Abscisic acid participates in melatonin-induced chilling tolerance of cucumber *via* regulating photosynthesis and the antioxidant system

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Abstract

Melatonin (MT) and abscisic acid (ABA) are crucial in regulating abiotic stress tolerance in cucumber. However, their roles as signaling molecules in regulating chilling tolerance remain ambiguous. The results showed that neither applying ABA nor removing endogenous ABA with sodium tungstate (Na_2WO_4, an inhibitor of ABA synthesis) has no effect on endogenous MT levels, however, MT-induced endogenous ABA content *via* activating the activities and mRNA levels of 9-cis-epoxycarotenoid dioxygenase (NCED) under normal conditions and inhibiting endogenous MT with p-chlorophenylalanine (p-CPA) blocked this effect. Further studies indicated that MT and ABA had a noteworthy effect on enhancing the chilling tolerance of cucumber seedlings in terms of lower chilling injury, EL, and MDA contents. Moreover, cucumber seedlings pretreated with MT and ABA showed lower ROS contents and higher antioxidant capacity than those in H_2O treatment under chilling stress. Meanwhile, MT and ABA alleviated the decline of chlorophyll content, photosynthesis, gene expression, and protein level of Rubisco and RCA caused by chilling stress as well as maintained photosynthetic electron transport *via* increasing heat dissipation, the levels of proteins PsbS and VDE as well as decreasing the chilling damage to donor and recipient side of electron transport chain under chilling stress. However, it was discovered that Na_2WO_4 and fluridone (Flu) blocked the chilling tolerance induced by MT, whereas p-CPA had little impact on ABA promotion on the chilling tolerance of cucumber. These findings imply that ABA may act as a downstream signal of MT in the regulation of chilling tolerance in cucumber.

Introduction

Low temperature is a significant environmental factor that restricts agricultural productivity and the distribution of plant species[1,2]. For horticultural crops, especially these in warm climates, like cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), chilling stress often occurrs during winter and spring cultivation in solar greenhouses in Northern China, which mainly affected photosynthetic capacity and resulted in the accumulation of reactive oxygen species (ROS) *via* down-regulating the activities and relative expression of photosynthetic enzymes, destroying the chloroplast and the electron transport chain in mitochondria, thereby causing a significant decline in the yield and quality of vegetable cultivators[3–5]. Thus, finding efficient pathways to alleviate the damage caused by chilling stress is meaningful for chilling-sensitive crops.

The phytohormone regulatory network is important for controlling the growth and the response to abiotic stress of plants. Among all these phytohormones, Melatonin (MT) and Abscisic acid (ABA) are widely reported to help promote the abiotic tolerance of plants[6,7]. As is known, MT is an indole compound that is present in animals, plants, and microorganisms[8–10]. It acts as a growth regulator involved in plant developmental processes, including seed germination, seedling growth, root development, leaf senescence, and fruit ripening[11–14]. In addition, several studies have shown that MT acts as a scavenger of free radicals, which protects plants from the damage caused by reactive oxygen species[15]. Furthermore, MT combats a range of abiotic and biotic stresses and improves plant resistance to

environmental stressors, such as bacterial pathogens, heavy metals, extreme temperatures, drought, and salinity[16–21]. Liu et al.[22] found that the application of MT had a significant positive effect on the growth status and photosynthesis of ozone-stressed grape leaves, as well as increasing the activity of antioxidant enzymes and reducing ROS production. In cucumber seedlings, the pretreatment of MT increased salt resistance by alleviating oxidative damage and salt-induced photosynthetic inhibition and up-regulating the expression of mitogen-activated protein kinase (MAPK) genes[21]. Furthermore, multiple studies indicate that MT enhances the growth and cold stress resilience of seedlings by regulating redox homeostasis, stomatal aperture, leaf photosynthetic activity, mineral nutrient accumulation, and inducing biosynthesis of osmotic regulators, primary and secondary metabolites[23–25]. More importantly, other plant hormones, such as auxin (IAA), cytokinins (CK), gibberellins (GA), and ABA are required for MT during the regulation on plant growth and abiotic resistance[26].

Abscisic acid (ABA), produced by zeaxanthin through a series of enzymatic reactions[27], is involved in several crucial plant developmental processes. It is also known to induce various abiotic stresses, including drought, salinity, and chilling stress[28–30]. A previous study has demonstrated that abscisic acid promotes the closure of stomata by inducing a rapid change in ion flux in defense cells, which leads to the changes in gene expression[31]. Additionally, the application of exogenous ABA has been shown to elevate the performance of photosystem II under cold conditions[32], mainly *via* increasing the electron transfer between the photosynthetic antenna uptake and PSII

receptor side QA, which is the key aspect of photosynthesis[33]. Our previous studies showed that the application of ABA could promote the chilling tolerance of cucumber seedlings through activating H_2O_2 signal[34], implying ABA works together with other plant regulators to enhance plant growth and abiotic stress tolerance. However, the relationship between ABA and MT was different with the variation of physiological processes or stresses. For instance, Xu et al.[35] discovered that the application of MT induced the synthesis of ABA during the ripening of grape berry, while, the use of MT down-regulated the endogenous ABA concentration and the stomatal behavior to promote the drought resistance of apple trees[36]. However, the interaction mechanisms underlying the ABA and MT in the regulation of chilling tolerance of cucumber has not been unveiled. Considering this, this research was conducted to examined the levels of MT and ABA, as well as their associated enzyme activities and gene expressions in cucumber seedlings subjected to chilling stress after pretreatment with MT, ABA, and their inhibitors and also analyzed the chilling tolerance of cucumber seedlings under different treatments.

Materials and methods

Plant material and treatments

'Jinyou 35'cucumber (*Cucumis sativus* L.) was used in this experiment and the cultivation of cucumber seedlings according to the method described in a previous study[37] and the environmental factors in the solar greenhouse were as follows: the average sunlight during the day (600 µmol·m^{-2}·s^{-1} PFD) and 25/18 °C day/night average temperature under a 12 h photoperiod.

At the two-leaf stage, 75 µM MT, 100 µM p-chlorophenylala-nine (p-CPA, biosynthesis inhibitor of MT synthesis), 100 µM ABA and 3 mM Na_2WO_4 (biosynthesis inhibitor of ABA synthesis) or deionized water (control) were applied to the leaves of uniformly growing cucumber seedlings, and samples were taken at 0, 3, 6, 9, 12, 18 and 24 h under normal conditions (12 h photoperiod with photon flux density (PFD) of 600 µmol·m^{-2}·s^{-1}, day/night, 25/18 °C) to analyze the response of endogenous MT or ABA to exogenous ABA or MT. At the same time, seedlings were sprayed with 75 µM MT, 100 µM ABA, 100 µM p-CPA+100 µM ABA, 3 mM Na_2WO_4+75 µM MT, 50 µM Flu (an inhibitor of ABA synthesis)+75 µM MT or H_2O. Inhibitor was sprayed first, followed by MT or ABA 12 h later. After 24 h, half of the H_2O treatments were maintained under normal conditions as the control and the other half and all the other seedlings were exposed to low temperatures (12 h photoperiod with PFD of 100 µmol·m^{-2}·s^{-1}, 8/5 °C) in a light incubator, all the exogenous substance concentrations sprayed above were derived from previous studies within this experiment[19,34,38]. After 24 or 48 h, the second leaves (from the bottom) were harvested to determine the parameters. Each treatment was repeated at least three times.

Determination of MT content and synthetic enzyme activity

MT was extracted from cucumber leaves as described in Feng et al.[37] and detected in cucumber leaves by high-performance liquid chromatography-mass spectrometry (HPLC-MS, Thermo Fisher Scientific, TSQ-Quantum Access, Waltham, MA, USA) following the method by Bian et al.[39] using an external standard method based on peak area.

The activities of plant tryptophan decarboxylase (TDC) and N-acetyl-5-hydroxytryptamine-O-methyltransferase (ASMT) were measured according to the instructions of the ELISA kit. (SU-B91337 and SU-B91345, Quanzhou Kenuodi Biotechnology Co., Ltd., Quanzhou, China).

Determination of ABA content and 9-cis-epoxycarotenoid dioxygenase activity

The ABA content was determined by using a high-performance liquid chromatography-triple quadrupole mass spectrometry method with reference to our previous study[34].

The 9-cis-epoxycarotenoid dioxygenase (NCED) activity was assayed using an NCED activity kit (DG95960Q, Beijing winter song Boye Biotechnology Co., Ltd., Beijing, China).

Measurement of the growth and semi-lethal temperature under chilling stress

The seedlings that were treated with low temperature and low light for 24 h were treated as follows. After 48 h of recovery at room temperature, leaf length was measured using a ruler, leaf area was calculated as described by Gong & Xiang[40] and dry matter was measured by weighing.

The semi-lethal temperature under chilling stress was determined as described by Zhu et al.[41] with minor changes. Leaf discs were obtained from cucumber seedlings of different treatments and then transferred to 0, −2, −4, −6, and −7 °C, respectively, for 30 min. Afterward, adding 20 mL of deionized water to the test tube with leaf discs treated by chilling stress, pumping air for 20 min, and then shaking cultivation for 1 h. Moreover, the EL1 of these leaf discs was measured firstly at 25 °C, then the EL2 was detected again after boiling for 5 min using a water bath. Finally, the EL of these leaf discs was calculated according to Dong et al.[42], which was used to fit the logistic equation to obtain the LT_{50}.

Measurement of EL, MDA, ROS content, and the activities of antioxidant enzymes

The methods used to analyze the electrolyte leakage rate (EL), malondialdehyde (MDA) have been described previously[42]. The content of hydrogen peroxide (H_2O_2) was measured according to the instructions specified in the H_2O_2 kit (A064-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The superoxide anion (O_2^-) were measured as described by Li & Gong[43]. The histochemical detection of H_2O_2 and O_2^- was carried out according to the method in our previous studies[44].

Superoxide dismutase (SOD) activity was measured using the method of Stewart & Bewley[45], peroxidase (POD) activity was detected according to the method of Omran[46] and ascorbate peroxidase (APX) activity was detected according to the method described in Nakano & Asada[47].

Glutathione and ascorbic acid content assays

The contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using a glutathione content kit (GSH-2-W, and GSSG-2-W, Suzhou CoMin Biotechnology Co., Ltd., Suzhou, China) according to the instructions. The contents of ascorbic acid (AsA) and dehydroascorbic acid (DHA) were estimated using the method of Law et al.[48].

Parameters of gas exchange and content of chlorophyll

Net photosynthetic rate (P_n), transpiration rate (T_r), intercellular CO_2 concentration (C_i) and stomatal conductance (G_s) of the second leaf (from the bottom) of cucumber seedlings were
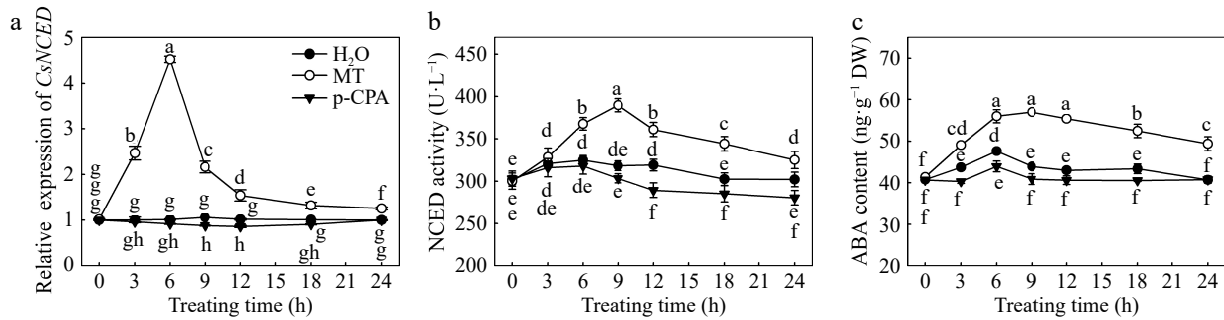
Fig. 1 Effect of MT on ABA content, activity and relative mRNA expression of *CsNCED* in cucumber seedlings under normal conditions (25/18 °C). Cucumber seedlings grown in a solar greenhouse were foliar sprayed with 75 μM MT, 100 μM p-chlorophenylalanine (p-CPA, an inhibitor of melatonin synthesis) and deionized water at the two-leaf stage, and samples from the second leaves (from the bottom) were collected at 0, 3, 6, 9, 12, 18, and 24 h for content, activity and relative mRNA expression. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).
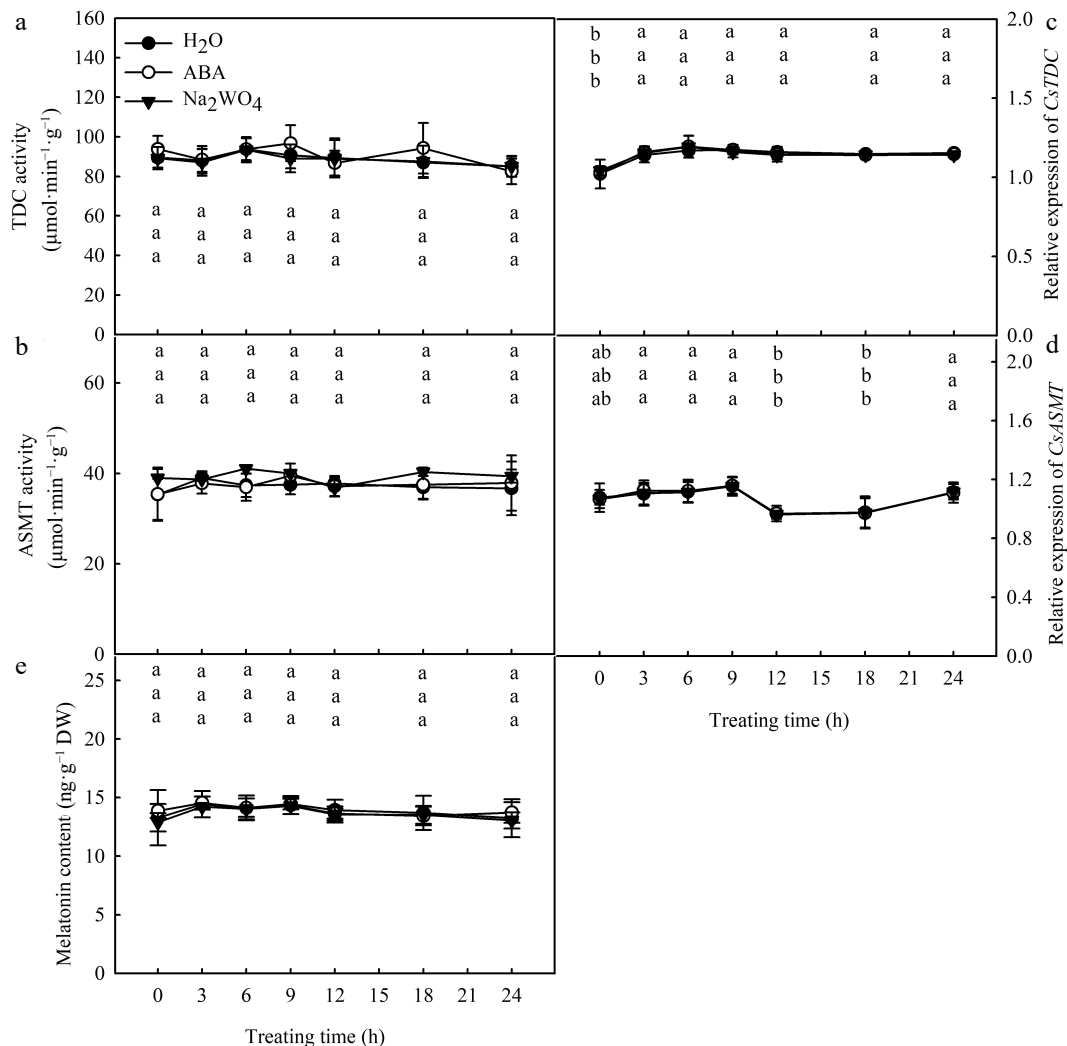

Fig. 2 Effects of ABA on MT content and activities and RNA expression of key MT biosynthesis enzymes in cucumber seedlings under normal conditions (25/18 °C). (a), (b) TDC and ASMT activities, respectively; (c), (d) *TDC* and *ASMT* mRNA expression levels; (e) MT content. Cucumber seedlings grown in a solar greenhouse were foliar sprayed with 100 μM ABA, 3 mM Na_2WO_4 (an inhibitor of ABA synthesis) and deionized water at the two-leaf stage, and samples from the second leaves (from the bottom) were collected at 0, 3, 6, 9, 12, 18, and 24 h for content, activity and relative mRNA expression. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).
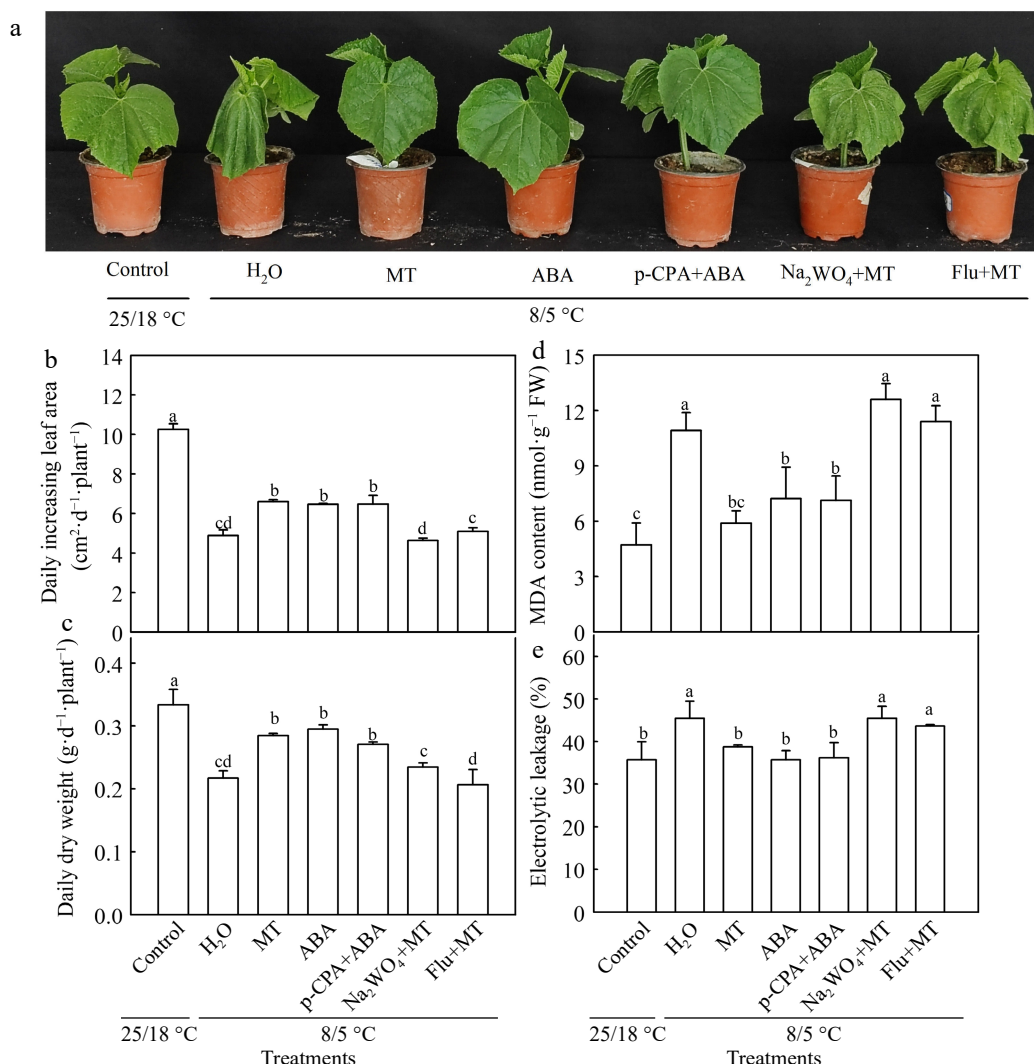
Fig. 3 Effects of MT and ABA on chilling tolerance of cucumber seedlings under low temperature stress. (a) Phenotype; (b) daily increasing leaf area; (c) daily dry weight; (d) MDA content and (e) electrolyte leakage of cucumber seedlings under chilling stress. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8/5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

measured using a PP-Systems Ciras-3 photosynthesizer from the USA. The related parameters during the measurement were set according to Feng et al.[37].

The determination of chlorophyll a, chlorophyll b and carotenoid of cucumber seedlings with 95% ethanol as described by Sartory & Grobbelaar[49].

Assay for the activities of photosynthetic enzymes

The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was measured by spectrophotometry using the Rubisco kit (RUBPS-2A-Y, Suzhou Comin Biotechnology Co., Ltd., Suzhou, China) Rubisco activase (RCA) activity was detected by ELISA using the RCA ELISA kit (SU-B91104, Quanzhou Knuodi Biotechnology Co., Ltd., Suzhou, China).

Parameters of the fluorescence

The FMS-2 pulse-modulated chlorophyll fluorometer (Hansatech, Norfolk, UK) was used to determine initial (F_0), maximum (F_m) and variable (F_v) fluorescence under dark adaptation for 30 min and steady-state (Fs), minimum (F_0'), maximum (F_m') and variable (F_v') fluorescence at 600 μmol·m^{-2}·s^{-1}. Following Demming-Adams & Adams[50] and the FMS-2 fluorometer manual, the meaning of each parameter and the photochemical efficiency were calculated using the following equations: actual photochemical efficiency of PSII (Φ_{PSII}) = (F_m' − F_s)/F_m'; maximum photochemical efficiency of PSII under dark adaptation (F_v/F_m) = (F_m − F_0)/F_m; photochemical quenching coefficient (qP) = (F_m' − F_s)/(F_m' − F_0'); non-photochemical quenching coefficient NPQ = (F_m − F_m')/F_m'.

OJIP and 820 nm absorbance curves of cucumber leaves were determined using MPEA-Senior (Hansatech, UK) according to Strasser et al.[51]. The JIP test was used to analyze and calculate the OJIP curves: Performance index based on absorbed light energy PI_{ABS} = RC/ABS · [$\Phi_{P0}/(1 − \Phi_{P0})$] × [$\psi_0$ × $(1 − \psi_0)$]; probability of the trapped exciton to release electrons to other electron acceptors after the QA (ψ_0) = ET_0/TR_0 = 1 − V_J; quantum for heat loss ratio Φ_{D0} = 1 − Φ_{P0} = (F_0/F_m).
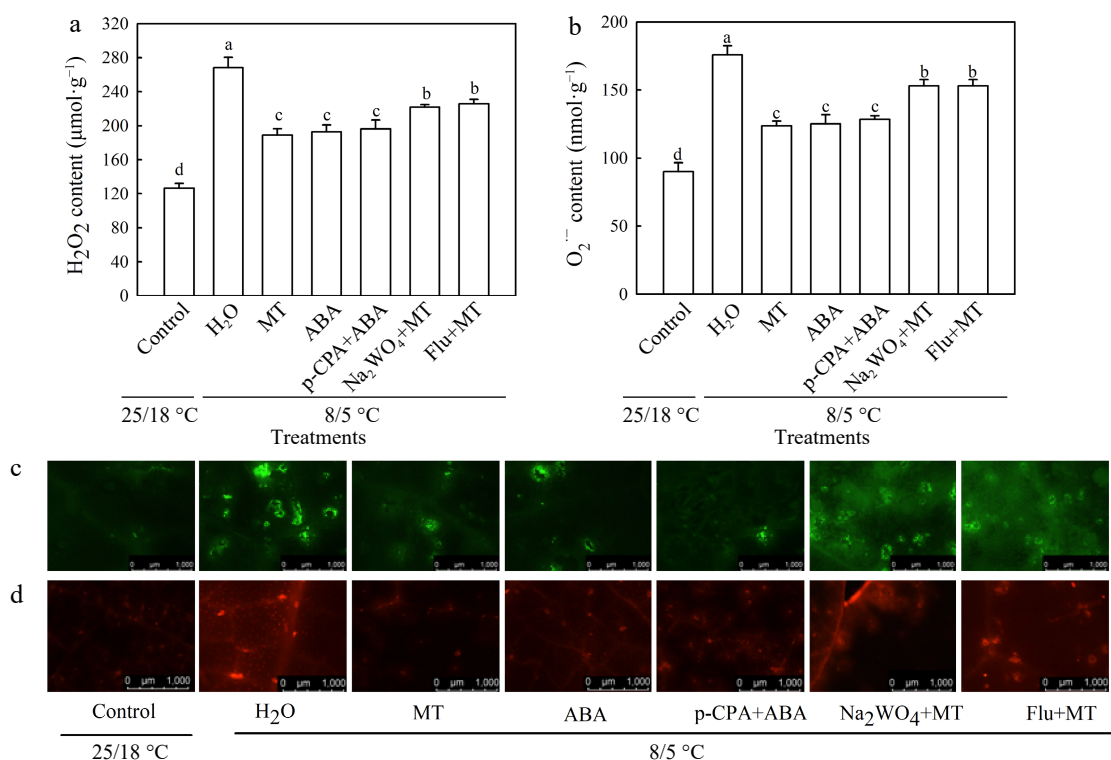
Fig. 4 Effects of different treatments on ROS content in cucumber seedlings under chilling stress. (a) H_2O_2 content; (b) $O_2^{·-}$ content; (c), (d) H_2O_2 and $O_2^{·-}$ inverted fluorescence microscope imaging. Bars = 1,000 μm. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O After 24 h, seedlings were exposed to 8/5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

Analysis of real-time quantitative PCR

Total RNA was extracted with TransZol reagent (Transgen, Beijing, China) and reverse transcribed using HiScriptRIII RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). Quantitative real-time PCR (qRT-PCR) for *NCED*, ABA receptor pyrabactin resistance 1-like gene (*PYL*), *ASMT*, *TDC*, the rubisco large subunit (*rbcL*) and *RCA* in cucumber seedlings was performed using ChamQTM Universal SYBR® qPCR MasterMix (Vazyme, Nanjing, China) according to the instructions. The synthesis of primers by RuiBio tech is shown in Supplemental Table S1.

SDS-PAGE and immunoblotting analysis

Total cucumber leaf protein was extracted as described in Feng et al.[37], loaded and separated on 8% (w/w) SDS-PAGE gels. The denatured protein complexes were transferred to PVDF membranes by wet transfer. They were blocked with 5% (w/w) skimmed milk powder. After blocking, the proteins were incubated for 1.5 h with specific I anti the RbcL, RCA, D1, PsbS, and VDE proteins (ATCG00490, AT2G39730, ATCG00020, AT1G44575, AT1G08550 PhytoAB Company, San Francisco, CA, USA) and then incubated for 1.5 h with horseradish POD conjugated anti-rabbit IgG antibody (ComWin Biotech Co., Ltd., Beijing, China). Finally, the color development reaction was performed by chemiluminescence. Detection was performed using a Shanghai Tanon Gel Imager Tanon 5200 series fully automated chemiluminescence image analysis system (Shanghai Tanon Life Sciences Co., Ltd., Shanghai, China). ImageJ image processing software for Western blot quantification analysis.

Statistical analysis

Microsoft Excel and SigmaPlot were used to process and plot the data. One-way ANOVA was performed on the data using DPS software and Duncan's test was used for multiple comparisons of significant differences ($p < 0.05$).

Results

Interaction of ABA and MT of cucumber seedlings under normal conditions

The transcriptome data analysis showed that the application of MT promoted the relative expression of ABA-related genes under chilling stress (Supplemental Fig. S1), thus it was speculated that an interaction between MT and ABA might exist during the response to chilling stress. Subsequently, an experiment was performed to verify the relationship between MT and ABA. As shown in Fig. 1, foliar spraying of MT significantly increased the activity and gene expression of *CsNCED* in cucumber seedlings under normal temperature conditions. In accordance to this, the higher endogenous ABA content was measured in MT-treated cucumber seedlings compared with that of the control, but the ABA content of seedlings treated with p-CPA, biosynthesis inhibitor of MT was similar to that of the control. Furthermore, the response of MT to exogenous ABA under normal temperature was also tested and it was found that the endogenous MT content, as well as the activities and gene expression of *CsTDC* and *CsASMT* (Fig. 2), which were the key MT biosynthetic enzymes, both displayed no obvious change in cucumber seedlings among H_2O-, ABA- and
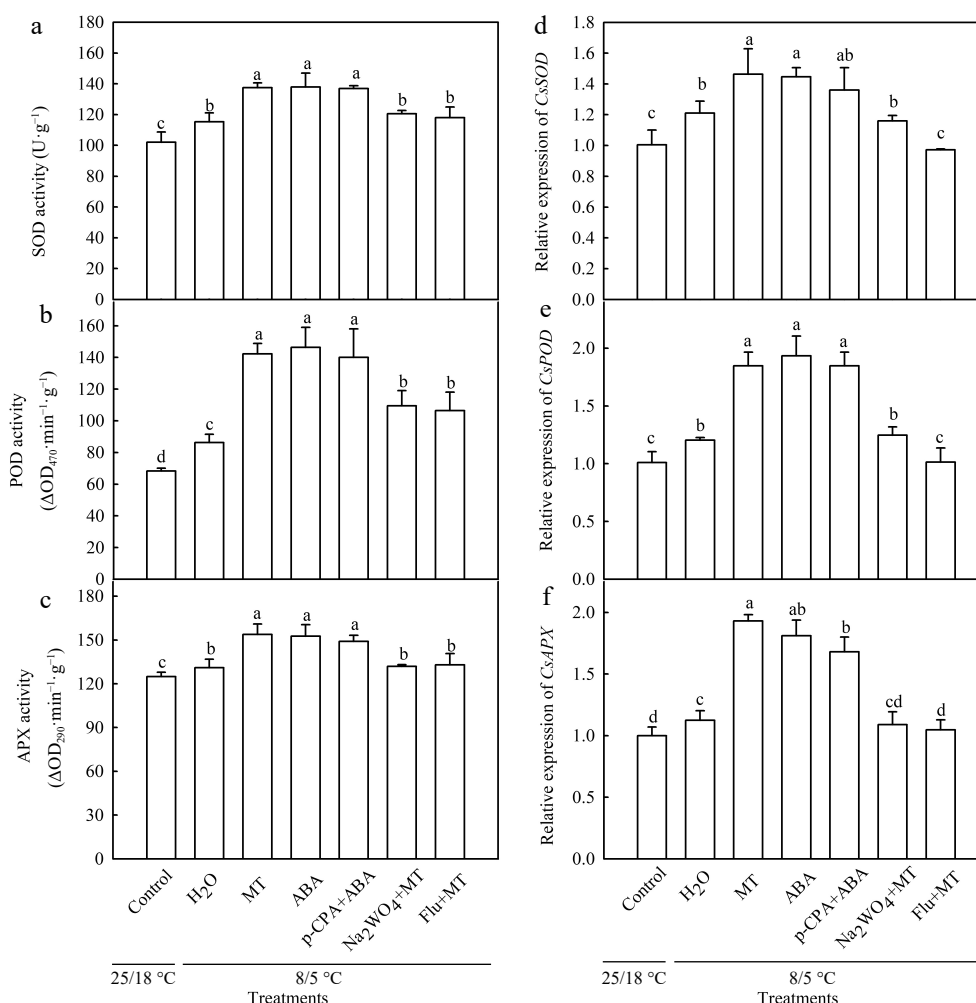
Fig. 5 Interactive effects of MT and ABA on activities and relative mRNA expression of antioxidant enzymes in cucumber seedlings under chilling stress. (a)–(c) Activities of superoxide dismutase (SOD), peroxidase (POD), ascorbate and peroxidase (APX), respectively. (d)–(f) Relative mRNA expression of *SOD*, *POD*, and *APX*, respectively. At the two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8/5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

Na_2WO_4 (biosynthesis inhibitor of ABA)-treatment under normal conditions, implying ABA may act as a downstream signal of MT.

Exogenous MT and ABA increased the chilling tolerance of cucumber seedlings

Our previous studies have proved the application of ABA and MT could improve the chilling tolerance of cucumber seedlings[34,37]. Here, the response of cucumber pre-treated with ABA and MT to chilling stress were measured and it was found that both of them increased the chilling tolerance of cucumber in terms of lower semi-lethal temperature (Supplemental Table S2), which was similar to our previous studies. In accordance with this, MT and ABA both alleviated the leaf wilting and decline of growth in cucumber seedlings caused by chilling stress (Fig. 3). To confirm whether ABA participates in the regulation of MT on chilling tolerance of cucumber, the changes of these above chilling parameters were also tested in cucumber pretreated with the inhibitors of MT and ABA. And the data showed that the application of p-CPA (biosynthesis inhibitor of MT) could not affect the chilling tolerance induced

by ABA, while, Na_2WO_4 and Flu (biosynthesis inhibitor of ABA) significantly decreased the chilling tolerance induced by MT. Taking MDA content as an example, compared to normal conditions, the MDA content increased by 131.43%, 25.10%, 52.95%, 51.14%, 167.07%, and 141.80% in cucumber seedlings of H_2O, MT, ABA, p-CPA+ABA, Na_2WO_4+MT, and Flu+MT treatments, implying ABA may act as a downstream signal of MT under chilling stress.

Effects of MT and ABA on the antioxidant capacity in cucumber seedlings under chilling stress

To explore the role of ABA in MT-regulated ROS accumulation in cucumber under chilling stress, the cucumber seedlings pretreated with ABA, MT, p-CPA, and Na_2WO_4, Flu were transferred to 8/5 °C for 48 h. As shown in Fig. 4a & b, chilling stress caused obvious increases in H_2O_2 and $O_2^{·-}$ contents, however, the cucumber seedlings treated by MT and ABA showed lower H_2O_2 and $O_2^{·-}$ contents than the seedlings treated by H_2O. The histochemical observations with inverted fluorescence microscope also supported the results of H_2O_2 and $O_2^{·-}$ contents assay (Fig. 4c, d). ROS-scavenging capacity is one of the
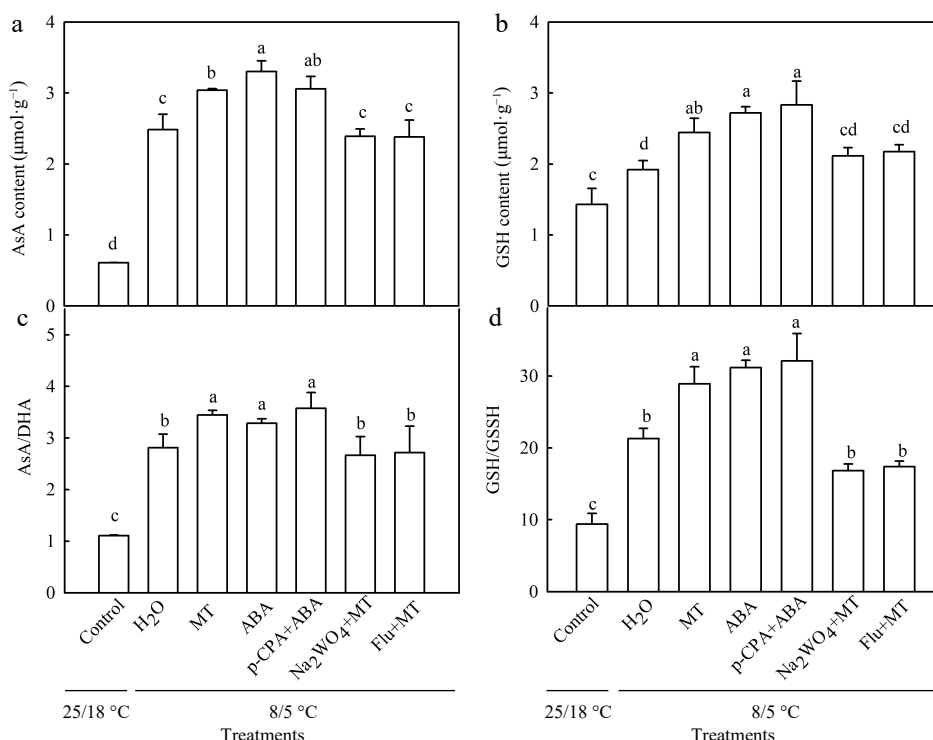
Fig. 6 Effects of different treatments on the contents of antioxidant substances in cucumber seedlings under chilling stress. (a) AsA content, (b) GSH content, (c) the ratio of AsA and DHA, (d) the ratio of GSH and GSSG. At the two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8/5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).
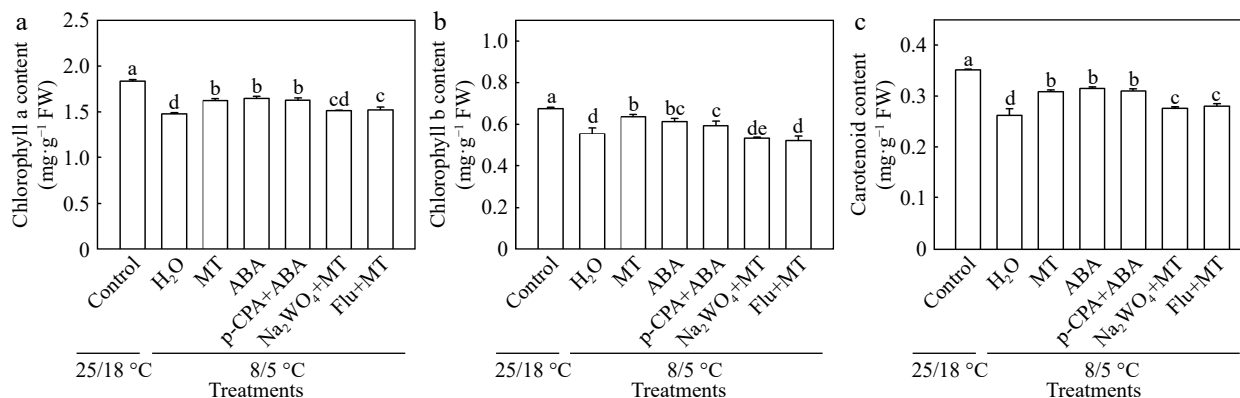

Fig. 7 Interaction between MT and ABA on the chlorophyll content of cucumber seedlings exposed to chilling stress. (a) Content of chlorophyll a; (b) Content of chlorophyll b; (c) Content of carotenoids. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8/5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

important mechanisms of relieving abiotic-induced oxidative damage. Here, it was noted that the activities and gene expression of *CsSOD*, *CsPOD*, and *CsAPX* were notably increased, which were even higher in MT- and ABA-treated cucumber seedlings after chilling stress (Fig. 5). Consistent with the observation of antioxidant enzyme activities, the application of MT and ABA also induced the content of AsA as well as the ration of AsA and DHA under chilling stress (Fig. 6). Interestingly, the inhibition of MT with p-CPA could not affect the ROS-scavenging capacity induced by ABA, whereas the inhibition of ABA

with Na_2WO_4 and Flu significantly decreased the ROS-scavenging capacity induced by MT, evidenced by higher ROS content and lower activities and gene expression of antioxidant enzyme and AsA content than MT single-treated seedlings. These data implied ABA acted as a downstream signal of MT to relieve oxidative damage caused by chilling stress.

Effects of MT and ABA on the photosynthesis in cucumber seedlings under chilling stress

After 24 h of chilling stress, the chlorophyll a, chlorophyll b and carotenoid contents of all the treatments reduced, but the

reductions of the MT, ABA, and p-CPA+ABA treatments were notably lower than those of the H_2O treatment (Fig. 7). But the Na_2WO_4 and Flu significantly decreased the mitigative effect of chlorophyll induced by MT. For instance, the chlorophyll a content of Na_2WO_4+MT and Flu+MT treatments reduced by 6.7% and 7.6%, respectively, compared with the MT treatment.

Chilling stress caused the decline of Pn in cucumber seedling, however, it was 73.5% and 119.4% higher in MT and ABA treatments, respectively, than in H_2O treatment. p-CPA+ABA treatment also had significantly higher Pn than that of H_2O treatment, and the difference was not significant compared with that of ABA treatment, whereas the Pn of Na_2WO_4 +MT and Flu+MT treatments were significantly lower than that of MT treatment (Fig. 8a). The similar change of Ci, Gs and Tr were observed in all these treatments under chilling stress (Fig. 8b–d), implying chilling caused the non-stomatal limitation of Pn and ABA was involved as a downstream signal in the regulation of MT on cucumber photosynthesis under chilling stress.

Subsequently, the variation of Rubisco and RCA of cucumber seedlings in different treatments after chilling stress were measured, and it was found that MT and ABA both alleviated the decrease of activities, gene expression, and protein level of Rubisco and RCA caused by chilling stress. In contrast, compared to MT treatment, Na_2WO_4, and Flu obviously decreased the activities, gene expression, and protein levels of Rubisco and RCA under chilling stress. For example, the expression of *CsrbcL* and *CsRCA* reduced by 22.8% and 23.1% in Na_2WO_4+MT, 37.1% and 16.5% in Flu+MT compared with MT, while the differences between p-CPA+ABA and ABA were not significant (Fig. 9).

Effects of MT and ABA on photoprotection in cucumber seedlings under chilling stress

To investigate whether the regulation of cucumber photosynthesis under chilling stress by MT and ABA is associated with photosynthetic electron transport, the degree of photoinhibition of PSII in seedlings of different treatments after chilling stress were compared. The results showed that Fv/Fm, ΦPSII, qP, and NPQ of cucumber seedling leaves significantly decreased under low-temperature stress (Fig. 10a–d), but Fv/Fm, ΦPSII, qP, and NPQ were higher in MT, ABA, and p-CPA+ABA pre-treated cucumber seedlings compared with H_2O-treated, whereas the differences between Na_2WO_4+MT, and Flu+MT treatments and H_2O treatment were not significant. Moreover, p-CPA had little effect on the alleviation of ABA-induced chilling photoinhibition. It can be seen that MT and ABA could enhance photoprotection to reduce the damage of PSII reaction center in cucumber seedlings under low temperature stress and the inhibition of ABA were able to reverse the promotion of photochemical efficiency induced by MT on the photochemical efficiency of cucumber seedlings.

Effects of MT and ABA on photosynthetic electron transport in cucumber seedlings under chilling stress

To further analyze whether ABA participates in the regulation of MT on the photosynthetic apparatus under chilling stress, the OJIP curve was measured. As shown in Fig. 11, low-temperature stress induced an increase in F_0 (O) and a decrease in Fm (IP), which increased the variable fluorescence (Fv) of O-J and J-I, but decreased I-P (Fm) at the late stage. The increases in O-J and J-I were significantly smaller in MT, ABA, and p-CPA +
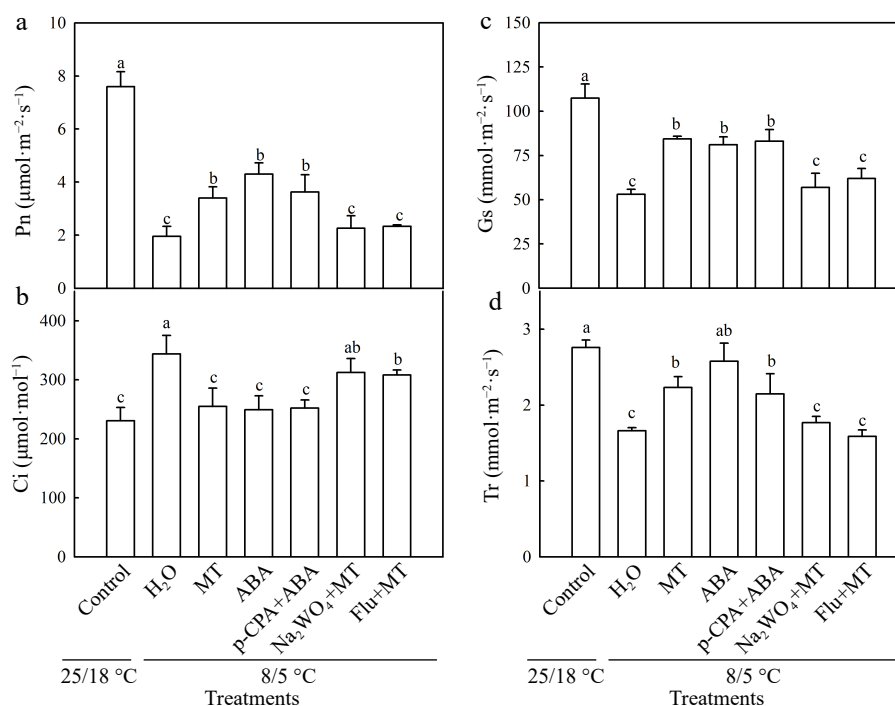

Fig. 8 Interaction between MT and ABA on gas exchange parameters in cucumber seedlings under chilling stress. (a) Net photosynthetic rate; (b) intercellular CO_2 concentration; (c) stomatal conductance; and (d) transpiration rate. At the two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8/5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are means ± SD (n = 3). Different letters indicate that the mean values are significantly different among the samples (*p* < 0.05).
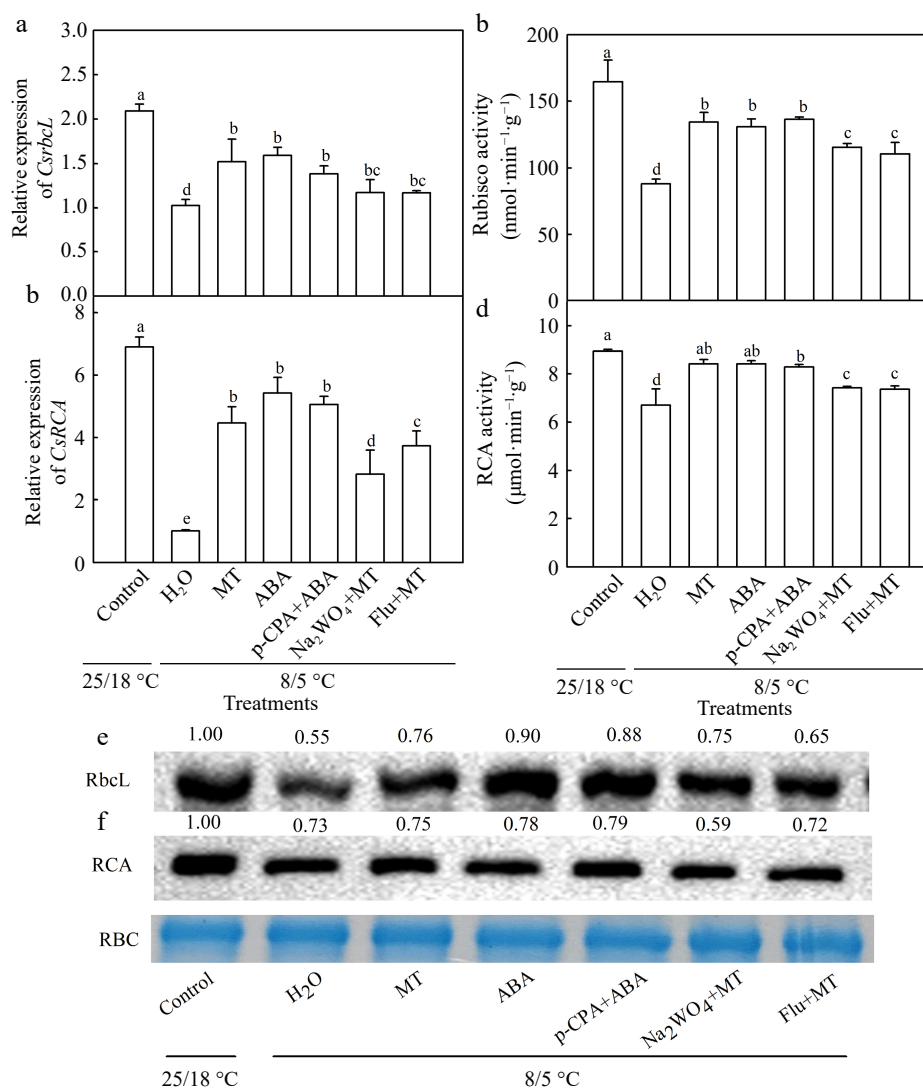
Fig. 9 Interactive effects of MT and ABA on the relative expression of *rbcL* and *RCA* in mRNA, and the activity of Rubisco and Rubisco activase (RCA) and protein levels in cucumber seedlings under chilling stress. (a), (b) Relative mRNA expression for *rbcL* and *RCA*, (c) Rubisco activity, (d) RCA activity and (e), (f) protein levels for RbcL and RCA. Rubisco (RBC) protein is shown as an equal loading control by Coomassie Brilliant Blue staining. The value above each line is the relative enrichment of the protein. Western blotting was performed three times with three independent biological samples and similar results were obtained. At the two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8/5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are means ± SD (n = 3). Different letters indicate that the mean values are significantly different among the samples ($p < 0.05$).

ABA treatments than in H_2O treatment. The J-point (2 ms) response in the chlorophyll a fluorescence transient is a specific indicator of limiting electron transfer from QA to QB and damage to the D1 protein, as seen from the ΔVt curves normalized to O-P (Fig. 11b), the J-points of the MT and ABA treatments were significantly less elevated than that of the H_2O treatment after low-temperature stress. Similarly, it was also found that MT and ABA significantly decreased the increase of K point (0.3 ms) (Fig. 11c), and Φ_{D0} and decrease of PI_{ABS} and ψ_0 (Fig. 11d–f), implying MT and ABA maintained normal photosynthetic electron transport under chilling stress. Unfortunately, the application of Na_2WO_4 and Flu decreased the positive effect of MT, whereas p-CPA had little effect on ABA-induced photosynthetic electron transport under chilling stress.

Protein levels of D1, PsbS, and VDE were analyzed in stressed seedlings to confirm the above results and to further investigate the effect of exogenous MT on photoprotection in response to chilling and the role of ABA in MT-induced photoprotection. Chilling stress significantly down-regulated the protein levels of D1, PsbS and VDE in all treated seedlings (Fig. 11g–i). Compared with H_2O-treated seedlings, MT- and ABA-treated seedlings showed a significant increase in the level of D1, PsbS, and VDE proteins after 24 h of chilling stress. The positive effect of MT on the accumulation of D1, PsbS, and VDE proteins were blocked by the application of Na_2WO_4 and Flu, whereas p-CPA was unable to block the effect of ABA on the protein levels of D1, PsbS and VDE.
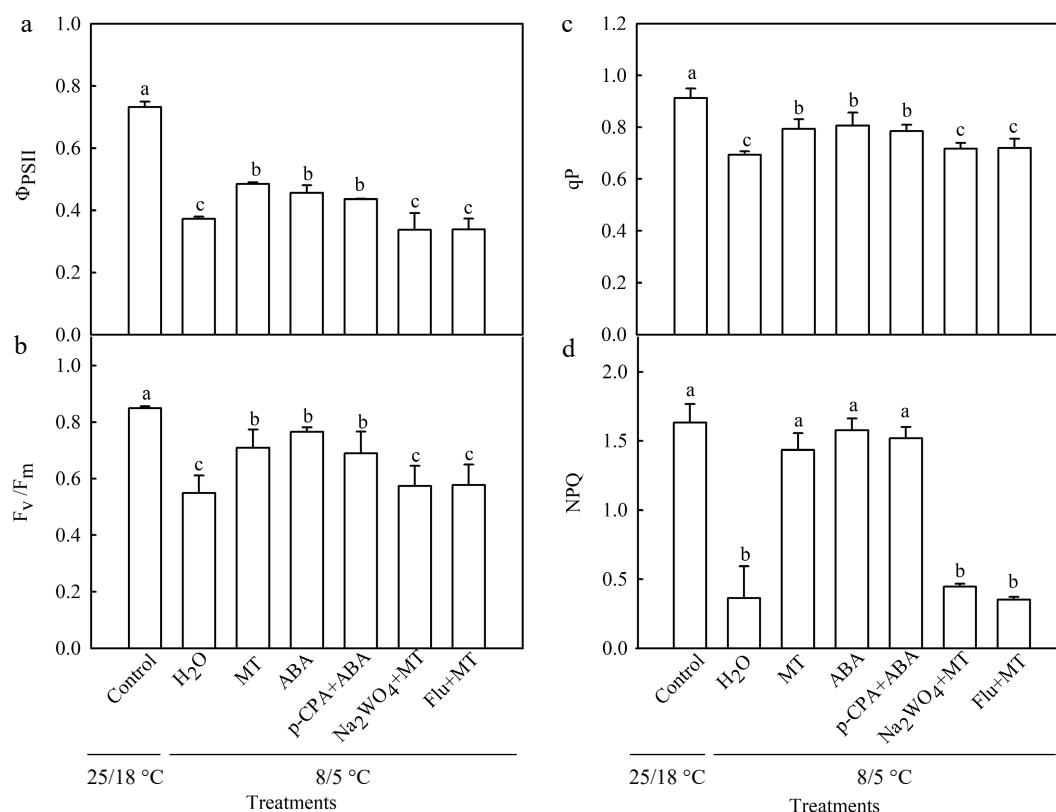
Fig. 10 Interactive effects of MT and ABA on photoprotection in chilling stressed cucumber seedlings. (a) ΦPSII; (b) Fv/Fm; (c) qP; (d) NPQ. At the two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8/5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are means ± SD (n = 3). Different letters indicate that the mean values are significantly different among the samples ($p < 0.05$).

Discussion

Lei et al.[52] first reported that MT participated in the chilling resistance with carrot suspension and also Kang et al.[53] found the heterologous expression of *SNAT* from humans in plants notably increased the endogenous MT content and promoted the chilling tolerance. Subsequently, several studies proved that MT was involved in the control of various physiological and molecular responses for inducing cold tolerance[25]. ABA is also a central regulator of plant defense against chilling stress[7]. Several studies have reported interactions existed between ABA and melatonin in the response of plants to abiotic stresses[54−55]. For instance, Li et al.[56] found that the application of MT promoted seed germination under salt stress *via* down-regulating the ABA synthesis gene *NCED* and upregulating the ABA catabolism gene *CYP707A* to decrease the ABA content. Meanwhile, MT was reported to repress the decrease expression of *NCED1*, *AAO3*, *PYL4* and *PP2Cs* to lower ABA level delaying loquat leaf senescence[57]. In contrast, MT stimulated the accumulation of ABA to enhance the antioxidant ability and then promoted the salt tolerance of Fragaria ananassa Duch[58]. In this study, 75 μM MT and 100 μM ABA both increased the chilling tolerance of cucumber, evidenced by the lower EL, MDA, and semi-lethal temperature. Moreover, it was found exogenous MT significantly increased the activity and the relative expression of *CsNCED* and ABA content in cucumber. In contrast, the inhibition of MT with p-CPA significantly decreased the NCED activity and *CsNCED* abundance, and then

caused the decline of endogenous ABA content. However, the application of ABA could not affect the endogenous MT content and the activities and expression of MT synthesis enzymes ASMT and TDC. In accordance with these results, the transcriptome data showed the relative expression of *CsCCD4*, *CsNCED3*, *CsNCED2*, and *CsPYL4* are involved in ABA synthesis, and the signal was higher in MT treatment than those in H_2O treatment. These data indicated that ABA may act as a downstream signal of MT in cucumber.

Photosynthesis is one of the physiological processes in plants that is most sensitive to adversity, and low-temperature stress affects light energy use and photosynthetic efficiency mainly by disrupting the electron transport chain in chloroplast and mitochondrion, leading to the accumulation of reactive oxygen species (ROS), which inhibits plant growth and development[3]. It is widely acknowledged that reactive oxygen species (ROS) encompass a diverse range of free and non-free radicals of oxygen origin, including superoxide radical (O_2^{--}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH·) and singlet oxygen ($^1O_2$). Each type of ROS exhibits unique and distinct chemical properties. For instance, hydrogen peroxide (H_2O_2) is relatively stable, with a half-life exceeding one millisecond, and is regarded as the predominant ROS involved in cellular signaling. Singlet oxygen ($^1O_2$) can oxidize lipids, proteins and guanidine residues of DNA, while superoxide (O_2^{--}) reacts with Fe-S proteins[59,60]. In this paper, similar results indicating that lower EL, MDA, and H_2O_2, and O_2^{--} contents as well as chlorophyll content and
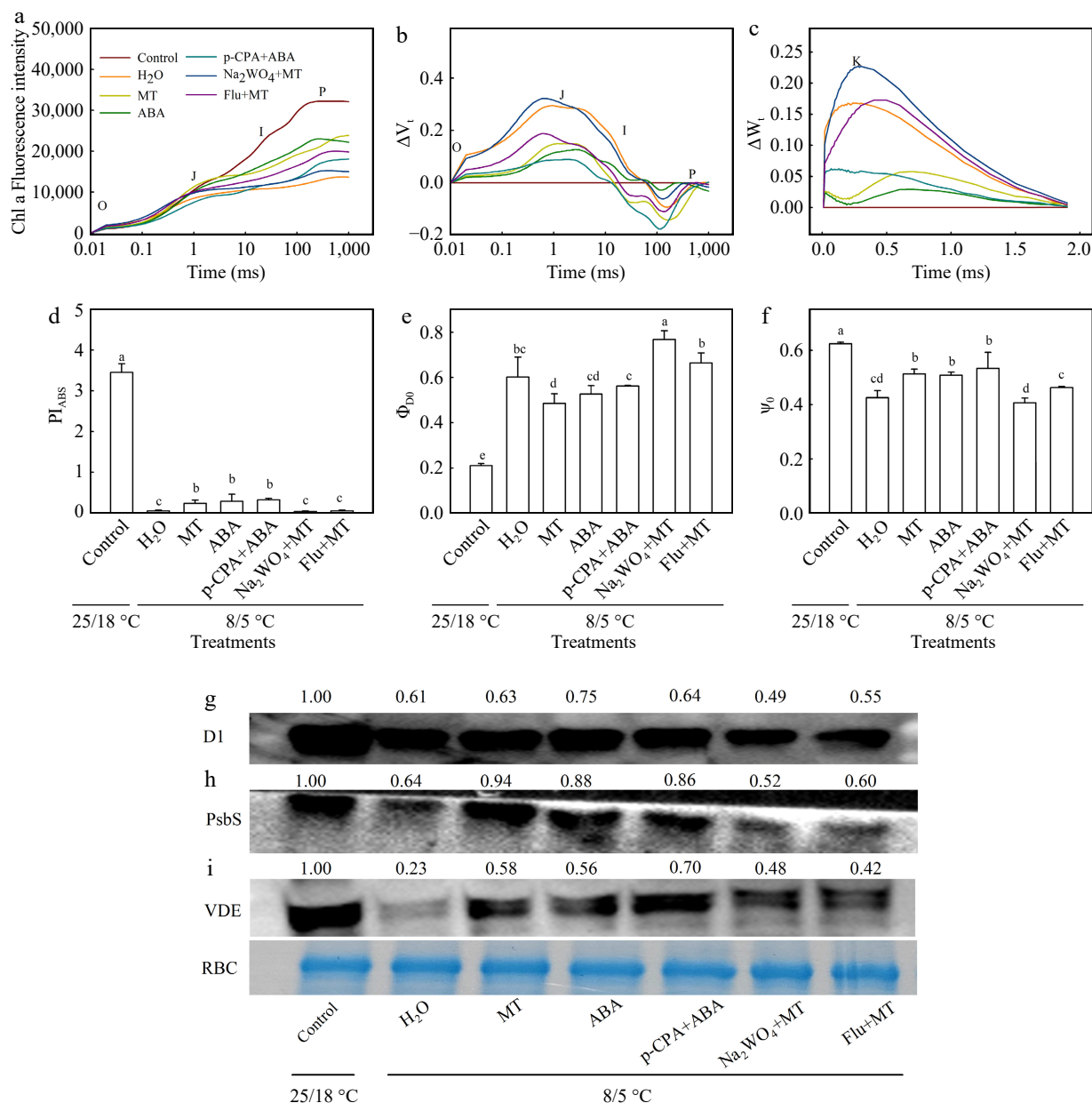
Fig. 11 Effects of MT and ABA on chlorophyll a fluorescence kinetics of the OJIP curve and PSII -related protein level of cucumber under chilling stress. (a) OJIP curve; (b)ΔV_t; (c) ΔW_t; (d) PI_{ABS} (e) Φ_{D0}; (f) ψ_0; (g) D1 protein; (h) PsbS protein; (i) VDE protein. Rubisco (RBC) protein is shown as an equal loading control by Coomassie Brilliant Blue staining. The value above each line is the relative enrichment of the protein. Western blotting was performed three times with three independent biological samples and similar results were obtained. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na$_2$WO$_4$+75 μM MT, 50 μM Flu+75 μM MT and H$_2$O. After 24 h, seedlings were exposed to 8/5 °C for 24 h. The half of H$_2$O pretreated seedlings under normal temperature (25/18 °C) were as the control. All values shown are means ± SD (n = 3). Different letters indicate that the mean values are significantly different among the samples ($p < 0.05$).

higher Pn were measured in MT- and ABA-pretreated seedlings than those in H$_2$O-pretreated seedlings were obtained. However, the inhibition of ABA with Na$_2$WO$_4$ and Flu blocked the positive effect induced by MT, while the inhibition of MT with p-CPA could not affect the ABA-induced positive effect under chilling stress, implying ABA participated in the regulation of chilling tolerance induced by MT. Many factors contribute to electron leakage and lipid peroxidation in plant cells under abiotic stress conditions. Studies have demonstrated that the production of excess reactive nitrogen species (RNS) by abiotic

stress can also be toxic to plants[61]. In the present study, the sole focus was on measuring the H$_2$O$_2$ and O$_2{}^-$ content, which explains the reason why the MDA content and EL had no significant difference, while H$_2$O$_2$ and O$_2{}^-$ contents were significantly declined in Na$_2$WO$_4$+MT and Flu+MT treatments, compared to H$_2$O treatment under cold condition. Moreover, chilling stress resulted in the decline of the activities of photosynthetic enzymes and photoinhibition, which was one important reason for the ROS accumulation and the decrease of Pn[5,62]. Here, we found MT and ABA promoted the activities of

Rubisco and RCA *via* upregulating the expression of *CsrbcL* and *CsRCA* as well as alleviating the degradation of RbcL and RCA protein under chilling stress. Meanwhile, the higher Fv/Fm and ΦPSII were observed in MT and ABA treatments. These results indicated that MT and ABA promoted the photosynthesis of cucumber seedlings under chilling stress *via* increasing the activities of photosynthetic enzymes and decreasing photoinhibition, which explained the lower ROS contents in seedlings pretreated with MT and ABA. Furthermore, the higher activities and expression of *CsSOD*, *CsPOD* and *CsAPX* as well as the AsA content was another important reason for lower ROS contents in MT and ABA treatments. But unfortunately, Na_2WO_4, and Flu also significantly repressed the increase of photosynthesis and antioxidant ability induced by MT. In addition to improve survival, plants have developed a range of adaptation processes, including thermal energy removal by NPQ[63], light repair by PSII[64], and transcriptional regulation of photosynthesis proteins[65]. PSII subunit S (PsbS) is a ubiquitous pigment-binding protein associated with photosystem II (PSII) of higher plants, which is involved in non-photochemical quenching. VDE is a violaxanthin-deepoxidase involved in the xanthophyll cycle and plants with mutations in either violaxanthin-deepoxidase (npq1) or PsbS protein (npq4) show reduced NPQ capacity[66]. Lou et al.[67] found that transgenic plants overexpressing *PeVDE* with higher NPQ capacity increased photoprotection under high light stress. Here, we found that the qP and NPQ and PsbS, VDE protein levels of MT and ABA treatments were significantly higher than those of other treatments under cold stress, indicating that MT and ABA can protect the cucumber photosynthetic apparatus by promoting thermal dissipation of excess excitation energy. Generally, D1 protein is used to reflect the PSII photoinhibition under abiotic stress[68]. The J point (2 ms) in the OJIP curve reflects a specific indicator of limited electron transfer from QA to QB and damage to D1 protein. The K point in the OJIP curve has often been used as a reflection of the degree of damage to the oxygen-excreting complex (OEC) on the donor side of PSII[69]. When both the donor and recipient sides are damaged simultaneously, the K point is elevated if the donor side of PSII is damaged more than the recipient side, and vice versa, the change in K point is insignificant[4]. The present data showed that chilling stress caused the increase of J point and the degradation of D1. Moreover, we also found higher K point, ΦD_0 and lower ψ_0 after chilling stress. In addition, MT and ABA pretreatments displayed reduced J point, K point, ΦD_0 and enhanced D1 protein level and ψ_0, implying MT and ABA maintained the photosynthetic electron transport under chilling stress. However, the ABA inhibition blocked the MT-enhanced photosynthetic electron transport, whereas, p-CPA pretreatment could not reverse the positive effect of ABA under chilling stress. These data showed that ABA may be a downstream signal of MT in the regulation of PSII photoinhibition induced by chilling stress. As the major regulatory module of cold acclimation, the inducer of CBF expression 1 (ICE1)–c-repeat binding factor (CBF)–cold-responsive gene (COR) significantly contributes to the cold stress signaling response pathway[70,71]. Previous studies have demonstrated that the ICE1-CBF-COR transcriptional cascade plays a role in the cold tolerance of cucumber seedlings induced by MT and ABA[18,34]. Therefore, we postulate that the ICE1-CBF-COR pathway may also represent an important mechanism underlying the involvement of ABA in MT-induced cold tolerance in cucumber. However, the specific molecular mechanisms remain to be elucidated.

Conclusions

In conclusion, the present results demonstrated that MT positively increases the chilling tolerance of cucumber seedlings. This is achieved by promoting the antioxidant ability to eliminate ROS accumulation, enhancing photoprotection and maintaining the photosynthetic electron transport, which alleviates the decline of photosynthesis. Furthermore, ABA, which is the downstream signal of MT, participates in the regulation of MT on chilling tolerance in cucumber seedlings. The present findings enrich the signal transduction pathway of MT in cucumber under chilling stress.

Author contributions

The authors confirm contributions to the paper as follows: study conception and design: Ai X; performed the experiments: Feng Y, Liu C; methodology and data analysis: Gong B; draft review and editing: Bi H. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

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Dates

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