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Pre treatment of melatonin rescues cotton seedlings from cadmium toxicity by regulating key physio-biochemical and molecular pathways

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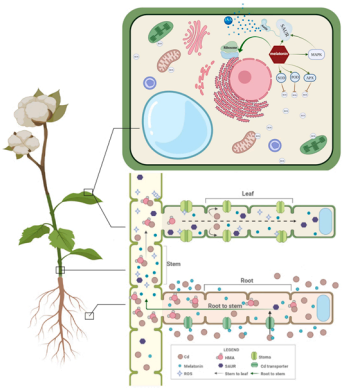
HIGHLIGHTS

- Excessive Cd ions impair plant growth through oxidative injury to cellular organelles.
- Melatonin represses metal transporter genes to inhibit Cd translocation to leaves.
- Melatonin upregulates antioxidant enzymes for sustaining rRNA biogenesis in Cd stressed leaves.
- Melatonin improves leaf photosynthesis by adjusting stomatal movement via MAPK signaling pathway.
- Melatonin regulates ROS generation and improves seedling growth.

GRAPHICAL ABSTRACT



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ABSTRACT

Melatonin, a plant/animal origin hormone, regulates plant response to abiotic stresses by protecting them from oxidative damage. This study identified physiochemical and molecular mechanism of melatonin-induced cadmium (Cd) stress tolerance and detoxification in cotton seedlings. Cotton seedlings, with or without melatonin (15 μM) pretreatment, were subjected to Cd (100 μM) stress in a hydroponic medium for eight days. We found that higher cellular Cd accumulation in leaf tissues significantly inhibited the growth and physiology of cotton seedlings. In contrast, melatonin-treated seedlings maintained leaf photosynthetic capacity, producing relatively higher fresh (17.4%) and dry (19.3%) weights than non-melatonin-treated plants under Cd-contaminated environments. The improved growth and leaf functioning were strongly linked with the melatonin-induced

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Leaf gas exchange
Cadmium sequestration

repression of Cd transporter genes (*LOC107894197*, *LOC107955631*, *LOC107899273*) in roots. Thus, melatonin induced downregulation of the Cd transporter genes further inhibited Cd ion transport towards leaf tissues. This suggests that the differentially expressed transporter genes (DEG) are key drivers of the melatonin-mediated regulation of Cd transportation and sequestration in cotton. Melatonin also protected cotton seedlings from Cd-induced oxidative injury by reducing tissues malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels and increasing the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) enzymes. Transcriptomic analysis revealed that melatonin activated mitogen-activated protein kinase (MAPK) signaling pathways to simulate stomatal adjustment and photosynthesis in Cd-stressed leaves. Further, melatonin protects intercellular organs, particularly ribosomes, from Cd-induced oxidative damage by promoting ribosomal biosynthesis and improving translational efficiency. The findings elucidated the molecular basis of melatonin-mediated Cd stress tolerance in plants and provided a key for the effective strategy of Cd accumulation in cotton.

1. Introduction

The exponential growth of industrialization in China over the last 30 years has significantly degraded the soil environment, resulting in severe heavy metal contamination, particularly in the southwestern regions (Wang et al., 2019). An agricultural soil survey found that approximately 20% of northwestern soils exceeded the environmental quality standard of the Ministry of Ecology and Environment (MEE). The major contribution (82.4%) of this contamination was contributed by heavy metals and metalloids, with cadmium (Cd) being the most prevalent (7%) of these metals (MEP, 2014). Due to high solubility and mobility in the soil system, Cd is readily absorbed by the plants (Ur Rehman et al., 2018). Cotton is an important economic crop, and China contributes 25.6% of global cotton production with 10.1% of the planted area (Arshad et al., 2021). However, cotton growth and productivity are seriously affected by Cd contamination. Thus, identifying efficient and environmentally friendly substances that protect plants from Cd toxicity would greatly benefit the global cotton industry.

High Cd concentrations in soil interfere with water, and nutrient uptake inhibits root respiration and impairs transcription and signaling pathways (Clemens and Ma, 2016; Yao et al., 2018). When translocation to the aboveground parts, Cd ions damage lipid membranous organelles, inhibiting leaf photosynthetic capacity, chlorophyll fluorescence and overall plant growth (An et al., 2019). At the molecular level, Cd exposure can induce the expression of stress-responsive genes in plant roots, i.e. jasmonic acid synthesis in plant roots (Lei et al., 2020). Some plants possess specific Cd detection, transport and detoxification mechanisms (Romero-Puertas et al., 2019). Specifically, Cd ions enter through roots, are loaded into the xylem tissue and then spread throughout the plant body, altering cellular redox homeostasis and inducing reactive oxygen species (ROS) in plant tissues (Das et al., 2015). To overcome these toxic ROS, plants have evolved an antioxidant defense system consisting of antioxidant enzymes and non-enzymatic components (Chen et al., 2016). However, this defense system may be ineffective against excessive Cd levels, and plants grown in chronically Cd-contaminated soils may experience significant reductions in growth and yield. Therefore, an efficient strategy is urgently needed to alleviate the effects of Cd stress on plant growth and development.

Melatonin, a potent bioactive and nontoxic molecule, is produced in the pineal glands of animals and various plant tissues (Kanwar et al., 2018). Exogenously applied melatonin can protect plants from stress-induced injury by increasing enzymatic antioxidant activity, regulating redox homeostasis and repairing damaged mitochondria (Wang et al., 2017; Zhang et al., 2017; Zheng et al., 2017; Lee and Back, 2018; Sharif et al., 2018; Ahammed et al., 2019). Due to its structural similarity to growth regulators, melatonin can regulate the metabolic activities of plants by binding to indoleacetic acid (IAA) receptors (Arnao and Hernández-Ruiz, 2018). For example, melatonin can promote root development by regulating IAA synthesis (Yang et al., 2021). Earlier studies suggested that exogenously applied melatonin can inhibit Cd transport gene IRT1, reducing Cd accumulation in plant tissues (Wang et al., 2021). Similarly, exogenous melatonin can re-establish redox homeostasis in stressed plants by activating several genes related to the antioxidant defense system, i.e. glutathione and phytochelatins and downstream signals, such as nitric oxide (NO), hydrogen peroxide (H_2O_2), and salicylic acid (SA) (Gu et al., 2021). Cd+melatonin treatments significantly upregulated ABC transporter and PCR2 transcripts in alfalfa and PDR8 and HMA4 in *Arabidopsis*. In contrast, it down-regulated Nramp6 transcripts and Cu/Zn superoxide dismutase genes in alfalfa seedlings (Gu et al., 2017). In safflower seedlings, a possible synergic interaction between SA and melatonin protected the plants from Cd-induced injury by accelerating the ascorbate-glutathione cycle (Amjadi et al., 2021). These findings suggest that melatonin can potentially inhibit Cd uptake and translocation in plant tissues (He et al., 2020). Despite studying the positive effects of melatonin on heavy metal-stressed plants (Clemens and Ma, 2016), the stress regulation mechanism is still thoroughly unexplored.

Furthermore, the underlying biochemical and molecular pathways involved in regulating Cd-stress tolerance, particularly when roots are the site of Cd exposure, have not been identified. This study aimed to (1) characterize the physio-biochemical and molecular pathways involved in the Cd stress response of cotton seedlings, (2) explore the potential mechanisms how melatonin alleviates Cd ion toxicity in cotton seedlings, and (3) identify the genes associated with the Cd stress response. Based on this study, we proposed a schematic regulatory network through which melatonin protects cotton seedlings from Cd-induced injury.

2. Materials and methods

2.1. Experimental details

A controlled environment experiment was performed at the Guangxi University experimental station in Nanning, Guangxi, China (22°49'0.0048'' N, 108°18'59.9976'' E). The cotton cultivar J-4B seeds were cultured in a mixture of peat, perlite and vermiculate in 100-cell plug trays. At leaf emergence, the seedlings were initially transplanted into a half-strength Hoagland solution for eight days and then raised in a full-strength Hoagland solution. The remaining seedlings were shifted to a hydroponic environment containing full-strength Hoagland solution with 15 μM of melatonin. After five days, half of the melatonin-treated and untreated seedlings were shifted to a full-strength nutrient solution either with or without Cd (100 μM). The Cd (100 μM) and melatonin (15 μM) concentrations were chosen based on the results of our preliminary experiments, where treatment with 15 μM of melatonin had the greatest impact on plant growth (Supplementary Fig. 1). In this study, we have four treatments, a control where plants were grown without cadmium and melatonin, a 15 μM melatonin, a 100 μM of Cd and a 15 μM of melatonin + 100 μM of Cd treatment. Cadmium chloride ($CdCl_2$) was used as the Cd source. The seedlings were exposed to Cd stress conditions for six days, and then root and leaf samples were taken for growth, physio-biochemical and molecular analysis. The nutrient solution was renewed every three days to prevent nutrient deficiency. The seedlings were grown in a greenhouse at a temperature of 30 °C and

relative humidity of 60–70%.

2.2. Cadmium uptake and translocation factor

The dried root and shoot samples from each treatment were digested with nitric-perchloric acid mineralization (HNO_3 /$HClO_4$ 4:1, v/v) (Liu et al., 2013). Tissue Cd content was assessed by an inductively coupled plasma atomic emission spectroscopy (ICP-AES; Fisons ARL Accuris, Ecublens, Switzerland). The Cd accumulation in tissues was determined based on dry mass. The bioconcentration factor (BCF) and translocation factor TF (Shi and Cai, 2009) were calculated to determine the Cd bioaccumulation and the potential capacity of phytoremediation, respectively.

$$BCF = [Cd]_{shoot\ or\ root} / [Cd]_{solution} \quad (1)$$

$$TF = \{[Cd]_{shoot} / [Cd]_{root}\} \times 100 \quad (2)$$

In the equation, [Cd] = total Cd concentration in the solution; $[Cd]_{shoot}$ = Cd content in leaves, and $[Cd]_{root}$ = Cd content in roots.

2.3. Leaf gas exchange

The uppermost functional, i.e. upper 4th leaf, was used for measuring leaf gas exchange traits, including stomatal conductance (gs), net photosynthesis rate (Pn), intercellular CO_2 (Ci) and transpiration rate (E). A portable infrared gas exchange analyzer (Li-6400, Li-Cor, Lincoln, NE, USA) was used, and measurements were taken between 11:00 h and 1:00 h Beijing time. The data were taken under 1000 μmol m^{-2} s^{-2} light intensity, 30 °C leaf temperature, and 415 mol mol^{-1} CO_2 concentration. Leaf chlorophyll content (SPAD) was assessed using a Minolta SPAD-502 Chlorophyll Meter.

2.4. Antioxidant enzyme activity and non-enzymatic antioxidant content

Antioxidant enzyme activity (SOP, POD, CAT and APX) was measured from the upper 4th leaf and root issues of four morphologically similar plants. The samples were analyzed using Nanjing Jiancheng Bioengineering Institute (NJBI) assay kits following the manufacturer's protocols. Specifically, the activities of SOD, POD, CAT and APX were assessed using NJBI assay kits A084–4, A084–3, A007–2 and A123, respectively. The upper leaf sample (0.2 g) from four morphologically similar plants was ground to powder using liquid nitrogen from each treatment. The powder was used for determining non-enzymatic antioxidants (H_2O_2 and MDA), with a buffer using NJBI's H_2O_2 and MDA kits (A064, A003–3) following producer instructions. The non-enzymatic antioxidant contents were determined at wavelengths 405 and 530 nm.

2.5. Transcriptome sequencing, gene expression quantification and-functional enrichment analysis

To assess the basis of increased Cd tolerance in melatonin-pretreated plants, the upper 4th leaves of four plants from control, Cd and Cd+melatonin treatments were sampled and frozen in liquid nitrogen. Total RNA (RIN ≥7) extraction and construction of the transcriptome library were done through an Ultra RNA Library Prep Kit (NEB, USA). Transcriptome sequencing was performed using an Illumina HiSeq4000 platform according to the manufacturer's specifications (BGI, Shenzhen, China). Gene expression was estimated by calculating the reads per kilobase per million reads (RPKM) based on reads mapped to the cotton reference genome (GenBank BioProject: PRJNA589982, accession number: GCA_000987745.1). Differentially expressed genes (DEGs) in pairwise comparisons of the control, Cd and Cd+melatonin treatments were identified using the DEGseq R package (1.34.1) (Wang et al., 2010) with a fold change cutoff of ≥ 2 and p-value cutoff of < 0.05. To identify functional classes of genes overrepresented among the DEGs, Gene Ontology (GO) and KEGG pathway enrichment analyses were carried out by OmicShare tools (http://www.omicshare.com/tools) with a corrected p-value cutoff (FDR) of < 0.05. All the data were submitted into the NCBI Sequence Read Archive (SRA) database (PRJNA589982).

2.6. Heavy metal associated (HMA) protein identified

Cotton genome data were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/genome/10704?genome_assembly_id=230443). Predicted HMA proteins were scanned from the cotton genome using HMMER 3.0 with the Hidden Markov model (HMM) corresponding to the Pfam HMA (PF00403.26) domains according to Lozano et al. (2015) and Kong et al. (2020) methods. Cotton-specific HMA, HMM was constructed using hmmbuild from HMMER 3.0 with predicted HMA proteins and HMA HMM. The specific HMA HMM was used, and all proteins with an E-value lower than 1e-5 were selected. Meanwhile, the amino sequences of all selected proteins were submitted to PFAM (http://pfam.xfam.org/) CDD (http://www.ncbi.nlm.nih.gov/cdd) and InterPro (http://www.ebi.ac.uk/interpro/) databases, only the sequences with HMA domains were considered as HMA proteins and used for further analyses.

2.7. Reverse-transcribed PCR (RT-PCR) and quantitative reverse-transcribed PCR (qRT-PCR)

A quantitative reverse-transcribed PCR was performed to analyze the expression of genes associated with Cd and melatonin treatment in cotton and to verify the expression of four DEGs identified from a pairwise comparison of the RNA-seq data from the different treatment conditions. Specifically, one μg microgram of total RNA was extracted and reverse transcribed into cDNA from the leaves of three technical replicates and three biological replicates from each condition using a Quantscript RT Kit (TIANGEN, Beijing, China). The qRT-PCR of the four DEGs was done by C1000 TouchTM Thermal Cycler (Bio-Rad, Hercules, CA, USA) and a SuperReal PreMix Plus Kit (TIANGEN, Beijing, China). The relative fold gene expression of the DEGs was calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). This study shows the primer pairs used (Supplementary 3). The PCR was run under these conditions, 95 °C for 30 s, 40 cycles of heating at 95 °C for 5 s annealing for 32 s at 60 °C. The temperature was increased to 95 °C at 4.4 °C/s (each temperature gradient was run for 5 s).

2.8. Statistical analysis

The data were analyzed in Microsoft Excel 2013. SPSS 22.0 (SPSS Inc., Chicago, USA) was used to assess the significant effects of different treatments. The treatment means were separated using the least significant difference (LSD) at the 5% probability level. Differences among treatments imply statistical difference ($p = 0.05$). The figures were made Via. Origin 9.5 software. Data represented are the standard deviations of the means (n = 3) for each treatment.

3. Results

3.1. Plant growth

Melatonin significantly improved the growth of Cd-stressed and non-stressed plants (Fig. 1). Melatonin-treated plants under non-stressed conditions (M) had 17.4% and 19.3% greater fresh and dry weights (Fig. 1**A & 1B**), respectively, compared with control plants. In contrast, Cd-stressed plants untreated with melatonin displayed 60.5% and 34.8% lower fresh and dry weights, respectively, compared with the control. In addition, Cd plants experienced a 39.7% reduction in relative chlorophyll content (SPAD) compared with the control (Fig. 1**C**). The plants pretreated with melatonin and subjected to Cd stress (M+Cd) produced significantly higher fresh and dry weights than the Cd only treated

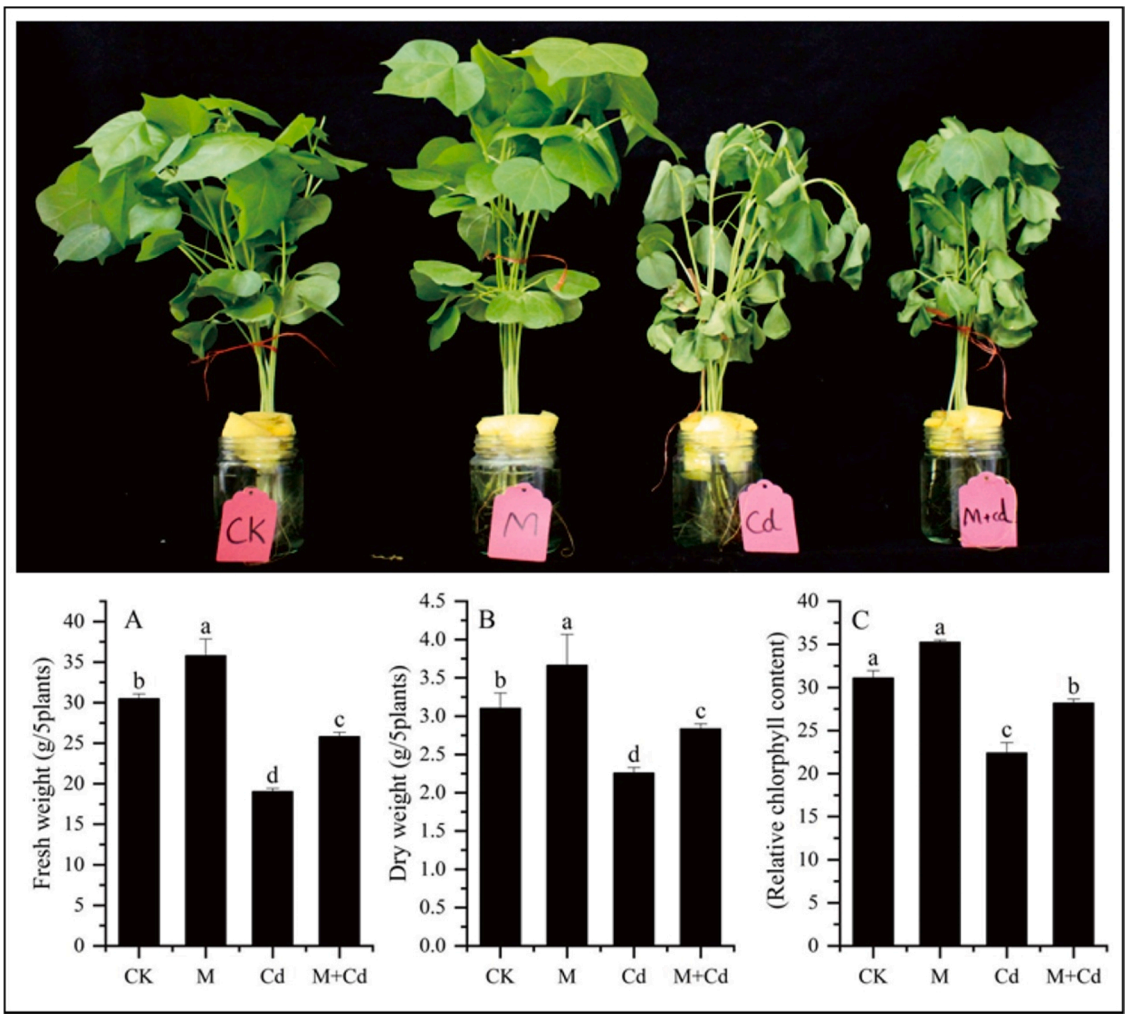

Fig. 1. Fresh mass (A) dry mass (B) and SPAD index (C) of cotton plants raised under different treatments, CK: plants raised in nutrients solution (control) M: seedlings treated with (15 μM) melatonin; Cd: seedlings raised in nutrient solution with (100 μM) cadmium; M+Cd: plants treated with melatonin containing cadmium under nutrient solution. Columns linked with different letters shows significantly differenrences ($P < 0.05$). Bars represent standard deviations of the means ($n = 3$).

seedlings (Fig. 1**A & 1B)**. Furthermore, melatonin application also improved the relative chlorophyll content of cotton plants under Cd stress conditions, with M+Cd seedlings maintaining 19% higher relative chlorophyll content than Cd-stressed seedlings (Fig. 1**C)**.

3.2. Cadmium uptake and translocation

Melatonin and Cd application strongly influenced the Cd concentration, BCF and TF of cotton seedlings (Table 1**)**. Cd-only treated seedlings accumulated relatively higher Cd concentrations (157.8 mg/kg) in their leaves than the plants pretreated with melatonin and subjected to Cd stress (M+Cd). Interestingly, the plants under M+Cd treatment accumulated significantly higher Cd ions in their roots (4976.2 mg/kg) than those grown under Cd-only conditions (4260.5 mg/kg). This indicates that despite the accumulating higher Cd in the roots, M+Cd treated plants translocated less to their leaves. Consequently, these grown plants had a significantly lower TF value (2.6) than those grown under Cd-only conditions (3.7).

Table 1
Cadmium concentration of cotton leaves and roots under normal and cadmium stress conditions.

Treatment	Leaf Cd content mg/kg DM	Root Cd content mg/kg DM	Leaf BCF	Root BCF	TF
CK	ND	ND	ND	ND	ND
M	ND	ND	ND	ND	ND
Cd	157.8a	4260.5b	1.58a	42.6b	3.7a
M+Cd	128.1b	4976.2a	1.28b	49.8a	2.6b
Source of variance					
CK	ND	ND	ND	ND	ND
M	ND	ND	ND	ND	ND
Cd	< 0.0001	0.0001	< 0.0001	0.0002	0.0007
M+Cd	< 0.0001	< 0.0001	< 0.0001	0.0004	0.0006

ND: not detected; Cd: cadmium; DM: dry matter; BCF: bi-concentration factor; TF: translocation factor. Values within the columns with different letters are statistically significant at ($P < 0.05$).

3.3. Hydrogen peroxide and malondialdehyde contents in plant tissues

Melatonin and Cd treatments significantly impacted the H_2O_2 and MDA contents of cotton leaves and roots (Fig. 2**A-D)**. Under non-stressed conditions, pretreatment melatonin had no significant effect on H_2O_2 and MDA contents. However, Cd stress significantly increased MDA and H_2O_2 contents in cotton tissues. For example, MDA and H_2O_2 contents of Cd-treated seedlings (averaged across root and leaf tissues) were 60% and 70% higher, respectively, than in the control seedlings. In contrast, the M+Cd seedlings had significantly lower H_2O_2 and MDA contents

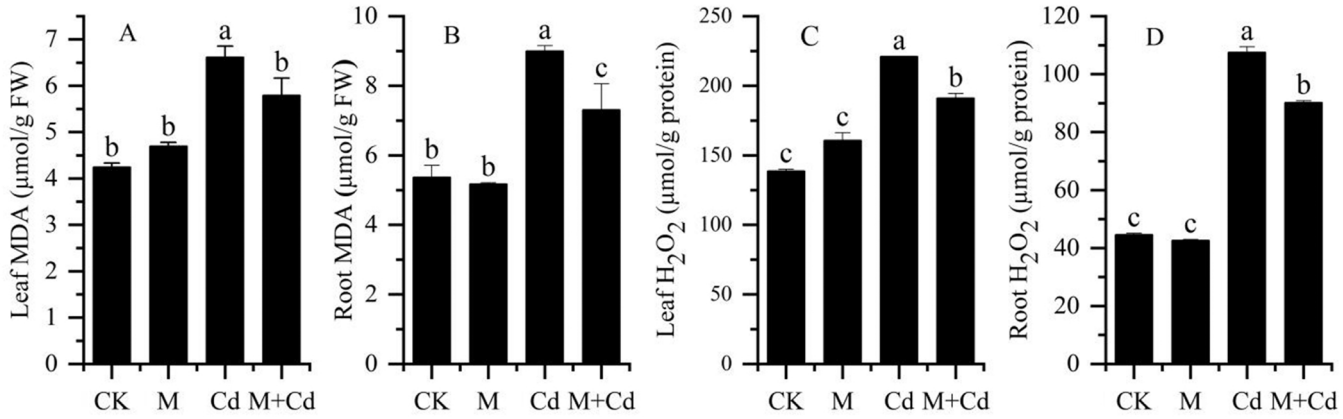

Fig. 2. Variations in malondialdehyde (MDA) (AB) and hydrogen peroxide (H_2O_2) content in root and leaves of cotton plants raised in normal and cadmium stress environment. CK: seedlings raised in nutrients solution (control); M: seedlings treated with (15 μM) melatonin; Cd: seedlings grown in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with melatonin containing cadmium under nutrient solution. Columns linked with different letters shows significantly differenrences ($P < 0.05$). Bars represent standard deviations of the means ($n = 3$).

than the Cd-only treatment.

3.4. Antioxidant enzymes

The Cd-stressed seedlings had a significantly lower antioxidant enzyme (SOD, POD, CAT and APX) activity than control seedlings (Fig. 3**A-H**). Under non-stressed conditions, melatonin significantly increased CAT (Fig. 3**C**) and APX (Fig. 3**DH**) activity in leaves and root tissue of cotton seedlings without affecting SOD and POD levels. However, M+Cd plants displayed significantly greater activities of all these protective enzymes in root and leaf tissues than Cd plants.

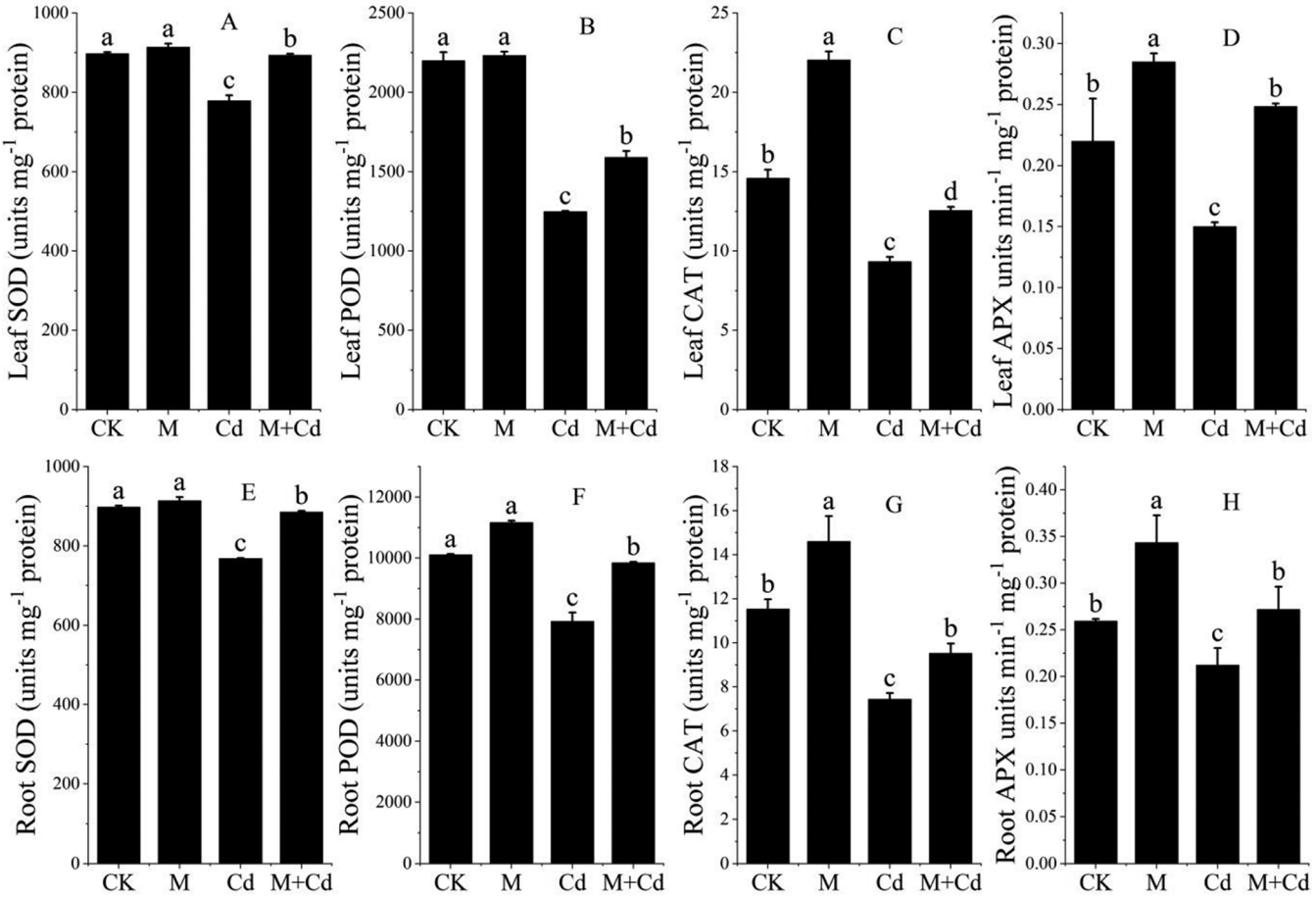

Fig. 3. Changes in superoxide dismutase (SOD) (AE) peroxidase (POD) (BF) catalase (CAT) (CG) ascorbate peroxidase (APX) (DH) activity of root and leaf of cotton plants grown in normal and cadmium stress environment. CK: seedlings grown in nutrients solution (control); M: seedlings treated with (15 μM) melatonin; Cd: seedlings raised in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with melatonin containing cadmium under nutrient solution. Columns linked with different letters shows significantly differenrences ($P < 0.05$). Bars represent standard deviations of the means ($n = 3$).

3.5. Leaf gas exchange attributes

Cd treatment significantly reduced the leaf gas exchange attributes in cotton seedlings, with the plants displaying a 66.3%, 47.6%, 69.1% and 69% reduction in Pn, gs, Ci and Tr, respectively, compared with the control. In contrast, M+Cd plants displayed significantly greater leaf Pn **(**Fig. 4**A)** and gs **(**Fig. 4**B)** compared with Cd-only stressed plants. By significantly reducing fresh and dry weights and relative chlorophyll contents **(**Fig. 1**),** Cd stress severely damaged the structure and functioning of cotton leaf tissues.

3.6. KEGG pathway enrichment of DEGs

In this study, among the DEGs identified by comparing the control and Cd-stressed plants, the genes associated with plant hormone signal transduction (ko04075), carotenoid biosynthesis (ko00906), circadian rhythm-plant (ko04712), zeatin biosynthesis (ko00908), flavonoid biosynthesis (ko00941), alpha-Linolenic acid metabolism (ko00592), sesquiterpenoid and triterpenoid biosynthesis (ko00909), fatty acid elongation (ko00062), ether lipid metabolism (ko00565), caffeine metabolism (ko00232), and glycine, serine and threonine metabolism (ko00260) were enriched **(**Fig. 5**A)**. When control and M+Cd were compared, genes related to 17 pathways were significantly enriched among the identified DEGs. These include leaf photosynthesis (ko00195), hormone signal transduction and MAPK signaling pathways (ko04016) **(**Fig. 5**B)**. Genes related to photosynthesis - antenna proteins (ko00196), photosynthesis (ko00195), carotenoid biosynthesis, porphyrin, and chlorophyll metabolism (ko00860), arachidonic acid metabolism, ribosome (ko00590), glycosphingolipid biosynthesis-lacto and neolacto series (ko00601), glyoxylate and dicarboxylate metabolism, zeatin biosynthesis (ko00630), and fructose and mannose metabolism (ko00051) were enriched among the DEGs of the Cd-only and M+Cd treatments **(**Fig. 5**C)**.

3.7. DEGs encoding heavy metal transporters

In total, 151 non-redundant HMA proteins were found in the cotton genome **(**Supplementary 1**)**. All HMAs were arranged into 14 groups based on the tree topology and bootstrap values **(**Fig. 6**)**. We mapped all HMA proteins to NCBI and found these proteins are associated with 134 genes. Of these 134 HMA genes, we identified 86 genes from our leaf transcriptomes that were expressed with an FPKM greater than 1 **(**Supplementary 2**)**. Among these 86 genes, 36, 54, and 23 DEGs encoding heavy metal transporter (HMA) proteins were identified (Supplementary 3**)**. We also identified 41 HMA protein-encoding genes that were differentially expressed between the control and Cd+M but not between the control and Cd, indicating their potential involvement in melatonin-induced alleviation of Cd stress.

3.8. DEGs involved in ROS reduction enzyme

Antioxidants such as SOD, POD, CAT and APX play an important role in regulating ROS generation. In this study, we identified 6, 8, 5 and 3 genes encoding SOD, POD, CAT and APX, respectively, from the DEGs. The expression profiles of the control, Cd and M+Cd treatments **(**Fig. 7**)** displayed significantly different trends in the expression of genes encoding antioxidants. Compared to the control, both Cd and Cd+M displayed down-regulation of genes encoding SODs, PODs and APXs and up-regulation of genes encoding CATs. Further, compared with Cd-only treatment, the genes encoding SODs and APXs were more significantly downregulated and genes for PODs were upregulated in Cd+M-treated plants, but the genes encoding CATs remained unaffected.

3.9. DEGs involved in ribosome

Genes associated with the ribosome pathway (ko03010) were highly enriched among the DEGs between Cd and Cd+M treated plants, and 391 genes impaired in the ribosome pathway were identified in cotton seedlings. Expression profile analysis of these ribosome-related genes revealed their high expression in control and low expression in Cd-only plants. Further, the ribosome-related genes had a relatively higher expression in Cd+M than in Cd-only treatments. This indicates that melatonin induces the recovery of ribosome genes in Cd-stressed plants **(**Supplementary fig. 2**)**. Further, the genes, such as 10796649 (encoding 60 S ribosomal protein L19–2), 107900848 (encoding 40 S ribosomal protein S13), and 107919844 (encoding 60 S ribosomal protein L12–3) also displayed a significantly greater expression in Cd+M than Cd-only treated plants. In contrast, genes such as 107938672 encoding 50 S ribosomal protein L17, 107901732 encodes 50 S ribosomal protein L34, and a novel gene BGI_novel_G000018 encoding small subunit ribosomal protein S12 had a significantly lower expression in Cd+M than in Cd-only treated plants.

3.10. DEGs involved in auxin response

When the control plants were compared with Cd-only and Cd+M treatments, genes linked with plant hormone signal transduction pathway (ko04075) and auxin (GO:0009733) were significantly

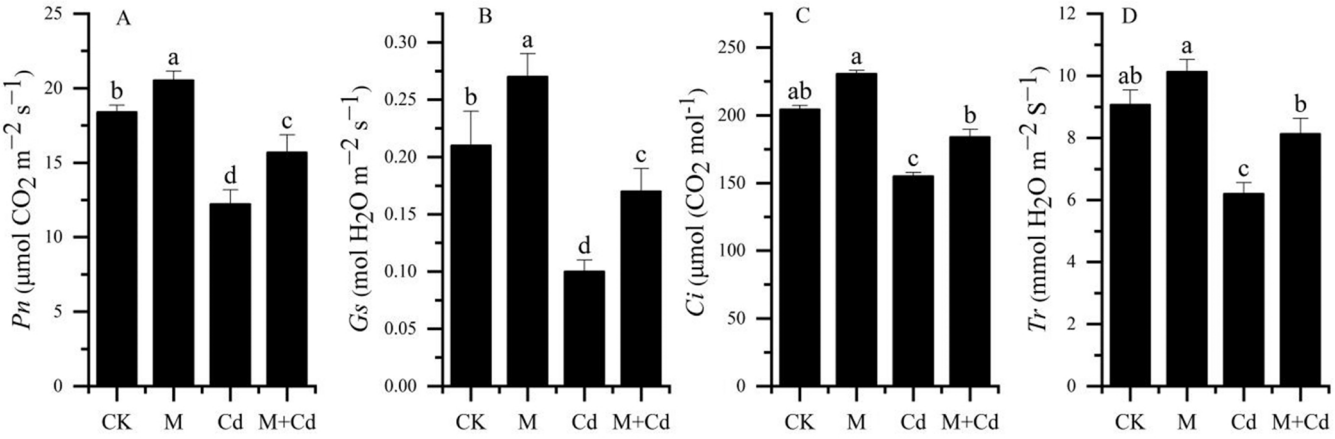

Fig. 4. Changes in leaf photosynthetic rate (A) stomatal conductance (B) intercellular CO_2 uptake (C) and transpiration rate (D) of plants raised in normal and cadmium stress environment. CK: seedlings grown in nutrients solution (control); M: seedlings treated with (15 μM) melatonin; Cd: seedlings raised in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with melatonin containing cadmium in nutrient solution. Columns linked with different letters shows significantly differenrences ($P < 0.05$). Bars represent standard deviations of the means ($n = 3$).

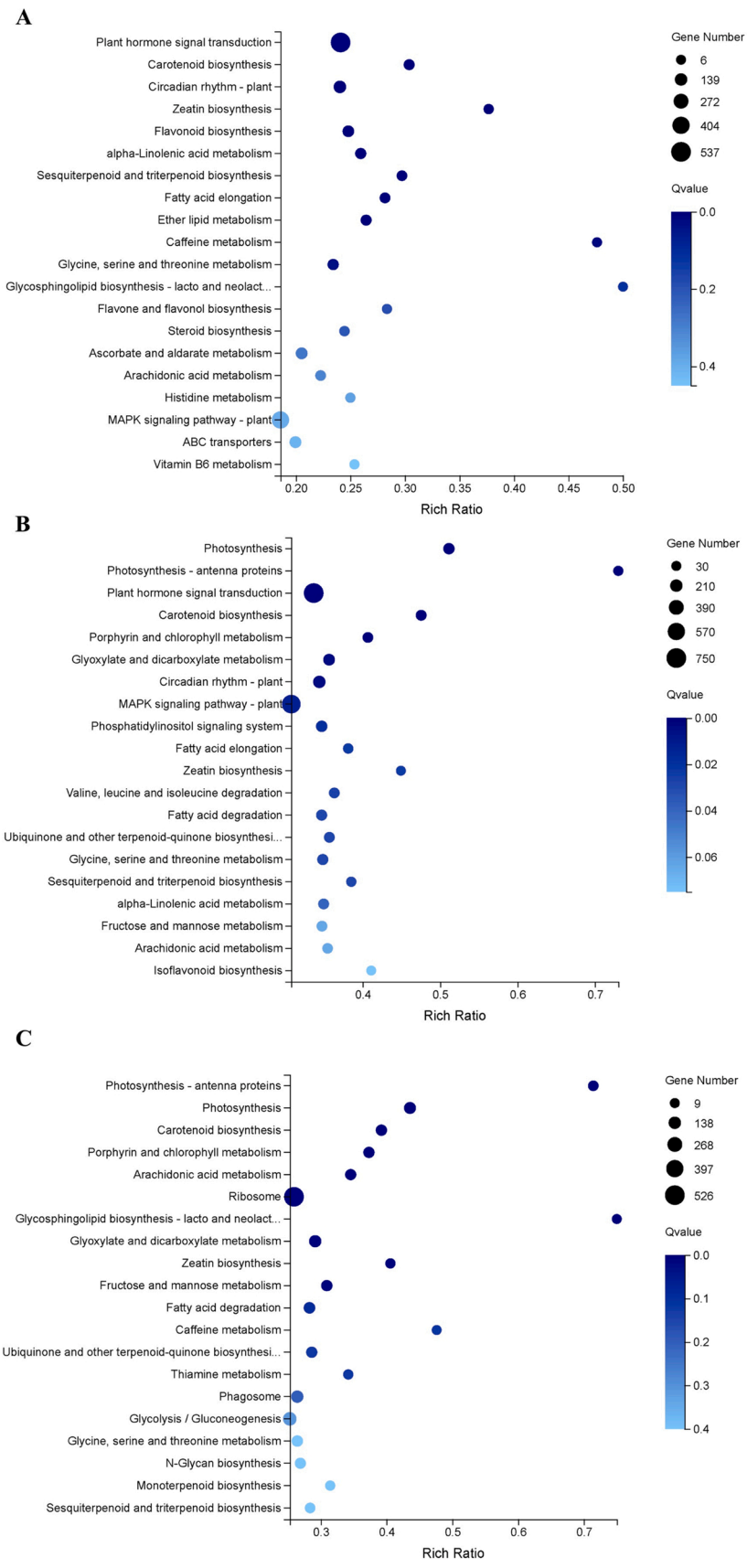

Fig. 5. The enrichment ratio of various KEGG pathways that were enriched among the DEGs identified from pairwise comparison of the CK and Cd treatments (A), CK and M+Cd treatments (B), and Cd and M+Cd treatments (C). The significance of the enrichment of each KEGG pathway corresponds with the color of the points, with light blue indicating a high Q-value and dark blue indicating a low Q-value. The size of the points corresponds with the total number of genes within each KEGG pathway. CK: seedlings raised in nutrients solution (control); Cd: seedlings raised in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with melatonin containing cadmium under nutrient solution.

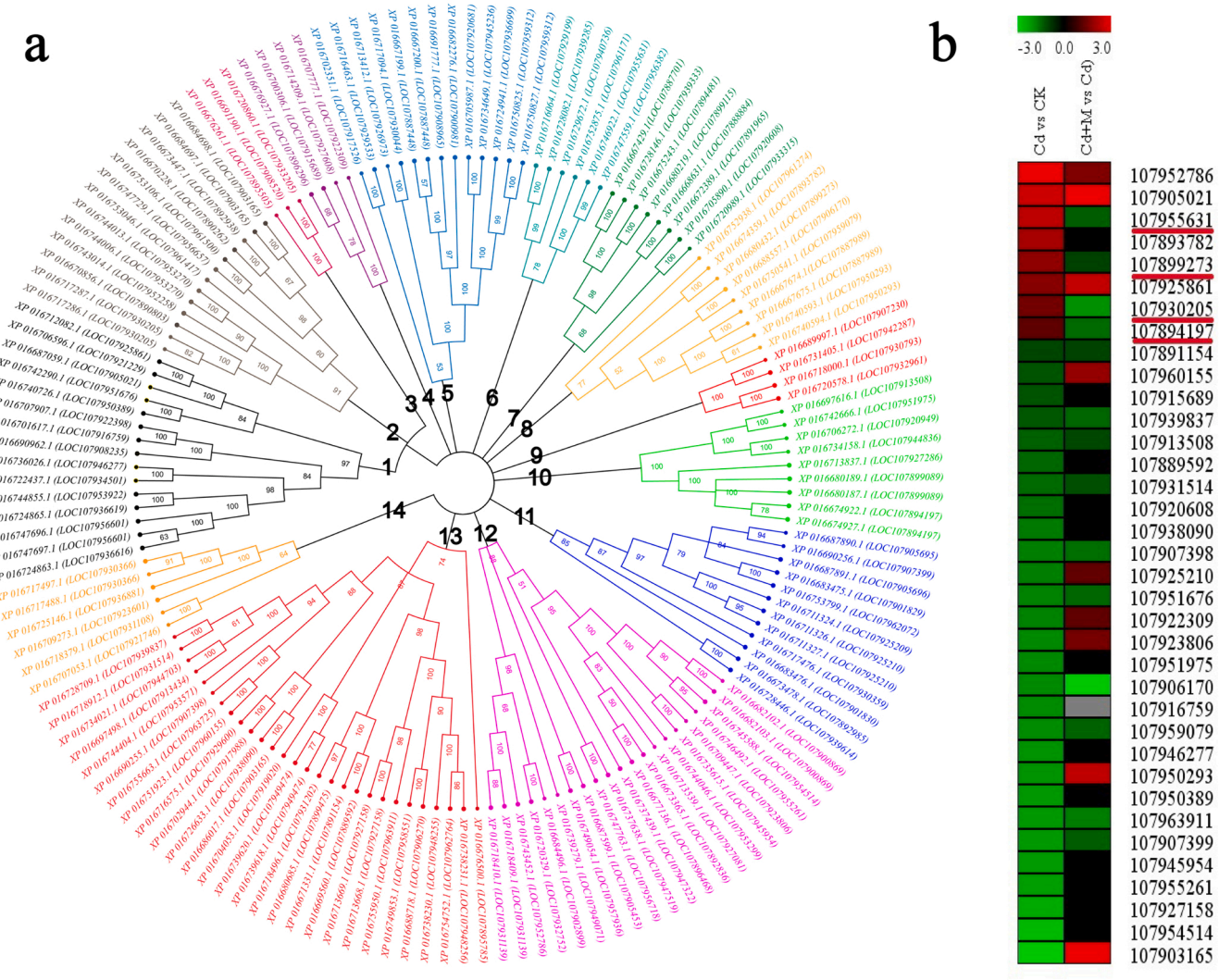

Fig. 6. **a** Phylogenetic analysis of HMA proteins. **b** heatmap of HMA gene expression between Cd and Ck, and between M+Cd and Cd. Heatmap colors show FPKM expression values. CK: seedlings raised in nutrients solution (control); Cd: seedlings raised in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with (15 μM) melatonin containing cadmium under nutrient solution.

enriched among the DEGs identified (Fig. 8). Alignment of all of the DEGs related to the response to auxin to NCBI, revealed that they encode three protein types, i.e. indole acetic acid (IAA), small auxin upregulated RNA (SAUR), and ADP-ribosylation factor (ARF). Expression level cluster analysis of the DEGs related to the response to auxin from a comparison of the control vs Cd, control vs Cd+M, and Cd vs Cd+M divided these genes into three expression patterns for the different comparisons: high-high-middle, low-low-middle and low-middle-high (Fig. 8). The expression patterns were further used for identifying Cd responsive genes in cotton plants. For example, the gene LOC107927898, which encodes a small auxin up-regulated RNA (SAUR) protein, was upregulated in Cd+M compared with Cd-only but down-regulated in Cd compared with control plants, indicating that this gene is involved in the alleviation of Cd stress by melatonin.

3.11. The expression of heavy metal transporter genes

Accumulatively, 151 non-redundant genes encoding HMA proteins were identified from the cotton genome database. Of these, four key genes (*LOC107955631*, *LOC107930205*, *LOC107899273*, and *LOC107894197*) were selected according to their expression in CK vs Cd and Cd vs Cd+M (Fig. 6) (Supplementary 3). To identify the expression level of these genes, qRT-PCR was performed on leaf and root tissue from the different treatments. *LOC107955631*, *LOC107899273*, and *LOC107894197* showed similar expression patterns in qRT-PCR in leaves and roots, which align with our transcriptome data. The fourth gene, *LOC107930205,* responded differently to the applied treatments in this study than the other three genes. For example, compared with the control, this gene was upregulated in Cd-stressed root tissues, but Cd+M upregulated it compared to the Cd-only treatment.

4. Discussion

4.1. Melatonin promotes plant growth and Cd tolerance in cotton

In this study, Cd (100 μM) stress significantly reduced the fresh and dry weight of cotton seedlings by impairing the biomass accumulation process (leaf chlorophyll content and photosynthesis). The negative effects of increasing Cd concentration on cotton growth have already been recorded (Daud et al., 2009). Cotton plants store higher Cd in roots than in foliar tissues to protect. However, increased cellular Cd ions in contaminated environments can impair leaf chloroplast function and development (Wan et al., 2011). Our study suggests that pretreatment of melatonin significantly protected cotton seedlings from Cd toxicity and sustained their growth (Fig. 1). This supports the hypothesis that pre-treatment of melatonin can protect plant growth and development

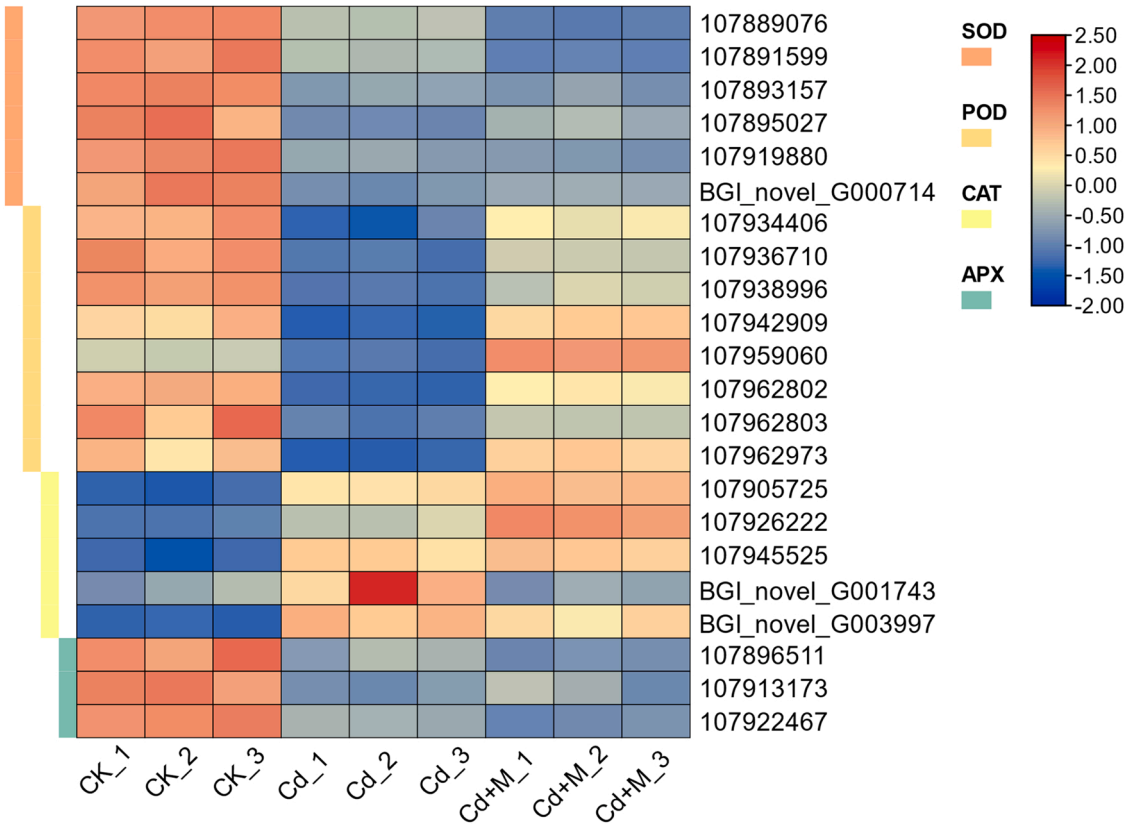

Fig. 7. Expression of DEGs related to ROS reduction enzymes in the three biological replicates from the Ck, Cd and Cd+M treatments. Heatmap colors show FPKM expression values. CK: seedlings grown in nutrients solution; (15 μM) melatonin; Cd: seedlings grown in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with (15 μM) melatonin containing cadmium under nutrient solution.

in Cd-contaminated environments. Our study showed that melatonin protected leaf physiological functioning of cotton seedlings from Cd stress by limiting Cd transport to the leaves (Table 1). Melatonin also regulated the cellular ROS levels of Cd-stressed plants and protected membranous organelles from cellular injury by modulating antioxidant enzymes. The ability of melatonin to protect plants from Cd-toxicity through the regulation of heavy metal transporters, antioxidant enzyme activities, and ROS scavenger generation has previously been observed in radish plants (Xu et al., 2020).

4.2. Melatonin reduces Cd ion translocation to leaves by down-regulating Cd ion transporters genes

We found that melatonin pretreatment reduced Cd transport to the leaves compared with the Cd-only treatment. This was further supported by the significantly down-regulated Cd ion transporters genes (*LOC107894197*, *LOC107955631*, *LOC107899273*) in Cd+M treated cotton roots than in Cd-only treatment (Fig. 9). Earlier studies also suggest the melatonin capacity to limit Cd accumulation in plant root, leaf and stem tissues (Menhas et al., 2022). Melatonin can selectively balance Cd-transporter genes, stimulate PC/Met biosynthesis, and sequester Cd in cells and vacuoles of stressed plants (Xu et al., 2020; Wang et al., 2019a, 2019b). For example, a higher *BnaHMA4c* expression can increase the Cd transfer coefficient, a primary mechanism for mitigating Cd-induced injury in sensitive plants (Zhang et al., 2020). The greater growth of Cd+M treated plants despite higher root Cd accumulation could result from detoxifying Cd ions in the root cells observed in the current study. Melatonin can reduce Cd-induced injury by regulating heavy metals (HM) chelators, transporters and antioxidants to scavenge ROS in radish plants (Xu et al., 2020).

4.3. Melatonin improves leaf gas exchange traits of Cd-fed plants

Higher Cd concentrations in cotton leaf tissues significantly reduced gas exchange characteristics (Fig. 4) through oxidative injury to the cellular organelles (Fig. 3). Higher leaf tissue Cd ion accumulation could be the major cause of inhibition of chloroplast and leaf functioning in cotton seedlings exposed to high levels of Cd (Daud et al., 2009). Our study suggests that melatonin can protect leaf tissues (structures and functions) from Cd toxicity by limiting Cd transport from root to leaves. This was supported by the repression of HMA transporter genes (Fig. 9) and lower TF values (Table 1) under Cd+M treatment (~30% reduction compared with Cd-only treatment). The decrease in Ci and Tr under Cd suggests that Cd exposure may impair stomatal conductance (Gao et al., 2021). It could be inferred that melatonin protects plant photosynthetic machinery from Cd toxicity, as previously observed in different plant species (Hoque et al., 2021; Menhas et al., 2022).

4.4. Melatonin regulates reactive oxygen species generation

Our study shows that increased cellular Cd ions, particularly in leaf tissues, are associated with ROS induction and oxidative stress (Table 1, Fig. 2**AD).** Unregulated ROS in plant cells can damage cellular integrity and functioning (Ismail, 2018). Increased ROS and cellular damage to Cd-stressed plants in this study were also evident from the 70% increase in H_2O_2 and a 60% increase in MDA contents under Cd conditions. The limited capacity of cotton seedlings to overcome excessive ROS was also evident from the down-regulation of DEGs associated with antioxidant enzymes (e.g., SODs, PODs, APXs). Our study showed that melatonin reduces Cd transport to the foliar tissues and significantly reduces oxidative cellular damage by activating antioxidant enzymes in cotton (Fig. 3**AH)**. SOD, POD CAT and APX up-regulation have also been

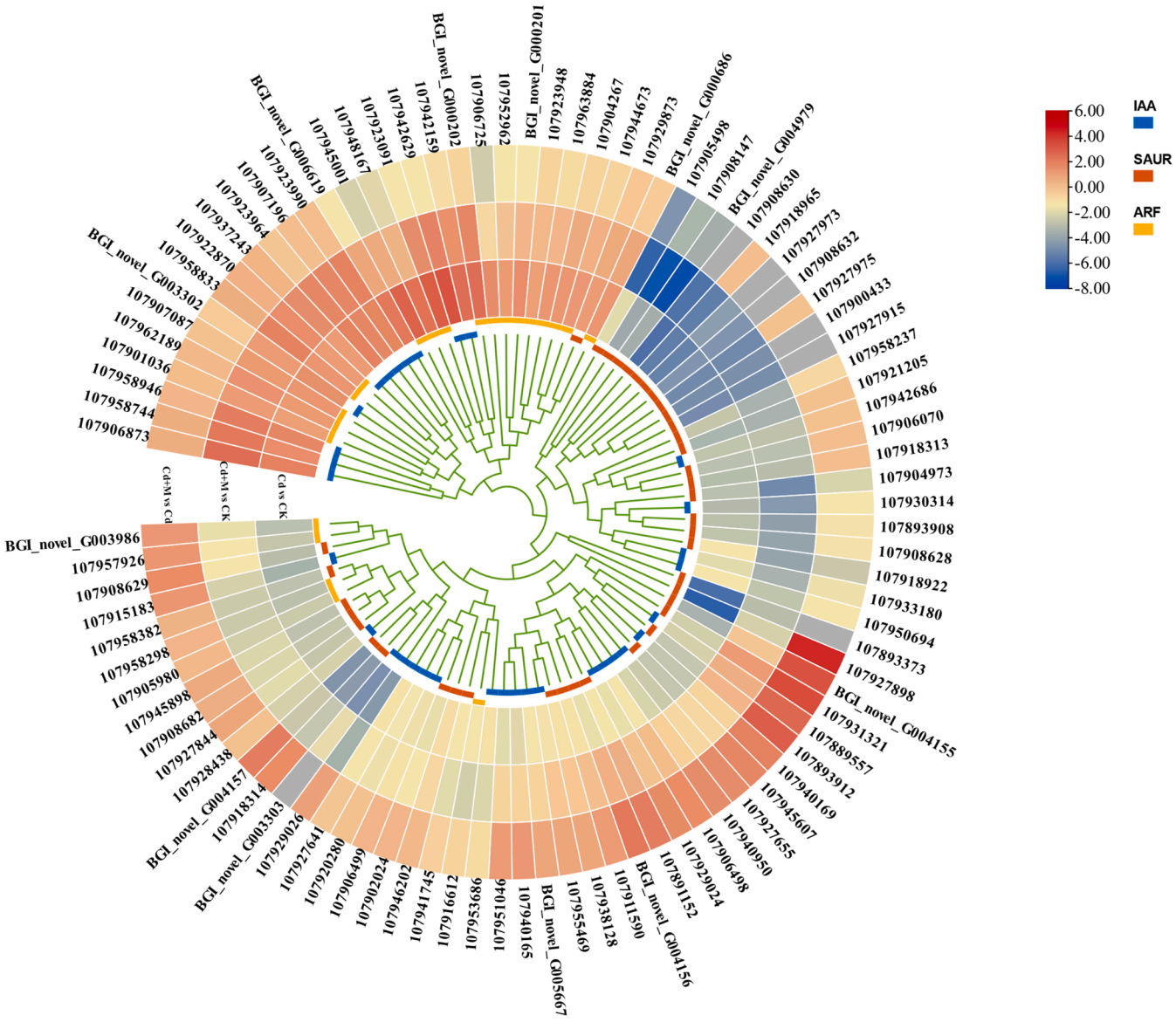

Fig. 8. Dendrogram showing the expression level clustering of DEGs related to the response to auxin from comparisons of CK vs Cd, CK vs Cd+M, and Cd vs Cd+M. The heatmap colors indicate the FPKM expression value of a gene between two treatments. The colored bars indicate the protein that the gene encodes. CK: seedlings grown in nutrients solution (control); (15 μM) melatonin; Cd: seedlings grown in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with (15 μM) melatonin containing cadmium under nutrient solution.

observed in melatonin-pretreated watermelon plants, enabling plants to sustain growth under vanadium toxicity (Nawaz et al., 2018).

4.5. *Melatonin activates the auxin pathway through MAPK signaling to regulate leaf stomatal movement*

Melatonin pretreated plants significantly affected genes allied with plant hormone signal transduction and MAPK signaling under Cd-stress conditions (ko04016). Cellular melatonin (endogenous or exogenous) levels in plants have been associated with the induction of tolerance to abiotic stress (Nawaz et al., 2018). Melatonin can directly regulate this tolerance mechanism by scavenging ROS (Bose and Howlader, 2020) and /or indirectly affecting stress response pathways (Zeng et al., 2022).

GO annotation and the expression profiles showed that all the DEGs related to the 'response to Auxin' encoded three kinds of proteins (IAA, SAUR, ARF) and were clustered into three expression models. In this study, a gene (107927898) encoding a SAUR protein was identified with a potential role in alleviating Cd stress by melatonin. The expression of this gene was significantly reduced by 98.9% in Cd-stressed plants and increased in Cd+M treatment, suggesting that 107927898 played a key role in regulating Cd toxicity **(**Fig. 8**).** SAUR (small auxin up-regulated RNA) constitutes a family of auxin-responsive genes, which also respond to internal and external signals (Stortenbeker and Bemer, 2019). The SAUR76 (107893373, 107929873)− 78 (107958382, 107906070) gene may also impair ethylene receptor signaling and boost plant performance in *Arabidopsis* (Li et al., 2015). SAUR could possibly have promoted PM H^+-ATPase activity and stomatal conductance of Cd-stressed cotton seedlings by inhibiting PP2C.D phosphatases (Haur et al., 2021).

4.6. *Melatonin enhances the translational efficiency of Cd-stressed plants by upregulating ribosomal biosynthesis*

The enrichment of genes from the ribosome pathway (ko03010) among the DEGs between Cd+M and Cd suggests that differences in the expression of ribosome-related genes may contribute to the observed

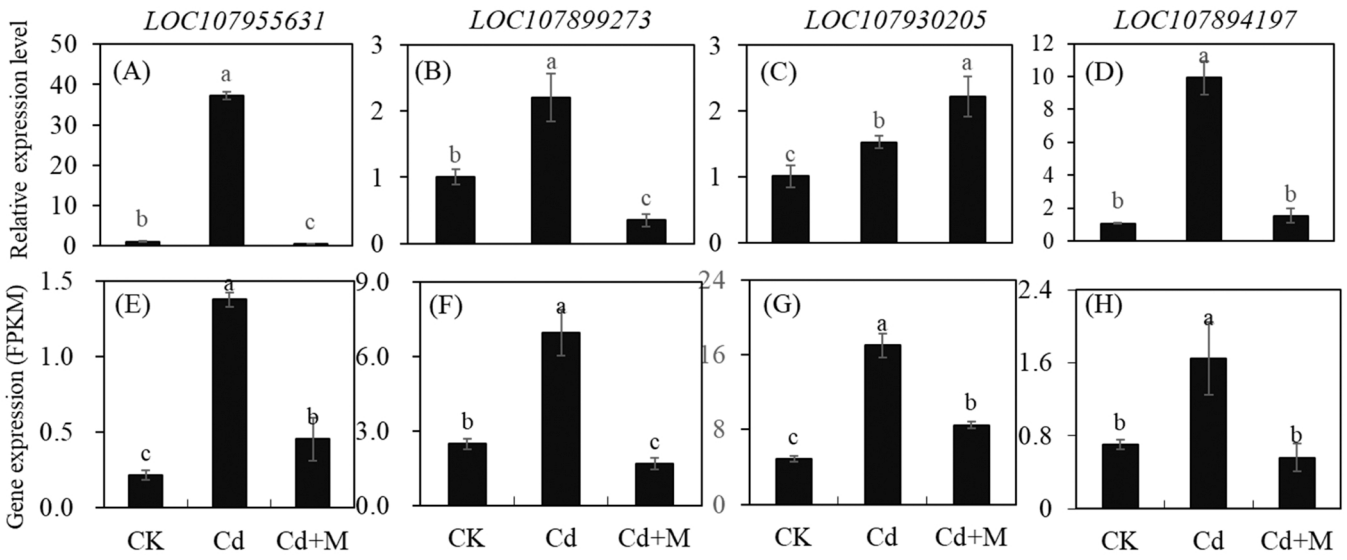

Fig. 9. Expression of four HMA genes in the roots (A-D) and leaves (E-H) of CK, Cd and Cd+M treatments using qRT-PCR and transcriptomic data, respectively. CK: seedlings raised in nutrients solution (control); Cd: seedlings raised in nutrient solution with (100 μM) cadmium; M+Cd: seedling treated with melatonin containing cadmium under nutrient solution. Columns linked with different letters are significantly different ($P < 0.05$). Bars indicate standard deviations of the means ($n = 3$).

physiological differences between Cd and Cd+M. In this study, the down-regulation of genes associated with the ribosome pathway in Cd-stressed seedlings suggests that Cd-induced oxidative injury impaired rRNA biogenesis (Supplementary Fig. 2). In contrast, melatonin restored ribosome biosynthesis in Cd-stressed plants and improved its translational efficiency. The ribosome plays an important role in plant metabolism by translating mRNA into functional proteins (Ramakrishnan, 2002). Abiotic stresses such as chilling and drought have been reported to negatively impact rRNA in crops such as rice and maize (Lei et al., 2015; Hang et al., 2018).

Phenotypic, physiological and gene expression data showed that melatonin promoted Cd accumulation in roots by down-regulating the key heavy metal transporter gene expression in the roots. In cotton leaves, melatonin also promoted ROS scavenging and enhanced the photosynthetic rate by initiating stomatal opening through the activation of auxin-responsive genes such as SAUR via MAPK signaling. In addition, melatonin also increases translational efficiency by up-regulating ribosomal biosynthesis genes. We propose that the combined effects of melatonin on these various processes mitigated the impact of Cd toxicity, improving the growth of cotton seedlings under Cd stress (Fig. 10).

5. Conclusion

It is the first attempt to identify the physiochemical and molecular basis of melatonin-induced Cd stress tolerance in cotton seedlings. We found that Cd ion accumulation in leaves impaired biomass assimilation and plant growth due to oxidative injury to the cellular organelles. Melatonin reduced Cd translocation to leaf tissues by down-regulating Cd ion transporter genes in root tissue resulting in improved seedlings growth. Several candidate DEGs encoding transporters were identified as key drivers in melatonin-mediated regulation of Cd transportation and sequestration in cotton. In addition, we also found that melatonin upregulates several antioxidant enzymes in Cd-stressed seedlings to scavenge ROS and sustain rRNA biogenesis. This study will facilitate the identification of the molecular mechanism explaining Cd acquisition and accumulation in plants and provides a key for the genetic management of Cd accumulation in cotton and other fiber crops.

Environmental implication

Heavy metal i.e. cadmium (Cd) contamination, pose a serious threat to ecosystem sustainability, agricultural productivity, and the human race, owing primarily to uncontrolled geogenic and anthropogenic activities around the globe. Heavy metal and metalloid contaminations in particular account for 82.4% of the contamination in 19.4% of agricultural soil samples collected from northwestern areas, with Cd being the most prevalent (7%) of these metals. Plants readily take up Cd from soil due to its great solubility and high mobility, resulting in its increased concentration in plant-derived food stuffs. Thus the identification of efficient and environmentally friendly substances such as melatonin that protect plants from Cd toxicity would greatly benefit the global cotton industry. We found that melatonin application under Cd stress maintained leaf physio-biochemical and molecular functioning by inhibition of Cd ion transport to the leaves by the repression of Cd transporter genes (*LOC107894197*, *LOC107955631*, *LOC107899273*) in roots. This consequenlty improved plant performance under Cd stress conditions. This suggests that these differentially expressed transporter genes are key participants in the melatonin-mediated regulation of Cd transportation and sequestration in cotton. The outcomes of this study will facilitate the identification of the molecular mechanism underlying Cd uptake and accumulation in plants, and provides a fundamental basis for efficient genetic management of Cd accumulation in cotton and other fiber crops.

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CRediT authorship contribution statement

A.K., R.Z., and X.Y. designed this work. **A.K. and Z.J.** performed the experiment. **K.X., Z.J., and A.K.** analyzed the data. **A.K. and Z.J.** wrote the manuscript. **X.Y., N.U., and A.S.** revised the paper.

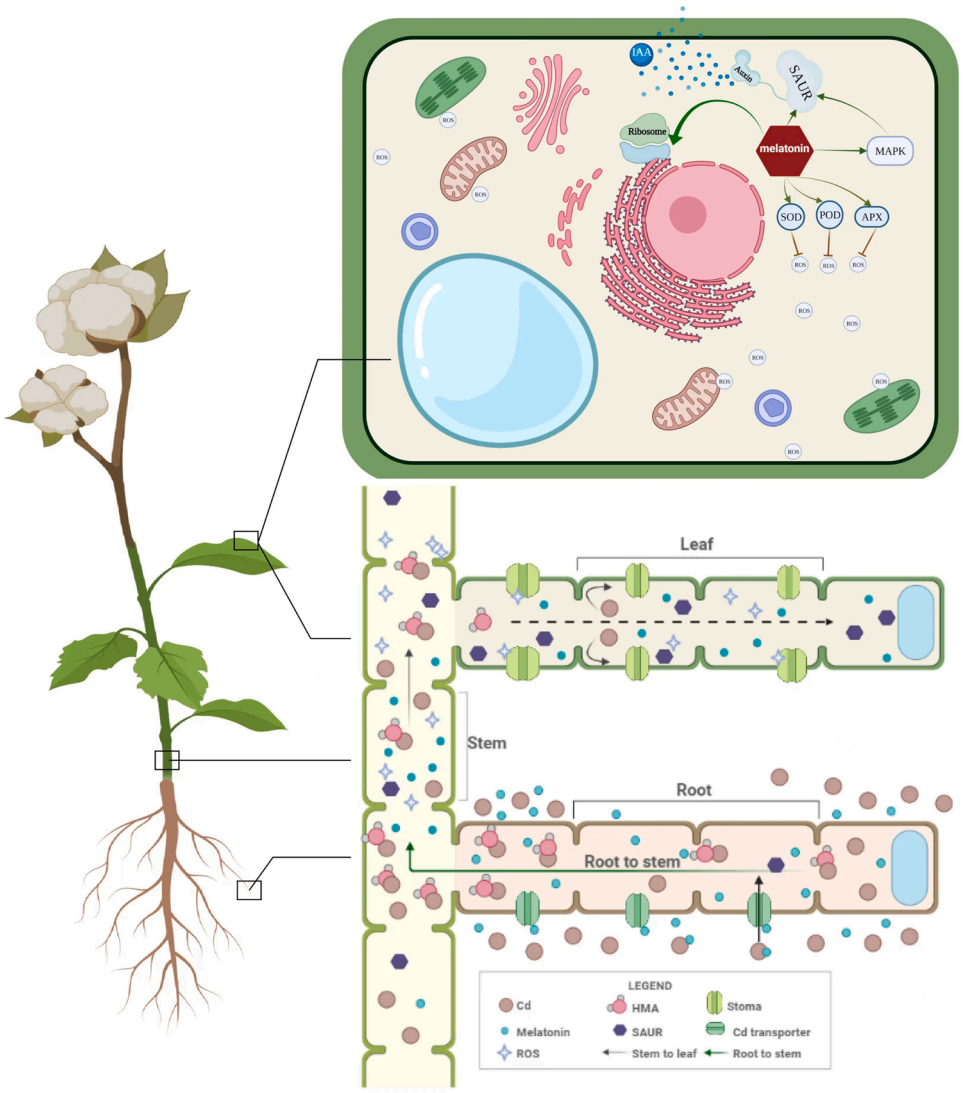

Fig. 10. Schematic diagram of the potential mechanisms involved in melatonin mediated Cd stress tolerance in cotton seedlings. Increased components are marked by an upwards arrow and decreased components are marked by a downwards arrow. Heavy metal associated (HMA), cadmium (Cd), reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK), small auxin up-regulated RNA (SAUR).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.130530.

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