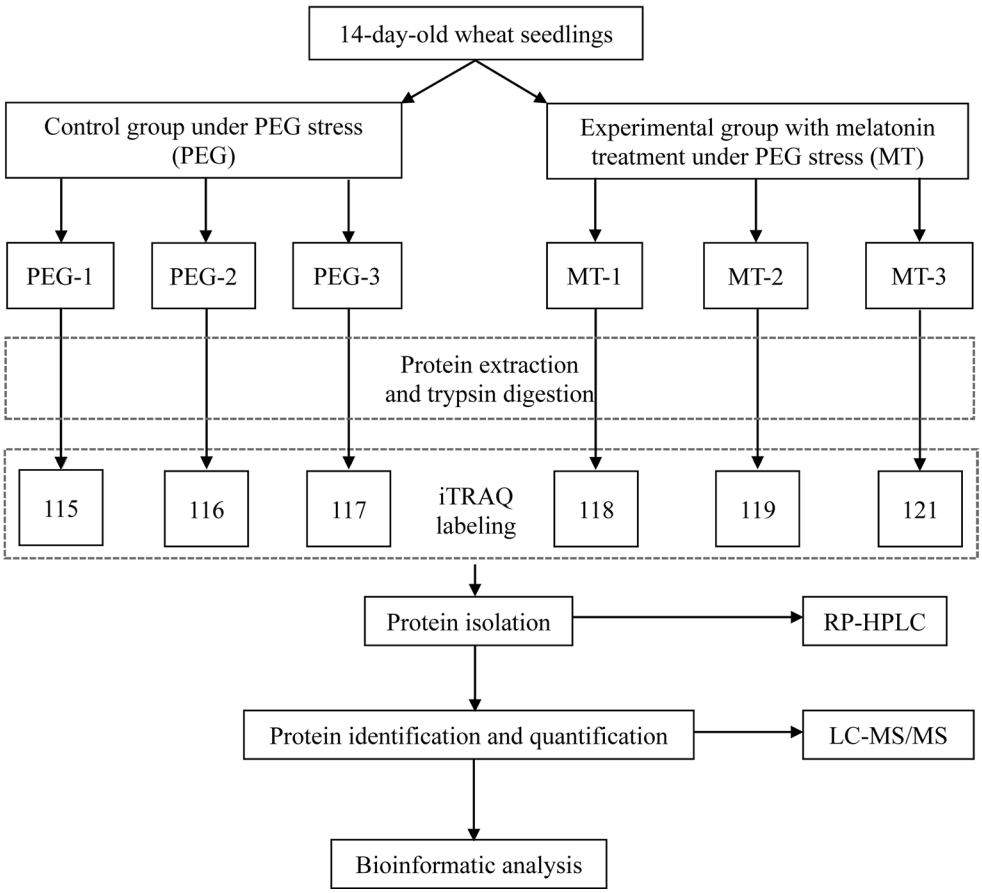

Fig. 1 iTRAQ labeling of wheat seedling protein. Wheat seedling proteins were extracted after from 14-days-old seedlings treated by 25% PEG-6000 for 72 h with or without 500 μM melatonin. PEG-1, PEG-2, and PEG-3 indicates three biological replicates of the control group under PEG stress. MT-1, MT-2, and MT-3 indicate three biological replicates of the experimental group with melatonin treatment (MT) under PEG stress. RP-HPLC, Reverse-phase high-performance liquid chromatography; LC–MS/MS, Liquid chromatography-tandem mass spectrometry

Protein isolation, identification and quantification

The sample protein was classified using high-pH reverse-phase high-performance liquid chromatography (RP-HPLC) with a Gemini-NX 3μ C18 110A column (150 mm × 2.00 mm). Labeled samples were re-dissolved in 100 μL of mobile phase A (2% acetonitrile, pH 10) and separated using a linear gradient [5–35% mobile phase B (98% acetonitrile, pH 10) for 30 min, 35–95% B for 30–32 min, 95% B for 32–37 min, 95–5% B for 37–39 min, and 5% B for 39–45 min]. The absorbance of the UA detector was 214 nm and the flow rate was 700 μL per minute. Fractions were collected starting at 5 min and every 1.5 min thereafter, and then dried by vacuum centrifugation.

Peptides were re-dissolved in 0.5% v/v formic acid and separated using capillary HPLC using a C18 trap column (C18 3 μm 0.10 mm × 20 mm), an analytical column (C18 1.9 μm 0.15 mm × 120 mm), and mobile phases A (0.1% v/v formic acid in water) and B (0.1% v/v formic acid in 80% v/v acetonitrile). The chromatographic column was balanced with 95% mobile phase A. Subsequently, peptides were separated using a linear gradient as follows: 6–9% B for 0–8 min, 9–14% B for 8–24 min, 14–30% B for 24–60 min, 30–40% B for 60–75 min, 40–95% B for 75–78 min, 95% B for 78–85 min, 95–6% B for 85–86 min, and 5% B for 86–90 min with a 600 nL/min flow rate. The MS/MS analysis was conducted using a Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A full mass spectrometry scan (300–1400 *m/z*) was required in the positive ion mode at a resolution of 120,000; an AGC value of 3e6; a maximum IT of 80 ms; one scan range; and a dynamic exclusion of 12.0 s. The data-dependent MS scan was acquired at a resolution of 15,000; an AGC value of 5e4; a maximum IT of 45 ms; an isolation window of 1.6 *m/z*; and a normalized collision energy of 30 eV.

Tandem mass spectra were searched using Mascot 2.1 (Matrix Science, Boston, MA, USA) containing Proteome Discover 1.4 against the Uniprot_Triticum Aestivum, FASTA (Uniprot sequences). The protein confidence threshold cutoff was set to 1.2, and the fold-changes > 1.2 or < 0.83 were set as cutoff values for significant alterations in protein expression.

Bioinformatic analysis

The differential proteins were subjected to gene ontology (GO) annotation using agriGO, and enrichment analysis

included molecular functions, cellular components, and biological processes (Du et al. 2010). The participant metabolic pathways of differential proteins were determined using Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (http://www.kegg.jp/kegg/) (Kanehisa et al. 2012). IBM SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA) and Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA) were used to process all other data.

RNA extraction and quantitative real-time polymerase chain reaction verification

The expression of genes related to antioxidant enzymes, amino acid metabolism, glycolysis, and autophagy was analyzed. RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific) according to manufacturer instructions. The fresh leaves were ground to powder in liquid nitrogen, and 50–100 mg powder was used for extraction. The extracted RNA was dissolved in 50 µL of RNase-free water. Genomic DNA was removed, and the first cDNA chain was reverse-transcribed using reverse transcription kits (HiScript II Q RT SuperMix for qPCR; Vazyme Biotech Co. Ltd., Nanjing City, China) with 2 µg of total RNA from each sample. The gene-specific primers were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA) and are shown in Supplementary Table S1. Quantitative real-time polymerase chain reactions (qRT-PCRs) were conducted in 20 µL of reaction mixtures containing 10 µL of AceQ qPCR SYBR Green Master Mix, 4 µL of template, 2 µL of primer mix (2 µM), and 4 µL of ROX Reference Dye 1 (5×). There were five biological replicates of every measurement.

Results

Effect of melatonin on wheat germination and seedling growth under PEG stress

The effects of melatonin on wheat germination and seedling growth are shown in Figs. 2 and 3. Seed germination and seedling growth under 25% PEG stress were significantly improved by melatonin (Figs. 2 and 3). The germination rate, coleoptile length, and primary root number (Fig. 2b, c, and d) and the fresh weight, dry weight, and water content (Fig. 3c, d, and e) of wheat seedlings under 25% PEG stress were increased by exogenous melatonin.

Effect of melatonin on the physiological parameters of wheat seedlings under PEG stress

The roles of melatonin in photosynthesis and water use in wheat seedlings under PEG stress were determined by measuring gas exchange parameters. Exogenous melatonin improved the photosynthetic rate, stomatal conductance, and transpiration rate (Fig. 4a, b, and d), but significantly decreased intercellular CO_2 (Fig. 4c). The instantaneous and intrinsic water use efficiencies in melatonin-treated seedlings under PEG stress were higher than those of the untreated seedlings under PEG stress (Fig. 4e and f).

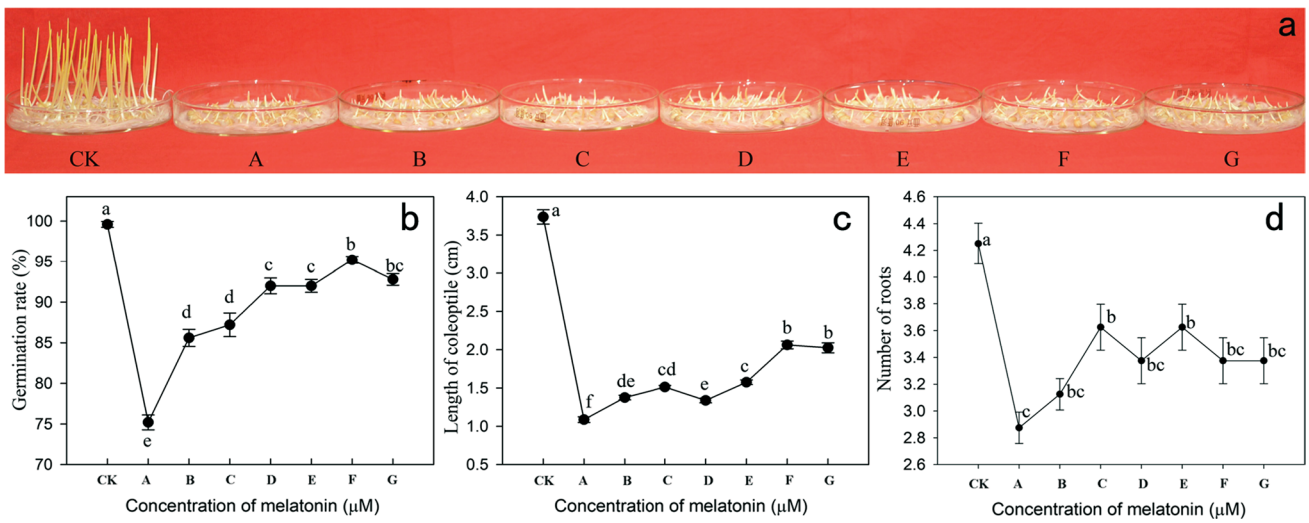

Fig. 2 Effect of exogenous melatonin on wheat seed germination. Seeds were first primed with different melatonin concentrations for 12 h. They were planted in 9-cm Petri dish with 3 layers of wet filter containing 5 mL of distilled water or 25% PEG-6000 solution and different melatonin concentrations at 20 °C in the dark. Germination rate, coleoptile length, and primary root number were measured 4 days after germination. **a–d** CK is the blank control without PEG or melatonin. The dishes A, B, C, D, E, F, and G are 0, 1, 10, 100, 300, 500, and 1000 µM melatonin with 25% PEG-6000, respectively. **b–d** Germination rates (**b**), coleoptile length (**c**), and number of roots (**d**). Different lowercase letters indicate significant differences (Duncan's multiple range, $P<0.05$, $n=5$). Bars indicate mean ± SE

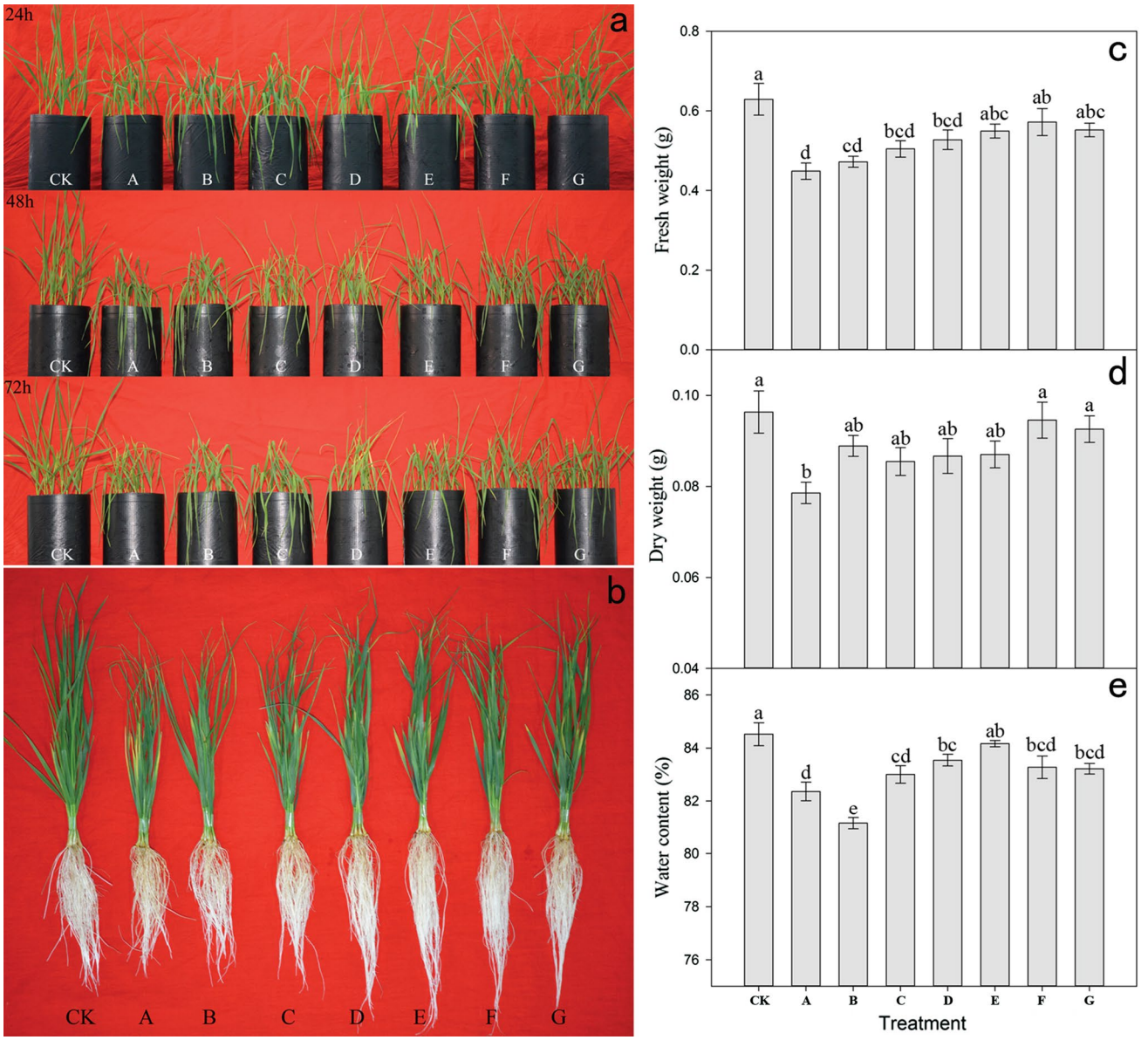

Fig. 3 Effect of exogenous melatonin on wheat seedling growth. 14-days-old wheat seedlings were treated with 25% PEG-6000 solution and different melatonin concentrations. After 72 h, seedling growth parameters were measured. **a–e** CK, blank control with 1/2 Hoagland's solution. A, B, C, D, E, F, and G are 0, 1, 10, 100, 300, 500, and 1000 μM with 25% PEG-6000 and 1/2 Hoagland's solution, respectively. **a** Wheat seedlings treated with melatonin for 24 h, 48 h, and 72 h. **b** Wheat seedlings treated with melatonin for 72 h. **c–e** Fresh weight (**c**), dry weight (**d**), and water content (**e**). Different lowercase letters indicate significant differences (Duncan's multiple range, $P < 0.05$, $n = 9$). Bars indicate mean ± SE

In this study, both H_2O_2 and $O_2^{\cdot-}$ in wheat seedlings under PEG stress were significantly decreased by melatonin treatment as per quantitative measurements (Fig. 5b and c). Melatonin also reduced MDA levels, which indicate membrane lipid peroxidation (Fig. 5a).

Protein profiling of melatonin-treated wheat leaf under PEG stress

In total, 2366 proteins extracted from the PEG control (PEG) and the PEG + melatonin treatment (MT) were identified by MS and database searches (Supplementary Table S2). There were 210 differentially accumulated

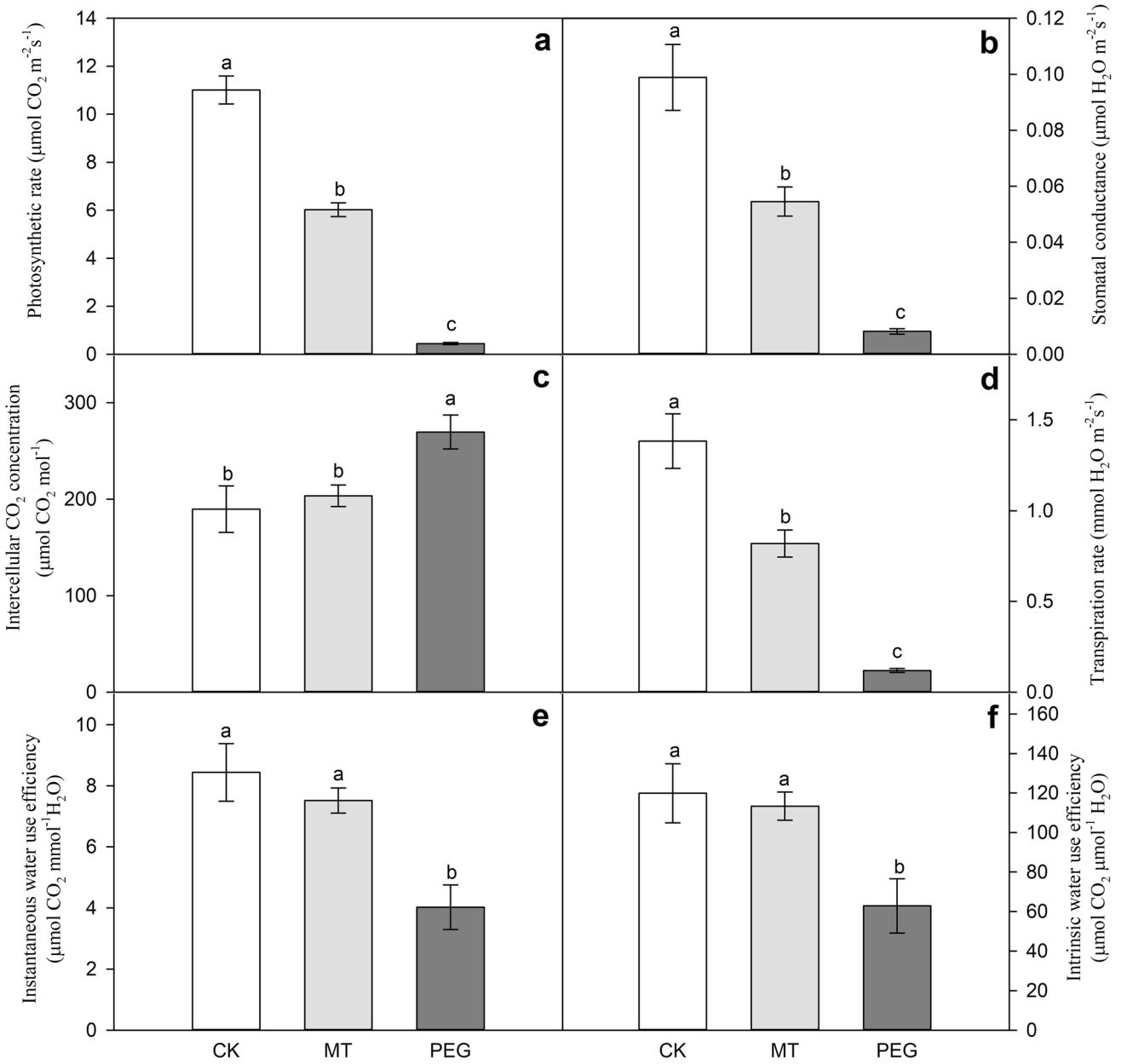

Fig. 4 Effect of melatonin on gas exchange parameters and water use efficiency. 14-days-old wheat seedlings were treated with 25% PEG-6000 solution and different melatonin concentrations. Measurements were performed 72 h after treatment. **a–f** CK, blank control (without PEG and melatonin); PEG, 25% PEG-6000; MT, 25% PEG-6000 with 500 μM melatonin. Photosynthetic rate (**a**), stomatal conductance (**b**), intercellular CO_2 concentration(**c**), transpiration rate (**d**), instantaneous water use efficiency (**e**), and intrinsic water use efficiency (**f**) are shown. Different lowercase letters indicate significant differences (Duncan's multiple range, $P < 0.05$, $n = 5$). Bars indicate mean ± SE

proteins (DP) between PEG and MT group, of which 96 proteins were up-accumulated and 27 were significantly down-accumulated in MT/PEG (Fig. 6, Supplementary Table S2). Moreover, 57 DP were significantly accumulated ($P < 0.05$), of which 30 DP were up-accumulated and 27 were down-accumulated (Fig. 6, Supplementary Table S2).

Gene ontology and KEGG enrichment of DP

The 210 DP of MT/PEG were searched in the gene ontology (GO) and KEGG databases. Overall, 179 and 112 DP were in the GO enrichment and the KEGG pathway, respectively (Supplementary Table S3 and S4).

The GO enrichment of DP in biological processes, cellular components, and molecular functions is shown in Fig. 7.

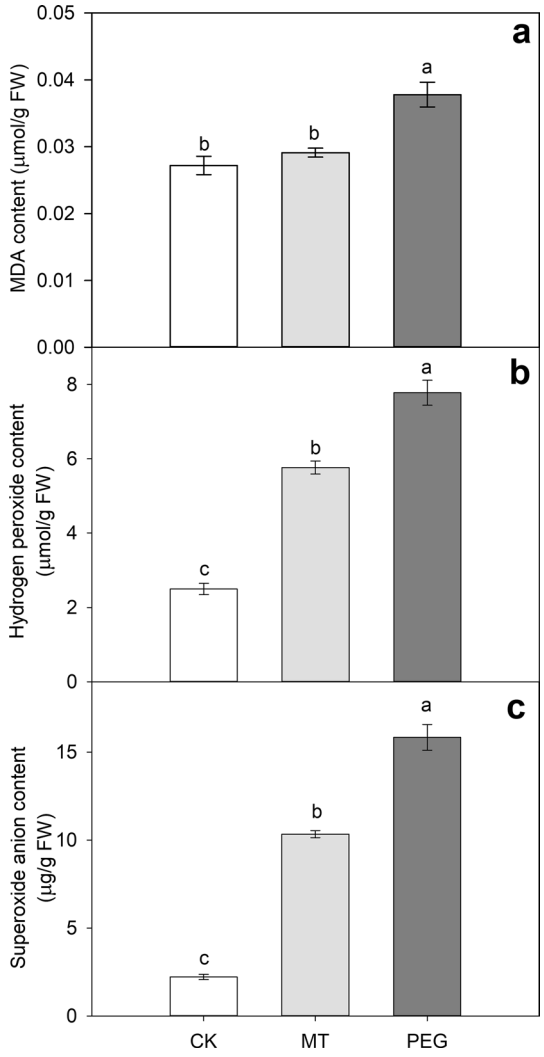

Fig. 5 Effect of melatonin on active oxygen level and membrane lipid peroxidation. 14-days-old wheat seedlings were treated with 25% PEG-6000 solution and 500 μM melatonin. Measurements were performed 72 h after treatment. **a–c** CK, blank control (without PEG and melatonin); PEG, 25% PEG-6000; MT, 25% PEG-6000 with 500 μM melatonin. MDA content (**a**), hydrogen peroxide (**b**), and superoxide anion content (**c**). Different lowercase letters indicate significant differences (Duncan's multiple range, $P < 0.05$, $n = 4$). Bars indicate mean ± SE

The biological processes of melatonin-induced DP included metabolism (36.94%), cellular processes (26.39%), single-organism processes (18.06%), localization (5.00%), cellular component organization or biogenesis (4.17%), responses to stimuli (2.78%), biological regulation (0.83%), signaling (0.28%), detoxification (0.28%), reproductive processes (0.28%), developmental processes (0.28%), and multicellular organismal processes (0.28%). The DP were localized mainly in the cell (23.96%), cell constituents (22.49%), organelles (15.98%), macromolecular complexes (13.61%), membranes (9.17%), membrane constituents (7.10%), organelle constituents (6.51%), extracellular regions (0.89%), and supramolecular complexes (0.30%). The DP were mostly associated with catalytic activity (46.08%), binding proteins (36.27%), structural molecule activity (10.78%), antioxidant activity (1.96%), transporter activity (1.96%), electron carrier activity (1.96%), molecular function regulation (0.49%), and nutrient reservoir activity (0.49%).

In the KEGG enrichment of DP, ≥ 10 DP were concentrated in the ribosomes, carbon metabolism, photosynthetic carbon fixation, and amino acid biosynthesis pathways (Fig. 8). The preferential pathways of upregulated DP expression were concentrated in carbon metabolism, amino acid biosynthesis, glycolysis/gluconeogenesis, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, protein processing in the endoplasmic reticulum (ER), phagosome, galactose metabolism, fatty acid degradation, endocytosis, sulfur metabolism, and oxidative phosphorylation. The downregulated expressions of DP were concentrated in the ribosome, photosynthetic carbon fixation, RNA degradation, photosynthesis, phenylpropanoid biosynthesis, pentose phosphate pathway, ubiquitin-mediated proteolysis, pyruvate metabolism, and proteasome.

qRT-PCR analysis of DP related to antioxidant metabolism, carbon fixation, carbon metabolism, amino metabolism, and autophagy

qRT-PCR was used to analyze the DP in melatonin-treated wheat seedlings. Under PEG stress, melatonin treatment in wheat seedlings significantly upregulated the transcription levels of genes related to antioxidant metabolism (*MSR*, *Z-ISO*, *SFGH*, *SOD*, *DHAR*, and *GME*), carbon fixation, and carbon metabolism (*RuBisCO-3*, *ENO*, and *HXK-1*) compared to those in untreated seedlings (Fig. 9). In addition, melatonin also significantly upregulated the genes participating in amino acid metabolism (*SALH*, *SP*, *PHGDH*, and *AP*) compared to those in untreated seedlings under PEG stress (Fig. 9). Three genes related to autophagy (*RAB5C*, *RAB11A*, and *SRPR*) were also upregulated by exogenous melatonin in wheat seedlings under PEG stress (Fig. 9).

Discussion

Morphological and physiological responses of wheat to melatonin at the germination and seedling stages

Melatonin is a pleiotropic molecule that participates in tolerance to abiotic stresses including drought, radiation, extreme temperature, and chemical stresses (Zhang et al. 2015). This study revealed that melatonin improved the response of wheat to PEG stress at both the germination and seedling

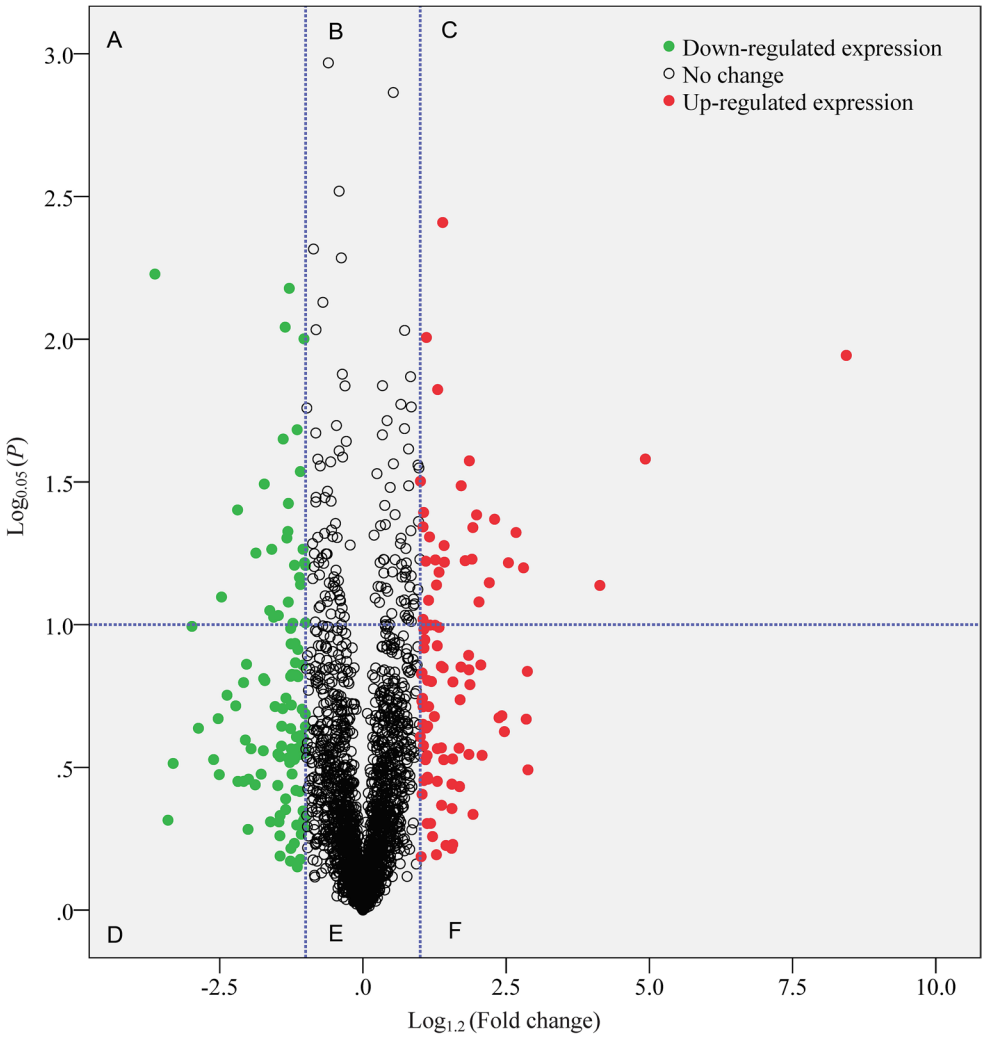

Fig. 6 Volcano plot of all proteins. Green and red filled points represent downregulated and upregulated proteins, respectively. Empty black points are unchanged proteins. Reference line at y-axis is the significance level of difference ($P = 0.05$). Reference line at the *x*-axis is fold change (MT/PEG = 1.200 or = 0.833). The points in section A and section C are significantly changed proteins

stages. Melatonin significantly ameliorated seed germination and seedling growth under PEG stress (Figs. 2 and 3). The findings of this study corroborate those reported for cucumber by Zhang et al. (2013). In addition, melatonin might participate in water retention, because the water content was higher in wheat seedlings treated with melatonin than in controls under PEG stress (Fig. 3e).

Exogenous melatonin also improved the photosynthetic capacity and water use efficiency in wheat seedlings (Fig. 4). The alterations in gas exchange parameters might be explained by a melatonin-induced increase in stomatal conductance. This effect removes the photosynthetic stomatal limit and results in more dry matter than that of untreated seedlings under the same water deficit condition (Figs. 3 and 4). This phenomenon has been reported in apple and cucumber (Zhang et al. 2013). Although increased stomatal conductance accelerates water loss, melatonin-treated seedlings might absorb and retain moisture by other means such as an enhanced solute potential. The melatonin-induced increases in amino acid synthesis support this hypothesis (Table 1). Further, ribulose bisphosphate carboxylase is the key CO_2 fixation enzyme, and melatonin significantly increased this enzyme at both the protein and transcription levels (RuBisCO-3 in Table 2 and Fig. 9). Melatonin also enhanced the production of glucose-1-phosphate adenylyl transferase (AGP), the rate-limiting starch synthesis enzyme (Table 2, Fig. 9). These effects might contribute to the increase in dry matter and maintenance of normal growth in melatonin-treated seedlings compared to untreated seedlings under PEG stress.

Melatonin regulates proteins associated with stress response and antioxidant enzymes

Plants have evolved various adaptive mechanisms in response to osmotic stress, including the synthesis of specialized proteins and antioxidant enzymes (Kosová et al. 2016). In this study, melatonin treatment induced the expression of nine stress-responsive proteins, including heat shock protein 70 (HSP70), late embryogenesis abundant protein

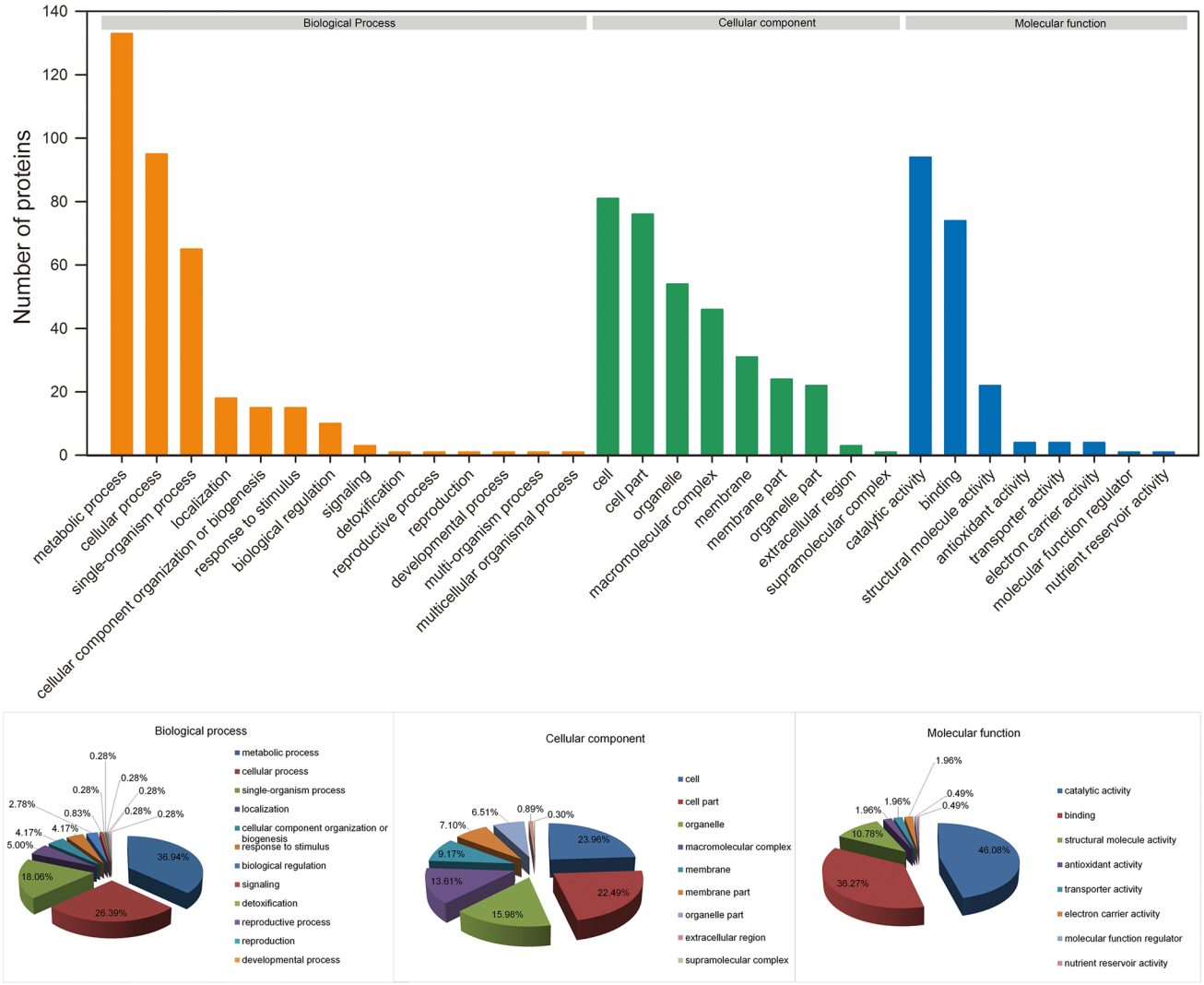

Fig. 7 GO categorization of differentially accumulated proteins identified from wheat seedlings treated by melatonin. GO analysis for protein function classification shows the number of proteins involved in biological process, cellular component and molecular function

(LEA), 15-*cis*-ζ-carotene isomerase (Z-ISO), methionine sulfoxide reductase (MSR), dehydroascorbate reductase (DHAR), glutathione transferase (GST), superoxide dismutase (SOD), *S*-formyl glutathione hydrolase (SALH), and GDP-D-mannose 3′,5′ epimerase (GME, Table 2). LEA and HSP70 play important roles in osmotic stress response (Kosová et al. 2014; Augustine 2016). In this study, melatonin treatment under PEG stress upregulated both LEA and HSP70, which in turn helped the wheat seedlings resist osmotic stress (Table 1).

Osmotic stress can generate excess ROS, including H_2O_2, and $O_2^{\cdot-}$, which can damage proteins, DNA, RNA, and enzymes, and reduce crop growth and yield (Mittler 2002). Melatonin treatment can obviously increase drought tolerance by eliminating excess ROS (Wang et al. 2013a; Liu et al. 2015). The present study showed that melatonin treatment decreased both ROS levels and plasma membrane injury in wheat seedlings compared to those in untreated controls under PEG stress (Fig. 5). Proteomic analysis revealed that seven up-accumulated DP are associated with antioxidant metabolism (Table 2). Carotenoids play multiple roles in plant development and defense against oxidative stress. A crucial enzyme in carotenoid synthesis is 15-*cis*-ζ-carotene isomerase (Z-ISO) (Beltrán et al. 2015). Melatonin treatment significantly increased the expression of the Z-ISO protein and gene (Table 2 and Fig. 9). DHAR, GST and SOD are important antioxidative enzymes (Hasanuzzaman et al. 2012). GME participates in ascorbate biosynthesis (Wolucka and Van Montagu 2003). *S*-Formylglutathione hydrolase (SFGH) is an important enzyme for glutathione-mediated detoxification (Harms et al. 1996), and MSR repairs oxidative protein damage (Romero et al. 2004). According

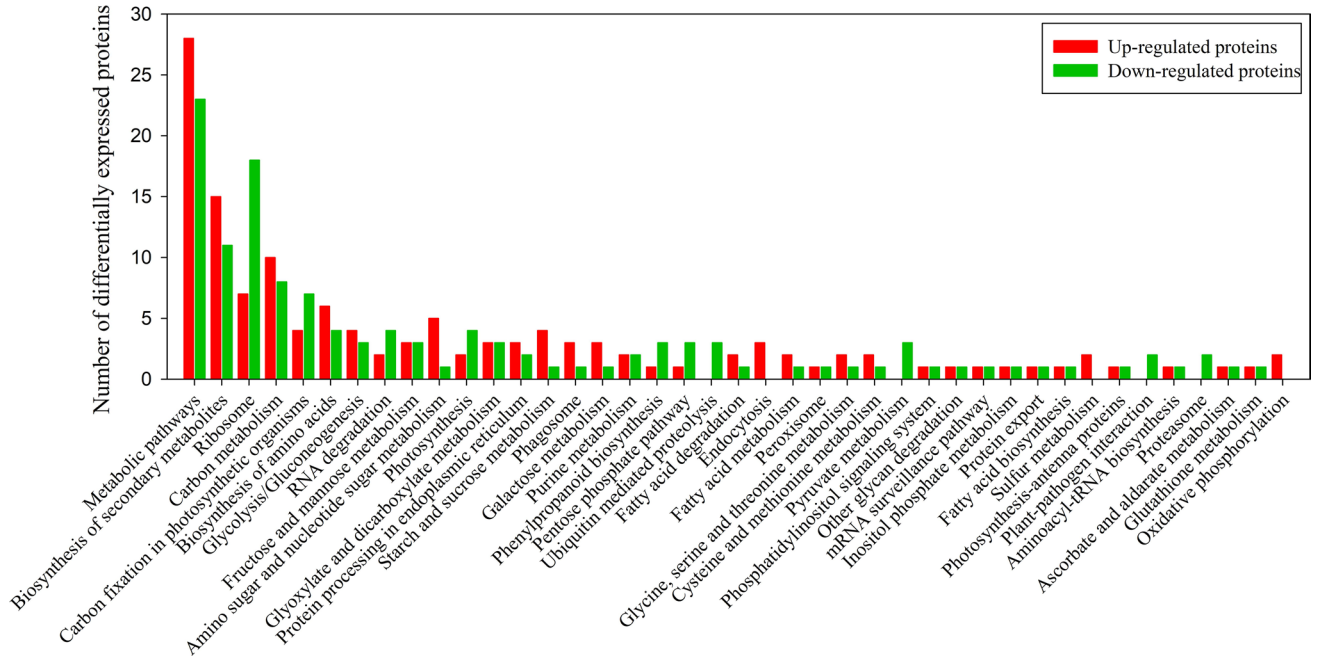

Fig. 8 KEGG pathway analysis of differentially accumulated proteins identified from wheat seedlings treated by melatonin. The red bars and green bars indicate the number of up-accumulated and down-accumulated proteins

to the qRT-PCR data, melatonin significantly upregulated the expression of *DHAR*, *SOD*, *GME*, and *MSR* (Fig. 9). Therefore, melatonin participated in antioxidant metabolism in wheat seedlings under PEG stress. Taken together, these findings suggest that melatonin treatment significantly increased antioxidant capacity, restored ROS homeostasis, and maintained normal biological function in wheat seedlings under PEG stress.

Effect of melatonin on carbon fixation and energy metabolism

Carbon fixation and energy metabolism are important physiological processes in plant growth and development. Nineteen DP were found to be involved in carbon fixation and carbon metabolism, including RuBisCO and AGP. The latter is a rate-limiting enzyme for carbon fixation and starch synthesis. Melatonin significantly increased the expression of RuBisCO-3 at the protein and transcription levels (Table 2, Fig. 9). Therefore, melatonin might participate in carbon fixation. It also increased starch synthesis by upregulating AGP in PEG-stressed wheat seedlings, thereby enabling them to produce more dry matter than that in untreated controls. These results indicate that melatonin improved CO_2 fixation and starch synthesis in wheat seedlings under PEG stress.

Active plant stress responses require metabolic energy. In this study, melatonin significantly affected glycolysis in PEG-stressed wheat seedlings (Fig. 10). Exogenous melatonin altered hexokinase (HXK), fructose-1,6-bisphosphate aldolase (FBA), glyceraldehydes phosphate dehydrogenase (GAPDH), pyruvate-phosphate dikinase (PPDK), and phosphopyruvate enolase (ENO) under PEG stress (Table 2). Similar findings were reported for germinating cucumber seeds under high salinity (Zhang et al. 2017). HXK catalyzes the first step in glucose phosphorylation and is involved in sugar sensing and gene expression (Granot 2008). FBA participates in glycolytic ATP synthesis (Uematsu et al. 2012) and ENO synthesizes phosphoenolpyruvic acid (Fig. 10). Melatonin treatment accumulated the proteins of HXK, FBA-4, and ENO, as well as the genes encoding HXK-1 and ENO (Table 2, Fig. 9). Melatonin also significantly increased the accumulation of the nicotinamide adenine dinucleotide transporter (NADT) and nicotinamide adenine dinucleotide binding protein (NADB) in treated seedlings compared to that in untreated seedlings under PEG stress (Table 2). Therefore, melatonin increases the nicotinamide adenine dinucleotide reaction rate in glycolysis, the Krebs cycle, and ATP synthesis. In addition, RuBisCO upregulation might increase the production of 3-phosphoglyceric acid, a key intermediate in glycolysis (Fig. 10). All of these results suggested that melatonin participated in energy metabolism and can provide more energy for osmotic stress response.

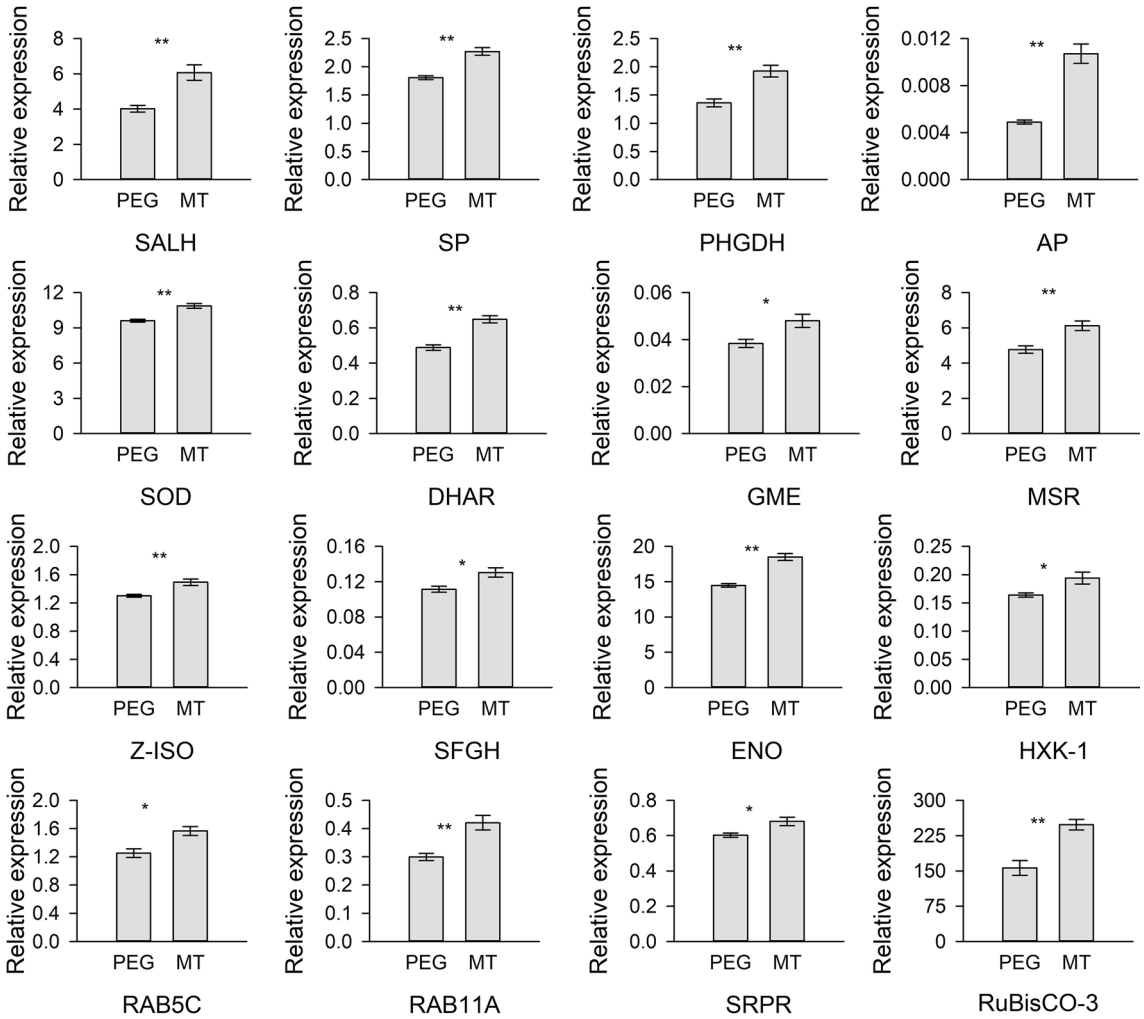

Fig. 9 Relative expressions of genes related to ROS homeostasis, carbon metabolism, amino acid metabolism, and autophagy. Actin was quantified as an internal control. Statistically significant differences (Student's *t* test, $*P<0.05$ and $**P<0.01$, $n=5$) are indicated by asterisks. SALH, *S*-adenosyl homocysteine hydrolase; SP, Serine protease-like protein; PHGDH, D-3-phosphoglycerate dehydrogenase; AP, Aspartyl protease-like protein; SOD, Superoxide dismutase; DHAR, Dehydroascorbate reductase; GME, GDP-D-mannose 3′,5′ epimerase; MSR, Methionine sulfoxide reductase; Z-ISO, 15-*cis*-ζ-carotene isomerase; SFGH, *S*-formylglutathione hydrolase; ENO, Phosphopyruvate enolase; HXK-1, Hexokinase-1; RAB5C, Rab-related protein-5C; RAB11A, Rab-related protein-11A; SRPR, Fused signal recognition particle receptor; RuBisCO-3, Ribulose bisphosphate carboxylase-3

Melatonin participates in amino acid metabolism and osmoregulation

Of the 210 DP, 11 are involved in amino acid metabolism and nine were up-accumulated by melatonin treatment. Free amino acids are important osmotic solutes. The overexpression of genes and proteins related to osmolyte biosynthesis might enhance stress tolerance (Nahar et al. 2016). Phosphoglycerate dehydrogenase (PHGDH) is the rate-limiting enzyme in serine synthesis (Willis and Sallach 1963). Melatonin significantly induced its expression at the transcription and protein levels (Table 1, Fig. 9). Cysteine synthase A (Cys-S) is the terminal enzyme in the cysteine synthesis pathway, and melatonin accumulated its protein (Table 1). Acetylglutamate kinase (AGK) and tryptophan synthase (Trp-S) participate in glutamate metabolism and tryptophan synthesis, respectively, and they were up-accumulated by melatonin under PEG stress (Table 1). Aspartyl protease-like protein (AP) and serine protease-like protein (SP) were both accumulated by melatonin treatment (Table 1, Fig. 9). Therefore, melatonin might participate in aspartyl, serine, cysteine, glutamate, and tryptophan metabolism, enhance the production of these amino acids, and increase the cellular osmotic potential. In addition, melatonin upregulated the expression of SALH at the transcription and translation levels (Table 1, Fig. 9); this protein is required for DNA methylation (Tanaka et al. 1997; Shu et al. 2006). Thus, melatonin might participate

Table 1 Enrichment analysis of differentially accumulated proteins related to amino acid metabolism, autophagy, and other metabolism

Protein ID	PEG	MT	MT/PEG	*P* value	Description	Abbreviations
Amino acid metabolism						
W5AQZ6	1.178	1.705	1.447	0.0394	*S*-Adenosyl homocysteine hydrolase	SALH
W5EFM4	0.866	1.229	1.419	0.0180	Omega-amidase	NIT2
A0A077RPJ4	0.646	0.859	1.331	0.5014	Tryptophan synthase	TrpS
W5BU82	1.119	1.457	1.302	0.5069	Acetyl-glutamate kinase	AGK
W5CJC6	0.905	1.153	1.274	0.0288	D-3-Phosphoglycerate dehydrogenase	PHGDH
Q5TLZ0	0.965	1.192	1.236	0.0199	Aspartyl protease-like protein	AP
W5FDX6	0.909	1.117	1.229	0.1451	Indole-3-acetaldehyde oxidase	IAO
W5B5P7	0.906	1.107	1.221	0.0257	Serine protease-like protein	SP
P38076	0.951	1.152	1.211	0.1183	Cysteine synthase A	Cys-S
W5EYV5	1.220	0.891	0.731	0.0114	Ornithine aminotransferase	OAT
W5DG92	1.171	0.961	0.820	0.1951	Histidinol phosphatase	HP
Autophagy						
W5F6B4	0.695	1.175	1.691	0.2294	Ras-related protein-5C	RAB5C
W5DF02	0.760	1.208	1.589	0.0261	Fused signal recognition particle receptor	SRPR
W5I0S7	1.155	1.640	1.420	0.3658	Ras-related protein-11A	RAB11A
Q9ZRA7	0.839	1.087	1.296	0.0259	Beta-tubulin 6 (Fragment)	TUBB
W5ENB3	1.010	1.269	1.255	0.1311	Heat shock protein 70	HSP70
W5H2P3	0.925	1.124	1.214	0.2563	Calnexin	CANX
Others						
W5ACX0	0.458	2.130	4.657	0.0030	Nicotinamide adenine dinucleotide transporter	NADT
W5A1U4	0.687	1.687	2.457	0.0088	Nicotinamide adenine dinucleotide binding protein	NADB
A0A080YTZ8	0.651	1.383	2.126	0.0331	Ubiquitin-fold modifier 1	UBQ-FM1
W5APP4	0.832	1.177	1.415	0.0251	Quinone reductase	QR
W4ZR97	0.942	1.305	1.385	0.0255	Late embryogenesis abundant protein	LEA
W5ADX4	0.928	1.114	1.201	0.0111	Ubiquitin	UBQ
W5EWB6	1.051	0.855	0.813	0.0862	Ubiquitin-activating enzyme E1	UBQ-AE1
W5BKX2	0.915	1.161	1.268	0.0042	Ubiquitin-mediated protease	UBQ-PT
A0A096UTX6	1.117	0.923	0.826	0.0226	*S*-Phase kinase-associated protein 1	SKP1

PEG 14-day-old wheat seedlings were treated with 25% PEG-6000 (1/2 Hoagland's solution) for 72 h, *MT* 14-day-old wheat seedlings were treated with 25% PEG-6000 (1/2 Hoagland's solution) and 500 μM melatonin for 72 h; *MT/PEG* the fold change of MT/PEG in protein expression

in DNA methylation regulation of wheat seedlings in response to PEG stress.

Melatonin participates in autophagy

Endocytosis is necessary for the regulation of cell division regulation, hormonal responses, nutrient uptake, and stress response in plants. Rab is the largest number of the guanosine triphosphate-binding protein family and is involved in vesicle formation, traffic, and fusion with the plasma membranes for endocytosis and secretion (Rehman and Di Sansebastiano 2014). The fused signal recognition particle receptor (SRPR) plays important roles in phagosome formation and function. Further, β-tubulin is the basic component of microtubules and supports phagosome formation. Heat shock protein 70 and calnexin are molecular chaperones of phagosome synthesis. In this study, melatonin accumulated rab-related proteins (RAB5C and RAB11A), SRPR, β-tubulin 6, HSP70, and calnexin. It also increased the expression of the genes encoding RAB5C, RAB11A, and signal recognition particle receptor (Table 1 and Fig. 9). Melatonin might thus participate in phagocytosis regulation. Wang et al. (2015) found that melatonin-induced autophagy activation without enhanced ROS production might be explained by the elimination of damaged proteins and other toxins induced by PEG stress in *Arabidopsis* seedlings. The increased expression of GST and proteases (SP, AP) also indicated an increase in toxins and damaged proteins (Table 1). Further, ubiquitin participated in the degradation of damaged proteins, and melatonin treatment altered the expression of ubiquitin-fold modifier 1, ubiquitin, ubiquitin-activating enzyme E1, and S-phase kinase-associated protein

Table 2 Enrichment analysis of differentially accumulated proteins related to antioxidant metabolism and carbon fixation and carbon metabolism

Protein ID	PEG	MT	MT/PEG	*P* value	Description	Abbreviations
Antioxidant metabolism						
W5F4P7	0.775	1.291	1.667	0.0275	15-*cis*-zeta-carotene isomerase	Z-ISO
W5AEY8	0.628	0.955	1.520	0.0165	Methionine sulfoxide reductase	MSR
W5HSM2	0.674	0.920	1.366	0.0779	Dehydroascorbate reductase	DHAR
W5E7A0	0.918	1.178	1.283	0.0774	Glutathione transferase	GST
C3VQ50	0.932	1.155	1.240	0.4022	Superoxide dismutase	SOD
W5CDK5	0.877	1.068	1.217	0.0586	*S*-Formylglutathione hydrolase	SFGH
A0A0C4BIT2	0.888	1.078	1.214	0.0525	GDP-D-mannose 3′,5′ epimerase	GME
Carbon fixation and carbon metabolism						
W5CEG1	1.091	0.733	0.671	0.0150	Fructose–bisphosphate aldolase	FBA-1
W5FL86	1.243	0.855	0.688	0.1677	Fructose–bisphosphate aldolase	FBA-2
W5H807	1.077	0.876	0.813	0.0647	Fructose–bisphosphate aldolase	FBA-3
W5D8S6	0.895	1.224	1.367	0.0116	Fructose–bisphosphate aldolase	FBA-4
W5AQS4	0.896	1.135	1.267	0.2586	Ribulose bisphosphate carboxylase	RuBisCO-1
W5I5M7	1.066	1.347	1.264	0.5588	Ribulose bisphosphate carboxylase	RuBisCO-2
W5HNC0	1.499	2.440	1.628	0.0190	Ribulose bisphosphate carboxylase	RuBisCO-3
G8D5C5	0.956	0.754	0.789	0.2024	Ribulose bisphosphate carboxylase	RuBisCO-4
W4ZS91	0.845	1.230	1.455	0.0762	Hexokinase	HXK-1
W5A7R2	0.920	1.223	1.330	0.2046	Hexokinase	HXK-2
W5F9H2	0.904	1.164	1.289	0.0007	Glucose-1-phosphate adenylyltransferase	AGP
W5FPN2	0.819	1.377	1.682	0.1347	Phosphopyruvate enolase	ENO
A0A077RUC1	0.878	1.353	1.542	0.1327	Beta-galactosidase	GS
A0A077RQT9	0.854	1.226	1.436	0.0158	Polygalacturonase	PGA
L0N593	0.994	0.821	0.826	0.0763	Beta-fructofuranosidase	6FEH
W5AGT4	1.101	0.906	0.823	0.1710	Pyruvate–phosphate dikinase	PPDK
A0A096UKG6	1.116	0.887	0.795	0.5228	Malate dehydrogenase	MDH
W5ETI9	0.574	0.428	0.745	0.3952	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
W5AQS1	1.343	0.953	0.710	0.2682	Aminomethyl transferase	AMT

PEG 14-day-old wheat seedlings were treated with 25% PEG-6000 (1/2 Hoagland's solution) for 72 h, *MT* 14-day-old wheat seedlings were treated with 25% PEG-6000 (1/2 Hoagland's solution) and 500 μM melatonin for 72 h, *MT/PEG* the fold change of MT/PEG in protein expression

1 (Table 1). Melatonin significantly accumulated ubiquitin-fold 1 and ubiquitin; thus, it might have a regulatory role in ubiquitin-mediated protein degradation. Overall, ubiquitin-mediated protein degradation is consistent with increased protease expression (AP and SP in Table 1 and Fig. 9) and autophagy. Hence, a metabolic cascade related to autophagy might be activated by melatonin under PEG stress in wheat seedlings (Fig. 11).

A number of previous proteomic studies have established the role of melatonin in plants, which is associated with delayed leaf senescence, stress tolerance, seed priming, and fruit ripening (Wang et al. 2014; Shi et al. 2015b; Kolodziejczyk et al. 2016; Sun et al. 2016; Xu et al. 2016a, b; Zhang et al. 2017). In this study, we used iTRAQ to identify the melatonin-induced the regulation of proteins under PEG stress. Proteomic analysis revealed that melatonin-induced proteins participate in many metabolism pathways including antioxidant metabolism, stress response, carbon fixation, carbohydrate metabolism, energy metabolism, amino acid metabolism, endocytosis, and so on (Fig. 8). Some pathways and proteins in our proteomic analysis were consistent with previous proteomic studies. These pathways included energy metabolism, stress response, proteasome, protein degradation, and ubiquitination regulation, which responded to melatonin treatment in the leaf, pericarp, and germinating seeds (Wang et al. 2014; Kolodziejczyk et al. 2016; Sun et al. 2016; Xu et al. 2016a, b; Zhang et al. 2017). The common protein targets following melatonin treatment in previous

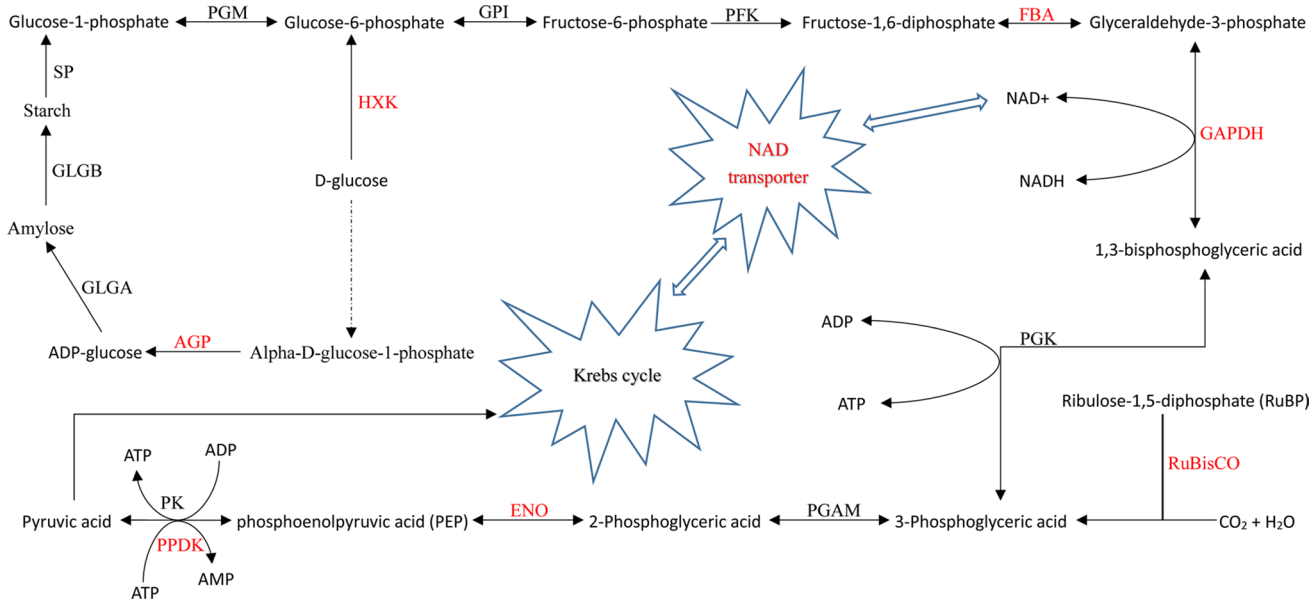

Fig. 10 Melatonin-induced proteins participating in energy production. Differentially accumulated enzymes shown in red font. *PGM* glucose-1-phosphate mutase, *GPI* glucose-6-phosphate isomerase, *PFK* phosphofructokinase, *FBA* fructose-1,6-bisphosphate aldolase, *GAPDH* glyceraldehyde phosphate dehydrogenase, *PGK* phosphoglycerate kinase, *RuBisCO* ribulose-bisphosphate carboxylase, *PGAM* phosphoglycerate mutase, *ENO* phosphopyruvate enolase, *PK* pyruvate kinase, *PPDK* pyruvate phosphate dikinase, *AGP* glucose-1-phosphate adenylyltransferase, *SP* starch phosphorylase, *GLGA* ADP-glucose glucosyltransferase, *GLGB* glucan-branching glycosyltransferase

studies and our studies included GAPDH, FBA, ENO, HXK, and malate dehydrogenase (MDH) in energy metabolism; and HSP70, GST, and SOD in stress responses. Moreover, tubulin, an important component of cell walls was observed in response to melatonin treatment under heat and salinity stress in previous and our studies (Xu et al. 2016a, b; Zhang et al. 2017). Some protease and ubiquitination proteins involved in protein modification and protein degradation were observed in previously reported proteomic analysis, as well as in our study (Wang et al. 2014; Shi et al. 2015b; Zhang et al. 2017). Then the denatured and damaged proteins were labeled by ubiquitin, coated by autophagosome, and degraded by lysosome subsequently (Fig. 11).

In summary, this study provides evidences for the positive effects of melatonin on seed priming and osmotic tolerance in wheat. In melatonin-treated wheat seedlings, osmotic tolerance by involving in carbon fixation, ROS homeostasis, stress response, energy metabolism, ubiquitin-mediated damaged protein degradation, and autophagy had been improved (Fig. 11). In stressed wheat seedlings, melatonin maintained seedling growth by improving carbon fixation and starch synthesis. Melatonin strengthened ROS homeostasis by increasing the expression of genes and proteins related to antioxidant enzymes in wheat seedlings under PEG stress. Further, melatonin ameliorated energy production by enhancing proteins, including NADT, NADB GAPDH, FBA, HXK, ENO, and MDH in PEG-stressed seedlings. In addition, ubiquitin-mediated damaged protein degradation and autophagosome formation might be another reason for melatonin-mediated osmotic tolerance in seedlings. Thus, this study showed that seed priming with melatonin provide an approach for improving drought tolerance in wheat seedlings, and also that melatonin is an effective exogenous tryptamine for improving drought tolerance in wheat production.

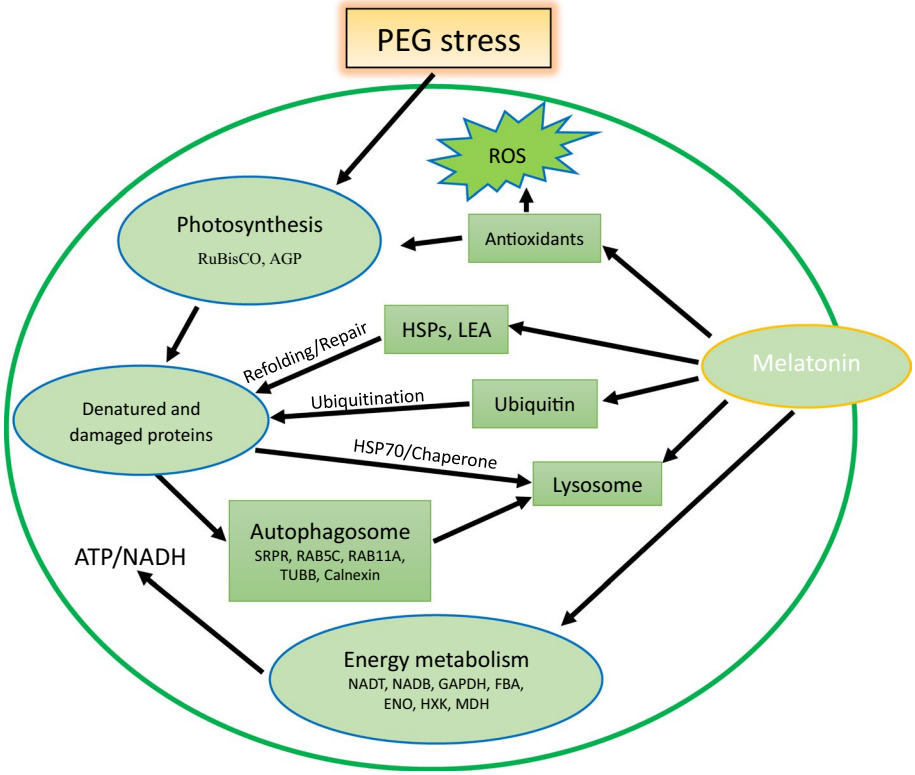

Fig. 11 Model showing the proposed mechanisms of osmotic tolerance by melatonin in wheat seedlings. Melatonin maintained seedling growth by improving carbon fixation and starch synthesis, as well as strengthening ROS homeostasis by increasing the accumulation of proteins related to antioxidants. Further, melatonin improved energy production for the biosynthesis of ATP by enhancing proteins including GAPDH, FBA, HXK, ENO, and MDH in PEG-stressed seedlings. In addition, the enhanced accumulation of NADT and NADB increased the transportation rate of NADH. The denatured and damaged proteins were labeled by melatonin-induced ubiquitin and were transported to the autophagosome and lysosome for next degradation. *RuBisCO* ribulose-bisphosphate carboxylase, *AGP* glucose-1-phosphate adenylyltransferase, *HSPs* heat shock proteins, *LEA* late embryonic abundant proteins, *SRPR* fused signal recognition particle receptor, *RAB5C* rab-related protein-5C, *RAB11A* rab-related protein-11A, *TUBB* tubulin, *NADT* nicotinamide adenine dinucleotide transporter, *NADB* nicotinamide adenine dinucleotide binding protein, *GAPDH* glyceraldehyde phosphate dehydrogenase, *FBA* fructose-1,6-bisphosphate aldolase, *ENO* phosphopyruvate enolase, *HXK* hexokinase, *MDH* malate dehydrogenase

Author contribution statement YX designed and directed this study and its experiments as well as drafted and revised the manuscript. GC performed the experiments and analyzed the data as well as drafted and revised the manuscript. FS analyzed the data as well as drafted and revised the manuscript. XG and KX measured the gas exchange parameters. CZ and SL improved the data analysis and revised the manuscript.

Acknowledgements We are grateful for the funding support for this study from The National Key Basic Research Program, China (2017YFD0100706), Protection and Utilization of Germplasm Resources of Shaanxi Province, China (20171010000004), Transgenic Major Projects of China (2014ZX0800203B), and Key Science and Technology Program of Shaanxi Province, China (2013K02-01-02).

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