**Comparative photosynthetic and metabolic analyses reveal
mechanism of improved cold stress tolerance in
bermudagrass by exogenous melatonin**

Zhengrong Hu^{1,2,†}, Jibiao Fan^{1,2,†}, Yan Xie¹, Erick Amombo^{1,2}, Ao Liu^{1,2},
Margaret Gitau Mukami^{1,2}, A. B. M. Khaldun³, Liang Chen^{1*}, Jinmin
Fu^{1*}

¹Key Laboratory of Plant Germplasm Enhancement and Specialty
Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences,
Wuhan, Hubei 430074, China

²University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing
100049, China

³ Plant Breeding Division Bangladesh Agricultural Research Institute
(BARI) Joydebpur, Gazipur-1701, Bangladesh

† These authors contributed equally to this study.

*Corresponding author E-mail:

chenliang1034@126.com

jfu@wbgcas.cn

23 Abbreviations: TBARS, TBA-reactive substances; SOD, superoxide
 24 dismutase; POD, peroxidase; OJIP curve, F_0 , minimal reliable recorded
 25 fluorescence, at 20 μ s with the pulse-amplitude modulation (PAM)
 26 fluorometer, F_J , fluorescence intensity at the J-step (2 ms) of OJIP, F_I ,
 27 Fluorescence intensity at the I-step (30 ms) of OJIP, F_P , maximal
 28 recorded fluorescence intensity, at the peak P of OJIP. ϕP_0 , maximum
 29 quantum yield of primary photochemistry; PI_{ABS} , PI (potential) for
 30 energy conservation from exciton to the reduction of intersystem
 31 electron; ϕE_0 , Quantum yield of the electron transport flux from QA to
 32 QB; γRC , Probability that a PSII Chl molecule functions as RC; ψE_0 ,
 33 Efficiency/probability with which a PSII trapped electron is transferred
 34 from QA to QB; ϕR_0 , Quantum yield for reduction of end electron
 35 acceptors at the PSI acceptor side; ABS/RC , Absorbed photon flux per
 36 RC; TP_0/RC , Trapped excitation flux per RC; DI_0/RC , dissipated photon
 37 flux per RC; ET_0/RC , Electron transport flux per RC, SC, cold-sensitive
 38 genotype treated with deionized water; SM, cold-sensitive genotype
 39 treated with melatonin; TC, cold-tolerant genotype treated with
 40 deionized water; TM, cold-tolerant genotype treated with melatonin.

Abstract

Melatonin (N-acetyl-5-methoxytryptamine) has been reported to participate in plant development and abiotic stress responses. The main objective of this study was to investigate the role of melatonin in the cold-sensitive (S) and the cold-tolerant (T) bermudagrass genotypes' response to cold stress. The genotypes were treated with 100 μ M melatonin and exposed to 4°C temperature for 3 days. In both genotypes, cold stress increased the endogenous melatonin levels, and more prominently in T than S. Physiological responses indicated that exogenous melatonin triggered antioxidant activities in both genotypes, while it alleviated cell damage in the T genotype response to cold stress. Melatonin treatment under cold stress increased fluorescence curve levels for both genotypes, and higher in T than S genotypes. In both genotypes, the alterations in photosynthetic fluorescence parameters after melatonin treatment highlighted the participation of melatonin in improving photosystem response to cold stress, particularly for the cold-tolerant genotype. The metabolic analyses revealed the alterations of 44 cold-responsive metabolites in the two genotypes, mainly including carbohydrates, organic acids and amino acids. After exogenous melatonin treatment under cold condition, there was high accumulation of metabolites in the cold-tolerant regimes than their cold-sensitive counterparts. Collectively, the present study revealed differential

modulations of melatonin between the cold-sensitive and the cold-tolerant genotypes in response to cold stress. This was mainly by impacting antioxidant system, photosystem II, as well as metabolic homeostasis.

Key words: Melatonin, Genotype, Bermudagrass, Cold stress, Photosystem II, Metabolism.

1. Introduction

Low temperature is a critical environmental factor that limits agricultural productivity, survival and geographical distribution of plants. Multiple physiological, biochemical, molecular and metabolic changes take place during plants' response to cold stress. These include alterations of membrane fluidity, enzyme activity, metabolism homeostasis and genes transcription (Thomashow 1999; Zhu et al. 2004; Chinnusamy et al. 2010; Knight and Knight, 2012).

Photosynthesis is sensitive to low temperature, which directly influences photosystem performance and photosynthetic apparatus activities (Smillie and Hetherington 1984). Under cold stress, the efficiency of photosynthetic electron transport in plant is vividly decreased, which leads to an excessive energy generation, and even triggers photoinhibition (Krause, 1994). Kee et al (1986) suggested that the cold-induced inhibition involves the whole chain electron transport

and PS II catalyzed photochemistry. Moreover, reactive oxygen species (ROS) is formed when plants are subjected to photoinhibitory conditions, which results in serious injury of PSII components (Osmond 1994). The extent of impairment relies on the balance between damage and repair of PSII components (Chen et al. 2013). However, there is limited information concerning the effects of melatonin on photosystem in plant under low temperature.

Metabolites are the end products of cellular regulatory processes, and their levels can be estimated as the ultimate response of biological systems to genetic or environmental changes (Fiehn, 2002). Many studies have revealed that small soluble metabolites, such as sucrose, fructan, and proline, participate in plants response to abiotic stress (Chen et al. 2002; Stitt et al. 2002). Sugars frequently accumulate in plants exposure to cold stress, especially if the temperature is insufficient to kill tissues (Levitt, 1980). In addition, soluble sugars, ROS and tetrapyrrole intermediate Mg-protoporphyrin (Mg-ProtoIX) are three major types of metabolic signals, which might be pivotal for cold signaling (Zhu et al. 2007).

Melatonin (N-acetyl-5-methoxytryptamine) is a ubiquitous and highly conserved molecule occurring even in evolutionarily distant organisms. Melatonin was first described in *Gonyaulax polyedra* in the year 1991. Subsequently, progressive research regarding its presence and

importance in plants has been rigorously conducted (Poeggeler et al., 1991; Posmyk et al., 2009). Melatonin was first discovered in higher plants by Dubbels et al. and Hattori et al. in 1995. Melatonin participates in multiple developmental processes including governing growth of roots and shoots (Zhang et al., 2014), circadian rhythms regulation, photosynthesis promotion, chlorophyll preservation (Arnao and Hernández-Ruiz, 2009; Tan et al., 2012), leaf senescence delay (Byeon et al., 2012), and alleviation of oxidative damage induced by reactive oxygen species (ROS) burst (Tan et al., 2012). Furthermore, has been reported to improve plants tolerance against multiple abiotic stresses, such as cold, drought as well as salt (Bajwa et al., 2014; Shi et al., 2015a; Wang et al., 2014)

Bermudagrass (*Cynodon dactylon* (L.) Pers.), is a major turf species for park, sport fields as well as lawns (Shi et al. 2015a; 2015b; Fan et al. 2014). As a widely cultivated warm-season turfgrass, low temperature is considered to be a major environmental factor limiting its usage (Fan et al. 2014). However, there is insufficient information regarding bermudagrass in response to cold stress. Thus, cold tolerance improvement in this botanical species is urgent for turf-grass engineering.

This study was designed to investigate the possible role of melatonin in bermudagrass response to cold stress, particularly focusing on

modulations of antioxidant enzymes activities, photosynthesis and metabolisms. Two bermudagrass genotypes that were filtrated by our team, the cold-sensitive (S) genotype ‘WBGg-17’ and the cold-tolerant (T) genotype ‘WBD128’, combined with exogenous melatonin treatment, were exposed to cold stress. The effects of exogenous melatonin on cell damage and underlying antioxidant responses were determined. Photosynthetic and comparative metabolic analyses were also performed. This study provided some insights into the possible mechanisms behind the differences in cold tolerance ability modulated by exogenous melatonin in bermudagrass.

2. Materials and Methods

2.1. Plant materials and growth conditions

Two wild genotypes of bermudagrass [*Cynodon dactylon* (L).Pers.] were used in this study. They are the cold sensitive ‘WBGg-17’ and the cold tolerant ‘WBD128’, which were collected from Xiaojiang city, Zhejiang province, China (N 27°34.258, E 120°27.383) and Baise city, Guangxi province, China (N24°51.397, E 106°33.288), respectively. Uniform stolons of the two cultivars were planted in plastic pots (7.5 cm in diameter and 9.0 cm deep) that were filled with matrix (brown coal soil). Several drainage holes were drilled at the bottom of the pot to allow

excess water to drain out and to enhance soil aeration. For bermudagrass establishment, the pots were kept in the greenhouse for 2 months with a growth condition of 12-h-light ($240 \mu\text{mol m}^{-2}\text{s}^{-1}$, 30°C) /12-h-dark (25°C) and 40% relative humidity. During the establishing period, bermudagrass was watered daily and fertilized every two weeks with full-strength Hoagland nutrient solution.

2.2. Treatments

After establishment, the plants were transferred into two growth chambers (LSC-339CF, Xingxing Group Co.,Ltd, Zhejiang, China) with different growth conditions. The condition of control chamber was 12-h-light ($260 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 30°C) / 12-h-dark (25°C) and 70% relative humidity, while cold-treated chamber were similar with control, except for 4°C (day/night) temperature. In the study, $100 \mu\text{M}$ melatonin was applied, while double distilled water was used as the control. The plants were subjected to four treatment regimes: (i) the cold-sensitive genotype treated with deionized water (SC), (ii) the cold-tolerant genotype treated with deionized water (TC), (iii) the cold-sensitive genotype treated with melatonin (SM) and (iv) the cold-tolerant genotype treated with melatonin (TM). The plants in each growth chamber were watered and sprayed daily with 100 mL of deionized water or melatonin for 3 days. After treatment, bermudagrass leaves were collected for

physiological and metabolomic assays, and chlorophyll (Chl) α fluorescence transient was recorded at 3 d of cold treatment.

2.3. Chlorophyll (Chl) α fluorescence transient

The data of chlorophyll (Chl) α fluorescence transient was measured by pulse-amplitude modulation (PAM) fluorometer (PAM 2500, Heinz Walz GmbH). The third fully expanded bermudagrass leaves were collected at 3 d of cold treatment. After 30 min pre-adaption in the dark, red light of $3000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ triggered the OJIP transients, which was aimed to ensure closure of all reaction centers of PSII to estimate a real maximal fluorescence intensity (Chen et al. 2014). Subsequently, the strong light pulses triggered the Chl α fluorescence emission that was subsequently measured and digitized between 10 μs and 320 ms. The data of OJIP transients was processed by using the JIP-test as reported by Chen et al. (2013).

2.4. Quantification of melatonin

Endogenous melatonin in bermudagrass was measured by enzyme-linked immunosorbent assay (ELISA). The extraction of melatonin from plant leaves was performed according to the method described by Pape and Lüning (2006). In brief, 0.3 g of leaf was ground into powder in liquid nitrogen, and then transferred to 10 ml tubes which contained 5 ml of mixed solution (acetone:methanol:water=89:10:1).

After 1 h of extensive homogenization on ice, the homogenate was centrifuged at 4000 g for 5 min at 4°C. The supernatant was transferred to 0.5 ml of 1% trichloric acid, centrifuged at 12000 g for 15 min at 4 °C. The extraction was used for quantification of endogenous melatonin in leaf by using the Melatonin ELISA Kit (EK-DSM; Buhlmann Laboratories AG, Schonenbuch, Switzerland) followed by the manufacturer's instruction.

2.5. Crude enzyme extraction

For crude enzyme, 0.2 g of leaves was grounded by using ice-cooled mortar and pestle with 4 mL of 150 mM sodium phosphate buffer (150 mM, pH 7.0). The homogenate was centrifuged for 25 min at 8000 rpm at 4°C, and then the supernatant was collected for physiological assays, comprising TBA-reactive substances (TBARS) content and enzyme activities, which were measured according to Fan et al. (2104) with some slight modifications.

2.6. TBA-reactive substances (TBARS) content

To measure the TBARS content, 1 mL of supernatant was added into 2 mL of reaction solution that contained 20% (v/v) trichloroacetic acid (TCA) and 0.5% (v/v) thiobarbituric acid (TBA). Then the mixed solution was heated at 95 °C in a water bath for 30 min. After cooling down to room temperature (25°C), the solution was centrifuged at 8000 rpm for 20 min. The changes in absorbance of the supernatant at 532 nm

and 600 nm were monitored by spectrophotometer. The TBARS content was calculated by using the following formula:
$$\text{TBARS } (\mu\text{mol.g}^{-1}\text{FW}) = [(\text{OD}_{532} - \text{OD}_{600}) * L] / (l * \epsilon * \text{FW})$$
(ϵ was the extinction coefficient of 155 $\text{mM}^{-1}\text{cm}^{-1}$).

2.7. Antioxidant enzyme activity:

For the SOD activity assay, 100 μL of crude enzyme extract was added into 2.9 mL solution which contained 50 mM sodium phosphate buffer (pH 7.8), 195 mM methionine, 3 μM ethylene diaminetetraacetic acid (EDTA), 60 μM riboflavin and 1.125 mM nitro blue tetrazolium (NBT). Then the mixture was illuminated under 4000 lx fluorescent lamp for 60 min for chromogenic reaction. The change of absorbance at 560 nm was determined with a spectrophotometer. One unit of SOD activity was defined as the inhibition of NBT reduction by 50%.

The POD activity was determined according to the method described by Fan et al (2014). Briefly, 50 μL of crude enzyme extract was added with 2.95 mL reaction solution that contained 0.1M sodium acetate-acetic acid buffer (pH 5.0), 0.25% guaiacol (dissolved in 50% ethanol solution), and 0.075% H_2O_2 and 50 μL enzyme extract. The changes in absorbance at 460 nm per minute were recorded for 3 min. A unit POD activity was defined as an increase by one unit of absorbance at 460 nm per minute.

The APX, GPX and GST activity was determined using APX Assay Kit (Nanjing Jiancheng Bioengineering Institute, China), GPX Assay

Kit (A005, Nanjing Jiancheng Bioengineering Institute, China), as well as GST Assay Kit (A004, Nanjing jiancheng Bioengineering Institute, China) respectively, according to the manufacturer's instructions. The oxidation of 1 μmol ASA per minute was defined as one unit of APX activity. One unit of GPX and GST activities were both defined as the reduction of 1 μmol GSH per minute, with deduction of non-enzymatic reactions.

2.8. Metabolite extraction and derivation

For metabolite assay preparation, about 0.1 g of fully expanded bermudagrass leaves were collected and frozen immediately in liquid nitrogen, then transferred into -80°C . Extraction and derivation of bermudagrass samples were performed by the method described by Roessner et al. (2000) with some modifications. Briefly, frozen leaves were grounded into a fine powder with ice-cooled mortar and pestle in liquid nitrogen. The powder was extracted in 1.4 mL of 80% (v/v) aqueous methanol for 2 h under ambient temperature at 200 rpm. Before incubation, 50 μL of ribitol (2 mg mL^{-1}) was added into the solution as internal standard. Then the mixture was heated at 70°C for 15 min, and then was centrifuged for 10 min at 12,000 g. The supernatant was transferred into new tubes containing 1.5 mL water and 0.75 mL chloroform. The mixture solution was vortexed completely for 15 s then centrifuged at 12000 g for 10 min. The 0.3 mL of polar phase was

transferred into HPLC vials and dried overnight by using a centrivap bench-top centrifugal concentrator (Labogene, Denmark). The dried polar phase was methoximated by adding 80 mL of 20 mg mL⁻¹ methoxyamine hydrochloride melted in pyridine at 30°C for 2 h, then adding 50 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) at 37°C for 2 h. The reagents used in this study were purchased from Sigma-Aldrich Co.Ltd. (Poole, UK).

2.9. Gas chromatography mass spectrometry (GC-MS) analysis

The metabolites were measured by using GC-MS (Agilent 7890A/5975C, Agilent Technologies, Palo Alto, CA, USA) according to the method described by Xie et al. (2014). Briefly, 1 µL of derivatized solution was transferred into a DB-5MS capillary (30 m×0.25 mm×0.25 mm, Agilent J&W GC column, USA). The procedure was set as the following: the inlet temperature was set at 280°C after 5 min of solvent delay, while the initial GC oven temperature was at 70°C. Subsequently, after 1 min of injection, GC oven temperature was increased to 280°C with a 5°C increase per min, and then maintained at 280°C for 10 min. The injection temperature was set at 280°C, while ion source temperature (230 °C) was simultaneously matched. Helium was applied as the carrier gas, and the constant flow rate was 1 mL·min⁻¹. The measurement was conducted at 2 scans·s⁻¹ with electron impact

ionization (70 eV) in a full scan mode (m/z 30-650).

2.10. Metabolite data processing and analysis

Metabolites were identified according to the retention time using the software of the Agilent MSD Productivity Chemstation, and coupled with the commercially available compound libraries (NIST 11) (Gaithersburg, MD, USA). Relative quantification of the metabolites was determined according to the value of internal standard ribitol. By using the MetaboAnalyst webpage (<http://www.metaboanalyst.ca/MetaboAnalyst/>) (Xia et al. 2009), the hierarchical clustering analysis (HCA) were performed. Statistical assessment was conducted after calculation of log-transformed response ratios for each identified metabolites,

2.11. Statistical analysis

The values were expressed as mean $\pm$ SD of three replicates. Statistical analyses were conducted by one-way analysis of variance (ANOVA) combined with independent-samples t test at a significant level of $P<0.05$, using the statistical package SPSS 16.0, and Excel 2010 for Windows.

3. Results

3.1. Alterations of endogenous melatonin level in the two

bermudagrass

To investigate how the melatonin levels altered in the two bermudagrass genotypes after melatonin treatment, the endogenous melatonin levels were quantified. Under control condition, exogenous melatonin treatment increased the levels of endogenous melatonin in both genotypes, and the level in the T genotype was higher than the S genotype (Fig.1). Compared with their **respective** control under normal condition, cold stress (4°C) significantly induced the melatonin accumulation in both genotypes, and the increase extents were further enhanced by exogenous melatonin treatment. In addition, under cold condition, the endogenous melatonin level in the TC regime was higher than SC regime, and similar result was observed between TM regime and SM regime.

3.2. Effects of melatonin on physiological properties of the two bermudagrass genotypes

TBARS content is **a** vital indicator of membrane stability. In the present study, oxidative damage to cell membrane triggered by cold stress was evaluated by TBARS content. Cold stress significantly increased the TBARS content in SC, SM and TC regimes, while that in TM regime **was maintained at a** constant level compared with their controls under normal condition, respectively (Fig. 2A). After 3 d of cold treatment (4°C), the TBARS content in TM regime was significantly lower than TC regime. Meanwhile, under low temperature, the TBARS contents in SC regime

and SM regime were remarkably higher than TC regime and TM regime, respectively. This observation indicated that there was more membrane damage in the S genotype than the T genotype response to cold stress. Exogenous melatonin significantly improved cell membrane stability in the cold-tolerant genotype.

To explore the relationship between ROS level and antioxidant enzymes activities, several antioxidant enzymes activities were measured, which included superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione peroxidase (GPX)) and glutathione S-transferase (GST). Under control condition, there was no difference in the activities of five antioxidant enzymes (SOD, POD, APX, GPX and GST) among all regimes (Figure 2B–F). However, cold stress significantly increased the activities of POD, SOD and APX in both genotypes, which were further enhanced by melatonin treatment (Fig.2 B-D). In addition, under low temperature, the T genotype had higher activities of antioxidant enzymes (POD, SOD and APX) than the S genotype, with or without melatonin treatment. Conversely,, cold stress reduced the GPX activity in the S genotype, which was rescued by melatonin treatment (Fig.2E). As for the T genotype, cold stress increased GPX activity, which was further enhanced by melatonin. Conversely, cold stress increased GST activity in both genotypes, while the cold-induced raise was suppressed in S genotype but maintained in the T genotype after

melatonin treatment (Fig.2F). Moreover, GPX activity in the T genotype was higher than the S genotype, with or without melatonin treatment, while **converse** result was observed for GST activity (Fig.2E-F).

3.3. Effects of melatonin on OJIP transient curves for the two bermudagrass genotypes

Under normal condition, there was no obvious difference in OJIP transient curves among the four regimes (Fig.3A). However, after 3 d of cold stress, the OJIP curves level for SM regime was higher than SC regime, **and similar results were** observed between TM regime and TC regime (Fig.3B). Besides, under cold condition, the OJIP transient curves for the T genotype exhibited higher levels compared to the S genotype, and the difference was more obvious after melatonin treatment.

To further investigate the alterations of photosynthetic behaviors in bermudagrass exposed to low temperature, multiple structural and functional parameters were calculated. As shown in Table.1, cold stress dramatically declined the ϕP_0 (maximum quantum yield of primary photochemistry) values for both genotypes, which were alleviated by melatonin treatment. Moreover, under cold condition, the ϕP_0 value for TC regime and TM regime were significantly higher than SC regime and SM regime, respectively. Similar result was observed for PI_{ABS} (performance index for energy conservation from photons absorbed by

PSII antenna to the reduction of QB). These results indicated that exogenous melatonin plays a protective role of photosynthetic performance in bermudagrass response to low temperature. Cold stress significantly decreased the ϕE_0 (quantum yield of the electron transport flux from Q_A to Q_B) and γRC (probability that a PSII Chl molecule functions as RC) values for both genotypes, which were alleviated by exogenous melatonin (Table.1). ΨE_0 is an indicator showing the open extent of activated reaction centers. In the present study, after cold stress application, the ΨE_0 value for SM regime was remarkably lower than SC regime, and the values for the T genotype (with/without melatonin treatment) were lower than the S genotype (Table.1). As for γRC , the values for SC, SM and TC were similarly lower than TM (Table.1). These results indicated that low temperature increased the open extent of activated reaction centers in the cold-sensitive genotype, which was alleviated by exogenous melatonin. Moreover, exogenous melatonin improves the probability that a PSII Chl molecule functions as RC in the cold-tolerant genotype response to cold stress.

Additionally, there was no distinct difference in energy fluxes for absorption (ABS/RC), trapping (TP_0/RC), dissipation (DI_0/RC), and electron transport (ET_0/RC) among all regimes under normal condition (Table.1). Low temperature increased ABS/RC and DI_0/RC values for both genotypes, which were inhibited by melatonin treatment (Table.1).

However, low temperature increased the ET_0/RC value for SC regime, while reduced that for TC and TM regimes. The ET_0/RC values in the T genotype significantly decreased as compared to the S genotype (with/without application of melatonin). These results indicated that cold stress might increase the energy fluxes for absorption and dissipation in two genotypes, which were eventually suppressed by exogenous melatonin.

3.4. Modulation of metabolic homeostasis by exogenous melatonin treatment in two bermudagrass genotypes

Photosynthesis is closely associated with metabolism. To explore further how exogenous melatonin modulates metabolic homeostasis in bermudagrass response to cold stress, GC- MS was applied to identify differentially expressed metabolites. Totally, 44 metabolites including organic acids (12), amino acids (8), carbohydrate (17), lipid (4) and others (3) were identified in the two bermudagrass genotypes (Fig.4).

As shown in Table.2, under normal condition, the amount of organic acids were similar among SC regime, SM regime and TC regime, while the amount in TM regime was decreased after melatonin treatment. Cold stress reduced the amounts of organic acids in SC regime and SM regime, but increased that in TM regime. Under cold exposure, the **quantity** of amino acids (i.e., glycine, norvaline, glutamic acid) in SM regime was

415 similar with SC counterpart, while that in TM regime was lower than TC
416 regime. Low temperature significantly increased the amount of
417 carbohydrates in all regimes. The carbohydrates level in TM regime was
418 notably higher than TC regime, while that in SM regime was lower than
419 SC regime. Under optimum conditions, the total lipid amount in the T
420 genotype was remarkably higher than the S one, whereas, cold stress
421 noticeably reduced the lipid amounts in the cold-tolerant genotype
422 (with/without melatonin treatment).

423 On one hand, analysis suggested that under optimal condition some
424 carbohydrates (talose, fructose, glucose, sorbose, sucrose), amino acids
425 (valine, glycine) and organic acid (pyruvic acid) were higher TM regime
426 compared to the control (TC regime). On the other hand, the contents of
427 some other carbohydrates (cellobiose, galactopyranoside), organic acids
428 (glyceric acid, gluconic acid, malic acid, citric acid) and amino acid
429 (serine) were lower than TC regime (Fig.4A). Generally, for the
430 cold-sensitive genotype, the metabolic profiles were similar with or
431 without melatonin application, under normal condition. When cold (4°C)
432 stress was applied, the metabolic homeostasis was altered in both
433 genotypes, which was modulated by exogenous melatonin. In detail, as
434 for the T genotype, the levels of some carbohydrates (xylulose, sorbose),
435 organic acids (galactaric acid, citric acid, threonic acid, glyceric
436 acid, gluconic acid), amino acid (threonine), lipid (glycerol) as well as

myo-inositol in TM regime increased compared to the control (TC regime) (Fig.4B). However, the metabolic profiles were remarkably altered after melatonin treatment in the S genotype. The identified metabolites including 2 amino acid (norvaline, glycine), 4 carbohydrates (galactinol, mannitol, mannobiose, sorbose) as well as 2 organic acids (glyceric acid, isocitric) in SM regime exhibited higher level than SC regime, while the contents of alanine, galactaric acid and some carbohydrates (xylopyranose, talose, glucopyranose) in SM regime were lower than SC regime.

Moreover, 21 metabolites including 6 carbohydrates, 8 amino acids, 5 organic acid, glycerol and myo-inositol were assigned to the carbon metabolic pathway, which comprised glycolysis, tricarboxylic acid (TCA) cycle, as well as oxidative pentose phosphate pathway (Fig.5). Under low temperature, the cold-tolerant genotype accumulated more sucrose, fructose, glucose and galatose after exogenous melatonin treatment. However, with application of melatonin, the levels of sucrose and fructose in the S genotypes significantly reduced, when responded to cold stress. Interestingly, after melatonin treatment, both genotypes accumulated more isocitrate and less malate, However, the citrate accumulation was up-regulated in TM regime and down-regulated in SM regime, when compared to their control (TC and SC regimes) respectively. These results indicated that melatonin modulated carbon

metabolic pathway in bermudagrass response to cold stress, and the regulated patterns differed between the two genotypes.

4. Discussion

As sessile organisms, botanical species have developed complex strategies to respond to cold stress that adversely affects growth and development. Recently, melatonin has been reported to play key roles in plant to response to cold stress (Bajwa et al., 2014; Shi et al. 2015a). Lei et al (2004) reported that cold-induced apoptosis in carrot cells was alleviated by exogenous melatonin treatment; while Posmyk et al (2009) determined that melatonin improved cucumber seeds germination under cold stress.

In the present study, different modulations of melatonin were revealed between the cold-sensitive and the cold-tolerant bermudagrass genotypes in response to cold stress. The notable induction of melatonin by low temperature suggested a vital role of melatonin in bermudagrass tolerating cold stress (Fig.1). This result was consistent with Shi and Chan (2014) and Shi et al (2015a). The T genotype exhibited higher endogenous melatonin level than the S genotype after cold treatment, and the trend was enhanced by exogenous melatonin. The relatively higher level of endogenous melatonin in TM regime might be closely correlated

with cold tolerance.

Cold stress can induce oxidative stress by rapid and excessive production of ROS. To protect plant cells from ROS damage, plants have developed complex antioxidant defense systems to scavenge the excessive ROS, including antioxidant enzymes (Apel and Hirt, 2004; Mittler, 2002; Mittler et al, 2004). SOD is a key antioxidant enzyme that converts superoxide anions to O₂ and H₂O₂, to scavenge toxic superoxide radicals. H₂O₂ is subsequently removed by APX, POD, CAT GPX or GST through different pathway (Apel and Hirt, 2004; Mittler, 2002; Mittler et al, 2004). Glutathione-S-transferase (GST) that catalyzes the conjugation of GSH with diverse electrophilic metabolites plays a critical role in defending against oxidative damage (Janda et al, 2003). Additionally, melatonin has been confirmed to be a **crucial** radical scavenger and a broad spectrum antioxidant by scavenging free radicals directly and stimulating the activities of antioxidant enzymes (Tan et al., 2000; Shi et al., 2015a). Consistently, it **was observed that** exogenous melatonin treatment alleviated oxidative stress-induced ROS accumulation and related oxidative damage (Shi et al., 2015b). In the present study, exogenous application of melatonin significantly activated ROS detoxification of antioxidants, including (SOD, POD, APX and GST) in both bermudagrass genotypes, and more prominent for the T genotype (Fig.2). Our result is consistent with Shi et al (2015a), and Chan

and Shi (2015). While the GPX activity is low, the GST is highest in the SC regime among the regimes tested. This might be a complementary strategy to develop a relative high antioxidant activity to tolerate low temperature. This result is in accordance with Janda et al (2003). Besides, cold-induced increase of TBARS content was reduced by melatonin treatment in the T genotype (Fig.2A), indicating that exogenous melatonin application alleviates cellular membrane damage of the T genotype. Collectively, these results suggested that endogenous production of melatonin improved cold tolerance in bermudagrass, especially for the cold-tolerant genotype, which was agreement with Kang et al (2010), Chan and Shi (2015).

Cold stress has been reported to limit photosynthetic electron transport efficiency in plant, leading to an excess of energy (Hodgson and Raison 1989). In the study, low temperature declined the OJIP transient curve levels for both genotypes, while the decreases in two genotypes were partly recovered by exogenous melatonin application, particularly for the T genotype (Fig.3). ϕP_0 , representing the maximum quantum efficiency of the PS II photochemistry, is revealed to be highly correlated with cold tolerance (Rizza et al. 2001; Mishra et al. 2011). PI_{ABS} is a sensitive indicator of photosynthetic performance based on light absorption. In this study, the cold-induced inhibitions of ϕP_0 and PI_{ABS} were partly recovered by exogenous melatonin in both genotypes (Table.1). In addition,

525 cold-triggered inhibitions of ϕE_0 were slightly recovered by melatonin in
526 both genotypes. These data illustrated that melatonin alleviates the
527 cold-triggered inhibition of quantum yield of the electron transport flux in
528 both genotypes, which is beneficial for maintenance of high level of
529 photosynthetic property in response to cold stress.

530 When energy absorption by chlorophyll exceeds the utilization
531 capacity of the organism, the trapped energy is utilized through
532 photosynthesis (photochemical quenching) or dissipated as heat
533 (non-photochemical quenching), resulting in PSII excitation pressure
534 increase, subsequently, ROS are formed (Huner et al. 1998). When
535 subjected to **prolonged cold stress**, photosynthetic acclimatization to high
536 PSII excitation pressure occurs due to the reduction in PSII antenna size,
537 which in turn reduces photosynthetic efficiency (Huner et al. 1998). In
538 the present study, exogenous melatonin reduces the level of openness of
539 reactive center (ΨE_0) in the cold-sensitive genotypes, but improves the
540 probability that a PSII Chl molecule functions as RC (γ RC) in the
541 cold-tolerant genotype, which are different patterns between two
542 genotypes in response to cold stress. In particular, melatonin treatment
543 inhibited the cold-triggered increases of values of ABS/RC and DI_0 /RC
544 for both genotypes, revealing melatonin inhibited the cold-triggered
545 energy absorption and dissipation in both genotypes to reduce the
546 excessive energy production (Table.1). In addition, exogenous melatonin

inhibited the cold-induced increase of ET_0/RC in the S genotype, while maintained the cold-triggered inhibition of the value in the T genotype. This results indicated that melatonin reduces energy fluxes for electron transport in the cold-sensitive genotype to weaken photosynthesis intensity in response to low temperature. Differently, the inherent inhibition of photosynthesis intensity in the cold-tolerant genotype may confer it stronger tolerance to cold.

Cold stress led to photosynthesis inhibition and induced further suppression of sucrose synthesis (Stitt and Hurry, 2002). Sugar is involved in synthesis of many compounds, energy production, membrane stabilization, r gene expression regulation and sugar-sensing signal system (Xie et al. 2014). Previous study has discovered a strong correlation between sugar levels and cold tolerance (Magel et al, 2001). Our results indicated that cold stress increased carbohydrates amount in both genotypes, while, the rise of carbohydrates (including sucrose, xylulose, cellobiose, sorbose) amount was further enhanced by melatonin in the cold-tolerant genotype, but not in the cold-sensitive regimes (Table.2 and Fig.4). Consistent with our results, Levitt (1980) suggested that sugars frequently accumulated in plants under cold condition.

Organic acids , such as citric acid, isocitric acid and malic acid that are critical in the Krebs cycle, are reported to play key roles in abiotic stresses tolerance. The levels of Krebs cycle intermediates are considered

569 as the indicators of mitochondrial activities which generate ATP and
570 reducing agents for the metabolism (Vasquez-Robinet et al. 2008). In the
571 present study, under cold condition, the amount of organic acids in the S
572 genotype was lower than the T genotype, especially after exogenous
573 melatonin treatment (Table.2). Moreover, after exogenous melatonin
574 application, citrate concentration increased in the T genotype, but not in
575 the S genotype under cold stress (Fig.5 and Supplement Table.1).
576 Previous study has indicated that organic acids may be vital in cold
577 tolerance (Shi et al. 2015a).

578 Additionally, in the present study, 21 metabolites assigned to
579 glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative pentose
580 phosphate pathway were identified (Fig.5). Under low temperature, the
581 up-regulations of glutamate, threonine, isocitrate and myo-inositol were
582 observed in both genotypes after exogenous melatonin application. This
583 result was consistent with the report of Shi et al. (2015a). Chan and Shi
584 (2015) suggested that small molecules, such as melatonin, induced the
585 accumulation of osmoprotectants, which is beneficial for protecting
586 bermudagrass from damages triggered by abiotic stresses, including cold
587 stress. Our results showed that the levels of some carbohydrates
588 (comprising sucrose, fructose, mannose, galatose and glucose), amino
589 acids (valine), organic acids (citrate) and glycerol in the cold-tolerant
590 genotype increased after melatonin treatment, whereas it decreased or

was unaltered in the cold-sensitive genotype response to cold stress (Fig.5). Conversely, the levels of several kinds of amino acids, (ie. glycine, norvaline, glutamic acid), galactinol, and lactic acid were higher in SM regime than that in SC regime, while opposite results were found between TM regime and TC regime. These data revealed differential modulations of metabolism homeostasis by melatonin in two genotypes response to cold stress. In the S genotype, decrease of carbohydrates and organic acids by melatonin leads to a decline of energy production, consequently, restraint of growth and developmental processes to adapt to cold stress. As for the T genotype, exogenous melatonin increases those metabolites to make plant maximize growth and developmental processes to maintain cellular homeostasis to response to cold stress. Similar conclusion was reported by Fitter and Hay (1981).

Generally, differential modulations of melatonin in the two bermudagrass genotypes response to cold were revealed. As compared with the S genotype, the T genotype exhibited higher levels of antioxidant system activity, photosystem II performance, as well as metabolic modulation, which conferred this genotype prominent tolerance to cold stress (Fig.6). Higher sensitivity to melatonin for the T genotype could induce more widespread alterations in antioxidant system, photosynthetic reaction as well as metabolism, which might be closely associated with stronger cold tolerance in the genotype.

5. Conclusions

The protective roles of melatonin in both bermudagrass genotypes response to cold stress were revealed in the present study. The improvement of cold tolerance by melatonin was more pronounced in the **T than the S genotype**. It might be attributed to differential adaptive responses to cold stress, including alterations of cell membrane stability, antioxidant enzymes activities, photosystem II (PSII) performance and metabolic homeostasis in plants.

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Figure Legends:

Fig.1. Endogenous melatonin content. There were four regimes, comprising SC, SM, TC, TM. They respectively represented the cold-sensitive bermudagrass genotype treated with deionized water or melatonin, the cold-tolerant genotype treated with deionized water or melatonin. The FW was fresh weight. Mean values and SD were calculated from three independent experiments. Columns marked with different letters indicate significant differences among different regimes at a given temperature based on Duncan's multiple range tests ($P < 0.05$). Columns marked with star represent statistical significance between 30°C and 4°C for a given regime based on Independent-samples *t* test ($P < 0.05$).

Fig. 2. Effects of exogenous melatonin on the physiological property for the two bermudagrass genotypes in response to cold stress. **(A)** TBA-reactive substances (TBARS) content; **(B)** Activities of superoxide dismutase (SOD); **(C)** Activities of peroxidase (POD); **(D)** Activities of ascorbate peroxidase (APX), **(E)** Activities of glutathione peroxidase (GPX); **(F)** Activities of glutathione S-transferase (GST). Plant leaves were treated with deionized water or 100 μ M melatonin. There were four regimes, comprising SC, SM, TC, TM. They respectively represented the cold-sensitive bermudagrass genotype treated with deionized water or melatonin, the cold-tolerant genotype treated with deionized water or

melatonin. The FW was fresh weight. Mean values and SD were calculated from three independent experiments. Columns marked with different letters indicate significant differences among different regimes at a given temperature based on Duncan's multiple range tests ($P < 0.05$). Columns marked with star represent statistical significance between 30°C and 4°C for a given regime based on Independent-samples t test ($P < 0.05$).

Fig. 3. Effects of the melatonin on polyphasic chlorophyll fluorescence transients of the two bermudagrass genotypes in response to cold stress. (A) Chlorophyll fluorescence transients of plants at 30°C. (B) Chlorophyll fluorescence transients of plants at 4°C. F_0 , minimal reliable recorded fluorescence, at 20 μ s with the pulse-amplitude modulation (PAM) fluorometer; F_J , fluorescence intensity at the J-step (2 ms) of OJIP; F_I , Fluorescence intensity at the I-step (30 ms) of OJIP; F_P , maximal recorded fluorescence intensity, at the peak P of OJIP. Plant leaves were treated with deionized water or 100 μ M melatonin. There were four regimes, comprising SC, SM, TC, TM. They respectively represented the cold-sensitive bermudagrass genotype treated with deionized water or melatonin, the cold-tolerant genotype treated with deionized water or melatonin.

Fig.4. Hierarchical cluster analysis of 44 metabolites modulated by exogenous melatonin in the two bermudagrass genotypes response to cold stress. (A) Hierarchical cluster analysis of metabolites at 30°C. (B)

Hierarchical cluster analysis of metabolites at 4°C. Plant leaves were treated with deionized water or 100 µM melatonin. There were four regimes, comprising SC, SM, TC, TM. They respectively represented the cold-sensitive bermudagrass genotype treated with deionized water or melatonin, the cold-tolerant genotype treated with deionized water or melatonin. Mean values and SD were calculated from three independent experiments. Columns marked with different letters indicate significant differences among different regimes at a given temperature based on Duncan's multiple range tests ($P < 0.05$). Columns marked with star represent statistical significance between 30°C and 4°C for a given regime based on Independent-samples *t* test ($P < 0.05$).

Fig.5. Metabolic pathways for metabolite shift modulated by exogenous melatonin in the cold-sensitive (S) bermudagrass genotype and the cold-tolerant (T) genotypes response to cold stress (corresponding to Supplement Table 1). Under cold condition (4°C), metabolites which have significantly higher or lower abundance in melatonin-treated leaves of the cold-sensitive genotype (left) or the cold-tolerant genotype (right) compared to their control ($P < 0.05$) were represented by red (higher) and green (lower) boxes, respectively. The pink boxes represented the metabolite abundance unchanged. The gray boxes represented the metabolite concentration undeterminable.

874 **Fig.6.** Model of differential modulations of exogenous melatonin
875 between the cold-sensitive (S) bermudagrass genotype and the
876 cold-tolerant (T) genotypes in response to cold stress. The left boxes
877 represented the cold-sensitive genotype (S), and the right ones
878 represented the cold-tolerant genotype (T). Different levels of alterations
879 were marked with different colors.

881 **Table.1** Photosynthetic parameters deduced by the JIP-test analysis of fluorescence transients in Fig.2

Photosynthetic	30°C				4°C				Definitions
parameters	SC	SM	TC	TM	SC	SM	TC	TM	
Specific energy fluxes (per active PSII reaction center)									
ABS/RC	2.67a	2.62a	2.62a	2.62a	3.09a*	2.78bc*	2.88b*	2.71c	Absorbed photon flux per RC
TP0/RC	1.98a	1.93a	1.95a	1.96a	2.21a*	2.00b	2.10ab*	1.98b	Trapped excitation flux per RC
ET0/RC	0.62a	0.61ab	0.60ab	0.62a	0.67a*	0.62b	0.57c*	0.57c*	Electron transport flux per RC
Quantum yields and efficiencies/probabilities									
ϕP_0	0.74a	0.74a	0.73a	0.73a	0.68c*	0.70b*	0.70b*	0.73a	Maximum quantum yield for primary photochemistry, namelyFV/FM
ϕE_0	0.31a	0.31a	0.31a	0.32a	0.24c*	0.29ab*	0.28b*	0.29a*	Quantum yield of the electron transport flux fromQAto QB
γRC	0.27a	0.28a	0.27a	0.27a	0.22b*	0.24b*	0.24b*	0.26a	Probability that a PSII Chl molecule functions as RC
ψE_0	0.25a	0.25a	0.25a	0.26a	0.42a*	0.34b*	0.27c	0.24c	Efficiency/probability with which a PSII trappedelectron is transferred fromQAto QB
ϕR_0	0.15a	0.15a	0.15a	0.15a	0.15c	0.17b*	0.17b	0.29a*	Quantum yield for reduction of end electronacceptors at the PSI acceptor side
Performance Indexes (PI, combination of parameters)									
PI _{ABS}	0.36a	0.37a	0.37a	0.37a	0.22c*	0.28b*	0.27b*	0.31a*	PI (potential) for energy conservation from excitonto the reduction of intersystem electron

882 The calculations of photosynthetic parameters are according to the method of Yusuf et al. (2010). Subscript “0” indicates that the parameter
883 refers to the onset of illumination. There are four regimes, comprising SC, SM, TC, TM. They represent the cold-sensitive bermudagrass
884 genotype treated with deionized water or melatonin, the cold-tolerant genotype treated with deionized water or melatonin, respectively. Values
885 are calculated from four independent experiments. Columns marked with different letters indicates significant differences among different
886 regimes at a given temperature based on Duncan’s multiple range tests ($P < 0.05$). Columns marked with star represents statistical significance
887 between 30°C and 4°C for a given regime based on Independent-samples t test ($P < 0.05$).

888

889 **Table 2.** Metabolites amounts in the cold-sensitive (S) genotype and the cold-tolerant (T) genotype

Metabolites	30°C				4 °C			
	SC	SM	TC	TM	SC	SM	TC	TM
Organic acids	3.19±0.44ab	3.21±0.13ab	3.68±0.23a	2.84±0.03b	2.73±0.16c*	2.77±0.10c*	3.86±0.13b	4.66±0.10a*
Amino acids	0.85±0.12b	1.11±0.08a	0.57±0.04c	0.23±0.02d	1.23±0.07a*	1.14±0.04a	0.96±0.17b*	0.49±0.02c*
Carbohydrates	5.46±0.54b	4.49±0.23c	5.26±0.13b	6.92±0.31a	11.29±1.62c*	6.07±0.37d*	14.90±0.71b*	31.70±1.09a*
Lipids	0.39±0.03b	0.37±0.07b	6.34±0.92a	6.61±0.47a	0.42±0.02bc	0.43±0.04b	0.37±0.01c*	0.52±0.03a*
Others	0.60±0.05b	0.88±0.07a	0.43±0.05c	0.41±0.03c	0.75±0.05b*	0.75±0.02b	0.72±0.03b*	0.84±0.02a*

891 Note: There are four regimes, comprising SC, SM, TC, TM. They represent the cold-sensitive bermudagrass genotype treated with deionized
892 water or melatonin, the cold-resistant genotype treated with deionized water or melatonin, respectively. Values are calculated from three
893 independent experiments. Columns marked with different letters indicates significant differences among different regimes at a given
894 temperature based on Duncan's multiple range tests ($P<0.05$). Columns marked with star represents statistical significance between 30°C and
895 4°C for a given regime based on Independent-samples t test ($P<0.05$).

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897 Figure 1

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