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Combined transcriptome and metabolome analyses reveal the mechanisms of ultrasonication improvement of brown rice germination

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ABSTRACT

This study investigated the effects of ultrasonication treatment on the germination rate of brown rice. Brown rice grains were subjected to ultrasound (40 kHz/30 min) and then incubated for 36 h at 37 °C to germinate the seeds. Ultrasonic treatment increased the germination rate of brown rice by up to ~28 % at 30 h. Transcriptomic and metabolomic analyses were performed to explore the mechanisms underlying the effect of ultrasonic treatment on the brown rice germination rate. Comparing the treated and control check samples, 867 differentially expressed genes (DEGs) were identified, including 638 upregulated and 229 downregulated), as well as 498 differentially accumulated metabolites (DAMs), including 422 up accumulated and 76 down accumulated. Multi-omics analysis revealed that the germination rate of brown rice was promoted by increased concentrations of low-molecular metabolites (carbohydrates and carbohydrate conjugates, fatty acids, amino acids, peptides, and analogues), and transcription factors (ARR-B, NAC, bHLH and AP2/EREBP families) as well as increased carbon metabolism. These findings provide new insights into the mechanisms of action of ultrasound in improving the brown rice germination rate and candidate DEGs and DAMs responsible for germination have been identified.

1. Introduction

Rice is a staple food for more than half of the global population and the second most consumed cereal grain in the world [1]. Brown rice is directly hulled from rough rice and consists of an endosperm (~90 %), bran layers (6 %–7%), and an embryo (2 %–3%) [2]. In addition to the basic nutritional components, brown rice contains many bioactive components compared with polished white rice, e.g., γ-oryzanol, γ-aminobutyric acid (GABA), and ferulic acid [3], and is preferred by health-conscious consumers. However, brown rice has some drawbacks as a health-food, such as its low digestion rate, unpleasant "bran" odor, poor textural profile, and hard mouthfeel after cooking [4], consequently the search for methods to improve the sensory quality of brown rice, without loss of nutrition, is attracting increasing research interest.

Germination is an inexpensive and effective technology, which has been adopted to enhance the nutritional value, mineral absorption, taste and flavor of cereals [5]. During germination, hydrolytic enzymes are activated and degrade polymers into low molecular weight compounds, such as the conversion of amylose and amylopectin (starch) into glucose, dextrin, and maltose by endogenous amylases, and the degradation of proteins into small peptides and amino acids, thus improving the digestibility and nutrient absorption rate of grains [6]. The types and contents of physiologically active substances continue to increase after germination. For example, the oryzanol content is thirteen times higher; the GABA content is ten times higher; the dietary fiber, vitamin E, niacin and lysine contents are about four times higher; and the vitamin B1,

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vitamin B6 and magnesium contents are nearly-three times higher in germinated brown rice than in white rice. In addition, germination reduces the hardness of brown rice, which is considered an advantage of germination [7].

The employment of chemical and physical methods to enhance seed-germination can cause structural damage and genetic differences, which may be undesirable, so there has been extensive research to investigate the influence of novel treatments on seed germination and growth rate [8], including high pressure [9], ultrasound [10], ultraviolet light [11], microwaves [12], ozone treatment [13], magnetic fields [14], pulsed electric fields [15], non-thermal plasma [16] and plasma activated water [17]. Ultrasound, ultraviolet and non-thermal plasma treatment can effectively enhance seed germination by surface modification of treated seeds, whereas pulsed electric field and microwave treatment trigger physiological changes that increase the germination rate by increasing water absorption during soaking [18]. Ozone treatment is thought to enhance seed germination by lowering the content of phytohormones responsible for maintaining dormancy [18]. These methods are potentially useful for improving the germination rate of brown rice and its nutrient bioavailability.

Recently, various omics techniques have been employed to improve understanding of the factors influencing grain germination. To study different seed treatments, metabolomic analysis of rice [19], wheat [20], and soybeans [21], was performed by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-MS, which provided useful information regarding the metabolic profile changes in these seeds. Advances in next-generation sequencing have paved the way for a new generation of different omics, including transcriptomics, genomics, and proteomics, which have been well-documented in food science. Multi-omics approaches with high throughput techniques can elucidate functions and networks of genes, proteins or metabolites in food materials [22]. Comprehensive multi-omics approaches with robust techniques have been applied to identify and explain the transformation of essential components under various treatments in importantly commercial crops, such as soybean, wheat, and millet [23]. Therefore, multi-omics studies are commonly used in improving the quality of food by analyzing the changes of substance in micro and macro levels during food producing and processing.

In this study, transcriptomic and metabolomic analyses were employed to elucidate the regulatory network and infer the molecular mechanism of brown rice germination. The effect of ultrasound treatment on germination rate was studied to elucidate the relationships between gene expression and metabolites related to rice germination. This will contribute to a comprehensive study and in-depth understanding of ultrasound treatment on the accumulation of germination-promoting factors in brown rice.

2. Materials and methods

2.1. Brown rice and ultrasound treatment

Brown rice cv. Koshihikari was grown and harvested at the experimental farm of the Liaozhong District nursery (122°73′ E, 41°51′ N) situated in Shenyang City, Liaoning Province, China. Rice seeds were incubated for 7 days at 50 °C to terminate dormancy [24], soaked in 1.5 % (v/v) sodium hypochlorite solution for surface sterilization, then flushed with sterile water. After soaking for 12 h, the seeds were treated with an ultrasonic cleaning bath, for different times, at 40 kHz (SK6210HP, overall dimensions: 570 × 160 × 310 mm, tank dimensions: 500 × 140 × 150 mm, capacity: 10.5 L, Shanghai Kedao Ultrasonic Instrument Co., Ltd, Shanghai, China), then placed in germination trays in a growth chamber for 36 hat 28 ± 1 °C. Brown rice without ultrasonic treatment served as the control check (CK). After germination, the samples were lyophilized and stored at 4 °C, protected from light, until needed for analysis.

2.2. Germination rate

To calculate the germination rate, the percentage of germinated grains in fifty grains was counted every 6 h. Seeds were considered to have germinated when a white embryo protrusion was visible [25]. The germination rate was calculated using the following formula:

$$Germination\ rate(\%) = The\ number\ of\ germinated\ seeds/Total\ number\ of\ seeds \times 100\%$$

2.3. Sample preparation for metabolomic analysis

Rice sample (~20 mg) was accurately weighed and added to methanol/acetonitrile (1:1 v/v, 400 μL, with 0.02 mg/mL L-2-chlorophenylalanine as internal standard) in a 2 mL Eppendorf tube containing a 6 mm grinding bead that can be used to completely extract sample. The blend was instantly ground in a cryogenic tissue homogenizer (Wonbio-96c, Shanghai Wanbo Biotechnology Co., Ltd., Shanghai, China) for 6 min at −10 °C, then placed in an ultrasonic cleaner (SBL-10TD, Ningbo Xinzhi Biotechnology Co., Ltd.) for 30 min at 5 °C, and left to stand for 30 min at −20 °C. After centrifuging at 13,000 g for 15 min, the supernatant was transferred to a sampling vial for LC-MS analysis. Each extraction was replicated six times.

Quality control samples were mixtures of equal volumes of UT and CK samples, to assess the reproducibility of MS results. One quality control sample was inserted in every 5–15 analyzed samples.

2.4. Metabolite analysis by LC-MS

Metabolite extracts were analyzed on an LC-MS system (UHPLC-Triple TOF, AB Sciex, Framingham, MA, USA). Rice grain extract (10 μL) was injected into an ACQUITY UPLC HSS T3 column (1.8 μm, 100 mm × 2.1 mm; Waters, Milford, MA, USA). The UHPLC mobile phases were 1:1 v/v acetonitrile/2-propanol (solvent B) and 95:5 v/v water/acetonitrile (solvent A), both containing 0.1 % formic acid. Elution was with a linear gradient: 0 min, 0 % B; 2.5 min, 25 % B; 9 min, 100 % B; 13.1 min, 0 % B; and 16 min 0 % B, at 0.40 mL/min flow rate and 40 °C column temperature. Data were obtained through continuous scanning in positive and negative ion modes. The electron spray ionization source operation parameters were as follows: mass range, 50–1000; ion source gas, 50 psi; curtain gas, 30 psi; source temperature, 550 °C; ion spray voltage floating, +5000 V and −4000 V; and collision energy, 40 ± 20 V.

2.5. Metabolomic data processing

Metabolomic data was analyzed using Progenesis QI (Waters) to acquire information on retention time, peak intensity, and mass-to-charge ratio. After identifying these metabolite features, MS and MS/MS fragmentation spectra were compared with biochemical databases, including the Metlin and Human metabolome databases (HMDB), for identification. The MS mass tolerance was set to <10 ppm. Metabolites with an MS/MS fragment score >30 were regarded as reliably identified, whereas those scoring <30 were only tentatively assigned. The operations of missing value recoding, normalization, quality control verified relative standard deviation ≤30 %, logarithmic process, and maintaining 80 % variability were adopted to eliminate or reduce the errors between two samples in the experiment and analysis.

2.6. Transcriptome sequencing

A Trizol reagent kit (Invitrogen, Carlsbad, CA) was applied to extract total RNA according to the manufacturer's instructions. The quality of the extracted RNA was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and analyzed using RNase-free agarose gel electrophoresis. After extracting total RNA, eukaryotic

mRNA was concentrated with Oligo(dT) beads (Cytiva Life Sciences, Goettingen, Germany), and prokaryotic mRNA was concentrated by eliminating rRNA using a ribo-ZeroTM Magnetic Kit (Epicentre, Madison, WI, USA). The enriched mRNA was cleaved into short fragments using fragmentation buffer and reverse transcribed to cDNA with random primers. Second-strand cDNA was synthesized by RNase H, DNA polymerase I, dNTP and buffer, purified with a QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end repaired, poly(A) added, and ligated to Illumina sequencing adapters. The ligation products were size-selected by agarose gel electrophoresis, PCR amplified, and sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.7. Bioinformatics analysis

Reads received from the RNA sequencers were filtered with fastp0.18.0, then mapped to the ribosomal RNA database of Nipponbare reference genome (ensemble release51, https://ftp.ensemblgenomes.org/pub/plants/release-51/fasta/oryza_sativa/dna/) using the alignment tool Bowtie 2.2.8 [26]. An index of the reference genome was constructed, and HISAT 2.2.4 was used for mapping paired-end clean reads to the rice reference genome [27]. For each transcript region, FPKM (fragment per kilobase of exon per million mapped fragments) values were computed to quantify expression levels and variations. DESeq2 software was applied to analyze differential expression of RNAs between different samples. Correlation analysis of replicates was performed by principal component analysis (PCA) with the R package gmodels. Genes with $|\log_2$ fold change (FC)$| > 1$ and false discovery rate <0.05 were considered as differentially expressed genes (DEGs) for Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses.

2.8. qRT-PCR

To verify the RNA sequencing results, 12 DEGs were selected for qRT-PCR and normalized by *OsACT-1* as internal reference [24]. The primers are shown in Supplementary Table S1. The threshold relative to the standard curve was employed for relative quantification. Three biological and technical replicates were conducted for each sample.

2.9. Statistical analysis

All data were presented as mean value ± standard deviation, from three biological triplicates. Student's *t*-test was used to compare significant differences, $p < 0.05$ indicated statistical significance.

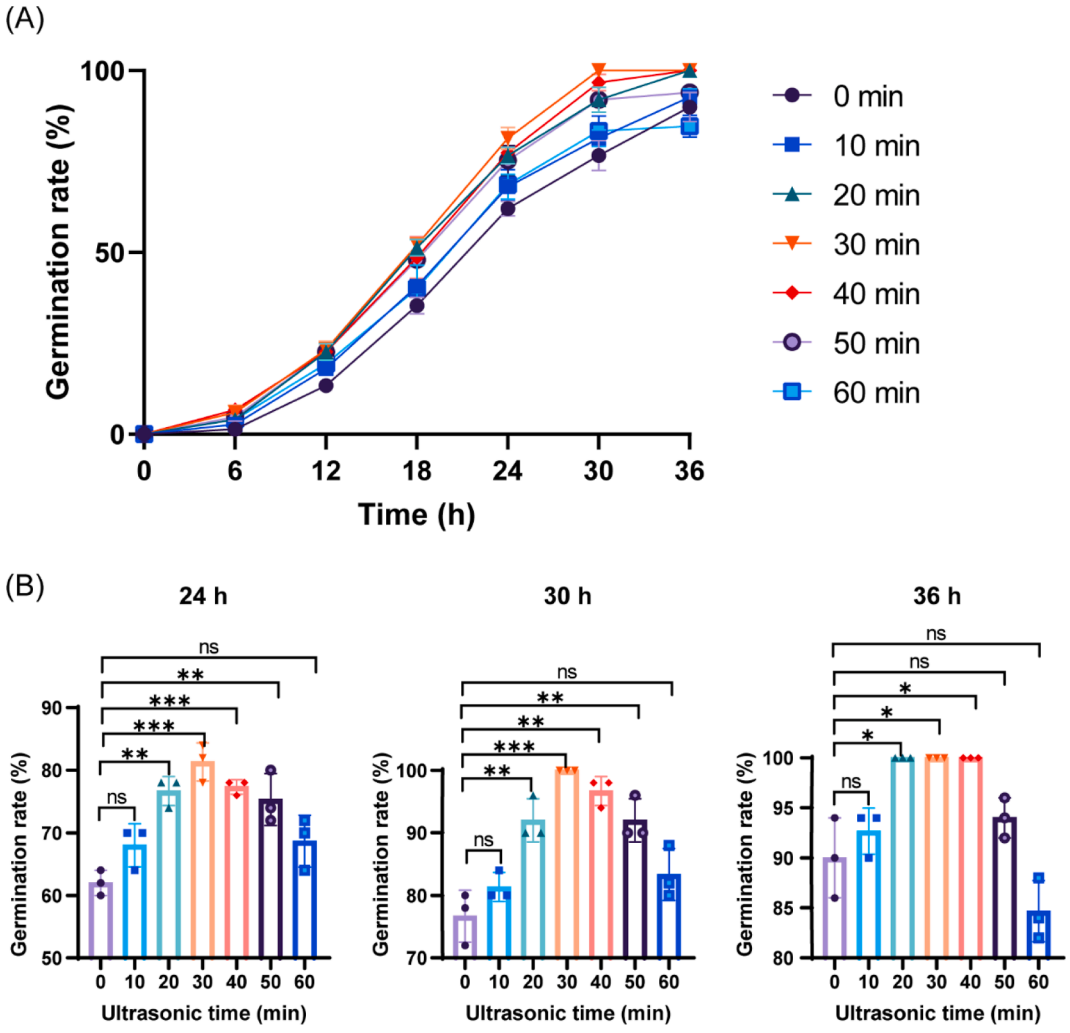

Fig. 1. Effects of ultrasonication on germination rates of brown rice. **A**: Different ultrasonication times (0–60 mins); B: Different germination times (24, 30, and 36 h). Student's *t*-test was used to compare significant differences, with * representing $p < 0.05$, ** representing $p < 0.01$, and *** representing $p < 0.001$.

3. Results

3.1. Germination rate

After soaking for 36 h, rice germination was determined (Fig. 1). The germination rate increased with germination time (Fig. 1A) and the best results (complete germination, i.e., 100 %) were obtained at 36 h, after ultrasonication pre-treatment for 20, 30, or 40 min of ultrasonic treatment (Fig. 1B). The germination rate of seeds was the highest after 30 min of ultrasonic treatment at 24 and 30 h of germination; germination was 100 % at 30 h, compared with ~ 77 % without ultrasonication. Therefore, germinated brown rice samples, pre-treated with, or without

Fig. 2. Differential chemotypes between UT and CK. **A**: PCA of metabolites detected in UT and CK. **B**: Correlation analysis heatmap of UT and CK; colors show relevance level of each sample, ranging from low (blue) to high (red). **C**: Volcano plots of differentially accumulated metabolites (DAMs) between UT and CK; up accumulated (red); down accumulated (blue); unchanged (grey). **D**: Most enriched KEGG terms. **E**: HMDB subclass categories of DAMs. CK = germinated rice without ultrasonic treatment; UT = germinated rice with 30 min ultrasonic treatment.

ultrasound for 30 min then soaked for 36 h, were used in the subsequent experiments and designated UT and CK, respectively.

3.2. Metabolite profile

The UT and CK treatments were subjected to metabolic profiling by untargeted LC-MS, which detected 1,057 metabolites. PCA analysis clearly separated UT and CK (Fig. 2A), with PC1 accounting for 88.6 % of the difference. Correlations between the treatments were analyzed using metabolite concentration data, which revealed that different samples were effectively distinguishable (Fig. 2B), indicating highly reliable metabolomic data. Overall, UT and CK had substantially different metabolic profiles.

3.3. Differentially accumulated metabolites (DAMs) in UT and CK

All metabolite analyses were performed using the following parameters, to verify the DAMs: variable importance in projection > 1; fold change (FC) > 1 determined as up accumulated; <1 determined as down accumulated. Overall, 498 DAMs were identified, including 422 up accumulated and 76 down accumulated (Supplementary Tab. S2, Fig. 2C). The top enriched KEGG terms (Fig. 2D) were pyrimidine metabolism (map00240), purine metabolism (map00230), ABC transporters (map02010), beta-alanine metabolism (map00410), arginine biosynthesis (map00220), phenylalanine, tyrosine and tryptophan biosynthesis (map00400), pentose phosphate pathway (map00030), and pantothenate and CoA biosynthesis (map00770). Notably, the 498 DAMs were categorized into >10 subclasses, mainly amino acids, peptides, and analogues, carbohydrates and carbohydrate conjugates, fatty acids and conjugates (Fig. 2E); these DAMs clearly have important functions during brown rice germination and may be related to energy metabolism, and therefore likely to be responsible for the increased germination rate of ultrasonicated brown rice.

3.4. Transcriptome profiling of UT and CK

RNA libraries constructed for UT and CK were sequenced to detect differentially expressed genes. The total number of reads was 111,783,141 (UT, 58,374,438; CK, 53,408,703; Supplementary Tab. S3). The removal of low-quality reads produced 111,189,574 clean reads (UT, 58,064,007; CK, 53,125,567). There were 105,268,220 (UT, 55,125,803; CK, 50,142,417) mapped reads generated, including 5,055,229 (UT, 2,665,226; CK, 2,390,003) multiple mapped, and 100,212,990 (UT, 52,460,576; CK, 47,752,414) uniquely mapped, to the reference genes. PCA analysis revealed close grouping of the three biological replicates of UT and CK, but wide separation between the groups (Fig. 3A), and correlation analysis indicated distinctly different relationships (Fig. 3B).

3.5. DEGs in UT and CK

To screen for candidate genes involved in brown rice germination, DEGs were selected by $|\log_2 FC| > 1$ in UT compared with CK, which detected 867 DEGs (Supplementary Tab. S4, Fig. 3C). In total, there were 638 upregulated and 229 downregulated genes in germinated brown rice after ultrasonic treatment (Fig. 3D). The 867 DEGs were categorized into three GO classifications: biological processes, molecular function, and cellular components (Fig. 4A). Metabolic process (364;

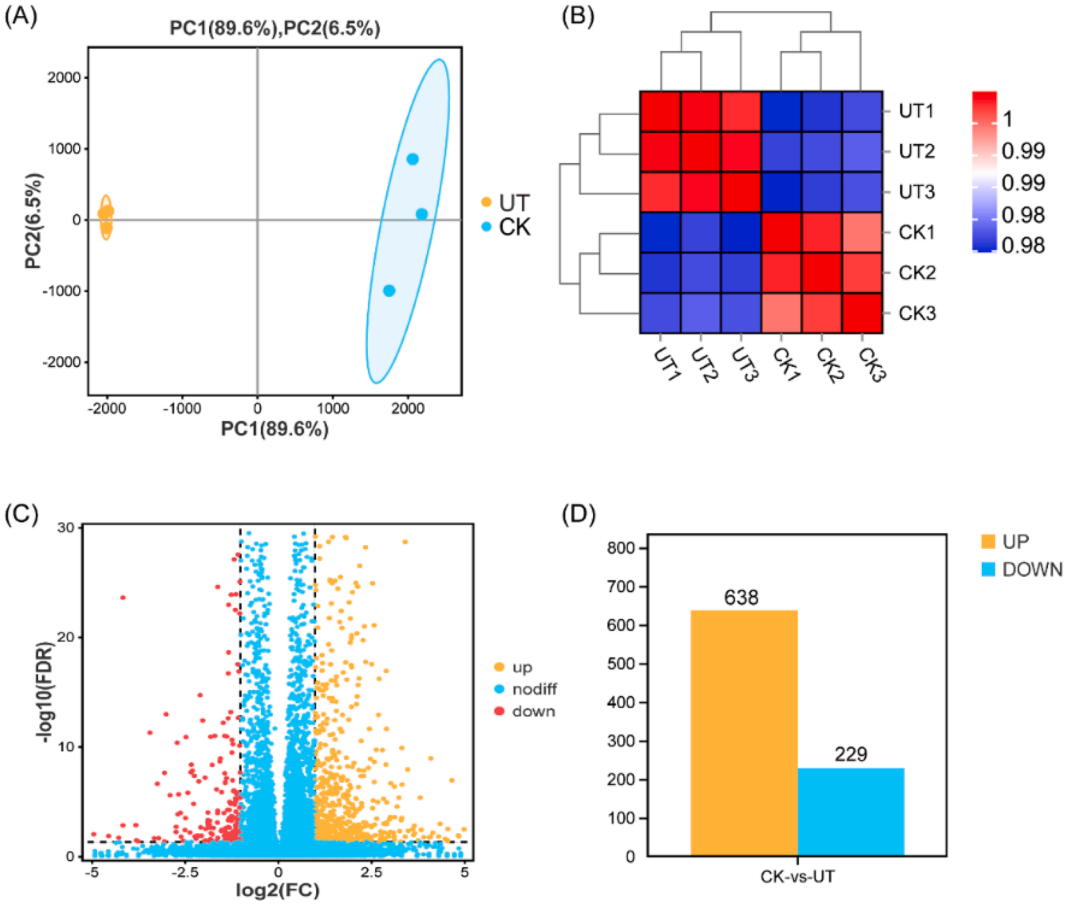

Fig. 3. Differential phenotypes between UT and CK. **A**: PCA of identified genes. **B**: Correlation analysis heatmap of UT and CK; colors show relevance level of each sample, ranging from low (blue) to high (red). **C**: Volcano plot of differentially expressed genes (DEGs); upregulated (yellow); downregulated (red); unchanged (blue) between UT and CK. **D**: Numbers of up- and downregulated genes in UT, compared with CK.

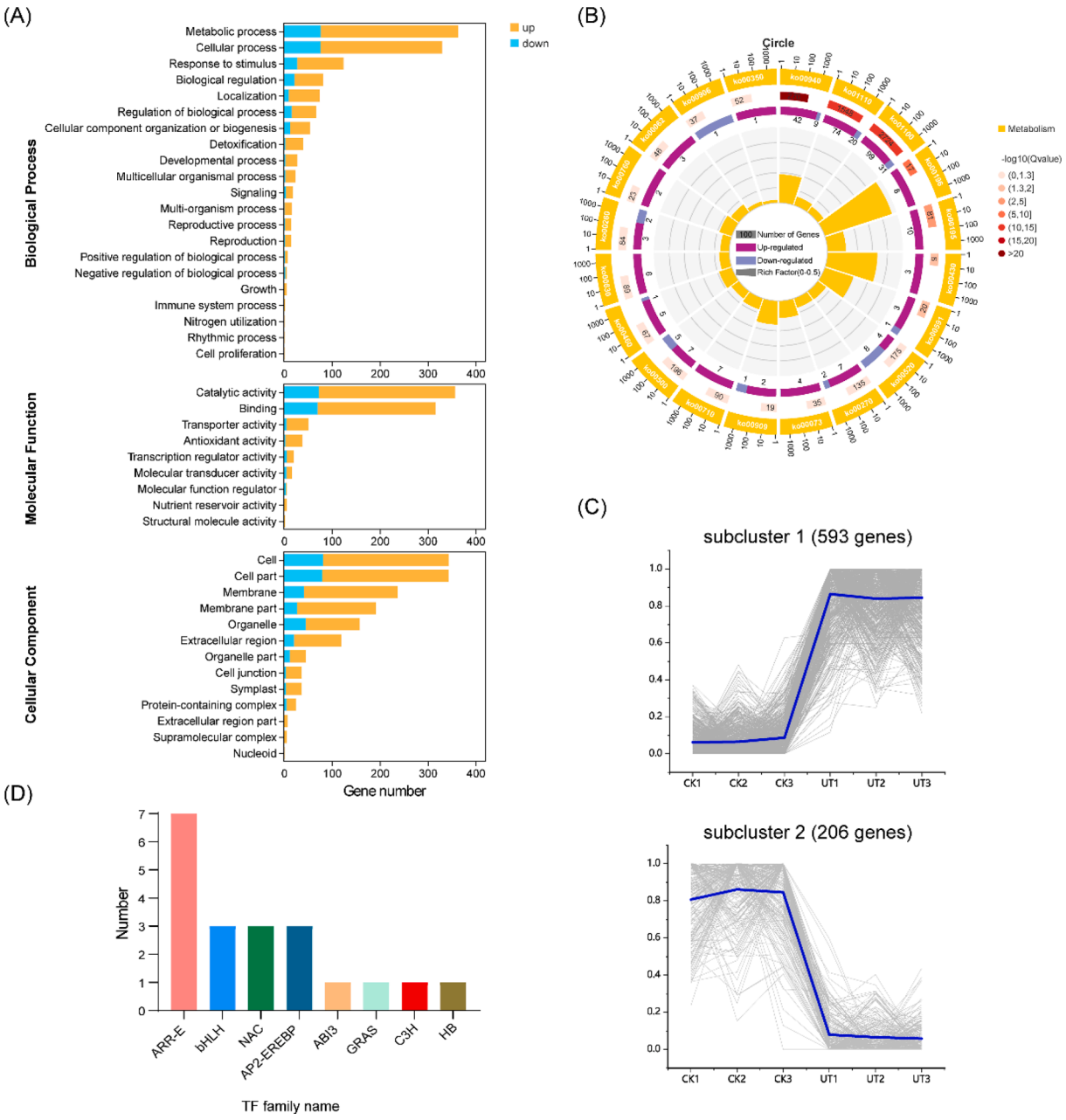

Fig. 4. Differential gene expression between UT and CK. **A**: GO enrichment. **B**: top 20 KEGG enriched pathways involving 867 DEGs. **C**: Two main clusters of DEGs; and **D**: Transcription factor (TF) families enriched in Cluster 1.

42.03 %) was the largest group, followed by catalytic activity (357; 41.22 %), cell (344; 37.72 %) and cell part (343; 39.61 %); relatively few DEGs were associated with nucleoid, structural molecule activity, nitrogen utilization, rhythmic process and cell proliferation. In the circular enrichment diagram (Fig. 4B), the innermost circle shows the top 20 enriched KEGG pathway terms; the second circle shows the background gene number in the pathway and Q values; the third circle shows the percentage of up- and downregulated genes; the outmost circle represents the value of enrichment factor for each pathway. The most enriched KEGG terms were metabolic pathways (map01100), biosynthesis of secondary metabolites (map01110), phenylpropanoid biosynthesis (map00940), amino sugar and nucleotide sugar metabolism (map00520), starch and sucrose metabolism (map00500), and photosynthesis (map00195).

To gain insights into the dynamic changes in DEGs during germination, cluster analysis was performed on the basis of log2 values (FPKM) to divide the DEGs into two main clusters (Fig. 4C). Cluster 1 included 593 genes with increased expression levels from CK to UT, indicating that they are positively associated with brown rice germination. These DEGs encode eight transcription factors (TFs; Fig. 4D), namely ARR-B (Type-B Arabidopsis Response Regulator), NAC (*N*-Acetyl Cysteine), bHLH (basic Helix-Loop-Helix), AP2-EREBP (APETALA2/ethylene-responsive element binding proteins), ABI3 (Abscisic Acid Insensitive3), GRAS (Gibberellic acid insensitive, Repressor of gibberellic acid insensitive, Scarecrow), C3H (CCCH Zinc Finger), and HB (Homeobox), which appear to be pivotal in regulating the expression levels of genes responsible for brown rice germination. In contrast, 206 genes in cluster 2 were negatively associated with brown rice germination, i.e., their expression levels decreased after ultrasound treatment. The expression levels of the other genes were not significantly different, therefore their relationship with brown rice germination is unclear.

3.6. qRT-PCR validation

To verify the accuracy of the RNA sequencing data, 12 DEGs related to rice germination, including those encoding SPS, SUS, RBCS, GDCH, NADP, Sub1B, MYB, and An-1, were screened by qRT-PCR. The expression levels of all 12 genes in UT were higher than those in CK (Supplementary Fig. S1). A heatmap revealed that the qRT-PCR results were consistent with the transcriptomic trends, which validated the reliability of the data (Supplementary Fig. S2).

Taken together, it appears that the profile of metabolite accumulation changes after ultrasonic treatment is closely related to differential gene expression.

4. Discussion

In this study, ultrasonic treatment increased the germination rate of brown rice (Fig. 1), which is consistent with previous reports on the application of ultrasound to improve the growth parameters of wheat grain [28], barley seed [29] and germination of sesame seed [30]. A possible mechanism of action is that the mechanical effects of ultrasound produced multiple pores in the seed outer coating and fissures in the pericarp, which increased water absorption [18]. Ultrasonic treatment also increases oxygen availability to cellular organelles and cellular metabolic activity, which promote germination [31]. However, the influencing factors and mechanism of ultrasound on brown rice germination are not yet clear. In this study, brown rice with or without ultrasonic pretreatment was compared by metabolomics and transcriptomics to identify DAMs and DEGs and thereby compare the physiological and biochemical changes between UT and CK.

During germination, macromolecular substances, namely starch, lipids and proteins accumulated in the mature seeds are mostly degraded by enzymes to provide precursors for growth and energy sources for germination [32]. Accordingly, UT contained markedly more metabolites, including amino acids, peptides and analogues, carbohydrates and carbohydrate conjugates, and fatty acids and conjugates, than CK (Fig. 2E). During grain germination, the stored proteins are catabolized into small peptides and amino acids by proteolytic enzymes, thereby increasing nutrient bioavailability [33]. The differential metabolite results showed that the amount of up accumulated amino acids, peptides, and analogues was almost 18 times that of the down accumulated ones (Fig. 2E), indicating more complete protein hydrolysis after ultrasound treatment. In addition, the starch in the endosperm, an energy supply for seed germination and seedling growth, is degraded by amylases secreted from the aleurone layer at the beginning of germination [34]. The starch is degraded by hydrolysis and phosphorolysis, then synthesized into sucrose by the sucrose phosphate phosphatase (SPP), sucrose phosphate synthase (SPS), and sucrose synthase (SUS) enzymes [35]. The transcriptomic results indicated that the genes encoding SPS (*Os11g0236100*) and SUS (*Os04g0309600*) were highly expressed in UT after ultrasonic treatment of the seeds (Supplementary Tab. S4). However, the amount of sucrose in UT was less than in CK (Supplementary Tab. S2), suggesting that sucrose in UT was degraded into monosaccharide, contributing to the energy needed for germination.

Lipid metabolism during seed germination involves lipase catalyzed hydrolysis of triacylglycerols to produce free fatty acids, which are then degraded through β-oxidation and the glyoxylate cycle, then further metabolized into sugars as energy and carbon sources. The differential metabolite results showed that up accumulated fatty acids and conjugates were four times more than down accumulated ones (Fig. 2E). As for other storage substances, lipolysis is also related to energy production during germination; the metabolic dynamics of fatty acids depend on lipase activity and pre-germination treatments of whole grains, with the levels of certain fatty acids progressively increasing or decreasing during germination [36].

Another promising finding was that carbon metabolism was a key process during brown rice germination. In this study, six substances involved in carbon metabolism were differentially accumulated, i.e., four up-accumulated (malate, gluconate, glucono-1,5-lactone and 3-hydroxy-propanoate) and 2 down-accumulated (glycerate and aspartate) in UT compared with CK, after ultrasonic treatment (Supplementary Tab. S2). Two metabolites also participated in the tricarboxylic acid (TCA) cycle, which is the final metabolic pathway for lipids, glucose and amino acids to produce energy for physiological modification, and is the most efficient energy metabolic pathway in higher animals and plants [37]. In this study, malate was up-accumulated, whereas aspartate was down-accumulated in the TCA cycle, after brown rice ultrasonic treatment. A high content of TCA cycle products activates malate dehydrogenase to generate oxaloacetate and nicotinamide adenine dinucleotide phosphate (NADP) [38], which supplies energy for germination. Aspartate and other amino acids are required for the TCA cycle [39]. A low level of aspartate is thought to replenish the TCA cycle, as a substrate for improving energy efficiency during germination. In our research, 8 up-regulated DEGs in carbon metabolism were participated in energy metabolism, amino acid metabolism, and carbohydrate metabolism to provide energy and primary metabolite (including amino acids and organic acids), which can promote germination of brown rice [40]. Moreover, gluconate can form lactobionic acid, which promotes metabolism and protects against oxidative damage to the cell membrane [41]. Above all, active carbon metabolism provides not only energy, but also primary metabolites and intracellular environment that promote germination.

In the transcriptome, there were 11 DEGs related to carbon metabolism, with 10 upregulated (*Os12g0291400; Os02g0698000; Os04g0106400; Os04g0459500; Os12g0292400; Os10g0516100; Os12g0274700; Os01g0723400; Os07g0621800; Os12g0291100*) and one downregulated (*Os05g0187100*), in UT compared with CK (Supplementary Tab. S4). Previous reports have demonstrated that up-regulation of genes related to carbon metabolism changes the concentrations of sugars, such as glucose and sucrose, which provide energy during germination [42].

Transcription factors are involved in rice germination, mainly ARR-B, NAC, bHLH, and AP2-EREBP families (Fig. 4D). In many cases, ARR-B family has an important function as a positive regulator in cytokinin signaling [43] and combines with other hormones to modulate coleoptile germination [44]. NAC family maintains membrane integrity under abiotic stresses [45] and modulates coleoptile germination in cross-talk with other hormones, for example, abscisic acid and jasmonic acid. bHLH family participates in regulation of seed germination and mediates the response of seed germination to temperature [46]. AP2/EREBP family genes are augmented at the early stage of rice seed germination and thought to be involved with water absorption and abscisic acid signaling during early rice seed germination [47]. In addition, correlation analysis of TF factor genes, DEGs and DAMs involved in carbon metabolism showed that all analyzed data had high relevance (Fig. S3). Therefore, the higher brown rice germination rate after ultrasonic treatment may result from involvement of TFs, such as ARR-B, NAC, bHLH and AP2/EREBP families. The discovery of the regulation of brown rice germination by TFs has opened up new avenues to understand the mechanisms underlying the effects of other non-thermal physical treatments on food structure and nutrient composition.

Based on the above findings, the factors influencing the improvement in brown rice germination resulting from ultrasonic treatment are summarized in Fig. 5. Firstly, the degradation of polymeric substances into oligomers and monomers releases large quantities of energy. Secondly, DEGs and DAMs involved in carbon metabolism and the TCA cycle provide energy indirectly for germination. Finally, transcription factors such as ARR-B, NAC, bHLH, and AP2-EREBP families promote germination. This study has revealed that physical energy, in the form of ultrasonic stress, can be effectively used to increase the germination rate of Koshihikari rice, so ultrasonic treatment has clear potential for improving germination rates of brown rice and other seeds in industrial applications. However, the diversity and complexity of seeds and

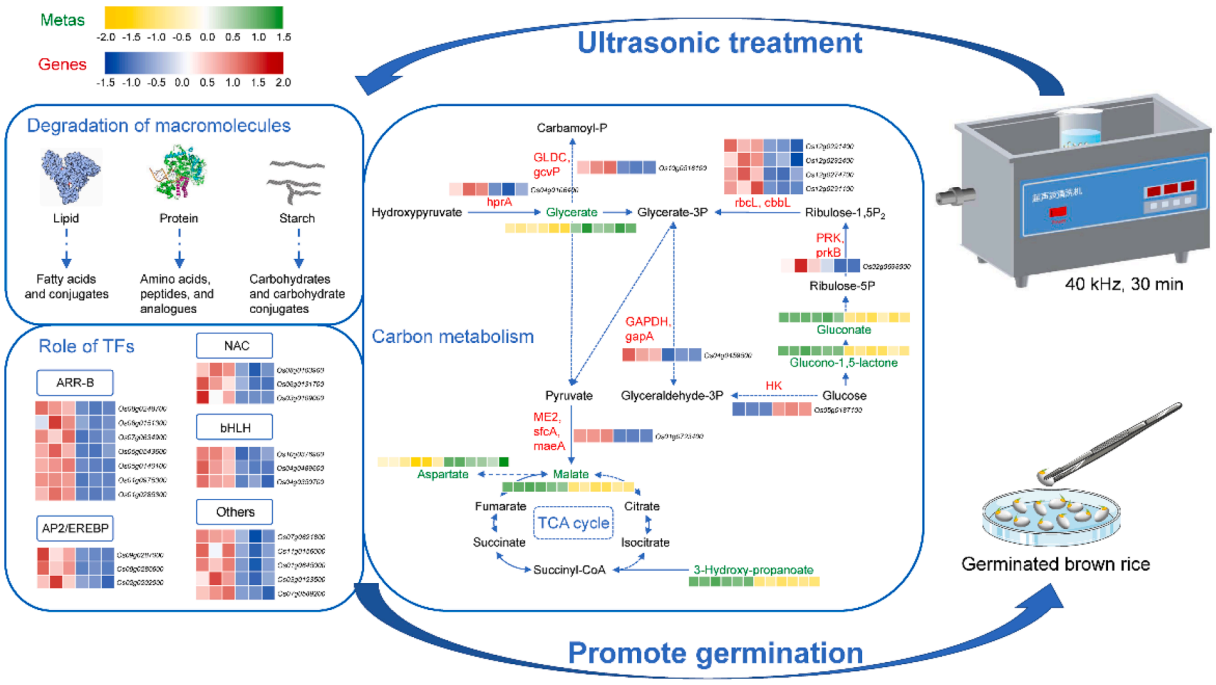

Fig. 5. Proposed mechanisms of action for effect of ultrasonic treatment on brown rice germination rate. Involved metabolites are marked in green; relative content of each metabolite is shown as a heatmap. Colors indicate the relevance level of each sample: low (yellow) to high (green). Involved enzymes are marked in red; gene expression level is shown as a heatmap. Colors indicate the relevance level of each sample: blue (downregulated) to red (upregulated). A set of rectangles corresponding to each metabolite, or gene indicates the corresponding content, or expression level using the respective color scales (from left to right: UT samples to CK samples).

differences in ultrasonic apparatus, seed quality and operating conditions means that conditions must be optimized on a case-by-case basis. Extensive future research will be needed to apply and test the differences in germination rate of more rice varieties under ultrasound pretreatment, and identify the most suitable seeds for germination. Enhancing germination rates has great potential for improving the nutrient content and bioavailability, and the digestibility of brown rice.

5. Conclusion

These findings support the applicability of ultrasonic pretreatment for enhancing the germination of brown rice grains and elucidated the mechanisms of action on the gene and metabolite level. Ultrasonic treatment (UT) prior to soaking resulted in a higher germination rate compared with the untreated control (CK). Transcriptomic analysis identified 638 upregulated genes and 229 downregulated genes; 422 up accumulated and 76 down accumulated metabolites were distinguished in UT compared with CK. Monomeric and oligomeric metabolites, such as carbohydrates and carbohydrate conjugates, amino acids, peptides, and analogues, fatty acids and conjugates, produced by enzymic hydrolysis of polymeric substances, provided not only energy, but also primary metabolites and the material basis for seed germination. Metabolomic and transcriptomic profiles demonstrated that the DEGs and DAMs related to energy metabolism, carbon metabolism and the TCA cycle were mainly responsible for the significant difference in brown rice germination rate with and without ultrasonic treatment. The increasing germination rate of rice coleoptiles in response to ultrasonic treatment appear to be related to a combination of mechanisms involving different TFs, such as ARR-B, NAC, bHLH and AP2/EREBP families. Overall, metabolomics and transcriptomics are introduced to understand the mechanisms behind the germination and biological responses derived from ultrasonic treatment, which is found to be a prospective technique in the agricultural food industry for shortening the seedling growth cycle, enhancing the sprouting efficiency, and reducing the production cost of GBR. In the future, more studies are encouraged to discover the accumulation of GABA and other health-favorable components in brown rice using ultrasonic treatment. In addition, due to the diversity and complexity of seeds, further studies are needed on understanding the overall regulatory mechanisms behind different cultivars.

CRediT authorship contribution statement

Guangchen Zhang: Formal analysis, Investigation, Methodology, Writing – original draft. **Jiaxin Xu:** Data curation, Formal analysis, Software, Writing – original draft. **Yiqiao Wang:** Visualization. **Xue Sun:** Methodology. **Shaosong Huang:** Software. **Lihua Huang:** Investigation. **Youhong Liu:** Conceptualization. **He Liu:** Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Jian Sun:** Data curation, Project administration, Resources.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2022.106239.

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