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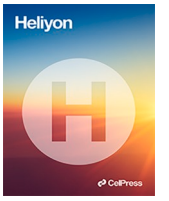

Research article

Comparative transcriptome analysis and meta-QTLs mapping reveal the regulatory mechanism of cold tolerance in rice at the budding stage

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ABSTRACT

Rice (*Oryza sativa* L.) is one of the most extensively farmed food crops, but its development and productivity are significantly impacted by cold stress during the budding period. In this study, transcriptome sequencing was conducted on two types of rice: the cold-sensitive *indica* rice A117 and the substantially cold-tolerant *japonica* rice B106 under control and cold treatments. Differentially expressed genes between the two materials under cold conditions were analyzed using GO and KEGG enrichment analyses. The results revealed that processes such as the TCA cycle, glycolysis/glycogenesis, oxidative phosphorylation, and glutathione metabolism contribute to B106's cold tolerance. Additionally, an enrichment analysis of cold-induced genes in each material and shared genes identified significant enrichment in pathways such as glutathione metabolism, phenylpropanoid biosynthesis, and photosynthesis-antenna proteins. Initial cold tolerance QTLs at the rice bud stage were collected from published literature, and meta-QTL mapping identified 9 MQTLs. Gene expression profiling led to the identification of 75 potential DEGs within the 9 MQTLs region, from which four candidate genes (Os02g0194100, Os03g0802500, Os05g0129000, and Os07g0462000) were selected using qRT-PCR and gene annotation. These findings provide genetic resources for further research on the molecular mechanisms underlying rice's response to cold stress during the bud stage.

1. Introduction

Rice (*Oryza sativa* L.) is a crucial staple crop, feeding over 60% of the population in China and providing 21% of the world's per capita energy intake [1]. Unlike cereals such as wheat and barley, rice is highly sensitive to low temperatures, which can significantly harm its growth [2]. Exposure to low temperatures during the bud bursting and seedling stages leads to poor plant establishment,

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delayed crop maturation, and reduced yields [3]. Around the world, including China, Japan and South Korea, there are about 15 million hectares of land prone to low temperature [4]. Especially in China, the annual loss of rice yield due to low temperature and cold damage is 3–5 million tons [5]. To improve efficiency, many rice-growing regions have adopted the technique of direct-seeded rice (DSR). However, DSR is extremely susceptible to cold stress during the germination and bud burst stages, posing a significant threat [6]. Therefore, breeding for cold resistance at the bud bursting stage is a crucial goal in rice breeding.

The best defence against cold damage is to breed cold-tolerant cultivars; however, this is challenging due to the polygenic nature of cold tolerance [7]. Over the past 20 years, gene mapping studies have identified multiple quantitative trait loci (QTLs) associated with cold tolerance in rice. For example, Zhang et al. [8] identified QTLs on chromosomes 3, 7, and 11 using a recombinant inbred line (RIL) population derived from Lemont and Teqing. Yang et al. [9] discovered three additional QTLs on chromosome 9 by resequencing RIL populations of cold-tolerant H335 and cold-sensitive CHA-1. Li et al. [10] identified twelve QTLs through genome-wide association studies (GWAS) by integrating 36,727 SNPs with phenotypic data from 211 rice varieties; seven were newly discovered, while five co-located with previously detected QTLs.

Despite identifying many QTLs for bud cold tolerance, only three genes—*LTT7*, *OsRab11C1*, and *LTG5*—have been cloned and characterized [11–13]. Liu et al. [14] recently discovered the primary gene *chilling-tolerance in Geng/japonica rice 3* (*COG3*), which provides chilling tolerance at both seedling and booting stages. A comprehensive understanding of the genetic architecture and distribution of relevant loci is essential for identifying targets for breeding or QTL cloning. Achieving a QTL meta-analysis can substantially aid in this synthesis. In order to facilitate the shrinking of QTL intervals and the identification of strong and dependable QTLs, meta-analysis of QTLs combines data from several QTL mapping investigations into a single consensus map [15]. The flowering times of maize [16] and wheat [17], soybean seed storage composition [18], blast resistance in rice [19], and fiber development in cotton [20] are a few examples of this type of investigation in plants. Yang et al. [21] investigated meta-QTLs using 189 public QTLs and discovered 47 cold-tolerant rice meta-QTLs.

The high-throughput RNA sequencing data has significantly facilitated the understanding the molecular foundation of rice's response to cold [22]. Under cold stress, the amount of ROS within cells increases rapidly, causing antioxidant defense systems to swiftly activate by upregulating the expression of genes involved in ROS scavenging [23]. As a crucial antioxidant defense system for scavenging reactive oxygen species (ROS), the ascorbate-glutathione AsA-GSH cycle is made up of six enzymes: ascorbate peroxidase (APX), glutathione reductase (GR), glutathione S-transferase (GST), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR). The two dominant non-enzymatic antioxidants in the cycle are GSH and AsA. The nitrogen recovery index, the amount of superoxide dismutase (SOD), and the amount of malondialdehyde (MDA) were among the metrics that Han et al. [24] found to be strongly linked with the cold tolerance of weedy rice seedlings using RNA-seq. Furthermore, under abiotic stress circumstances, when greater rates of glycolysis and activities of fermentative enzymes were seen in plants [25,26], it has been reported that the ATP synthesis rates are adversely hindered [27]. In this case, plants with low energy levels may employ complementary responses to maintain a stable energy balance. For instance, they could derepress ATP-regenerating pathways such as glycolysis to optimize energy generation while simultaneously delaying ATP-utilizing pathways to conserve ATP [25].

Two cultivars—B106, a *japonica* cultivar highly cold-tolerant, and A117, an *indica* cultivar sensitive to cold—were subjected to transcriptome analysis to investigate the cold stress mechanism. Finally, mapping meta-QTLs led to a more specific identification of the differentially expressed genes (DEGs). Investigating the gene expression patterns under low-temperature stress will aid in developing a regulatory network for cold tolerance in rice. This study will also identify numerous potential genes that may be used in breeding programs to improve cold tolerance during the budding stage of rice growth.

2. Materials and methods

2.1. Plant materials and treatments

The *indica* variety A117 and the *japonica* variety B106, which were cultivated in the paddy field base, were provided by Zhaoqing Academy of Agriculture and Forestry Sciences for this study. Seeds were gathered on the 40th day following the heading, considering seed maturity influences germination. The seeds were then stored at −20 °C after being air-dried for five days at 42 °C. The seeds were first dried at 50 °C in an oven and then subjected to a seven-day dry-air treatment to break dormancy. After being disinfected for 20 min with 20% bleach (6–7% NaOCl), the seed surfaces were rinsed three times with distilled water to ensure sterility. Ten milliliters of sterilized distilled water were added to each Petri dish after roughly 100 seeds were coated with 9-cm circular filter paper. For every sample, three separate biological replicates were made. For 72 h, each Petri dish was cultured with an 8-h light (200 μmol $m^{-2}s^{-1}$) and a 16-h dark cycle at 30 °C. After three days, about thirty buds measuring 0.5–1 cm were removed from each Petri dish using 10 ml of distilled water. Afterwards, we placed culture dishes (30 buds per dish, three dishes for each variety) in a 4 °C incubator and cultured for four days before sampling (samples include buds and seeds). A117-NT and B106-NT were the names of the six samples that were treated at normal temperature (30 °C, NT), while A117-LT and B106-LT were the names of the six samples that were treated at low temperature (4 °C, LT). In preparation for the subsequent RNA extraction procedure, all samples were stored at −80 °C.

2.2. RNA extraction, illumina transcriptome library preparation, and sequencing

Following standard protocols, total RNA was extracted using a mortar and pestle from the A117 and B106 under control and cold treatment using a Plant RNA Extraction kit (Tiangen Bio Co., Ltd). We performed quality control on the extracted RNA to guarantee the accuracy of the sequencing data, and the database experiment could only proceed with samples whose RNA detection outcomes

satisfied the database's sequencing specifications. Following sample qualification, the eukaryotic mRNA was randomly disrupted by fragmentation buffer after being enriched using magnetic beads and Oligo (dT). Six-base random hexamers were used to create the first-strand cDNA using the mRNA template. Second-strand cDNA was created after adding buffer, dNTPs, and DNA polymerase I. AMPure XP beads were then used to purify the double-stranded cDNA. Following end-repair, a tail was inserted, and sequencing joints were attached to the purified double-stranded cDNA. After selecting the fragment sizes using AMPure XP beads, the library creation was finished by performing real-time fluorescence quantitative PCR with Applied Protein Technology Ltd. (Shanghai, China). Transcriptome sequencing was carried out using an Illumina HiSeq™ 2500 system.

2.3. Transcriptome data analysis

The clean reads were chosen for the RNA-seq analysis after removing adaptor sequences, low-quality reads (average base mass value less than 20), and reads with more than 5% N bases (bases unknown). Using the default Pythonpackage-HTSeq parameters, gene expression was measured by calculating the fragments per kilobase of FPKM once all clean reads had been mapped to the IRGSP-1.0 reference genome. The DEGs were identified by analyzing the expression of each gene between the groups using the DESeq2 package in R 1.10.1, with FDR of less than 0.05 and a fold change of more than 2. Using OmicShare tools, all DEGs were subjected to GO and KEGG enrichment studies to assess annotations and pathway enrichments. We employed the p-value <0.05 to measure enrichment in KEGG pathways, and GO terms of DEGs with FDR <0.05 were significantly enriched [24]. Using REVIGO and a 0.7 criterion, redundant phrases were eliminated from the enriched GO terms [28].

2.4. Meta-QTLs mapping

To identify the initial quantitative trait loci (QTLs), we searched on the Web of Science (http://www.webofknowledge.com) for published research articles on cold tolerance in rice during the budding stage. We excluded articles from this list that discussed comparable treatments, such as the placement of 0.5–1 cm buds at a temperature of 4 °C for four days. In addition, we categorized these initial QTLs as QTLs identified through biparental genetic population QTL mapping and GWAS. The following essential data was gathered for each initial QTL: (1) the type of QTL mapping population (RILs, $F_{2:3}$, SSSLs, and Nature population); (2) the QTL characteristics (the number of QTLs included in articles, QTL id, and chromosome); (3) flanking or closely linked marker; and (4) the physical intervals where the molecular markers are located. However, some of these were not followed in the study, so we located them using the gramene database (http://www.gramene.org) to identify the sequence information. In addition, the initial QTLs' physical locations in IRGSP-1.0 were determined by the online blast tool (https://rapdb.dna.affrc.go.jp/tools/blast) on the RAP-DB website (https://rapdb.dna.affrc.go.jp/download/irgsp1.html). The sequences of the SSR markers must be a complete match. In this study, the physical intervals detected once, twice, or more were classified as non-repetitive QTL physical intervals based on their number of repetitions. The physical intervals detected three or more times were called Multiple-QTL (MQTL). Consequently, we gathered the candidate genes' gene ID, FPKM, function annotations, and supplementary data.

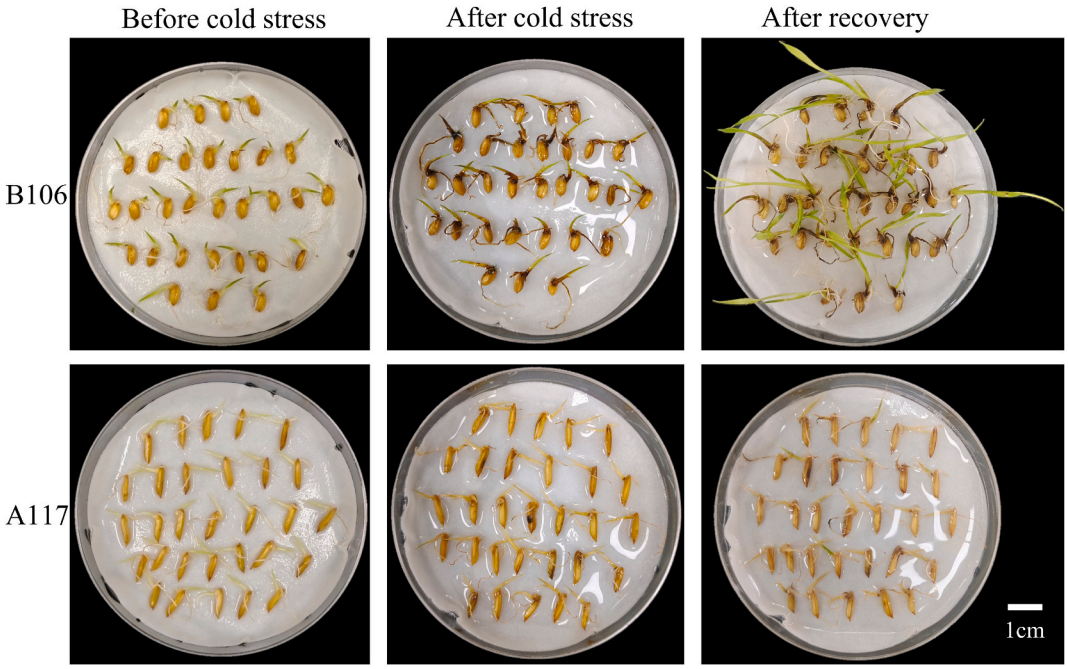

Fig. 1. Evaluation of cold tolerance of *japonica* rice B106 and *indica* rice A117 at budding stage.

2.5. Validation of candidate genes by real-time quantitative RT-PCR

The RNA samples were converted into complementary DNA (cDNA) using the high-capacity cDNA archive kit from Applied Biosystems, USA. The AceQ qPCR SYBR Green Master Mix Kit from Vazyme Biotech was utilized for qRT-PCR using standard protocols, and the StepOnePlus System (Applied Biosystems, USA) was employed to quantify the levels of gene expression. Three duplicates were employed for every treatment. The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the relative expression values, and Actin was used as an endogenous control to standardize the acquired Ct values. Gene-specific primers were created using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences for each of the ten potential genes are listed in Table S1.

3. Results

3.1. Quality assessment and differential expression analysis of transcriptome sequencing

The seeds were treated at a low temperature for 4 days before returning to room temperature. The survival rate (SR) of *indica* rice A117 and *japonica* rice B106 was examined after 7 days of recovery (Fig. 1). The results showed that the SR of *japonica* rice B106 was more than 97.13%, while that of *indica* rice A117 was 14.90%. The results indicated that the cold tolerance of *japonica* rice B106 was stronger than that of *indica* rice A117.

After all reads satisfied the filtering requirements, twelve samples produced 83.32 Gb clean bases (Table S2). The mapped read ratios for each sample ranged from 91.26% to 94.09% (Table S3). Furthermore, calculations were established for the GC content, Q20, Q30, clean bases, and quantity of clean reads. The Q30 and GC ranges, 92.87% and 54%, respectively, suggested that the sequencing data quality was adequate for additional transcriptome investigation (Table S2). Matrix files containing 33,966 gene expression profiles were generated from transcriptome sequencing of all tissues, including seeds and buds (Table S4). To assess each sample's quality, we performed principal component analysis (PCA) using the transcript per million mapped reads (FPKM) values for each gene. The results showed strong sample repeatability across all replicates and a reflection of genotype differences (Fig. S1a).

Fig. S1a shows that a higher percentage of PC_1 (58.82%) and PC_2 (26.11%) interpreted temperature differences than material differences. Furthermore, under control and cold treatments, there was a strong correlation between the three biological duplicates of the A117 and B106 samples, as demonstrated in Fig. S1b. In addition, each treatment's three biological replicates were categorized based on a Pearson correlation coefficient approaching 1.

Two materials subjected to cold and control treatments, respectively, underwent transcriptome analysis (Fig. 2). A117 had 15,265 genes under the two treatments, with 7,419 genes displaying up-regulation and 7,846 showing down-regulation. Under normal temperature treatment, 4,323 genes were found between B106 and A117 (1,481 upregulated and 2,842 down-regulated). B106 had 14,804 genes under the control and cold treatments, of which 7,345 were upregulated, and 7,459 were downregulated. When B106 and A117 were exposed to cold, a total of 7,868 genes were found, of which 3,667 showed up-regulation and 4,201 showed down-regulation (Fig. 2a). This explained why more differentially expressed genes were detected in both A117 and B106 under two treatments, demonstrating that temperature had a stronger impact on rice than rice type—*indica* or *japonica*. Interestingly, the results resemble principal component analysis (Fig. S1a).

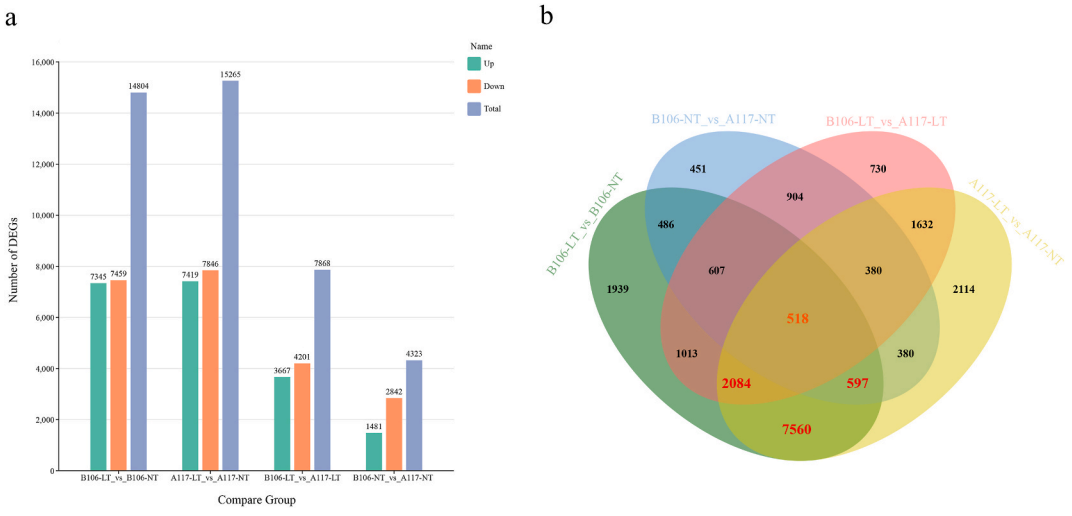

Fig. 2. Differential expression analysis of transcriptome. (a) The number of DEGs under control and cold stress in B106 and A117. Genes up- or down-regulated are shown by green and orange bars, respectively. Gene totals are shown by blue bars. (b) Venn diagram showing the DEGs unique to and shared by B106 and A117 under cold and control treatments. The red-colored number represents the core genes' reaction to cold. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

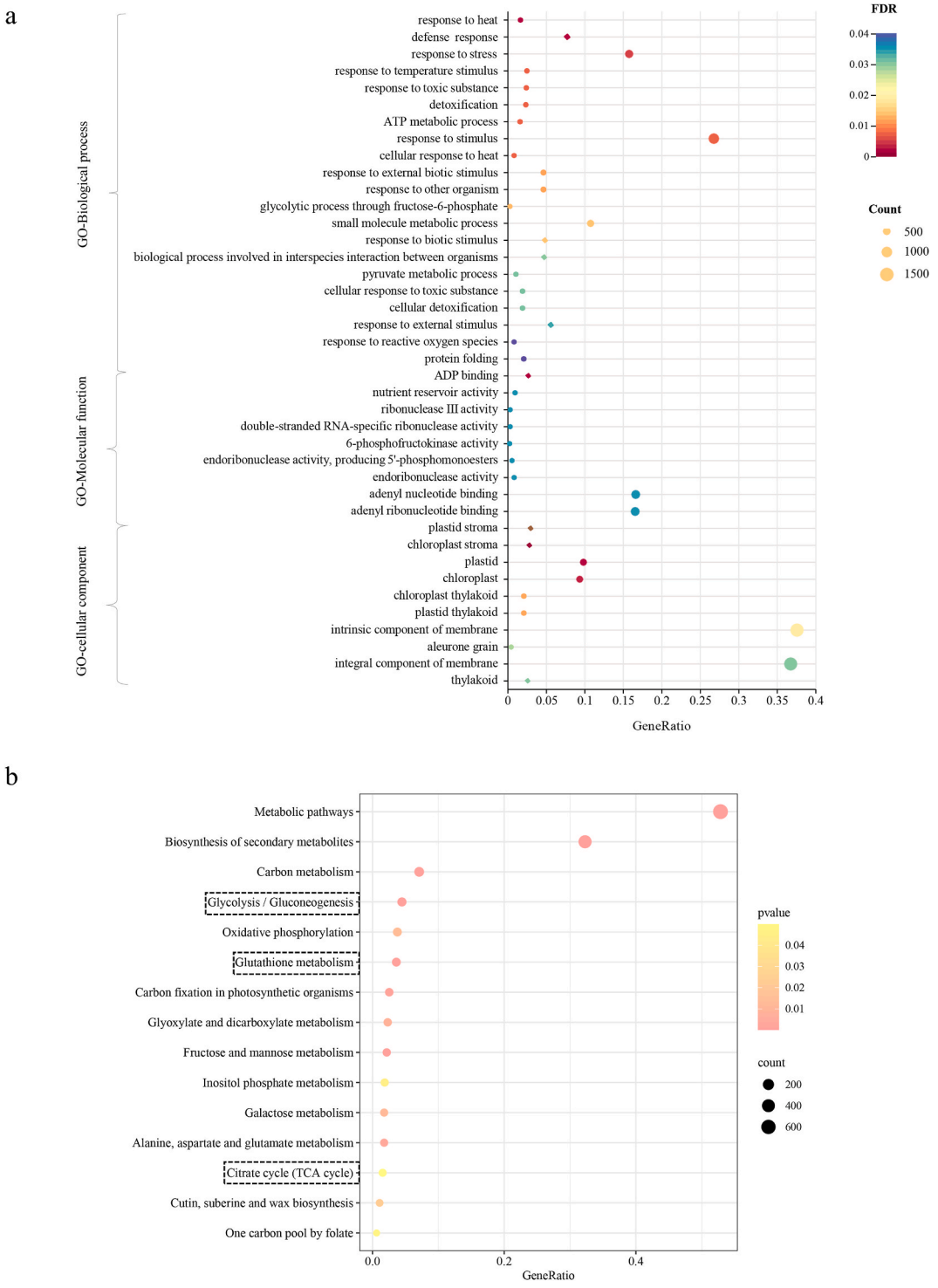

Fig. 3. GO and KEGG enrichment analyses for the DEGs between B106 and A117 under cold stress. (a) DEGs' GO enrichment. The circle's size, ranging from blue to red depicts the FDR from large to small, indicating the number of DEGs. (b) DEGs' KEGG enrichment. The circle size represents the number of DEGs, and the yellow to pink circles indicate the *p*-value from large to small. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Transcriptome difference analysis of two materials after cold treatment

GO and KEGG enrichment analyses were performed using 7,868 DEGs between the two materials under cold treatment to evaluate the functional and biological process categories of the DEGs in B106 and A117 under cold stress conditions (Fig. 3). The GO enrichment threshold was set at a false discovery rate (FDR) of less than 0.05, while the KEGG enrichment threshold was set at a *p* value less than 0.05. GO analysis revealed 40 pathways where the DEGs were significantly enriched in total (Fig. 3a). Within the biological process (BP) category, 21 items exhibiting significantly enrichment were identified. These terms included response to temperature stimulus, pyruvate metabolic process, fructose-6-phosphate-mediated glycolysis, and ATP metabolic process. These DEGs were primarily significantly involved in 9 items in the molecular function (MF) category, which included ADP binding, endoribonuclease activity, and

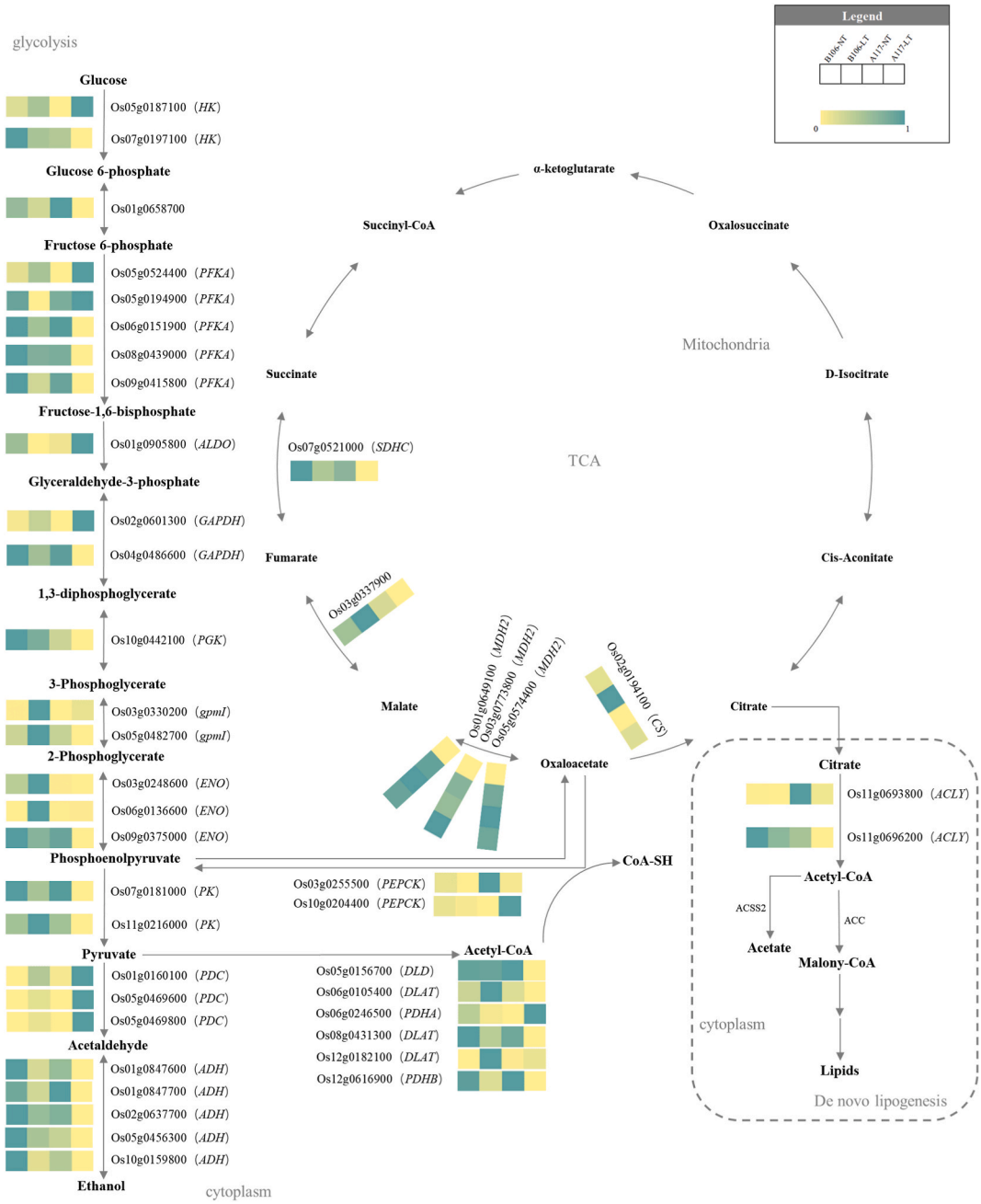

Fig. 4. Variations in DEG expression in the glycolysis pathway and TCA cycle were observed between B106 and A117. The four boxes in a row represent B106-NT, B106-LT, A117-LT and A117-NT, and the color from yellow to green indicates that the FPKM is from small to large. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

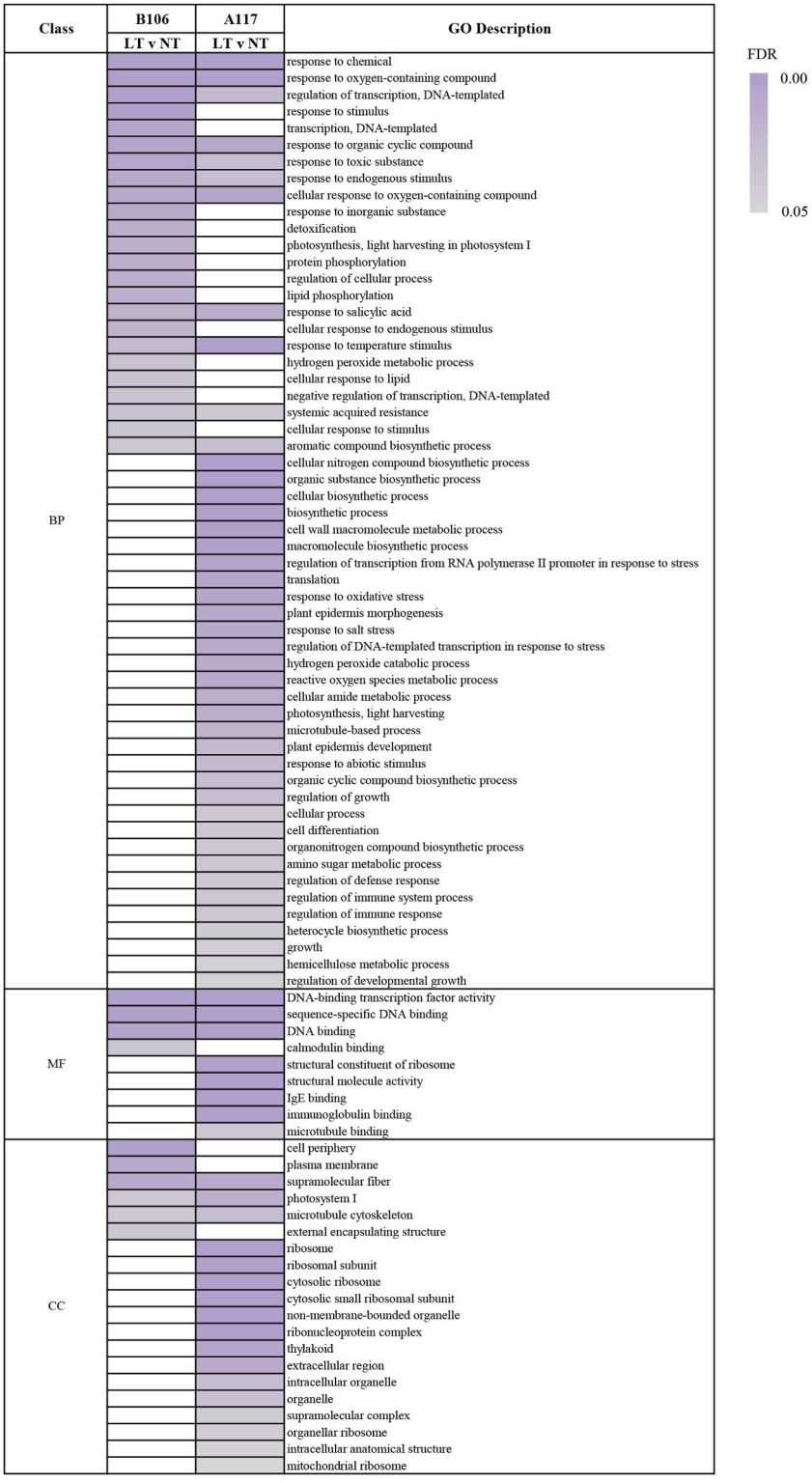

Fig. 5. Heatmap showing the common and unique GO classifications for B106 and A117 under cold and control treatments. The color from purple to grey indicates that the FDR is from small to large. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

6-phosphofructokinase activity. In the cellular component (CC) category, the DEG enrichment was evident in 10 processes, including plastid stroma, chloroplast stroma, and chloroplast thylakoids. The TCA cycle, glycolysis/gluconeogenesis, glutathione metabolism, oxidative phosphorylation, glyoxylate and dicarboxylate metabolism, carbon fixation in photosynthetic organisms, and glyoxylate and dicarboxylate metabolism were the main pathways related to the differences in cold stress tolerance between *indica* and *japonica* rice, according to KEGG enrichment analyses (Fig. 3b).

According to findings from the KEGG and GO enrichment investigations, the TCA cycle and glycolysis pathway may be important in controlling cold tolerance in the B106 and A117 cultivars. As a result, a comprehensive analysis was conducted to assess the DEG expression patterns associated with the TCA cycle and glycolysis pathway (Fig. 4). Most DEG-encoded key enzymes involved in the aerobic oxidation process' production of glucose to pyruvate were strongly expressed in B106 as opposed to A117. These genes included hexokinase (*HK*: Os07g0197100), phosphofructokinase (*PFKA*: Os05g0194900, Os06g0151900, Os09g0415800), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*: Os04g0486600), enolase (*ENO*: Os03g0248600, Os06g0136600, Os09g0375000), and pyruvate kinase (*PK*: Os07g0181000, Os11g0216000). The pyruvate dehydrogenase complex catalyzes the conversion of the product pyruvate into acetyl-CoA, which initiates the TCA cycle. Pyruvate dehydrogenase complex DEGs (*PDHB*: Os12g0616900, *DLAT*: Os06g0105400, Os08g0431300, Os12g0182100, *DLD*: Os05g0156700) had greater expression levels in B106 compared to A117. Most DEGs involved in the TCA cycle, the second stage of aerobic oxidation, were expressed at a higher level in B106 than in A117. These DEGs primarily included the malic dehydrogenase genes (*MDH2*: Os01g0649100, Os03g0773800, Os05g0574400), fumarase gene (Os03g0337900), citrate synthase gene (*CS*: Os02g0194100), and succinodehydrogenase gene (*SDHC*: Os07g0521000). Importantly, the enzymes encoded by the majority of the aforementioned genes are involved in the crucial energy production steps of the TCA cycle, and B106 showed greater expression levels of all the enzymes encoded by DEGs than A117 did. Therefore, we presume that B106 obtains its energy mostly from aerobic respiration and that this process is more active in B106 than in A117. It could lead to B106's higher survival rate than A117.

This theory is reinforced by the finding that acetyl-CoA combines with oxaloacetic acid in mitochondria to produce citric acid, which is then transported to the cellular solution by a tricarboxylic acid carrier in the inner membrane. While oxaloacetic acid is reduced to form malic acid under the catalysis of malate dehydrogenase, citrate lyase uses ATP to break down citric acid back into oxaloacetic acid and acetyl-CoA, which can be used for the synthesis of fatty acids. Malidase then catalyzes the transformation of malic acid into pyruvate. The intimal carrier transports the pyruvate back to the mitochondria, where pyruvate carboxylase catalyzes its regeneration into oxaloacetic acid, completing the replenishment process. This allows the pyruvate to be used again in the TCA cycle. Based on our expression profile, B106 exhibited more expression of the citrate lyase gene (*ACLY*: Os11g0696200) than A117.

The phosphoenolpyruvate carboxylase (PEPC) in plant cells catalyzes the conversion of phosphoenolpyruvate to oxaloacetic acid. Conversely, phosphoenolpyruvate carboxykinase (PEPCK) regenerates phosphoenolpyruvate when exposed to oxaloacetic acid, and then glycolysis proceeds to generate pyruvate for anaerobic respiration. According to our expression profile, two *PEPCK* genes (Os03g0255500, Os10g0204400) had significantly higher expression levels in A117 than in B106. These findings indicate that A117 is more active than B106 during anaerobic respiration. Additionally, glycolysis yields far less energy than the TCA cycle and generates harmful substances such as alcohol and acetaldehyde, leading to accelerated plant mortality. Pyruvate is primarily utilized in the production of acetaldehyde, facilitated by the action of pyruvate decarboxylase (PDC) as a catalyst. As predicted, A117 had significantly higher expression of the three *PDC* genes (Os01g0160100, Os05g0469600, and Os05g0469800) than B106 (Fig. 4). Overall, A117 exhibits greater activity than B106 in the context of anaerobic respiration. These factors may explain why A117's survival rate is poorer than that of B106.

3.3. Cold response transcriptome analyses in two rice cultivars

To develop a deeper understanding of the mechanism that underlies the cold tolerance of rice during the bud stage. After subjecting

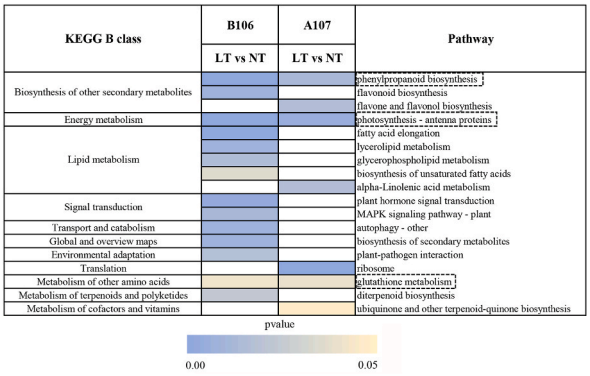

Fig. 6. Heatmap of KEGG pathways unique to and shared by B106 and A117 under cold and control treatments. The color from blue to yellow indicates that the *p* value is from small to large. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

A117 and B106 to low temperature treatment, we conducted gene enrichment analysis on the DEGs. During the GO enrichment analysis, employing a FDR threshold of less than 0.05, a combined total of 14,804 DEGs in B106 and 15,265 DEGs in A117 were categorized into three primary groups. Following the use of REVERGO for condensation, 85 enriched terms were identified (Fig. 5). Ten terms, such as oxygen-containing compound, response to toxic substance, response to temperature stimulus, etc.—that were shared by B106 and A117 under two treatments in the BP aspect. In addition, 32 terms were exclusive to A117, and 13 items were exclusive to B106. In the aspect of MF, B106, and A117 shared three pathways: sequence-specific DNA binding, DNA binding, and DNA-binding transcription factor activity. The single pathway unique to B106 was calmodulin binding, while A117 had five distinct items. Meanwhile, photosystem I, supramolecular fiber, and microtubule cytoskeleton were prevalent CC pathways. B106 had three unique pathways, whereas A117 contained 14 separate pathways.

We performed KEGG enrichment analyses for two gene sets with a p value < 0.05. According to the KEGG analysis, B106 DEGs (LT v NT) were most significantly involved in 14 pathways. In comparison, the DEGs (LT v NT) of A117 were most significantly engaged in 7 pathways (Fig. 6). The 3 pathways that both materials shared were glutathione metabolism, phenylpropanoid biosynthesis, and photosynthesis-antenna proteins.

3.4. GO and KEGG enrichment analysis of core genes in response to cold

The varieties B106 and A117 belong to two subspecies. As a result, these two materials should exhibit distinct responses to cold. The intersection of the 15,265 DEGs in A117 under control and cold treatments with the 14,804 DEGs in B106 under control and cold treatments resulted in a total of 10,759 DEGs (Fig. 2b). These overlapping DEGs, in our opinion, are the "Core DEGs" required for rice seed buds to be able to withstand cold stress.

To better understand the functions and processes by which core DEGs respond to cold during the budding stage of rice growth. Afterwards, the 10,759 genes were then subjected to KEGG (p value < 0.05) and GO (FDR <0.05) enrichment analyses. After removing redundant terms using REVIGO, 60 terms were enriched in GO (Table S5). The terms encompassed various metabolic processes such as amino acids, sugars, lipids, light harvesting, photosynthesis, hydrogen peroxide catabolism, reactive oxygen species metabolism, reaction to oxygen-containing compounds.

Table S6 presents the enrichment of ten terms identified by the KEGG pathways. These terms primarily comprised of biosynthesis of diterpenoid, fatty acid elongation, photosynthesis-antenna proteins, glutathione metabolism, phenylpropanoid biosynthesis, and biosynthesis of unsaturated fatty acids. Surprisingly, this result aligns with the discoveries made in transcriptome studies of the cold response in two varieties of rice (Fig. 6). These studies show that the regulation mechanism of cold tolerance in rice seeds during the budding stage involves the formation of phenylpropanoid chemicals and photosynthetic-antenna protein, specifically glutathione metabolism. A thorough profiling was conducted to evaluate the expression patterns of DEGs implicated in the glutathione metabolism pathway (Fig. 7).

Ascorbate peroxidase (APX) is an enzyme involved in the glutathione metabolism pathway that scavenges H_2O_2 and senses changes in the redox status of plant cells [29]. Five *APX* genes were found in our expression profile; among the five, one gene (Os03g0285700) expressed itself relatively abundantly under cold stress. Specifically, we discovered that two genes encoding glutamate-cysteine ligase (*GSHA*) (Os05g0129000, Os07g0462000) and two genes encoding glutathione reductase (*GSR*) (Os02g0813500, Os10g0415300) were both highly expressed under cold treatment. This occurrence resulted in the heightened synthesis of GSH, particularly the elevated proportion of reduced to oxidized states (GSH/GSSG), thereby enhancing REDOX homeostasis. Two crucial enzymes, GPX and APX, catalyze the reduction of H_2O_2 to prevent potential cellular harm induced by H_2O_2. The overexpression profile results revealed that two glutathione peroxidases (*GPX*) genes (Os04g0556300, Os04g0683850) exhibited significantly higher expression levels when subjected to cold treatment. A substantial number of glutathione S-transferases (*GST*) genes (Os01g0369700, Os01g0692000,

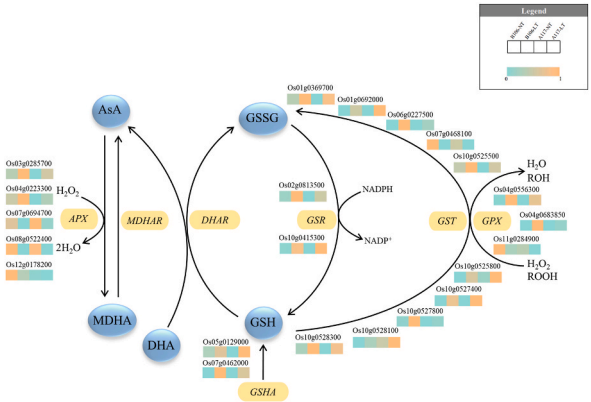

Fig. 7. Variations in DEGs expression in the glutathione metabolism pathway between B106 and A117 under control and cold treatments. The four boxes in a row represent B106-NT, B106-LT, A117-LT and A117-NT. The color from blue-green to orange indicates that the FPKM is from small to large. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Os06g0227500, Os10g0525500, Os10g0527800, and Os10g0528300) exhibited high expression levels at low temperature in B106 and A117, providing further evidence for this finding. These findings suggested that rice's ROS-scavenging mechanism is more active when low temperatures stress the plant.

Furthermore, during cold stress, most of the expression profiles of the genes *GPX*, *APX*, *GSHA*, *GST*, and *GSR* were higher in B106 than in A117. This showed that B106, which is cold-resistant, has a greater capacity for scavenging ROS than A117, which can lessen damage in cold temperatures.

3.5. Transcriptome combined with meta-QTLs analysis to mine key cold response genes

To find more potential genes that respond to cold stress during the bud stage of rice, we carefully chose 13 relevant studies that primarily published mostly from 2005 to 2023—and used a similar cold treatment to induce initial QTL acquisition in buds measuring 0.5–1 cm in length at a temperature of 4 °C for four days. A comprehensive collection of 108 QTLs associated with cold resistance during budding was compiled from 13 published investigations on QTLs. These QTLs indirectly contribute to the development of cold tolerance. Table S7 lists the fundamental details of these QTLs, such as the mapping population type (RILs, $F_{2:3}$, SSSLs, and Nature population), the QTLs' characteristics (number of QTLs included in articles, QTL id, and chromosome), flanking or closely linked marker, and the physical intervals where the molecular markers are located. Out of the 108 original QTLs, 53 were obtained via biparental genetic population QTL mapping and 55 by GWAS (Fig. 8a). Specific QTL counts are as follows: chromosome 3 has the most, followed by chromosome 1, and chromosome 11 has the least (Fig. 8b; Fig. 9).

Following manual screening, the physical intervals containing these initial QTLs were divided into physical intervals detected once, twice, third, and fourth based on chromosomal repeat times. These physical intervals were identified as non-repetitive QTL physical intervals; Fig. 8c and Table S8 show that 88 physical intervals were detected once, 31 times, eight times, and only once in the fourth period. Multiple-QTL (MQTL) refers to the physical intervals equal to or greater than three times (Table 1; Table S8). On several chromosomes, the nine MQTLs were irregularly distributed (Fig. 9; Table 1). Chromosome 3 included a total of four MQTLs. Among these, three MQTLs (MQTL3, 4, 6) were detected three times, while one MQTL (MQTL5) was detected four times. Furthermore, two MQTLs were dispersed on chromosome 1 and two MQTLs on chromosome 6. Interestingly, the MQTLs' distribution on chromosomes was mostly in agreement with the initial QTL distribution, indicating the stability and dependability of MQTLs for confirming the presence of the important cold response genes.

By aligning the MQTL overlapping interval with the reference genome, 224 potential genes were discovered (Table S9). This helps narrow down the candidate gene pool that responds to cold stress. To further narrow down the range of candidate genes that react to cold stress, it is intended to intersect the genes inside the MQTL intervals with the 10,759 core cold-responsive genes identified through transcriptome analysis. Ultimately, the initial set of 10,759 core DEGs was narrowed down to a final selection of 75 genes as shown in Fig. S2. Among the two materials, the majority of these 75 genes exhibited increased expression at low temperature compared to normal temperature. Furthermore, after cold treatment, B106's expression levels of these 75 genes were generally much greater than A117's. Using the gene annotation, we identified four of the most plausible candidates to help rice develop its cold tolerance. These included an *ATPase* (Os03g0802500), a *glutamate-cysteine ligase* (Os07g0462000), a *citrate synthase* (Os02g0194100), and an *OsECS* (Os05g0129000) *glutamate-cysteine ligase*. Next, we confirmed that these four genes were expressed using qRT-PCR (Fig. 10). The qRT-PCR and RNA-seq data agreed, indicating the latter's dependability.

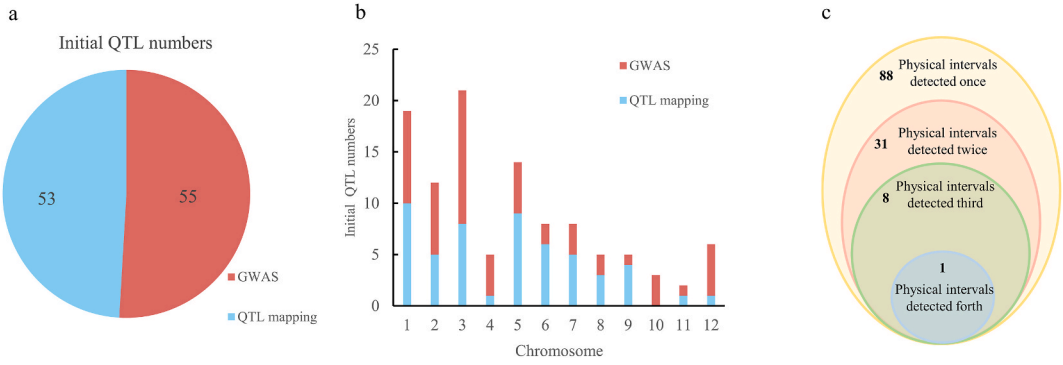

Fig. 8. The initial cold tolerance QTL information from earlier QTL mapping investigations was utilized in the meta-QTL study of rice budding. (a) The percentage of initial bud cold tolerance QTLs classified by biparental genetic population QTL mapping and GWAS. (b) The chromosomal distribution of the initial QTLs. (**c**) QTL physical intervals that are non-repetitive.

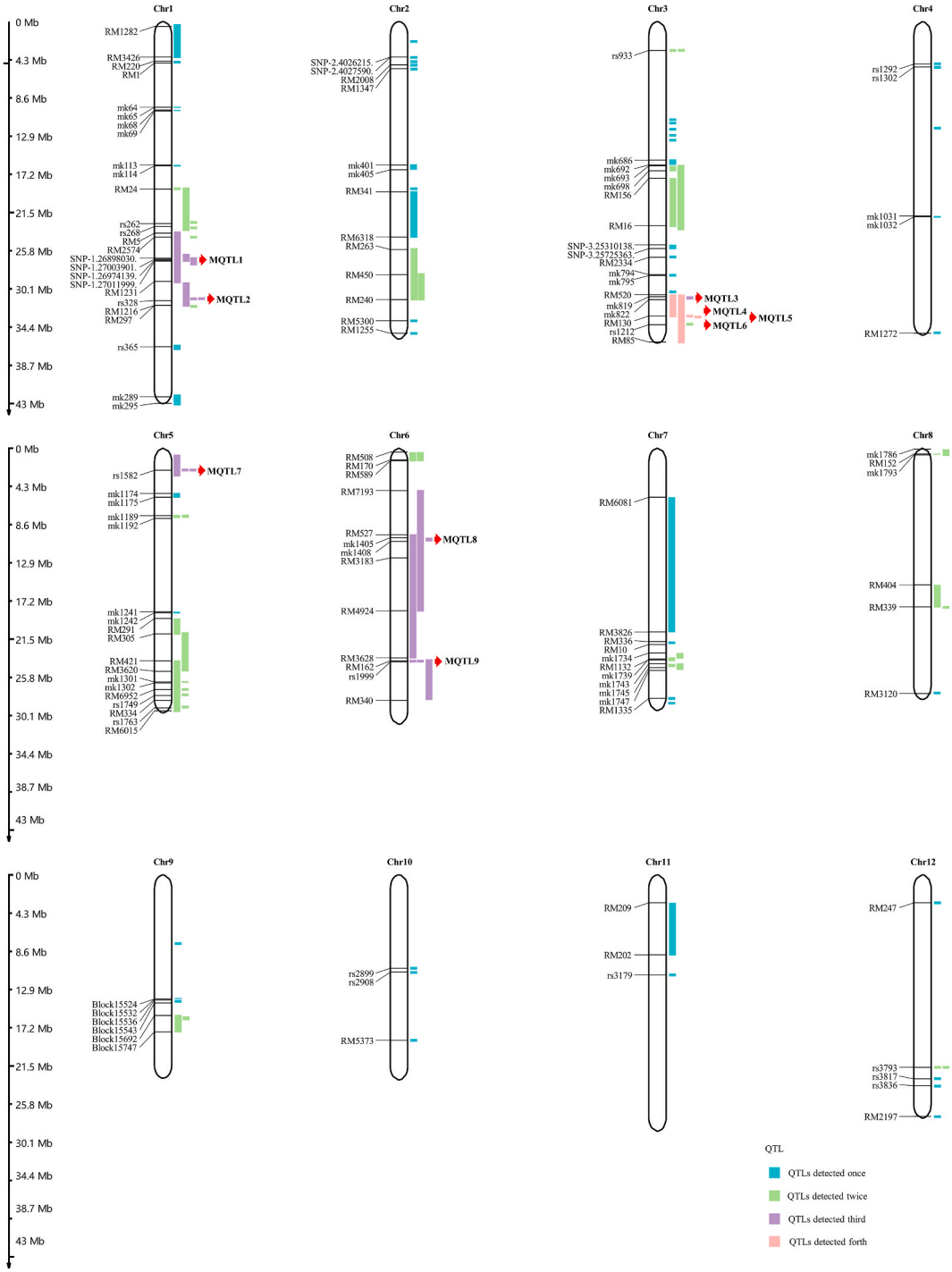

Fig. 9. Nine MQTLs and 108 original QTLs are distributed across chromosomes. Different colored squares are used to indicate different non-repetitive QTL physical intervals. The left axis represents the physical distance (Mb).

4. Discussion

4.1. Glutathione metabolism, glycolysis pathway and TCA cycle may be involved in mediating cold tolerance of the two materials at budding stage

In this study, under control and cold treatments, transcriptome sequencing analysis was carried out on cold-sensitive *indica* rice

Table 1
Large-scale integration of meta-QTL reveals the overlapping intervals and candidate genes for cold tolerance during rice budding.

MQTL	Chr.	Overlapping intervals	Initial QTLs	No. of candidate genes in the interval
MQTL1	1	26,904,946–26,999,075	*qSCT-1-1*, *qCTB-1-1*, *qCTB-1-2*	12
MQTL2	1	31,509,584–31,709,584	*qSCT-1-2*, *qCTSR1-2*, *qCTSD1-2*	34
MQTL3	3	31,150,000–31,450,000	*qGS3.4*, *qSCT-3*, *qSCT-3(t)*	50
MQTL4	3	33,293,569–33,344,741	*qSCT-3*, *qLTSSvR3-4*, *qSCT-3(t)*	13
MQTL5	3	33,344,741–33,393,548	*qSR3-7*, *qLTSSvR3-4*, *qSCT-3(t)*, *qSCT-3*	12
MQTL6	3	33,393,548–33,493,548	*qSR3-7*, *qLTSSvR3-4*, *qSCT-3(t)*	19
MQTL7	5	2,398,170–2,598,170	*qCTSR5-1*, *qCTSD5-1*, *qCTBB5*	28
MQTL8	6	10,150,000–10,550,000	*qSR-6*, *qSCT-6*, *qSCT-6(t)*	21
MQTL9	6	24,146,421–24,346,421	*qCTSR6-1*, *qCTSD6-1*, *qCST6*	35

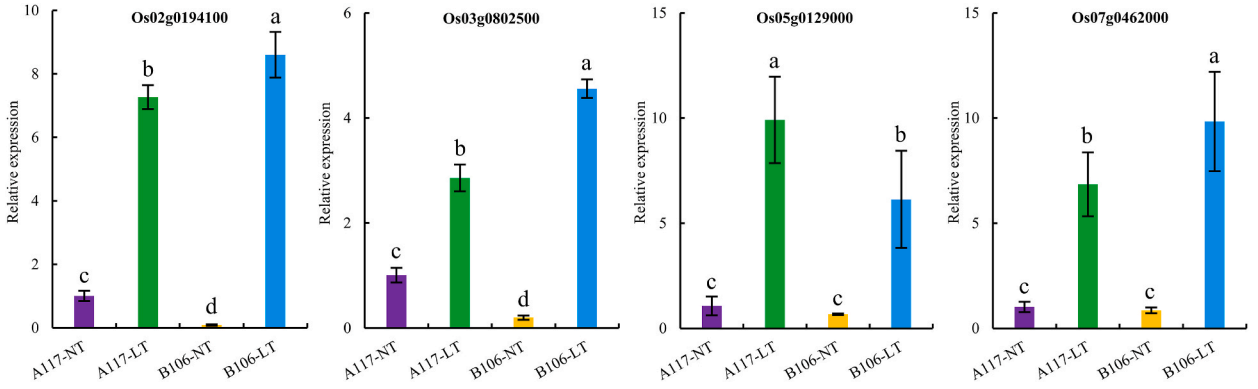

Fig. 10. Relative expression levels of the four most plausible candidate genes. Different letters indicate individual groups for multiple comparisons with significant differences (one-way ANOVA, Duncan, $p < 0.05$).

A117 and very cold-tolerant *japonica* rice B106. Following that, we performed gene enrichment analysis on the following four gene sets: ①B106-LT_vs_A117-LT, ②A117-NT_vs_A117-LT, ③B106-NT_vs_B106-LT, and ④Core DEGs (A117-NT_vs_A117-LT ∩ B106-NT_vs_B106-LT). Surprisingly, the glutathione metabolic pathway showed substantial enrichment in all four gene sets. That is to say, the differential in cold tolerance between *indica* rice A117 and *japonica* rice B106 during the budding stage is related to the glutathione route. Furthermore, it shares a close relationship with cold stress. It is commonly known that the AsA-GSH pathway is crucial for controlling reactive oxygen species (ROS) within the plant antioxidant system [30]. However, ROS are a consequence of metabolism and can induce oxidative damage and interfere with normal cell activity, especially in unfavorable environmental conditions [29]. Thus, we hypothesized that B106 and A117's varying cold tolerance during the budding stage was due to their ability to scavenge ROS directly or indirectly. The expression patterns of the AsA-GSH pathway genes were thoroughly examined (Fig. 7). Two *GSHA* genes (Os05g0129000 and Os07g0462000), which are involved in GSH production, showed increased expression in response to cold stress, and the expression of *japonica* rice B106 was higher than that of *indica* rice A117 under low temperature stress, which markedly raised reduced glutathione levels. Additionally, GSR keeps the correct GSH/GSSG ratio, which is required for H_2O_2 detoxification [31]. In this case, two *GSR* genes (Os02g0813500 and Os10g0415300) exhibited strong expression at low temperatures and had greater expression in B106 than in A117. Therefore, the expression of *GSHA* and *GSR* genes indicated that the ability of *japonica* rice B106 to convert oxidized glutathione (GSSG) to reduced glutathione (GSH) was stronger than that of *indica* rice A117, which resulted in enhanced REDOX homeostasis. In addition, the GPXs utilize reduced glutathione (GSH) and TRXs to scavenge H_2O_2, reducing lipid peroxides and organic hydroperoxides [32]. The overexpression of GST decreased the accumulation of H_2O_2 and malondialdehyde (MDA) produced by oxidative damage [33]. In our study, the majority of the *GST* and *GPX* genes were also stimulated by low temperatures, leading to a marked rise in their expression levels. Upon closer examination of the gene expression in the two materials subjected to cold stress, we were astonished to discover that three *GPX* genes (Os04g0556300, Os04g0683850, Os11g0284900) and a substantial number of *GST* genes (Os01g0369700, Os01g0692000, Os06g0227500, Os07g0468100, Os10g0525500, Os10g0527800, and Os10g0528300) exhibited higher expression levels in B106 compared to A117. Meanwhile, we observed increased expression of the *japonica* rice B106 compared to the *indica* rice A117 for most of the *APX* genes (Os03g0285700, Os04g0223300, Os12g0178200) we collected. Based on the results, B106 demonstrated superior ability in scavenging ROS, which is consistent with the findings of the gene enrichment analysis. According to Wei et al. [34], H_2O_2 in chloroplasts mainly depends on the clearance of APX, GPX, and GST in the AsA-GSH cycle in tobacco. Additionally, the contents of GPX, GST, and GSH in cold-tolerant chickpeas increased significantly on the 6th day, while those in cold-sensitive chickpeas did the opposite [35].

According to Koç et al. [36], cold stress in *Arabidopsis* first caused the mobilization of energy from glycolysis and ethanol breakdown to increase TCA cycle activity via acetyl-CoA. Furthermore, it was discovered that the TCA cycle and glycolysis pathways contributed to the cold tolerance of pepper [37], *Xanthoceras sorbifolia* [38], and *P. pratensis* [39]. As a common step of aerobic and

anaerobic respiration in plants, the glycolysis pathway provides acetyl-CoA for the TCA cycle. The TCA cycle is the most efficient way for plants to oxidize sugar or other substances for energy. This study focused on analyzing the genes expressed differently in B106 and A117 under cold stress conditions, emphasizing genes involved in the TCA cycle and glycolysis. The genes *HK*, *PFKA*, *DLD*, *DLAT*, *PDHB*, *CS*, and *MDH2* were identified as DEGs with higher expression levels in the cold-tolerant variety B106 when exposed to cold stress, in comparison to the cold-sensitive variety A117 (Fig. 4). These genes are important in the energy generation process. This suggested that the TCA cycle and glycolysis may strengthen rice budding's capacity for cold tolerance.

4.2. Transcriptome combined with meta-QTLs mapping can effectively lock candidate genes related to cold stress tolerance in rice

According to Lv et al. [40], transcriptomics is a useful technique for examining the cold tolerance mechanism in rice. Nonetheless, the majority of research focuses mostly on the following stages: germination [41], booting [42], and seedling [22,24,43], while relatively few reports on the budding stage. In this instance, transcriptome sequencing was performed on B106 and A117 during the budding stage following four days of 4 °C treatment. The results of GO and KEGG enrichment analysis suggest that the cold resistance of B106 at the bud stage is strongly associated with the TCA cycle, glycolysis/gluconeogenesis, oxidative phosphorylation, glutathione metabolism, and other KEGG pathways. Additionally, it is linked to the response to temperature stimulus, response to reactive oxygen species, ADP binding, ATP metabolic process, and other GO items (Fig. 3). On the other hand, gene enrichment analysis was conducted utilizing 10,759 core genes in response to cold. For example, cold stress significantly increased the breakdown of hydrogen peroxide, the metabolic activity of reactive oxygen species (ROS), and photosynthesis. Furthermore, we discovered significantly enhanced KEGG pathways associated with glutathione metabolism, photosynthesis-antenna proteins, and the production of phenylpropanoid compounds. Furthermore, following a low temperature treatment, the majority of the genes in these pathways exhibit substantial upregulation (Fig. 4; Fig. 7). Reactive oxygen species (ROS) can be effectively removed, and energy metabolism can be increased by low-temperature resistant materials, which helps them tolerate stress caused by low temperatures.

The cloning of cold-tolerant genes is an efficient and essential method for producing cold-tolerant cultivars and deciphering the genetic control mechanism of cold tolerance at the budding stage. Many rice bud stage QTLs that are cold tolerant have been found in recent years. For instance, based on the bud stage and low temperature treatment settings, we gathered 108 QTLs from 13 papers associated with cold tolerance in the rice budding stage. However, out of the 108 QTLs, only three genes—*LTT7*, *OsRab11C1*, and *LTG5*—have been cloned, and their functions have been established [11–13]. In other words, these published results can provide us with more valuable information. QTL meta-analysis is a method for identifying precise and robust QTL [44]. For example, using meta-QTLs analysis, Yang et al. [21] successfully lowered the physical distances of MCqtl1-4 and MCqtl6-3 to 1.0 and 2.7 Mb, respectively. In a similar vein, Kong et al. [45] discovered that following DEGs meta-QTL mapping, two previously cloned genes, *SNAC2* and *OsSPX1*, were highlighted in MCqtl1-4 and MCqtl6-3. In this study, we decreased the 224 MQTL gene candidates to 75 by obtaining 9 stable and reliable MQTLs from the 108 initial QTLs. We then paired these MQTLs with gene expression patterns to identify 4 most likely candidate genes by gene annotation and qRT-PCR. It significantly raises our candidate gene identification efficiency.

4.3. Four candidate genes in MQTLs involved in ROS scavenging and ATP production potentially regulate cold tolerance during rice budding

According to Han et al. [24], cold stress results in various cellular dysfunctions, including membrane damage, the generation of reactive oxygen species (ROS), protein denaturation, and the buildup of toxic chemicals. Given that it can either directly or indirectly scavenge ROS, GSH is one of the most significant cellular antioxidants [46]. The two main enzymes of the GSH biosynthesis pathway in plants are glutathione synthetase (GS) and γ-glutamylcysteine synthetase (γ-ECS) [47]. Surprisingly, two of our four candidate genes (*OsECS*: Os05g0129000 and Os07g0462000) encode the first rate-limiting enzyme in GSH production, γ-glutamylcysteine synthetase. Furthermore, transgenic rice that overexpresses *OsECS* improves redox homeostasis through an increased GSH pool, increasing tolerance and germination rate in the face of abiotic stress [48]. In the natural environment, overexpression of *OsECS* can increase rice yield [48]. Furthermore, their expression levels dramatically increased at low temperatures, as shown by our expression profile results (Fig. 7).

For plants to tolerate abiotic stress, they must maintain energy homeostasis. This leads to an energy deficit that impedes the plant's ability to grow and develop [49]. Based on the gene enrichment results and comprehensive expression profile analysis of this study, it was shown that *japonica* rice B106 had higher active aerobic respiration (Fig. 3; Fig. 4). Os03g0802500 and Os02g0194100, the other two potential genes found, are strongly associated with energy metabolism. Os03g0802500 encodes ATPase among them. The final gene, *CS* (Os02g0194100), was found in the TCA cycle and encodes the citrate synthase, which is essential to energy metabolism (Fig. 4). In summary, these genes provide a foundation for understanding the regulatory mechanism of cold tolerance during the budding stage of rice.

5. Conclusions

The current study investigated the differences in cold tolerance between cold-tolerant *japonica* rice B106 and cold-sensitive *indica* rice A117 during the budding stage under both normal temperature and cold stress. The impact of cold stress on rice was greater than that of rice itself, regardless of whether it was *japonica* or *indica*, as indicated by RNA-seq data. Transcriptome sequencing also showed that the TCA cycle, the glycolysis pathway, and glutathione metabolism may mediate the two materials' cold tolerance during the budding stage. We performed meta-QTLs analysis on the cold resistance of rice during budding and obtained 224 genes from 9 MQTLs. By combining cold-tolerant meta-QTL mapping with RNA-seq, 75 potential DEGs were identified. Finally, the four most probable

candidate genes were identified through qRT-PCR, providing valuable target genes for the future propagation of cold stress-resistant rice while the rice is still in the process of budding.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

CRediT authorship contribution statement

Nan Li: Writing – original draft, Visualization, Software. **Jiahao Miao:** Validation, Investigation. **Yichao Li:** Validation. **Faru Ji:** Formal analysis, Data curation. **Min Yang:** Software, Investigation. **Kunyan Dai:** Investigation, Formal analysis. **Zixian Zhou:** Software, Investigation. **Die Hu:** Investigation. **Haiyang Guo:** Visualization. **Hong Fang:** Resources, Data curation. **Hongyang Wang:** Writing – review & editing, Methodology, Formal analysis. **Maohui Wang:** Resources, Investigation. **Jing Yang:** Writing – review & editing, Formal analysis, Data curation, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37933.

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