Exogenous melatonin promotes rice seed germination under salinity through regulating antioxidants and metabolic homeostasis

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Abstract

Background Melatonin plays important roles in multiple plant developmental processes and stress responses. However, little is known about the role and putative mechanism of exogenous melatonin in regulating rice seed germination under salt stress.

Main Body Here, we revealed that the exogenous application of melatonin can significantly promote rice seed germination under salinity. Its putative molecular mechanisms are further investigated through metabolomic and transcriptomic analyses. The results revealed that the phytohormone concentrations in germinating seeds are reprogrammed, the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are enhanced, and the total antioxidant capacity under salinity is activated by exogenous melatonin. In addition, rice seeds pre-treated with melatonin exhibit higher concentrations of glycosides than non-treated seeds under salinity. Furthermore, exogenous melatonin alleviates the accumulation of fatty acids under salinity. Genome-wide transcriptomic profiling is used to identify 7160 transcripts which are differentially expressed under salt (NaCl), salt + melatonin (MT100), and control treatments. Pathway and GO enrichment analyses reveal that the genes involved in the response to oxidative stress, hormone metabolism, heme building, mitochondrion, and tricarboxylic acid transformation are altered after melatonin pre-treatment under salinity.

Conclusion This study provides evidence for exogenous melatonin increasing rice seed germination under salt stress, mainly through the activation of antioxidants and modulation of metabolic homeostasis.

Background

Seed germination, which refers to the process of reactivation of the metabolic machinery resulting in the emergence of radicle and plumule, is the first critical step in plant growth and development (Penfield 2017). In the germination stage, the seed is very susceptible to multiple abiotic stresses. Among them, soil salinity is a world-wide severe abiotic stress factor for crop production (Negrao et al. 2017), and the hyperosmotic stress caused by high salinity leads to nutrient deficiency, ion imbalance, and further secondary oxidative stress (Daur 2018). To address these harmful effects, using exogenous substances to promote seed germination under salinity is an efficient method in crop production (Leubner-Metzger 2001).

Melatonin, also known as N-acetyl-5-methoxytryptamine, is involved in many biological activities in animals, such as circadian rhythm, sexual behavior, senility, antioxidant activities, and antidepressant (Hardeland 2012; Pytka et al. 2017). In addition, melatonin has also been found in different plant species (Arnao and Hernandez-Ruiz 2013, 2009a; Murch et al. 2009). As an important modulator in plant growth and development, melatonin plays subtle roles in chlorophyll preservation, photosynthesis promotion, root system architecture stimulation and regeneration, and delayed foliar senescence (Arnao and Hernandez-Ruiz 2009b; Wang et al. 2012; Tan et al. 2012). Melatonin also protects against multiple

abiotic stresses in plants, such as salt, drought, cold, and high temperature (Posmyk et al. 2009b; Li et al. 2012a; Byeon and Back 2014). Remarkably, melatonin scavenges reactive oxygen/nitrogen species (ROS/RNS), protecting cells, tissues, and organisms from oxidative damage (Galano et al. 2011; Tan et al. 2012). More importantly, melatonin has the potential ability to improve crop production, and transgenetic plants with increased melatonin concentrations may contribute to the increased production of crops (Tan et al. 2012). However, much attention has paid to economic plants, and rarely has it been suggested that melatonin could protect against stresses in grain crops, especially in rice (Liu et al. 2015; Meng et al. 2014; Posmyk et al. 2009a).

Rice (Oryza sativa L.) is one of the world's staple crops, supporting over 3.5 billion people (Wing et al. 2018). Multiple abiotic stresses which are unfavorable for growth and development commonly occur in major rice-producing areas. Among them, salinity is a frequent menace (Zhu 2016). The role of melatonin in alleviating salt damages have been confirmed in many crops. For instance, exogenous melatonin improves abiotic stress resistance in Bermuda grass (Shi et al. 2015), and the seed germination of cucumber under salinity (Wang et al. 2016). Every farmer knows that salinity affects rice growth during all developmental stages; however, whether exogenous melatonin alleviates the damage to rice seed germination caused by salinity has not been established. A relevant experiment was, thus, set up in this study, from which we noticed that exogenous melatonin application promoted rice seed germination under salinity. The effects of melatonin in regulating antioxidants and homeostasis were found to be the major mechanisms in improving seed germination under salinity. Our investigations provide insights into the molecular background underlying salt tolerance in rice.

Materials And Methods

Plant materials and growth conditions

Fresh and holonomic Nipponbare seeds were used in this study. First, the Nipponbare seeds were stratificated at 4 ℃ for 2 days in darkness, then steeped in corresponding solution at 28 ℃ for 24 hours in a darkroom. Second, Nipponbare seeds were sown in germinating boxes with three layers of absorbent paper at the bottom in an artificial illumination incubator, which was controlled at temperature 28 ± 0.5 ℃, light intensity of 0, and relative humidity (RH) of about 60 ± 5%.

Germination test of rice seed

To test the effects of exogenous melatonin on the seed germination rate of rice, 0.5 μM, 1 μM, 5 μM, 10 μM, 50 μM, and 100 μM concentration melatonin solutions were used to soak the sterilized seeds. After 24 hours, some seeds pre-treated with deionized water were used as a normal control by sowing in sprouting boxes (Size: 25 cm * 25 cm * 2 cm), and the remaining pre-treated seeds tested under salinity. Four kinds of salinities were subjected to the primed seeds, with concentrations of 50 mM^{-1}, 100 mM^{-1}, 150 mM^{-1}, and 200 mM^{-1}. In addition, seeds with melatonin-pre-treatment were sown in sprouting boxes

with distilled water. We set up three replicates per treatment and took notes of the germination number of rice seeds under different treatments per two hours until the budding rate was constant.

Extraction and quantification of hormones by HPLC analysis

The extraction procedure was operated as previously described (Tal et al. 2011). Quantification of the extractive was carried out using the method of high-performance liquid chromatography (HPLC). System analysis was as reported by Susanne Burkhard (Burkhardt et al. 2001). The specific methods were as follows: the rice seed samples (2 g), including rind, were homogenized in 1 mL of 0.05 M potassium phosphate buffer (pH = 8.0). The homogenates were centrifuged at 4 ℃ and 3000 rpm for 5 min. Then, 500 μL of the supernatant was mixed with 25 μL of chloroform and the samples were horizontally shaken for 10 min. The water phase was discarded and the chloroform phase was dried under vacuum. The residues were dissolved in 120 μL of the HPLC mobile phase and 30 μL was injected into the HPLC-EC system.

Determination of hydrogen peroxide (H_2O_2), antioxidants, Hydroxyl radical scavenging rate, and Total sulfhydryl group (T-SH)

As one of main indicators of reactive oxygen species (ROS), H_2O_2 content was quantified using the titanium sulphate method, as previously mentioned. The activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), total antioxidant capacity (T-AOC), and T-SH were assayed using relevant Assay Kits. The spectrophotometric method was used for reaction activity by examining the absorbance of the centrifuged supernatant at 560 nm, 470 nm, 240 nm, and 412 nm (Ponsgen and Betz 1986; Doubnerova et al. 2007; Sokol et al. 1959). Quantification of the Hydroxyl radical scavenging rate was operated as described by Cai (Cai et al. 2012).

Determination of α, β-amylase (α, β-AL)

The activities of α, β-AL (EC 3.2.1.1, EC 3.2.1.2) were assayed using an α-AL Assay Kit and a β-AL Assay Kit, as previously mentioned (Hayashi et al. 1990).

Quantification of Isocitrate lyase (ICL) and NADP-malic enzyme (NADP-ME)

The ICL activity was determined using the method modified by Ranaldi (Ranaldi et al. 2000), assayed at 25 °C and the absorbance was monitored at 324 nm. The activity of NADP-ME was measured using spectrophotometric assays, as described previously (Pengelly et al. 2010), and was calculated by monitoring the decrease/increase of $NADH^+$ absorbance at 340 nm.

Quantification of protein, starch, and soluble sugar

Protein, starch, and soluble sugar content were extracted using bicinchoninic acid (BCA), acid hydrolyzation, and anthrone-sulfuric, respectively, then quantified by determining the absorbance of the supernatant at 540–595 nm and 620 nm, as previously described (Stobbe et al. 1997; Wang et al. 2010).

Metabolite profiling and data analysis

Metabolites extracted from the sampled rice seeds were measured using UPLC-Q-TOF MS technology combined with data-dependent acquisition (Zhou et al. 2009). The XCMS software was used for peak extraction and metabolite identification, and multidimensional statistical analysis of the mass spectrometry data was carried out by the SIMCA software. Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to filter out some of the noise unrelated to classified information, which improved the analytical ability and effectiveness of the model.

RNA extraction, library construction, and sequencing

Sequencing libraries were generated using a NEBNext® Ultra TM RNA Library Prep Kit for Illumina® (NEB, USA), following the manufacturer's recommendations, and index codes were added to attribute sequences for each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. In order to select cDNA fragments of preferentially 250–300 bp in length, the library fragments were purified with an AMPure XP system (Beckman Coulter, Beverly, USA). Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Finally, the PCR products were purified (AMPure XP system) and library quality was assessed using the Agilent Bioanalyzer 2100 system. Approximately 253 million reads were used for the RNA-Seq reads de novo assembly, from which 240 million reads were selected for further analysis, after removing adapter reads and poorly expressed reads.

Quantification and analysis of gene expression level

The Feature Counts v1.5.0-p3 software was used to count the read numbers mapped to each gene. Then, the FPKM was calculated based on the length of a gene and read count mapped to the gene. FPKM, the expected number of Fragments Per Kilobase of transcript sequence per Million base pairs sequenced, considers the effect of sequencing depth and gene length for the read count at the same time.

Differential expression analysis of two groups was performed using the DESeq2 R package (1.16.1). The resulting P-values were adjusted using the Benjamini-Hochberg approach for controlling the false discovery rate. Genes with an adjusted P-value less than 0.05, as found by DESeq2, were assigned as differentially expressed. Gene Ontology (GO) enrichment analysis was implemented by the cluster Profiler R package, as mentioned in AgriGO. KEGG is a database resource for understanding the high-level functions and utilities of biological systems (http://www.genome.jp/kegg/). We used the cluster Profiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

Quantitative real-time PCR

A set of nine DEGs was selected to confirm the expression level of microarray results using real-time PCR (RT-PCR). The sequences of exons for each gene were used to design the RT-PCR primers using the Primer 5.0 software; specific primers for RT-PCR are shown in Table S6. RT-PCR was performed as described by Li et al. (Li et al. 2019).

Results

Effect of exogenous melatonin on seed germination under salt stress

In order to illustrate the effects of pre-treatment with melatonin on rice seed germination rate, six melatonin concentrations (0.5, 1, 5, 10, 50, and 100 μM^{-1}) were used to compare the germination rate with non-treated seeds. The results revealed that both low (0.5 and 1 μM^{-1}) and high melatonin concentrations (50 and 100 μM^{-1}) significantly inhibited germination. The 10 μM^{-1} melatonin pre-treated seeds had no significant difference in germination rate, compared with CK (purified water). However, we noticed that the 5 μM^{-1} melatonin pre-treated seeds possessed a higher rate than other treatments within 10–32 h and, therefore, this concentration was selected for further analysis (Fig. 1a). To evaluate the effect of salt stress on seed germination, four NaCl concentrations (50, 100, 150, and 200 mM^{-1}) were used. With increasing NaCl concentration, seed germination was significantly inhibited: 91.5% of seeds germinated after 32 h under CK, whereas the germination ratio was 86.5%, 71.5%, 60.5%, and 34.4% under 50, 100, 150, and 200 mM^{-1} NaCl concentrations, respectively (Fig. 1b).

To verify the putative effect of exogenous melatonin in promoting rice seed germination under salt stress, three treatments were conducted: CK, NaCl (100 mM^{-1}), and MT100 (5 μM^{-1} melatonin and 100 mM^{-1} NaCl). The results revealed that the exogenous melatonin promoted seed germination under salt stress. The average germination ratio was 86.5%, 58.5%, and 79% for CK, NaCl, and MT100 after 32 h, respectively (Fig. 1c).

Exogenous melatonin altered germinating seed ROS accumulation and phytohormone concentrations under salinity

A total of seven oxidation indicators were investigated for the three treatments. Compared with CK, the activities of T-AOC, SOD, POD, CAT, and H_2O_2 were decreased under salt treatment (NaCl), while the hydroxyl radical scavenging rate was increased. When the pre-treatment of melatonin (MT100) was used, the activities of T-AOC, SOD, POD, and CAT, as well as the hydroxyl radical scavenging rate, were increased, while the content of H_2O_2 showed no obvious change (Figs. 2a–e). The T-SH content was highly consistent with the hydroxyl radical scavenging rate (Fig. 2g). These observations revealed that the pre-treatment of melatonin could improve rice seed germination through improving antioxidant capacity under salinity.

Six major phytohormones were quantified for the three treatments. We noticed that salt stress activated the endogenous melatonin, and exogenous melatonin further induced the level of endogenous melatonin (Fig. 2h). In addition, salt stress plays a role in suppressing the synthesis of indole-3-acetic acid (IAA), gibberellin (GA), zeatin (ZT), and jasmonic acid (JA), and in promoting the synthesis of abscisic acid (ABA), compared with non-treatment of NaCl. We further noticed that the content of IAA and ZT increased with melatonin pre-treatment under salinity. However, the MT100 treatment had a lower content of ABA

and JA than the NaCl treatment (Figs. 2i–m), suggesting that the synthesis of these two phytohormones was suppressed by the pre-treatment of melatonin.

Additionally, the activities of NADP-ME and ICL in NaCl treatment were significantly higher than those of CK. However, with pre-treatment of melatonin, the activities of these two enzymes were quite different. Melatonin has the role of suppressing the activity of NADP-ME, while promoting that of ICL. We further noticed that pre-treatment with melatonin did not influence α- and β-AL in germinating seeds under salinity. In addition, the content of protein in the germinating seeds under the MT100 treatment was higher than that of NaCl. However, there was no statistical difference in the content of starch and soluble sugar between the MT100 and NaCl treatments.

Effects of exogenous melatonin on metabolism under salinity

In order to gain more insights into the changes in metabolites induced by exogenous melatonin under salinity, metabolomic profiling was performed to quantify the differently expressed primary metabolites by GC-TOF-MS. OPLS-DA is a modified supervised method of data analysis, which is usually applied to reflect the variability between sample groups. The model evaluation parameters (R2Y, Q2) in Table S1 are more than 0.5, indicating the stability of the OPLS-DA models. Figs. S1a and S1b demonstrate that the CK, NaCl, and MT100 treatments were clearly separate from each other, and that metabolomic profiling was statistically significant.

A total of 222 primary metabolites were examined in the CK, NaCl, and MT100 treatments (Fig. 3a), and 86 differentially expressed metabolites (VIP > 1, $P_{adj} < 0.05$) included 55 positive ions and 31 negative ions were detected (Figs. S2a, b). These metabolites were composed of various sugars, amino acids, and some molecular components (Tables S2 and 3). In positive ions, NaCl treatment resulted in the accumulation of various glycosides, including dimethyl guanosine, 2'-O-methylinosine, N6-methyladenosine, adenosine, and 5'-deoxyadenosine, indicating that a wide variety of sugars were combined with non-sugars under salinity. When the melatonin pre-treatment was applied, the glycosides content decreased clearly (Fig. S2c). In negative ions, some organic acids were detected to have prominent changes; in particular, palmitic acid, arachidic acid, succinate, and mevalonic acid. The contents of palmitic acid and arachidic acid were decreased after NaCl treatment, whereas they significantly increased under MT100 treatment. The levels of succinate and mevalonic acid also showed a similar expression pattern (Fig. S2d).

Enrichment analysis was used to list the first 50 enrichment types, based on 222 metabolites (Fig. 3b). Most metabolites were enriched by catecholamine biosynthesis, malate-aspartate shuttle, galactose metabolism, and protein biosynthesis. Fig. S3 shows the biosynthesis network of plant secondary metabolites. We found that many primary metabolites were precursors involved in catecholamine biosynthesis, alkaloid biosynthesis, antibiotic biosynthesis, and hormone biosynthesis. For example, linolenic acid is the substrate of JA synthesis, which was decreased under NaCl treatment compared with

CK, and which was much lower in MT100 (Table S2). This phenomenon may be one of the reasons for the change in JA concentration.

In addition, some specially expressed metabolites were noticed in this study, which may contribute to the phenotypic differences among NaCl and MT100 treatments. Four metabolites were exclusively detected under NaCl treatment, including 1,2-Benzenedicarboxylic acid, Azelaic acid, beta-Estradiol 3-sulfate, and 3,4-Dihydroxybenzoate. Among them, Azelaic acid was down-regulated and 1,2-Benzenedicarboxylic acid was up-regulated. Under MT100 treatment, Oleic acid and cholecalciferol were distinctly reduced, whereas only pyridoxal 5'-phosphate was induced significantly (Fig. 3c).

In the biosynthesis network of plant secondary metabolites, many differentially expressed metabolites were involved in the processes of phytohormone synthesis. Assignment of 16 metabolites from the 71 assayed differentially expressed metabolites were directly or indirectly involved in phytohormone synthesis (Fig. 3d). These results indicate that exogenous melatonin plays an exceptional role in the regulation of phytohormones.

Functional annotation of differential expressed genes (DEGs)

As melatonin pre-treatment increased the germination rate and salt tolerance of rice under salinity, transcriptomic analysis was used to reveal the effects of melatonin on the vitality of rice seeds under salinity. In total, 7160 DEGs were identified, based on the criteria of significance at $P_{adj} < 0.05$ in t-tests (Table S4). Venn diagrams show overlapping DEGs (Fig. 4a), where 6379 DEGs were screened under NaCl treatment, including 2565 up- and 3814 down-regulated DEGs (Fig. 4b). Only 340 DEGs were identified between MT100 and CK, which consisted of 107 up- and 233 down- regulated DEGs (Fig. 4c), a drop of nearly 96% and 94%, respectively. Furthermore, 4794 DEGs were observed in MT100 vs NaCl, an obvious decrease in comparison with that in NaCl vs CK; especially down-regulated DEGs (Fig. 4d). This seems to imply that melatonin regulated the gene expression responding to salinity. In the detected data set, 130 DEGs were caught for co-expression, most DEGs were decreased, and only 14 DEGs were induced in NaCl treatment, compared with CK. When pre-treatment of melatonin was used, most DEGs were induced and only 13 DEGs were inhibited, compared with NaCl treatment, including Peroxidases, resistance-related genes, and transcription factors (Table S5).

To check the credibility of the RNA-Seq data, we randomly selected nine genes which were differently expressed between control and treated condition, which were estimated by quantitative real-time PCR. Consistently, the results of real-time PCR showed the same trend and were in good agreement with the RNA-Seq data ($R^2 = 0.8562$; Fig. S4).

GO enrichment analysis was used to understand the functional meanings of the identified DEGs. All transcripts were differentiated into three branches: Biological Processes (BP), Molecular Functions (MF), and Cellular Components (CC). In comparison with CK, more than 10,000 DEGs were significantly enriched in 208 teams after NaCl treatment (Table S7). DEGs enriched in BP were mainly involved in response to oxidative stress (GO:0006979), reactive oxygen species metabolic processes (GO:0072593),

response to toxic substances (GO:0009636), hydrogen peroxide catabolic processes (GO:0042744), and related ROS detoxification; in MF, were mainly enriched in structural molecular activity (GO:0005198), structural molecule activity (GO:0005198), peroxidase activity (GO:0020037), oxidoreductase activity, acting on peroxide as acceptor (GO:0016684), and antioxidant activity (GO:0016209); and in CC, were mainly related to ribosomal subunits (GO:0044391), cytosolic ribosomes (GO:0022626), and cytosolic parts (GO: 0044445) (Fig. 4e). In addition, the z-score (value 0) exhibited that most GO terms were mainly composed of down-regulated genes, implying that salt damage in germinating seeds due to DEGs related to oxidation processes and detoxification were inhibited and blocked cell activities.

When the rice seeds were pre-treated with 5 μM^{-1} melatonin, quite a number of genes were changed, compared with CK. Only 650 DEGs were significantly enriched in 62 GO terms in MT100 vs CK (Table S7), and the most over-represented terms included peroxidase activity (GO:0004601), response to oxidative stress (GO:0006979), oxidoreductase activity (GO:0016684), antioxidant activity (GO:0016209), and some plasma membrane maintenance related terms (Fig. 4f). There were 4794 DEGs significantly enriched in 127 teams between the MT100 and NaCl treatments (Table S7). DEGs that were enriched in BP were mainly involved in response to oxidative stress (GO:0006979), reactive oxygen species metabolic processes (GO:0072593), and response to abiotic stimulus (GO:0009628); in MF, were mainly enriched in the structural constituent of ribosomes (GO:0003735), structural molecule activity (GO:0005198), and peroxidase activity (GO:0004601); and in CC, were mainly related to cytosolic ribosomes (GO:0022626), ribosomal subunits (GO:0044391), and related ribosomal subunits (Fig. 4g). GOplot showed that the z-score values of most terms tended to zero but were clearly increased with melatonin pre-treatment, suggesting more up-regulated DEGs were induced by melatonin. These results indicate the importance of exogenous melatonin in maintaining cell structural stability and activating ROS detoxification-related genes to improve antioxidative ability and stress resistance under salinity.

To further investigate the putatively biological functions of DEGs, KEGG was performed to analyze the DEG pathways. We found that DEGs were enriched significantly in phenylpropanoid biosynthesis, ribosomes, and biosynthesis of amino acids among the three treatments (Figs. S5a–c). The number of DEGs that involved in plant hormone signal transduction, carbon metabolism, and the biosynthesis of amino acids were most abundant, except phenylpropanoid biosynthesis and ribosomes when melatonin was applied (Figs. S5b, c). In addition, DEGs in MT100 vs NaCl were also involved in the metabolism of amino acids (β − alanine metabolism, alanine, aspartate, and glutamate metabolism) and phenylpropanoid biosynthesis (Fig. S5c).

DEGs in hormonal signaling and ROS

In the present study, hormone-responsive DEGs were mainly classified into seven categories, including JA, IAA, ABA, ethylene, GA, and Benzyl aminopurine (BA) pathways. A total of 109 DEGs were identified to be involved in hormone signal transduction (Fig. 5a), where only 16 DEGs were involved in hormone biosynthesis. As expected, the levels of most enzyme-coding genes in ABA biosynthesis were up-regulated under NaCl treatment, compared with CK, such as the NECD9 class (Os12g0617400,

Os07g0154100), a kind of oxidase, which were significantly induced (10.6- and 4.5-fold, respectively). On the contrary, three genes coding NECDs were significantly restrained, including NCED3 (Os03g0645900) in MT100 treatment compared with NaCl treatment. GA plays a regulatory role in seed germination by co-regulation with ABA signal pathways under abiotic stresses (Sun 2010). Three genes encoding the GA2 family (gibberellin 2-oxidase) were detected in GA biosynthesis. Among these, the levels of OsqSD1-2 (Os01g0883800) and OsGA2OX7 (Os01g0209700) were down-regulated in NaCl vs CK, whereas they were up-regulated in MT100 vs NaCl; one of which was induced up to 32-fold, approximately. All ten DEGs involved in JA biosynthesis were divided into three classes—LOX, AOS, and OPR—which are pivotal rate-limiting enzymes in the biosynthesis of JA (Vick and Zimmerman 1984), which were down-regulated obviously in NaCl vs CK and were induced, except for Os05g0355800 (a similar lipoxygenase-coding gene), in MT100 vs NaCl. The remaining 109 DEGs were mainly involved in hormone transduction pathways. These findings indicate that one of reasons for depressed seed germination can be attributed to the inhibition of hormone biosynthesis and signal transduction enzyme-coding genes under salinity. Moreover, most DEGs in hormone synthesis have been found to encode oxidoreductase.

Peroxiredoxin (PRX) belongs to a peroxidase family of antioxidant enzymes which has been found to be distributed ubiquitously in aerobic organisms (Fujii and Ikeda 2002). A total of 52 DEGs encoding peroxidases were identified in this study (Fig. 5b). In NaCl treatment, the levels of most genes were downgraded; however, the expression of three putative peroxidase-related genes were slightly increased. These downregulated genes included rare cold inducible genes (RCI3) and PRX4-, PRX6-, and PRX10-encoding genes. After the application of exogenous melatonin, all detected PRX-encoding genes were exceedingly increased, and most peroxidase-related genes were raised significantly, compared with NaCl treatment; but with no statistical difference in MT100 vs CK. Empirical studies have shown that the PRX protein family affects intracellular H_2O_2 levels and regulates cell signaling pathways (Rhee 2006). These results fully prove that melatonin plays a catalytic role in activating genes which code antioxidant enzymes.

Expression pattern of transcription factors

Transcription factors (TFs) play important roles in regulating various mechanisms for abiotic stress resistance in plant. With reference to the Plant TF Database v4.0, about 400 DEGs encoding TFs were found (Table S8). Most TFs were down-regulated in NaCl vs CK (Fig. 6a), whereas the expression of quite a number of TFs were induced in MT100 vs NaCl (Fig. 6c). In addition, the number of TFs were reduced significantly in MT100 vs CK (Fig. 6b). All TFs were divided into 71 categories, where the main TF families included 44 MYB, 27 AP2/E2EBP, 19 bHLH, 16 WRKY, 12 bZIP, 12 HSF, 19 C_2H_2, and so on (Fig. 7d). Interestingly, many TFs are involved in hormone transduction, such as NAC, a gibberellin signal regulator (Olsen et al. 2005); WRKY, a brassinolide signal regulator (Chen et al. 2017); and AUX/IAA, an auxin signal regulator (Dunlap et al. 1986). In this study, the MYB family was the largest TF family among all detected TFs. A total of 44 MYB were detected, including 21 up-regulated and 23 down-regulated DEGs in NaCl vs CK; and 10 up-regulated and 13 down-regulated DEGs in MT100 vs NaCl. Moreover, only one down-regulated DEG was detected in MT100 vs CK; the number of DEGs was reduced

by nearly 100% (Fig. 6e), suggesting the pivotal role of melatonin in activating resistance mechanisms and maintaining the metabolic balance.

Discussion

Exogenous melatonin promotes rice seed germination under salinity

Salt stress is a very common adversity globally, in which various physiological and biochemical processes in plants are damaged by salinity, including withered leaves, low germination rate, sterility, and so on (Zhu 2016). Germination is the first stage of a plant's growth, playing a critical role in the development and morphogenesis of a plant (Rajjou et al. 2012). In the past two decades, the mitigated roles of exogenous melatonin have been corroborated in stress responses (Lee and Back 2016). Rice is an important grain crop, the germination and future physiological process of which are seriously affected by salt stress (Alam et al. 2004). Melatonin has been previously reported to improve the germination of cucumber seeds under abiotic stress, such as chilling, water, and high salinity (Zhang et al. 2017b). In the present study, melatonin pre-treatment significantly increased the germination rate of rice seeds and significantly promoted the elongation of embryo under salinity. This provides direct evidence indicating that melatonin could enhance the tolerance of rice seeds to salinity. Therefore, physiological, metabolomic, and transcriptomic analyses were performed to interpret the effects of melatonin on rice seed germination under salt stress.

Melatonin activates antioxidants and reduces accumulation of ROS in rice seeds under salinity

ROS is composed of H_2O_2 and O_2^-. Strangely, the H_2O_2 content was at a low level in NaCl and MT100 treatments, and no significant difference was observed between melatonin pre-treated and non-treated seeds under salinity, indicating that O_2^- was likely the main ROS in seed germination under salinity. Melatonin, as an antioxidant in animals and plants, has been confirmed to stimulate the activities of antioxidant enzymes (Li et al. 2012b). In this study, the ultimate results showed that exogenous melatonin catalyzed the activities of SOD, CAT, and POD under salt stress. In vivo, T-SH, consisting of a glutathione sulfhydryl group and a protein sulfhydryl group, has been considered as a powerful scavenger and protein repair agent (Circu and Aw 2010). The T-SH content and capability for scavenging ROS were increased by melatonin under salt stress. In addition, catecholamine, as a neurotransmitter, has been identified as an important reactive oxygen scavenger in plants (Kulma and Szopa 2007). Dopamine and tyrosine, the intermediate products in catecholamine biosynthesis, were down-regulated under NaCl treatment and up-regulated by melatonin. Modulation and balance of ROS is vital for maintaining the normal metabolism of plants. As ROS mediators, large number of organic acids and amino acids were accumulated under the MT100 treatment, which is consistent with previous findings (Igamberdiev and Bykova 2018). For example, Azelaic acid, a main oxidation product of Oleic acid, was specially down-regulated in NaCl treatment and was adjusted to maintain the normal level by exogenous melatonin. Many studies have identified that Azelaic acid can inhibit the hydroxylation of aromatic compounds induced by ROS and the peroxidation of arachidonic acid in vitro (Passi et al. 1991; Noureddini and

Kanabur 1999). The change of protein content was consistent with the metabolite profile, further implying the significance of amino acids in germination. In addition, many of the detected metabolites had certain antioxidant capacities, such as cholecalciferol, an important liposoluble vitamin in animals, which has been proved to have a strong antioxidant capacity (Wang et al. 2008). In the transcriptional profile, enrichment analysis showed that most down-regulated transcripts were enriched in the oxidative process, energy metabolism, and cell structure-related terms in NaCl vs CK; whereas application of exogenous melatonin resulted in the up-regulation of more genes involved in ROS detoxification, carbohydrate formation, and plasmalemma formation. In this study, peroxidase-coding genes were recoded under NaCl and MT100 treatments, where the expression levels showed high consistency with the activities of antioxidant enzymes. This suggests that oxidation is a key mechanism for germination under salinity and that exogenous melatonin increases the tolerance of rice seeds under salinity through ROS mediation.

Melatonin treatment reprogrammed phytohormone assignment to advance germination under salinity

Phytohormones play important roles in a plant's response to various biotic and abiotic stresses and can effectively regulate the growth and development of plants (Kumar 2013). Previous research has showed that melatonin can affect plant growth by reprogramming IAA, BR, GA, ET, JA, ABA, ZT, and so on (Arnao and Hernandez-Ruiz 2018). In this study, six common hormones were determined and 127 DEGs involved in hormone biosynthesis and signal transduction were screened, including 33 IAA-related genes, three cytokine-related genes, eight GA-related genes, ten JA-related genes, ten BA-related genes, 13 ABA-related genes, and 50 ethylene-related genes. It is also known that IAA, GA, and ZT play vital roles in promoting the development and elongation of the mesocotyl in seeds and enhance the salt stress tolerance of plants (Ryu and Cho 2015; Kim et al. 2006; Miransari and Smith 2014). Comparative analyses showed that most of the IAA, cytokine, and GA-related genes were down-regulated by NaCl treatment and up-regulated after melatonin pre-treatment. Meanwhile, a large number of MYB were activated after melatonin treatment, which has been identified to induce GA-related genes (Martin and Paz-Ares 1997). In addition, AP2/EREBP and AUX/IAA, as the intermediates of Auxin and cytokinin signaling transduction, respectively, were also inhibited in NaCl treatment and up-regulated by melatonin (Table. S7). ABA is a diapause hormone which can inhibit the germination of seeds by affecting the cell cycle (Sánchez et al. 2004). In this study, most of the ABA-related genes were decreased in NaCl vs CK and increased in MT100 vs NaCl. These results are positively correlated with the contents of IAA, GA, ZT, and ABA in rice seeds. JA, one of the most important signaling molecules (as an oxylipin), has been reported to participate in a large number of different physiological processes in plants, such as senescence induction and growth inhibition (Wei et al. 2016; Santino et al. 2013). We found that the DEGs related to JA biosynthesis showed more sensitivity than other phytohormone-related genes; further, 2 F-box family genes OsFbox131 (Os03g0196500), OsFbox319 (Os06g0655500) and some fatty acid oxygenase-coding genes were detected in JA synthesis. A previous study has indicated that the F-box family genes can influence plant growth and development through affecting the IAA and JA signal pathways (Xu et al. 2009). However, the expression patterns of JA synthesis genes seemed to show some contradictions with JA content in NaCl and MT100 treatments. Previous study showed that the traumatin synthesis pathway is a

process for forming calluses by a series of physiological responses to injury in plants (Singh et al. 2000); fatty acid hydroperoxides, the initial reaction products of JA biosynthesis, are the precursors for traumatin synthesis (Gardner 1998). These features indicate that melatonin can mediate the activation of phytohormone signaling to advance the germination of rice seeds in response to salt stress, and we postulated that more mechanisms were involved in response to salinity by exogenous melatonin.

Melatonin is a possible regulator of ion balance under salinity

Salt stress is always accompanied by osmotic stress and drought stress in rice (Djanaguiraman et al. 2006; Pandey et al. 2004). NADP-ME, as an enzyme relying on a metal ion, plays a vital role in the regulation of osmotic pressure balance inside plant cells (Song et al. 2001). In this study, the transformation of NADP-ME suggested the importance of melatonin in maintaining an ion balance during seed germination under salinity. Many stress-induced genes were identified to have low expression under NaCl treatment but were increased in the MT100 treatment, such as OsABF1 (Os01g0859300), a bZIP TF, which has been proved to enhance the resistance to drought in rice (Zhang et al. 2017a); and OsERF48 (Os08g0408500), a member of the AP2/EREBP TF family, which plays a positive role in resistance to drought stress and the enhanced root growth of rice through the regulation of OsCML16 (Jung et al. 2017). In addition, MYBs, as potential salt-tolerance genes can regulate not only GA signals, but also secondary metabolic pathways, such as cutin biosynthesis (Li et al. 2019). Previous research has indicated the importance of cutin biosynthesis for shuttling phospholipid and fatty acid groups between cell membranes (Domínguez et al. 2015). Notably, more melatonin-responsive DEGs in CC were involved in plasmalemma formation with the application of melatonin. We carefully conclude that the membrane lipid fluidity of the cell membrane is inhibited by salinity, but melatonin may enhance lipid transport. In summary, as a stable regulator, melatonin could maintain higher integrity of the cell wall and stabilize the cellular environment to promote seed germination under salinity.

Conclusions

In this work, we revealed that exogenous melatonin possessed the function in promoting rice seed germination under salt stress. Comparative transcriptomic and metabolic analyses suggested that the putative mechanisms of this include the activation of antioxidants and the modulation of extensive metabolic homeostasis. Taken together, the decreased germination of rice seeds under salt stress was mainly due to the accumulation of ROS and hormonal changes. However, exogenous melatonin modulated the level of antioxidants and phytohormones, as well as stabilizing the cell environment under salinity (Fig. 7). Our results provide clear evidence for the roles of melatonin in promoting the germination of rice seeds under salinity.

Declarations

Availability of Data and Materials

All data have been uploaded to the National Coalition Building Institute Gene Expression Omnibus with accession number GSE143922 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143922). BioProject: PRJNA602156.

Abbreviations

Abbreviations	Full names
α-AL	α-amylase
β-AL	β-amylase
ABA	abscisic acid
BA	benzyl aminopurine
BCA	bicinchoninic acid
BP	biological processes
CAT	catalase
CC	cellular components
DEGs	differential expressed genes
GO	gene ontology
GA	gibberellin
GA2	gibberellin 2-oxidase
HPLC	high-performance liquid chromatography
H_2O_2	hydrogen peroxide
IAA	indole-3-acetic acid
ICL	isocitrate lyase
JA	jasmonic acid
MF	molecular functions
NADP-ME	NADP-malic enzyme
OPLS-DA	orthogonal partial least squares discriminant analysis
POD	peroxidase
PRX	peroxiredoxin
RCI3	rare cold inducible genes
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
RH	relative humidity
SOD	superoxide dismutase
T-AOC	total antioxidant capacity

T-SH	total sulfhydryl group
TFs	transcription factors
ZT	zeatin

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Contributions

ZY, YZ and CX conceived and designed the research. LH, HF PL, YX, and RC conducted the experiments. LH, EZ and SY performed the metabolomic and transcriptomic analyses. HL, EZ, CX and ZY wrote the manuscript. YY and YZ edited the manuscript. All authors read and approved the manuscript for publication.

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Ethics declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Figures

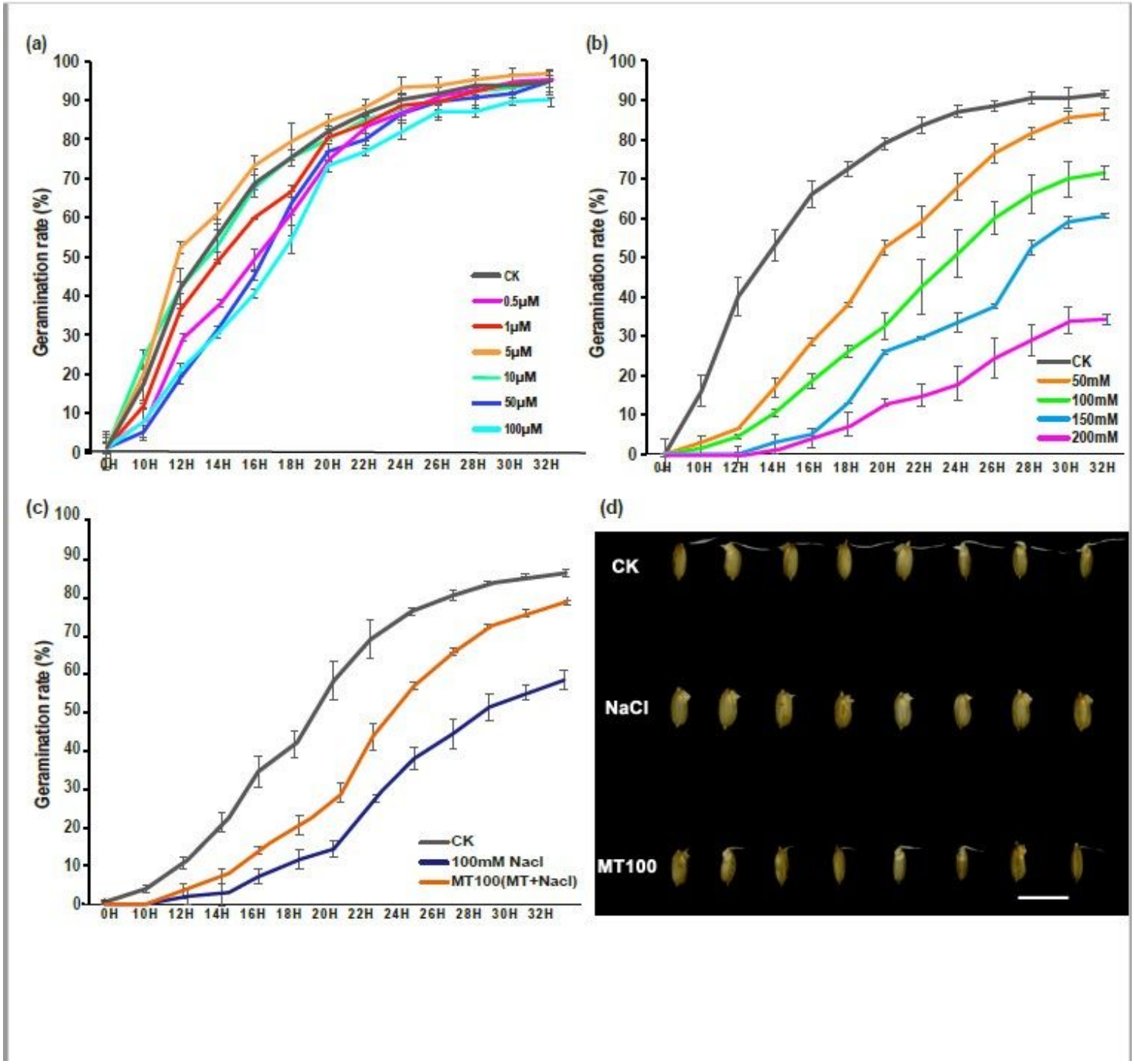

Figure 1

The germination rate of rice seeds under different treatments, including CK, NaCl, and MT100 treatments: (a) The germination rate of rice seeds with and without melatonin pre-treatments. Rate of rice seeds germination per two hours. CK, without melatonin pre-treatment and control conditions. (b) The germination rate of rice seeds with control and different salt stresses. (c) Scanning images of rice seeds under different treatments after 38 hours.

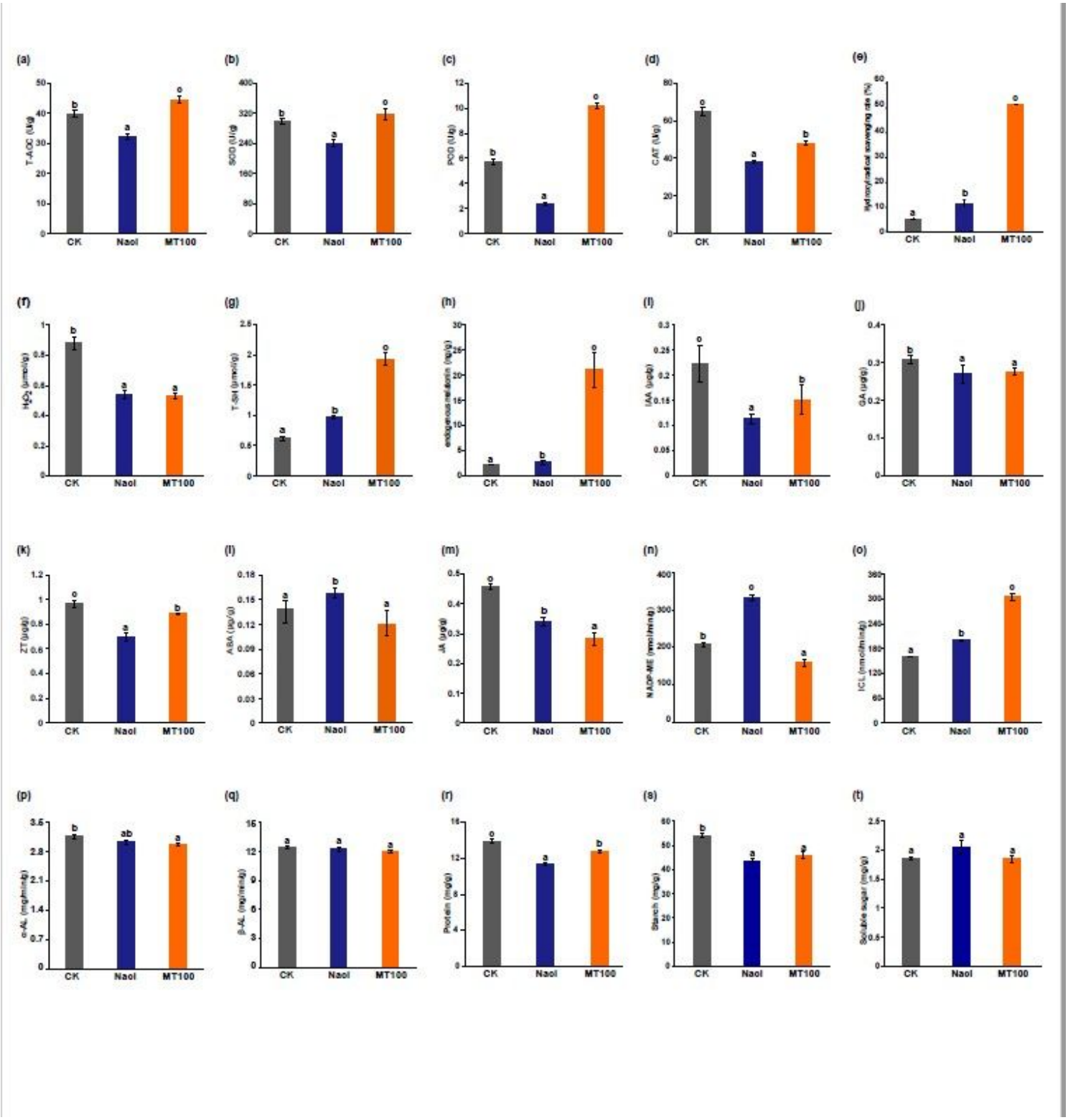

Figure 2

The relative secondary metabolites in rice seed germination measured under CK, NaCl, and MT100 conditions. Results are shown as means ± SD. (a) T-AOC of the rice seeds measured after treatments. (b) SOD activity of rice seed measured after treatments. (c) POD activity of rice seed measured after treatments. (d) CAT activity of rice seed measured after treatments. (e) Hydroxyl radical scavenging rate of rice seed measured after treatments. (f) H2O2 contents of rice seed measured after treatments. (g)

Endogenous melatonin concentrations of rice seed measured after treatments. (h) IAA concentrations of rice seed measured after treatments. (i) GA concentrations of rice seed measured after treatments. (j) ZT concentrations of rice seed measured after treatment. (k) ABA concentrations of rice seed measured after treatments. (l) JA concentrations of rice seed measured after treatments. (m) NADP-ME activity of rice seed measured after treatment. (n) ICL activity of rice seed measured after treatment. (o, p) α, β-AL activity of rice seed measured after treatment. (q) Protein content of rice seed measured after treatment.

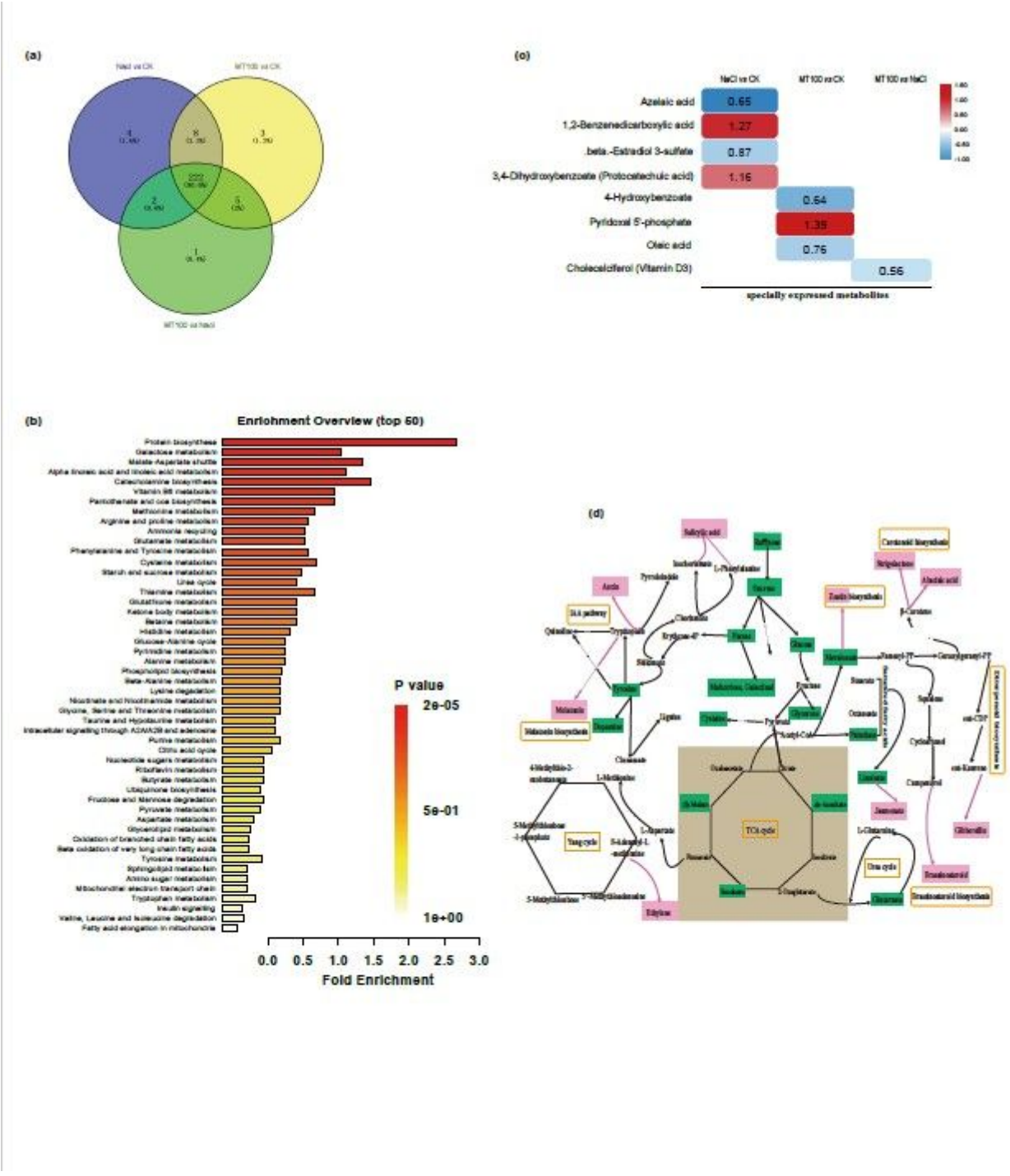

Figure 3

Comparison of metabolites identified in MT100, salt, and control conditions. (a) Three-way Venn diagram indicating the number of primary metabolites in different treatments. Hierarchical cluster analysis. (b) The top 50 enrichment terms from common detected metabolites under three conditions. (c) Primary metabolites especially expressed between NaCl vs CK, MT100 vs CK, and MT100 vs NaCl. (d) The assignment of the 16 primary metabolites from 71 assayed metabolites to the carbon metabolic pathway and major metabolic pathways between MT100 vs NaCl vs CK.

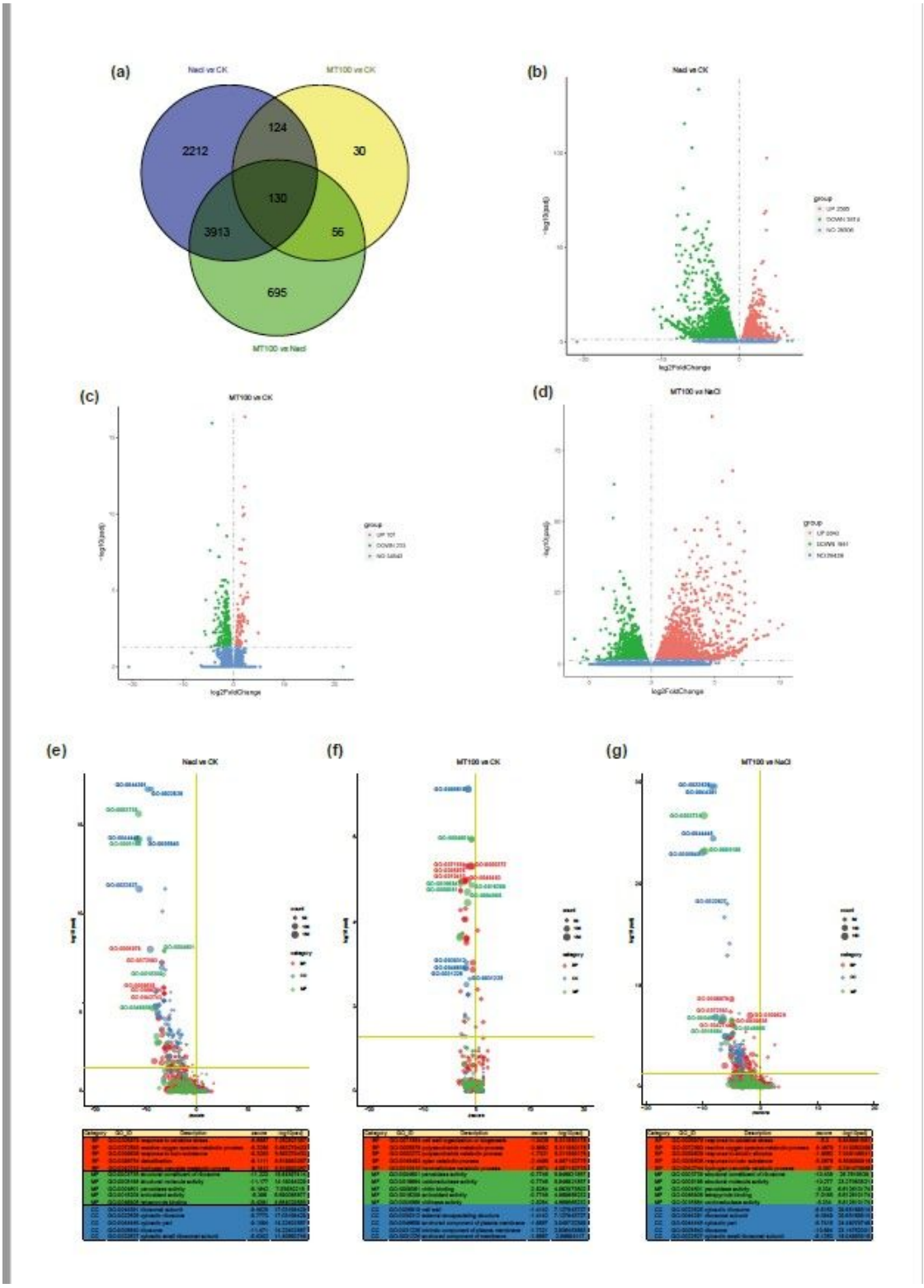

Figure 4

DEGs identified in this study (Padj < 0.05, Fold Change > 1). (a) Three-way Venn diagram indicating the number of common DEGs and distinctive DEGs under different treatments. (b, c, d) The scatter plot of DEGs in NaCl vs CK, MT100 vs CK, and MT100 vs NaCl. The X-axis represents the 2-based logarithm of fold change value (log2FC) and the Y-axis represents the 10-based logarithm of Padj. (e) GO enrichment terms in NaCl vs CK, as well as a list of the top five significant terms. (f) GO enrichment terms in MT100 vs CK, list of top five significant terms. (g) GO enrichment terms in MT100 vs NaCl, list of top five significant terms.

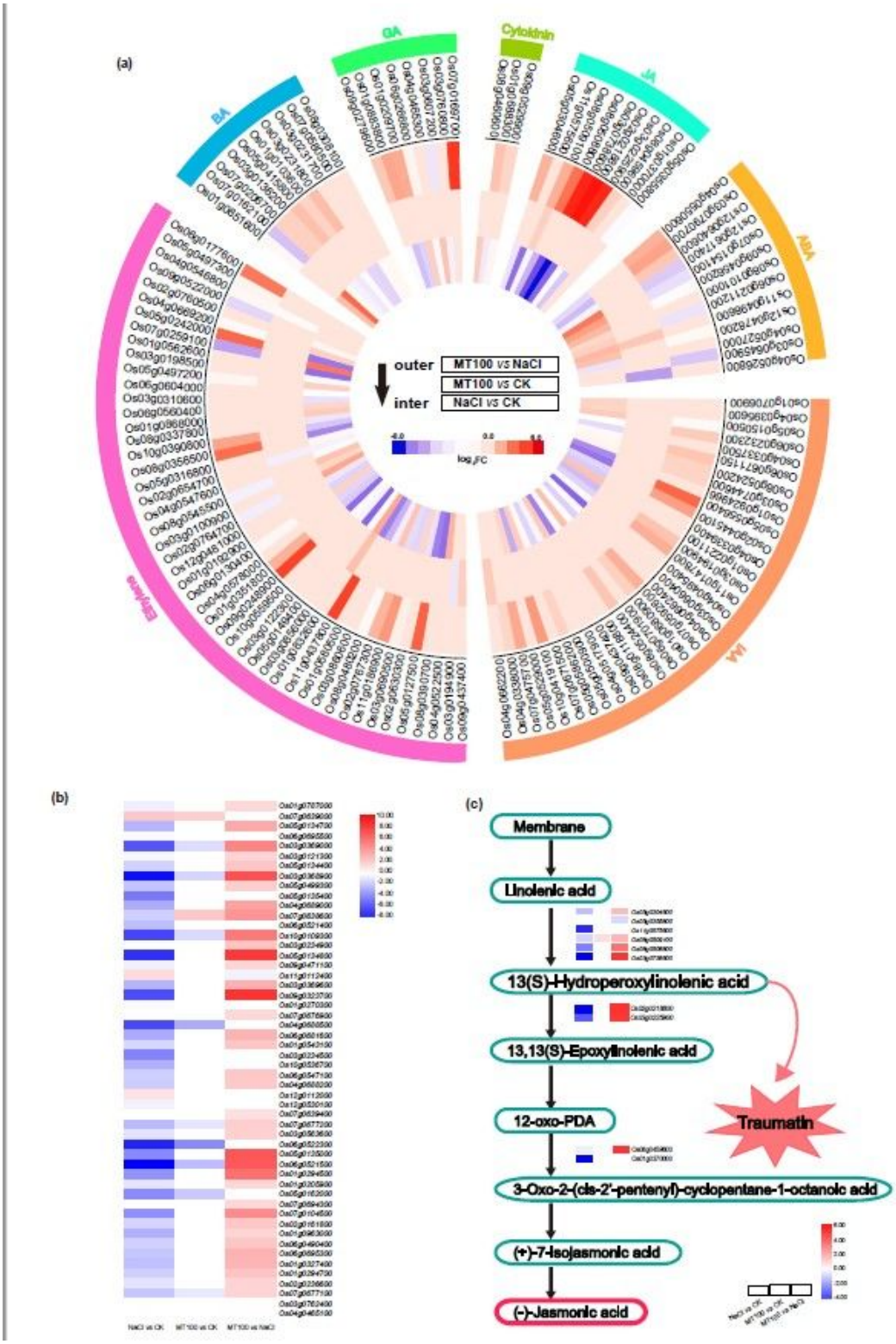

Figure 5

RNA-seq analysis of transcriptome related to hormones and ROS. The 2-based logarithm of fold change value (Log2FC) is denoted. (a) The changes in hormone-related gene expression profile shown using a bubble diagram. (b) The changes in ROS-related gene expression profile shown using a bubble diagram. (c) Changes in the expression of JA biosynthesis-related genes. The pathway diagram of the expression patterns of JA biosynthesis-related genes was similarly created using TBtools.

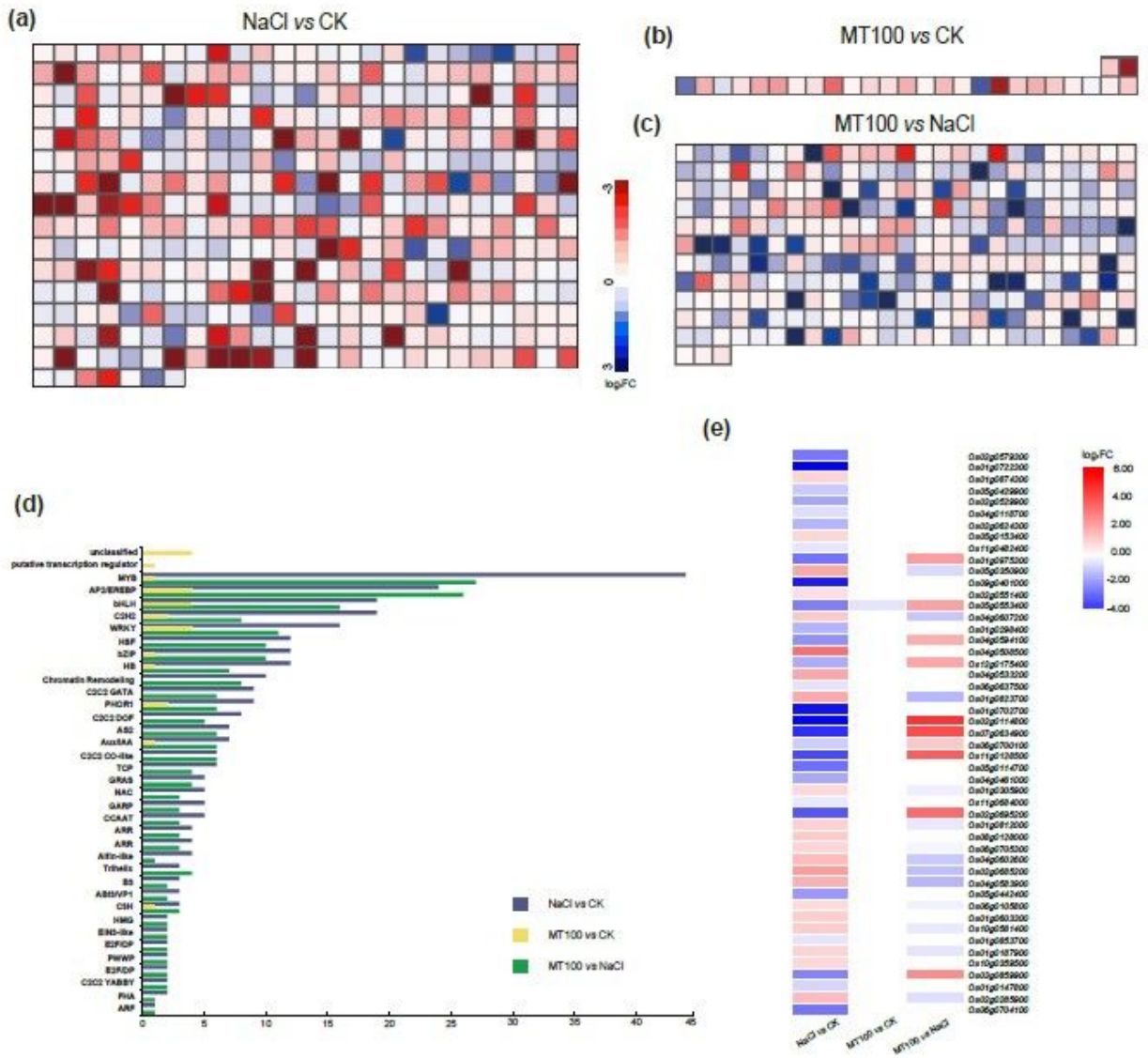

Figure 6

Changes of transcription factors in different treatments. (a, b, c) The expression patterns of transcription factors in combination NaCl vs CK, MT100 vs CK, and MT100 vs NaCl. The transcripts are represented by a set of closely connected cubes. (d) The classification of transcription factor families under different conditions. Statistics are displayed by Excel 2016. (e) Changes in the MYB gene family expression profile.

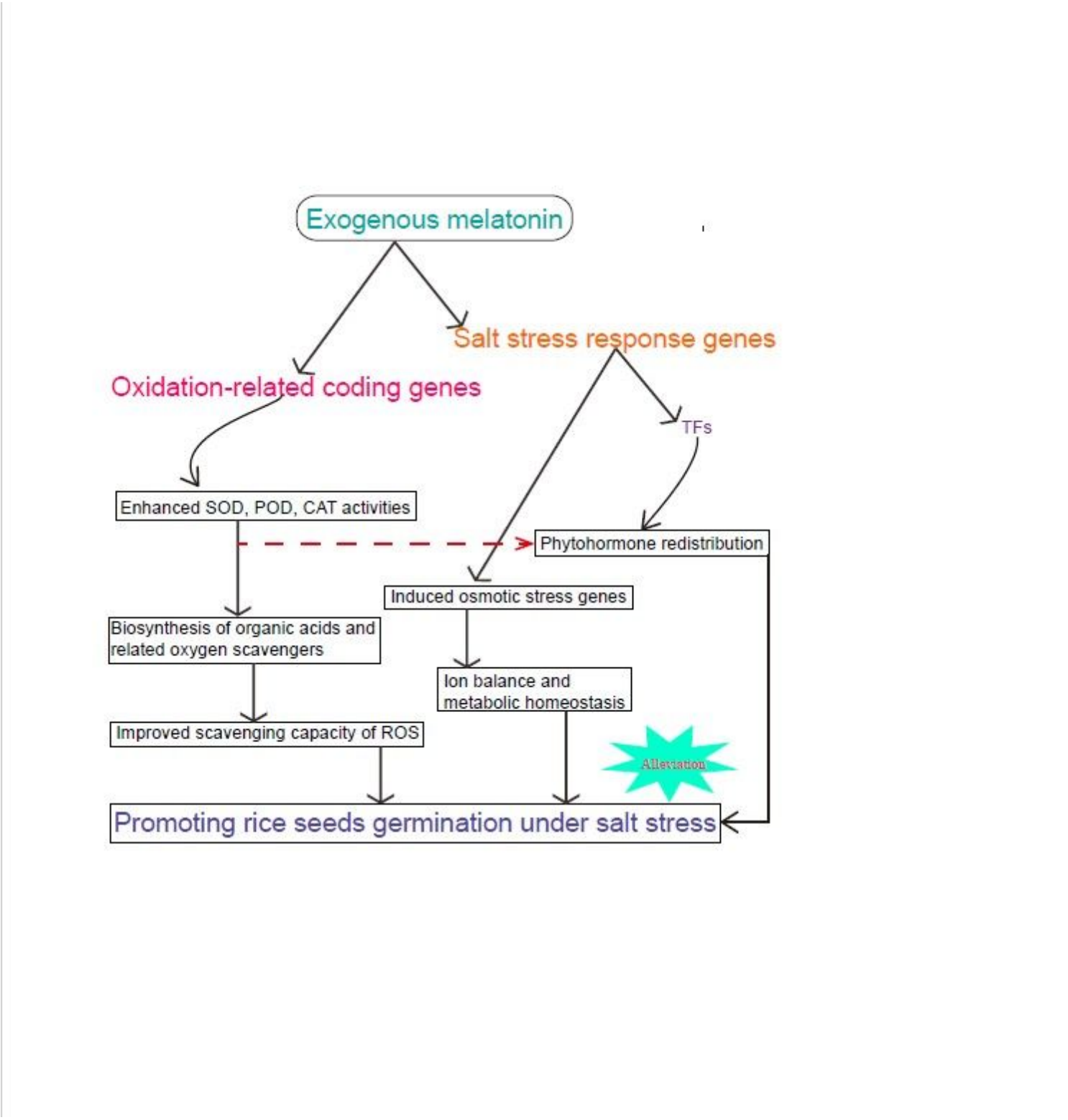

Figure 7

Proposed model of exogenous melatonin improving rice seed germination under salinity.

Supplementary Files

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- FigureS5.pdf
- FigureS3.pdf
- FigureS1.pdf
- FigureS2.pdf
- TableS4.xlsx
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- TableS7.xlsx
- TableS2.xlsx
- TableS1.xlsx
- Table.S8.xlsx
- TableS6.xlsx
- FigureS4.pdf