



Melatonin Improves Drought Resistance in Maize Seedlings by Enhancing the Antioxidant System and Regulating Abscisic Acid Metabolism to Maintain Stomatal Opening Under PEG-Induced Drought

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Abstract

Maize (*Zea mays* L.) is highly sensitive to drought stress, resulting in large losses in yield; therefore, strategies aimed at enhancing drought tolerance are essential. Melatonin improves stress tolerance in plants; however, its mechanism in maize seedlings under drought stress remains unknown. Therefore, we investigated the effects of foliar-sprayed melatonin (100 $\mu\text{mol L}^{-1}$) on the antioxidant system, photosynthetic gas exchange parameters, stomatal behavior, endogenous melatonin and abscisic acid (ABA)-related gene expression in maize seedling leaves under 20% polyethylene glycol (PEG)-induced drought stress. PEG treatment resulted in oxidative stress and stomatal closure, resulting in chlorophyll degradation and inhibition of photosynthesis; thereby, reducing seedling biomass. Melatonin pretreatment significantly improved the relative water content, photosynthetic gas exchange parameters and stomatal behavior; thereby, maintaining chlorophyll contents and photosynthesis. Melatonin also stimulated the antioxidant system, enhancing the clearance of reactive-oxygen species, preventing severe damage under PEG-induced drought. Pre-treatment also increased endogenous melatonin and inhibited up-regulation of *NCED1*, an ABA synthesis-related gene, as well as selectively up-regulating ABA catabolic genes *ABA8ox1* and *ABA8ox3*, reducing ABA accumulation and inducing stomatal reopening. Overall, these findings suggest that melatonin pre-treatment alleviated the inhibitory effects of drought stress on photosynthesis, enhancing tolerance in maize seedlings.

Keywords Maize · Drought stress · Melatonin · Photosynthesis · Abscisic acid · Stomata

Introduction

As sessile organisms, plants are inevitably exposed to various environmental stresses (Gururani et al. 2015), of which drought is one of the most common, occurring annually in many regions around the world and subsequently inhibiting

plant growth and development (Avramova et al. 2015). When plants experience drought stress, transpiration rates decrease and active transport is impaired; thereby, affecting nutrient transport from the roots to the shoots and seriously reducing overall biomass (Griffiths and Parry 2002). Drought stress also causes a significant decrease in the relative water content (RWC) of the leaves, directly inducing toxicity in plant cells (Meher et al. 2018).

Stomata regulate the flow of gases and water in and out of plants, which plays an important role in overall survival (Peterson et al. 2010). In order to reduce water losses under drought stress, the stomata close as a result of changes in the turgidity of guard cells relative to epidermal cells; thereby, reducing the transpiration rate and limiting the diffusion of CO_2 into intercellular spaces, ultimately inhibiting photosynthesis (Cornic 2000). Meanwhile, drought also triggers other factors that have an inhibitory effect on photosynthesis

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(Chaves and Oliveira 2004). The antioxidant defense system removes reactive-oxygen species (ROS) in response to the external environment and aging, resulting in a dynamic balance. However, redox imbalance due to drought causes over production and accumulation of ROS, which induce membrane lipid peroxidation and impair membrane function, ultimately leading to chlorophyll degradation and loss of photosynthetic activity (Ippolito et al. 2011; Li et al. 2011; Anjum et al. 2017). In this case, plants activate their antioxidant system to cope with ROS-induced oxidative stress, employing enzymatic [superoxide dismutase [SOD], peroxidase (POD) and catalase (CAT)] and non-enzymatic antioxidants (glutathione (GSH)] (Anjum et al. 2011; Ashraf et al. 2015). Abscisic acid (ABA) is a hormone that regulates plant growth and development as well as the responses to stress (Kushiro et al. 2004). Drought stress triggers ABA production via 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key enzyme in the ABA biosynthesis pathway. Meanwhile, the main pathway of ABA catabolism is mediated by cytochrome P450 monooxygenase ABA 8'-hydroxylase (ABA8ox), also known as CYP707A (Qin and Zeevaert 1999; Saito et al. 2004; Nambara and Marionpoll 2005). The accumulation of ABA in plant cells is closely associated with ROS production, with drought stress stimulating the activation of Ca²⁺ channels in the plasma membrane, inducing ABA accumulation to regulate stomatal closure (Hu et al. 2008; Liu et al. 2010; Zhou et al. 2014).

Melatonin (*N*-acetyl-5-methoxytryptamine) was first discovered in the bovine pineal gland (Lerner et al. 1958) and has since been found in a variety of plant species, gaining widespread attention among biologists (Paredes et al. 2009; Tan et al. 2012; Arnao and Hernandezruiz 2014). Participation in various physiological processes, such as seed germination (Tiryaki and Keles 2012), root growth (Zhang et al. 2013) and leaf senescence has so far been demonstrated (Wang et al. 2014). Melatonin has also been shown to improve tolerance to drought, salinity and chilling stress (Park 2011; Janas and Posmyk 2013; Wei et al. 2015), as well as acting directly as an effective antioxidant, enhancing plant resistance by decreasing ROS accumulation (Arnao and Hernandezruiz 2015; Liu et al. 2015). In maize seedlings, melatonin increased drought tolerance by alleviating drought-induced photosynthetic inhibition and oxidative damage (Ye et al. 2016), while in hickory it preserved functionality of photosynthetic apparatus by stimulating antioxidant activity and expression levels of antioxidant enzymes under drought stress (Wang et al. 2019a). Wang et al. (2013) also revealed that exogenous application of melatonin delayed senescence of apple leaves under long-term drought stress, while Li et al. (2015) showed that melatonin-maintained drought tolerance in apple plants by regulating ABA metabolism and stomatal behavior. Melatonin has also been shown to prevent chlorophyll degradation by

down-regulating chlorophyll-degrading enzymes in tomato leaves (Wang et al. 2019b), while application effectively reduced drought stress in wine grapes (Meng et al. 2014). Meanwhile, in a recent study, melatonin delayed MeJA-induced senescence in tomato leaves, while pre-soaking of wheat seeds with melatonin improved yield by delaying leaf senescence and promoting root development (Ye et al. 2020).

Maize is one of the most important cereals in the world as well as being extremely sensitive to drought stress. Drought stress during maize growth can therefore lead to significant reductions in yield (Ziyomo and Bernardo 2013; Lobell et al. 2014), highlighting the need for scientific research aimed at improving drought tolerance. Studies suggest that melatonin could effectively improve drought tolerance in crops; however, few studies have targeted important crops such as maize. As a result, not much is known about the mechanism of melatonin in maize seedlings. Polyethylene glycol (PEG) is often used to simulate drought stress in experimental studies since PEG molecules with a molecular weight of more than 3000 are not absorbed by plant cells, preventing toxic side effects (Emmerich and Hardegee 1990). In this study, maize seedling leaves were pre-treated with melatonin before being subjected to PEG-induced drought stress. The effects on morphological characteristics, antioxidant enzyme activities, photosynthetic parameters, stomatal behavior and expression levels of key enzyme genes involved in the ABA metabolic pathway were then examined. The protective mechanism of melatonin on maize seedlings under drought stress was subsequently discussed from a physiological and metabolic perspective.

Results

Melatonin Improved the Biomass of Maize Seedlings Under Drought Stress

PEG-induced drought had severe toxic effects on the phenotype of the maize seedling leaves; however, pre-treatment with melatonin alleviated this damage, with relatively significant changes on day 3 and 4 (Fig. 1a). When compared with the control (CK), PEG treatment (D) caused a significant reduction in plant height, leaf length and width, and the dry weight of the maize seedlings. Meanwhile, melatonin pre-treatment (MT + D) effectively alleviated the PEG-induced negative impact on all traits (Table 1). No significant differences were observed between CK and melatonin pre-treatment alone (MT); however, compared with D treatment, the height of the seedlings increased by 8.93, 7.29, 9.93 and 10.42% under MT + D treatment on day 1, 2, 3 and 4, respectively. Moreover, leaf length increased by 5.33, 8.24, 10.06 and 12.80%, leaf width increased by 10.24, 11.38, 15.38

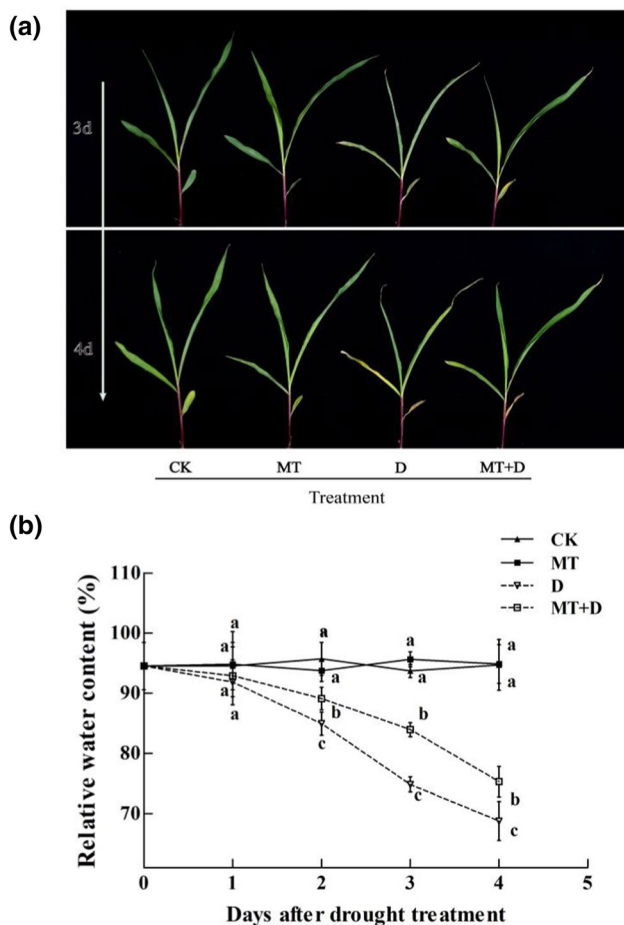


Fig. 1 Melatonin pretreatment alleviated the damage of maize seedling under drought stress. **a** Growth phenotype on day 3 and 4 for each treatment. **b** mean relative water content (%) of maize seedling leaves on day 1, 2, 3 and 4, respectively. Two seedlings were randomly selected per treatment and the third leaf was excised for determination. (i) control (CK): grown in Hoagland solution only; (ii) melatonin treatment (MT): pre-treated with $100 \mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution only; (iii) drought treatment (D): grown in Hoagland solution with 20% PEG-6000; and (iv) melatonin plus drought treatment (MT+D): pre-treated with $100 \mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution with 20% PEG-6000. The data are expressed as the mean \pm SD of three independent biological replicates. Different lower cases letters indicate significant differences at the same time point ($P < 0.05$; Duncan's multiple range test)

and 24.3%, and biomass increased by 8.46, 12.50, 13.27 and 11.39%, respectively.

Melatonin Increased the RWC of PEG-Treated Maize Seedling Leaves

As shown in Fig. 1b, melatonin pre-treatment in the absence of PEG (MT) had no significant effect on the RWC of the maize seedling leaves, unlike PEG treatment (D), which had an increasing negative effect. When compared with CK, D treatment decreased the RWC of the leaves by 2.94, 12.69,

25.21 and 37.69% on day 1, 2, 3 and 4, respectively. Meanwhile, pre-treatment with melatonin under PEG-induced drought (MT + D) effectively improved the RWC of the leaves, with the most significant effect on day 3 (12.17% increase compared to D treatment). These findings suggest that melatonin pre-treatment could improve the RWC of the leaves under drought conditions; thereby, helping maintain cell pressure.

Melatonin Reduced the Level of Reactive-Oxygen Species (ROS) in PEG-Treated Maize Seedling Leaves

Levels of ROS (H_2O_2 and O_2^-) accumulation in the maize seedling leaves increased under PEG treatment; however, melatonin pre-treatment suppressed these levels (Fig. 2a, b). No significant differences were observed between CK and MT treatment, with the exception of O_2^- concentration on day 3. The accumulation of H_2O_2 and O_2^- increased gradually with time under PEG-induced drought (D treatment), and although the changes were similar under MT + D treatment, the concentrations of H_2O_2 and O_2^- were significantly lower. These findings suggest that pre-treatment with melatonin could reduce ROS levels under drought conditions.

Melatonin Stimulated Antioxidant Activity in PEG-Treated Maize Seedling Leaves

Melatonin pre-treatment under non-drought conditions (MT) had no effect on activities of SOD, CAT and POD. In contrast, PEG -treatment (D) stimulated activity of all three enzymes, while MT + D treatment did so further (Fig. 2c–e). When compared with D treatment, SOD activity increased by 17.77, 15.36, 24.35 and 20.71%, CAT activity increased by 16.32, 23.02, 41.88 and 40.21%, and POD activity increased by 19.29, 10.25, 23.81 and 24.27% under MT + D on day 1, 2, 3 and 4, respectively. The general trend in GSH levels was similar to that of SOD, CAT and POD (Fig. 2f), with an upward trend from days 1 to 4 under drought stress. When compared with D treatment, the GSH content of the leaves under MT + D treatment increased by 26.11, 35.25, 36.76 and 39.14% on day 1, 2, 3 and 4, respectively. These findings suggest that pre-treatment with melatonin could increase antioxidant activity under drought conditions.

Melatonin Improved the Levels of Photosynthetic Pigment in PEG-Treated Maize Seedling Leaves

Melatonin pre-treatment alone (MT) had no significant effects on chlorophyll *a* (Chl *a*), Chl *b*, carotenoid (Car) and total chlorophyll (Chl *t*) (Fig. 3); however, D treatment caused a significant decrease in all four parameters, especially on day 3, with a reduction of 26.98, 36.63, 30.83 and 33.78%, respectively, compared with CK.

Table 1 Effects of melatonin on plant height, leaf length, leaf width and the dry weight of the shoots in PEG-treated

Time	Treatment	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Dry weight of shoot (g/plant)
1d	CK	27.27 ± 0.90a	20.00 ± 0.75a	1.43 ± 0.08ab	0.219 ± 0.009a
	MT	28.40 ± 0.66a	19.50 ± 0.66a	1.45 ± 0.13a	0.231 ± 0.004a
	D	22.40 ± 0.89c	16.87 ± 0.40b	1.27 ± 0.03b	0.189 ± 0.005c
	MT + D	24.40 ± 0.62b	17.77 ± 0.85b	1.40 ± 0.09ab	0.205 ± 0.009b
2d	CK	28.00 ± 0.96a	20.23 ± 0.96b	1.50 ± 0.09a	0.234 ± 0.007a
	MT	29.23 ± 0.85a	21.53 ± 0.70a	1.48 ± 0.03a	0.233 ± 0.004a
	D	22.90 ± 0.72c	17.00 ± 0.46c	1.23 ± 0.08c	0.192 ± 0.007c
	MT + D	24.57 ± 0.80b	18.40 ± 0.50d	1.37 ± 0.03b	0.216 ± 0.009b
3d	CK	28.67 ± 0.67b	20.70 ± 0.95a	1.52 ± 0.03a	0.237 ± 0.009a
	MT	30.13 ± 0.38a	21.97 ± 0.57a	1.57 ± 0.03a	0.245 ± 0.009a
	D	23.47 ± 0.67c	16.90 ± 0.62c	1.17 ± 0.06c	0.196 ± 0.007c
	MT + D	25.80 ± 0.66d	18.60 ± 0.62b	1.35 ± 0.05b	0.222 ± 0.006b
4d	CK	29.13 ± 0.81a	21.37 ± 0.55a	1.57 ± 0.03a	0.244 ± 0.005a
	MT	30.47 ± 0.59a	22.23 ± 0.61a	1.62 ± 0.08a	0.248 ± 0.008a
	D	23.70 ± 0.46c	16.40 ± 0.56c	1.07 ± 0.12c	0.202 ± 0.006c
	MT + D	26.17 ± 0.91b	18.50 ± 0.66b	1.33 ± 0.03b	0.225 ± 0.009b

Values represent the mean ± standard error of three replicates

Different lowercases letters indicate significant differences among treatments ($P < 0.05$; Duncan's multiple range test)

Moreover, although each parameter was significantly lower under MT + D compared with CK treatment, the contents of Chl *a*, Chl *b* and Chl *t* were significantly higher than under D treatment (increases of 17.61, 33.33 and 22.26%, respectively). In contrast, there was no significant difference in Car between the two treatments. These findings suggest that pre-treatment with melatonin could alleviate chlorophyll degradation under drought conditions.

Melatonin Improved Photosynthetic Gas Exchange Parameters in PEG-Treated Maize Seedling Leaves

Photosynthetic gas-exchange parameters directly reflect the photosynthetic carbon assimilation ability. As shown in Fig. 4, melatonin pre-treatment alone (MT) had no significant effects on P_n , G_s , C_i or T_r . In contrast, under D treatment, all four parameters decreased to a varying degree, with significant reductions compared to CK of 29.8, 38.1, 45.5 and 47.4%, respectively. Although MT + D treatment also caused a significant reduction in all four parameters compared with CK, an increase of 22.02, 23.1, 30.2 and 13.5 was observed compared with D treatment, respectively. These findings suggest that pre-treatment with melatonin could significantly increase photosynthesis and gas exchange in maize seedling leaves under drought conditions, thereby helping maintain normal photosynthesis.

Melatonin Changed the Stomatal Ultrastructure and Induced Stomatal Opening in PEG-Treated Maize Seedling Leaves

The lower surfaces of the leaf samples were subsequently scanned under a SEM at × 1500 magnification (Fig. 5a). Under control conditions (CK), the epidermal cells had a smooth surface and the stomata were arranged neatly, with semilunar stomatal guard cells and large apertures. No significant differences were observed under MT treatment; however, under D treatment, the epidermal cells became smaller and the guard cells contracted, and most of the stomata were closed. Meanwhile, melatonin pre-treatment (MT + D) effectively alleviated these PEG-induced effects, causing the stomata to reopen.

Melatonin pre-treatment alone (MT) had no significant effects on stomatal density, length, width or aperture size (Fig. 5b–e). However, in comparison, the density of the stomata increased by 34.5%, the width decreased by 14.7%, and the stomatal aperture decreased by about 79.7% under D treatment. In contrast, no significant differences in the length of the stomata were observed. Although MT + D treatment decreased the width of the stomata and reduced the stomatal aperture compared to CK, the aperture and width of stomatal increased by 197.36% and 8.04% compared with D treatment. These findings suggest that pre-treatment with melatonin could improve stomatal conditions and induce stomatal reopening.

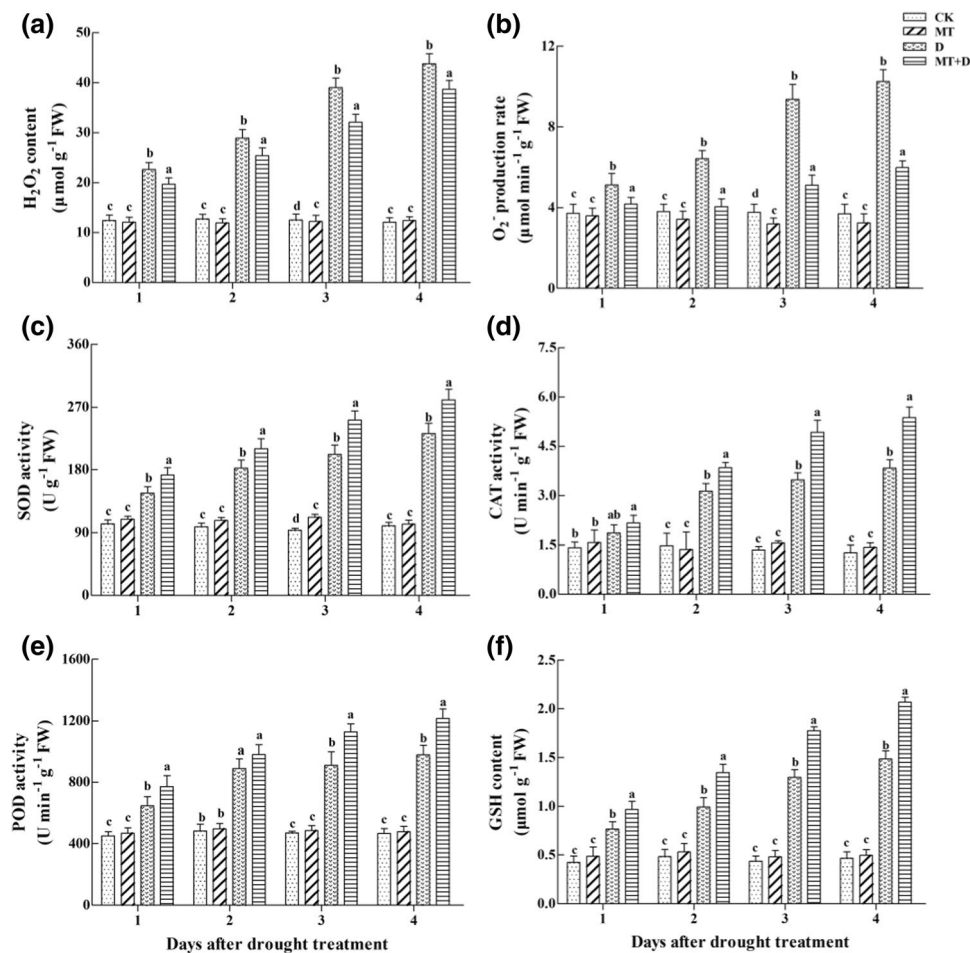


Fig. 2 Effects of melatonin on reactive-oxygen species (ROS) and antioxidant system in PEG-treated maize seedling leaves. **a, b** Mean levels of H_2O_2 (**a**) and O_2^- (**b**) in maize seedling leaves under different treatments on day 1, 2, 3 and 4, respectively. **c–f** Mean levels of SOD (**c**), CAT (**d**), POD (**e**) and GSH (**f**) in maize seedling leaves under different treatments on day 1, 2, 3 and 4, respectively. (i) control (CK): grown in Hoagland solution only; (ii) melatonin treatment (MT): pre-treated with $100 \mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution only; (iii) drought treatment (D): grown in

Hoagland solution with 20% PEG-6000; and (iv) melatonin plus drought treatment (MT+D): pre-treated with $100 \mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution with 20% PEG-6000. Two seedlings were randomly selected per treatment and the third leaf was excised for determination. The data are expressed as the mean \pm SD of three independent biological replicates. Different lowercase letters indicate significant differences among treatments ($P < 0.05$; Duncan's multiple range test)

Exogenous Melatonin Decreased Abscisic Acid and Increased Endogenous Melatonin Levels in PEG-Treated Maize Seedling Leaves

MT treatment significantly increased endogenous melatonin levels by 21.98% compared with the control (CK). Meanwhile, D treatment caused a significant increase in endogenous melatonin compared with CK, while melatonin pre-treatment under PEG-induced drought (MT+D) caused a further improvement of 36.71% compared with D treatment (Fig. 6a). MT treatment had no effect on ABA levels (Fig. 6b), while D treatment caused a significant increase of 164.74%. Meanwhile, MT+D treatment significantly suppressed this stress-related increase in ABA

production, with a decrease of 30.12% compared with D treatment.

Melatonin Decreased ABA Levels by Regulating the Expression of Genes Related to the ABA Metabolic Pathway in PEG-Treated Maize Seedling Leaves

To validate the involvement of melatonin in the regulation of ABA metabolism, expression of ABA biosynthesis- and catabolism-related genes was examined. D treatment caused significant up-regulation of the *NCED1* gene, with an 18.4-fold increase in expression compared with CK on day 3 of PEG treatment. However, MT+D treatment significantly

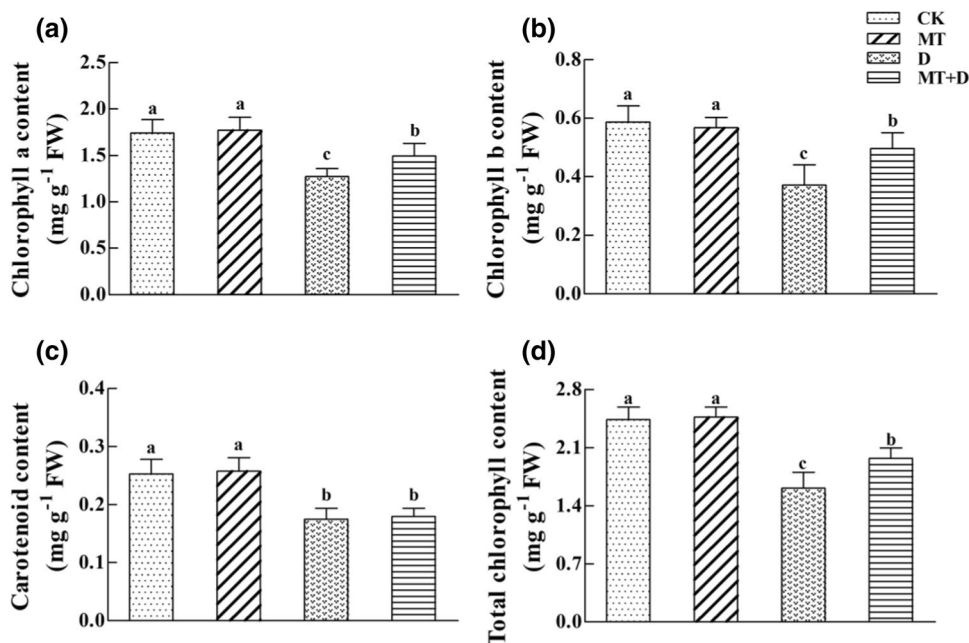


Fig. 3 Effects of melatonin on chlorophyll content in PEG-treated maize seedling leaves. **a–d** Mean levels of chlorophyll a (**a**), chlorophyll b (**b**), carotenoid (**c**) and total chlorophyll (**d**) in maize seedling leaves under different treatments on day 1, 2, 3 and 4, respectively. (i) control (CK): grown in Hoagland solution only; (ii) melatonin treatment (MT): pre-treated with 100 $\mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution only; (iii) drought treatment (D): grown in Hoagland solution with 20% PEG-6000; and (iv) melatonin plus

drought treatment (MT+D): pre-treated with 100 $\mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution with 20% PEG-6000. Two seedlings were randomly selected per treatment and the third leaf was excised for determination. The data are expressed as the mean \pm SD of three independent biological replicates. Different lowercase letters indicate significant differences among treatments ($P < 0.05$; Duncan's multiple range test)

suppressed up-regulation, with a decrease in the expression of 63.80% compared with D treatment (Fig. 6c). As shown in Fig. 6d–g, four ABA catabolism-related genes (*ABA8ox1a*, *ABA8ox1b*, *ABA8ox3a* and *ABA8ox3b*) were down-regulated under D treatment, with a decreased in the expression of 16.42, 76.55, 67.26 and 93.40%, respectively, compared with CK. In contrast, MT + D treatment caused a significant 2.2-, 3.1- and 12.3-fold increase in *ABA8ox1b*, *ABA8ox3a* and *ABA8ox3b* expression, but a decrease in *ABA8ox1a* expression of 36.83% compared with D treatment.

Discussion

It is well known that drought stress adversely effects physiological and biochemical processes in plants, leading to a reduction in overall productivity (Reddy et al. 2004; Sanchita et al. 2015). As higher plants, they have developed different strategies to response various environmental stress (Kim and Kim 2020). Melatonin, as a kind of new plant growth regulator, is thought to be involved in abiotic stress responses (Zhang et al. 2015). In our study, the positive protective role of melatonin in maize seedlings against drought stress was investigated. The PEG-induced drought stress

caused a decrease in the leaf area and relative water content of maize seedlings, and the severity of these effects was intensified with time. Meanwhile, melatonin pre-treatment significantly improved both parameters under drought stress (Table 1, Fig. 1). Ye et al. (2016) reported that 100 μm melatonin pretreatment improved the shoot dry weight and leaf area of maize seedlings under drought stress, which is consistent with our results. In the present study, it is worth noting that melatonin-treated plants maintained higher biomass especially on the third day (Table 1), which is constant with the trend of relative water content, suggesting that melatonin may enhance plant root water uptake ability and alleviate the inhibitory effects of drought on maize seedling growth.

Photosynthesis occurs in the chloroplasts, which is also the leaf organelle that is most sensitive to abiotic stress. They are also the main site of ROS generation in plant cells. The chlorophyll content of the chloroplast therefore largely determines whether a plant is under good growth conditions as well as the photosynthetic capacity of the leaves (Pandey et al. 2009; Shapiguzov et al. 2012; Croft et al. 2017). Drought can induce accumulation of ROS in plants, with excessive ROS promoting chlorophyll degradation and reducing the chlorophyll content (Yordanov et al. 2000). In this study, drought stress caused a significant

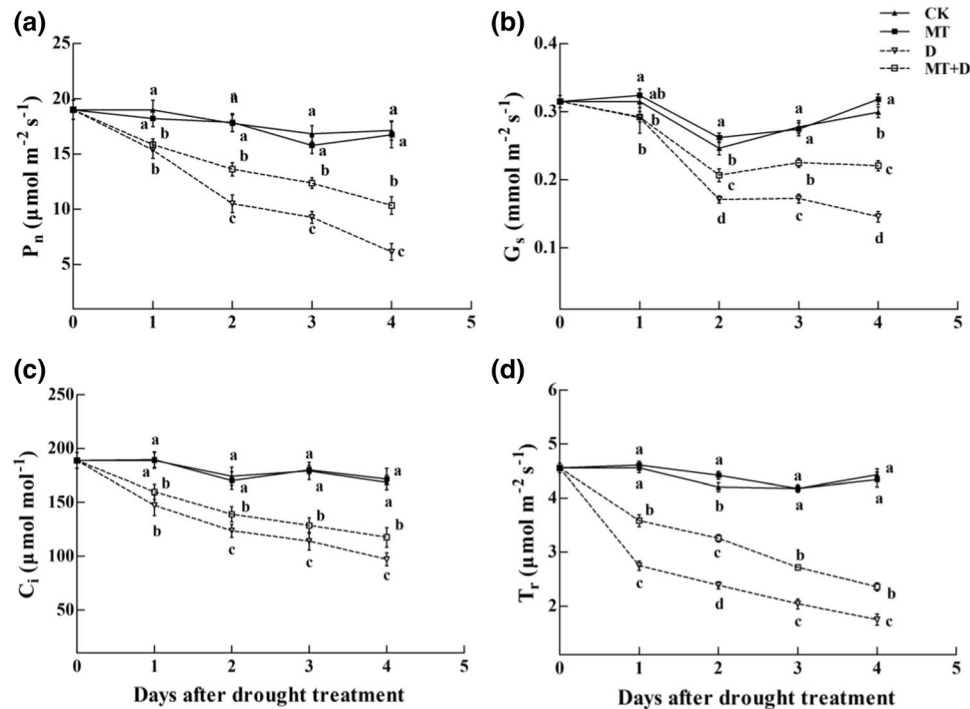


Fig. 4 Effects of melatonin on gas exchange in PEG-treated maize seedling leaves. **a–d** Mean levels of P_n (**a**), G_s (**b**), C_i (**c**) and T_r (**d**) in maize seedling leaves under different treatments on day 1, 2, 3 and 4, respectively. (i) control (CK): grown in Hoagland solution only; (ii) melatonin treatment (MT): pre-treated with 100 μmol L⁻¹ melatonin then grown in Hoagland solution only; (iii) drought treatment (D): grown in Hoagland solution with 20% PEG-6000; and (iv) melatonin

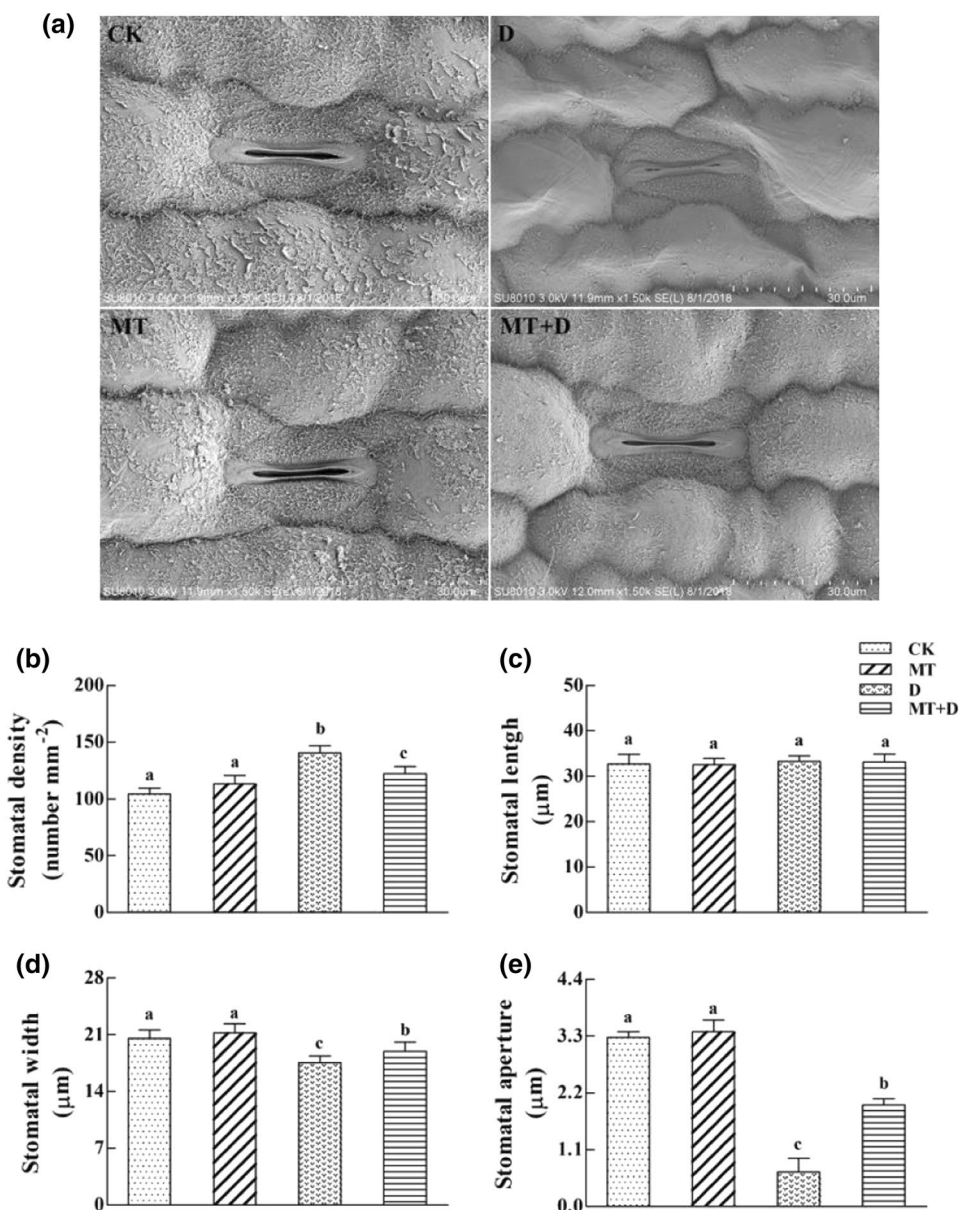
plus drought treatment (MT + D): pre-treated with 100 μmol L⁻¹ melatonin then grown in Hoagland solution with 20% PEG-6000. Two seedlings were randomly selected per treatment and the third leaf was excised for determination. The data are expressed as the mean ± SD of three independent biological replicates. Different lowercase letters indicate significant differences among treatments ($P < 0.05$; Duncan's multiple range test)

increase in H₂O₂ and O₂⁻, as a result, the chlorophyll content of the leaves was lower than under control conditions (Fig. 3). However, melatonin pre-treatment reduced the H₂O₂ and O₂⁻ content, as well as improving chlorophyll levels. These findings suggest that melatonin acts as an antioxidant, directly reducing ROS levels and inhibiting chlorophyll degradation (Tan et al. 2007; Ma et al. 2018). There is further evidence that melatonin reduces the degradation of chlorophyll by down-regulating expression of chlorophyll degradation-related genes during MeJA-induced senescence (Wang et al. 2019b). SOD, POD and CAT are important protective enzymes in the enzymatic defense system, effectively decreasing levels of H₂O₂ and decomposing ROS. Moreover, GSH is an important non-enzymatic antioxidant, scavenging O₂⁻ and reducing oxidative damage under stressful conditions. Activity of all four components therefore ensures stress tolerance in plants, including those subjected to long-term drought stress (Liu et al. 2009; Kang et al. 2013). In this study, antioxidant enzyme activity (SOD, POD and CAT) and the GSH content of the maize seedling leaves increased significantly, mainly due to the response of maize seedlings to drought stress (Anjum et al. 2017). Meanwhile, pre-treatment with melatonin further improved

antioxidant enzyme activity and GSH levels under drought conditions (Fig. 2c–f), increasing ROS clearance (Fig. 2a, b). These findings suggest that pre-treatment with exogenous melatonin reduced ROS accumulation by further activating the antioxidant defense system and alleviating oxidative damage in maize seedlings under drought stress.

Important physiological processes in the leaves, such as respiration, photosynthesis and transpiration, are regulated by stomata, the opening and closing of which is regulated by water balance and complex signal transduction pathways (Gray 2005; Zou and Kahnt 2008). When subjected to drought stress, plants maintain their cellular moisture content by regulating stomatal closure and reducing the transpiration rate (Schroeder et al. 2001). In this study, the lower surfaces of the leaves were scanned at × 1500 magnification, revealing a significant decrease in the lengths and widths of the stomata under drought stress, as well as a decrease in the aperture opening (Fig. 5a). However, stomatal density increased significantly under drought stress, with contraction of the guard cells. Moreover, pre-treatment with melatonin increased the stomatal length and width under drought stress, although measurements were lower than in the control leaves (Fig. 5b–e). In contrast to our findings, Li et al. (2015)

Fig. 5 Effects of melatonin on the stomatal of PEG-treated maize seedling leaves. **a** The scanning electron microscopy (SEM) images of seedling leaves on the third day of drought treatment. **b–e** Mean levels of stomatal density (**b**), stomatal length (**c**), stomatal width (**d**) and stomatal aperture (**e**) in maize seedling leaves under different treatments on the third day. (i) control (CK): grown in Hoagland solution only; (ii) melatonin treatment (MT): pre-treated with $100 \mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution only; (iii) drought treatment (D): grown in Hoagland solution with 20% PEG-6000; and (iv) melatonin plus drought treatment (MT + D): pre-treated with $100 \mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution with 20% PEG-6000. Two seedlings were randomly selected per treatment and the third leaf was excised for determination. The data are expressed as the mean \pm SD of three independent biological replicates. Different lowercase letters indicate significant differences among treatments ($P < 0.05$; Duncan's multiple range test)

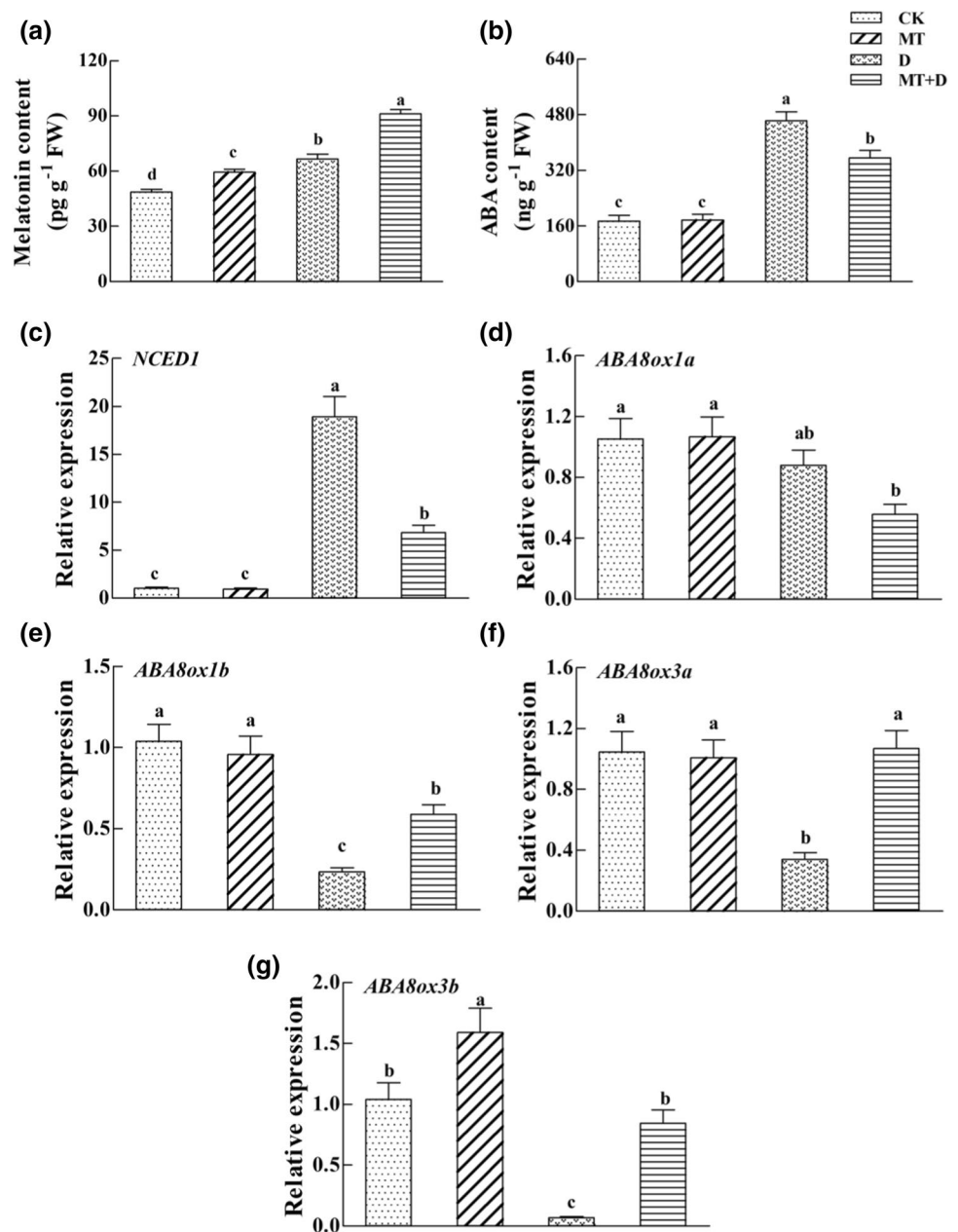


suggested that drought did not alter the stomatal density in apple leaves, however, pre-treatment with exogenous melatonin did maintain high turgor pressure and keep the stomata open. Similarly, Meng et al. (2014) revealed no obvious differences in the stomatal aperture between grape plants pre-treated with melatonin and stressed control plants. Although not completely consistent with our findings, this may be due to differences in the regulatory mechanism of melatonin in different species.

ABA is an important plant hormone that plays pivotal roles in conferring tolerance to various environmental stressors (Fujita et al. 2005). It is involved in a number of physiological processes, such as plant growth and development, responses to abiotic stress and regulation of stomatal closure (Finkelstein et al. 2002; Timothy and Brodrribb

2013). Stress stimulates the accumulation of ABA in plants, reducing transpiration by regulating stomatal closure to prevent cellular water losses (Zhang et al. 2006). Studies have shown that ABA levels in plants are associated with genes involved in ABA synthesis and degradation, such as *NCED1* (ABA biosynthesis), *ABA8ox1* and *ABA8ox2* (ABA catabolism) (Li et al. 2015). Our study focused on ABA biosynthesis and metabolic pathways. PEG-induced drought stress significantly increased ABA levels in maize seedling leaves (Fig. 6b). Consistently, the expression of *NCED1* was also significantly up-regulated, while expression of *ABA8ox1* and *ABA8ox3* was significantly down-regulated. Meanwhile, melatonin pre-treatment repressed *NCED1* up-regulation and selectively up-regulated *ABA8ox1* and *ABA8ox3*, ultimately reducing

Fig. 6 Effects of melatonin on endogenous melatonin, abscisic acid content and expression of key ABA synthesis and metabolism-related genes in PEG-treated maize seedling leaves. **a, b** Mean levels of endogenous melatonin (**a**) and abscisic acid (**b**) in maize seedling leaves under different treatments on the third day. **C–G** Mean expression of *NCED1* gene (**c**), *ABA8ox1a* (**d**), *ABA8ox1b* (**e**), *ABA8ox3a* (**f**) and *ABA8ox3b* (**g**) in maize seedling leaves under different treatments on the third day. (i) control (CK): grown in Hoagland solution only; (ii) melatonin treatment (MT): pre-treated with 100 $\mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution only; (iii) drought treatment (D): grown in Hoagland solution with 20% PEG-6000; and (iv) melatonin plus drought treatment (MT+D): pre-treated with 100 $\mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution with 20% PEG-6000. Two seedlings were randomly selected per treatment and the third leaf was excised for determination. The data are expressed as the mean \pm SD of three independent biological replicates. Different lowercase letters indicate significant differences among treatments ($P < 0.05$; Duncan's multiple range test)



ABA levels (Figs. 6c–g). These findings suggest that melatonin acts as a negative regulator of ABA biosynthesis, inducing stomatal reopening by regulating ABA metabolism (Li et al. 2015). In addition, analysis of endogenous melatonin levels in the maize seedling leaves revealed a much higher level after melatonin pre-treatment compared with control plants. This suggests that exogenously applied melatonin promotes biosynthesis of melatonin de novo via increased expression of melatonin biosynthesis-related genes (Zheng et al. 2017). More notable is that endogenous melatonin in the leaves of plants pre-treated with exogenous melatonin was 21.98% higher than that

in the control plants, while under drought stress, levels were 36.71% higher after pre-treatment with melatonin than under drought stress (Fig. 6a). These findings suggest lead us to the following hypothesis: under drought stress, exogenous melatonin induces stomatal re-opening via regulation of ABA metabolism, thereby restoring transpiration and active transport. The bioactive melatonin molecules are subsequently transported via an active transport mechanism or specialized channel (Erland et al. 2018), subsequently increasing levels of endogenous melatonin. Further studies are now needed to confirm this hypothesis.

Conclusions

To summarize, this study suggests that melatonin mitigates photosynthetic damage in maize seedling under drought stress in two ways: (i) by maintaining a high water content in the leaves and enhancing the antioxidant system, thereby effectively reducing the accumulation of ROS, preventing chlorophyll degradation and maintaining photosynthesis; (ii) by significantly promoting ABA decomposition and inhibiting synthesis, thereby reducing accumulation, inducing stomatal reopening and helping maintain photosynthesis. A schematic representation of this hypothesis is provided in Fig. 7. Overall, therefore, the results of this study provide preliminary evidence that melatonin protects photosynthesis and enhances drought tolerance in maize seedlings under drought stress.

Materials and methods

Experimental Materials

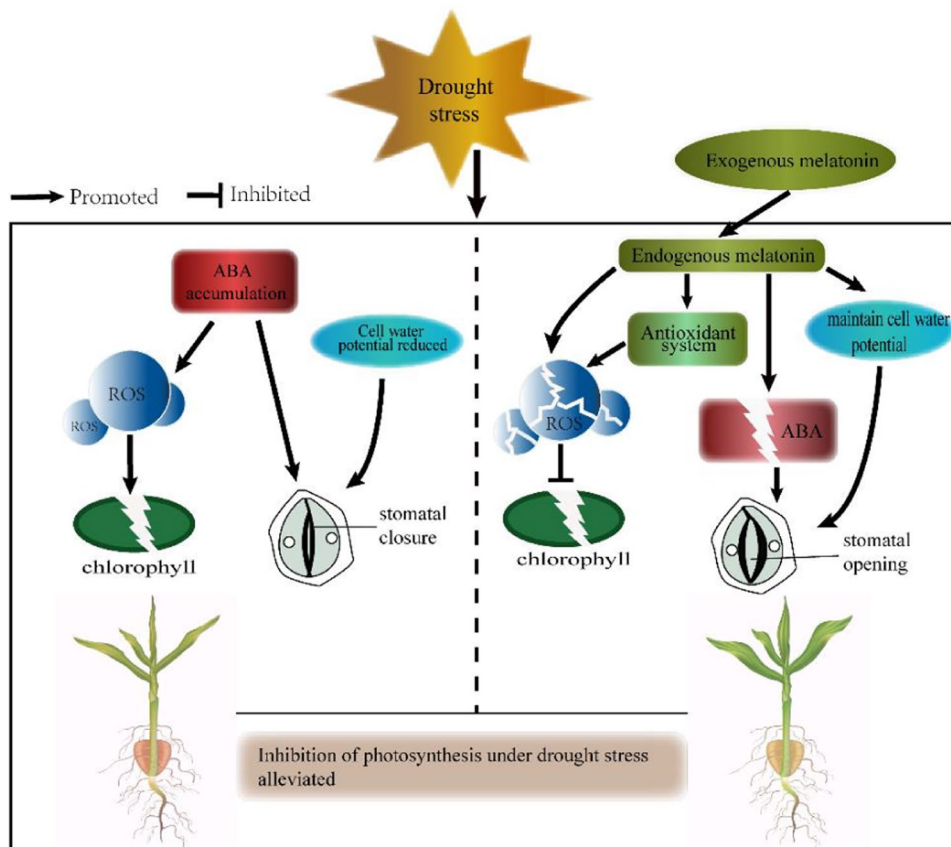
Zhengdan 958, a widely used maize (*Zea mays* L.) cultivar in China, was used in this study. Uniform seeds were

selected to accelerate germination in a greenhouse at 25 °C. After germination, the seedlings were placed in an artificial climate chamber (75% humidity, 25/15 °C day/night) and grown via hydroponics in Hoagland solution. Light was provided by sodium lamps during a 14-h photoperiod (photon flux density of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The nutrient solution was changed daily.

Experimental Design

Preliminary experiments were conducted to determine the optimal melatonin concentration. Leaves were sprayed with five concentrations of melatonin (0, 50, 100, 150 and 200 $\mu\text{mol L}^{-1}$) once a day for 4 consecutive days then before treatment with PEG for four days. The rate of photosynthesis under each concentration was then measured. Based on this preliminary experiments, 100 $\mu\text{mol L}^{-1}$ melatonin was selected for the final experimentation. When seedlings reached the trifoliate stage, half of all uniformly growing plants were sprayed with 100 $\mu\text{mol L}^{-1}$ melatonin at 8:00 am and 20:00 pm daily for 5 consecutive days. The remaining plants were treated with distilled water as a control at the same time points. After a pre-cultivation period, the control and melatonin pre-treated seedlings were each randomly divided into two subgroups: normal control and

Fig. 7 Regulatory effect of melatonin on photosynthesis in maize seedlings under drought stress



drought treatment. Under drought treatment, 20% PEG-6000 was used to induce drought treatment for four days. A total of four treatments were therefore examined: (i) control (CK): grown in Hoagland solution only; (ii) melatonin treatment (MT): pre-treated with 100 $\mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution only; (iii) drought treatment (D): grown in Hoagland solution with 20% PEG-6000; and (iv) melatonin plus drought treatment (MT + D): pre-treated with 100 $\mu\text{mol L}^{-1}$ melatonin then grown in Hoagland solution with 20% PEG-6000. Each treatment was carried out in triplicate with 36 seedlings per treatment. Seedlings were observed after 1, 2, 3 and 4 days of treatment, and third leaves from the bottom of the stem were sampled for subsequent analysis.

Measurement of Plant Traits

Plants were collected from each treatment group (CK, MT, D, MT + D) after 1, 2, 3 and 4 days treatment for measurement of plant height, leaf length and width and dry weight. Plant height was determined as the height of the above-ground part using a ruler. Leaf length and width were determined as the maximum length and width of the third leaf using a ruler. The dry weight of the aboveground tissues was determined after drying in an oven at 80 °C until a constant weight was reached. At least three independent biological replications were performed for each experiment.

Measurement of RWC

The RWC was estimated according to the method of Gaxiola et al. (2001). Briefly, two seedlings were randomly selected per treatment and the third leaf was excised, divided into two equal parts then the fresh weights (F_w) of each were recorded immediately. One part was floated in deionized water for 12 h then the rehydrated weight (S_w) was determined. The remaining part was oven-dried at 70 °C until reaching a constant weight then the dry weight was determined (D_w). The RWC was then calculated as follows:

$$(\text{RWC}) = (F_w - D_w) / (S_w - D_w) \times 100\%, \quad (1)$$

Measurement of H_2O_2 and O_2^-

The production rate of superoxide O_2^- was determined as described by Schneider et al. (1981). Briefly, the fresh samples of the third leaf (0.5 g) were homogenized with 65 mM phosphate buffer (pH 7.8) then centrifuged at 5000 g for 15 min at 4 °C. Next, 0.5 mL supernatant, 0.5 mL phosphate-buffered saline (PBS; 65 mM, pH 7.8) and 1.0 mL 10 mM hydroxylamine hydrochloride were mixed together and incubated at 25 °C for 1 h before adding 1.0 mL 17 mM

p-aminobenzene sulfonic acid and 1.0 mL 7.0 mM naphthylamine to the mixture and incubating at 25 °C for a further 20 min. The change in absorbance at 530 nm was then determined. A standard curve of NO_2^- was used to calculate the production rate of O_2^- from the chemical reaction of O_2^- and hydroxylamine, while the H_2O_2 content was assayed according to Thordal-Christensen et al. (1997).

Measurement of Antioxidant Activity

Superoxide dismutase (SOD) activity was determined using the nitro blue tetrazolium (NBT) illumination method (Kakkar et al. 1984). The amount of enzyme required for 1 mg of tissue protein in 1 ml of reaction mixture to raise the SOD inhibition rate to 50% at 550 nm was defined as SOD activity. Peroxidase (POD) activity was determined according to the method of Upadhyaya et al. (1985), whereby the absorbance of reaction mixture containing 30 μl enzyme extract, 50 mM phosphate buffer (pH 7.8), guaiacol and 30% H_2O_2 was recorded then the amount of enzyme required to change the absorbance by 0.01 was defined as POD activity. Catalase (CAT) activity was assayed according to Hamurcu et al. (2013), whereby one unit of CAT activity was determined as the amount of enzyme required to decompose 1 $\mu\text{mol H}_2\text{O}_2$ at 405 nm s^{-1} in 1 mg fresh tissue protein. The glutathione (GSH) content was measured according to the method of Ma and Cheng (2003).

Measurement of Gas Exchange Parameters

Gas exchange parameters [the rate of photosynthetic (P_n), stomatal conductance (G_s), transpiration rate (T_r) and intercellular CO_2 concentration (C_i)] were recorded using the fully expanded third leaf at 10:00 am with a portable photosynthesis system (LI-6400XT; LI-COR Biosciences, Lincoln, NE, USA). A 6 cm^2 (2×3) leaf chamber was used to carry out measurements at 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, with a constant airflow rate of 500 $\mu\text{mol s}^{-1}$, a CO_2 concentration of 450 $\mu\text{mol mol}^{-1}$ and 60% relative humidity. Each treatment was carried out in triplicate.

Measurement of Chlorophyll Concentrations

The chlorophyll content was determined according to the method of Arnon (1949) with slight modifications. Briefly, the third leaf samples were selected from each treatment and cut up then soaked in a graduated tube with 80% acetone to prevent light from affecting the results. When chlorophyll extraction was complete. The supernatant was then separated and placed in a new tube, and absorbance was recorded at wavelengths of 663, 645 and 470 nm to determine chlorophyll a (Chl *a*), Chl *b*, carotenoid (Car) content. As a blank control, 80% acetone was used.

Table 2 Primers used for qRT-PCR analysis

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
<i>NCED1</i>	CCACGCACACCAGAGTTACA	GCTGGGCGCCTTTCTACTAA
<i>ABA8ox1a</i>	ATGCTCGTGCTCTTCCACCACCT	TATACCGCCATACCATATCCATCCGCC
<i>ABA8ox1b</i>	ATGCTCGTGCTCTTCCACCACCT	GGAAGCGGTTTTTCGCGTTCCTGG
<i>ABA8ox3a</i>	ACAGAAAGGGGCGTGAGACCGA	AGGCGAGCAAAGAAGAATTTCAA
<i>ABA8ox3b</i>	ACAGAAATGGCCGCCATGAGACCGA	ATTTCTTCTCCCCCTCAAGGTAAT

Observations of Stomata via Scanning Electron Microscopy

On the third day of drought treatment, one seedling was selected per treatment and third leaf samples were cut into 1×2 mm rectangular pieces and immediately fixed in 4% glutaraldehyde solution with 0.1 M PBS (pH 7.4). After rinsing five times in PBS, they were then dehydrated in a graded ethanol series, dried to the critical point of CO₂ and gold coated. The stomata were then observed under a JSM-6360LV microscope (JEOL Ltd., Tokyo, Japan).

Measurement of Endogenous Melatonin and Abscisic Acid

First, 0.1 g maize leaf was ground into powder with liquid nitrogen and extracted with 1.0 mM phosphate buffer (pH 7.4) in 1.5 mL centrifuge tube. Secondly, the mixed sample was placed on ice for 1 min, then centrifuged at 4 °C for 20 min at 10,000×g and collect supernatant. The contents of endogenous melatonin and abscisic acid were assayed using a melatonin enzyme-linked immunosorbent assay (ELISA) kit (ml024033; Mlbio, Shanghai, China) and plant hormone abscisic acid (ABA) ELISA kit (ml-E2607; Mlbio, Shanghai, China) according to the manufacturer's instructions, respectively.

qRT-PCR Analysis

Total RNA was extracted from the leaves according to the methods of Faccioli et al. (1995). Primer sequences were obtained from the GeneBank database and specific primers were designed using Primer Express Software v2.0 (Biosoft International, Palo Alto, CA, USA). β -actin was used as a reference gene. All primers used are listed in Table 2. QRT-PCR was carried out on an ABI ViiA 7 Real-Time PCR System using ABI SYBR® Select Master Mix (4,472,908, Mingyangkehua BioTechnology Co., Ltd, Beijing). Two technical repeats were carried for each sample. Relative expression levels were then calculated using the $2^{-\Delta\Delta C_p}$ method (Livak and Schmittgen 2001).

Statistical Analysis

An one-way analysis of variance (ANOVA) was carried out using SPSS 17.0 software (IBM, Armonk, NY, USA). Multiple pairwise comparisons using Duncan's multiple range tests at $\alpha=0.05$ were used to determine significant differences among treatments. mks were generated with Origin 9.0 (OriginLab, Northampton, MA, USA) and standard errors of the means were calculated and presented in each graph. The regulatory network was generated using Adobe Illustrator 5.1 (Adobe Systems Inc., San Jose, California, USA).

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Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflict of interest.

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