Vegetable Research

ARTICLE

Open Access

https://doi.org/10.48130/vegres-0024-0024

Vegetable Research **2024**, in press

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

Yiqing Feng, Chaoyue Liu, Biao Gong, Xizhen Ai and Huangai Bi*

Key Laboratory of Crop Biology and Genetic Improvement of Horticultural Crops in Huanghuai Region; College of Horticulture Science and Engineering, Shandong Agricultural University, Tai'an, Shandong, 271018 China

* Corresponding author, E-mail: bhg163@163.com

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, the 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 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.

Citation: Feng Y, Liu C, Gong B, Ai X, Bi H. 2024. Abscisic acid participates in melatonin-induced chilling tolerance of cucumber via regulating photosynthesis and antioxidant system. *Vegetable Research* https://doi.org/10.48130/vegres-0024-0024

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 live in warm climate, like cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), chilling stress often occurred during winter and spring cultivation in solar greenhouse in Northern China, which mainly affected photosynthetic capacity and resulted 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 the significant decline of the yield and quality of vegetable cultivators[3–5]. Thus, finding the efficient pathways to alleviate the damage caused by chilling stress is of 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 phytohormone, Melatonin (MT) and Abscisic acid (ABA) are widely reported that help promote the abiotic tolerance of plants[6,7]. As we 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, a number of 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 important, other plant hormones, such as auxin (IAA), cytokinins (CK), gibberellins (GA), ABA were 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]. 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 of 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 process 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, we conducted this experiment 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

The 'Jinyou 35'cucumber (*Cucumis sativus* L.) was used in this experiment and the cultivation of cucumber seedlings according to the method described in our 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 °C /18 °C day/night average temperature under a 12 h photoperiod.

At the two-leaf stage, 75 μM MT, 100 μM p-chlorophenylalanine (p-CPA, biosynthesis inhibitor of MT synthesis), 100 μM ABA and 3 mM Na_2WO_4 (biosynthesis inhibitor of ABA synthesis) or deionised 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 °C /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 24h, 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 (12h photoperiod with PFD of 100 μmol·m^{-2}·s^{-1}, 8 °C /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 a NCED activity kit (DG95960Q, Beijing winter song Boye Biotechnology Co., Ltd., Beijing, China).

The 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 in Gong et al.[40] and dry matter was measured by weighing.

The semi-lethal temperature under chilling stress was determined as described in Zhu et al.[41] with minor change. Leaf discs was obtained from cucumber seedlings of different treatments and then transferred to 0, −2, −4, −6, and −7 °C, respectively, for 30 min. Afterwards, adding 20 ml 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, 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) were described previously[41]. 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 ($O_2^{\cdot-}$) were measured as described by Li et al.[43]. The histochemical detection of H_2O_2 and $O_2^{\cdot-}$ was carried out according to the method in our previous studies[44].

Superoxide dismutase (SOD) activity was measured with the method of Stewart and 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 and Asada[47].

Glutathione and ascorbic acid content assays

We measured the contents of reduced glutathione (GSH) and oxidized glutathione (GSSG) 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 measured using a PP-Systems Ciras-3 photosynthesizer from the USA. The the related parameter during the measurement was set according to Feng et al.[37].

The determination of chlorophyll a, chlorophyll b and carotenoid of cucumber seedlings with 95% ethanol as described in Sartory and 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 minutes and steady-state (Fs), initial (F_0), maximum (F_m') and variable (F_v') fluorescence at 600 μmol·m^{-2}·s^{-1}. Following Demming-Adams and 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 analyse and calculate the OJIP curves: Performance index based on absorbed light energy PI_{ABS} = RC/ABS · [Φ_{P0}/(1 – Φ_{P0})] · [ψ_0 · (1 – ψ_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).

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 Supplementary 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 colour 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

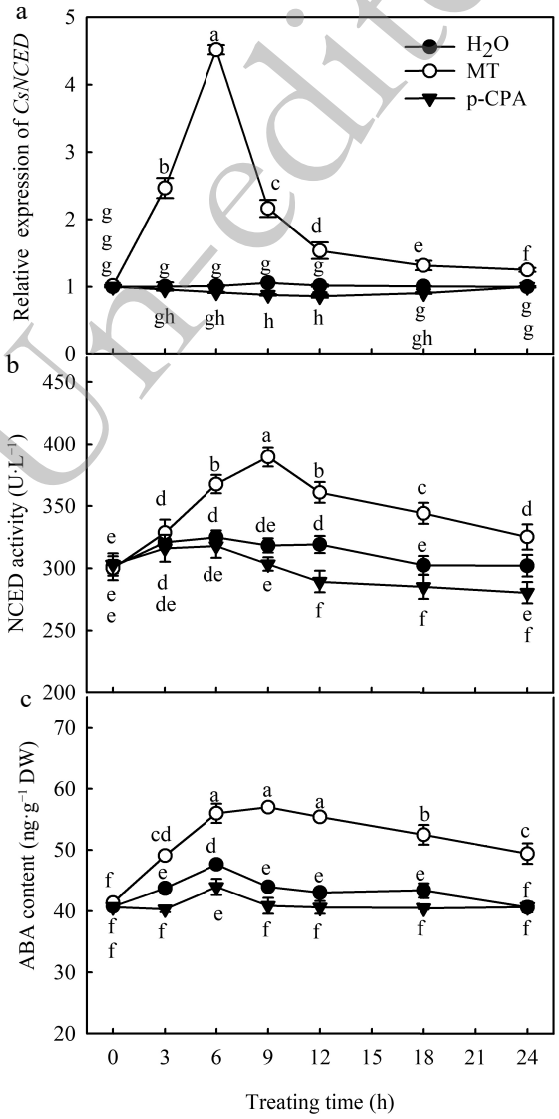

Fig. 1 Effect of MT on ABA content, activity and relative mRNA expression of *CsNCED* in cucumber seedlings under normal conditions (25 °C /18 °C). Cucumber seedlings grown in a solar greenhouse were foliar sprayed with 75 μM MT, 100 μM p-chlorophenylalanine (p-CPA, an inhibitor of melatonin synthesis) 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 ($p < 0.05$).

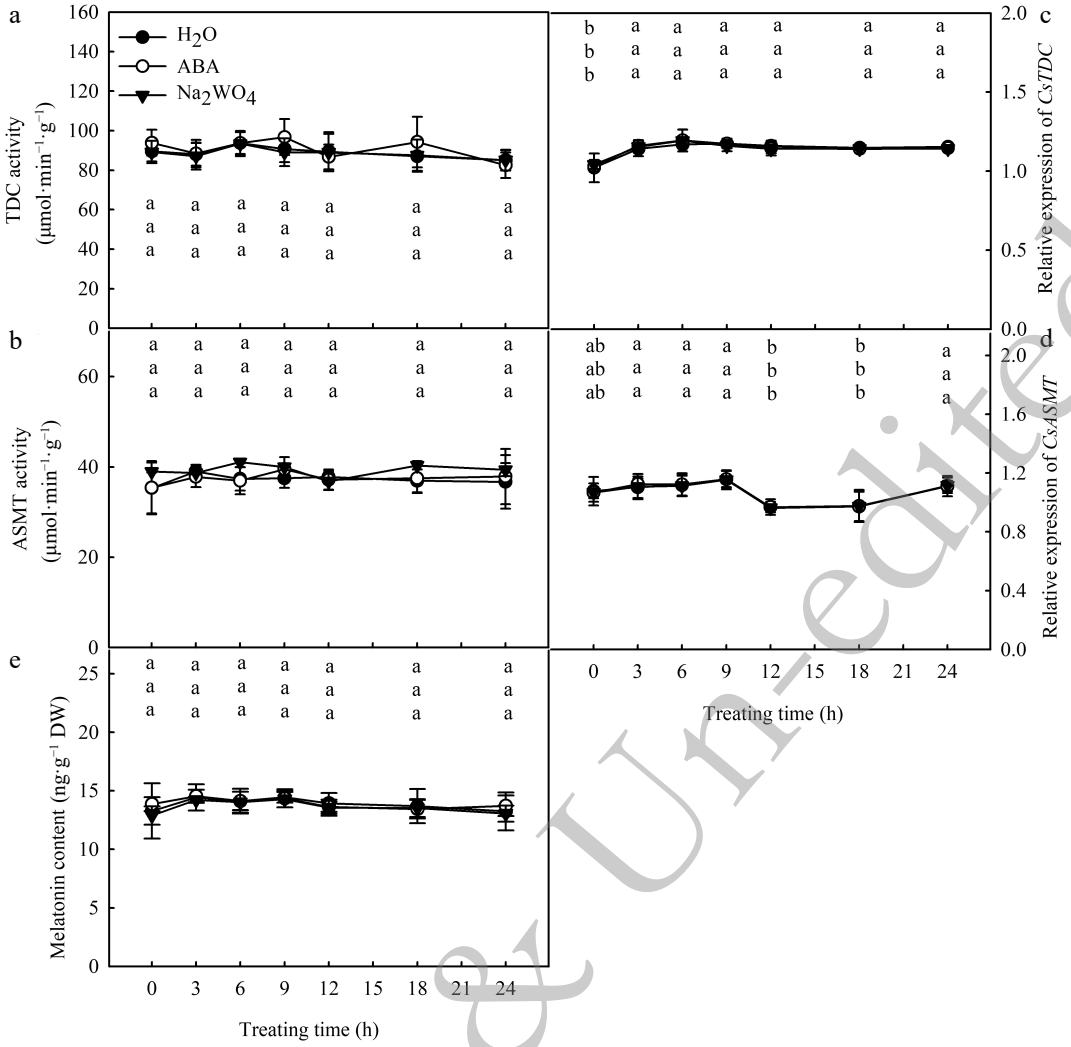

Fig. 2 Effects of ABA on MT content and activities and RNA expression of key MT biosynthesis enzymes in cucumber seedlings under normal conditions (25 °C /18 °C). (a, b) TDC and ASMT activities, respectively; (c, d) *TDC* and *ASMT* mRNA expression levels; (e) MT content. Cucumber seedlings grown in 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 ($p < 0.05$).

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

The interaction of ABA and MT of cucumber seedlings under normal conditions

Our transcriptome data analysis showed that the application of MT promoted the relative expression of ABA-related genes under chilling stress (Supplemental Fig.S1), thus we speculated that an interaction between MT and ABA might exist during the response to chilling stress. Subsequently, we carried an experiment 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, we also tested the response of MT to exogenous ABA under normal temperature and we found 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 Na_2WO_4 (biosynthesis inhibitor of ABA)-treatment under normal condition, 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, we measured the response of cucumber pre-treated with ABA and MT to chilling stress and found both of them increased the chilling tolerance of cucumber in terms

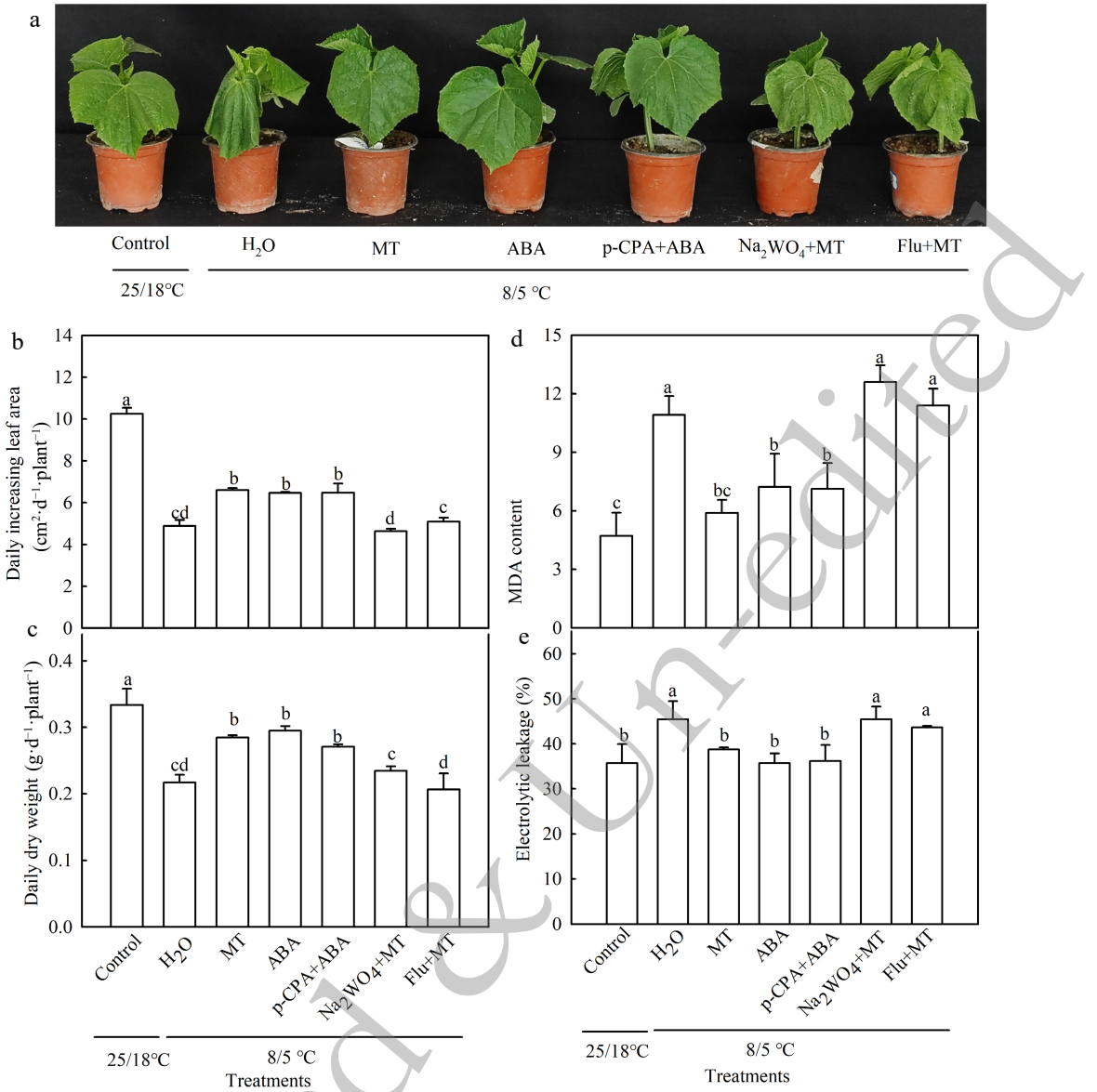

Fig. 3 Effects of MT and ABA on chilling tolerance of cucumber seedlings under low temperature stress. (a) phenotype; (b) daily increasing leaf area; (c) daily dry weight; (d) MDA content and (e) electrolyte leakage of cucumber seedlings under chilling stress. At two-leaf stage, cucumber seedlings were 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 °C /5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

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 the regulation of MT on chilling tolerance of cucumber, we also tested the changes of these above chilling parameters 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. Take MDA content as an example, compare to normal condition, 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 °C /5 °C for 48 h. As shown in Fig. 4a, b, chilling stress caused obvious increases of H_2O_2 and $O_2^{\cdot-}$ contents, however, the cucumber seedlings treated by MT and ABA showed lower H_2O_2 and $O_2^{\cdot-}$ contents than the seedlings treated by H_2O. The histochemical observations with inverted fluorescence micro-

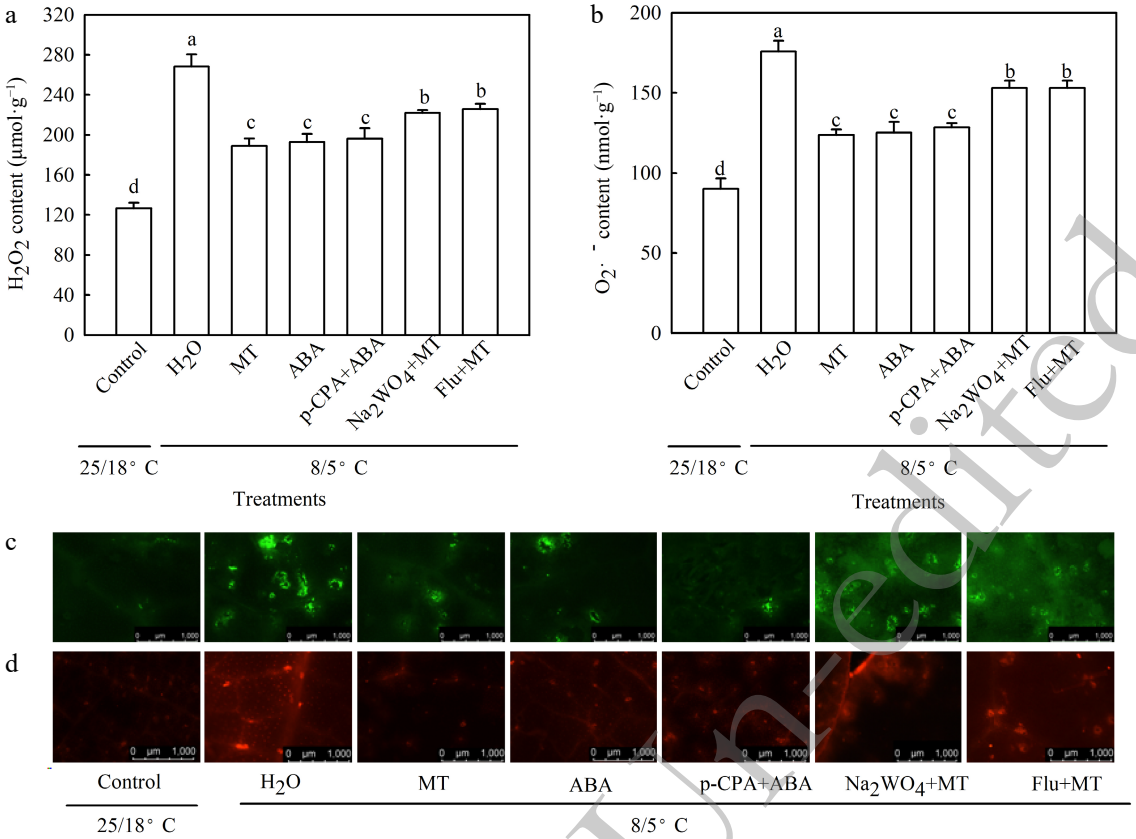

Fig. 4 Effects of different treatments on ROS content in cucumber seedlings under chilling stress. (a) H_2O_2 content; (b) $O_2^{\cdot-}$ content; (c, d) H_2O_2 and $O_2^{\cdot-}$ inverted fuorescence microscope imaging. Bars = 1000 μm. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 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 °C /5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

scope also supported the results of H_2O_2 and $O_2^{\cdot-}$ contents assay (Fig. 4c, d). ROS-scavenging capacity is one of the important mechanisms of relieving abiotic-induced oxidative damage. Here, we 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 24h 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, c & 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, we measured the variation of Rubisco and RCA of cucumber seedlings in different treatments after chilling stress and 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 level 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

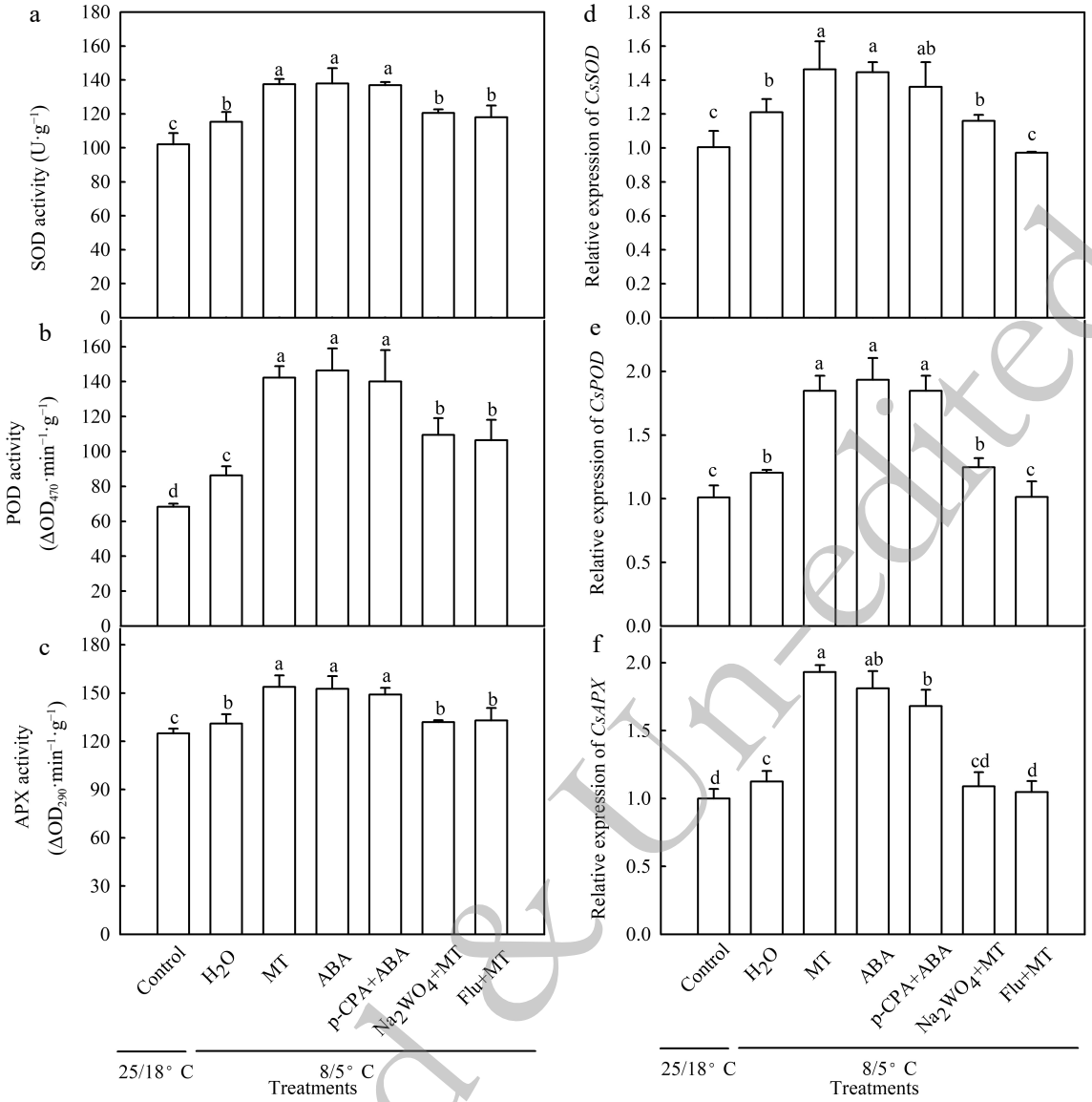

Fig. 5 Interactive effects of MT and ABA on activities and relative mRNA expression of antioxidant enzymes in cucumber seedlings under chilling stress. (a–c) Activities of superoxide dismutase (SOD), peroxidase (POD), ascorbate and peroxidase (APX), respectively. (d–f) Relative mRNA expression of *SOD*, *POD*, *APX*, respectively. 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 Na2WO4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8 °C /5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

significant (Fig. 9).

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

In order investigate whether the regulation of cucumber photosynthesis under chilling stress by MT and ABA is associated with photosynthetic electron transport, we compared the degree of photoinhibition of PSII in seedlings of different treatments after chilling stress. The results showed that Fv/Fm, *Φ*PSII, qP and NPQ of cucumber seedling leaves significantly decreased under low-temperature stress (Fig. 10a–d), but Fv/Fm, *Φ*PSII, 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 in this paper. As shown in Fig. 11, low temperature stress induced an increase in F_0 (O)

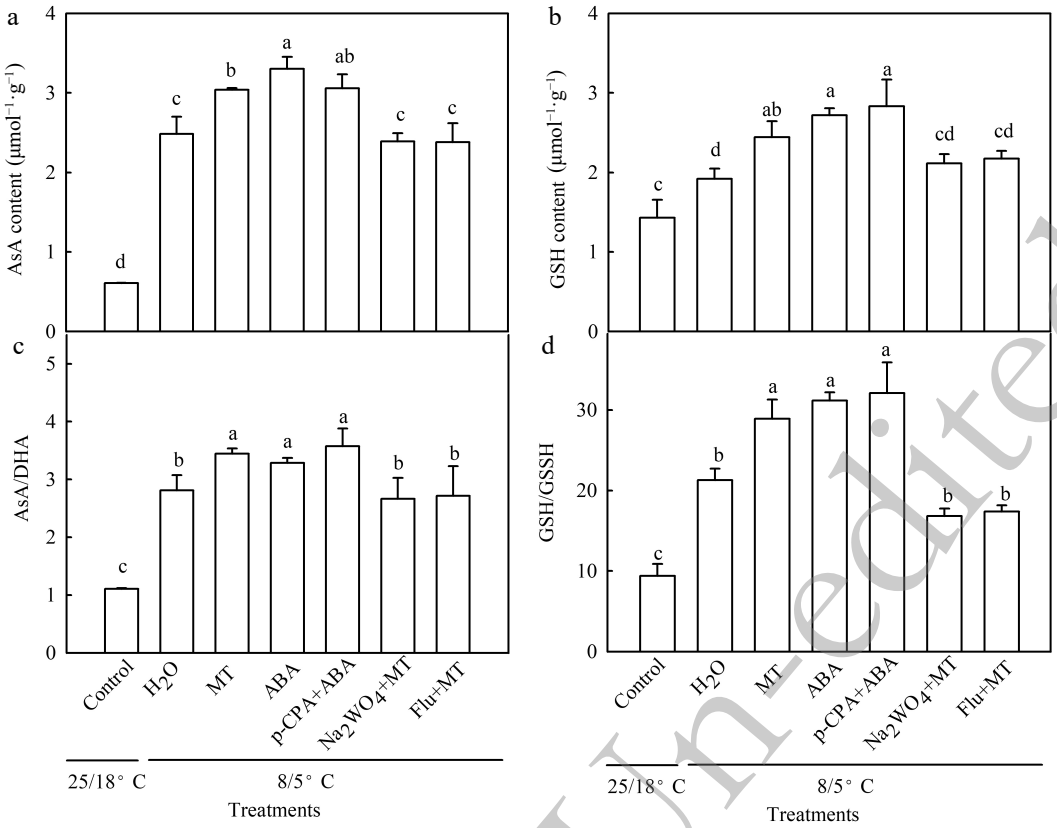

Fig. 6 Effects of different treatments on the contents of antioxidant substances in cucumber seedlings under chilling stress. (a) AsA content, (b) GSH content, (c) the ratio of AsA and DHA, (d) the ratio of GSH and GSSG. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM 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 °C /5 °C for 48 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /18 °C) were as the control. All values shown are mean ± SD (n = 3). Different letters indicate significant differences among treatments ($p < 0.05$).

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 significant smaller in MT, ABA and p-CPA+ABA treatments than in H_2O treatment. The J-point (2ms) 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, and 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. Similar, we also found 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 the 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 was blocked by 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.

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 human in plants notably increased the endogenous MT content and promoted the chilling tolerance. Subsequently, a number of studies proved that MT was involved in the control of various physiological and molecular responses for inducing cold tolerance[54]. ABA is also a central regulator of plant defense against chilling stress[55]. Several studies have reported interactions existed between ABA and melatonin in the response of plants to abiotic stresses[56–57]. For instance, Li et al.[58] found that the application of MT promoted the seed germination under salt stress via down-regulating the ABA synthesis gene *NCED* and upregulating 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[59]. In contrast, MT stimulated the accumulation of ABA to enhance the antioxidant ability and then promoted the salt tolerance of Fragaria ananassa Duch[60]. In this study, 75 μM MT and 100 μM ABA

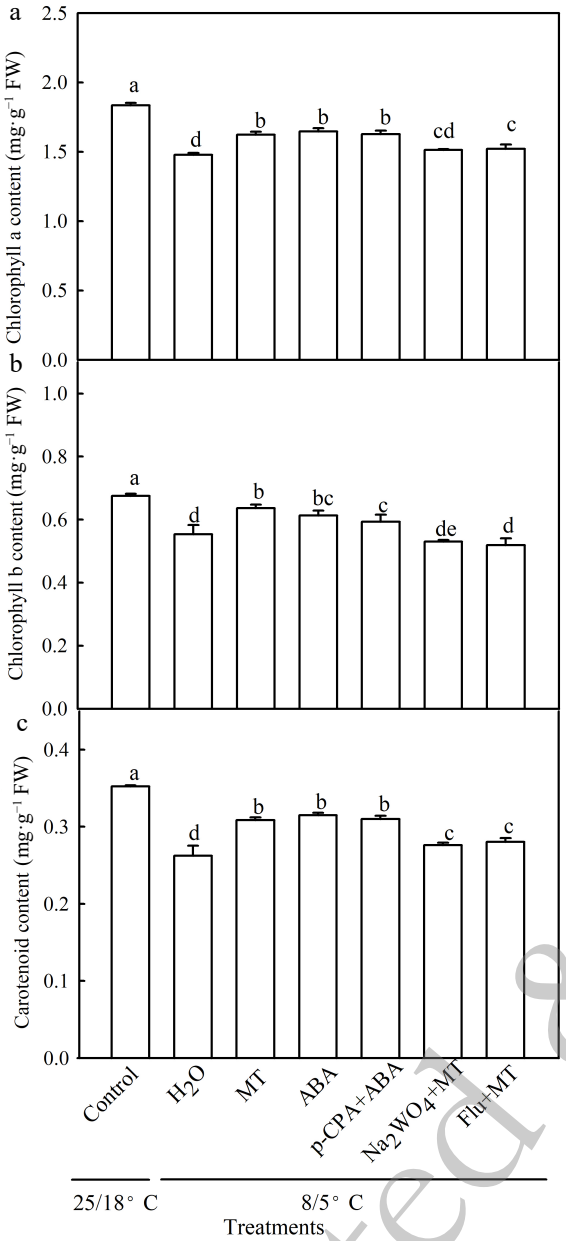

Fig. 7 Interaction between MT and ABA on the chlorophyll content of cucumber seedlings exposed to chilling stress. (a) Content of chlorophyll a; (b) Content of chlorophyll b; (c) Content of carotenoids. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100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

both increased the chilling tolerance of cucumber, evidenced by the lower EL, MDA and semi-lethal temperature. Moreover, we 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 to these results, the transcriptome data showed the relative expression of *CsCCD4*, *CsNCED3*, *CsNCED2*, *CsPYL4* involved in ABA synthesis and 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[61]. 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 ($O_2\cdot^-$), 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 signalling. Singlet oxygen ($^1O_2$) has the capacity to oxidise lipids, proteins and guanidine residues of DNA, while superoxide ($O_2\cdot^-$) reacts with Fe-S proteins[62–63]. In this paper, we obtained the similar results that lower EL, MDA and H_2O_2 and $O_2\cdot^-$ contents as well as chlorophyll content and higher Pn were measured in MT- and ABA-pretreated seedlings than those in H_2O-pretreated seedlings. However, the inhibition of ABA with Na_2WO_4 and Flu obviously blocked the positive effect induced by MT, while the inhibition of MT with p-CPA could not affect 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[64]. In the present study, we have solely focused on measuring the H_2O_2 and $O_2\cdot^-$ content. which have explained the reason why the MDA content and EL had no significant difference, while H_2O_2 and $O_2\cdot^-$ contents were significant decline in Na_2WO_4+MT and Flu+MT treatments, compared to H_2O 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[5,65]. 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 ΦPS Ⅱ 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 well 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

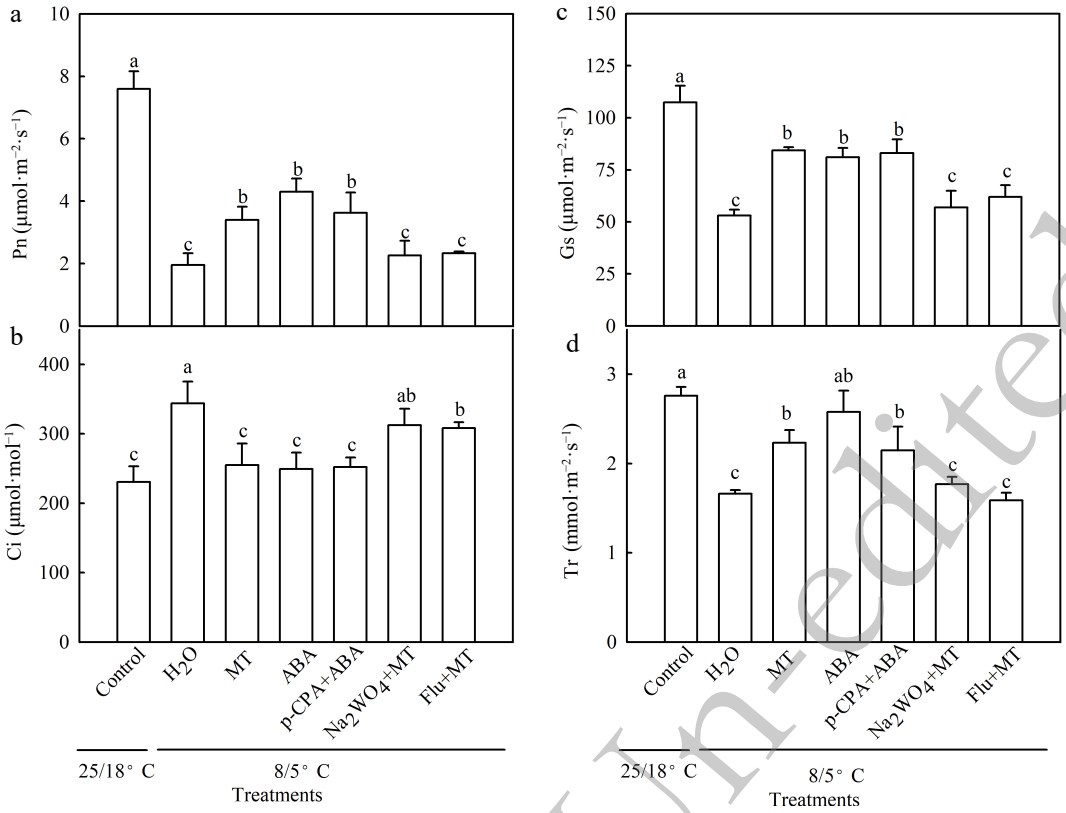

Fig. 8 Interaction between MT and ABA on gas exchange parameters in cucumber seedlings under chilling stress. (a) photosynthetic rate; (b) intercellular CO2 concentration; (c) stomatal conductance; and (d) transpiration rate. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 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 °C /5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /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 ($p < 0.05$).

adaptation processes, including thermal energy removal by NPQ[66], light repair by PSII[67], and transcriptional regulation of photosynthesis proteins[68]. 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[69]. Lou et al.[70] found that transgenic plants over-expressing *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[71]. 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[72]. 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]. Our 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[73,74]. 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, our results demonstrated that MT positively increases the chilling tolerance of cucumber seedlings. This is achieved by promoting the antioxidant ability to eliminate ROS

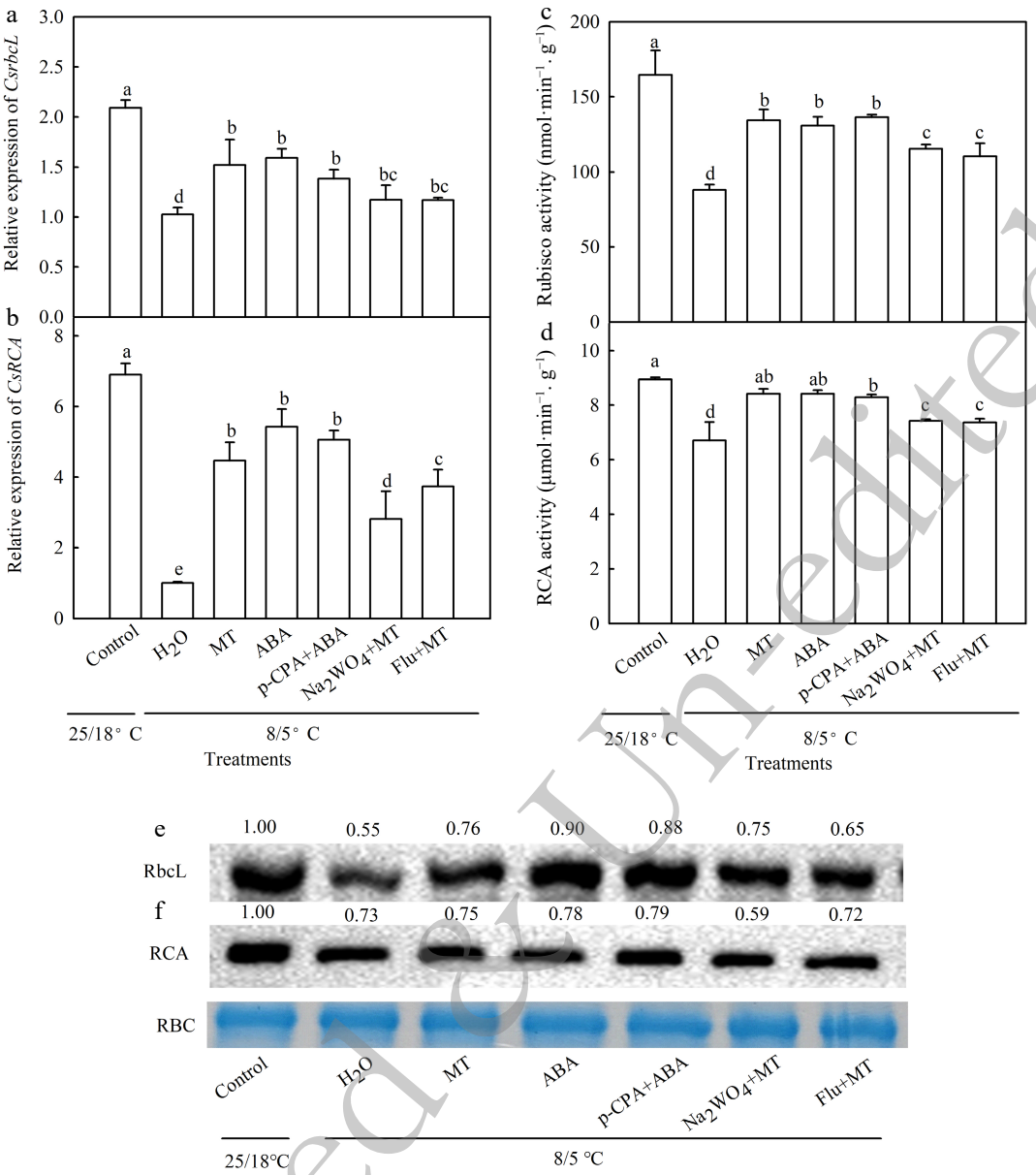

Fig. 9 Interactive effects of MT and ABA on the relative expression of *rbcL* and *RCA* in mRNA, and the activity of Rubisco and Rubisco activase (RCA) and protein levels in cucumber seedlings under chilling stress. (a, b) Relative mRNA expression for *rbcL* and *RCA*, (c) Rubisco activity, (d) RCA activity 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 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 °C /5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /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 ($p < 0.05$).

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. Our 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 XZ; performed the experiments: Feng YQ, Liu CY; methodology and data analysis: Gong B; draft review and editing: Bi HG. All authors reviewed the final version of this draft and granted approval for its publication.

Data availability

All data generated during this study are included within the article or its supplementary files.

Acknowledgments

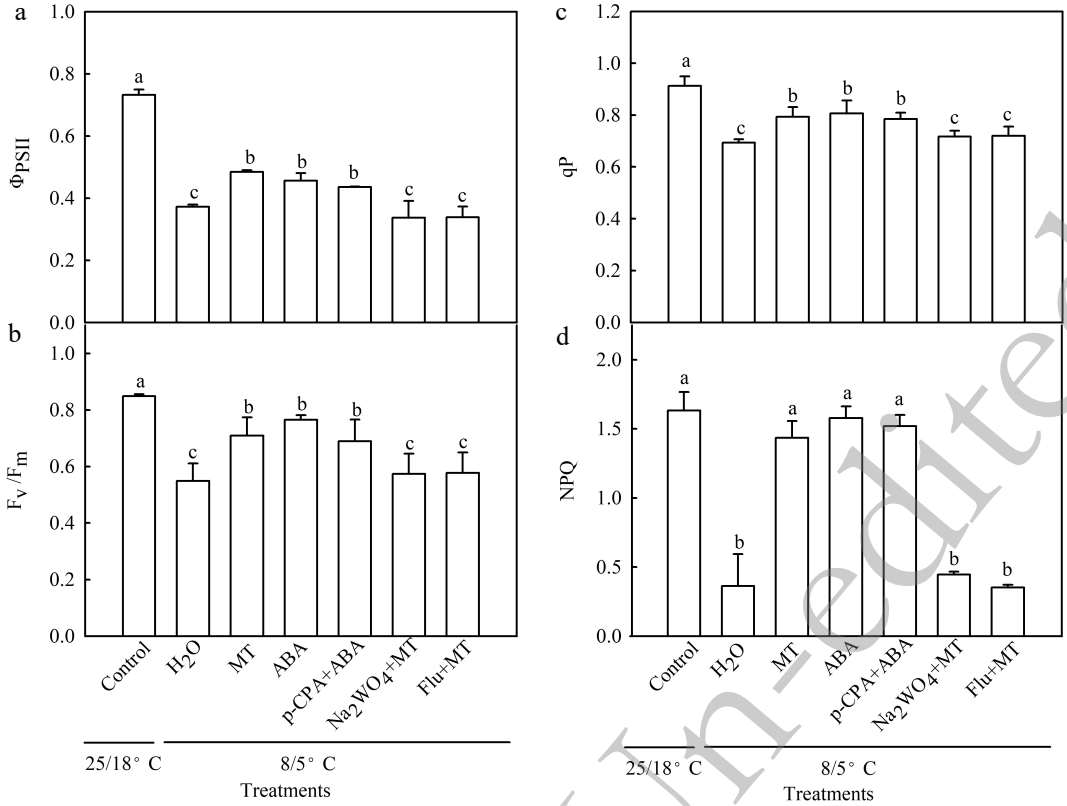

Fig. 10 Interactive effects of MT and ABA on photoprotection in chilling stressed cucumber seedlings. (a) *Φ*PSII; (b) Fv/Fm; (c) qP; (d) NPQ. At two-leaf stage, cucumber seedlings were foliar sprayed with 75 μM MT, 100 μM ABA, 100 μM p-CPA+100 μM ABA, 3 mM Na2WO4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8 °C /5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /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 ($p < 0.05$).

This work was supported by the National Key Research and Development Program of China (2018YFD1000800); the National Science Foundation of Shandong province (ZR2023MC183); the Special Fund of Modern Agriculture Industrial Technology System of Shandong Province in China (SDAIT-05-10).

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (XXXXXX)

Dates

Received 10 May 2024; Accepted 11 June 2024; In press 23 June 2024

References

1. Allen DJ, Ort DR. 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends in Plant Science* 6(1):36–42
2. Janská A, Marsík P, Zelenková S, Ovesná J. 2010. Cold stress and acclimation - what is important for metabolic adjustment? *Plant Biology* 12(3): 395–405
3. Fan JB, Hu ZR, Xie Y, Chan ZL, Chen K, et al. 2015. Alleviation of cold damage to photosystem II and metabolisms by melatonin in Bermudagrass. *Frontiers in plant science* 6:925
4. Bi HG, Li FD, Dong XB, Ai XZ. 2017. Effects of transketolase gene silencing on photosynthesis in cucumber seedlings under high temperature stress. *Plant Physiology Journal* 53(10):1859–66
5. Bi HG, Li FD, Wang HG, Ai XZ. 2019. Overexpression of transketolase gene promotes chilling tolerance by increasing the activities of photosynthetic enzymes, alleviating oxidative damage and stabilizing cell structure in *Cucumis sativus* L. *Physiologia plantarum* 167(4):502–15
6. Ahmad I, Song X, Hussein Ibrahim ME, Jamal Y, Younas MU, et al. 2023. The role of melatonin in plant growth and metabolism, and its interplay with nitric oxide and auxin in plants under different types of abiotic stress. *Frontiers in plant science* 14:1108507
7. Singh A, Roychoudhury A. 2023. Abscisic acid in plants under abiotic stress: crosstalk with major phytohormones. *Plant Cell Reports* 42(6):961–74
8. Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. 1958. Isolation of melatonin, the pineal gland factor that lightens melanocytes. *Journal of the American Chemical Society* 80(10):2587
9. Hattori A, Migitaka H, Iigo M, Itoh M, Yamamoto K, Ohtani-Kaneko R, Hara M, Suzuki T, Reiter RJ. 1995. Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. *Biochemistry and Molecular Biology International* 35(3):627–34
10. Balzer I. Hardeland R. 1996. 1996. *Melatonin in algae and higher plants: possible new roles as a phytohormone and antioxidant, Botanica Acta* 109(3):80–83
11. Simlat M, Ptak A, Skrzypek E, Warchoł M, Morańska E, et al. 2018.

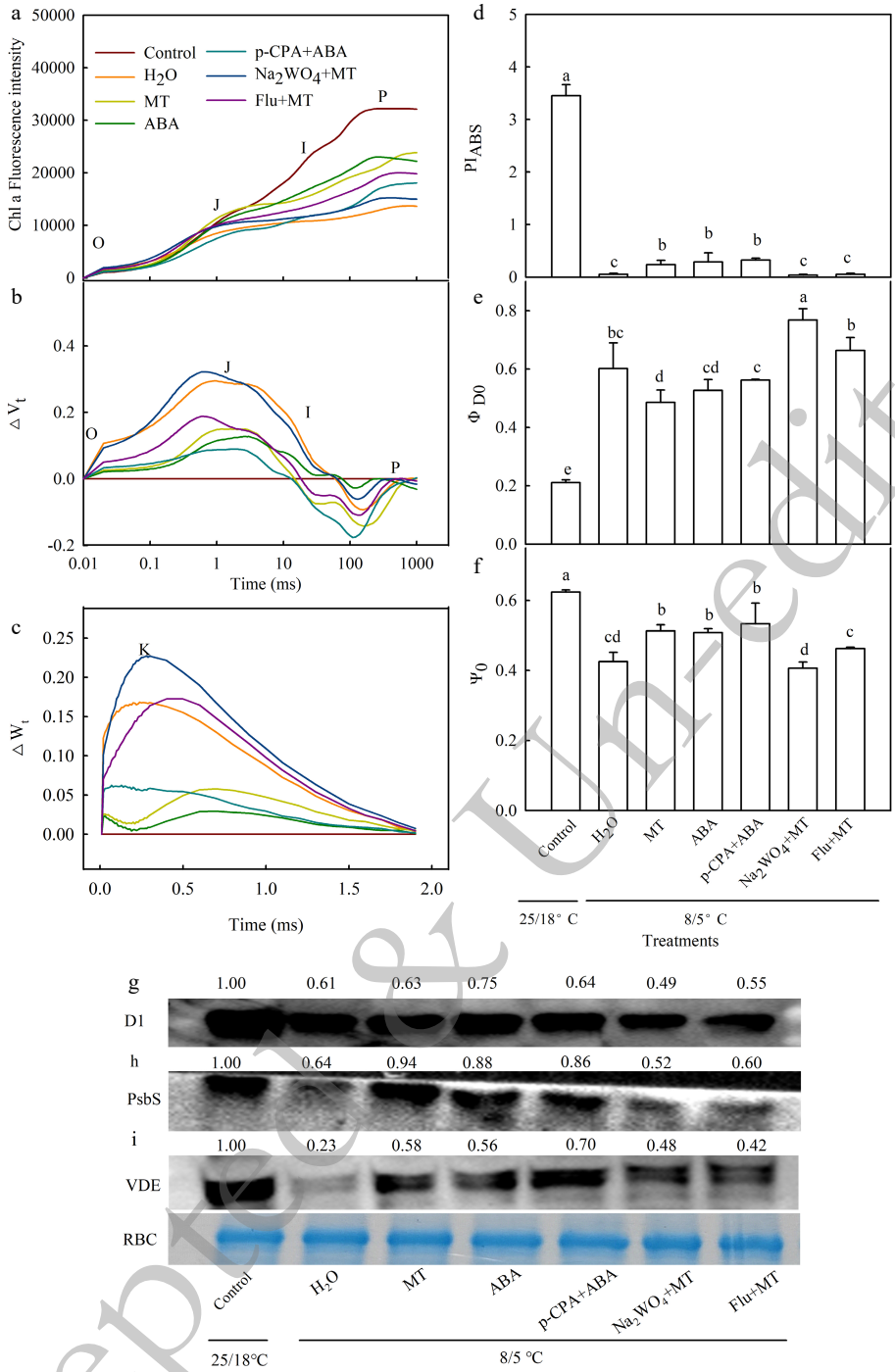

Fig. 11 Effects of MT and ABA on chlorophyll a fluorescence kinetics of the OJIP curve and PSII -related protein level of cucumber under chilling stress. (a) OJIP curve; (b) Vt; (c) ΔVt; (d) PIABS (e) ΦD0; (f) ψ0; (g) D1 protein; (h) PsbS protein; (i) VDE protein. Rubisco (RBC) protein is shown as an 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_2WO_4+75 μM MT, 50 μM Flu+75 μM MT and H_2O. After 24 h, seedlings were exposed to 8 °C /5 °C for 24 h. The half of H_2O pretreated seedlings under normal temperature (25 °C /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 ($p < 0.05$).

Melatonin significantly influences seed germination and seedling growth of Stevia rebaudiana Bertoni. *PeerJ* 6:e5009

12. Mao J, Niu C, Li K, Chen S, Tahir MM, Han M, Zhang D. Melatonin promotes adventitious root formation in apple by promoting the function of *MdWOX11*. *BMC Plant Biology* 20(1): 536
13. Liu K, Jing TT, Wang YN, Ai XZ, Bi HG. 2022. Melatonin delays leaf senescence and improves cucumber yield by modulating chlorophyll degradation and photoinhibition of PSII and PSI. *Environmental and Experimental Botany* 200:104915
14. Arnao, M. B., Hernández-Ruiz, J. 2020. Melatonin in flowering, fruit

set and fruit ripening. *Plant Reproduction* 33(2):77–87
15. Shi HT, Jiang C, Ye TT, Tan DX, Reiter RJ, et al. 2015. Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L). *Pers.] by exogenous melatonin. Journal of Experimental Botany* 66(3):681–94
16. Shi HT, Chen YH, Tan DX, Reiter RJ, Chan ZL, et al. 2015. Melatonin induces nitric oxide and the potential mechanisms relate to innate immunity against bacterial pathogen infection in *Arabidopsis*. *Journal of Pineal Research* 59(1):102–8
17. Nawaz MA, Jiao YY, Chen C, Shireen F, Zheng ZH, et al. 2018. Melatonin pretreatment improves vanadium stress tolerance of watermelon seedlings by reducing vanadium concentration in the leaves and regulating melatonin biosynthesis and antioxidant-related gene expression. *Journal of Plant Physiology* 220:115–27
18. Zhang XW, Feng YQ, Jing TT, Liu XT, Ai XZ, et al. 2021. Melatonin Promotes the Chilling Tolerance of Cucumber Seedlings by Regulating Antioxidant System and Relieving Photoinhibition. *Frontiers in plant science* 12:789617
19. Xu CX, Zhang XY, Liu CY, Liu K, Bi HG, et al. 2022. Alleviating effect of exogenous melatonin and calcium on the peroxidation damages of cucumber under high temperature stress. *Chinese Journal of Applied Ecology* 33(10):2725–35
20. Sharma A, Wang JF, Xu DB, Tao SC, Chong, SL, et al. 2020. Melatonin regulates the functional components of photosynthesis, antioxidant system, gene expression, and metabolic pathways to induce drought resistance in grafted *Carya cathayensis* plants. *The Science of Total Environment* 713:136675
21. Zhang TG, Shi ZF, Zhang XH, Zheng S, Wang J, et al. 2020. Alleviating effects of exogenous melatonin on salt stress in cucumber. *Scientia Horticulturae* 262:109070
22. Liu C, Kang H, Wang YF, Yao YX, Gao Z, et al. 2021. Melatonin relieves ozone stress in grape leaves by inhibiting ethylene biosynthesis. *Frontiers in plant science* 12:702874
23. Han QH, Huang B, Ding CB, Zhang ZW, ChenYE, et al. 2017. Effects of melatonin on anti-oxidative systems and photosystem II in cold-stressed rice seedlings. *Frontiers in plant science* 8:785
24. Korkmaz, A, Karaca, A, Kocacinar F, Cuci, Y. 2017. The effects of seed treatment with melatonin on germination and emergence performance of pepper seeds under chilling stress. *The Journal of Agricultural Science* 23:167–76
25. Qari SH, Hassan MU, Chattha MU, Mahmood A, Naqve M, et al. 2022. Melatonin Induced Cold Tolerance in Plants: Physiological and Molecular Responses. *Frontiers in plant science* 13:843071
26. Zhang ZX, Zhang Y. 2021. Melatonin in plants: what we know and what we don't. *Food Quality and Safety* 5(3):213–21
27. Nambara E, Marion-Poll A. 2005. Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* 56:165–85
28. Huang XZ, Hou LY, Meng JJ, You HW, Li Z, et al. 2018. The antagonistic action of abscisic acid and cytokinin signaling mediates drought stress response in *Arabidopsis*. *Molecular Plant* 11:970–82
29. Abu-Ghosh S, Iluz D, Dubinsky Z, Miller G. 2021. Exogenous Abscisic Acid Confers Salinity Tolerance in *Chlamydomonas reinhardtii* During Its Life Cycle. *Journal of Phycology* 57(4):1323–34
30. Zhang YY, Fu X, Feng YQ, Zhang XW, Bi HG, et al. 2022. Abscisic Acid Mediates Salicylic Acid Induced Chilling Tolerance of Grafted Cucumber by Activating H_2O_2 Biosynthesis and Accumulation. *International Journal of Molecular Sciences* 23(24):16057
31. Parida AK, Das AB. 2005. Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety* 60(3):324–49
32. Huang XB, Shi HY, Hu ZR, Liu A, Amombo E, et al. 2017. ABA Is Involved in Regulation of Cold Stress Response in Bermudagrass. *Frontiers in plant science* 8:1613
33. Daszkowska-Golec A, Collin A, Sitko K, Janiak A, Kalaji HM, Szarejko I. 2019. Genetic and Physiological Dissection of Photosynthesis in Barley Exposed to Drought Stress. *International Journal of Molecular Sciences* 20(24):6341
34. Lv CY, Li FD, Ai XZ, Bi HG. 2022. H_2O_2 participates in ABA regulation of grafting-induced chilling tolerance in cucumber. *Plant cell reports* 41(4):1115–30
35. Xu LL, Yue QY, Xiang GQ, Bian FE, Yao YX. 2018. Melatonin promotes ripening of grape berry via increasing the levels of ABA, H_2O_2, and particularly ethylene. *Horticulture Research* 5:41
36. Li C, Tan DX, Liang D, Chang C, Jia DF, et al. 2015. Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behaviour in two Malus species under drought stress. *Journal of Experimental Botany* 66(3):669–80
37. Feng YQ, Fu X, Han LJ, Xu C, Liu CY, et al. 2021. Nitric Oxide Functions as a Downstream Signal for Melatonin-Induced Cold Tolerance in Cucumber Seedlings. *Frontiers in plant science* 12:686545
38. Qiu SN. 2022. Mitigative Effects of Melatonin on Chilling Stress of Cucumber in Solar Greenhouse. Thesis. University of Shandong Agricultural, CN.
39. Bian FE, Xiao QH, Hao GM, Sun YJ, Lu WL, et al. 2018. Effect of root-applied melatonin on endogenous melatonin and chlorophyll fluorescence characteristics in grapevine under NaCl stress. *Scientia Agricultura Sinica* 51(5):952–63
40. Gong JH, Xiang J. 2001. Study on non-destructive rapid measurement method of cucumber leaf area. *Chinese Vegetables* 2001(4):7–9
41. Dong XB, Bi HG, Wu GX, Ai XZ. 2013. Drought-induced chilling tolerance in cucumber involves Membrane stabilisation improved by antioxidant system. *International Journal of Plant Production* 7:67–80
42. Zhu GH, Liu ZQ, Zhu PR. 1986. A study on Determination of lethal temperature with logistic function. *Journal of Nanjing Agricultural University* 3:11–16
43. Li ZG, Gong M. 2005. Improvement of measurement method for superoxide anion radical in plant. *Acta Bot Yunnanica* 27(2):211–16
44. Zhang XW, Liu FJ, Zhai J, Li FD, Bi HG, et al. 2020. Auxin acts as a downstream signaling molecule involved in hydrogen sulfide-induced chilling tolerance in cucumber. *Planta* 251:69
45. Stewart RR, Bewley JD. 1980. Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiology* 65(2):245–48
46. Omran R G. 1980. Peroxide levels and the activities of catalase, peroxidase, and indoleacetic acid oxidase during and after chilling cucumber seedlings. *Plant Physiology* 65(2):407–8
47. Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology* 22(5):867–80
48. Law MY, Charles SA, Halliwell B. 1983. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. *the effect of hydrogen peroxide and of Paraquat. The Biochemical Journal* 210(3):899–03
49. Sartory DP, Grobbelaar JU. 1984. Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. *Hydrobiologia* 114:177–87
50. Demming-Adams B, Adams III W W. 1992. Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Biology* 43:599–626.
51. Strasser RJ, Tsimilli-Michael M, Qiang S, Goltse V. 2010. Simultaneous in vivo recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. *Biochimica et Biophysica Acta* 1797:1313–26
52. Lei XY, Zhu RY, Zhang GY, Dai YR. 2004. Attenuation of coldin-duced apoptosis by exogenous melatonin in carrot suspension cells: the possible involvement of polyamines. *Journal of Pineal Research* 36:126–31
53. Kang K, Lee K, Park S, Kim YS, Back K. 2010. Enhanced production of melatonin by ectopic overexpression of human serotonin N-acetyltransferase plays a role in cold resistance in transgenic rice seedlings. *Journal of Pineal Research* 49:176–82

54. Qari SH, Hassan MU, Chattha MU, Mahmood A, Naqve M, et al. 2022. Melatonin induced cold tolerance in plants: Physiological and molecular responses. *Frontiers in plant science* 13:843071
55. Singh A, Roychoudhury A. 2023. Abscisic acid in plants under abiotic stress: crosstalk with major phytohormones. *Plant Cell Reports* 42(6):961–974
56. Zhang J, Shi Y, Zhang XH, Du HM, Xu B, et al. 2017. Melatonin suppression of heat-induced leaf senescence involves changes in abscisic acid and cytokinin biosynthesis and signaling pathways in perennial ryegrass (*Lolium perenne* L.). *Environmental and Experimental Botany* 138:36–45
57. El-Yazied AA, Ibrahim MFM, Ibrahim MAR, Nasef IN, Al-Qahtani SM, et al. 2022. Melatonin Mitigates Drought Induced Oxidative Stress in Potato Plants through Modulation of Osmolytes, Sugar Metabolism, ABA Homeostasis and Antioxidant Enzymes. *Plants* 11(9):1151
58. Li H, Mo YL, Cui Q, Yang XZ, Guo YL. 2019. Transcriptomic and physiological analyses reveal drought adaptation strategies in drought-tolerant and-susceptible watermelon genotypes. *Plant Science* 278:32–43.
59. Wang D, Chen QY, Chen WW, Guo QG, Xia Y, et al. 2021. Physiological and transcription analyses reveal the regulatory mechanism of melatonin in inducing drought resistance in loquat (*Eriobotrya japonica* Lindl.) *seedlings. Environmental and Experimental Botany* 181:104291
60. Zahedi SM, Hosseini MS, Abadía J, Marjani M. 2020. Melatonin foliar sprays elicit salinity stress tolerance and enhance fruit yield and quality in strawberry (*Fragaria* × *ananassa* Duch.). *Plant Physiology and Biochemistry* 49:313–323
61. Fan JB, Hu ZG, Xie Y, Chan ZL, Chen K, et al. 2015. Alleviation of cold damage to photosystem II and metabolisms by melatonin in Bermudagrass. *Frontiers in plant science* 6:925
62. Hasanuzzaman M, Bhuyan MHMB, Zulfiqar F, Raza A, Mohsin SM, et al. 2020. Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. *Antioxidants (Basel)* 9(8):681.
63. Waszczak C, Carmody M. Kangasjärvi J. 2018. Reactive oxygen species in plant signaling. *Annual Review of Plant Biology* 69:209–36.
64. Zhang X, Du H, Shi QH, Gong B. 2022. Loss of *GSNOR* increases abiotic stress sensitivity via regulating *MAPK-ethylene* cascade signaling in *Solanum lycopersicum* L. *Environmental and Experimental Botany* 199:104872
65. Zhang ZS, Yang C, Gao HY, Zhang LT, Fan XL, et al. 2014. The higher sensitivity of PSI to ROS results in lower chilling-light tolerance of photosystems in young leaves of cucumber. *Journal of Photochemistry and Photobiology. B, Biology* 137:127–34
66. Ruban AV. 2016. Nonphotochemical Chlorophyll Fluorescence Quenching: Mechanism and Effectiveness in Protecting Plants from Photodamage. *Plant Physiology* 170(4):1903–16
67. Nixon PJ, Michoux F, Yu J, Boehm M, Komenda J. 2010. Recent advances in understanding the assembly and repair of photosystem II. *Annals of Botany* 106(1):1–16
68. Li X, Wang HB, Jin HL. 2020. Light Signaling-Dependent Regulation of PSII Biogenesis and Functional Maintenance. *Plant Physiology* 183(4):1855–68
69. Acebron K, Matsubara S, Jedmowski C, Emin D, Muller O, et al. 2021. Diurnal dynamics of nonphotochemical quenching in Arabidopsis npq mutants assessed by solar-induced fluorescence and reflectance measurements in the field. *The New Phytologist* 229(4):2104–19
70. Lou YF, Sun HY, Zhu CL, Yang KB, Li XP, et al. *PeVDE*, a violaxanthin de-epoxidase gene from moso bamboo, confers photoprotection ability in transgenic *Arabidopsis* under high light. *Frontiers in plant science* 13: 927949
71. Aro EM, Virgin I, Andersson B. 1993. Photoinhibition of photosystemII inactivation, protein damage and turnover. *Biochimica et Biophysica Acta* 1143:113–34
72. Srivastava A, Guissé B, Greppin H, Strasser RJ. 1997. Regulation of antenna structure and electron transport in Photosystem II of Pisum sativum under elevated temperature probed by the fast polyphasic chlorophyll a fluorescence transient: OKJIP. *Biochimica et Biophysica Acta* 1320(1):95–106
73. Medina J, Catalá R, Salinas J. 2011. The CBFs: three arabidopsis transcription factors to cold acclimate. *Plant Science* 180:3–11
74. Zhao C, Zhang Z, Xie S, Si T, Li Y, Zhu JK. 2016. Mutational Evidence for the Critical Role of CBF Transcription Factors in Cold Acclimation in *Arabidopsis*. *Plant Physiology* 171(4):2744–59