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Research Article

Transcriptomic and physiological analyses reveal drought adaptation strategies in drought-tolerant and -susceptible watermelon genotypes

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ABSTRACT

Drought stress has become one of the most urgent environmental hazards for horticultural crops. In this research, we analyzed watermelon adaptation strategies to drought stress in drought-tolerant (M20) and -susceptible (Y34) genotypes via transcriptomic and physiological analyses. After drought stress, a total of 6228 and 4311 differentially expressed genes (DEGs) were observed in Y34 and M20, respectively. Numerous DEGs were involved in various defense responses such as antioxidation, protein protection, osmotic adjustment, wax accumulation, hormone signaling, and melatonin biosynthesis. Accordingly, the contents of ABA, melatonin, wax, and some osmoprotectants were increased by drought stress in both Y34 and especially M20. Exogenous application of melatonin or ABA induced wax accumulation and drought tolerance and melatonin may function upstream of ABA. In comparison to Y34, M20 was more able to activate ABA signaling, melatonin biosynthesis, osmotic adjustment, antioxidation, and wax accumulation under drought stress. These stronger responses confer M20 tolerance to drought. Photosynthesis and most DEGs involved in photosynthesis and cell growth were decreased by drought stress in both M20 and especially Y34. For drought-susceptible genotypes, growth retardation may be an important mechanism for saving and redistributing resources in order to reprogram stress signaling networks.

1. Introduction

Watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, is one of the most economically important crops as it is economically valuable and nutritious. According to FAOSTAT, the cultivated area of watermelon in 2016 was approximately 3.51 million hectares worldwide, and the annual global production of watermelon is about 117.20 million tons, making it among the top five most consumed fresh fruits (http://www.fao.org/). The gross production value of watermelon in 2014 was $38.56 billion. Watermelon is a high water-consuming plant, especially during the fruit development stage [1]. In recent years, drought has become a major abiotic stress on watermelon growth and productivity [2]. According to previous studies [3–6], watermelon has evolved various strategies to adapt to drought stress: accumulation of citrulline and elongation of roots to avoid water loss; modification of photosynthetic proteins to protect photosystem II from photoinhibition; and changes in the proteome and transcriptome to regulate tolerance. However, the physiological and molecular mechanisms of watermelon adaption to drought stress are still unclear.

The responses and tolerance of plants to drought stress have been studied intensively [7,8]. Under drought stress, plants can induce stomatal closure, accumulation of compatible solutes, and biosynthesis of wax to avoid water loss; enhance antioxidant abilities to alleviate oxidative damage; and induce some molecular chaperones to protect proteins from damage [9]. At the cellular and molecular levels, a generic signal transduction pathway starts with stress perception by molecular sensors and thereby generates secondary signals, and activates different transcriptional regulators, via the activation of phosphoprotein kinases [10]. Eventually, the metabolic changes result in the activation of an assortment of genes and proteins involved in particular tolerance responses such as protein protection, antioxidation, osmotic adjustment, and stomatal adjustment [9]. To minimize the deleterious effects of drought, plants also reduce their growth and development as a way to save and redistribute resources that can become limited under water deficit conditions [11,12].

The various drought responses are mediated by some important

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regulators such as phytohormones and phytomelatonin. Plant hormones, especial abscisic acid (ABA), play critical roles in regulating plant growth and defense under drought stress [13]. Accumulating data indicates that ABA functions as a central integrator that links and reprograms the complex stress adaptive signaling cascades in the drought stress response [13]. Melatonin (*N*-acetyl-5-methoxytryptamine), a pleiotropic and highly conserved molecule, is ubiquitous throughout the animal and plant kingdoms [14]. Since the discovery of melatonin in vascular plants in 1995 [15,16], numerous subsequent studies have demonstrated its important roles in regulating plant growth, development, and defense against various environmental stresses, including extreme temperatures, excess copper, salinity, and drought [17].

Until now, most drought tolerance-related genes have been identified in model plants such as *Arabidopsis* and *Oryza sativa*. The gene regulation networks behind drought adaption strategies in black poplar and grapevine have been reported recently [18,19]. Since drought tolerance is a highly complex trait, a whole-genome perspective is required to identify genes related to drought tolerance in different plant species. In 2013, a high-quality draft genome sequence of the east Asia watermelon cultivar '97103' was reported [20]. Later, Wang et al. used deep sequencing of mRNAs (RNA-seq) to assay the transcriptomic response to drought stress in the leaves of *Citrullus colocynthis*, a drought-tolerant species that is closely related to watermelon [6]. They found that many transcription factors, stress signaling factors, and genes involved in phytohormone signaling, antioxidation, and citrulline metabolism were upregulated by drought stress in *Citrullus colocynthis*. However, the responses of drought-inducible genes are closely related to genotypes. Thus, to compare transcriptome responses of drought-tolerant and -susceptible genotypes under drought stress enables the comprehensive analysis of watermelon adaption strategies to drought stress.

In our previous work [21], a wild watermelon germplasm (*C. lanatus* var. *citroide*, M20) was identified as a drought-tolerant genotype, and a Chinese domesticated watermelon (*C. lanatus* var. *lanatus*, Y34) was identified as a drought-susceptible genotype. In this study, we used RNA-seq to assay the responses of M20 and Y34 to drought stress at a genome-wide level. Based on the RNA-seq results, we analyzed the physiological responses of these two genotypes to drought stress, including changes in plant hormones, melatonin, antioxidants, osmoprotectants, wax accumulation, and photosynthesis. Furthermore, we analyzed the interaction effects between melatonin and ABA and the effects of ABA and melatonin on drought tolerance and wax biosynthesis.

2. Materials and methods

2.1. Plant materials, growth conditions and treatments

Two watermelon germplasms, M20 (a drought-tolerant genotype) and Y34 (a drought-susceptible genotype) were used. Uniformly germinated seeds were directly sown into plastic pots (10 × 8.5 × 7 cm) filled with a mixture of sand and a commercial peat-based compost (3/1, *v/v*). All pots initially contained the same weight of dry growth media (approximately 350 g). The seedlings were grown in a greenhouse at Northwest A&F University, Yangling (34° 283′ N, 108° 067′ E), China, with the following environmental conditions: a temperature ranging from 18.0 °C to 36.0 °C, a relative humidity range of 65–80%, and natural light. Before the experiments began, all plants were well watered (200 mL) each day and fertilized weekly with ½-strength Hoagland's solution (200 mL).

2.1.1. Experiment 1

At the four- or five-leaf stage, half of Y34 and M20 seedlings were unwatered for 4 days and 8 days, and these treatments were designated as YDS-4, MDS-4, YDS-8, and MDS-8 according to their genotypes and days of drought treatment. The control plants (CK) were well watered to 75 ± 5% field capacity (FC) on the basis of soil weight every evening and were similarly designated as YCK-4, MCK-4, YCK-8, and MCK-8. Each treatment included three biological replications with 12 plants per replication. Leaf sampling was randomly conducted; the second topmost fully expanded leaves from four plants were sampled in each biological sample, and three biological replicates per treatment were used for biochemical analyses. The harvested samples were frozen immediately in liquid nitrogen and stored at −80 °C.

2.1.2. Experiment 2

Y34 seedlings at the three-leaf stage were pretreated with 150 μM melatonin on their roots or 100 μM ABA on their leaves three times (once every two days). The melatonin and ABA solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [1/10000, ethanol/water (*v/v*)]. After melatonin or ABA pretreatment, plants were unwatered for 4 days. Leaf samples (from the second fully expanded leaf beneath the apical bud) were harvested after measuring the gas exchange, relative water content, and relative electric conductivity. Harvested samples were rapidly frozen in liquid nitrogen and stored at −80 °C.

2.2. Analysis of leaf water status, relative electric conductivity, and lipid peroxidation

The leaf relative water content (RWC) was determined according to the method of Barrs and Weatherley [22]. The fresh weight of leaf sample (1 g) was determined immediately after being collected. The leaves were then floated on water in a Petri dish. After 4 h, surface-dried leaves were re-weighed to determine their turgid weights. The dry weight was then determined. The RWC was calculated according to the following equation: (turgid weight - fresh weight) / (turgid weight - dry weight) * 100.

The relative electric conductivity (REC) was measured and calculated according to the method developed by Zhou and Leul [23]. The fresh leaf samples (1.0 g) were cut into 0.5-cm circles using a punch and placed in a test tube (50 mL) containing 25 mL of deionized water. The test tubes were vacuumed for 10 min, the leaf samples were immersed and vibrated 20 min, and then the conductivity of the solution (C1) was measured using a conductivity meter. Afterward, samples were boiled for 10 min and the conductivity (C2) was measured again when the solution was cooled to room temperature. REC was calculated as C1 / C2 * 100%.

The level of lipid peroxidation in leaves was estimated by measuring the malondialdehyde (MDA) content with 2-thiobarbituric acid (TBA) [24]. Leaf tissue (0.5 g) was homogenized in 5 mL of 5% (*w/v*) trichloroacetic acid (TCA). After centrifugation at 10,000 rpm for 15 min, 2 mL of crude extract was mixed with 2 mL of 0.5% (*w/v*) thiobarbituric acid (TBA) solution containing 5% (*w/v*) TCA. The mixture was heated at 100 °C for 15 min, cooled quickly, and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was measured at 532, 600, and 450 nm.

2.3. Observation of stomata and epidermal wax crystal by scanning electron microscopy

Four leaves were collected from each treatment for scanning electron microscopy (SEM). Specimens for observation of stomata were prepared as described by Wu et al. [25]. The surface of the lower leaf epidermis was observed and photographed using a JSM-6360LV microscope (JEOL Ltd., Tokyo, Japan). Stomata were counted in 15 randomly selected microscope visual fields. For epidermal wax crystal examination, the leaf samples were frozen in liquid nitrogen, and then freeze dried. The freeze-dried materials were sputter-coated with gold film and viewed using a Hitachi S4800 scanning electron microscope (Hitachi, Tokyo, Japan) [26].

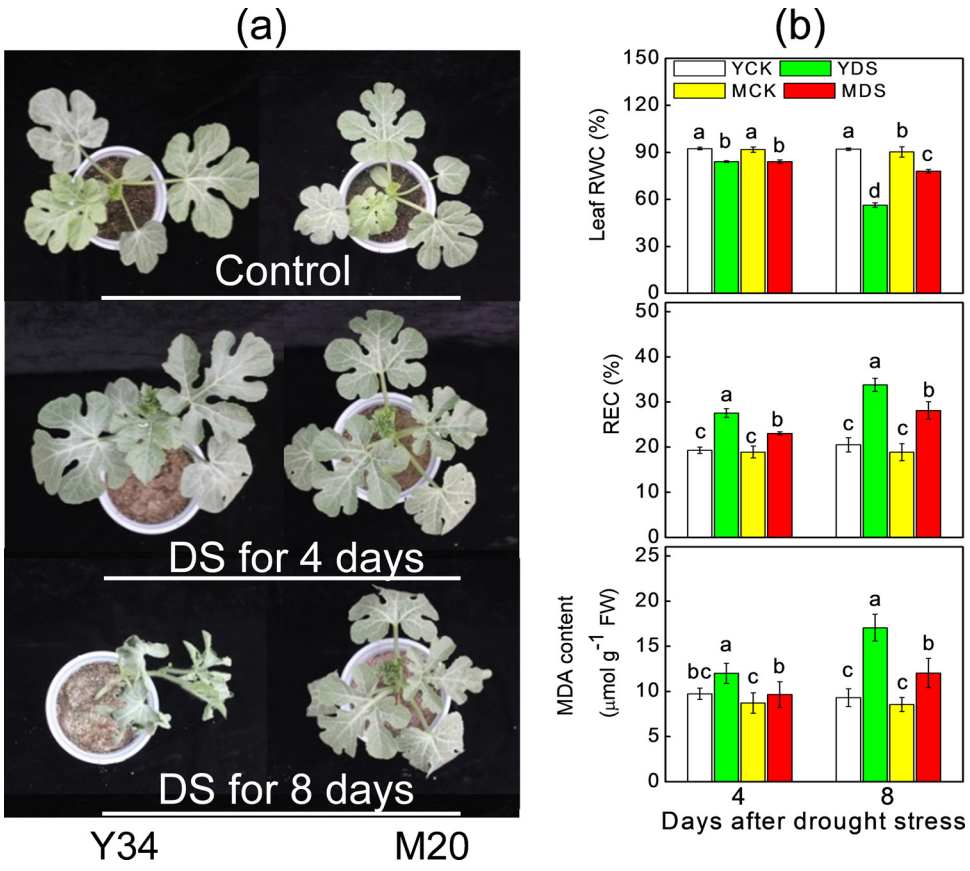

Fig. 1. Effects of drought stress on watermelon (a) phenotypes and (b) leaf relative water content (RWC), relative electrical conductivity (REC), and malondialdehyde (MDA) content. At the four- or five-leaf stage, seedlings of both M20 and Y34 were exposed to drought stress for 4 and 8 days, with well-watered seedlings used as a control. The data shown are the means of three replicates (± SD). Means denoted with the same letter did not significantly differ at $P < 0.05$. DS, drought stress; YCK, control of Y34; MCK, control of M20; YDS, Y34 with drought treatment; MDS, M20 with drought treatment; RWC, relative water content; REC, relative electric conductivity; MDA, malondialdehyde.

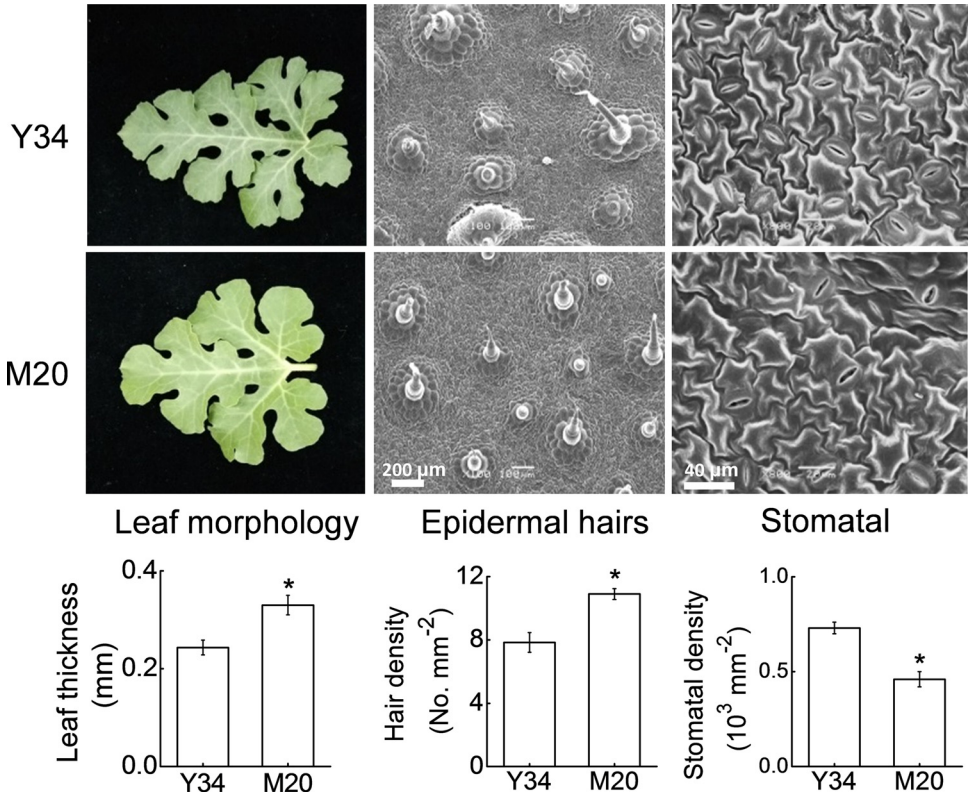

Fig. 2. Comparison of the morphology, epidermal hair density, and stomatal density between the leaves of Y34 and M20. The leaf epidermis and stomata were observed and photographed using a JSM-6360LV microscope (JEOL Ltd., Tokyo, Japan). The data shown are the means of three replicates (± SD). Means denoted with asterisk (*) differed significantly at $P < 0.05$.

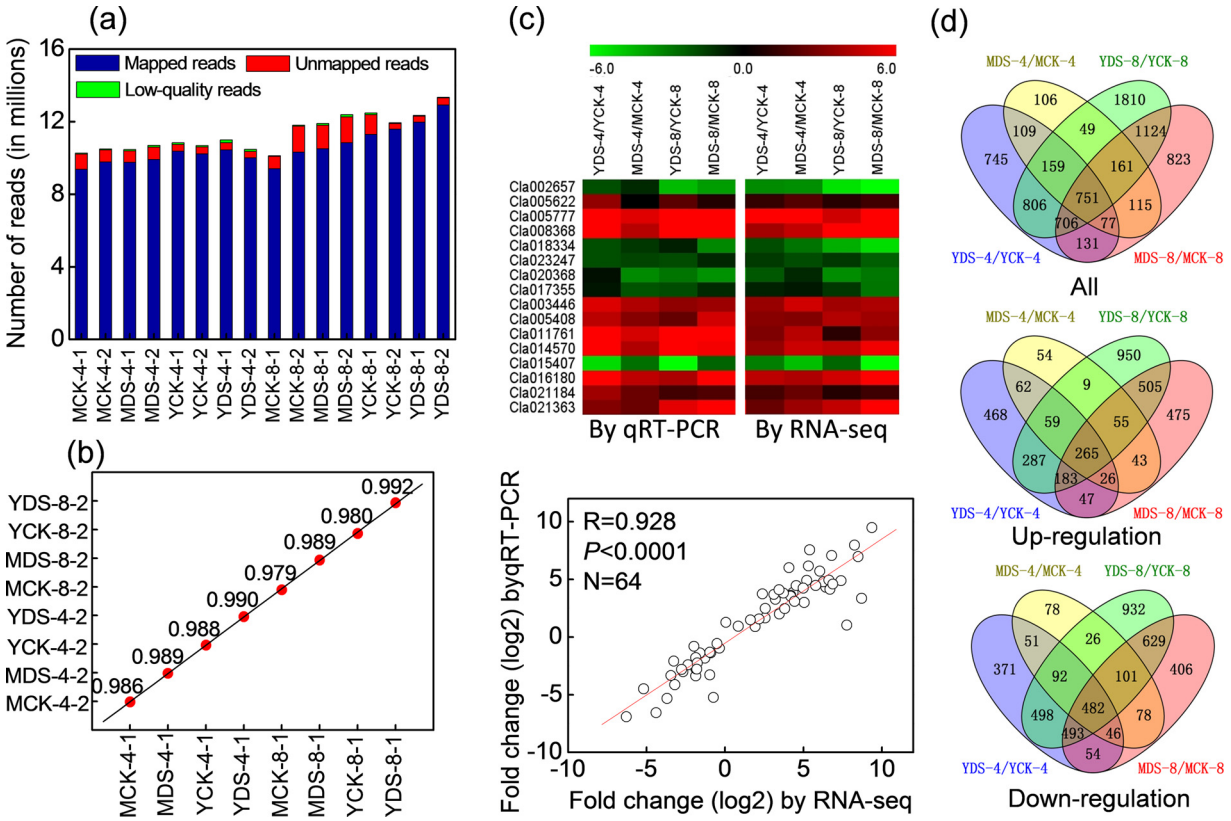

Fig. 3. Effects of drought stress on the genome-wide expression profiles of Y34 and M20 based on RNA-seq. Seedlings treatments and the sample harvesting were conducted as described in Fig. 1. (a) Read abundance of mRNAs in each treatment. (b) The correlation between two biological replicates. (c) The expression analysis of genes based on qRT-PCR. (d) The correlation analysis of gene expression values obtained from the RNA-seq and qRT-PCR analyses. (e) Venn diagram illustrating the numbers of up- and down-regulated genes. YCK-4, control of YDS-4; YDS-4, Y34 with drought for 4 days; MCK-4, control of MDS-4; MDS-4, M20 with drought for 4 days; YCK-8, control of YDS-8; YDS-8, Y34 with drought for 8 days; MCK-8, control of MDS-8; MDS-8, M20 with drought for 8 days.

2.4. Cuticular wax extraction and chemical characterization

Cuticular waxes were extracted from leaves by immersing tissues for 1 min in chloroform containing *n*-tetracosane (C24 alkane) as an internal standard [26]. Extracts were dried under N_2 gas and derivatized with 100 μL of bis-*N*, *N*-(trimethylsilyl) trifluoroacetamide and 100 μL of pyridine for 60 min at 70 °C. Solvent was again evaporated under N_2 gas and the waxes were dissolved in chloroform for analysis. Samples were analyzed using a capillary gas chromatograph equipped with a Rxi-5 ms column (30 m length, 0.25 mm id, 0.25 μm thickness) and attached to a mass spectrometer (GCMS-QP2010, Shimadzu, Kyoto, Japan) with helium as the carrier gas. The initial temperature of 50 °C was held for 2 min, increased by 20 °C min^{-1} to 200 °C, held for 2 min at 200 °C, increased again by 2 °C min^{-1} to 320 °C, and held for 15 min at 320 °C. Injector and detector temperatures were set at 250 °C. Quantification was based on flame ionization detector peak areas and the internal standard. The total amount of cuticular wax was expressed per unit of leaf surface area. Areas were determined using ImageJ software (http://rsb.info.nih.gov/ij/) based on digital images of leaves.

2.5. Quantification of abscisic acid, auxin, gibberellin, and brassinosteroids

Plant hormones were extracted according to the method of Yang et al. [27] and were measured using an immunoassay kit (China Agricultural University, Beijing, China) according to the manufacturer's instructions. Colorimetric recording was conducted using a Multimode Plate Reader M200 pro (Tecan, Männedorf, Switzerland).

2.6. Quantification of melatonin

Melatonin was extracted according to the method of Pape and Lüning [28] and was measured using an enzyme-linked immunosorbent assay [29]. Briefly, leaf samples (1.0 g) were extracted in 10 mL of extraction mixture (89:10:1, acetone:methanol:water). The extract was centrifuged (4500 × *g*, 4 °C) for 5 min and 2 mL of 1% trichloric acid was added to the supernatant for protein precipitation. The extraction was repeated twice, and the combined supernatants were concentrated in a vacuum concentrator to a total volume of 10 mL, which was then adjusted with distilled water to a total volume of 50 mL. After centrifugation (10,000 × *g*, 10 °C) for 15 min, the supernatants were then filtered through a Sep-Pak C18 cartridge (Millipore, Billerica, MA, USA) for the purification of melatonin. Melatonin was measured using an immunoassay kit (Shanghai Lanpai Biotechnology Co., Ltd, Shanghai, China) following the manufacturer's instructions. Colorimetric recording was conducted using a Multimode Plate Reader M200 pro (Tecan, Männedorf, Switzerland).

2.7. Glutathione and ascorbic acid contents determination

For the measurement of glutathione and ascorbic acid contents, 0.3 g of leaf tissue was homogenized in 2 mL of 5% metaphosphoric acid containing 2 mM EDTA and centrifuged at 4 °C for 15 min at 12,000 × *g*. Total glutathione and oxidized glutathione (GSSG) contents were determined from the supernatants by the 5,5′-dithio-bis(2-nitrobenzoic acid)–GSSG reductase recycling method [30]. The reduced glutathione (GSH) concentration was obtained by subtracting the GSSG concentration from the total concentration.

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) were

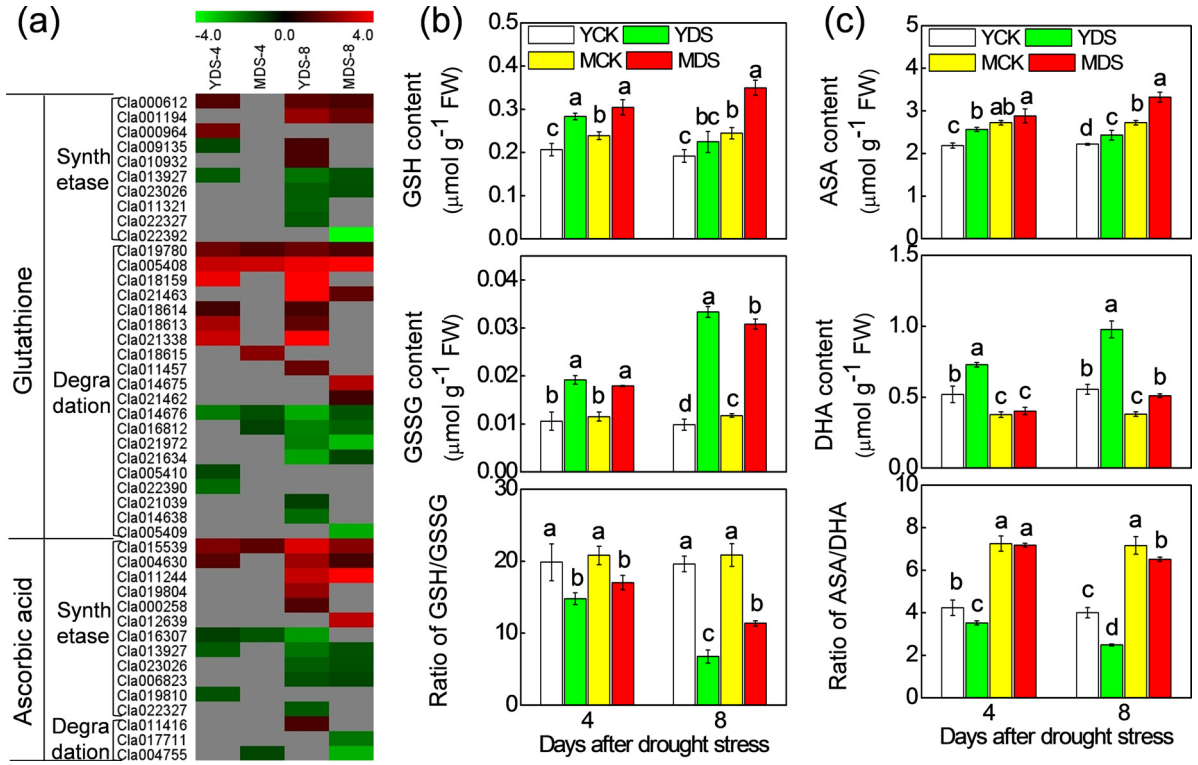

Fig. 4. Responses of glutathione and ascorbic acid under drought stress. Seedlings treatments and the sample harvesting were conducted as described in Fig. 1. (a) The differentially expressed gene transcripts involved in glutathione and ascorbic acid metabolism. (b) Analysis of the content and redox status of glutathione. (c) Analysis of the content and redox status of ascorbic acid. Data in (b and c) are the means of three replicates (± SD), and means denoted with the same letter did not significantly differ at $P < 0.05$. YDS-4, Y34 with drought for 4 days; MDS-4, M20 with drought for 4 days; YDS-8, Y34 with drought for 8 days; MDS-8, M20 with drought for 8 days; GSH, glutathione; GSSG, oxidized glutathione; AsA, ascorbic acid; DHA, dehydroascorbic acid.

measured according to the method developed by Law et al. [31]. For the total ascorbate (AsA + DHA) content assay, the extract was incubated with 150 mM phosphate buffer solution (pH 7.4) and 10 mM DTT for 20 min to reduce all DHA to AsA and then 0.5% (*w/v*). *N*-ethylmaleimide (NEM) was added to remove excess DTT. For the AsA content assay, deionized H_2O was substituted for DTT and NEM. Then the reaction mixtures were added to TCA, phosphoric acid, α,α′-dipyridyl, and $FeCl_3$ and were incubated at 37 °C for 60 min. The absorbance was recorded at 525 nm. The DHA concentration was then calculated by deducting the estimated AsA content from the total ascorbate content.

2.8. Proline and soluble sugar content determination

The proline content was measured following the method of Bates et al. [32]. The leaf tissue (0.3 g) was homogenized in 3% aqueous sulfosalicylic acid. After centrifugation, the homogenate was filtered through Whatmann No.1 filter paper. Then, the supernatant (2 mL) was added to 2 mL of acid ninhydrin and 2 mL of glacial acetic acid and was boiled at 100 °C for 1 h. After termination of the reaction in an ice bath, the reaction mixture was extracted with 4 mL of toluene, and the absorbance was recorded at 520 nm.

The soluble sugar content was measured using the anthrone and sulfuric acid colorimetric methods [33]. Leaf tissue (0.3 g) was ground with 3 mL of 80% ethyl alcohol and the extracts were maintained at 80 °C for 30 min. After centrifugation at 5000 rpm for 10 min, 1 mL of supernatant was mixed with 5 mL of 0.1% (*w/v*) anthrone solution containing 98% sulfuric acid and the mixtures were then incubated at 100 °C for 20 min. After cooling for 20 min at room temperature, the absorbance was recorded at 620 nm.

2.9. Analysis of gas exchange, chlorophyll content, and initial Rubisco activity

The net photosynthetic rate (Pn) was measured on the second fully expanded leaf beneath the apical bud using a Li-COR-6400 infrared gas analyzer (LI-COR Inc., Lincoln, NE, USA) equipped with a red/blue LED light source (6400-02B; Li-COR). Six plants were used for each treatment. The measurement was performed within the time period from 8:00 am to 11:00 am at their growth temperature with a photosynthetic photon flux density (PPFD) of 1000 μmol m^{-2} s^{-1} and a CO_2 concentration of 400 μmol mol^{-1}.

The total chlorophyll content (Chl a + b) was determined colorimetrically according to the method of Arnon [34]. Leaf tissue samples (0.3 g) were homogenized in 8 mL of 80% acetone. After filtration using filter paper, the extract was used to measure the absorbance at 663 and 646 nm.

To analyze initial Rubisco activity, 0.3-g leaf samples were homogenized with cooled extraction buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$, 12.5% (*v/v*) glycerin, 10% polyvinylpyrrolidone (PVP) and 10 mM? ?-mercaptoethanol. The homogenates were centrifuged at 15,000 × *g* for 15 min at 4 °C. The Rubisco activity was measured by recording the nicotinamide adenine dinucleotide oxidation at 340 nm using a spectrophotometer [35].

2.10. RNA-seq library construction and sequencing

Sixteen independent RNA-Seq libraries from eight treatments with two biological replicates in each were constructed and sequenced by Novogene Co. (Beijing, China). After total RNA was extracted using the RNA extraction kit (Promega, USA), mRNA was enriched by oligo (dT) beads and then cleaved into short fragments using fragmentation

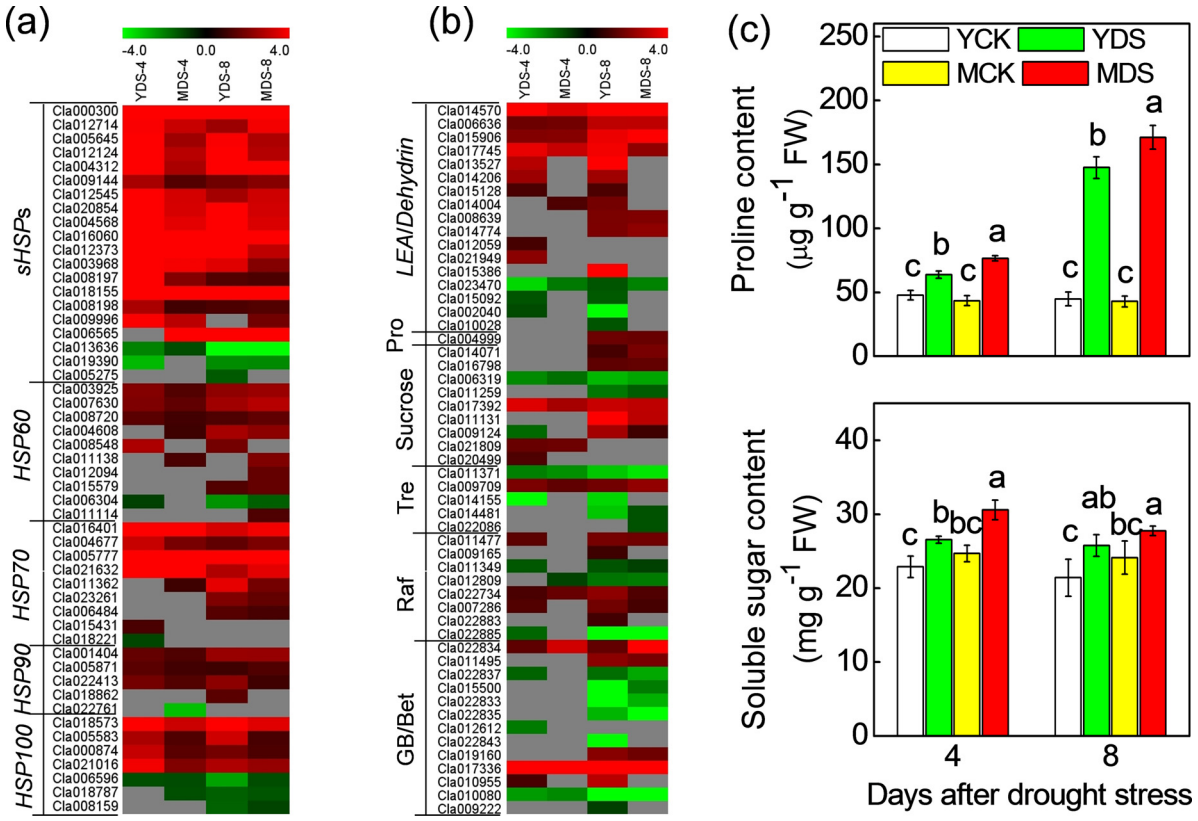

Fig. 5. Responses of protein protecion and osmotic adjustment under drought stress. Seedlings treatments and the sample harvesting were conducted as described in Fig. 1. (a) The abundance of differentially expressed genes (DEGs) involved in HSP biosynthesis. (b) The abundance of DEGs involved in LEA/dehydrin and osmotic adjustment. (c) Analysis of the proline and soluble sugar contents. Data in (c) are the means of three replicates (± SD), and means denoted with the same letter did not significantly differ at $P < 0.05$. YDS-4, Y34 with drought for 4 days; MDS-4, M20 with drought for 4 days; YDS-8, Y34 with drought for 8 days; MDS-8, M20 with drought for 8 days; *LEA*, *late embryogenesis abundant*; *HSP*, *heat shock protein*; Pro, proline; Tre, trehalose; Raf, raffinose; GB, glycine betaine; Bet, betanin.

buffer. The first-strand cDNA was synthesized using random hexamer primers and SuperScript II. Second-strand cDNA synthesis was subsequently performed using DNA polymerase I, RNase H, dNTP, and buffer. Then the cDNA fragments were purified with QiaQuick PCR extraction kit (Qiagen, Hilden, Germany), and end repaired, followed by the addition of poly-(A) tails and ligation to Illumina sequencing adapters. The ligation products were size selected using AMPure XP beads (Beckman Coulter, Brea, CA, USA) and PCR amplified, and single-end reads (SE50) were sequenced on an Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA). The sequences have been deposited into the NCBI Sequence Read Archive database (SRP143549).

2.11. Sequence analysis

Raw reads were first processed using in-house Perl scripts to remove reads containing adapter sequences, reads containing poly-N sequences, and low quality reads. After filtering, the obtained clean reads were mapped to the watermelon reference genome (ftp://cucurbitgenomics.org/pub/cucurbit/genome/watermelon/) using TopHat2. The expression level of each gene was calculated based on the length of the gene and the number of reads mapped to this gene to generate RPKM (Reads Per Kilobase of exon model per Million mapped reads). Analysis of DEGs was performed using the DESeq R package, and the resulting *P*-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Thresholds for significantly differential expression were set to an adjusted *P*-value (padj) < 0.05 and fold change ≥ 2. Pathway enrichment analysis of differentially expressed gene transcripts (DEGs) was performed using the Kyoto Encyclopedia of Genes and Genome (KEGG, http://www.genome.jp/kegg/).

2.12. Total RNA extraction and gene expression analysis

Total RNA from leaves was extracted using an RNA extraction kit (Axgen, Union City, CA, USA). Residual DNA was removed with a DNase Mini Kit (Qiagen, Hilden, Germany). The integrity and quality of RNA extracts were analyzed using 2.0% agar gel electrophoresis and a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One microgram of total RNA was used for reverse transcription using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). According to RNA-seq analysis, the transcripts of 16 mRNAs with different expression patterns were randomly selected for qRT-PCR analysis. The primers for qRT-PCR are listed in supplemental Table S1, and the watermelon *β-actin* gene (*ClACT*) was used as the internal control [36]. The qRT-PCR assay was performed using an iCycler iQ TM Multicolor PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative gene expression levels were analyzed according to the method of Livak and Schmittgen [37].

2.13. Statistical analysis

All data were analyzed using variance (ANOVA) and significant differences (at $P < 0.05$) among treatments were determined using Tukey's test. The results are presented as the mean ± standard deviation (SD) of three independent biological replicates.

3. Results

3.1. M20 showed more tolerant to drought stress than Y34

As shown in Fig. 1a and b, drought stress caused leaf wilting,

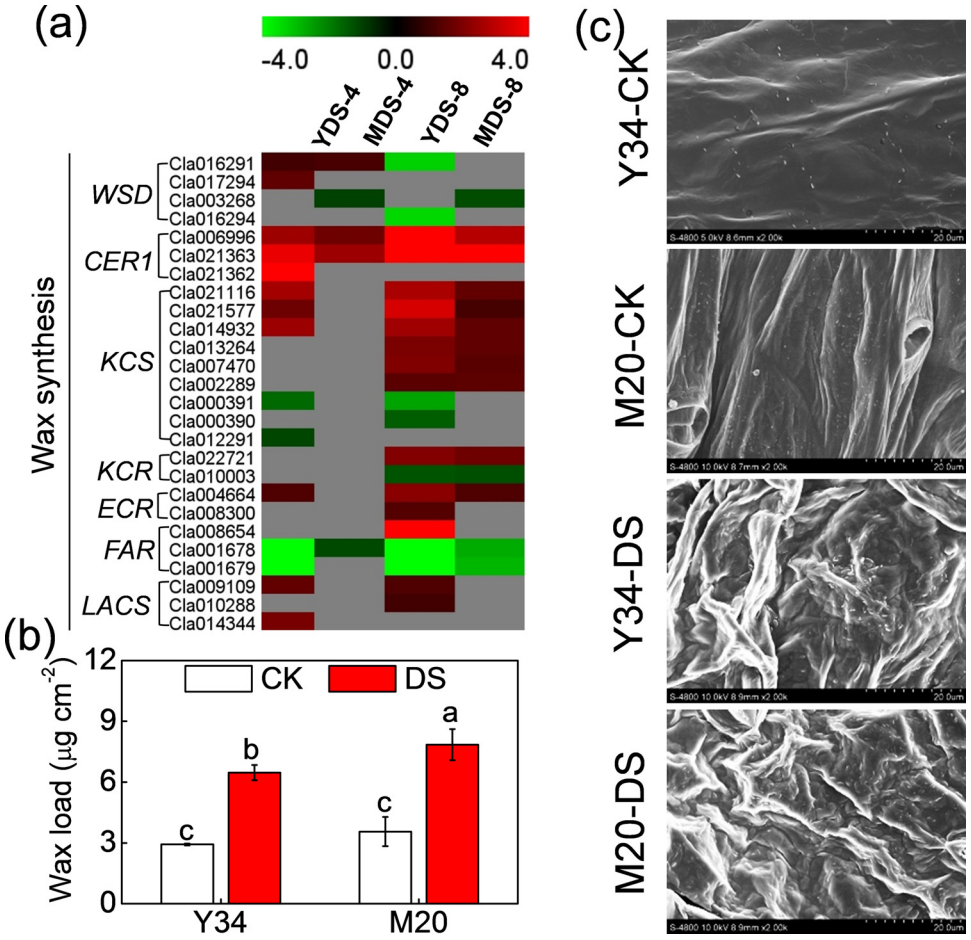

Fig. 6. Responses of wax biosynthesis under drought stress. Seedlings treatments and the sample harvesting were conducted as described in Fig. 1. (a) Differentially expressed genes involved in wax synthesis. (b) Analysis of the wax content and (c) observation of epidermal wax crystal using a Hitachi S4800 scanning electron microscope. Data are the means of three replicates (± SD), and means denoted with the same letter did not significantly differ at $P < 0.05$. YDS-4, Y34 with drought for 4 days; MDS-4, M20 with drought for 4 days; YDS-8, Y34 with drought for 8 days; MDS-8, M20 with drought for 8 days; CK, control; DS, drought stress; *WSD*, *wax synthase*; *CER1*, *ECERIFERUM1*; *KCS*, *3-ketoacyl-CoA synthase*; *KCR*, *3-ketoacyl-CoA reductase*; *ECR*, *trans-2-enoyl-CoA reductase*; *FAR*, *fatty acyl coA reductase*; *LACS*, *long-chain fatty acyl-CoA synthetase*.

reduced leaf relative water content (RWC), and increased leaf relative electric conductivity (REC) and lipid peroxidation in both Y34 and M20. RWC was significantly reduced by 38.78% and 13.68%, but MDA content was increased by 83.05% and 40.89% in Y34 and M20, respectively, after drought stress for 8 days. M20 showed higher tolerance to drought stress than Y34, based on its higher RWC but lower REC and lipid peroxidation.

To understand the differences between these two genotypes, we compared their leaf morphologies. The leaves of M20 were thicker with higher epidermal hair density, but lower stomatal density than those of Y34 (Fig. 2). For example, the leaf thickness and epidermal hair density of M20 were 35.80% and 39.03% higher than those of Y34, respectively, whereas the leaf stomatal density of M20 was 36.99% less than that of Y34.

3.2. Responses of genome-wide expression profiles under drought stress based on mRNA-seq data

To reveal the molecular regulatory strategies in watermelon adaptation to drought stress, RNA-seq analysis was performed. By mapping all unique sequences to the watermelon genome, a total of 168.61 million mapped reads were obtained (Fig. 3a, Table S2). There was a strong correlation ($R^2 \geq 0.98$) between the two biological replicates for each treatment, suggesting that the sequencing results are highly reliable (Fig. 3b). To further validate the efficiency of the RNA-seq analysis, we analyzed the transcripts of 16 mRNAs with different expression patterns by qRT-PCR. Among these transcripts, 10 mRNA transcripts were significantly upregulated but six mRNA transcripts were downregulated by drought stress in both Y34 and M20, with some exceptions. The changes in 16 mRNA transcripts assessed by qRT-PCR were similar ($R = 0.928$; $n = 64$, $P < 0.0001$) to those based on RNA-seq analysis, suggesting the validity of the RNA-seq data (Fig. 3c). In total, 2916 DEGs in Y34 and 1783 DEGs in M20 were upregulated, while 3775 DEGs in Y34 and 2536 DEGs in M20 were downregulated after drought stress (Fig. 3d). Additionally, more DEGs were observed after drought stress for 8 days in both Y34 and M20 than those observed after drought stress for 4 days.

3.3. Responses of glutathione and ascorbic acid under drought stress

Numerous DEGs involved in glutathione (GSH) and ascorbic acid (AsA) metabolism were up- or down-regulated by drought stress in both Y34 and M20 (Fig. 4a, Table S3). Moreover, more GSH and AsA metabolism-related gene transcripts were affected by drought stress in Y34 relative to those in M20. The ratio of reduced/oxidized glutathione (GSH/GSSG) was decreased by 65.58% and 45.52% in Y34 and M20, respectively, after drought stress for 8 days (Fig. 4b). Similarly, the ratio of ascorbic/dehydroascorbic acid (AsA/DHA) was decreased by 37.75% and 9.08% in Y34 and M20, respectively, after drought stress for 8 days (Fig. 4c). Consequently, the ratios of GSH/GSSG and AsA/DHA in M20 were 68.30% and 161.45% higher than those in Y34, respectively, after drought stress for 8 days.

3.4. Responses of protein protection and osmotic adjustment under drought stress

Most DEGs encoding late embryogenesis abundant (LEA) proteins, dehydrin, and heat shock proteins (HSPs) were upregulated, suggesting their positive roles in drought tolerance (Fig. 5a, b, Table S4). In particular, most transcripts encoding small HSPs were increased in both

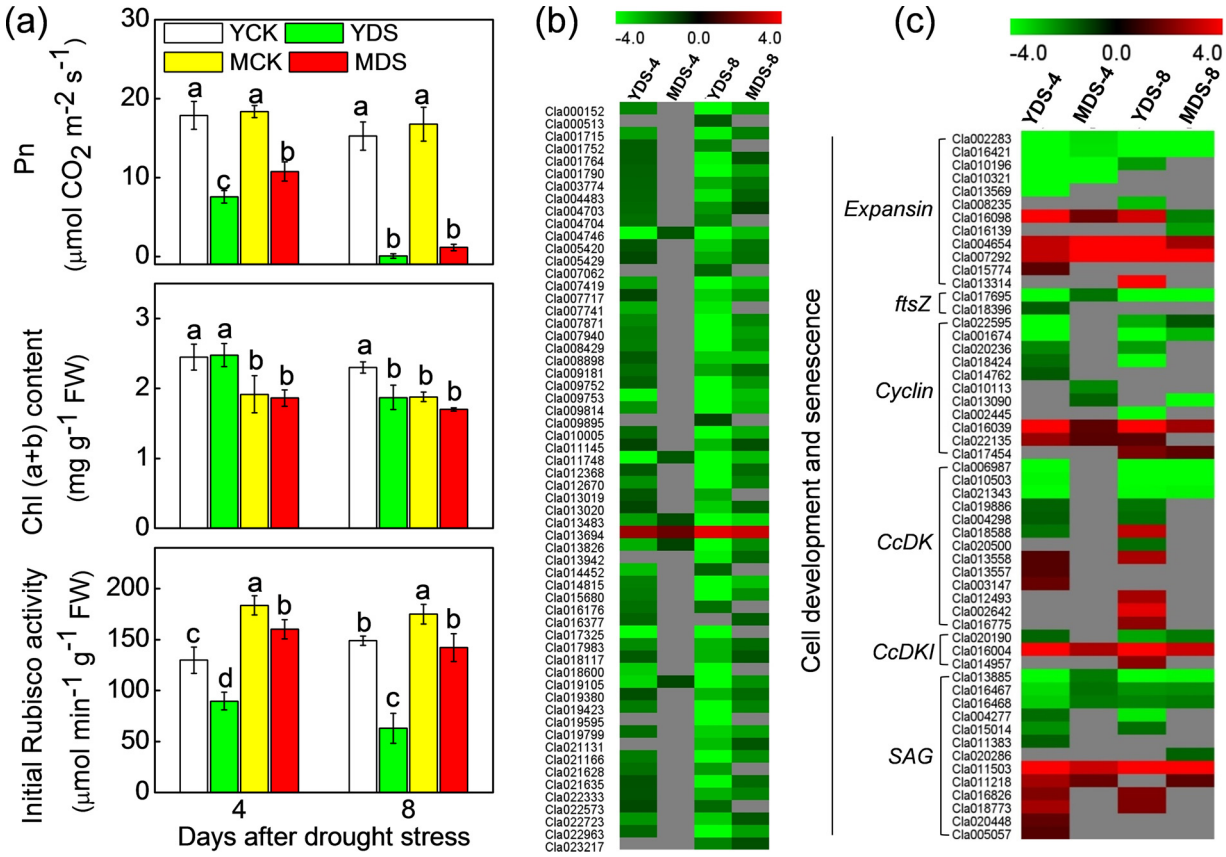

Fig. 7. Responses of (a) photosynthesis and genes related to (b) photosynthesis and (c) cell growth under drought stress. Seedlings treatments and the sample harvesting were conducted as described in Fig. 1. Data in (a) are the means of three replicates (± SD), and means denoted with the same letter did not significantly differ at $P < 0.05$. YDS-4, Y34 with drought for 4 days; MDS-4, M20 with drought for 4 days; YDS-8, Y34 with drought for 8 days; MDS-8, M20 with drought for 8 days; Pn, The net photosynthetic rate; Chl, chlorophyll; *ftsZ*, *filamentous temperature sensitive protein Z*; *CcDK*, *cyclin-dependent kinase*; *CcDKI*, *CcDK inhibitor*; *SAG*, *senescence-associated protein*.

Y34 and M20 after drought stress for both 4 and 8 days. Additionally, some DEGs involved in the biosynthesis of osmoprotectants, including proline and soluble sugar, were upregulated. The proline content was significantly increased by 2.29- and 3.00-fold in Y34 and M20, respectively, after drought stress for 8 days (Fig. 5c). The soluble sugar content was significantly increased by 20.36% and 15.18% in Y34 and M20, respectively, after drought stress for 8 days.

3.5. Responses of wax biosynthesis under drought stress

Cuticle accumulation on leaf surfaces is an important drought response that prevents water loss by reducing transpiration [38]. Most DEGs involved in wax biosynthesis were induced by drought stress in both Y34 and M20 (Fig. 6a, Table S5). Consistently, wax accumulation was significantly induced in both Y34 and M20 after drought stress for 8 days (Fig. 6b, c). Although there was no significant difference in wax content between Y34 and M20 under normal conditions, more wax accumulation was observed in M20 than that in Y34 under drought stress. The wax content of M20 was 21.33% higher than that of Y34 under drought stress.

3.6. Responses of photosynthesis and genes involved in photosynthesis and cell growth under drought stress

The net photosynthetic rate (Pn) and initial Rubisco activity were reduced by drought stress in both Y34 and M20 (Fig. 7a). In comparison to M20, Y34 showed lower Pn after 4 days of drought stress and lower initial Rubisco activity after both 4 and 8 days of drought stress. Pn was reduced by 57.78% and 41.31%, while the initial Rubisco activity was reduced by 30.91% and 12.64% in Y34 and M20, respectively, after drought stress for 4 days. The chlorophyll content was almost unchanged in both genotypes after drought stress for 4 days, but was obviously decreased by 18.70% in Y34 after drought stress for 8 days. Most DEGs involved in photosynthesis and cell cyclin and expansion were downregulated by drought stress in both Y34 and M20 (Fig. 7b, c, Table S6). After 4 days of drought stress, fewer downregulated DEGs involved in photosynthesis and cell growth were observed in M20 relative to those in Y34.

3.7. Responses of plant hormones and hormone signaling-related genes under drought stress

In the abscisic acid (ABA) pathway, ABA biosynthesis-related gene (e.g., *NCED*) transcripts were upregulated, but most ABA metabolism-related gene (e.g., *CYP707A1*) transcripts were downregulated in both M20 and Y34 under drought stress (Fig. 8a, Table S7). Consistently, ABA content was increased by 113.15% and 132.74% in Y34 and M20, respectively, and ABA in M20 was 17.36% higher than that in Y34, after drought stress for 4 days (Fig. 8b). Finally, most ABA-responsive element binding factor (*ABF*) transcripts were upregulated by drought stress in both Y34 and M20. In auxin, gibberellin (GA), cytokinin (CTK), and brassinosteroid (BR) pathways, the transcripts of most biosynthesis-related genes and downstream signal genes were downregulated by drought stress in both Y34 and M20, with some exceptions. GA_3 levels in both Y34 and M20 were almost unchanged after drought stress for 4 days, but were increased after drought stress for 8 days. IAA levels in Y34 were increased by drought for 8 days; however, those in M20 were decreased by drought for 4 days. BR content in Y34 was obviously

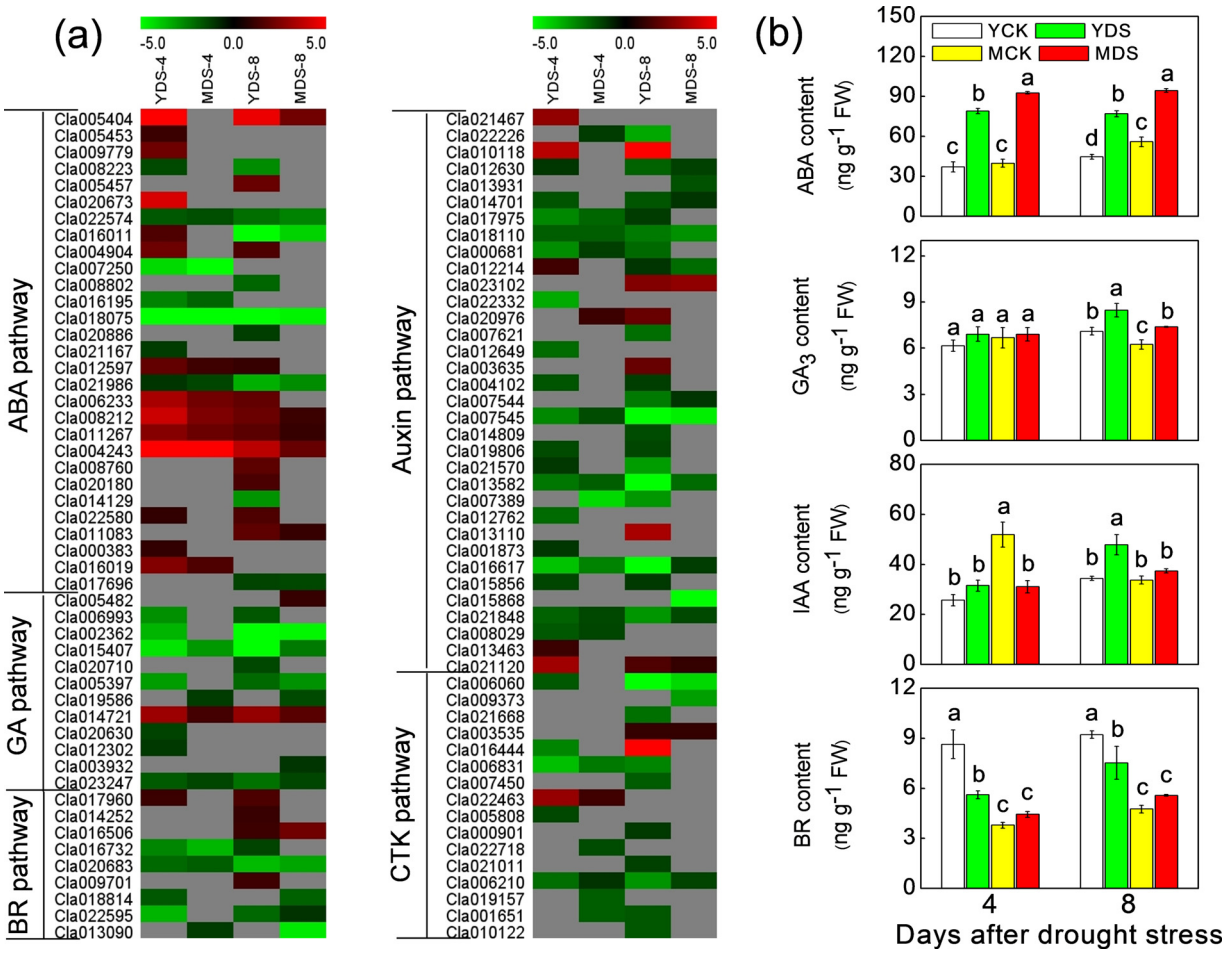

Fig. 8. Responses of (a) genes involved in hormonal signaling and (b) analysis of hormone levels under drought stress. Seedlings treatments and the sample harvesting were conducted as described in Fig. 1. Data in (b) are the means of three replicates (± SD), and means denoted with the same letter did not significantly differ at $P < 0.05$. ABA, abscisic acid; BR, brassinosteroid; CTK, cytokinin; GA, gibberellin; YDS-4, Y34 with drought for 4 days; MDS-4, M20 with drought for 4 days; YDS-8, Y34 with drought for 8 days; MDS-8, M20 with drought for 8 days.

reduced by 35.00% and 18.33% under drought for 4 and 8 days, respectively, but was almost unchanged in M20.

3.8. Response of melatonin content under drought stress and the interaction effects between melatonin and ABA

The transcripts of three *GCN5-related N-acetyltransferase* (*GNAT*) superfamily genes, probable key rate-limiting genes (e.g., *serotonin N-acetyltransferase*, *SNAT*) for plant melatonin biosynthesis, were upregulated in both Y34 and M20 under drought stress (Fig. 9a, Table S7). Two and four *GNAT* transcripts in Y34 were decreased after 4 and 8 days of drought stress, respectively, while only one *GNAT* transcript in M20 was decreased by drought stress for 8 days. Melatonin content was induced by 27.68% and 44.04% in Y34 and M20, respectively, and melatonin in M20 was 20.79% higher than that in Y34, after drought stress for 4 days (Fig. 9b). However, melatonin content was significantly decreased after 8 days of drought stress in both M20 and especially Y34.

To understand the interaction effects between melatonin and ABA, we analyzed the changes in ABA and melatonin content after melatonin and ABA pretreatment, respectively, under normal and drought conditions. Application of ABA had almost no effect on the melatonin content under normal or drought conditions (Fig. 9c). However, melatonin pretreatment obviously increased ABA contents under both normal and drought stress (Fig. 9d). ABA contents were increased by 20.00% and 15.44% under normal and drought stress, respectively, after melatonin pretreatment.

3.9. The effects of ABA and melatonin on drought tolerance and wax biosynthesis

ABA and melatonin had little effect on leaf RWC, Pn, and REC under normal growth conditions, except that Pn was increased by melatonin treatment (Fig. 10a). After 4 days of drought stress, the RWC and Pn values were significantly reduced but REC was increased. However, pretreatment with ABA or melatonin obviously alleviated the drought-induced reduction of RWC and Pn and increase of REC. For example, the Pn was decreased by 49.32% and 50.72% in ABA- and melatonin-pretreated plants, respectively, after drought stress, far less than the changes of 75.79% observed in control plants.

As well as the expression of *CER1* (*Cla006996*), wax contents were significantly increased by ABA and melatonin under normal conditions (Fig. 10b). Two *CER1* transcripts and wax accumulation were significantly induced after exposure to drought stress. Importantly, the magnitudes of the increases in the transcripts of these two genes and wax content were much greater in ABA- and melatonin-pretreated plants than those in control plants. After drought stress, 95.15% and 118.23% increases in wax contents were observed in ABA- and melatonin-pretreated plants, respectively, far greater than those in control plants.

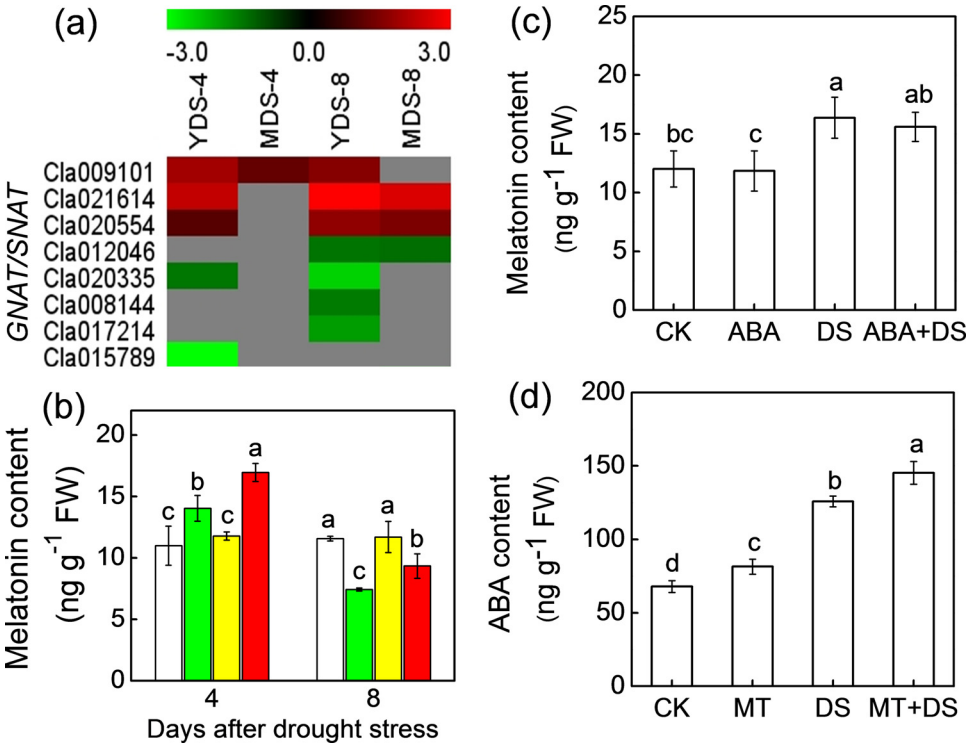

Fig. 9. (a) Response of melatonin content and (b) the interaction effects between melatonin and ABA under drought stress. In (a) and (b), seedlings treatments and the sample harvesting were conducted as described in Fig. 1. Seedlings at the three-leaf stage were pretreated with (c) 100 μM ABA or (d) 150 μM melatonin three times (once every two days). Then, plants were unwatered for 4 days. The data shown are the means of three replicates (± SD), and means denoted with the same letter did not significantly differ at $P < 0.05$. YDS-4, Y34 with drought for 4 days; MDS-4, M20 with drought for 4 days; YDS-8, Y34 with drought for 8 days; MDS-8, M20 with drought for 8 days; ABA, abscisic acid; MT, Melatonin; DS, drought stress; GNAT/SNAT, GCN5-related N-acetyltransferase superfamily genes/serotonin N-acetyltransferase.

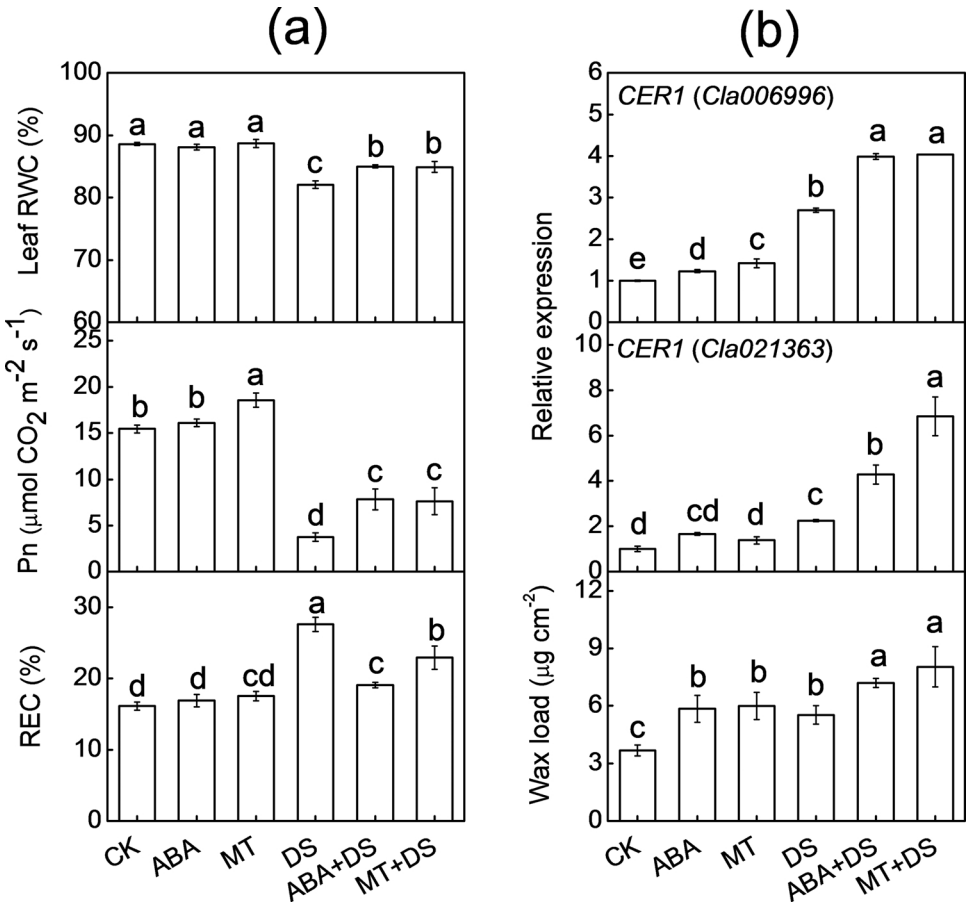

Fig. 10. The effects of ABA and melatonin on (a) drought tolerance and (b) wax biosynthesis. Seedlings treatments and the sample harvesting were conducted as described in Fig. 9c and d. Data are the means of three replicates (± SD), and means denoted with the same letter did not significantly differ at $P < 0.05$. ABA, abscisic acid; MT, Melatonin; DS, drought stress; RWC, relative water content; Pn, the net photosynthetic rate; REC, relative electric conductivity; *CER1*, *ECERIFERUM 1*.

4. Discussion

Consistent with our previous study [21], M20 displayed higher tolerance to drought stress than did Y34 (Fig. 1). Leaf thickness is an important character imparting drought tolerance to plants by reducing water loss, and thicker leaves are thus related to greater drought tolerance [39]. As well as stomatal density and size, leaf hairs are considered an effective barrier to the diffusion of H_2O [40,41]. Therefore, the tolerance of M20 may be partly associated with its specific leaf traits, such as its much thicker, more numerous epidermal hairs, and lower stomatal density (Fig. 2).

4.1. Antioxidation, molecular chaperones, osmotic adjustment, and wax accumulation correlate with drought tolerance

To minimize the effects of drought-induced peroxidation damage, plants have evolved a highly efficient antioxidant system [9]. M20 showed higher ratios of reduced GSH and AsA than did Y34 under drought stress (Fig. 4). Thus, drought tolerance of M20 is closely associated with strong antioxidant abilities. As important molecular chaperones, heat shock proteins (HSPs) are responsible for protein folding, assembly, translocation and degradation in many normal cellular processes and can stabilize proteins and membranes and facilitate protein refolding under stress conditions [42]. Similarly, late embryogenesis abundant (LEA) proteins play crucial roles in protecting plants against stresses by stabilizing proteins, membranes, and thus cellular homeostasis [43]. Most DEGs encoding LEA proteins and HSPs were upregulated by drought stress, especially by 8 days of drought stress, suggesting that they are possibly involved in protein protection under drought stress (Fig. 5a, b).

In addition to protein protection and antioxidation, water retention is another important strategy for plant adaption to drought stress. Osmotic adjustment is widely recognized to have a role in plant adaptation and survival under drought stress through maintaining turgor and protecting specific cellular functions [44]. Proline and soluble sugars are not only important compounds involved in protein synthesis and energy production but also important osmoprotectants involved in plant tolerance to osmotic stresses [9,45]. Accompanied with the upregulation of some biosynthesis-related DEGs, proline and soluble sugar levels were significantly increased by drought stress in both Y34 and especially M20 (Fig. 5b, c). Thus, we speculated that proline and soluble sugars might play an important role in the adaption of both Y34 and especially M20 to drought stress.

As a waterproof barrier, the leaf cuticle protects plants against desiccation [39]. Most DEGs involved in the biosynthesis of wax (a major cuticle component) and wax accumulation was increased in both Y34 and especially M20, suggesting that wax plays a positive role in watermelon adaptation to drought stress (Fig. 6). Therefore, the greater abilities in regulating antioxidation, protein protection, osmotic adjustment, and wax accumulation may confer the higher tolerance of M20 to drought stress.

4.2. Growth retardation correlates with plant avoidance of drought stress

Apart from drought tolerance, the set of regulatory genes reduced most DEGs involved in photosynthesis and cell cyclin, division, and growth (Fig. 7b, c). Moreover, photosynthesis was obviously inhibited by drought stress in both Y34 and especially M20 (Fig. 7a). These results suggest that the inhibition of plant photosynthesis and growth is attributed to both regulation of related gene expression and damage caused by drought stress. Watermelon, especially the drought-susceptible Y34 genotype, may actively reduce growth and development as a way in order to allocate resources to reprogram stress signaling networks [46]. M20, with high tolerance, alleviated drought caused damage and thus showed relatively lower reduction of growth and related gene expressions under drought stress.

4.3. Various plant hormone signaling correlates with drought adaptation

An increasing body of evidence indicates that ABA plays a crucial role in governing signaling events and inducing tolerance responses under drought stress [47]. ABA is well recognized to mediate early responses to soil drying by limiting stomatal apertures in order to prevent water loss [48]. Moreover, ABA can induce antioxidant activities, LEA protein and HSP accumulation, and cuticular wax biosynthesis to scavenge excessed reactive oxygen species, protect proteins, and prevent water loss, respectively, under drought stress [9,49–51]. As the transcripts involved in ABA biosynthesis and metabolism were up- and down-regulated, respectively, ABA levels in both Y34 and M20 increased after drought stress (Fig. 8). Moreover, exogenous ABA application increased wax biosynthesis and drought tolerance (Fig. 10). Thus, ABA plays a critical role in watermelon tolerance to drought stress by regulating various processes such as antioxidation, protein protection, and wax accumulation. Notably, M20 showed higher ABA levels than Y34 under drought stress, suggesting that higher ABA levels confer M20 tolerance to drought stress.

In addition to ABA, auxin, BR, and CTK have been shown to play important roles in improving plant tolerance to drought stress [13,52]. However, most DEGs involved in auxin, GA, BR, and CTK signaling were downregulated by drought stress in both Y34 and M20. It is well known that these hormones also play important roles in regulating plant growth and development [13]. Moreover, multiple hormones regulate some physiological responses to drought stress through synergistic or antagonistic interactions [13]. For instance, auxin and CTK are antagonistic with ABA and counteract stomatal closure [53]. Thus, hormonal balance is required for plant adaptation to drought stress [27]. Inhibition of auxin, GA, CTK, and BR signaling may be implicated in reducing plant growth under drought stress.

4.4. Melatonin induces wax biosynthesis and drought tolerance by interacting with ABA

Numerous studies have demonstrated that melatonin plays an important role in regulating plant tolerance to drought stress [54]. Consistent with previous studies [55,56], melatonin levels in both Y34 and M20 were increased after drought stress, and exogenous melatonin application increased wax biosynthesis and drought tolerance (Figs. 9 and 10). These results suggested that melatonin plays an important role in watermelon tolerance to drought stress and its positive role correlates with the induction of wax biosynthesis.

As universal abiotic stress regulators, the crosstalk between ABA and melatonin in various stresses has been studied. The effect of melatonin on ABA content is still controversial. For example, exogenous melatonin reduced ABA content in drought-stressed apple trees by down-regulating *MdNCED3* and up-regulating *MdCYP707A* [57]; however, in cold-stressed *Elymus nutans* and cucumber, ABA production was increased by exogenous application of melatonin [58,59]. Additionally, a recent study provides evidence that ABA did not affect endogenous melatonin content [58]. Our results have shown that ABA had almost no effect on melatonin content, though melatonin increased ABA levels under both normal and drought conditions. Thus, melatonin may function upstream of the ABA pathway in inducing wax accumulation and drought tolerance.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.10.016.

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