

Regulatory effect of exogenous melatonin on the expression of enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice seedlings under NaCl stress

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Abstract

In this study, the regulatory effect of exogenous melatonin on the expression of genes related to the AsA-GSH cycle and nitrogen metabolism in seedlings under NaCl stress was determined; the homology between enzyme genes and similar genes in other species was analyzed by applying different concentrations of exogenous melatonin to rice seedlings under NaCl stress; and the morphology and physiological indicators of seedlings were determined. Exogenous melatonin was found to upregulate the expression of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings under NaCl stress, which enhanced the enzyme activities of the AsA-GSH cycle and nitrogen metabolism, ultimately reducing the content of reactive oxygen species (ROS) and improving the photosynthetic rate and nitrogen efficiency. A high homology was found when rice *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* were compared with similar genes in *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*. Further, the location of the gene on the chromosome, the similar gene structure, GO annotation, and KEGG metabolic pathways were explained by the functions and metabolic processes of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1*. Overall, our findings indicate that exogenous melatonin participates in the regulation of enzyme gene expression during the AsA-GSH cycle and nitrogen metabolism, ultimately affecting the activities of related enzymes and alleviating the oxidative damage induced by ROS in seedlings.

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Abstract

In this study, the regulatory effect of exogenous melatonin on the expression of genes related to the AsA-GSH cycle and nitrogen metabolism in seedlings under NaCl stress was determined; the homology between enzyme genes and similar genes in other species was analyzed by applying different concentrations of exogenous melatonin to rice seedlings under NaCl stress; and the morphology and physiological indicators of seedlings were determined. Exogenous melatonin was found to upregulate the expression of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings under NaCl stress, which enhanced the enzyme activities of the AsA-GSH cycle and nitrogen metabolism, ultimately reducing the content of reactive oxygen species (ROS) and improving the photosynthetic rate and nitrogen efficiency. A high homology was found when rice *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* were compared with similar genes in *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*. Further, the location of the gene on the chromosome, the similar gene structure, GO annotation, and KEGG metabolic pathways were explained by the functions and metabolic processes of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1*. Overall, our findings indicate that exogenous melatonin participates in the regulation of enzyme gene expression during the AsA-GSH cycle and nitrogen metabolism, ultimately affecting the activities of related enzymes and alleviating the oxidative damage induced by ROS in seedlings.

KEYWORDS

rice; melatonin; NaCl stress; AsA-GSH cycle; nitrogen metabolism

1 | INTRODUCTION

Rice (*Oryza sativa* L.) is a widely cultivated food crop. Food security is of vital importance for ensuring the survival of human beings worldwide. However, salt stress severely affects rice yield globally (Zörb et al., 2019; Liu et al., 2022; Sengupta & Majumder, 2010). Rice is a moderately salt-sensitive crop, and salt stress can induce the accumulation of ROS, such as superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), in rice seedlings. Excess ROS usually weakens various physiological functions of cells, resulting in a decrease in the photosynthetic rate, blockage of protein synthesis, disturbance of the nitrogen metabolism pathway, and cell death in severe cases (Michard & Simon, 2020; Ullah et al., 2019). Therefore, low levels of ROS can be used as a signaling factor for rice to respond to stress. However, the accumulation of large amounts of ROS seriously affects the normal growth and development of rice.

Melatonin (N-acetyl-5-methoxytryptamine, MT) is an indole compound that plays an important role in life activities and is widely found in most animals and plants (Tan et al., 2012). Owing to the successful isolation of the pineal gland of cattle in 1958, the pineal hormone was identified and named melatonin (Lerner et al., 1958). However, trace amounts of melatonin were not found in plants until 1995 (Hattori et al., 1995; Dubbels et al., 1995). Based on an in-depth study, melatonin is an important antioxidant that plays an important role in promoting seedling growth (Liu et al., 2021; Samanta et al., 2021), increasing photosynthetic rate (Sezer et al., 2021; Yan et al., 2021), and enhancing metabolic activity (Yu et al., 2018; Wang et al., 2021).

Salt stress disrupts the balance of ROS in plants, leading to disorders of plant physiology and metabolism. To restore ROS content to normal levels, the antioxidant mechanism of ROS scavenging is necessary. The ascorbate-glutathione (AsA-GSH) cycle is an important link in plant antioxidant mechanisms that can effectively eliminate the accumulation of ROS in plants (Ye et al., 2015). Ascorbic acid (AsA), glutathione (GSH), ascorbic acid oxidase (AAO), dehydroascorbic acid reductase (DHAR), glutathione reductase (GR), and glutathione peroxidase (GPX) are key components of the plant AsA-GSH cycle (Vasseur et al., 2011). Other antioxidants, including polyamines, proline, and glycine betaine, also contribute to the stabilization of ROS balance. Under the catalysis of key enzymes of the AsA-GSH cycle, excess H_2O_2 and $O_2^{\cdot-}$ in the plant are converted into water, which is finally absorbed and utilized by plant cells. According to related studies, melatonin can reduce the ROS content in plants by increasing the activities of key enzymes in the AsA-GSH cycle of sugar beets and resisting oxidative damage caused by stress (Zhang et al., 2021). Melatonin can also

regulate the expression of key enzyme genes in the AsA-GSH cycle to affect the activities of key enzymes and enhance the tolerance of plants to adverse stress (Lv et al., 2019; Ma et al., 2016).

Nitrogen metabolism is a necessary normal metabolic activity in the life process of plant cells, and its pathway plays a certain role in all functional regions of plants (Duan et al., 2018). Plants absorb nitrogen-containing ions in the soil and convert them into nitrogen, which is stored in plants and then used by plants as nitrate nitrogen and ammonium nitrogen. Key enzymes involved in nitrogen metabolism play important roles in nitrogen assimilation. Nitrate reductase (NR) is the first key enzyme and an important rate-limiting enzyme that catalyzes the reduction of NO_3^- to NO_2^- in nitrogen metabolism. Glutamine synthetase (GS) and glutamate synthase (GOGAT) constitute the GS/GOGAT pathway, which participates in the primary assimilation and re-assimilation of ammonia and is an indispensable enzyme in ammonia assimilation (Wang, 2013). Under salt stress, the nitrogen metabolism pathway is hindered, mainly by changing the expression of key nitrogen metabolism enzyme genes, ultimately affecting the activities of key nitrogen metabolism enzymes (Debouba et al., 2013). Exogenous melatonin was reported to induce the expression of key enzyme genes for nitrogen metabolism in maize and alfalfa under drought stress, and enhance the activities of key enzymes in nitrogen metabolism, thereby ensuring the normal progress of nitrogen metabolism (Ren et al., 2021; Antoniou et al., 2017).

The regulatory effects of melatonin on the antioxidant system of rice and other crops under stress conditions have been widely reported (Jahan et al., 2020; Zhang et al., 2021). However, only few studies sought to evaluate the effects of melatonin on the rice AsA-GSH cycle and nitrogen metabolism under salt stress. Further, the mechanism by which melatonin regulates the rice AsA-GSH cycle and nitrogen metabolism remains unclear. Therefore, in this study, exogenous melatonin was used to treat rice seedlings under NaCl stress. Thereafter, the morphological, physiological, and molecular indices were measured, and the regulatory mechanism of exogenous melatonin on the expression of related enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice seedlings under NaCl stress was investigated.

2 | MATERIALS AND METHODS

2.1 | Rice material

The rice ‘glutinous rice 89-1’ was selected as the experimental material and was obtained from the laboratory of Chongqing Engineering Research Center of Specialty Crop Resource, Chongqing Normal University. Glutinous rice 89-1 is an overwintering rice variety, as its axillary buds are dormant during the winter and can germinate and regenerate the following spring. The yield of overwintering regeneration season is equivalent to that of current season (Deng et al., 2018; Chen et al., 2021). After cleaning and disinfection, 100 rice seeds were selected and placed in the germination box (10 rows \times 10 columns), which was transferred to a light incubator under the conditions of 16 h light/8 h dark, 30 °C, and 70% humidity for 10 d. Seedlings were cultivated for three- and one-heart periods, and seedlings with consistent growth were used for the next treatment. The following seven experimental treatments were administered: (1) control check treatment (CK); nutrient solution treatment for 8 d; (2) NaCl treatment: nutrient solution treatment for 3 d and then 80 mmol·L⁻¹ NaCl solution containing nutrient solution treatment for 5 d; (3) melatonin pretreatment using different concentrations (50, 100, 200, 400, and 800 $\mu\text{mol}\cdot\text{L}^{-1}$) of melatonin and nutrient solution for 3 d, with the corresponding concentrations represented by MT50, MT100, MT200, MT400, and MT800, respectively, and then treatment with 80 mmol·L⁻¹ NaCl solution containing nutrient solution for 5 d.

2.2 | Determination of seedling height, root length, fresh weight, and dry weight

The plant height and root length of rice seedlings were measured with an accurate scale ruler (cm), and the fresh and dry weights of the rice seedlings were measured with an electronic balance (g).

2.3 | Determination of chlorophyll, malondialdehyde (MDA), H_2O_2 , and O_2^- contents in seedlings

Chlorophyll, MDA, H_2O_2 , and O_2^- contents were determined according to the method of Zhang and Li (2016); MDA content was determined according to the absorbance at 450, 532, and 600 nm; and chlorophyll

a and b contents were determined via acetone extraction.

2.4 | Determination of AsA, GSH, AAO, DHAR, GR, and GPX indices of seedlings

The contents of AsA and GSH were determined according to the method of Griffith (1980); the activities of AAO and DHAR were determined at 265 nm following the method of Nakano and Asada (1981); and the activities of GR and GPX were determined by the method of Nagalakshmi and Prasad (2001) at an absorbance of 340 nm.

2.5 | Determination of nitrate-nitrogen content, ammonium nitrogen content, NR, GS, and GOGAT indices of seedlings

The nitrate and ammonium nitrogen contents in fresh leaves were determined using the method of Gao (2000); the NR activity was determined using the method described by Barro et al. (1991); and the activities of GS and GOGAT were determined according to the method described by Hao et al. (2004).

2.6 | Determination of enzyme gene expression for the AsA-GSH cycle and nitrogen metabolism in seedlings

2.6.1 | Design of RT-qPCR Primers

We downloaded the AsA-GSH cycle enzyme genes (*OsGR3* and *OsGPX1*) and nitrogen metabolism enzyme genes (*OsNR2*, *OsGS1*, and *OsGOGAT1*) of rice seedlings from NCBI (<https://www.ncbi.nlm.nih.gov>) and referred to the sequence of the gene, *OsActin1*. Primers were designed using Primer-BLAST sequence alignment. Thereafter, the specificity of the primers was verified to obtain gene-specific primers, and the sequence number of the genes and the size of the product were obtained (Table 1).

TABLE 1 Primers for the enzyme genes and reference gene in the AsA-GSH cycle and nitrogen metabolism in rice seedlings

Gene Name	Accession Number	Forward Primer (5'-3')	Reverse Primer (5'-3')	Prod
<i>OsGR3</i>	XM.015757646	F: CTCCAACGACCACGTATCCA	R: AGCCATGGATCCTCAACACC	143
<i>OsGPX1</i>	XM.015780454	F: AGCGGAAAAGAGGTGAACCT	R: TCCAAACTGATTGCACGGGA	171
<i>OsNR2</i>	XM.015767224	F: AGAGGCTAGCGGTTGATTCC	R: CCTACTGCTTCTCTCCCAAACCT	142
<i>OsGS1</i>	XM.015770616	F: TCGCCATTATATTGCCGCCT	R: CGAGATCGGTGAGAGAAGCC	121
<i>OsGOGAT1</i>	XM.015793756	F: GGGTGGTGGAGAGATGGTTG	R: TAGCCAACACAGACAAGCGT	112
<i>OsActin1</i>	XM.015774830	F: GCGTCTGGATTGGTGGTTCT	R: ACCGCTCTACAACTTGGCA	142

2.6.2 | RNA extraction and RT-qPCR analysis

Total RNA was extracted using TRIzol reagent, and the cDNA template was obtained by reverse transcription. The reaction for fluorescence quantitative PCR consisted of 12.5 μ L of SYBR fluorescent dye, 1 μ L of cDNA template, 0.5 μ L of forward and reverse primers, and ddH₂O to a final volume of 25 μ L. The following PCR cycling condition was employed: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. The relative gene expression values were calculated using the $2^{-\Delta\Delta^T}$ method (Livak & Schmittgen, 2001).

2.7 |

Chromosomal locations and exon-intron structures of enzyme genes in the AsA-GSH cycle and nitrogen metabolism in seedlings

The location distribution map and exon-intron structure maps of genes on chromosomes were generated using MapGene2Chromosome_v2.1 (MG2C_v2.1) (Chao et al., 2015) and Gene Structure Display Server 2.0 (GSDS2.0) online software (Hu et al., 2015).

2.8 | GO annotations, KEGG metabolic pathways, and heat maps of the enzyme genes in the AsA-GSH cycle and nitrogen metabolism in seedlings

The GO annotations of genes were obtained from the Ensembl database (Kinsella et al., 2011), KEGG metabolic pathways for the genes were obtained using DAVID online software (Huang et al., 2009), and heat maps of gene expression were constructed using HemI1.0 software (Deng et al., 2014).

2.9 | Statistical analysis

The data were statistically analyzed using Excel and SPSS 21.0, plotted using Origin8.5, and tested for significance using the Duncan's multiple comparison method ($p < 0.05$ is the level of significance).

3|

RESULTS AND ANALYSIS

3.1 | Basic characteristics of the enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice, and similar genes in other species

The *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* sequences of rice seedlings were downloaded from NCBI, and the gene name, gene symbol, and Os ID were obtained. The protein size was between 190 and 2200 aa, and the molecular weight was within the range of 39-240 kD (Table 2). The rice gene sequences mentioned above were compared with nucleotide-blastn sequences, which revealed the presence of similar genes in *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor* with high consistency and low E-value (Table 3).

TABLE 2 Basic characteristics of the enzyme genes in the AsA-GSH cycle and nitrogen metabolism

Gene Name	Gene Symbol	Os ID	Protein Size (aa)	Molecular Weight (kD)
<i>OsGR3</i>	LOC4348623	Os10g0415300	388	41.808
<i>OsGPX1</i>	LOC4336627	Os04g0556300	198	21.666
<i>OsNR2</i>	LOC4330867	Os02g0770800	890	98.639
<i>OsGS1</i>	LOC4330649	Os02g0735200	357	39.201
<i>OsGOGAT1</i>	LOC4324398	Os01g0682001	2168	236.895

TABLE 3 Enzyme genes in the AsA-GSH cycle and nitrogen metabolism of rice that are similar to genes found in *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*

	<i>Arabidopsis thaliana</i>	<i>Hordeum vulgare</i>	<i>Zea mays</i>	<i>Sorghum bicolor</i>
Gene Name		Gene Symbol		
<i>OsGR3</i>	<i>ATGR ATGR1</i>	LOC123449517	LOC541986	LOC8059472
<i>OsGPX1</i>	<i>ATGPX6</i>	LOC123427543	LOC100273297	LOC8073934
	<i>ATGPX8</i>		LOC100280060	LOC8056585
<i>OsNR2</i>	<i>ATNIA1 ATNIA2</i>	LOC123404242	LOC103643134	LOC8078943
		LOC123404584	LOC109939975	
<i>OsGS1</i>	<i>ATGSR1 ATGSR2</i>	LOC123449274	LOC542401	LOC110434374
	<i>ATGLN1.3</i>	LOC123401521	LOC542214	
	<i>ATGLN1.4</i>		LOC542520	
<i>OsGOGAT1</i>	<i>ATGLT1</i>	LOC123444122	LOC103636185	LOC8068026
			LOC103651348	LOC8074568

3.2|

Chromosomal localization of enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice and similar genes in other species

The results of chromosome mapping of the enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice and similar genes in *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor* are shown in (Figure 1a–e). *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* were found to be unevenly distributed on four rice chromosomes. In this study, similar genes in *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor* were employed as examples, and the above gene symbols were replaced with the abbreviations of species Latin names and the first letters of gene names. *OsGR3* is located on chromosome 10, *ATGR* is located on chromosome 3, *HvGR* is located on chromosome 4H, and *ZmGR* and *SbGR* are located on chromosome 1, with differences found in their distribution. *OsGPX1* is located on chromosome 4, *ATGPX* is

located on chromosomes 1 and 4, *HvGPX* is located on chromosome 2H, *ZmGPX* is located on chromosomes 2 and 10, *SbGPX* is located on chromosomes 4 and 6, and most of the genes are located on chromosomes 2 and 4. *OsNR2* and *OsGS1* are located on chromosome 2; *ATNIA* is located on chromosome 1; *ATGSR* and *ATGLN* are located on chromosomes 1, 3, and 5; *HvNR* and *HvGS* are located on chromosomes 4H and 6H; *ZmNR* and *ZmGS* are located on chromosomes 1, 4, and 5; and *SbNR* and *SbGS* are located on chromosome 4. The NR and GS genes were found to be distributed on different chromosomes of rice, *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*, and located on the same chromosome in the same species. *OsGOGAT1* is located on chromosome 1, *ATGLT1* is located on chromosome 5, *HvGOGAT* is located on chromosome 3H, *ZmGOGAT* is located on chromosomes 3 and 8, *SbGOGAT* is located on chromosomes 3 and 9, and most of the genes are located on chromosome 3.

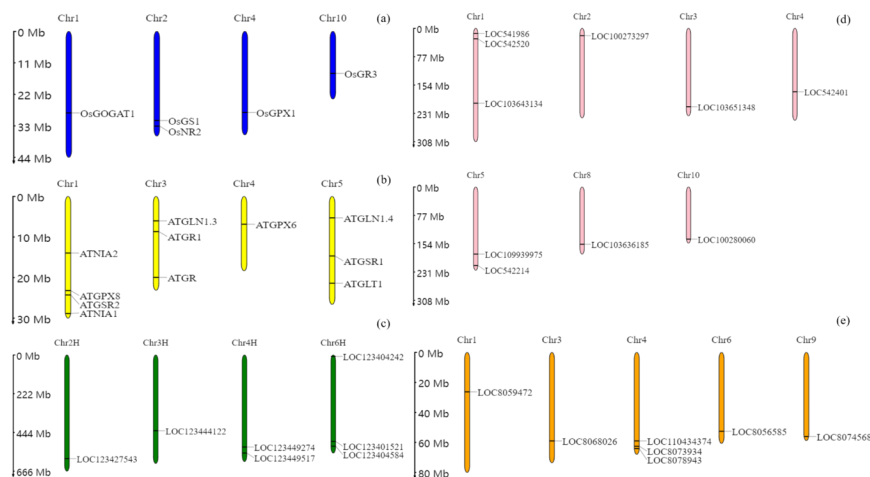


FIGURE 1 Chromosomal locations of the enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice and similar genes in other species. (a), (b), (c), (d), and (e) represent the chromosomes of rice, *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*, respectively.

3.3| Exon-intron structure of enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice and similar genes in other species

(Figure 2a–e) show the exon-intron structure and the ratio of intron phases 0, 1, and 2 to all introns of the enzyme genes in the AsA-GSH cycle and nitrogen metabolism in rice, and similar genes in *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*. The exon numbers of *ATGR* and *ATGR1* were 10 and 16, respectively; and the GR gene has 10 exons in rice, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*. The exon number of the GPX genes was the same in all species, with six exons. Except for *ATNIA1* and *ATNIA2*, there were four and three exons, and two and three exons in *Hordeum vulgare* NR genes; and four exons in rice, *Zea mays*, and *Sorghum bicolor* NR genes, respectively. The GS gene exon

number was 11 in rice. Further, *Arabidopsis thaliana* GS genes had 9, 12, 9, and 10 exons; *Hordeum vulgare* GS genes had 10 and 11 exons; *Zea mays* GS genes had 10, 10, and 11 exons; and *Sorghum bicolor* GS gene had 10 exons. There were 22 exons of the GOGAT gene in all species, except *ATGLT1*, which had 20 exons. From the intron phase results of (Figure 2f-j), intron phase 0 of each species was found to account for 56%-60%, which served as the largest proportion. Intron phase 1 was 16%, 13%, 19%, 13%, and 16% in rice, *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor*, respectively; and the proportion of intron phase 2 was 23%-30% in each species. Based on an analysis of the intron phase of each species, the intron phases were found to account for 0, 1, and 2 of all introns in rice, *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays*, and *Sorghum bicolor* have similar results.

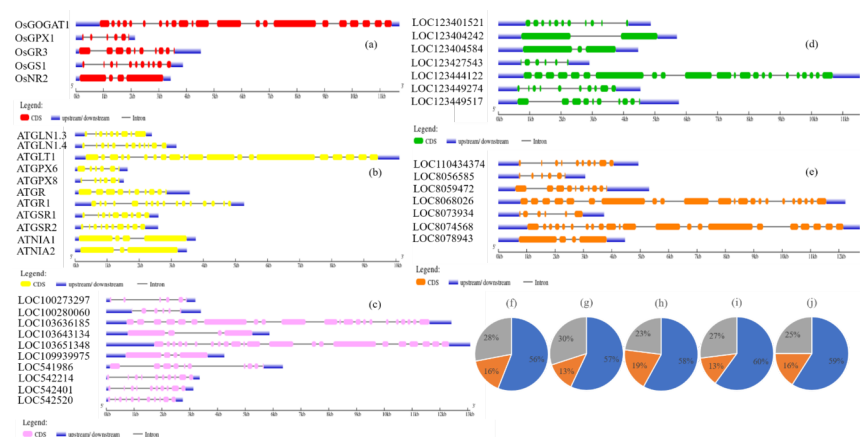
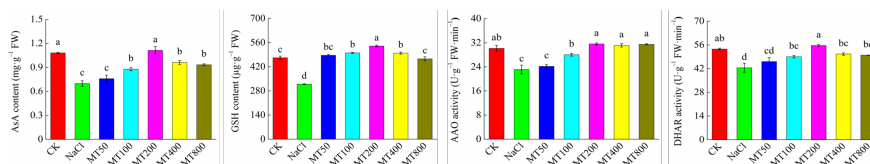


FIGURE 2 Exon-intron structure and intron phases 0, 1, and 2 accounted for the proportion of all introns of AsA-GSH cycle enzyme genes and nitrogen metabolism enzyme genes in rice and similar genes in other species. (a) and (f), (b) and (g), (c) and (i), (d) and (h), (e) and (j) represent the exons and intron phases 0, 1, and 2, which accounted for the proportion of all introns in rice, *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor*, respectively. The blue rectangle represents the 5'-UTR and 3'-UTR, and the line represents introns; the unit of exon length is kb.

3.4| Effects of exogenous melatonin on the expression of enzymes involved in the AsA-GSH cycle and nitrogen metabolism in NaCl-stressed seedlings

As shown in (Figure 3a-d), NaCl treatment significantly decreased AsA, GSH, and ammonium nitrogen content and AAO, DHAR, GR, GPX, NR, GS, and GOGAT activities in seedlings, but significantly increased nitrate-nitrogen content. The results of exogenous melatonin at different concentrations (50–800 $\mu\text{mol} \cdot \text{L}^{-1}$) opposed those of NaCl treatment. The heat maps of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* represented by Log_2 FC values among the different treatments were constructed (Figure 4a-b). Compared with control treatment, the color of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* on the heat map was green under NaCl stress, indicating that gene expression was significantly downregulated; the darker the green, the greater the degree of downregulation (Figure 4a-b). In other melatonin-pretreated samples, the color of the genes on the heat map was reddish, even red, indicating that the expression of the genes was upregulated; the darker the red, the higher the degree of upregulation (Figure 4a-b). Simultaneously, the expression levels of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings were detected by RT-qPCR. The results of gene expression were found to be similar to those on the heat map.



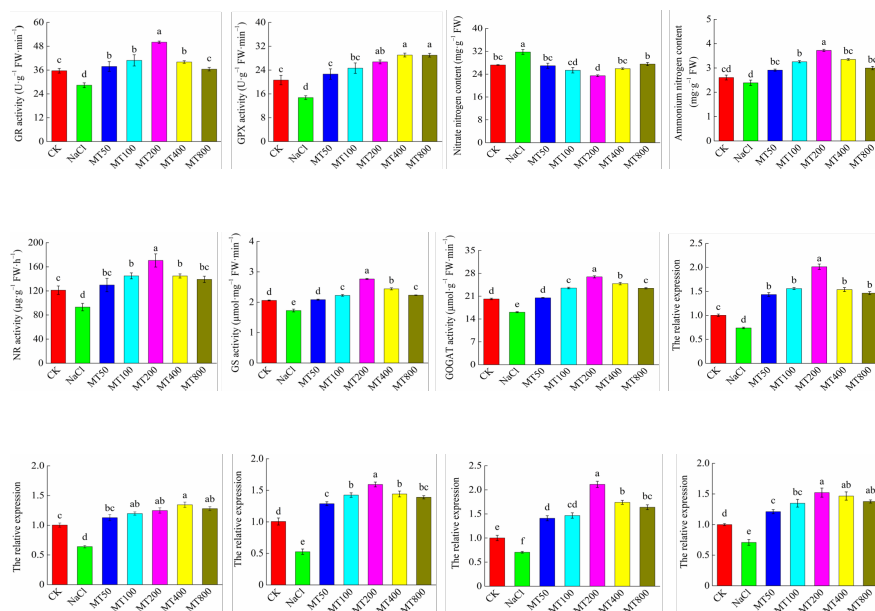


FIGURE 3 Effects of exogenous melatonin on the activities of enzymes in the AsA-GSH cycle and nitrogen metabolism and enzyme gene expression in seedlings under NaCl stress. Data are presented for (a): AsA, GSH, AAO, and DHAR, (b): GR, GPX, nitrate nitrogen content, ammonium nitrogen content, (c): NR and GS, GOGAT and *OsGR3*, (d): *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1*. Different lowercase letters indicate significant difference ($p < 0.05$).

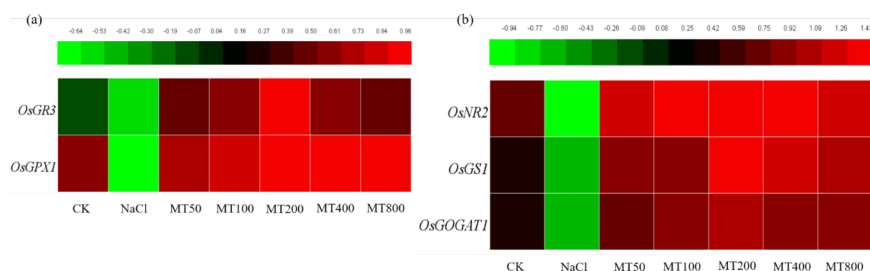


FIGURE 4 Heat map of exogenous melatonin regulating the expression of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings under NaCl stress. (a) indicates the expression of the *OsGR3* and *OsGPX1*, (b) indicates the expression of the *OsNR2*, *OsGS1* and *OsGOGAT1*.

3.5 | Expression regulation, GO annotation, and KEGG metabolic pathway of exogenous melatonin for enzymes involved in the AsA-GSH cycle and nitrogen metabolism in seedlings under NaCl stress

GO functional annotations can be divided into three categories: biological processes, molecular functions, and cell composition (Dong et al., 2021). Based on the results of GO annotations and KEGG metabolic pathways of rice *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1*, these genes are involved in biological processes, such as redox and cytosolic composition; functions, such as oxidoreductase activity; and three metabolic pathways, including nitrogen metabolism, alanine, aspartate and glutamate metabolism, and glutathione metabolism (Figure 5a, b). Exogenous melatonin regulated gene expression in the AsA-GSH cycle and nitrogen metabolism pathway in seedlings under NaCl stress (Figure 6). NR reduces nitrate to nitrite, and then converts it to ammonia, which is

converted under the action of GS and GOGAT for Gln and Glu. Further, exogenous melatonin regulates the activities of NR, GS, and GOGAT by upregulating the expression of *OsNR2*, *OsGS1*, and *OsGOGAT1*, ultimately affecting the nitrogen metabolism whole process. Glu can produce GS through the action of glutamate cysteine ligase (GCLM) and glutathione synthetase (GS II). In contrast, GSH can synthesize Glu via γ -glutamyltranspeptidase (γ -GT), which connects the AsA-GSH cycle with nitrogen metabolism, forming a complete metabolic channel network (Fig. 6). Under the action of GR and GPX, a balance between GSSG and GSH can be achieved, and the circulation and regeneration of GSH can be promoted. Exogenous melatonin may upregulated the expression of *OsGR3* and *OsGPX1*, therefore, upregulated the activity of GR and GPX, and participated in the AsA cycle. The purpose of the AsA-GSH cycle is to eliminate excessive ROS and reduce the toxic effects of NaCl stress on seedlings.

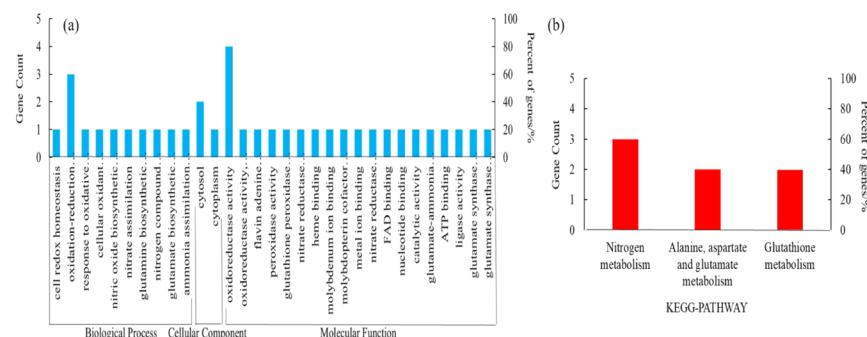


FIGURE 5 GO annotations and KEGG metabolic pathways results for the enzyme genes. (a) GO annotations for the genes, and (b) KEGG metabolic pathways for the genes.

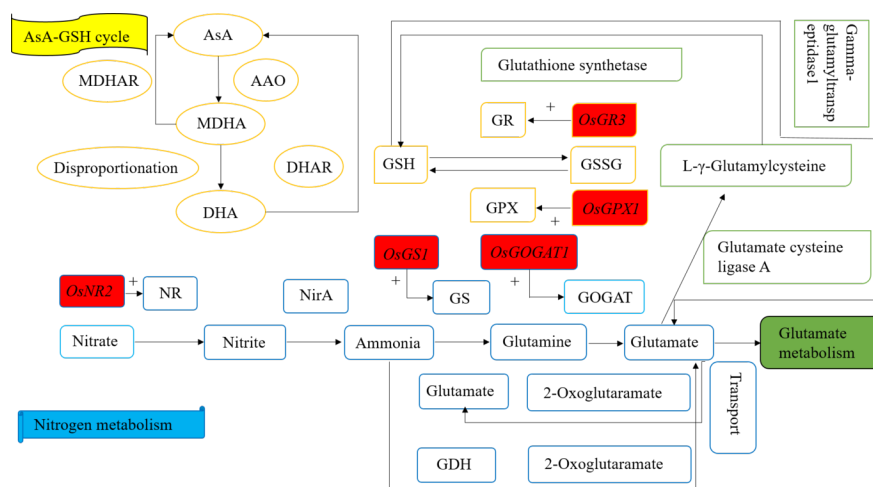


FIGURE 6 Exogenous melatonin regulates enzyme gene expression in the AsA-GSH cycle and nitrogen metabolism pathway in seedlings under NaCl stress. (+) represents the upregulation of gene expression. MDHAR, Monodehydroascorbate reductase, MDHA, Monodehydroascorbate, DHA, Dehydroascorbate, GSSG, Glutathione disulfide, GDH, Glutamate dehydrogenase, NirA, Nitrite reductase A, GS, Glutathione synthetase, Gamma-GT1, Gamma-glutamyltranspeptidase1, L- γ -GC, L- γ -Glutamylcysteine, GCL A, Glutamate cysteine ligase A

3.6|

Effects of exogenous melatonin on the morphology, photosynthetic pigment, and reactive oxygen species parameters of rice seedlings under NaCl stress

As shown in Table 4, under NaCl stress, plant height (Figure 7), root length, fresh weight, dry weight, chlorophyll a, chlorophyll b, and total chlorophyll (chlorophyll a+b) content of seedlings were significantly reduced compared to those of the control. Further, the H_2O_2 , $O_2^{\cdot-}$, and MDA contents increased by 47.404%, 57.277%, and 36.815%, respectively. Compared with salt treatment alone,

exogenous melatonin at different concentrations (50-800 $\mu\text{mol}\cdot\text{L}^{-1}$) significantly increased plant height (Figure 7), root length, fresh weight, dry weight, chlorophyll a, chlorophyll b, and total chlorophyll content (Table 4), whereas H_2O_2 , $O_2^{\cdot-}$, and significantly decreased MDA content to different degrees. The higher the melatonin concentration, the less obvious the alleviation effect under NaCl stress. These results indicate that exogenous melatonin could increase the chlorophyll content of rice seedlings under NaCl stress to a certain extent, enhance the photosynthetic rate and biomass of leaves, reduce the ROS content in seedlings, and promote the growth and development of seedlings.

TABLE 4 Effect of melatonin on the parameters of morphological, photosynthetic, and reactive oxygen species of rice seedlings under NaCl stress

	Treatments	Treatments	Treatments
Parameters	CK	NaCl	MT50
Morphological parameters	Morphological parameters	Morphological parameters	Morphological parameters
plant height /cm	12.407 \pm 0.255 ^{bc}	11.440 \pm 0.061 ^e	11.917 \pm 0.061 ^e
root length /cm	6.110 \pm 0.056 ^a	4.293 \pm 0.258 ^d	4.760 \pm 0.258 ^d
fresh weight /g	0.142 \pm 0.003 ^{bc}	0.121 \pm 0.006 ^d	0.134 \pm 0.006 ^d
dry weight /g	0.012 \pm 0.001 ^b	0.009 \pm 0.001 ^c	0.011 \pm 0.001 ^c
Photosynthetic pigment parameters	Photosynthetic pigment parameters	Photosynthetic pigment parameters	Photosynthetic pigment parameters
Chl a ($\text{mg}\cdot\text{g}^{-1}$ FW)	0.890 \pm 0.034 ^{bc}	0.551 \pm 0.022 ^d	0.952 \pm 0.022 ^d
Chl b($\text{mg}\cdot\text{g}^{-1}$ FW)	0.392 \pm 0.022 ^{bc}	0.294 \pm 0.030 ^c	0.358 \pm 0.030 ^c
Chl a+b ($\text{mg}\cdot\text{g}^{-1}$ FW)	1.282 \pm 0.041 ^b	0.845 \pm 0.024 ^c	1.310 \pm 0.024 ^c
Oxidative damage parameters	Oxidative damage parameters	Oxidative damage parameters	Oxidative damage parameters
H_2O_2 ($\mu\text{mol}\cdot\text{g}^{-1}$ FW)	36.381 \pm 1.072 ^e	53.627 \pm 1.229 ^a	47.082 \pm 1.229 ^a
$O_2^{\cdot-}$ ($\mu\text{mol}\cdot\text{g}^{-1}$ FW)	4.480 \pm 0.110 ^e	7.046 \pm 0.178 ^a	6.495 \pm 0.178 ^a
MDA ($\mu\text{mol}\cdot\text{g}^{-1}$ FW)	15.499 \pm 0.607 ^d	21.205 \pm 1.002 ^a	19.550 \pm 1.002 ^a

Note: The data are expressed as mean \pm standard error, and different lowercase letters indicate significant differences ($p < 0.05$).

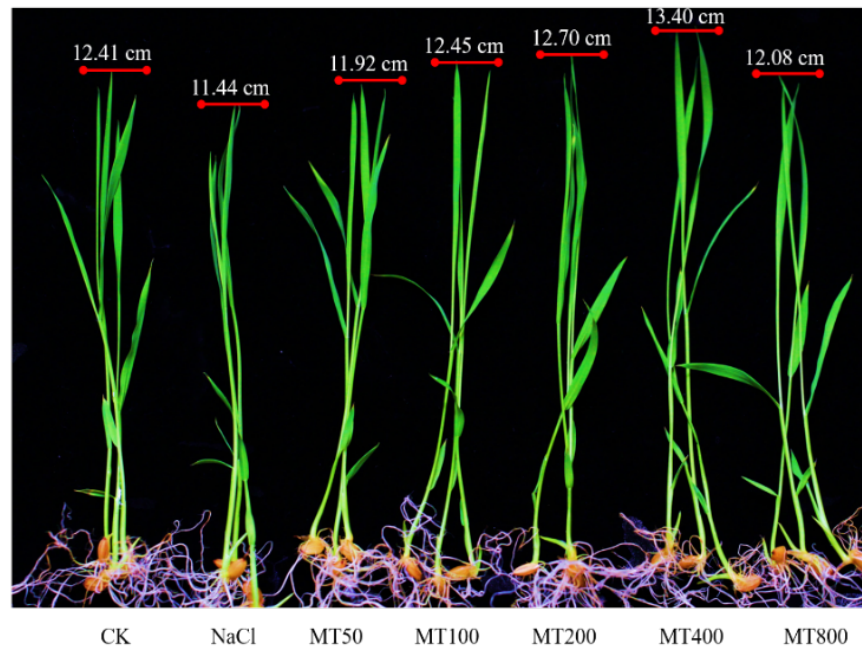


FIGURE 7 Effect of exogenous melatonin on the height of rice seedlings under NaCl stress. The red line represents the top of the seedlings, and the white numbers represent the height of the seedlings between different treatments

4|

DISCUSSION

Nucleotide-blastn homologous sequence alignment revealed that the sequences of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* of rice seedlings were markedly similar to those in *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor*, with a consistency of more than 80%. Such findings indicate that the sequences could be homologous sequences. Based on chromosome mapping, the genes had a similar location distribution on the chromosomes of rice, *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor*, aligning with the results of other studies (Zhou et al., 2018). The analysis of gene exon-intron structure showed that genes in rice, *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor* had similar gene structures, but different fragment size, which may be caused by partial deletion or addition of genes in the process of evolution (He et al., 2021; Li et al., 2021). The proportions of intron phases 0, 1, and 2 to all introns were roughly the same in rice, *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor*, with a proportion of 56%-60% for intron phase 0. Therefore, the evolution of genes in rice, *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor* might be largely conserved and may carry out similar functions. Other studies revealed a high proportion of intron phase 0 and more conserved evolution of genes (Tyagi et al., 2017; Xie et al., 2002). Overall, rice seedling genes (*OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1*) had similar gene functions to *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor* genes and may play the same regulatory role in the synthesis of the AsA-GSH cycle and nitrogen metabolism-related enzymes in various species.

In the AsA-GSH cycle, oxidative stress is alleviated by degrading H_2O_2 and maintaining the cellular redox balance through multi-step enzymatic reactions (Roychoudhury et al., 2012). Nitrogen assimilation can directly affect crop biomass and grain yield, and is closely related to crop growth and development. In this study, NaCl stress significantly downregulated the expression of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings, whereas AsA and GSH contents, enzyme activities in the AsA-GSH cycle, and

nitrogen metabolism were significantly decreased. Such findings may be due to NaCl stress downregulating the expression of enzyme genes in the AsA-GSH cycle and nitrogen metabolism, resulting in changes in related enzyme activities, which ultimately affect the ROS scavenging ability of the AsA-GSH cycle and nitrogen metabolism efficiency. Similar results were obtained for other crops under different stresses (Wang et al., 2012; Wang et al., 2018; Zhang et al., 2020; Li et al., 2010). After treatment with different concentrations of exogenous melatonin, the expression levels of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings were upregulated, and the activities of enzymes in the AsA-GSH cycle and nitrogen metabolism were significantly enhanced, suggesting that exogenous melatonin treatment changed the inhibitory effect of NaCl stress on seedlings to a certain extent. This change significantly improved the ability of the AsA-GSH cycle to scavenge ROS and the efficiency of nitrogen metabolism, which notably improved the ROS scavenging ability of the AsA-GSH cycle and the efficiency of nitrogen metabolism. In other studies of melatonin under other stress conditions, melatonin resisted adverse stress by enhancing the antioxidant capacity and nitrogen metabolism level of seedlings and regulating the expression of antioxidant and nitrogen metabolism genes (Xu et al., 2010; Zhang et al., 2017; Zhou et al., 2020). The results of RT-qPCR and heat map analysis showed that exogenous melatonin regulated the expression of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings under NaCl stress, affecting the activities of enzymes in the AsA-GSH cycle and nitrogen metabolism, accelerating the AsA-GSH cycle and nitrogen generation process, and reducing the damage caused by NaCl stress in seedlings.

Seedlings *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* are essential for the life process of AsA-GSH cycle and nitrogen metabolism under NaCl stress, which could affect the normal growth and development of rice seedlings (Gu, 2019). Gene GO annotations and KEGG metabolic pathways revealed that the genes were involved in redox and other processes, including nitrogen metabolism, glutamate metabolism, and glutathione metabolism, and regulated the synthesis of key metabolic enzymes and combined with coenzymes. Such findings indicate that these genes are crucial to the antioxidant system, ROS scavenging, and metabolism. Based on the RT-qPCR and heat map results, exogenous melatonin affected the process of the AsA-GSH cycle and nitrogen metabolism by regulating the expression of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings under NaCl stress, reducing the content of ROS, improving the utilization rate of nitrogen, and promoting the growth and development of seedlings. Similar reports on beans and other crops have been published (Wei et al., 2015; Erdal, 2019; ElSayed et al., 2021).

In this study, the plant height, root length, fresh weight, dry weight, and chlorophyll content of seedlings were significantly decreased, indicating that NaCl stress notably inhibited the growth of seedlings. NaCl stress may inhibit the activities of enzymes in the AsA-GSH cycle and nitrogen metabolism in seedlings, resulting in the accumulation of a large amount of ROS in seedlings that could not be eliminated. The content of MDA was significantly increased and the efficiency of nitrogen assimilation slowed down, which markedly inhibited the growth of seedlings. Related studies have reported that high salt stress is not conducive to the growth of seedlings. In fact, high salt stress destroys the structure of cells and significantly weakens their metabolic activity (Zhan et al., 2019). On one hand, treatment with different concentrations of exogenous melatonin improved the activity of enzymes in the AsA-GSH cycle and nitrogen metabolism in seedlings, restored normal ROS levels in seedlings under NaCl stress, decreased the content of MDA in seedlings, and accelerated the efficiency of nitrogen assimilation, thereby increasing the biomass of seedlings. On the other hand, exogenous melatonin can reduce the degradation of chlorophyll in seedlings under NaCl stress, improve the photosynthetic efficiency of seedlings, and accelerate the generation rate of nutrients. Of note, similar results were found in maize and cucumber seedlings. Overall, exogenous melatonin promoted seedling growth by enhancing the activity of enzymes in the AsA-GSH cycle, nitrogen metabolism, and photosynthesis in seedlings (Li et al., 2021; Ahmad et al., 2021).

5 | CONCLUSIONS

In the present study, rice *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* were found to have high homology with similar genes in *Arabidopsis thaliana*, *Zea mays*, *Hordeum vulgare*, and *Sorghum bicolor* based on sequence comparison. The localization of genes on chromosomes and similar gene structures were

also revealed herein. Through GO annotation and KEGG metabolic pathway analysis, the functions of genes and their participation in metabolic processes were identified. Exogenous melatonin regulates the expression of *OsGR3*, *OsGPX1*, *OsNR2*, *OsGS1*, and *OsGOGAT1* in seedlings under NaCl stress to influence the efficiency of the AsA-GSH cycle and nitrogen metabolism, reduce the ROS content of seedlings, improve nitrogen utilization efficiency, enhance leaf photosynthetic rate, and promote seedling growth.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA

AVAILABILITY STATEMENT

Data will be available from the corresponding author on request.

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