Exogenous melatonin enhances salt stress tolerance in maize seedlings by improving antioxidant and photosynthetic capacity

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Melatonin (N-acetyl-5-methoxytryptamine) is an important biological hormone in many abiotic stress responses and developmental processes. In this study, the protective roles of melatonin were investigated by measuring the antioxidant defense system and photosynthetic characteristics in maize under salt stress. The results indicated that NaCl treatment led to the decrease in plant growth, chlorophyll contents and photochemical activity of photosystem II (PSII). However, the levels of reactive oxygen species increased significantly under salt stress. Meanwhile, we found that application of exogenous melatonin alleviated reactive oxygen species burst and protected the photosynthetic activity in maize seedlings under salt stress through the activation of antioxidant enzymes. In addition, 100μM melatonin-treated plants showed high photosynthetic efficiency and salinity. Immunoblotting analysis of PSII proteins showed that melatonin application alleviated the decline of 34kDa PSII reaction center protein (D1) and the increase of PSII subunit S protein. Taken together, our study promotes more comprehensive understanding in the protective effects of exogenous melatonin in maize under salt stress, and it may be involved in activation of antioxidant enzymes and regulation of PSII proteins.

Introduction

As a cereal plant of the family Poaceae, Maize (Zea mays L.) is one of the most important crops throughout the world, and it is the staple food in many areas. At the same time, it is also very importantly raw materials of biofuels and industry in the world (Nuss and Tanumihardjo 2010). Salt is one of the environmental cues that often limits the growth of plant and reduces the productivity of agricultural crops because most of plants are sensitive to soil salinity. Salt stress influences plants in many ways: ion (Na\textsuperscript{+}) toxicity, physiological water deficit, nutritional imbalance, oxidative damage, metabolic disorder, the alteration of major cytosolic enzyme activities and photoinhibition (Chen et al. 2007, Cuin and Shabala 2007, Munns and Tester 2008, Pandolfi et al. 2012, Tang et al. 2015). More than 45 million hectares of agricultural land has been impaired by salinity and sodicity. In addition, about 1.5 million hectares are unsuitable for cultivation due to high levels of salinity in the soil (Munns and Tester 2008). With the salinized agricultural areas increasing, maize plants often suffer from salt stress. Therefore, the efforts to increase salt tolerance of crop plants become more and more important, and we can get more output

Abbreviations

- ΦPSII, quantum yield of PSII electron transport; AsA, ascorbic acid; EL, electrolyte leakage; MDA, malondialdehyde; NPQ, non-photochemical quenching coefficient; qP, photochemical quenching; RWC, relative water content; SOD, superoxide dismutase.

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from the salinized agricultural land through improving salt tolerance of maize plants.

Many plant growth-regulating substances have been used to improve salt tolerance of the crop plants for agricultural sustainability, mainly including the application of glycinebetaine (Yang and Lu 2005, Kreslavski et al. 2017), salicylic acid (Bastam et al. 2013, Song et al. 2014) and nitric oxide (Zhang et al. 2006). N-acetyl-5-methoxytryptamine (Melatonin) is a natural indoleamine compound and an important animal hormone that takes part in numerous vital life processes, including sleep physiology, body temperature regulation, mood, circadian rhythms, immunological enhancement, seasonal reproduction, sexual behavior and antioxidative activity (Barrett and Bolborea 2012, Venegas et al. 2012, Calvo et al. 2013, Galano et al. 2013). Although melatonin was initially identified in animal pineal gland (Lerner et al. 1958), it has also been commonly found in various plant species (Hattori et al. 1995, Byeon and Back 2014, Shi and Chan 2014). Many studies showed that melatonin might take vital functions in growth, development and various stress responses of plants (Kolar and Macháˇcková 2005, Tan et al. 2012, Uchendu et al. 2013, Zhang et al. 2014, Wei et al. 2015, Shi et al. 2015a, Li et al. 2016). One of the most frequently reported roles of melatonin in plant is to protect against abiotic and biotic stress as an antioxidant (Tan et al. 2012, Szafranska et al. 2016), mainly including salinity (Jiang et al. 2016), drought (Wang et al. 2013), heavy metal (Posmyk et al. 2008) and low temperature stress (Shi et al. 2015a). Although the roles of melatonin in the antioxidant defense system have been widely studied under different environmental stresses in different plants (Posmyk et al. 2008, Wang et al. 2013, Bajwa et al. 2014, Wei et al. 2015, Shi et al. 2015a, Jiang et al. 2016), the detailed role of exogenous melatonin in protecting photosystem II (PSII) against salt stress is still very limited in maize.

In this study, we mainly aim to investigate the effects of different melatonin concentration on plant growth, reactive oxygen species (ROS) accumulation, antioxidant defense system and photosynthetic capacity in maize seedling exposed to salt stress. Furthermore, we also want to clarify the protective role of exogenous melatonin in PSII of maize under salt stress. This study provides some new insights into the improvement of salt resistance by melatonin in plants.

Materials and methods

Plant materials and treatments

Maize (Zea mays L.) seeds of uniform size were surface-sterilized using 0.1% (m/v) HgCl₂ for 10 min and rinsed twice with tap water. After that, maize seeds were germinated in Petri dishes with filter papers for 48 h at room temperature in the dark. Then, maize seedlings were cultured in plastic pots with 1/2 strength Hoagland’s solution (Chen et al. 2009a, 2009b). The plants were grown in a controlled growth room with a photoperiodic cycle of 16 h light/8 h dark at 25/22 °C, approximately 75% relative humidity and 180 μmol m⁻² s⁻¹ light intensity.

Two-week-old (the beginning of third-leaf stage) maize seedlings with uniform sizes were subjected to different treatments. Salt stress treatments were carried out as described by Jiang et al. (2016) with slight modifications. All maize seedlings were randomly divided into six groups: (1) 1/2 Hoagland’s solution (CK); (2) 1/2 Hoagland’s solution + 150 mM NaCl (Na); (3) 1/2 Hoagland’s solution + 20 μM melatonin (M1); (4) 1/2 Hoagland’s solution + 100 μM melatonin (M2); (5) 1/2 Hoagland’s solution + 150 mM NaCl (Na) + 20 μM melatonin (M1 + Na); (6) 1/2 Hoagland’s solution + 150 mM NaCl (Na) + 100 μM melatonin (M2 + Na). After 7 days of treatment, physiological and biochemical parameters were measured. For each treatment, at least three independent experiments were performed.

Photosynthetic pigment content, relative water content, soluble sugar and proline content

Chlorophyll (Chl) and carotenoid were extracted from fresh leaves with 80% (v/v) acetone and measured spectrophotometrically by the previously described method (Arnon 1949). Relative water content (RWC) was calculated according to the following formula: 
\[
\text{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100\% 
\]
 (Chen et al. 2015). Soluble sugar was extracted from the leaves in boiling water and its content was quantified with the method of Arthur-Thomas (1977). Proline contents were determined as described previously by Bates et al. (1973) with minor modifications. The extraction of proline was performed with 3% sulfosalicylic acid and then filtered. An aliquot of the filtrate was supplemented by adding 1 ml of ninhydrin and glacial acetic acid reagent. The reaction mixture was boiled (95°C) for 1 h. After stopping the reaction by putting the mixture on ice, the absorbance of the samples was determined at 520 nm by using a UV spectrophotometer.

Quantitation of endogenous melatonin

The extraction of melatonin from maize leaves was performed according to Pape and Lüning (2006). Maize
leaves (1 g) were ground in liquid nitrogen and homogenized fully in extraction solution (5 ml of 89/10/1 acetone/methanol/water). After 5 min, 4500 g centrifugation at 4°C, the resulting supernatant was transferred to a new EP tube with of 1% trichloric acid (0.5 ml) for protein precipitation. Then, 10 min 12000 g centrifugation at 4°C was performed. After centrifugation, the quantification of endogenous melatonin in the extracts was performed with melatonin enzyme-linked immunosorbent assay (ELISA) Kit (EK-DSM; Buhlmann Laboratories AG, Schonenbuch, Switzerland) following manufacturer’s instruction.

**Determination of Na⁺ and K⁺ contents**

The concentrations of Na⁺ and K⁺ were determined following the method of Chen et al. (2018). The root apices and leaves were thoroughly rinsed three times with distilled deionized water, and then oven-dried at 75°C for 48 h. The dried samples (100 mg) were incubated with HNO₃ (68%) and then were digested in a microwave digestion system (Ethos 900, Milestone Srl. Sorisole, Italy) for 30 min. The contents of Na⁺ and K⁺ were measured by inductively coupled plasma mass spectrometry (Optimal 2100DV, PerkinElmer Instruments, Waltham, MA).

**Measurement of endogenous abscisic acid contents**

Determination of abscisic acid (ABA) concentrations in maize leaves was performed according to Carvajal et al. (2017) with minor modifications. The leaves were ground in liquid nitrogen and homogenized with 80% methanol. After 30 min incubation at 4°C, the samples were centrifuged for 10 min at 12 000 g at 4°C and the pellets were extracted. The extracts were freeze-dried using the Scientz-10N (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China) and then were resolved in 20% methanol followed by filtration through 0.2 µm syringe filters. ABA was measured by liquid chromatography system (1290 LC, Agilent Technologies Inc., Santa Clara, CA) couple to a mass spectrum system (6470 LC-MS/MS, Agilent Technologies Inc., Singapore).

**Determination of electrolyte leakage and malonaldehyde content**

Electrolyte leakage (EL) values of maize leaves were measured with a conductivity meter (DDSJ-308A, Shanghai Precision Instruments Co. Ltd., Shanghai, China) as described by Chen et al. (2015). The relative EL was obtained according to the ratio of the initial conductivity to the absolute conductivity. Malonaldehyde (MDA) was extracted from maize leaves with thiobarbituric acid solution. After centrifugation, the absorbance of the supernatant was recorded at 450, 532 and 600 nm (Chen et al. 2015). The extinction coefficient is 155 mM⁻¹ cm⁻¹.

**Measurement of reactive oxygen species**

Hydrogen peroxide (H₂O₂) and superoxide (O₂—) in maize leaves were visually observed using 3,3-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), respectively. The second leaves were excised and soaked in 0.5 mg ml⁻¹ NBT or 2 mg ml⁻¹ DAB solution. Following NBT or DAB staining for 2–8 h with vacuum infiltration in the dark, the detached leaves were then decolorized using 95% ethanol for 0.5–2 h in a boiling water bath (Chen et al. 2015). At least three leaves were used for each treatment. The content of O₂— was quantified according to the previous method through recording the nitrate formation from hydroxylamine (Elstner and Heupel 1976). H₂O₂ content of leaves was quantified by the previous method of Okuda et al. (1991).

**Assay of antioxidants**

For determination of antioxidant enzymes, 0.5 g fresh maize leaves were ground in the chilled extraction buffer that contained 25 mM Hepes (pH 7.8), 2% (w/v) polyvinylpyrrolidone, 2 mM ascorbate and 0.2 mM Ethylenediaminetetraacetic acid (EDTA) on ice. After centrifugation (12 000 g for 30 min at 4°C), the supernatants were used for the subsequent enzyme and non-enzymatic antioxidant assays. To measure the activity of superoxide dismutase (SOD), the extraction buffer had no 2 mM ascorbate. The activities of antioxidant enzymes, namely catalase (CAT, EC 1.11.1.6), SOD (EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.8.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11), were assayed following the methods of Elstner and Heupel (1976), Foyer and Halliwell (1976), Nakano and Asada (1981), Egley et al. (1983), Flohé and Günzler (1984) and Cakmak and Marschner (1992), respectively. One unit of SOD activity was defined as the amount of enzyme that inhibited 50% reduction of NBT. One unit of POD activity was defined as the amount of the enzyme that caused an absorbance change of 0.01 per minute, and 1 unit of GR activity was defined as the amount of the enzyme that consumed 1 µmol of NADPH per minute.
The concentrations of dehydroascorbate (DHA) and reduced ascorbic acid (AsA) in 5% (w/v) of trichloroacetic acid were analyzed as described by Cakmak and Marschner (1992). The concentrations of oxidized glutathione/reduced glutathione (GSSG/GSH) were measured according to the method of Griffith (1980).

**Analysis of gas exchange and chlorophyll fluorescence**

Measurements of gas exchange parameters were conducted using the GSF-3000 photosynthesis system (Heinz-Walz Instruments, Effeltrich, Germany). CO₂ assimilation rate (Pn) was measured at a CO₂ concentration set to 360 μmol mol⁻¹ and 1000 μmol photons m⁻² s⁻¹, 70% relative humidity at room temperature (Yamori et al. 2006). The photosynthetic rates were determined at least 30 min after the attainment of the temperature. Internal conductance was measured for intact leaves by the concurrent measurements of the gas exchange rate and stable carbon isotope ratio. Gas exchange parameters were calculated as described previously (von Caemmerer and Farquhar 1981).

Modulated chlorophyll fluorescence was analyzed using the Imaging PAM M-Series Chlorophyll Fluorescence System (Heinz-Walz Instruments) following manufacturer’s instructions. Maize samples were adapted in dark for at least half-an-hour before fluorescence measurements. The actinic light intensity was set to 180 μmol m⁻² s⁻¹, and the saturated flash intensity was at 6000 μmol m⁻² s⁻¹ (620 nm) and duration of the saturation pulse was 0.8 s. The maximum efficiency of PSII (Fv/Fm), the quantum yield of PSII electron transport (ΦPSII), the photochemical quenching (qP) and the non-photochemical quenching coefficient (NPQ) values were obtained as described previously (Maxwell and Johnson 2000). The representative image data obtained from each experiment were normalized to a false color scale.

**Trypan blue staining**

The second leaves after different treatments were used for trypan blue staining following the protocol described by Koch and Slusarenko (1990). After staining, the leaves were cleared by 2.5 g ml⁻¹ chloral hydrate reagent.

**Protein western blotting analysis**

For thylakoid protein immunoblotting analyses, thylakoid membranes from leaves were prepared based on the method of Chen et al. (2016). The Chl concentration of the isolated thylakoid membranes was measured according to Porra et al. (1989). The thylakoid membrane proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were shifted to polyvinylidifluoride membranes (Immobilon, Millipore, Darmstadt, Germany), and then antisera to the D1, D2, Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6 and PsbS (Agrisera, Umea, Sweden) were applied. Then, the horseradish peroxidase-conjugated secondary antibody (Agrisera, Umea, Sweden) and a chemiluminescent detection system (ECL, GE Healthcare, Buckinghamshire, UK) were adopted for detecting the signals of western blotting. The quantitative analysis of signal amplitude was performed through Quantity One software (Bio-Rad Co., Hercules, CA).

**Statistical analysis**

In this study, all data were shown as the mean values ± SD (standard deviations). At least three independent replicates were conducted for each experiment. Statistical analysis was performed using the SPSS Statistics 19.0 software (IBM, Chicago, IL), and the means were compared using Duncan’s multiple range test. Different letters above the columns of figures indicated to be statistically significant at the 0.05 level among treatments.

**Results**

**Plant growth, chlorophyll and carotenoid content, and endogenous melatonin level under salt stress**

To test the effects of exogenous melatonin on plant growth, maize plants were grown in three different concentrations of melatonin (0, 20 and 100 μM). After 7 days, maize seedlings showed no significant differences between the melatonin-treated and not treated seedlings under normal condition (Fig. 1A). When salt stress was applied, the growths of salt-treated plants with or without melatonin were all inhibited. However, non-melatonin-treated seedlings were significantly shorter than 20 and 100 μM melatonin-treated plants (Fig. 1A). Consistently, Chl and carotenoid of melatonin-treated maize seedlings were nearly equivalent to those of control plants. Application of 100 μM melatonin significantly improved the chlorophyll and carotenoid level under 150 mM NaCl (Fig. 1B, C). These results indicated that exogenous melatonin treatment could promote growth of maize plants exposed to salinity.

To dissect how salt stress is affected by melatonin concentration, the endogenous melatonin concentration in maize plants was measured after treatments with
150 mM NaCl for 7 days. Melatonin content was approximately 4 pg g⁻¹ fresh weight in the control plants. When subjected to melatonin treatment and salt stress, the content of melatonin in maize leaves was remarkably induced by 67.5 and 567.5%, respectively (Fig. 1D). The significant increase in endogenous melatonin level under NaCl treatment suggested an in vivo role of melatonin in maize responses to salt stress.

**Effects of exogenous melatonin on Na⁺ and K⁺ contents and cell death under salt stress**

As shown in Fig. 2A-B, Na⁺ contents significantly increased while K⁺ contents remarkably decreased in maize leaves and roots exposed to salt stress. Under salt stress, 100 μM melatonin significantly increased the concentrations of K⁺ and decreased Na⁺ contents in leaves and roots. Although K⁺/Na⁺ ratio significantly decreased in maize leaves and roots under salt stress compared with control, melatonin application did not obviously alleviate the decline (Fig. 2C). To test whether melatonin alleviated the degree of cell death, maize leaves treated with exogenous melatonin were exposed to salt stress. Then, the second leaves were stained with trypan blue. The results revealed that salt stress resulted in intensely stained areas compared to the control plants (Fig. 2D). However, 100 μM melatonin-treated plants presented less blue stains than non-treated seedlings.

**Effects of exogenous melatonin on RWC, soluble sugar, free proline and abscisic acid under salt stress**

Compared with control, salt stress led to a significant decline in RWC, but this was alleviated by the melatonin application. Noticeably, 100 μM melatonin improved RWC more than 20 μM melatonin (Fig. 3A). Proline and soluble sugar usually act as important osmotic regulators in plants (Xu and Huang 2010). Compared to the control plants, the levels of soluble sugar and proline significantly increased in the leaves under salt stress. Application of exogenous melatonin decreased the contents of these osmotic regulators under salt stress (Fig. 3B, C). Furthermore, we found that ABA contents were significantly higher than that in the control plants. However, the melatonin application resulted in a significant decrease of ABA in salt-stressed plants (Fig. 3D). In addition, we found that RWC, two osmotic regulators and ABA content of exogenous melatonin-treated plants were generally equivalent to the control without salt stress (Fig. 3).

**Effects of exogenous melatonin on ROS accumulation and lipid peroxidation**

As important indicators of oxidative damage under environmental stresses, H₂O₂, O₂⁻, MDA and EL were...
Fig. 2. Effects of melatonin on K⁺ content (A), Na⁺ content (B), K⁺/Na⁺ ratio (C) and cell death (D) in salt-stressed maize seedlings. Bars represent the means ± s (n = 3). Columns labeled with different letters indicate significant differences with P < 0.05. The legend is as follows: Control, 0 NaCl + 0 melatonin (CK), 150 mM NaCl + 0 melatonin (Na), 0 NaCl + 20 μM melatonin (M1), 0 NaCl + 100 μM melatonin (M2), 150 mM NaCl + 20 μM melatonin (M1 + Na) and 150 mM NaCl + 100 μM melatonin (M2 + Na).

Fig. 3. Effects of melatonin on relative water content (A), proline (B), soluble sugar (C), and ABA contents (D) in maize seedlings under salt stress. Bars represent the mean ± s (n = 3). Different letters are significant differences at P < 0.05 according to Duncan’s multiple range test. The legend in A–C is as follows: Control, 0 NaCl + 0 melatonin (CK), 150 mM NaCl + 0 melatonin (Na), 0 NaCl + 20 μM melatonin (M1), 0 NaCl + 100 μM melatonin (M2), 150 mM NaCl + 20 μM melatonin (M1 + Na) and 150 mM NaCl + 100 μM melatonin (M2 + Na).

measured. NBT and DAB staining documented that there were no obvious differences between the control and melatonin-treated seedlings without salt stress (Fig. 4A, B). However, both O₂⁻ and H₂O₂ levels of the leaves were increased under salt stress. Accumulation of O₂⁻ and H₂O₂ decreased by 20.9 and 21.3% in the presence of 100 μM melatonin under salt stress, respectively (Fig. 4A, B). To verify these results, the contents of O₂⁻ and H₂O₂ in maize seedlings were further determined in the presence or absence of melatonin under salt stress. Exogenous melatonin showed no obvious fluctuations on H₂O₂, O₂⁻, MDA content and EL under control condition (Figs 4 and S1). When salt treatment was performed, melatonin-treated plants presented remarkably lower contents of H₂O₂, O₂⁻, MDA and EL in comparison with no-melatonin-treated plants, especially in 100 μM melatonin-treated plants (Figs 4 and S1). These facts indicated that exogenous melatonin might alleviate salt stress-induced ROS accumulation and the corresponding oxidative damage in maize plants.
Exogenous melatonin influenced enzymatic and non-enzymatic antioxidant activities under salt stress

Six antioxidant enzyme (CAT, SOD, POD, GR, GPX and APX) activities and the concentrations of non-enzymatic antioxidants (AsA, DHA, GSH and GSSG) in maize plants under salt stress in the situation of different melatonin concentrations were measured. Compared to the control, 20 and 100 μM melatonin did not result in significant oscillations in the activities of antioxidant enzymes and the contents of non-enzymatic antioxidants under control condition (Figs 5 and S1). Under salt stress condition, the activities of APX, CAT and GPX were greatly enhanced by 21.0, 68.2 and 49.4% respectively, while the activities of GR were significantly declined by 29.2% (Fig. 5B–F) compared to the control in the absence of melatonin. In addition, melatonin-treatment effectively improved the activities of six antioxidant enzymes compared with non-treated seedlings under salt stress (Fig. 5A–F). Additionally, the DHA and GSSG contents were significantly increased, while AsA and GSH contents were greatly decreased under salt stress (Fig. S2). When salt treatment was applied, melatonin-treated plants presented significantly higher contents of AsA and GSH, and lower contents of DHA and GSSG in comparison with non-treated seedlings. These data indicated that melatonin might have important effects on antioxidant enzymes and non-enzymatic antioxidants, which could be involved in alleviating salt stress-induced oxidative damage in maize.

Effects of exogenous melatonin application on photosynthesis under salt stress

To explore the protective role of melatonin in maize photosystem against salt stress, photosynthetic parameters were examined. Melatonin treatment displayed no obvious effects on Fv/Fm, NPQ, qP and ΦPSII under non-stressful condition (Fig. 6). However, salt stress led to a significant increase of NPQ and a noticeable decrease in qP and ΦPSII compared to the control. Under salt stress, 100 μM melatonin-treated plants presented significantly lower level of NPQ and higher value of qP and ΦPSII compared to non-melatonin-treated plants. Moreover, the net photosynthetic rate (Pn), intercellular CO₂ concentration, stomatal conductance and transpiration rate were investigated. The results showed that in the control seedlings, Pn, intercellular CO₂ concentration, transpiration rate and stomatal conductance were significantly higher than those in the salt-treated seedlings (Fig. 7). Without salt stress, Pn, transpiration rate, intercellular CO₂ concentration and stomatal conductance were not altered by 20 μM melatonin treatment but 100 μM melatonin application improved these parameters. Under salt stress condition, 100 μM melatonin-treated plants exhibited higher level of Pn, transpiration rate, intercellular CO₂ concentration and stomatal conductance than non-treated plants (Fig. 7). Taken together, these findings suggested that melatonin treatment played a positive role in protecting plant photosynthetic apparatus against environmental stress.
Effects of melatonin application on PSII proteins under salt stress

To understand in more detail modulation of thylakoid membrane proteins in the presence of exogenous melatonin under salt stress conditions, immunoblotting of thylakoid polypeptide composition was performed. The amount of almost all analyzed PSII proteins except for PsbS and D1 protein in melatonin-treated and non-treated seedlings were similar under control and salt stress conditions (Fig. 8). The amount of D1 protein in salt-stressed seedlings was remarkably decreased compared with the control (Fig. 8B). Interestingly, the level of PsbS protein significantly increased by approximately 70% in salt-stressed plants compared with control plants (Fig. 8A, B). However, exogenous melatonin application significantly alleviated these negative influences under salt stress. In the absence of salt stress, exogenous melatonin application did not lead to the marked differences in the levels of D1 and PsbS compared to the control plants. It is worth noting that the amount of Lhcb4/CP29 did not change significantly among these treatments, but Lhcb4 was strongly phosphorylated in salt-stressed plants in the presence or absence of melatonin (Fig. 8A).

Discussion

It is well known that salt stress markedly inhibits plant growth and result in the decrease of crop yield (Munns and Tester 2008). As higher plants, they have developed different strategies to response to various environmental stresses. Melatonin, as a kind of new plant growth regulator, is thought to be involved in different biotic and abiotic stress responses (Tan et al. 2012, Park et al. 2013, Yin et al. 2013, Wei et al. 2015, Shi et al. 2015a). In the present experiment, the positive protective role of melatonin in maize plants against salt stress was investigated.
Fig. 6. Effects of melatonin on chlorophyll fluorescence parameters (Fv/Fm, the maximum efficiency of PSII photochemistry; NPQ, non-photochemical quenching coefficient; qP, photochemical quenching and \( \Phi_{PSII} \), quantum yield of PSII electron transport) in maize seedlings under salt stress. Quantitative values (±SD) are shown below the individual fluorescence images. Values are mean ± SD (n = 3). The legend is as follows: Control, 0 NaCl + 0 melatonin (CK), 150 mM NaCl + 0 melatonin (Na), 0 NaCl + 20 \( \mu \)M melatonin (M1), 0 NaCl + 100 \( \mu \)M melatonin (M2), 150 mM NaCl + 20 \( \mu \)M melatonin (M1+Na) and 150 mM NaCl + 100 \( \mu \)M melatonin (M2+Na).

Previous works have indicated that melatonin might play an important regulatory role in plant growth and development (Chen et al. 2009a, 2009b, Tan et al. 2012, Wei et al. 2015). In this study, the growth of maize was notably inhibited when exposed to salt stress, but the inhibition of growth was obviously relieved by melatonin application. Shi et al. (2015a) reported that 20 and 100 \( \mu \)M melatonin pretreatment improved the height of bermudagrass under abiotic stresses, which is consistent with our results. In addition, many studies have indicated that melatonin might inhibit the decline of the photosynthetic pigments under different environmental stresses (Wei et al. 2015; Shi et al. 2015a). These findings were further confirmed by our data. Under salt stress condition, the application of exogenous melatonin showed higher content of photosynthetic pigments than the control (Fig. 1B, C). This observed decrease in pigments might be attributed not only to an increased degradation but also to an inhibited synthesis of chlorophylls due to salinity. It is well known that sodium ions could interact with the negatively charged plasma membrane, which would lead to depolarization of the transmembrane potential and changes of \( H^+ \)-ATPase activity, and subsequently disrupt the uptake of some cations, such as \( Mg^{2+} \), impacting chlorophyll biosynthesis.

Fig. 7. Effects of melatonin on maize seedlings under salt stress on net photosynthetic rate (A), transpiration rate (B), intercellular CO2 concentration (C) and stomatal conductance (D). \( P_{n} \), net photosynthetic rate. Values are mean ± SD (n = 3). Different letters are significant differences at \( P < 0.05 \) level (Duncan's multiple range test). The legend in A–D is as follows: Control, 0 NaCl + 0 melatonin (CK), 150 mM NaCl + 0 melatonin (Na), 0 NaCl + 20 \( \mu \)M melatonin (M1), 0 NaCl + 100 \( \mu \)M melatonin (M2), 150 mM NaCl + 20 \( \mu \)M melatonin (M1+Na) and 150 mM NaCl + 100 \( \mu \)M melatonin (M2+Na).
A previous study indicated that melatonin application significantly decreased the accumulation of Na\(^+\) and resulted in an obvious increase of K\(^+\) contents in maize shoots under salinity (Jiang et al. 2016). However, our study demonstrated that this phenomenon exists in both roots and leaves under salt stress. In addition, Jiang et al. (2016) also found that 1 \(\mu\)M melatonin improved the photosynthetic and antioxidant capacities and enhanced salt tolerance in maize seedlings. However, we found that 2 \(\mu\)M melatonin could not increase significantly salt tolerance of maize seedlings (data not shown). The differences may be due to the concentration of melatonin, which will additionally act as an antioxidant at high concentrations in plants under environmental stresses.

In addition, the melatonin level in vivo was markedly induced by salt stress in maize, suggesting that melatonin may play a regulator role or antioxidants in response to environmental stresses in plants (Tan et al. 2012, Shi and Chan 2014, Shi et al. 2015a). In the present study, slight differences in the increase of the activities of antioxidant enzymes activity were observed between 20 and 100 \(\mu\)M melatonin treatment indicating that melatonin might play a regulatory role. However, 100 \(\mu\)M melatonin was more effective in decreasing the accumulation of ROS than 20 \(\mu\)M melatonin, suggesting that melatonin at high concentrations might act as an antioxidant and be directly involved in the scavenging of ROS. Furthermore, the remarkable high melatonin concentrations under salt stress in the presence of exogenous melatonin may be due to the synergetic effect of melatonin and environmental stresses. Therefore, further research is required to elucidate the detailed reasons.

It has been known that abiotic stress usually causes the decrease in leaf water status and the increase in osmotic regulators (Xu and Huang 2010, Jiang et al. 2016). In accordance with these results, we observed that salt stress led to a decline in RWC and an increased level of soluble sugar and proline (Fig. 3). In addition, it has been demonstrated that ABA can induce the accumulation of osmotic regulators such as proline and soluble sugar under environmental stresses (Urano et al. 2009). In the present study, the level of ABA content increased proportionally with the increase in osmoprotectant under salt stress. It has been previously reported that exogenous melatonin may reduce ABA contents under drought stress (Li et al. 2015). This result was further demonstrated in our experiment. However, melatonin application alleviated the decline in leaf water status, indicating
that melatonin might improve the water absorbing or holding capacity, probably through regulating the level of ABA and decreasing the accumulation of Na+ (Jiang et al. 2016).

Multiple publications have indicated that melatonin alleviates ROS production triggered by abiotic stress (Cano et al. 2006, Wei et al. 2015, Zhang et al. 2015, Shi et al. 2015a, 2015b). A previous study indicated that exogenous melatonin significantly suppressed the production of hydroxyl radicals and H$_2$O$_2$ (Li et al. 2012). Consistently, our research suggested that salt stress resulted in ROS accumulation in maize leaves, but the application of exogenous melatonin alleviated ROS accumulation (Fig. 4A, B). Under environmental stresses, the accumulation of ROS usually leads to damaging the integrity of cell membrane and cell death (Smirnoff 1993). MDA and EL are thought to be important indicators of oxidative damage impacting the membrane’s integrity (Zhang et al. 2014). In the present experiment, lower levels of EL and MDA were observed in melatonin-treated seedlings under stress condition, which was consistent with the decrease of ROS generation, further indicating that melatonin might protect cell membranes against oxidative damage induced by salt stress (Jiang et al. 2016). In addition, our study also showed that a lower rate of cell death was found in melatonin-treated plants under salt stress, indicating that melatonin could alleviate cell death by decreasing ROS accumulation under environmental stresses in plants.

To reduce stress-triggered ROS accumulation, plants have evolved an effective antioxidant system, including enzymatic and non-enzymatic antioxidants. In plants, melatonin has also been suggested to be a crucial antioxidant that can scavenge oxygen free radicals effectively (Cano et al. 2006, Wei et al. 2015, Shi et al. 2015a, 2015b). Many studies have indicated that exogenous melatonin application can increase some antioxidant enzyme (such as POD, SOD and APX) activities under abiotic stress in plants (Li et al. 2012, Zhang et al. 2014, Shi et al. 2015a, Jiang et al. 2016). Consistently, we showed that melatonin application increased the activities of antioxidant enzymes in maize seedlings under salt stress (Fig. 5A–F). In the present experiment, the high activities of CAT, POD, GR and APX under salt stress in the presence of melatonin may mainly be due to the synergistic effects of the combination of salt stress and exogenous melatonin. Moreover, the AsA–GSH cycle is a vital antioxidant system against oxidative stress in plants (Zhang et al. 2015). Previous works showed that melatonin treatment resulted in the maintenance of higher contents of AsA and GSH (Wang et al. 2013, Shi et al. 2015a). In accordance with these reports, our study suggested that AsA and GSH content were markedly induced in melatonin-treated seedlings under salt stress. These findings suggested that exogenous melatonin application dramatically activated active oxygen scavenging system including enzymatic and non-enzymatic antioxidants to maintain a relatively low level of ROS, further conferring salt stress resistance to maize. Therefore, the positive effects of exogenous melatonin on the active oxygen scavenging system could improve stress resistance of plants.

It has been shown that melatonin can preserve chlorophyll and improve the photosynthetic efficiency in plants under stressful conditions (Yin et al. 2013, Zhao et al. 2015, Jiang et al. 2016, Li et al. 2018). Consistent with these findings, our results indicated that 100 μM melatonin application significantly improved the levels of qP and ΦPSII under NaCl stress (Fig. 6). But 20 μM melatonin did not present as remarkable effects on photochemical efficiency. The decrease in qP in the presence of melatonin under salt stress was probably because exogenous melatonin affects the water-splitting complex or because it disturbs the organization of thylakoid protein complex. Under salt stress combined with melatonin treatment, the slight reduction in ΦPSII could be due to less downregulation of the linear electron flow, suggesting that melatonin acts as a protective mechanism that either match the lower demand for NADPH or avoid the excessive accumulation of ROS. However, the lower level of NPQ in the presence of melatonin under salt stress indicates that melatonin plays a regulatory role in the dissipation of light energy. Furthermore, the increase of photosynthetic capacity was also confirmed by high Chl content, stomatal conductance and intercellular CO$_2$ concentration. These findings were in line with the recent report of tomato under salt stress (Zhao et al. 2015). Therefore, the protective effect of exogenous melatonin on the photosynthetic efficiency in plants seems to depend on the melatonin concentration.

Numerous works have indicated that D1 protein is the key target under environmental stresses (Murata et al. 2007, Yamamoto et al. 2008, Su et al. 2014, Chen et al. 2016). In accordance with these reports, we found that the content of D1 was significantly decreased under salt stress. This decrease may be one of the main reasons for the photoinhibition. Melatonin has been observed to protect proteins against oxidative damage and regulate the levels of senescence-associated proteins (Byeon et al. 2012, Zhang et al. 2015). In this study, the application of exogenous melatonin made those plants maintain significantly high content of D1 protein under salt stress (Fig. 8), indicating that melatonin could counteract the reduction of D1 protein under environmental stresses, thereby possibly contributing to greater stress resistance.
H2O2 may result in the generation of the highly reactive hydroxyl radical (Hancock et al. 2005). In addition, superoxide and hydroxyl groups and reduce the photosynthetic CO2 assimilation and inhibit the activities of enzymes possessing sulfhydryl groups. Therefore, these results indicated that the PSII supercomplex is probably disassembled to some extent in the presence of melatonin. A recent research showed that the level of PsbS was markedly increased in Arabidopsis plants under water stress (Chen et al. 2016). In accordance with this work, our result showed that salt stress induced the upregulation in the level of PsbS, which is a key regulator of the energy dissipation process (Johnson and Ruban 2010). However, exogenous melatonin application alleviated the increase of PsbS protein content under NaCl stress, suggesting that melatonin plays a certain role in the regulation of excitation energy dissipation by changing the level of PSII proteins under environmental stresses.

It is well demonstrated that the major generation site of ROS in the thylakoid membrane is PSI and PSII (Gill and Tuteja 2010). Among ROS, H2O2 in chloroplast is an important inhibitor of the Calvin cycle. It may inhibit the activities of enzymes possessing sulfhydryl groups and reduce the photosynthetic CO2 assimilation (Hancock et al. 2005). In addition, superoxide and H2O2 may result in the generation of the highly reactive hydroxyl radical responsible for extensive damage to photosynthetic membranes and proteins through the metal-catalyzed Haber-Weiss reaction (Gill and Tuteja 2010). However, melatonin and its metabolite might bind to Fe ion involved in this reaction, and thus prevent catalysis (Limson et al. 1998, Maharaj et al. 2003). Therefore, melatonin application may alleviate the extensive damage caused to the photosynthetic apparatus by regulating the levels of reactive hydroxyl radical under environmental stresses.

Taken together, our data provide the detailed protective evidence of exogenous melatonin in maize against salt stress by investigating the antioxidative defense system and photosynthetic machinery. This protective role may be involved in the activation of antioxidant defense system, the elimination of ROS and the protection of photosynthetic apparatus.

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Author contributions
Y.-E.C., J.-J.M. and L.-Q.S. performed the experiments. Y.-E.C. and Y.M. designed the experiments and wrote the article. S.Y. and Z.-W.Z. participated in the design of the study. B.H. and Y.G. participated in the measurement of chlorophyll fluorescence. J.-Q.L. and C.H. helped to measure the concentrations of melatonin. C.-B.D. participated in the analyses of the data.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Effects of melatonin under salt stress on superoxide anion radicals (O₂⁻) production rate and hydrogen peroxide (H₂O₂) content in maize seedlings.

Fig. S2. Effects of melatonin under salt stress on the level of non-enzymatic antioxidants in maize seedlings.