Melatonin Improves Waterlogging Tolerance of *Malus baccata* (Linn.) Borkh. Seedlings by Maintaining Aerobic Respiration, Photosynthesis and ROS Migration

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Waterlogging, one of the notorious abiotic stressors, retards the growth of apple plants and reduces their production. Thus, it is an urgent agenda for scientists to identify the suitable remedies for this problem. In the current study, we found that melatonin significantly improved the tolerance of apple seedlings against waterlogging stress. This was indicated by the reduced chlorosis and wilting of the seedlings after melatonin applications either by leaf spray or root irrigation. The mechanisms involve in that melatonin functions to maintain aerobic respiration, preserves photosynthesis and reduces oxidative damage of the plants which are under waterlogging stress. Melatonin application also enhances the gene expression of its synthetic enzymes (MbT5H1, MbAANAT3, MbASMT9) and increases melatonin production. This is the first report of a positive feedback that exogenous melatonin application promotes the melatonin synthesis in plants. A post-transcriptional regulation apparently participated in this regulation. When exogenous melatonin meets the requirement of the plants it is found that the protein synthesis of MbASMT9 was suppressed. Taken together, the results showed that melatonin was an effective molecule to protect plant, particularly apple plant, against waterlogging stress.

**Keywords:** melatonin, waterlogging, ROS, oxidative stress, aerobic respiration, photosynthesis

**INTRODUCTION**

Waterlogging, is a major agricultural constraint that limits crop growth and reduces their yield (Xu et al., 2013). It is frequently encountered during the raining seasons in many areas worldwide. The excessive waterlogging causes root damage, impairs the water uptake and, finally leads to chlorosis and wilting of the plants (Arbona et al., 2008). It was estimated that waterlogging stress resulted in nearly 40–80% of the crop yield loss in the area greater than 17 million km² (Voesenek and Sasidharan, 2013; Shabala et al., 2014).

The reactive oxygen species (ROS) is believed to play a critical role in the response of plant to waterlogging stress. At the early stage during waterlogging, the elevated ROS molecules functions as an important second messenger in signaling for response. Following the prolonged waterlogging, the increased anaerobic respiration of root and the responsive stomata closure in leaves induce a burst of excessive ROS production. If the excessive ROS is not migrated properly, it will cause plant oxidative damage and finally, it leads to roots rotting and leaves wilting (Hossain et al., 2009).
Plants have already developed a series of antioxidant mechanisms to defend themselves against oxidative stress. These include small molecule antioxidants (SMA) and antioxidant enzymes (Apel and Hirt, 2004; Mittler et al., 2004). SMA includes ascorbic acid, carotenoids, tocopherol, glutathione, polyphenol, etc. They can scavenge ROS with different chemical reactions (Sмирнов, 2000; Kuzniak and Sklodowska, 2001; Foyer and Noctor, 2005). The antioxidant enzymes are mainly those of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), etc. Both SMA and antioxidant enzymes work coordinately to keep the oxidative stress in check (Hossain et al., 2009). In addition to the SMA mentioned above, melatonin (N-acetyl-5-methoxytryptamine) is a potent free radical scavenger and an antioxidant (Tan et al., 1993, 2012). Melatonin was identified in plants in Dubbels et al. (1995) and Hattori et al. (1995). Since then it has been reported to exist in many plants and plant products (Ramakrishna et al., 2012; Yip et al., 2013; Shi et al., 2015b; Zhao et al., 2015a; Li C. et al., 2016; Ma et al., 2016; Xu et al., 2016). Different from other antioxidants, it is an amphiphilic molecule which makes it distribute in all cellular compartments including cytosol, membrane, mitochondria and chloroplasts (López et al., 2009; Byeon et al., 2013; Back et al., 2016). Also, melatonin as well as its metabolites can eliminate different kinds of ROS including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH), singlet oxygen (¹O$_2$), peroxynitrite anion (ONOO⁻) and nitric oxide (NO) (Tan et al., 2013). Melatonin has been reported to protect plants against a variety of abiotic and biotic stresses (Zuo et al., 2014; Liang et al., 2015; Liu et al., 2015; Shi et al., 2015a; Wang et al., 2015; Zhao et al., 2015b; Li C. et al., 2016; Xu et al., 2016). The transgenic Arabidopsis plants, ecotopically expressing melatonin synthetic gene MzASMT1, had higher endogenous melatonin production and significantly lower ROS than that of their wild types. The melatonin enriched transgenic Arabidopsis had a greater tolerance to drought stress than the wild types (Zuo et al., 2014). The exogenous melatonin also improved tolerance of tomato plants against alkaline stress by migrating O$_2^-$ and H$_2$O$_2$ (Liu et al., 2015). Melatonin application to apple leaves alleviated the drought-induced inhibition of photosynthesis (Wang et al., 2012). However, the effects of the exogenous melatonin application on apple plants which are under the sustained waterlogging condition have not been reported yet.

Reduced apple yield caused by waterlogging stress is a worldwide problem to be solved. Apple is a perennial woody plant, thus, the damage from waterlogging stress not only reduces apple yield of the current year, but also suppresses the tree vigor which also leads to the yield loss in the following years (Qu et al., 1999). Malus baccata (Linn.) Borkh. is used as a rootstock, which frequently suffers from the waterlogging stress (Wang et al., 2013). Therefore improvement of its waterlogging resistance by melatonin application will provide a potential cultivation method for apple production.

In current study, we investigated the protective effects of melatonin on M. baccata seedlings, which were subjected to the waterlogging stress. Furthermore, the potential mechanisms of these protections were also explored and discussed.

**MATERIALS AND METHODS**

**The Cultivation of Plant Material**

Seeds of apple (M. baccata) were sown in sopy vermiculite. Two weeks later, the seedlings were watered with half-strength Hoagland’s nutrient solution (Li M.Q. et al., 2016). When the seedlings developed to have four leaves, they were watered with complete nutrient solution. The plants were kept in green house with the temperature at a constant 22 ± 2°C and a 16/8 h light/dark cycle. The light intensity was approximately 100 µmol.m$^{-2}$.s$^{-1}$.

**Waterlogging Stress/Melatonin Treatment and Sample Collection**

After the M. baccata seedlings developed to have four leaves, a total of 96 seedlings were transplanted into the glass container with sterilized matrix soil. They were divided into eight groups. Plants in group I were watered with the 200 mL normal nutrient solution. Totally 25 mL normal nutrient solution was added every 3 days as control. The waterlogging stress was conducted in the remaining seven groups by keeping the soil being covered with 300 mL the nutrient solution and added 25 mL normal nutrient solution every 3 days.

The seedlings from group II was waterlogging stressed without supply of exogenous melatonin. The waterlogging stressed seedlings from group III to VIII were treated with different concentration of melatonin by spraying or irrigation. Melatonin was dissolved in 100% ethanol at a concentration of 10 mM and stored at −20°C as a stock solution. When use, melatonin was then diluted into 50, 100, and 200 µM, respectively with deionized water. These different concentrations of melatonin were sprayed to the leaves of seedlings every other day in group III, IV, and V, respectively. Two pieces of hardboard were used to avoid sprayed melatonin dropping into the soil. In group VI, VII, and VIII, melatonin was directly supplemented to the nutrient solution at the concentrations of 200, 400, and 600 µM, respectively. Groups I and II was also applied with equal volume of ethanol. The experiments were independently repeated three times. Photos were taken before and after 9 days of waterlogging stress/melatonin treatments.

The leaves and roots were collected from the all groups of seedlings, respectively, before and after 9 days of waterlogging stress/melatonin treatment.

**Melatonin Measurement**

The leaves collected from the seedlings were immediately frozen at −80°C for future melatonin detection. Around 1 g of frozen leaves of M. baccata was ground to a fine powder in liquid nitrogen. The powder was mixed with 10 mL methanol and ultrasonicated (80 Hz) for 35 min at 45°C. The sample preparation and HPLC detection of melatonin were performed as described by Zhao et al. (2013). Each experiment was independently repeated three times.

**RNA Extraction and RT-PCR Analysis**

Total RNA was isolated from the leaves of seedlings of the eight groups, respectively, before and after 9 days of waterlogging stress/melatonin treatment.
stress/melatonin treatment, using the EASY spin Plant RNA Rapid Extraction Kit (Biomed, Beijing, China). The first-strand cDNA was synthesized following the protocol of Kit (Promega, Madison, WI, USA). The cDNAs were used as template for RT-PCR. The specific primers were designed according to the sequence of melatonin synthesized enzyme genes MbASMT9 (KJ156531), MbAANAT3 (KJ156532), and MbT5H1, respectively, by Primer 5 software and checked by BLAST search in the apple genome.

Western blot was stained by Coomassie Brilliant Blue. The MbASMT9 antibody preparation were reviewed and approved by Beijing Municipal Science and Technology Commission. The experimental protocols of the GST-MbASMT9 antibodies in rabbit. The preparation of GST-MbASMT9 was used as an antigen to raise polyclonal antibodies with antibody of MbASMT9 (rabbit, 1:3000). The recombinant MbASMT9 was confirmed by SDS–PAGE. The total protein was isolated from leaves of seedlings of the GST-MbASMT9 protein was confirmed by SDS–PAGE.

Protein Extraction and Western Blot Analysis

The total protein was isolated from leaves of seedlings according to Wang et al. (2006). Western blot was applied with antibody of MbASMT9 (rabbit, 1:3000). The recombinant GST-MbASMT9 was used as an antigen to raise polyclonal antibody of MbASMT9 antibodies in rabbit. The preparation of MbASMT9 antibody was carried out in accordance with relevant guidelines and regulations. The experimental protocols of the MbASMT9 antibody preparation were reviewed and approved by Beijing Municipal Science and Technology Commission. The chemiluminescent signals were detected using an ECL detection kit (Amersham-Pharmacia, USA). The loading control of the Western blot was stained by Coomassie Brilliant Blue.

Detection of Antioxidant Enzyme Activities

A total of 0.3 g leaves or 0.3 g roots of M. baccata seedlings were ground with 8 mL chilled 50 mM phosphate buffer (pH 7.8), then they were transferred into 10 mL tubes and centrifuged at 4°C for 15 min at 10,000 g. The supernatants were diluted with phosphate buffer to 10 mL and used for enzyme activity detection. The enzyme activity of SOD was detected according to the method of Stewart and Bewley (1980). CAT activity was measured as the absorbance at 240 nm wave length according to the method of Ma et al. (2016). POD ( Peroxidase) activity was measured by the changes in absorbance at 470 nm due to guaiacol oxidation according to the method of Shi et al. (2015b). Each experiment was independently repeated at least three times.

Measurements of Enzyme Activities of Alcohol Dehydrogenase (ADH) and Succinate Dehydrogenase (SDH)

A total of 0.3 g roots for each group were collected to detect the enzyme activity of ADH and SDH before and after 9 days of waterlogging stress/melatonin treatment to identify the alterations of anaerobic and aerobic respiration. ADH activity was measured as reported by Yamashita et al. (2015). SDH activity was detected according to Landi et al. (2009). Each experiment was independently repeated at least three times.

Detections of ROS Level and Malondialdehyde (MDA) Content

The ROS level in roots was detected according to the method modified from Zuo et al. (2014). The intact roots were collected from each group and the fresh roots were immediately used for ROS detection before and after 9 days of waterlogging stress/melatonin treatment. Simply, the entire roots were incubated with the 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) solution for 20 min and washed with distilled H2O to remove excess CM-H2DCFDA. The fluorescence images were obtained with a Leica stereoscope (Leica, Wetzlar, Germany) (×10).

In addition to roots, the ROS level in leaves was also detected. The leaves were collected from the each group, respectively before and after 9 days of waterlogging stress/melatonin treatments and used for detection of O2•− and H2O2 immediately. For O2•− measurement, the leaf histochemical staining was vacuum infiltrated with 0.1 mgmL−1 nitroblue tetrazolium in 25 mM K-HEPES buffer (pH 7.9) for 40 min. Then the samples were kept at 25°C in dark for an additional 4 h. For H2O2 detection the leaves were vacuum infiltrated with 0.1 mg·mL−1 DAB in 50 mM Tris-acetate (pH 3.8) and were incubated at 25°C in dark for 24 h. Then leaves for either O2•− or H2O2 detection were washed in 80% ethanol every 10 min at 80°C until the leaves lost green color completely (Gong et al., 2014). The MDA detection in the leaf samples were performed according to Zhao et al. (2013). Each experiment was independently repeated at least three times.

1https://www.rosaceae.org/species/malus/all
2http://en.wikipedia.org/wiki/ImageJ

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Analyses of Chlorophyll (Chl) Content and Photosynthetic Rate (Pn) in Leaves of Seedlings

The extraction of Chl was conducted according to Porra et al. (1989). A total of 0.2 g leaves were homogenized in 2–3 mL 80% acetone. After filtration with four layers of gauze (1 mm × 1 mm), the homogenate was diluted with 80% acetone to 25 mL. The absorbance of the extract was measured at 645 and 663 nm. Chl concentration was calculated from the following equations: Chl a = 12.72 × OD663 − 2.59 × OD645; Chl b = 22.88 × OD645 − 4.67 × OD663; chl = chl a + chl b (Arnao and Hernández-Ruiz, 2009).

The photosynthetic rate was measured by LI-6400XT (LI-COR, Lincoln, NE, USA) according to the producer’s protocol. The light intensity was at 800 µmol·m^{-2}·s^{-1}. The humidity was about 50% and the temperature was 23°C. Each experiment was independently repeated three times.

Statistical Analysis

The data are expressed as means ± SD. One-way ANOVA was used for the normality evaluation followed by a Tukey–Kramer multiple comparison test. The statistical significant difference was set up when P < 0.05. Statistical evaluations were carried out using SPSS software (IBM, Armonk, NY, USA).

RESULTS

Melatonin Applications Improved the Tolerance of M. baccata Seedlings against Waterlogging Stress

The results showed that waterlogging stress significantly impaired the M. baccata seedling growth. Those plants which were under waterlogging stress for 9 days wilted severely compared to the normal controls (Figure 1A). When melatonin was sprayed to
the seedlings at the concentrations of 50, 100, and 200 µM, respectively, it was apparent that melatonin spraying improved tolerance of seedlings against waterlogging stress. The protective effects of melatonin indicated a dose-responsive manner. The seedlings treated with 200 µM of melatonin, even under the waterlogging stress, showed a similar phenotype as the normal controls. The similar results were also observed in the seedlings those melatonin was irrigated (Figure 1B). When melatonin was irrigated to the seedlings at the concentrations of 200, 400, and 600 µM, respectively, it also increased waterlogging resistance of seedlings as a dose-dependent manner. The irrigation with 600 µM of melatonin, gained a similar phenotype as spraying with 200 µM melatonin. The percentage of leaf chlorosis was also analyzed. It was clearly to see that the percentage of leaf chlorosis was significantly increased after waterlogging treatment. After melatonin was applied to the seedlings under waterlogging, the percentage of leaf chlorosis were significantly decreased (Supplementary Figure 1).

The Effects of Waterlogging Stress and Melatonin Application on De novo Melatonin Synthesis in Seedlings

To find the potential effects of waterlogging stress and melatonin application on the endogenous melatonin synthesis, the melatonin level and its synthetic gene expression were detected. The results showed that waterlogging stress significantly elevated plants melatonin levels in leaves (87 vs. 21 ng/g FW of control) and in roots (73 vs. 16 ng/g FW of control) (Figure 2A).

When exogenous melatonin was applied to the stressed seedlings, their endogenous melatonin was further increased compared to the plants under waterlogging stress alone (Figure 2A). For example, the endogenous leaf melatonin level in melatonin (200 µM) sprayed plants was 256 ng/g FW, which was 2.9 times higher than that in leaves of waterlogged seedling alone (87 ng/g FW). The similar results were observed in roots and also in melatonin irrigated seedlings. In accordance with the elevated melatonin production, the gene expressions of melatonin synthetic enzymes including ASMT (acetylserotonin O-methyltransferase), AANAT (aralkylamine N-acetyltransferase) and T5H (tryptamine 5-hydroxylase), were slightly upregulated under the waterlogging stress and melatonin treatment compared to the controls (Figure 2B). The upregulated gene expression of ASMT9, which is the supposed melatonin synthetic rate-limiting enzyme, failed to result in the increase in its protein level. In contrast, its protein level was declined when compared to the controls (Figure 2C). Obviously, the post-transcriptional regulation occurred for ASMT9.

The Effects of Melatonin on Antioxidant Enzymes

The activities of the antioxidant enzymes including SOD, POD, CAT were measured in four groups of M. baccata seedlings. These groups included the normal control group, waterlogging stress alone, waterlogging stress treated with melatonin (200 µM spray and waterlogging stress treated with melatonin (600 µM) irrigation. Waterlogging stress significantly suppressed all antioxidant enzymes tested in both levels and roots of the seedlings. However, melatonin treatment could recover the activities of these antioxidant enzymes suppressed by waterlogging stress in a great degree. For example, melatonin (200 µM) spray recovered the activity of CAT in leaves to the level comparable to the control seedlings (Figure 3).

Effects of Melatonin on Aerobic and Anaerobic Respiration of M. baccata Seedlings under Waterlogging Stress

The activities of ADH and SDH indicate the anaerobic and aerobic respirations, respectively. It was found that the ADH activity (11.71 U/mg), the index of anaerobic respiration, was significantly higher in the roots of waterlogging stressed seedlings
than that of normal control (7.01 U/mg). Both melatonin spray and irrigation reduced the anaerobic respiration indicated by decreased ADH activity (Figure 4A). In contrast, waterlogging stress suppressed the aerobic respiration indicated by the significantly reduced SDH activity compared to the control seedlings. This tendency was reversed by melatonin treatment in a great degree (Figure 4B).

The Effect of Melatonin on \( \text{O}_2^{\cdot-} \) and \( \text{H}_2\text{O}_2 \) Production and the Oxidative Damage Induced by Waterlogging Stress

Waterlogging stress significantly elevated the \( \text{O}_2^{\cdot-} \) and \( \text{H}_2\text{O}_2 \) production both in leaves and in roots of the seedlings. These were indicated by the increased fluorescent staining intensities on them. Melatonin treatment significantly reduced \( \text{O}_2^{\cdot-} \) and \( \text{H}_2\text{O}_2 \) productions, no matter melatonin was sprayed to the leaves or irrigated to the roots of the seedlings (Figures 5A,C). Accordantly, waterlogging stress caused oxidative damage in both leaves and roots of the seedlings. This was indicated by the increased content of MDA. It was expected that melatonin treatment significantly reduced the MDA levels in leaves and also in roots (Figures 5B,D).

The Effect of Melatonin on the Chlorophyll Content and Photosynthetic Rate

Waterlogging stress dramatically reduced the Chl content in the leaves of seedlings. After 9 days of waterlogging stress, the Chl content of these plants (2.4 mg/g FW) was only roughly half of the value of control seedlings (4.1 mg/g FW). The photosynthetic rate of the waterlogging stressed plants was 1.67 µmol·m\(^{-2}\)·s\(^{-1}\). The value was significantly lower than that of control seedlings (2.67 µmol·m\(^{-2}\)·s\(^{-1}\)). Melatonin treatments (spray or irrigation) improved both Chl content and photosynthetic rate which were suppressed by the waterlogging stress (Figures 6A,B).

DISCUSSION

Waterlogging, caused by poor drainage, flooding, and long periods of rainfall, usually occurs in summer and autumn, which hampers apple tree growth and results in yield loss (Yu et al., 2015). Because of the global warming effect, it is predicted that this climate change will bring more rainfall yearly and thus
FIGURE 5 | Effects of melatonin on ROS levels and oxidative damage in M. baccata seedlings which were under waterlogging stress. (A) ROS level in roots. (B) MDA production in roots. (C) The levels of superoxide anion and hydrogen peroxide in leaves. (D) MDA production in leaves. The waterlogging stress lasted for 9 days. Scale bars in (A) represent 0.5 cm. Scale bars in (C) represent 1 cm. The data are means ± SD of triplicate experiments. Different letters indicate significant differences from the control ($P < 0.05$).

FIGURE 6 | Effects of melatonin on the chlorophyll content and photosynthetic rate of the M. baccata seedlings before and after waterlogging stress. (A) Chlorophyll content. (B) Photosynthetic rate. The data are means ± SD of triplicate experiments. Different letters indicate significant differences from the control ($P < 0.05$).

the waterlogging will be frequently encountered by the crops worldwide. This will bring severe problem for these crops which are intolerant to the waterlogging stress including the apple trees. To solve this problem, it has become an urgent agenda for scientist.

In the current study, a unique molecule, melatonin, was tested with this purpose. Melatonin was reported to enhance plant tolerance against various abiotic stressors including cold, hot, drought, salinity, and chemical pollutants (Bajwa et al., 2014; Wei et al., 2014; Liang et al., 2015; Liu et al., 2015; Shi et al., 2015b;
expression of the melatonin synthetases and increased melatonin support this consideration. Waterlogging induced the gene et al., 2015). Our observation provided new evidence to as self-defense of organisms against external insults (Tan 2012; Li C. et al., 2016; Xu et al., 2016). This is considered production in plants as well as in animals (Byeon et al., 2012). Application significantly increased melatonin level of the plants. [Figure 3, 5]. This observation provided valuable information as to use of exogenous melatonin to improve plant tolerance to against stressors.

There are accumulated reports described the waterlogging caused transition from aerobic to anaerobic respiration in root, which is also confirmed by us (Figure 4). Our results also uncovered the role of melatonin to maintain aerobic respiration under waterlogging stress, by efficient suppression of the ROS burst and subsequent mitochondria degradation. The chlorosis is a typical sign unavoidably happening after severe waterlogging. Due to the reason that waterlogging leads to water shortage and subsequent quick stomata closure, the high concentration of \(O_2\) can’t be released out and photosynthetic electron transportation is blocked in chloroplasts. Therefore, the ion leakage from the electron transport chain would induce in over-produced \(O_2^-\) and \(H_2O_2\), which destroy chlorophyll and lead to the disintegration of chloroplasts (Musgrave, 1994; Smethurst and Shabala, 2003). The protective effects of melatonin on Chl decay, photosynthetic capacity and stomata configurations have been reported previously in other stressors such as in drought and hot (Wang et al., 2012; Xu et al., 2016). Actually, chloroplasts, as one of the most suffered organelles from ROS, were proved to be the major site for melatonin production. A large amount of melatonin is needed to maintain its structure and function. Therefore absorbed and in \textit{vivo} synthesized melatonin can function together to migrate waterlogging induced ROS and help to survive the stress. Here we reported that melatonin application preserved the Chl content and maintained the photosynthetic rate in seedlings suffered from waterlogging stress. The high content of Chl and efficient photosynthesis are required for high yield of apple production (Li et al., 2000). It is our
speculation that melatonin application in the field will increase the tolerance of apple tree and reduces apple yield loss against waterlogging stress. The speculated mechanisms are summarized in Figure 7.

AUTHOR CONTRIBUTIONS

Designed the studies: JK. Undertook the experimental work: XZ, JZ, NW, LW, and DS. Contributed to figures and manuscript preparation: XZ, D-XT, and JK. All authors read and approved the final manuscript.

REFERENCES

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399. doi: 10.1146/annurev.arplant.55.031903.141701

Arbona, V., Hossain, Z., López-Climent, M. F., Pérez-Clemente, R. M., and Gómez-Cadenas, A. (2008). Antioxidant enzymatic activity is linked to waterlogging stress tolerance in citrus. *Physiol. Plant.* 132, 452–466. doi: 10.1111/j.1399-3054.2007.01029.x

Arnau, M. B., and Hernández-Ruiz, J. (2009). Protective effect of melatonin against chlorophyll degradation during the senescence of barley leaves. *J. Pineal Res.* 46, 58–63. doi: 10.1111/j.1600-079X.2008.00625.x

Back, K., Tan, D. X., and Reiter, R. J. (2016). Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. *J. Pineal Res.* 61, 426–437. doi: 10.1111/jpi.12364

Baijwa, V. S., Shukla, M. R., Sherif, S. M., Murch, S. J., and Saxena, P. K. (2014). Role of melatonin in alleviating cold stress in *Arabidopsis thaliana*. *J. Pineal Res.* 56, 238–245. doi: 10.1111/j.1600-079X.2011.12115

Byeon, Y., Lee, H. Y., Lee, K., Park, S., and Back, K. (2013). Cellular localization and kinetics of the rice melatonin biosynthetic enzymes SNAT and ASMT. *J. Pineal Res.* 56, 107–114. doi: 10.1111/j.1600-079X.2012.00976.x

Cowley, M., Wood, A. J., Böhm, S., Schulz, R., and Oakey, R. J. (2012). Epigenetic control of alternative mRNA processing at the imprinted Herc3/Nap1l5 locus. *Nucleic Acids Res.* 40, 8917–8926. doi: 10.1093/nar/gks654

Dubbels, R., Reiter, R. J., Klenke, E., Goebel, A., Schnakenberg, E., Ehlers, C., et al. (1995). Melatonin in edible plants identified by radioimmunoassay and by high performance liquid chromatography–mass spectrometry. *J. Pineal Res.* 18, 28–31. doi: 10.1111/j.1600-079X.1995.tb00136.x

Foyer, C. H., and Noctor, G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17, 1866–1875. doi: 10.1105/tpc.105.033589

Fukao, T., and Bailey-Serres, J. (2004). Plant responses to hyposia–is survival a balancing act? *Trends Plant Sci.* 9, 449–456. doi: 10.1016/j.tplants.2004.07.005

Gong, B., Li, X., Vandenbergkeng, K. M., Wen, D., Sun, S., Wei, M., et al. (2014). Overexpression of S-adenosyl-L-methionine synthetase increased tomato tolerance to alkali stress through polyamine metabolism. *Plant Biotechnol. J.* 12, 694–708. doi: 10.1111/pbi.12173

Hattori, A., Migitaoka, H., Iigo, M., ltoh, M., Yamamoto, K., Ohtani-Kaneko, R., et al. (1998). Identification of melatonin in plants and its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. *Biochem. Mol. Biol. Int.* 35, 627–634.

Hossain, Z., López-Climent, M. F., Arbona, V., Pérez-Clemente, R. M., and Gómez-Cadenas, A. (2009). Modulation of the antioxidant system in citrus under waterlogging and subsequent drainage. *J. Plant Physiol.* 166, 1391–1404. doi: 10.1016/j.jplph.2009.02.012

Kang, K., Lee, K., Park, S., Byeon, Y., and Back, K. (2013). Molecular cloning of rice serotonin N-acetyltransferase, the penultimate gene in plant melatonin biosynthesis. *J. Pineal Res.* 55, 7–13. doi: 10.1111/j.1600-079X.2012.00976.x

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SUPPLEMENTARY MATERIAL

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Voesenek, L., and Sasidharan, R. (2013). Ethylene–and oxygen signalling–drive Tan, D. X., Manchester, L. C., Liu, X., Rosales-Corral, S. A., Acuna-Castroviejo, Tan, D. X., Manchester, L. C., Esteban-Zubero, E., Zhou, Z., and Reiter, R. J. (2015). Melatonin Shi, H., Chen, Y., Tan, D. X., Reiter, R. J., Chan, Z., and He, C. (2015a). Melatonin Shiabala, S., Shabala, L., Barcelo, J., and Poschenrieder, C. (2014). Membrane transporters mediating root signalling and adaptive responses to oxygen deprivation and soil flooding. Plant Cell Environ. 37, 2216–2233. doi: 10.1111/ jpe.12339 Shi, H., Chen, Y., Tan, D. X., Reiter, R. J., Chan, Z., and He, C. (2015a). Melatonin induces nitric oxide and the potential mechanisms relate to innate immunity against bacterial pathogen infection in Arabidopsis. J. Pineal Res. 59, 102–108. doi: 10.1111/jpi.12244 Shi, H., Wang, X., Tan, D. X., Reiter, R. J., and Chan, Z. (2015b). Comparative physiological and proteome analyses reveal the actions of melatonin in the reduction of oxidative stress in Bermuda grass (Cynodon dactylon (L.) Pers.). J. Pineal Res. 59, 120–131. doi: 10.1111/jpi.12246 Smehurst, C. F., and Shabala, S. (2003). Screening methods for waterlogging tolerance in lucerne; comparative analysis of waterlogging effects on chlorophyll fluorescence, photosynthesis, biomass and chlorophyll content. Funct. Plant Biol. 30, 335–343. doi: 10.1071/FP02192 Smirnoff, N. (2000). Ascorbic acid: metabolism and functions of a multi-facetted molecule. Curr. Opin. Plant Biol. 3, 229–235. doi: 10.1016/S1369-5268(00)00069-8 Stewart, R. B., and Bewley, J. D. (1980). Lipid peroxidation associated with accelerated aging of soybean axes. Plant Physiol. 65, 245–248. doi: 10.1104/pp. 65.2.245 Takahashi, H., Yamauchi, T., Rajhi, I., Nishizawa, N. K., and Nakazono, M. (2015). Transcript profiles in cortical cells of maize primary root during ethylene-induced lysigenous aerenchyma formation under aerobic conditions. Ann. Bot. 115, 879–894. doi: 10.1093/aob/mcv018 Tan, D. X., Chen, L. D., Poeggeler, B., Manchester, L. C., and Reiter, R. J. (1993). Melatonin: a potent, endogenous hydroxyl radical scavenger. Endocr. J. 1, 57–60. Tan, D. X., Hardeland, R., Manchester, L. C., Korkmaz, A., Ma, S., Rosales-Corral, S., et al. (2012). Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science. J. Exp. Bot. 63, 577–597. doi: 10.1093/jxb/eru199 Tan, D. X., Manchester, L. C., Esteban-Zubero, E., Zhou, Z., and Reiter, R. J. (2015). Melatonin as a potent and inducible endogenous antioxidant: synthesis and metabolism. Molecules 20, 18886–18906. doi: 10.3390/molecules2010 18886 Tan, D. X., Manchester, L. C., Liu, X., Rosales-Corral, S. A., Acuna-Castroviejo, D., and Reiter, R. J. (2013). Mitochondria and chloroplasts as the original sites of melatonin synthesis: a hypothesis related to melatonin’s primary function and evolution in eukaryotes. J. Pineal Res. 54, 127–138. doi: 10.1111/jpi. 12026 Voosenek, L., and Sasidharan, R. (2013). Ethylene–and oxygen signalling–drive plant survival during flooding. Plant Biol. 15, 426–435. doi: 10.1111/plb.12014 Wang, L., Wang, Z., and Li, X. (2013). Optimization of ultrasonic-assisted extraction of phenolic antioxidants from Malus baccata (Linn.) Borkh. using response surface methodology. J. Sep. Sci. 36, 1652–1658. doi: 10.1002/jssc. 201300622 Wang, P., Sun, X., Wang, N., Tan, D. X., and Ma, F. (2015). Melatonin enhances the occurrence of autophagy induced by oxidative stress in Arabidopsis seedlings. J. Pineal Res. 58, 479–489. doi: 10.1111/jpi.12233 Wang, P., Yin, L. H., Liang, D., Li, C., Ma, F. W., and Yue, Z. Y. (2012). Delayed senescence of apple leaves by exogenous melatonin treatment: toward regulating the ascorbate–glutathione cycle. J. Pineal Res. 53, 11–20. doi: 10.1111/j.1600-079X.2011.00966.x Wang, W., Vignani, R., Scali, M., and Cresti, M. (2006). A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Electrophoresis 27, 2782–2786. doi: 10.1002/elps.200500722 Wei, L., Qi, T. S., Chu, Y. N., Reiter, R. J., Yu, X. M., Zhu, D. H., et al. (2014). Melatonin enhances plant growth and abiotic stress tolerance in soybean plants. J. Exp. Bot. 66, 695–707. doi: 10.1093/jxb/eru392 Xu, Q. T., Yang, L., Zhou, Z. Q., Mei, F. Z., Qu, L. H., and Zhou, G. S. (2013). Process of aerenchyma formation and reactive oxygen species induced by waterlogging in wheat seminal roots. Planta 238, 969–982. doi: 10.1007/s00425-013-1947-4 Xu, W., Cai, S. Y., Zhang, Y., Wang, Y., Akhammer, G. J., Xia, X., et al. (2016). Melatonin enhances thermotolerance by promoting cellular protein protection in tomato plants. J. Pineal Res. 61, 457–469. doi: 10.1111/jpi.12359 Yamashita, H., Goto, M., Matsui-Yuasa, I., and Kojima-Yuasa, A. (2015). Ecklonia cava polyphenol has a protective effect against ethanol-induced liver injury in a cyclic AMP-dependent manner. Mar. Drugs 13, 3877–3891. doi: 10.3390/ md13058877 Yip, H. K., Chang, Y. C., Wallace, C. G., Chang, L. T., Tsai, T. H., Chen, Y. L., et al. (2013). Melatonin treatment improve adipo-derived mesenchymal stem cell therapy for acute lung ischemia–reperfusion injury. J. Pineal Res. 54, 207–221. doi: 10.1111/jpi.12020 Yu, F., Han, X. S., Geng, C. J., Zhao, Y. X., Zhang, Z. X., and Qiu, F. Z. (2015). Comparative proteomic analysis revealing the complex network associated with waterlogging stress in maize (Zea mays L.) seedling root cells. Proteomics 15, 135–147. doi: 10.1002/pmc.201400156 Zhao, H., Su, T., Huo, L., Wei, H., Jiang, Y., and Xu, L. (2015a). Unveiling the mechanism of melatonin impacts on maize seedling growth: sugar metabolism as a case. J. Pineal Res. 59, 255–266. doi: 10.1111/jpi.12258 Zhao, H., Xu, L., Su, T., Jiang, Y. H., Hu, L., and Ma, F. (2015b). Melatonin regulates carbohydrate metabolism and defenses against Pseudomonas syringae pv. tomato DC3000 infection in Arabidopsis thaliana. J. Pineal Res. 59, 109–119. doi: 10.1111/jpi.12245 Zhao, Y., Tan, D. X., Lei, Q., Chen, H., Wang, L., Li, Q. T., et al. (2013). Melatonin and its potential biological functions in the fruits of sweet cherry. J. Pineal Res. 55, 79–88. Zheng, X., Tan, D. X., Allan, A. C., Zuo, B., Zhao, Y., Reiter, R. J., et al. (2017). Chloroplastic biosynthesis of melatonin and its involvement in protection of plants from salt stress. Sci. Rep. 7:41236. doi: 10.1038/srep41236 Zuo, B. X., Zheng, X. D., He, P. L., Wang, L., Lei, Q., Feng, C., et al. (2014). Overexpression of MzASMT improves melatonin production and enhances drought tolerance in transgenic Arabidopsis thaliana plants. J. Pineal Res. 57, 408–417. doi: 10.1111/jpi.12180 Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Copyright © 2017 Zheng, Zhou, Tan, Wang, Wang, Shan and Kong. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.