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Effects of Melatonin on Growth, Physiology and Gene Expression in Rice Seedlings Under Cadmium Stress

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Abstract: Melatonin (MLT) is a hormonal substance found in many organisms and can improve plant stress resistance. In this study, the *japonica* rice variety Y32 and *indica* rice variety NJ6 were cultivated in hydroponics under different concentrations of $CdCl_2$ at the two-leaf stage. The growth, physiological and biochemical responses of the seedlings and the expression of cadmium (Cd)-related genes under exogenous melatonin (MLT) treatment were assessed. The results indicated that Cd stress destroyed the dynamic balance between reactive oxygen species (ROS) production and removal, resulting in ROS accumulation, membrane lipid peroxidation, and impaired growth and development. Following the application of exogenous MLT to rice seedlings, increases in plant biomass including both underground and above-ground areas were observed. MLT also scavenged the inhibition of superoxide dismutase (SOD) and peroxidase (POD) in a concentration dependent manner in response to Cd stress. Catalase (CAT) activity and malondialdehyde (MDA) expression also decreased following MLT treatment. Amongst the six Cd-related genes assessed, five genes were down-regulated and one was up-regulated in response to MLT treatment. Taken together, these data demonstrate that MLT improves the resilience of rice seedlings at the biochemical, physiological, and molecular levels, and diminishes the damage caused by Cd stress.

Keywords: Rice; melatonin; cadmium stress; physiological and biochemical index; gene expression

1 Introduction

Cadmium (Cd) is a highly toxic and widely distributed heavy metal. Approximately 30,000 tons of Cd per year enter the environment due to human factors of which 82%-94% are discharged into the soil [1]. China's Central, Southern, and Southwestern regions are contaminated with heavy metals, of which Cd constitutes > 7.0% and is considered the most prominent pollutant [2]. Approximately 1.13×10^6 hm^2 of cultivated land in China is thought to be Cd polluted. Up to 12 million tons of grains are polluted by heavy metals each year, of which 10 million tons of grains are lost, with economic losses of up to 20 billion Yuan RMB [3,4]. Cd in cultivated soil is easily absorbed by crops, accumulates in the body, and is biomagnified, seriously afflicting crop yields [5]. Strategies to improve Cd-resistance and plant yields under Cd stress conditions are therefore highly sought.

Rice (*Oryza sativa* L.) is globally one of the most important cultivated crops feeding nearly half of the world's population [6]. Due to the strong ability of rice plants to absorb Cd, rice Cd pollution is severe [7,8]. The toxicity of Cd in plants leads to photosynthetic damage, a loss of mineral nutrient pathways, and a disruption of carbohydrate metabolism. Furthermore, Cd toxicity leads to a reduction in plant height, reduced branch numbers, lower chlorophyll content, and a loss of total biomass [9,10]. Normal growing rice

has an active protective enzyme system that maintains the homeostasis of active oxygen production and clearance. Cd contamination disrupts this dynamic balance [11], resulting in increased tissue reactive oxygen species (ROS), enhanced membrane lipid peroxidation, increased biofilm voids, enhanced permeability, and disordered cell metabolism, hindering the growth and development of rice seedlings [12].

Melatonin (MLT) is a steroidal tryptamine, a hormonal substance found in many organisms, from algae to humans. In recent years, MLT has been detected in angiosperms of different countries and regions [13,14]. In addition to acting as a dark signal and a regulator of plant growth, MLT has antioxidant activity and can scavenge ROS, protecting plants from internal and environmental oxidative stress [15,16]. Studies have shown that in response to MLT induction, the root length of seedlings increases by 20%, and disruption to the cell ultrastructural is reduced [17]. MLT also promotes drought tolerance in apples through the regulation of ABA metabolism and free radical scavenging [18]. The combination of these results indicates that MLT improves plant stress resistance. To-date, the synthetic pathways of MLT synthesis are well understood, but the relationship between MLT and plant gene expression and the subsequent molecular benefits of its activity, remain poorly understood.

In this study, we assessed the effects of MLT on the growth index, protective enzyme activity, membrane lipid peroxidation, and other physiological and biochemical indices in rice seedlings under Cd stress. We reveal novel Cd-related genes the expression of which are regulated by MLT, and likely contribute to plant Cd resistance. These data provide theoretical support for the cultivation of rice with a high resistance to Cd stress.

2 Materials and Methods

2.1 Plant Materials and Culture Condition

Rice varieties Y32 (a *japonica* rice) and NJ6 (an *indica* rice) were selected as the materials. Rice seeds were sterilized in 3% H_2O_2 for 10 min, rinsed with deionized water 5 times, soaked in water for 24 h, and transferred to petri dishes with wet filter paper. Seeds were germinated for 48 h in the dark (temperature 30°C) and transferred into light culture incubator (culture temperature 30°C, relative humidity 70%, photoperiod 14 h/10 h, photosynthetically active radiation 16000 lx) in 96-well plates. Rice seedlings were hydroponically cultured with rice whole nutrient solution [19]. Cd stress and exogenous MLT treatment were performed when the seedlings had two leaves and one apical bud. A total of 7 groups were tested and repeated 3 times: (1) CK (0); (2) 10 Cd (10 μM $CdCl_2$); (3) 10 Cd + 10 MLT (10 μM $CdCl_2$, 10 μM MLT); (4) 10 Cd + 50 MLT (10 μM $CdCl_2$, 50 μM MLT); (5) 50 Cd (50 μM $CdCl_2$); (6) 50 Cd + 10 MLT (50 μM $CdCl_2$, 10 μM MLT); (7) 50 Cd + 50 MLT (50 μM $CdCl_2$, 50 μM MLT). Phenotypic and physiological data were measured one week post-treatment.

2.2 Growth Indicators

Rice plants were separated into roots, stems, and leaves, and washed in deionized water. Plants were blotted with filter paper to absorb excess liquid, and fresh roots and leaves were independently weighed and then dried at 80°C for 3 days to constant weight and weighted again.

2.3 Physiological and Biochemical Indicators Determination

For the determination of malondialdehyde (MDA) content, catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) activity, the methods described by Zhang et al. were employed [20].

2.3.1 MDA Content

Rice plants flesh tissue (1 g) was homogenized with 5 mL of 10% trichloroacetic acid. The mixture was then centrifugedat 4000×g for 10 min. 1 mL supernatant was absorbed, and add 4 mL of 10% trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was then heated at 100°C for 15 min. After rapid cooling, the mixture was centrifugedat 4000×g for 10 min. The absorbance of the

supernatant was measured at 450 nm and 600 nm. The MDA content was expressed on a fresh weight basis as $nmol \cdot g^{-1}$.

2.3.2 CAT Activity

CAT activity of catalase was determined by ultraviolet absorption method: take 100 μL enzyme solution, add 2800 μL PBS (25 mM, pH 7.0, +2 mM EDTA), and 100 μL H_2O_2 (300 mM, 1.53 mL 30% H_2O_2 constant volume at 50 mL), shake well, and transfer quickly into a cuvette. The absorbance was measured at 240 nm. The CAT activity was expressed on a fresh weight basis as $U \cdot g^{-1}FW \cdot min^{-1}$.

2.3.3 SOD Activity

Reaction solution (3 mL) included: 50 mM pH7.8 phosphate buffer, 13 mM methionine, 75 μM NBT, 10 μM EDTA, 2 mM riboflavin and 200 μL crude enzyme extract. The absorbance at 560 nm was measured after the reaction solution was exposed to light for 15 min. SOD activity was calculated by inhibiting 50% of NBT photochemical reduction reaction as an enzyme activity unit. The SOD activity was expressed on a fresh weight basis as $U \cdot g^{-1}FW$.

2.3.4 POD Activity

The activity of the enzyme was expressed as A470 change per minute. Reaction solution (3 mL) includes: 2.7 mL 25 mM phosphate buffer (pH 7.0, containing 2 mM EDTA), 100 μL enzyme solution, 100 μL guaiacol (1.5%, v/v) and 100 μL H_2O_2 (300 mM). H_2O_2 was added to start the reaction, and the absorption value was measured at 470 nm after the oscillator was fully mixed. The POD activity was expressed on a fresh weight basis as $U \cdot g^{-1}FW \cdot min^{-1}$.

2.4 Analysis of Differentially Expressed Cd-Related Genes

2.4.1 Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the rice leaves with the reference RNA plant extraction kit (QIANGEN). DNA contaminants were removed through DNase I treatment (RNase free). Total RNA was reverse-transcribed to generate cDNA using the TOYOBO®ReverTra Ace qPCR RT Kit. cDNA products were diluted 5-10 folds and stored at -20℃ prior to qRT-PCR analysis.

Table 1: Primers of Cd related genes

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (3'-5')
OsPCR1	AACTGCGTCTACTCCTGCTTCT	CCATCCGAGGTTCATGTCGAAG
OsLCD	CAAGGCCTCATGACTTGGTGT	ACTTCTGCGAGTGTGAGCAA
OsHMA3	GGGTGGAGATGTTCTTGAATCACTTGG	CCATTGGGTGGCTTGACTTGCTCT
OsNramp1	GGAAACTGGAGGTTGTGGTC	TTTGCTGATGCGGGTGTATT
OsIRT2	TGCATGATGTATAGGTGAAGGTG	CGGCAGAAGCTGGTCTTTATTA
OsNAS2	CGTCTGAGTGCGTGCATAGTA	GAAGCACAAACACAAACCGATA
OsActin	CAGGCCGTCCTCTCTCTGTA	AAGGATAGCATGGGGGAGAG

2.4.2 Primer Design

Select six Cd-related genes in Tab. 1, download the target gene sequence from NCBI (https://www.ncbi.nlm.nih.gov/), and use Primer Premier version 5.0 software to design each gene quantitative primers according to the real-time fluorescent quantitative PCR primers design principle.

2.4.3 qPCR Analysis

qPCR analysis of each gene was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). RT-PCR reactions were as follows: (total volume: 10 μL): 5 μL SYBR Green Fast

qPCR Master Mix (BBI); 0.8 μL upstream/downstream primer; 1 μL cDNA; 3.2 μL DDW. The PCR conditions consisted of denaturation at 95℃ for 10 min followed by 40 cycles of denaturation at 95℃ for 15 s, annealing at 57℃ for 25 s. Each sample was repeated 3 times. The relative quantitative method was used for experimental data analysis. *OsActin* was selected as reference gene.

2.5 Data Processing

Data were analyzed for significance using SigmaPlot 10.0 and IBM SPSS Statistics 20 statistical software. The single-sample t-test was used to test significance ($p < 0.05$ indicates significant difference, indicated by *; $p < 0.01$ indicates high significance, indicated by **).

3 Results Analysis

3.1 Effects of MLT on the Biomass of Rice Seedlings Under Cd Stress

Tab. 2 shows that the fresh weight of rice seedlings changed significantly under Cd stress. Compared to the CK group, the weight of the roots decreased first and then increased in response to exogenous MLT in the presence of 10 μM $CdCl_2$. After treatment with 50 μM $CdCl_2$, the fresh weight of roots increased first then decreased with increasing exogenous MLT concentrations. Following $CdCl_2$ treatment, the dry weight of the roots significantly decreased, and then increased in the presence of MLT. The effects of 10 μM MLT were significant. The fresh weight of the stems of the two rice seedlings decreased under Cd treatment. The NJ6 significantly decreased in the 10 Cd group, reaching 49.4%. The fresh weight of the stems significantly increased following MLT treatment. Compared to the CK group, the stem weight of NJ6 in the 10 Cd and 50 Cd groups decreased by 59.6% and 42.3% respectively. The dry weight of the Y32 stems decreased by 39.5% and 36.8% respectively. Different concentrations of MLT led to varying levels of recovery on the same varieties of rice. Compared to the fresh weight of roots, the fresh weight of leaves decreased under Cd stress. Under the same Cd stress, 50 μM MLT increased the fresh weight of NJ6 leaves, and decreased the fresh weight of Y32 leaves. That indicated that the same concentration of MLT had different mitigation effects on the different varieties of rice. The variation tendency of the dry weight of leaves was similar to the fresh weight of leaves. Cd stress reduced the biomass accumulation of above-ground and root of rice, and after applying the exogenous MLT, Cd stress in rice was effectively diminished, which proved that MLT is effective in promoting plant growth while harmful in other aspects and is consistent with the previous research.

Table 2: Biomass of rice seedlings under Cd stress and MLT treatment

	Treatment (μM)	Fresh weight (mg)			Dry weight (mg)		
		Root	Stem	Leaf	Root	Stem	Leaf
NJ6	CK	36.00	64.33	43.33	6.500	8.667	9.667
	10 Cd	41.50	27.50**	30.40	4.000	3.500	7.500
	10 Cd + 10 MLT	31.33	34.33**	34.00	5.000*	4.000	8.000
	10 Cd + 50 MLT	40.00	37.67*	33.67	4.333	3.333	7.000
	50 Cd	25.25	26.00**	27.00*	3.250**	5.000	6.500
	50 Cd + 10 MLT	36.00**	30.67**	32.33*	6.000	5.333	8.667
	50 Cd + 50 MLT	19.67	30.00**	27.33	4.333*	6.333	6.333
Y32	CK	34.00	49.20	16.00	5.800	7.600	2.400
	10 Cd	42.80	38.60**	17.40	4.000**	4.600*	3.800
	10 Cd + 10 MLT	32.33	33.00	16.67	5.667	4.667	2.333
	10 Cd + 50 MLT	42.67**	41.50	14.17	5.667	5.833*	3.333
	50 Cd	26.20	36.80*	16.40	5.000	4.800	3.400

50 Cd + 10 MLT	31.83	26.17**	12.00	5.500	5.500	3.333
50 Cd + 50 MLT	30.75**	30.25	11.00*	5.000	5.000	2.250

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level.

3.2 Effects of MLT on the Antioxidant Index of Rice Seedlings Under Cd Stress

As shown in Fig. 1, plants treated with MLT had higher levels of SOD and POD activity and lower levels of CAT activity than those in the 10 Cd and 50 Cd groups. There were no significant differences in SOD activity between the two varieties of rice seedlings in the CK group. The activity significantly decreased under Cd stress. Compared to the 10 Cd group, the SOD activity of Y32 and NJ6 increased by 51.7% and 3.7% in the 10 Cd + 10 MLT group, and 1.63 and 1.46-fold in the 10 Cd + 50 MLT group, respectively, which showed a significant difference. The SOD activity of Y32 in the 10 Cd + 10 MLT group increased by 51.7% compared to the 10 Cd and 50 Cd + 10 MLT groups that displayed a 2.49-fold increase over the 50 Cd group (Fig. 1(A)), which proved that the effects of MLT were impacted by the Cd^{2+} concentration. When not subjected to Cd stress, the POD activity of Y32 (a *japonica* rice) was low, whilst NJ6 (an *indica* rice) levels were high. From Fig. 1(B), it can be seen that the POD activity of the two rice plants significantly decreased under Cd stress, indicating damage to the antioxidant system. POD activity increased significantly following MLT treatment, indicating its ability to enhance antioxidant enzyme activity in response to Cd stress. Compared to the CK group, CAT activity increased after 7 days of Cd treatment (Fig. 1(C)). Compared to the 10 Cd and 50 Cd groups, CAT activity in rice significantly decreased following MLT treatment.

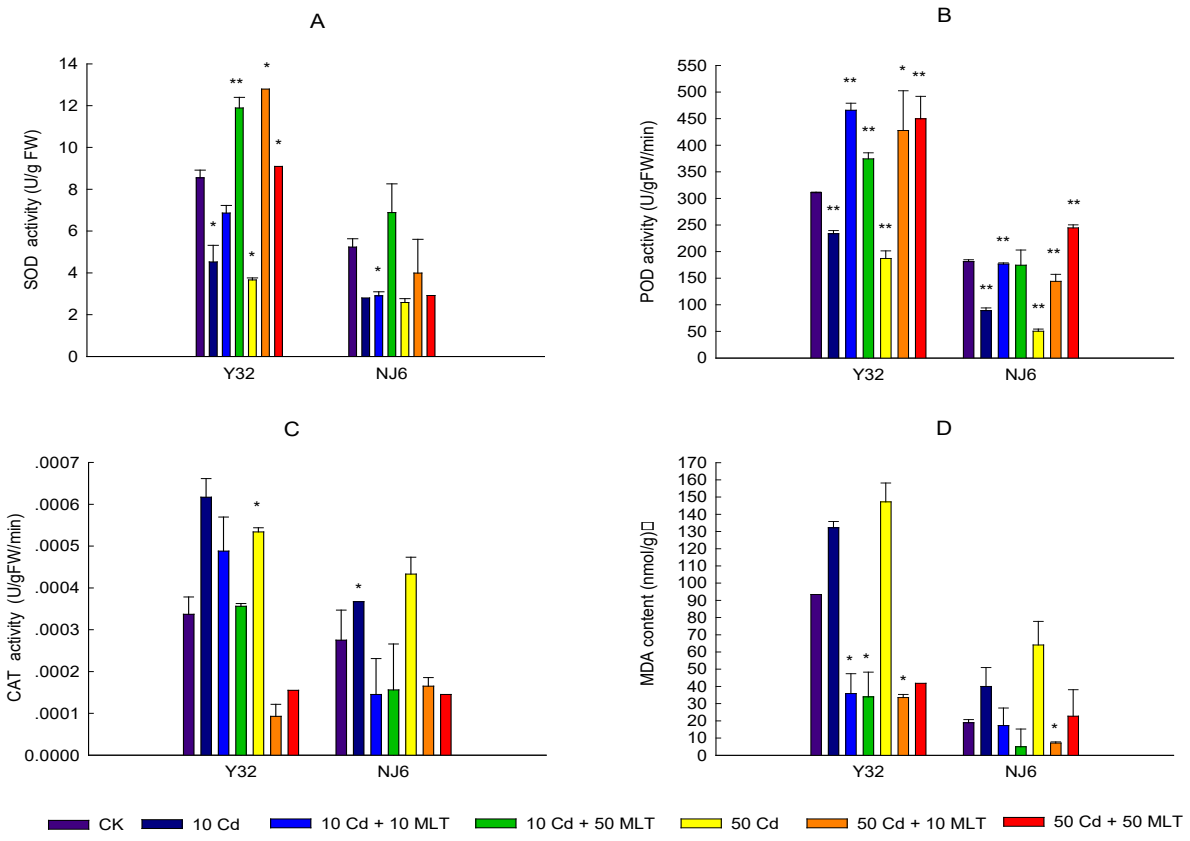

Figure 1: Antioxidant index of rice seedlings under Cd stress and MLT treatment. (A) SOD activity. (B) POD activity. (C) CAT activity. (D) MDA content. *Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level

From Fig. 1(D), it can be seen that the MDA content in rice significantly increased under Cd stress, indicative of cell membrane damage. Exogenous MLT significantly inhibited the increase in MDA in response to Cd stress. Compared to the CK group, the MDA content in Y32 and NJ6 increased by 41.6% and 112% in the 10 Cd group, and increased by 57.7% and 239% in the 50 Cd group, respectively, indicating cell membrane damage. The MDA content was significantly reduced by MLT, indicating reduced membrane damage and enhanced stress resistance. MLT diminished oxidative stress in rice under Cd stress. The effects of MLT were more pronounced in Y32 compared to NJ6, indicating genotype differences (Fig. 1).

3.3 Effects of MLT on the Expression of Cd-Related Genes in Rice Seedlings Under Cd Stress

Six Cd-related genes were selected and qRT-PCR was used to detect changes in gene expression in the Y32 and NJ6 treatment groups (CK, 50 Cd, 50 Cd + 50 MLT). Cd treatment influenced the expression of Cd-related genes in rice, and genotypic differences were observed. Following Cd treatment, the expression of *OsPCR1*, *OsLCD*, *OsIRT2* and *OsNAS2* in Y32 increased, whilst the expression of *OsNramp1* decreased compared to the CK group. The expression of *OsHMA3* showed no significant changes. The expression of all genes in NJ6 in the 50 Cd group were higher than the CK group. Following MLT treatment, 5 genes in the 50 Cd + 50 MLT were down-regulated compared to the 50 Cd group, whilst the expression of a single gene was up-regulated and the levels differed according to the variety of plants. *OsNramp1* was up-regulated in Y32 in the 50 Cd + 50 MLT group, which was significantly higher than the 36.0% increase in the 50 Cd group ($p < 0.01$) (Fig. 2(A)). The reduction in *OsPCR1*, *OsIRT2* and *OsNAS2* were 52.6%, 51.0%, and 50.6% lower in response to MLT, respectively, whilst the expression levels of *OsLCD* and *OsHMA3* did significantly differ from the CK group ($p > 0.05$). Although the *OsPCR1* of NJ6 was up-regulated, the difference was not significant ($p > 0.05$), and the expression levels of the 5 down-regulated genes were less than those in Y32 (Fig. 2(B)). Interspecies differences in the expression of genes were also observed. The expression of *OsNramp1* significantly changed in Y32, whilst the expression of *OsIRT2* was comparable to *OsNAS2*. The expression of *OsIRT2* was high in the 50 Cd group in both rice strains, and the responses of these groups to MLT treatment were similar (Fig. 2).

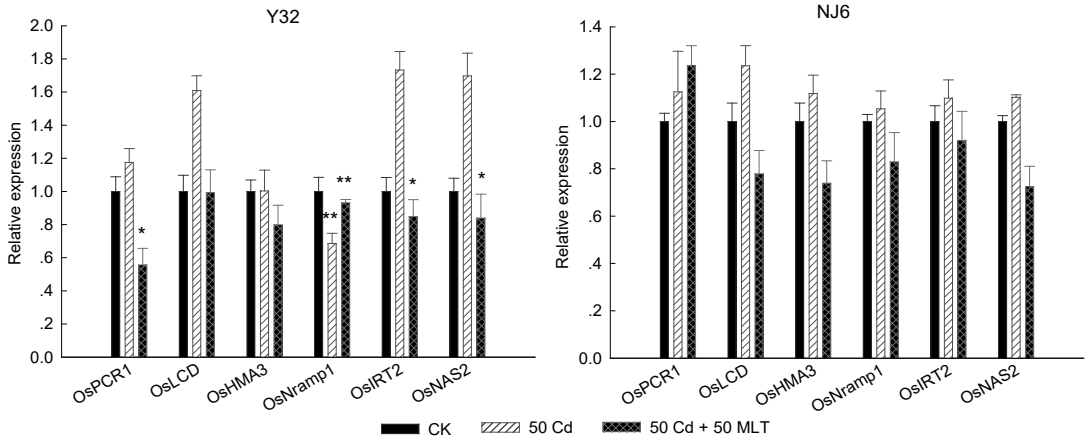

Figure 2: Expression of Cd-related genes in rice seedlings under Cd stress and MLT treatment. (A) Y32. (B) NJ6. *Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level

4 Discussion

Heavy metal ions in the soil, such as Cu and Zn, are crucial to the ability of plants to maintain normal growth and development. However, if these ions are in excess, the structure and function of plant proteins are damaged, the dynamic balance of active oxygen production and elimination in plants is

disrupted, and plant growth is endangered. In recent years, MLT has been shown to regulate antioxidant enzyme activity and can scavenge ROS in plants under salt, drought, and heavy metal stress [21,22].

An important manifestation of plants suffering from environmental stress is slow growth, leading to a loss of plant height, decreased stem diameters, and reduced fresh and dry weights of the aerial and underground plant regions. In this study, when plants under Cd stress were treated with MLT, the fresh weight of NJ6 initially increased and then decreased, but remained higher than the CK group, reflecting the beneficial effects of MLT. The fresh and dry weights of the roots, stems and leaves of the rice varied, indicating that MLT differentially influenced specific plant regions. It should however be noted that the fresh weights of the roots of NJ6 and Y32 in the Cd treatment group were higher than the CK group. This may have been due to the rice plants possessing their own protective mechanisms, permitting growth stimulation in response to low Cd concentrations [23]. Consistent with our findings, it has been shown that the exogenous application of MLT can range from meaningful improvement to ineffectiveness and toxicity, dependent on the MLT concentration [24]. In the experimental range assessed, Cd stress led to a reduction in plant biomass in the shoots and roots of rice, which could be diminished through the addition of exogenous MLT. Whilst MLT was generally protective to the plant growth, it was harmful in some cases.

SOD can scavenge free radicals, catalyze the conversion of O^{2-} into H_2O_2 with weak oxidation, and synergize with POD and CAT to protect plants from external stress. In this study, in response to 10 μM $CdCl_2$ treatments, the SOD activity of *japonica* Y32 and *indica* NJ6 gradually increased with increasing MLT concentrations. Following 50 μM $CdCl_2$ treatment, the SOD activity of both rice strains significantly increased then decreased. This indicated that MLT has variable mitigating effects under different degrees of Cd stress. In plants under Cd stress that were treated with MLT, the POD activity of NJ6 gradually increased, whilst the POD activity of Y32 increased sharply and then decreased. This indicated that the sensitivity of rice to MLT differed between each species. Shi et al. found that MLT, as an antioxidant, not only directly removes H_2O_2, but also stimulates the activity of SOD, CAT and POD [25,26]. MDA can be used as an indicator of the degree of peroxidation to indirectly determine the degree of damage to the membrane system and stress resistance of the plants [27]. A higher MDA content indicates higher levels of membrane lipid peroxidation. In this study, MLT significantly reduced the MDA content in rice seedlings under Cd stress, indicating that the Cd induced toxicity was diminished. These results were similar to the experimental results of MLT treatment which significantly reduced the MDA content of peaches [28]. In summary, MLT improves the antioxidant activity of plants under adverse conditions and promotes plant growth and development, alleviating the toxic effects of Cd stress.

A series of physiological and biochemical responses to Cd stress occur as a result of complex intrinsic resistance mechanisms. To understand the role of MLT in relieving Cd stress, we investigated their effects on the expression of Cd-related genes in rice. Previous studies demonstrated a role for MLT in the regulation of gene expression and antioxidant activity [29,30]. In this study, 6 genes were selected: *OsPCR1*, *OsLCD*, *OsHMA3*, *OsNramp1*, *OsIRT2* and *OsNAS2*. Five genes were down-regulated in the treatment group supplemented with MLT. OsNRAMP [31,32] and OsHMA families [33] have a strong Cd transshipment capability. OsHMA3 is located on the tonoplast membrane that pumps Cd^{2+} into the vacuole in the cytoplasm, not only regulating the transport of Cd^{2+} from the root to the aerial regions, but influencing plant Cd tolerance [34]. OsLCD participates in the accumulation of Cd in rice, which is expressed in the vascular bundles and phloem companion cells of rice roots [35]. Yeast mutants expressing *OsIRT1* and *OsIRT2* are more sensitive to Cd and are associated with Cd uptake [36]. Deoxygenated wheat root treatment is beneficial to inhibit Cd uptake of roots. OsNAS2 is an important enzyme in the synthesis of deoxygenated wheat root precursors, which indirectly regulates Cd uptake [37]. *OsNramp1* is involved in the uptake and transport of Fe^{2+} and Cd^{2+} in rice, and participates in Cd transport of roots. Elevated *OsNramp1* expression contributes to the higher absorption capacity of Cd by rice roots, and higher Cd accumulation in leaves. Studies on tomatoes have reported that the overexpression of the Nramp family increases the tolerance to Fe stress [38]. In this experiment, *Nramp* expression in the 50 Cd group of Y32 was lower than that in the CK group. The expression in the 50 MLT group was 36.0% higher than the 50 Cd group, indicating that MLT-mediated stress resistance is

related to *OsNramp1*. This supports a role for MLT in stimulation of other antioxidant activities. In this case, the activation of *OsNramp1* by MLT enhances the ROS-associated antioxidant system to protect plants from oxidative damage. The Cd resistance family gene *OsPCR1* is related to plant Cd resistance and participates in the transfer of Cd^{2+} from the inside to the outside of cells [39]. The expression of this gene increases in NJ6 under Cd treatment, and increased by 9.8% following MLT addition, suggesting that MLT is an important regulator of genes related to non-biological tolerance. MLT has a variety of physiological functions, including cytoskeletal effects and membrane receptor regulation, but its influence on plant cell physiology is less well understood. The up-regulation of *OsPCR1* in NJ6 by MLT also suggests a potential role in plant stress signaling. The role of *OsPCR1* in plant signaling has rarely been reported and warrants further investigation in future studies.

It is speculated that under different stresses, rice seedling leaves stimulate differential gene expression to promote Cd transport according to pathway requirements. *OsNramp1*, *OsIRT2* and *OsNAS2* were induced in both Y32 and NJ6, consistent with the findings of Zhou et al., which may be related to the mechanism of Cd uptake by plants [40]. In the experimental group, all genes excluding *OsNramp1* and *OsPCR1* were down-regulated in Y32 and NJ6. The result showed that MLT could reduce the accumulation of Cd in seedlings by reducing the transcription of Cd uptake and transport related genes.

In summary, we have preliminarily explored the effects of MLT on the physiological and biochemical indexes and Cd-related genes in rice. We found that the exogenous application of MLT plays an important role in protecting rice under Cd stress. The analysis of specific stress response genes supports the hypothesis that MLT protects plant cells from abiotic stresses. It also provides theoretical support for the cultivation of rice varieties with excellent stress resistance traits. Whilst the relevant regulatory mechanisms are complex, further studies on the specific molecular and biochemical pathways affected by these gene products will provide new information on both the direct and indirect mechanisms of MLT activity in plants.

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References

1. Ditoppi, S., Lambardi, L., Pazzagl, M. L., Cappugi, G., Durante, M. et al. (1998). Response to cadmium in carrot *in vitro* plants and cell suspension cultures. *Plant Science, 137(2),* 119-129.
2. Zhao, F. J., Ma, Y., Zhu, Y. G., Tang, Z., Mcgrath, S. P. et al. (2015). Soil contamination in China: current status and mitigation strategies. *Environmental Science & Technology, 49(2),* 750-759.
3. Wong, S. C., Li, X. D., Zhang, G., Qi, S. H., Min, Y. S. (2002). Heavy metals in agricultural soils of the Pearl River Delta, South China. *Environmental Pollution, 119(1),* 33-44.
4. Cheng, W., Yao, H., Wu, W., Zhang, G. P. (2005). Heavy metal pollution and its countermeasures in soil-rice system. *Review China Agricultural Science and Technology, 7(4),* 51-54.
5. Wang, F. J., Zhang, Y. T., Guo, Q. X. Tan, H. F., Han, J. H. et al. (2018). Effects of exogenous 5-aminolevulinic acid and 24-epibrassinolide on Cd accumulation in rice from Cd-contaminated soil. *Rice Science, 25(6),* 320-329.
6. Kalita, J., Pradhan, A. K., Shandilya, Z. M., Tanti, B. (2018). Arsenic stress responses and tolerance in rice: physiological, cellular and molecular approaches. *Rice Science, 25(5),* 235-249.
7. Xue, D., Jiang, H., Deng X., Zhang, X., Wang, H. et al. (2014). Comparative proteomic analysis provides new insights into cadmium accumulation in rice grain under cadmium stress. *Journal of Hazardous Materials, 280,* 269-278.
8. Wang, J., Fang, Y., Tian, B., Zhang, X., Zeng, D. et al. (2018). New QTLs identified for leaf correlative traits in rice seedlings under cadmium stress. *Plant Growth Regulation, 85(2),* 329-335.
9. Moya, J. L., Ros, R., Picazo, I. (1993). Influence of cadmium and nickel on growth, net photosynthesis and

carbohydrate distribution in rice plants. *Photosynthesis Research, 36(2),* 75-80.

10. Zhang, X., Chen, H., Jiang, H., Lu, W., Pan, J. et al. (2015). Measuring the damage of heavy metal cadmium in rice seedlings by SRAP analysis combined with physiological and biochemical parameters. *Journal of the Science of Food and Agriculture, 95(11),* 2292-2298.
11. Fornazir, R. F., Ferreira, R., Vitoria, A. P., Molina, S. M. G., Lea, P. J. et al. (2002). Effect of cadmium on antioxidant enzyme activities in sugar cane. *Biologia Plantarum, 45(1),* 91-97.
12. Hasan, M. K., Ahammed, G. J., Yin, L., Shi, K., Xia, X. et al. (2015). Melatonin mitigates cadmium phytotoxicity through modulation of phytochelatins biosynthesis, vacuolar sequestration, and antioxidant potential in *Solanum lycopersicum* L. *Frontiers in Plant Science, 6,* 601.
13. Cao, J., Murch, S. J., Obrien, R., Saxena, P. K. (2006). Rapid method for accurate analysis of melatonin, serotonin and auxin in plant samples using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A, 1134(1-2),* 333-337.
14. Pape, C., Luning, K. (2006). Quantification of melatonin in phototrophic organisms. *Journal of Pineal Research, 41(2),* 157-165.
15. Reiter, R. J., Tan, D., Zhou, Z., Maria, C., Lorena, F. B. et al. (2015). Phytomelatonin: assisting plants to survive and thrive. *Molecules, 20(4),* 7396-7437.
16. Li, X., Yu, B., Cui, Y., Yin, Y. (2017). Melatonin application confers enhanced salt tolerance by regulating Na^+ and Cl^- accumulation in rice. *Plant Growth Regulation, 83(3),* 441-454.
17. Szafrańska, K., Glińska, S., Janas, K. M. (2013). Ameliorative effect of melatonin on meristematic cells of chilled and re-warmed *Vigna radiata* roots. *Biologia Plantarum, 57(1),* 91-96.
18. Li, C., Tan, D. X., Liang, D., Chang, C., Jia, D. et al. (2015). Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behavior in two Malus species under drought stress. *Journal of Experimental Botany, 66(3),* 669-680.
19. Yoshida, S., Forna, D. A., Cock, J. H. (1976). *Laboratory manual for physiological studies of rice*, pp. 62-63. International Rice Research Institute.
20. Zhang, X., Wu, K. L., Xue, D. W. (2009). Effects of waterlogging stress on antioxidative enzyme system in different barley genotypes. *Journal of Zhejiang University (Agriculture and Life Sciences), 35(3),* 315-320.
21. Bonnefont-Rousselot, D., Collin, F. (2010). Melatonin: Action as antioxidant and potential applications in human disease and aging. *Toxicology, 278(1),* 55-67.
22. Liu, J., Wang, W., Wang, L., Sun, Y. (2015). Exogenous melatonin improves seedling health index and drought tolerance in tomato. *Plant Growth Regulation, 77(3),* 317-326.
23. Shi, J., Pan, G. X., Xia, Y. S., Zhang, N. M. (2013). Effects of Cd on different rice growth and antioxidant enzyme system. *Ecology Environmental Science, 22(5),* 832-837.
24. Chen, Q., Qi, W. B., Reiter, R. J., Wei, W., Wang, B. M. (2009). Exogenously applied melatonin stimulates root growth and raises endogenous indoleacetic acid in roots of etiolated seedlings of Brassica juncea. *Journal of Plant Physiology, 166(3),* 324-328.
25. Shi, H., Jiang, C., Ye, T., Tan, D. X., Reiter, R. J. et al. (2015). Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L). Pers] by exogenous melatonin. *Journal of Experimental Botany, 66(3),* 681-694.
26. Li, C., Wang, P., Wei, Z., Liang, D., Liu, C. et al. (2012). The mitigation effects of exogenous melatonin on salinity-induced stress in *Malus hupehensis*. *Journal of Pineal Research, 53(3),* 298-306.
27. Salin, M. L. (1988). Toxic oxygen species and protective systems of the chloroplast. *Physiologia Plantarum, 72(3),* 681-689.
28. Gao, H., Zhang, Z. K., Chai, H. K., Cheng, N., Yang, Y. et al. (2016). Melatonin treatment delays postharvest senescence and regulates reactive oxygen species metabolism in peach fruit. *Postharvest Biology & Technology, 118,* 103-110.
29. Rodriguez, C., Mayo, J. C., Sainz, R. M., Antolín I, Herrera, F. et al. (2010). Regulation of antioxidant enzymes: a significant role for melatonin. *Journal of Pineal Research, 36(1),* 1-9.
30. Reiter, R. J. (1998). Oxidative damage in the central nervous system: protection by melatonin. *Progress in Neurobiology, 56(3),* 359-384.

31. Sasaki, A., Yamaji, N., Yokosho, K., Ma, J. F. (2012). Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. *Plant Cell, 24(5),* 2155-2167.

32. Ishikawa, S., Ishimaru, Y., Igura, M., Kuramata, M., Abe, T. et al. (2012). Ion-beam irradiation, gene identification, and market-assisted breeding in the development of low-cadmium rice. *Proceedings of the National Academy of Sciences USA, 109(47),* 19166-19171.

33. Yamaji, N., Xia, J., Mitani-Ueno, N., Yokosho, K., Ma, J. F. (2013). Preferential delivery of zinc to developing tissues in rice is mediated by P-type heavy metal ATPase *OsHMA2*. *Plant Physiology, 162(2),* 927-939.

34. Miyadate. H., Adachi, S., Hiraizumi, A., Tezuka, K., Nakazawa, N. et al. (2011). *OsHMA3*, a P1B-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. *New Phytologist, 189(1),* 190-199.

35. Shimo, H., Ishimaru, Y., An, G., Yamakawa, T., Nakanishi, H. et al. (2011). *Low cadmium (LCD)*, a novel gene related to cadmium tolerance and accumulation in rice. *Journal of Experimental Botany, 62(15),* 5727-5734.

36. Nakanishi, H., Ogawa, I., Ishimaru, Y., Mori, S., Nishizawa, N. K. (2006). Iron deficiency enhances cadmium uptake and translocation mediated by the Fe^{2+} transporters *OsIRT1* and *OsIRT2* in rice. *Soil Science and Plant Nutrition, 52(4),* 464-469.

37. Inoue, H., Higuchi, K., Takahashi, M., Nakanishi, H., Nishizawa, N. K. (2003). Three rice nicotianamine synthase genes, *OsNAS1*, *OsNAS2*, and *OsNAS3* are expressed in cells involved in long-distance transport of iron and differentially regulated by iron. *Plant Journal, 36(3),* 366-381.

38. Bereczky, Z., Wang, H. Y., Schubert, V., Ganal, M., Bauer, P. (2003). Differential regulation of *nramp* and *irt* metal transporter genes in wild type and iron uptake mutants of tomato. *Journal of Biological Chemistry, 278(27),* 24697-24704.

39. Huang, Z. X., Wang, F. J., Jiang, H., Li, Z. L., Ding, Y. F. et al. (2014). Comparison of Cadmium-accumulation-associated genes expression and molecular regulation mechanism between two rice cultivars (*Oryza sativa* L. subspecies *japonica*). *Acta Agronomica Sinica, 40(4),* 581-590.

40. Zhou, Q., Wang, H., Zhang, Y. X., Dong, Q., Meng, S. et al. (2016). Changes in cadmium concentration in rice plants under different cadmium levels and expression analysis of genes related to cadmium regulation. *Chinese Journal of Rice Science, 30(4),* 380-388.