An association between the nitrogen metabolism pathway and cold tolerance in rice was identified using comparative transcriptome and proteome profiling

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Abstract

Background

Rice (*Oryza sativa* L.), one of the most important crops cultivated in both tropical and temperate regions, has a high sensitivity to cold stress. Chilling stress limits the N uptake and nitrogen metabolism in rice. To identify the genes and pathways involved in cold tolerance, and specifically associations with the nitrogen metabolism pathway, we have compared the gene and protein expression changes between a cold-tolerant cultivar, Dongnong428 (DN), and a cold-sensitive cultivar, Songjing10(SJ).

Results

Using absolute quantification (iTRAQ) with high-throughput mRNA sequencing (RNA-seq) techniques, we identified 5,549 genes and 450 proteins in DN and 6,145 genes and 790 proteins in SJ, that were differentially expressed during low-water temperature (T_w) treatment. There were 354 transcription factor (TF) genes (212 down, 142 up), 366 TF genes (220 down, 146 up), including 47 gene families, differentially expressed in the DN under control (CKDN) vs. DN under low-T_w (D15DN) and CKSJ vs. D15SJ, respectively. These results indicated that TF genes play a major role in post-translational regulations. Genes related to rice cold-related biosynthesis pathways, particularly the MAPK signaling pathway, zeatin biosynthesis, and plant hormone signal transduction pathways, were significantly differentially expressed in both rice cultivars. Differentially expressed proteins (DEPs) related to rice cold-related biosynthesis pathways and particularly glutathione metabolism were significantly differentially expressed in both rice cultivars. Transcriptome and proteome analysis of the nitrogen metabolism pathways showed that major genes and proteins were down-regulated that participated in γ-aminobutyric acid (GABA) and glutamine synthesis.
Conclusion

Under cold stress conditions during reproductive growth, genes and proteins related to the biosynthesis pathways of cold stress, were significantly differentially expressed in the DN and SJ. The present study confirmed the known cold stress-associated genes and identified a number of putative new cold-responsive genes. It has also revealed that translational regulation under cold stress plays an important role in cold-tolerant DN. Low-Tᵢ treatments affect the N uptake and N metabolism in rice, and promote Glu metabolism, and the synthesis of ornithine and proline in cold-sensitive SJ.

Background

Rice (Oryza sativa L.) is one of the most important crops cultivated in both tropical and temperate regions and is characterized by a high sensitivity to cold stress. Cold temperatures cause huge agricultural losses and are one of the most important environmental factors that affect rice growth and development (Ding et al, 2019). Chilling stress (0-20°C) is the primary cold stress in tropical and subtropical climatic zones (Xu et al, 2008, Suh et al, 2010), especially in northeastern China, in parts of northern Japan (Shimono et al, 2007b), and Australia (Farrell et al, 2006), and it influences the yield and quality of important food crops, such as rice (Shimono et al, 2002, Suzuki et al, 2008, Thakur et al, 2010, Wang et al, 2013, Zhao et al, 2013). Several previous studies have shown that the reproductive stage is the most sensitive period for cold damage in rice production (Jia et al, 2015, Wang et al, 2013, Sipaseuth et al, 2007, Jacobs and Pearson, 1999, Shimono et al, 2007a). Low root zone temperatures inhibit the occurrence and elongation of functional roots, which also affects rice growth (Ahamed et al, 2012, Kramer and Boyer, 1995). However, there is still no clear understanding of the response mechanisms of rice roots to low temperature stress during the reproductive stage.
The key challenge for crops is to maintain an adequate nutrition supply under abiotic stress conditions (Mcallister et al, 2012). Previous study has demonstrated that there are many metabolic changes that occur in Arabidopsis to enhance freezing tolerance, such as those involving the Calvin cycle, nitrogen metabolism, starch synthesis, and sugar synthesis (Stitt and Hurry, 2002). Nitrogen (N) plays an integral role in plant growth and development (Kraiser et al, 2011, Mcallister et al, 2012). Previously, the inhibition of growth at decreased temperatures of the desA and desB mutants of Synechocystis was found to correlate with the inhibition of their nitrate uptake (Sakamoto and Bryant, 1999), and was relieved by application of urea (Sakamoto and Bryant, 1999). Chilling stress has been found to generally limit the N uptake in rice (Zia et al, 1994, Shimazaki et al, 1963, Lu et al, 2005). Water temperature, not air temperature, is the main environmental factor regulating the growth and N uptake of rice (MATSUSHIMA et al, 1964a, MATSUSHIMA et al, 1964b). Several studies have shown that low water temperature (T_w) stress reduced N uptake in rice (Shimazaki et al, 1963, Shimono et al, 2012). In addition, the N uptake ability in rice is significantly reduced due to the reduced activity of enzymes and transporters in low root temperature water conditions (Feng et al, 2011).

Glutamic acid (Glu) plays a central role in the amino acid metabolism of plants (Seifi et al, 2013), which may orchestrate crucial metabolic functions under abiotic stress, such as GABA and proline (Pro) biosynthesis, which perform key roles in plant defense mechanisms (Forde and Lea, 2007, KISHOR et al, 2014, Hayat et al, 2012, Zhao et al, 2013). Cold stress resulted in increased free amino acid levels in barley and wheat (Mazzucotelli et al, 2006, Kovács et al, 2011). There have been a number of previous studies into the GABA shunt response to abiotic stress (Kinnersley and Turano, 2000, Bouche et al, 2003, Miyashita and Good, 2008, Song et al, 2010), and GABA and Pro, as a kind of osmotic regulator, could reduce the oxidative damage of crops under abiotic stresses (Stitt and Hurry, 2002, Bouche...
and Fromm, 2004, Fait et al, 2008). These results indicated that GABA and Pro may have the potential to enhance the cold tolerance of plants. In light of this, the acclimation of nitrogen metabolism may be an essential prerequisite for the adaptation and performance of plants in low temperatures. However, the mechanisms of N metabolism in the roots of rice under low-Tw during the reproductive stage are not yet completely understood.

A number of studies have shown that the MAPK signaling pathways played beneficial roles in plants when exposed to cold conditions(Shi et al, 2018, Nakagami et al, 2005, Zhu, 2016). Homologous genes of ionotropic glutamate receptors (iGluRs) exist in rice(Singh et al, 2014), and glutamate receptor-like (GLR) channels may be essential components of plant signal transduction pathways, affecting the metabolism levels of plants in stress conditions (Forde et al, 2013, Michard et al, 2011, Vincill et al, 2013, Li et al, 2006, Mousavi et al, 2013). Moreover, a significant impact of plant GLRs was observed in MAPK activation and the accumulation of defense gene transcripts in Arabidopsis plants (Kwaaitaal et al, 2011). Transcription factors (TFs) also play important roles in plant responses to low temperatures(Chinnusamy et al, 2007). WRKY TFs are involved in the mitogen-activated protein kinase (MAPK) signaling pathway (Tsuneaki et al, 2002). Likewise, OsWRKY71 which is a cold-responsive rice WRKY gene, acts as a repressor for Glutamate decarboxylase (LOC_Os03g13300) (Kim et al, 2016). Furthermore, WRKY genes regulate Pro biosynthesis in stress conditions. The transcription factor FcWRKY40 functions positively in Pro biosynthesis by directly regulating SOS and P5CS1 homologs under salt stress(Dai et al, 2018). CsWRKY46 confers cold resistance in transgenic-plants by regulating Pro accumulation and a set of cold-stress responsive genes in an ABA-dependent manner (Zhang et al, 2016). There is ample evidence that many metabolites in glutamate metabolism are involved in plant responses to low temperature stress, though their reconfiguration in stress conditions is complex and involves multiple molecular
pathways (Guy et al., 2008, Hamid et al., 2013, Zhao et al., 2013, Dai et al., 2018, Kim et al., 2016, Zhang et al., 2016).

RNA sequencing (RNA-Seq) and MS-based shotgun proteomics are powerful high-throughput technologies for identifying and quantifying RNA transcripts and proteins, respectively (Wang et al., 2014). Transcriptome and proteome analysis of gene and protein expression, respectively, has already contributed greatly to our understanding of cold stress and identified a large number of cold-responsive genes in rice (Zhang et al., 2018, Sperotto et al., 2018, Cen et al., 2018, Ji et al., 2017). RNA-seq provides comprehensive information on mRNA abundance, alternative splicing, nucleotide variation, and structure alterations. Meanwhile, proteomics data provides essential confirmations on the validity and functional relevance of novel findings from RNA-seq data. As the mRNA expression levels do not necessarily directly correspond to the abundance of the corresponding protein species. The relationship between protein and mRNA expression levels provides information on the combined outcomes of translation and protein degradation, which are, in addition to transcription and mRNA stability, essential contributors to gene expression regulation (de Sousa Abreu et al., 2009, Maier et al., 2009). Combined analysis of the transcriptome and proteome can generate data that are complementary to each other, and when integrated appropriately, they could potentially accelerate biological discoveries (Wang et al., 2014).

In this study, we explored the molecular mechanisms involved in rice cold tolerance, using high-throughput mRNA sequencing (RNA-seq) and isobaric tags for relative and absolute quantification (iTRAQ), to analyze the transcriptome and proteome, respectively. Cold-tolerant cultivar DN and cold-sensitive cultivar SJ at the reproductive stage of growth, were exposed for 15 days to low-T_w or control treatments. We compared the mRNA and protein expression profiles of the rice from the control and cold stress conditions, which
demonstrated low expression correlation. We determined the functions of differentially expressed genes (DEGs) and proteins (DEPs) using enrichment analysis of the gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. We then compared transcriptome and proteome data between the different rice cultivars under normal and cold stress conditions, and selected the DEGs with inverse change trends, and analyzed the nitrogen metabolism responses of rice during low-Tw treatment, which will provide an association between the nitrogen metabolism pathway and cold tolerance in rice.

Results

Phenotypic and physiological responses of rice during cold stress

The cold-stress phenotypes of rice are often too unstable to be surveyed in the field. To precisely measure the cold tolerant phenotypes of rice, we used 150 in-field plots for cold water treatments (Fig. 1a). Each plot was 9 m$^2$ and could grow 90 individual rice plants (Fig.1a). We selected 50 widely planted japonica rice cultivars with similar flowing times to measure their phenotypic responses to cold stress at the reproductive stage. For each cultivar, we grew 1620 individual plants in 6 plots with an approximately 20 cm water layer depth. When the plants reached their reproductive stage, with approximately 1 cm young panicles (da Cruz et al, 2006), we independently treated 3 plots with 17°C low-water temperature for 15 days, which is close to the temperature that causes cold damage to rice during reproductive growth in Heilongjiang Province, China. The other 3-plots of rice severed as controls. In total, we systematically measured the phenotypes of 81000 individual plants of rice. We identified 21 cold-sensitive cultivars and 29 cold-sensitive cultivars. Compared with those of the control, the cold-sensitive cultivars had significantly reduced yields and biomass; whereas those of the cold-tolerant cultivars were only slightly
We selected the cold-tolerant cultivar Dongnong428 (DN) and cold-sensitive cultivar Songjing 10 cultivar (SJ) to utilize in the following experiments (Fig. 1a). The yield of CKDN was 880 g/m², that of CKSJ was 810 g/m². The 15 d of low-Tₜw treatment decreased the yields in comparison to the controls, by 41% for D15DN (515 g/m²) and 51% for D15SJ (395 g/m²).

We measured the physiological indicators and nitrogen content of the samples that experienced chilling damage. Compared with the untreated samples, the nitrate reductase (NR) activity of the root was significantly decreased in D15DN (37.8 %) and D15SJ (51.5 %), while the glutamate synthase (GOGAT), glutamine synthetase (GS), glutamate dehydrogenase (GDH), aspartate aminotransferase (GOT), and alanine aminotransferase (GPT) activities were significantly increased in D15DN and D15SJ. This indicated that under the low-Tₜw treatment, the NO₃⁻ reducing ability in the roots of the rice was decreased under low-Tₜw treatment, but the NH₄⁺ assimilation ability was enhanced; which was the main reason for the increased of N concentration in the roots (21.6%, DN; 14.1%, SJ). However, due to the low-Tₜw treatment, the biomass in the roots of the rice was significantly decreased (30.4%, DN; 36.4%, SJ), which leads to decreased N content in the roots (15.4%,DN; 27.4%,SJ) (Fig. 1b).

Amino acid content analysis showed that the glutamate acid (Glu) content in D15DN was significantly reduced and the Pro content was slightly increased, compared to the CKDN. In D15SJ, the content of Glu increased and the content of Pro decreased slightly, compared to the CKSJ. Compared with D15SJ, the Glu content in D15DN was 64.7% less, while the Pro content was increased by 42.9% (Fig. 1c).

**Genome-wide expression changes of mRNA and protein expression under cold**
stress

There were significant phenotypic differences in the dry matter accumulation and N metabolism related physiological indicators of the roots between the DN and SJ (Fig.1b). In order to reveal the regulatory mechanisms of the cold stress and explore new regulators, cold responsive transcriptome and proteome changes in the roots of DN and SJ were studied using RNA-seq and iTRAQ. Cold-tolerant cultivar DN and cold-sensitive cultivar SJ were grown normally (Control; CK) and in low-T_w conditions (D15). Four samples (CKDN; CKSJ; D15DN; D15SJ) with three biological replicates each, were used for transcriptome analysis. Then, 12 cDNA libraries were prepared from these samples and subjected to paired-end sequencing, and gene expression was calculated as the fragments per kilobase, per million reads (FPKM). For RNA-seq, each library obtained clean reads, and ranged from 31.40 to 46.94 million reads. We mapped the clean reads onto the reference genome via HISAT (hierarchical indexing for spliced alignment of transcripts) (Kim et al, 2015) and most of the clean reads (73.02-88.02%) of each library were perfectly mapped to the rice reference genome. The reads that could not be mapped to the rice genome were discarded, and only the mapped reads were analyzed further. FPKM was calculated to measure the expression levels of the transcripts, and showed high correlations (Spearman correlation coefficient (SCC) = 0.84-0.98) among the biological replicates. A total of 43,704 transcripts were detected in at least one sample.

Changes in the proteomes of the roots were quantitatively cataloged using the iTRAQ technology. There were three biological replicates for each sample in the proteome analysis. The search algorithm Mascot was used to identify the proteins (Hirosawa et al, 1993). There were 1,184,867 spectra generated from the roots of the two rice varieties. A total of 265,517 of the spectra were matched to known spectra, and 212,755 spectra were matched to unique spectra. There were also 11,666 mapped peptides, 32,750 mapped...
unique peptides, and 7,281 mapped proteins, with a 1% false discovery rate (FDR). The repeatability of the proteomic analyses according to the CV (coefficient of variation) revealed that 95-100% of the proteins could be found in all the identified proteins, when the CV value was less than 50 % among the replicates.

Of the proteins, 88.8% were detected based on iTRAQ, and corresponding transcripts could be detected in the transcriptome, accounting for 14.8% of the total number of transcripts detected (Fig. 2a). To explore the relationship between the proteins and their corresponding genes, we matched all expressed proteins with their corresponding genes in each treatment and observed a weak correlation, with r-values ranging from 0.14 to 0.31, demonstrating that gene expression cannot fully account for the abundance of protein species, and strong post-translational regulations may exist in the process of protein species production.

**Cold response transcriptome changes in the two rice cultivars**

Differential gene expression was examined using the R package DEseq2 to quantify and analyze all control and treatment combinations (Love et al, 2014). Over the 15 days of cold exposure, a total of 5,549 genes were differentially expressed in the roots between D15DN and CKDN of which 3,539 genes were down-regulated and 2,010 up-regulated. There were 6,145 genes that were differentially regulated in the roots between D15SJ and CKSJ of which 3,690 were down-regulated and 2,455 were up-regulated (Fig. 2b). The number of differentially expressed genes in the two varieties of rice was close after the cold treatments, indicating that there is some similarity between the two varieties in their responses to the cold treatments.

To classify the plant systems influenced during the cold stress, the DEGs were searched against the GO database for enrichment analysis. The GO database enrichment analysis was separated into three ontologies: molecular function (MF), cellular component (CC),
and biological process (BP). 19 and 16 GO terms were significantly changed (P ≤ 0.05) in CKDN vs. D15DN and CKSJvs. D15SJ comparisons, respectively.

In terms of molecular function, the DEGs of CKDN vs. D15DN and CKSJ vs. D15SJ enriched terms focused on kinase, phosphotransferase (alcohol group as acceptor), and transferase activities. The specific term of enrichment for the CKDN vs. D15DN was calmodulin binding (as well as ion binding), Oxidoreductase activity, acting on single donors with the incorporation of molecular oxygen (incorporation of two atoms of oxygen) in CKSJ vs. D15SJ (Fig. 3b).

In terms of the cellular components, the DEGs of CKDN vs. D15DN and CKSJ vs. D15SJ enriched the following terms, intrinsic component of membrane, membrane, integral component of membrane and membrane part. The specific term of enrichment for CKDN vs. D15DN was cell periphery and plasma membrane (Fig. 3b).

In terms of biological processes, no term was enriched in both the DEGs of CKDN vs. D15DN and CKSJ vs. D15SJ. The specific terms of enrichment for CKDN vs. D15DN were diterpenoid metabolic process, cellular ion homeostasis, cellular cation homeostasis, terpenoid metabolic process, ion homeostasis, cellular metal ion homeostasis, metal ion transport; for CKSJ vs. D15SJ the terms of enrichment also included trehalose metabolic process; ion transport; cellular protein modification process; and protein modification (Fig. 3b).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted to determine the biological pathways involved in cold stress. Previous studies have demonstrated that there are many metabolic changes in Arabidopsis to enhance tolerance to freezing conditions, impacting areas such as the Calvin cycle, nitrogen metabolism, starch synthesis, and sugar synthesis (Stitt and Hurry, 2002). By applying a cut-off criterion of Q-value ≤ 0.05, the results showed that six and one pathways were
significantly enriched in the CKDN vs. D15DN and CKSJ vs. D15SJ group, respectively (Fig. 3a).

Pathways enriched between the CKDN vs. D15DN were those for the MAPK signaling pathway, diterpenoid biosynthesis, plant-pathogen interaction, plant hormone signal transduction, limonene and pinene degradation, and zeatin biosynthesis. While the pathway enriched between the CKSJ vs. D15SJ was for plant-pathogen interactions. A number of studies have shown that the MAPK signaling pathway played beneficial roles in plants when exposed to cold conditions (Shi et al, 2018, Nakagami et al, 2005, Zhu, 2016). There were 184 DEGs involved in the MAPK signaling pathway in CKDN vs. D15DN, of which only 164 were DEGS in CKSJ vs. D15SJ (Fig. 3a). There were 20 genes that were specifically up-regulated and 55 specifically down-regulated in the MAPK signaling pathway in CKDN vs. D15DN, including two ethylene receptors (Cht9, ERS2), three LRR receptor-like serine/threonine-protein kinases (Os01g0191200, 107275384, 107278972), and two WRKY transcription factors (Os08g0500300, 107281845).

**Cold response proteome changes in contrasting rice cultivars**

Applying the cut-off threshold of a 1.2-fold change for increased accumulations and a 0.83-fold change for decreased accumulations, together with the number of unique peptides ≥2, a total of 450 proteins were differentially expressed in the roots between D15DN and CKDN of which 215 were down-regulated and 235 were up-regulated; whereas 790 proteins were differentially regulated in the roots between D15SJ and CKSJ, and 454 were down-regulated and 336 were up-regulated (Fig. 2c).

Gene Ontology enrichment analysis was used to identify the over-represented terms from the GO (Ashburner et al, 2000) ontologies for differentially expressed proteins. A full list of all the enriched terms with p values ≤0.05 can be found (Table S5).

The molecular function term enrichment analysis of the DEPs of CKDN vs. D15DN and CKSJ
vs. D15SJ enriched terms both focused on oxidoreductase activity; heme binding; tetrapyrrole binding; cation binding; catalytic activity; nutrient reservoir activity; and antioxidant activity. The specific terms of enrichment for CKDN vs. D15DN were iron ion binding; metal ion binding, ent-copalyldiphosphate synthase activity, glutamate synthase (NADH) activity; for CKSJ vs. D15SJ the terms of enrichment also included hydrolase activity, acting on glycosyl bonds; oxidoreductase activity, and acting on CH-OH group of donors (Fig. 3b).

In cellular component terms, the DEPs of CKDN vs. D15DN and CKSJ vs. D15SJ were enriched in terms focused on cytoplasmic membrane-bounded vesicle; vesicle; extracellular region; and the cytoplasm. Specific terms enriched for CKDN vs. D15DN were cell surface, cytoplasmic part; for CKSJ VS. D15SJ the terms of enrichment also included external encapsulating structure and plasma membrane (Fig. 3b).

The biological process terms associated with the DEPs of CKDN vs. D15DN and CKSJ vs. D15SJ enriched terms both focused on oxidation-reduction process; single-organism metabolic process; diterpenepytoalexin metabolic process; terpenoid metabolic process; generation of precursor metabolites and energy; carbohydrate metabolic process; response to cold; response to oxidative stress. Specific terms of enrichment for CKDN vs. D15DN were glycogen biosynthetic process, and for CKSJ VS. D15SJ there was one term, response to cadmium ion (Fig. 3b).

The KEGG pathways enriched using the DEPs in CKDN vs. D15DN and CKSJ vs. D15SJ were diterpenoid biosynthesis, phenylpropanoid biosynthesis, flavonoid biosynthesis, cutin, suberin, and wax biosynthesis, amino sugar and nucleotide sugar metabolism; biosynthesis of amino acids; ascorbate and aldarate metabolism, carbon metabolism, and flavone and flavonol biosynthesis. The specific pathway enrichments for the CKDN vs. D15DN were carotenoid biosynthesis, as well as glutathione metabolism, cysteine and
methionine metabolism, carbon fixation in photosynthetic organisms, fatty acid biosynthesis in CKSJ vs. D15SJ (Fig. 3b).

There have been previous reports on the genes involved in diterpenoid biosynthesis and the phenylpropanoid biosynthesis pathways, in response to cold stress (Fang et al., 2017). Diterpenoid biosynthesis was enriched in the transcriptome (39 genes) and the proteome (9 genes) data. Os04g0178400 (CYP99A3), Os12g0491800 (KSL10), Os02g0570700 (CYP71Z7), Os01g0561600, and Os04g0178300 (CPS4) were both up-regulated in the transcriptome and proteome data.

**Combined transcriptome and proteome analyses of the differences in cold response mechanisms of the two rice cultivars**

Venn analysis showed that in the transcriptome data, 1015 DEGs were up-regulated in the CKDN \D15DN group, and 904 were down-regulated (Fig. 4a, b). Venn analysis showed that in the proteome data, only 38 DEPs were up-regulated in the CKDN\D15DN group, and 50 were down-regulated (Fig. 5a, b). There were only 5 up-regulated and 9 down-regulated in both proteome and transcriptome data in the CKDN \D15DN group, indicating a strong post-translational regulation and the complementarity of the transcriptome and proteome analysis. The up-regulated genes contained an aspartyl protease family protein (Os06g0304600); tuliposide A-converting enzyme b6 (Os09g0455900); probable polygalacturonase (Os06g0106800); and enolase 1 (Os06g0136600); lichenase-2 (Os05g0375400). The down-regulated genes contained FAR1; PRXIIIE-2; CYP76M7; CXE15; CPS2; AIG2LD; ALDH2C4; uncharacterized isomerase BH0283 (Os01g0266500); and basic secretory protease (Os10g0491000). CYP76M7 participated in the GO terms of oxidoreductase activity; terpenoid biosynthetic process; lipid metabolic process; phytoalexin metabolic process; oxidation-reduction process. CPS2 participated in the GO terms of magnesium ion binding; lyase activity; terpenoid biosynthetic process; lipid
metabolic process; and phytoalexin metabolic process. Enolase 1 participated in the GO terms of magnesium ion binding; lyase activity; oxidoreductase activity; oxidation-reduction process and CYP76M7, CPS2, and enolase 1 may play important roles in resisting cold stress.

Combined DEGs and DEPs together, we found 1,048 up-regulated and 945 down-regulated genes found in proteome or transcriptome data in the CKDN \D15DN group (Fig. 4a, b). GO enrichment analysis of the up-regulated and down-regulated genes indicated responses to heat; electron transport chain; flavonoid glucuronidation; and inorganic anion transport in biological process. There were seven genes identified that were involved in the “response to heat” term: Os06g0104100, Os03g0268400, Os01g0688900, Os07g0517100, Os05g0334000, Os01g0136100, and Os05g0519700, that may also participate in cold stress resistance. There were eight genes identified that were involved in the “inorganic anion transport” term: Os01g0645900, Os06g0324800, Os01g0704100, Os01g0547600, Os10g0420400, Os01g0304100, Os03g0196000, and Os07g0524000. The terms cellular component, chloroplast thylakoid membrane; chloroplast; ATP-binding cassette (ABC) transporter complex; plastid envelope; extracellular region; and microtubule cytoskeleton were also enriched. Os01g0836600 and Os01g0393400 were associated with the term ATP-binding cassette (ABC) transporter complex. In molecular functions, polysaccharide binding; rRNA binding; protein tyrosine kinase activity; inorganic anion transmembrane transporter activity; quercetin 3-O-glucosyltransferase activity; O-acetyltransferase activity; terpene synthase activity; plant-type vacuole membrane; GTP binding; and protein serine/threonine kinase activity. There were seven genes involved in “rRNA binding”: Os03g0356300, Os02g0489400, Os12g0133050, Os03g0122200, Os08g0130500, Os07g0628400, and Os05g0270000 (Fig. 4c).

Identification of transcription factors (TFs) responding to cold response
Study has shown that transcription factors (TFs) play important roles in plant responses to low temperatures (Chinnusamy et al, 2007), as they regulate downstream genes in response to cold stress. Identification of TF genes can provide insight into the molecular mechanisms in cold stress response systems. 1,869 TFs were expressed in at least one cultivar. There were 354 TF genes (212 down, 142 up), 366 TF genes (220 down, 146 up), including 47 gene families, differentially expressed in CKDN vs. D15DN and CKSJ vs. D15SJ, respectively (Fig. 6).

There were 101 TFs (42 down, 59 up) that were only differentially expressed in the CKDN vs. D15DN group. 5 genes of C2C2-Dof family were up-regulated, 3 genes of C2C2-GATA family were up-regulated, 3 genes of mTERF family were up-regulated, 13 members of MYB family were up-regulated. In the WRKY family, 2 genes were up-regulated and 9 genes were down-regulated. In the GRAS family, 1 gene was up-regulated and 3 genes were down-regulated.

The Dof family of transcription factors are one of the most important families of transcriptional regulators in higher plants and are involved in plant growth, development, and responses to abiotic stresses. The following genes: Os03g0821200, Os07g0685000, Os10g0406300, Os05g0112200, and Os12g0569900 genes belonging to the C2C2-Dof family were only up-regulated in the CKDN vs. D15DN group. All 5 genes participated in the go terms of regulation of the RNA biosynthetic process, regulation of gene expression, and DNA binding.

MtERF genes are involved in developmental regulation and environmental responses. The Os05g0403400, 9268165, and Os02g0749900 genes belonging to the MtERF family were only up-regulated in the CKDN vs. D15DN group. The Os05g0403400 and Os02g0749900 genes were grouped with the GO terms for mitochondrion and nitrogen compound metabolic processes.
The MYB family is large and involved in controlling various processes, like responses to biotic and abiotic stresses, development, differentiation, metabolism, defense, etc. The Os08g0178900, Os05g0140100, Os11g0180900, Os08g0151300, Os07g0634900, Os01g0192300, Os01g0274800, Os02g0685200, Os09g0538400, Os01g0128000, Os11g0684000, Os06g0105800 and Os11g0207600 genes belonging to the MYB family were only up-regulated in the CKDN vs. D15DN group.

The WRKY transcription factors are one of the largest families of transcriptional regulators in plants. WRKY genes are not only found to play significant roles in biotic and abiotic stress responses, but also regulate growth and development. The Os07g0111400, Os12g0597700, Os01g0586800, Os06g0146250, Os12g0116700, Os08g0235800, 10727782, Os11g0117500, Os11g0685700, Os06g0158100, and BGI_novel_G000104 genes belonging to the WRKY family, were only up-regulated in the CKDN vs. D15DN group. The Os07g0111400, Os12g0597700, Os01g0586800, Os06g0146250, Os08g0235800, Os11g0685700, Os06g0158100, and BGI_novel_G000104 genes were involved in the MAPK signaling pathway. The WRKY TFs were involved in the mitogen-activated protein kinase (MAPK) signaling pathways (Tsuneaki et al, 2002), which were involved in stress-induced defensive responses (Asai et al, 2002).

The plant-specific GRAS gene family plays important roles in plant growth, development, and stress responses. The Os03g0433200, Os11g0705200, Os06g0127600 and Os03g0263300 genes belonging to the WRKY family were only up-regulated in the CKDN vs. D15DN group. All 4 genes, which were in the KEGG pathway of the plant hormone signal transduction, regulate gibberellic acid (GA) signaling.

**Metabolism, transcriptome, and proteome analyses of nitrogen metabolism responses to cold stress**

Due to the low-$T_w$ treatment, the N content in the roots decreased by 15.4% in the DN and
27.4% in the SJ. The amino acid content analysis showed that the glutamate content in the D15DN was significantly reduced and the Pro content was slightly increased, compared to the control. In D15SJ, the content of glutamic acid increased, and the content of Pro decreased slightly, compared to the control. Compared with D15SJ, the glutamate content in D15DN was 64.7% less, while the Pro increased by 42.9%.

Transcriptome analysis of the nitrogen metabolism pathways, identified 9 genes that were down-regulation in the D15SJ-vs-CKSJ group (Fig. 7a, b), including 3 glutamate decarboxylase genes involved in GABA synthesis, 2 glutamine synthetase genes involved in glutamine synthesis, 1 glutamate synthase gene involved in glutamate synthesis from glutamine, 2 delta-1-pyrroline-5-carboxylate synthetase genes involved in L-Glutamyl 5-phosphate synthesis, and 1 amino-acid N-acetyltransferase gene involved in in N-Acetyl-L-glutamate synthesis. There were 6 genes that were down-regulation in the D15DN-vs-CKDN group, including 3 glutamate decarboxylase genes involved in GABA synthesis, 2 glutamine synthetase genes involved in glutamine synthesis, and 1 delta-1-pyrroline-5-carboxylate synthetase gene involved in L-glutamyl 5-phosphate synthesis. Compared to the D15SJ, no genes showed significant up- or down-regulation in the D15DN.

Proteome analysis of the nitrogen metabolism pathways, identified 5 proteins that were down-regulation or down-regulation in the D15SJ-VS-CKSJ group (Fig. 7 b, c), including 2 glutamate decarboxylase proteins involved in GABA synthesis, 1 arginase gene involved in L-Ornithine synthesis from arginine, 1 glutamate synthase gene involved in glutamate synthesis and 1 ornithine carbamoyl transferase gene involved in L-ornithine synthesis were up-regulated in the D15SJ-vs-CKSJ group. In the D15DN-vs-CKDN group, 2 glutamate synthase genes involved in glutamate synthesis were up-regulated. Compared to the D15SJ, 1 ornithine carbamoyl transferase gene involved in L-ornithine synthesis, showed significant up-regulation in D15DN.
Discussion

**Transcriptome analysis cannot fully represent the proteome analysis**

Comparative analyses of the mRNA and protein abundances in a steady state and the expression alterations that occur with different environmental conditions, have demonstrated that transcription is only half the story (Plotkin, 2010). Discordant mRNA and protein changes, revealed the genes responsible for the changes in protein-level regulation and the potential regulators responsible for the changes have been identified (Wang et al, 2014). In our study, 43,704 transcripts and 7,281 proteins were detected in the transcriptome and proteome data. While transcriptome sequencing can detect more gene expression, more than 80% of the differentially expressed proteins were not differentially expressed in the transcriptome. We also matched expressed proteins with their corresponding genes in each treatment and observed a weak correlation, demonstrating that gene expression cannot fully represent the abundance of protein species, and strong post-translational regulations may exist in the process of protein species production. A total of 43,704 transcripts were detected in at least one sample.

**7,281 mapped proteins**

**The role of transcription factors in rice responses to cold stress**

The key of plant tolerance to cold stress is a complex regulatory network involving TFs and other regulatory genes that control enzymes, regulatory proteins, and metabolites. In this study, genes from five TF families including the C2C2-Dof, C2C2-GATA, WRKY, GRAS and MtERF were identified as COR genes (Zhang et al, 2009). In previous studies, over-expressed plants such as OsCOIN, OsMYB2, OsMYB4, OsMYB3R-2, and OsZFP245 showed significant increases in Pro content and enhanced their tolerance to low temperatures (Liu et al, 2007, Yang et al, 2012, PARK et al, 2010, Vannini et al, 2004, Ma et al, 2009, Huang et al, 2009). In this study, compared with D15SJ, the Pro content in D15DN was increased...
by 42.9%. The Os08g0178900, OsMYB2P-1, Os11g0180900, OsMYB103, Os07g0634900, Os01g0192300, CSA, Os02g0685200, Os09g0538400, Os01g0128000, OsJAMyb, Os06g0105800, and Os11g0207600 genes belonging to the MYB family, were only up-regulated in the CKDN vs. D15DN group. The expression of OsMYB2P-1 has been shown to be induced by cold, salt, and osmotic stress conditions (Xu et al., 2014), indicating that OsMYB2P-1 is associated with Pro accumulation in rice under low temperature stress.

The Dof family of transcription factors, are one of the most important families of transcriptional regulators in higher plants, and involved in plant growth, development, and responses to abiotic stresses. The OsDof16, OsDof24, OsDof27, OsDof19 and OsDof29 genes belonging to the C2C2-Dof family, were only up-regulated in the CKDN vs. D15DN group.

The WRKY TFs are involved in the mitogen-activated protein kinase (MAPK) signaling pathway, which is involved in stress-induced defensive responses (Asai et al., 2002). In this study, the OsWRKY29, OsWRKY83, OsWRKY27, OsWRKY73, OsWRKY64, OsWRKY25, WRKY117, 107277882, OsWRKY40, OsWRKY93, and BGI_novel_G000104 genes, belonging to the WRKY family, were only up-regulated in the CKDN vs. D15DN group, and OsWRKY29, OsWRKY83, OsWRKY27, OsWRKY73, OsWRKY25, OsWRKY61, OsWRKY93 and BGI_novel_G000104 were involved in the MAPK signaling pathway. Furthermore, WRKY TFs have previously been found to be involved in Glu and Pro metabolism under abiotic stress (Kim et al., 2016, Mirabella et al., 2015, Dai et al., 2018). The transcription factor FcWRKY40 of Fortunella crassifolia functions positively in Pro biosynthesis by directly regulating SOS2 and P5CS1 homologs under salt stress (Dai et al., 2018). OsWRKY40 may confer cold resistance in transgenic-plants by regulating the Pro accumulations and a set of cold-stress responsive genes in the MAPK signaling pathway.

The CBF-COR regulatory signaling pathway is highly complex and requires further in-depth
investigation (Ding et al., 2019). RNA-seq analysis of the triple mutants in previous investigations revealed that the expression of c. 10~20% of the COR genes were CBF-dependent (Jia et al., 2016, Zhao et al., 2016, Ding et al., 2019). In our study, CBF1 was up regulated in D15DN more than that of D15SJ. ICE1 activates the expression of CBF genes by directly binding to their promoters under cold stress conditions. Mutations of ICE1 impairs the cold-induced CBF expression and decreases freezing tolerance (Chinnusamy et al., 2003, Ding et al., 2015). In our experiment, ICE1 (LOC_Os01g0928000) was up regulated in D15DN more than that in D15SJ.

Many WRKY proteins have been proven to respond to various abiotic stresses, such as cold (Zhang et al., 2012, Hwang et al., 2005). Several CBF-independent regulators which were cold-induced transcription factors, function in a similar manner to CBFs, and can induce the expression of COR genes under cold stress, including WRKY33 and MYB73 (Park et al., 2015, Ding et al., 2019). In this study, we found that more WRKY and MYB genes were up regulated in CKDN vs. D15DN than in CKSJ vs. D15SJ.

**Nitrogen metabolism responses to cold stress**

Although the actual roles of Glu in plant osmo tolerance remain controversial, there is supporting evidence for a positive effect on enzyme and membrane integrity, osmotic adjustment, and free radical scavenging. Still, some authors have argued that Glu accumulation under stress is a product of, and not an adaptive response to stress. Previous study has found that the N uptake of rice was severely affected by the low-T<sub>w</sub> treatments during reproductive growth (Jia et al., 2019) and that Glu content and glutamate dehydrogenase (GDH) activity were important traits that influenced the grain yield and spikelet sterility, respectively (Jia et al., 2015). In this study, we found that with the low-T<sub>w</sub> treatment, the Glu content in D15DN was significantly reduced, and the Pro content was slightly increased, compared to the control. In D15SJ, the trends of Glu and
Pro content were opposite to those of D15DN. Compared with D15SJ, the Glu content in D15DN was 64.7% less, while the Pro was increased by 42.9%. These results could be attributed to several factors. As indicated by the key enzyme activities of nitrogen metabolism and amino acid content (Fig. 1 b, c), the increase of GOGAT activity and GS activity in D15SJ (69.0% and 59.3%) was greater than that of D15DN (43.8% and 29.6%), while the increase of GDH activity in D15SJ (58.0%) was less than that of D15DN (85.3%), compared with the control. The GDH activity was demonstrated to confer resistance to various stress conditions (Dubois et al., 2003, Robredo et al., 2011) and GDH catalyzes the reversible oxidative deamination of Glu, to supply 2-oxoglutarate and ammonium (Aubert et al., 2001). Secondly, Glu plays a central role in the amino acid metabolism of plants (Seifi et al., 2013), which may orchestrate crucial metabolic functions under abiotic stresses, such as GABA and Pro biosynthesis, which each perform key roles in plant defense mechanisms (Forde and Lea, 2007). The finding that a large amount of the Glu of D15DN was used in the biosynthesis of Pro, which leads to the Glu content in D15DN was less, while the Pro was more than that of D15DN.

Moreover, Compared to D15SJ, the 1 ornithine carbamoyl transferase gene involving in L-ornithine synthesis, showed significant up-regulation in D15DN. Indicating that low-$T_w$ treatments may promote Glu metabolism, synthesis of ornithine, and Pro in D15DN.

**Conclusion**

Cold stress during reproductive growth, resulted in the genes and proteins related to the biosynthesis pathways of cold stress being significantly differentially expressed in both rice cultivars investigated (DN and SJ). The DN was more efficient in N metabolism, the MAPK signaling pathway, and the regulation of transcription factors. The present study confirmed the known cold stress-associated genes and identified a number of putative
new cold-response genes. The study revealed that the translational regulation under cold stress plays an important role in the cold-tolerant DN. The low-$T_w$ treatment affects the N uptake and N metabolism in rice, and promotes Glu metabolism, synthesis of ornithine, and Pro in cold-sensitive SJ.

Materials and methods

**Plant material and growth conditions**

Two *japonica* rice (*Oryza sativa* L. subsp. *japonica*) cultivars, Dongnong428 (DN) and Songjing10 (SJ), which are currently used for local rice production in China, were utilized in this investigation. DN is a cold resistant cultivar, while SJ is a cold-sensitive cultivar. A split-plot design was used in this experiment. The whole plot factor had two levels, normal irrigation (CK) and 15 days of low-$T_w$ irrigation (D15) during the reproductive growth stage of the rice. The cultivar of the rice (DN and SJ) was the subplot factor, the area of each subplot was 9 m$^2$, and there were three replicates of the design. Approximately 30- to 35-day-old seedlings were transplanted with one seeding per hill at a spacing of 13.3 cm (hill space) by 30.0 cm (row space). The application of the low-$T_w$ treatment was started at the beginning of the reproductive growth stage, when the young panicle was approximately 1 cm in length (da Cruz et al, 2006). The low water temperature for the treatment was 17°C with an approximately 20 cm water layer depth, 17°C is close to the temperature that causes cold damage to rice during reproductive growth in Heilongjiang Province, China (Jia et al, 2015). The cold water (at 17°C) was generated automatically using a temperature controlled cold water irrigation system. The low-$T_w$ irrigation was applied from 6:00 AM to 8:00 PM every day for the 15-day period. The experimental approach used for measuring the gross root growth was the previously reported mesh bag method (Steen, 1991). For each root sample, according to the planned
area of each hill, an iron tube (diameter 10 cm, length 30 cm) was inserted into the ground before transplanting. The soil in the tube was dug up with a spade. The mesh bag (diameter 10 cm, length 30 cm, mesh size 3 mm) was pulled onto the iron tube, and then filled with soil. At the end of the stress period, the mesh bags were pulled out of the soil, and the roots were carefully rinsed and separated from their nodal bases. The temperature of the water used for washing is consistent with the water temperature of each treatment. Ten grams of roots from three samples were immediately frozen in liquid nitrogen for 10 min and then stored at -80°C for RNA isolation and the N metabolism enzyme activity measurements. Ten grams of roots from another three samples were used for dry matter measurement.

**Biomass and nitrogen metabolism related indicators**

After being fixed at 105°C for 30 mins, the samples were oven dried at 80°C to a constant weight, and the dry weights of roots were measured. An amino acid analyzer (HITACHI L-8900, Japan) was used for analysis of amino acids. The GS activity was measured based on the method described by O'Neal and Joy (O’Neal and Joy, 1973). The GOGAT activity was determined based on the method by Singh and Srivastava (Singh and Srivastava, 1986). The GDH activity was determined according to Loulakakis and Roubelakis-Angelakis (LOULAKAKIS and Roubelakis-Angelakis, 1990).

**RNA extraction, Illumina transcriptome library preparation, and sequencing**

Total RNA was extracted from the rice shoots using a TranZol Up RNA kit (TransGen Biotech, Beijing, China). All samples were treated with DNase I (TransGen Biotech, Beijing, China). RNA quality was evaluated by gel electrophoresis on 1% agarose gels. RNA samples were further quantified and analyzed by the Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). Then, 1μg of total RNA from each sample was used to construct the cDNA library using the NEBNextUltraTM RNA Library Prep Kit for
Illumina (NEB), following the manufacturer's protocols, and index codes were added to attribute sequences to each sample. The mRNA was purified by Oligo(dT)-attached magnetic beads from total RNA. Purified mRNA was fragmented into small pieces with fragment buffer at 20°C. The RNA fragments were primed with random hexamers and reverse transcribed into the first strand of cDNA, and then the second strand was synthesized. The cDNA was purified by AMPure XP Beads, and after, an A-Tailing Mix and RNA Index Adapters were added by incubating for end the repair. The cDNA fragments with adapters were amplified by PCR, and the products were purified by AMPure XP Beads, then dissolved in EB solution. The quality and quantity of the cDNA library was assessed using an Agilent 2100 bioanalyzer and real-time quantitative PCR. The qualified library was amplified on cBot (Meyer and Kircher, 2010) to generate the cluster on the flow cell. After cluster generation, the libraries were sequenced on an Illumina Novaseq 6000 platform, according to the manufacturer's instructions, and paired-end reads were obtained.

**Transcriptome data analysis**

The raw reads were filtered to obtain clean reads, by removing those without adaptor sequences and the low-quality reads (those containing over 10% N, or when the quality score of over 50% of the base was lower than 10); the clean reads were used for downstream analysis. The clean reads were aligned to the rice genome using Tophat (v2.0.9). The gene expression levels were calculated using the FPKM (fragments per kilobase of transcript per million mapped reads) and the Python package HTSeq with default settings. The DESeq R (1.10.1) package was used to evaluate the differential gene expression between the two groups. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (Anders, 2010). The differential expression of the genes was determined using the criteria of the false
discovery rate (FDR) ≤ 0.01 and the fold change (FC) ≥ 2.

**Protein preparation**

Total protein from tissue was extracted using a Plant Total Protein Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA), in accordance with the manufacturer’s instructions. The samples from the tissues were frozen in liquid nitrogen, homogenized to powders with 10% polyvinylpolypyrrolidone, and homogenized in lysis buffer (8 M urea and 40 mM Tris-HCl, 2 mM EDTA, and 10 mM DTT, pH 8.5) containing 1 mM PMSF. Homogenates were sonicated for 5 min on ice. Samples were then centrifuged at 15,000 g for 20 min. The supernatant was transferred to a new tube and 5 volume of 10% TCA/cold acetone added, 10 mM DTT. The tubes were then kept at -20 °C for 2 h or over night, to precipitate the protein. After, tubes were centrifuged at 15,000 g for 20 min, and the supernatants discarded. Then 1 ml of cold acetone and 10 mM DTT were added to the tubes and suspended the sediment. After 30 min on ice, the samples were centrifuged at 25,000 g for 20 min, and the supernatants discarded. This step was repeated until the supernatant was colorless.

The proteins were air dried and resuspended in lysis buffer. The samples were then sonicated on ice for 5 minutes (2sec/3sec) to improve protein dissolving. After the centrifuge, the supernatants were incubated at 50 °C for 1 hour for reduction and alkylated by 55 mM iodoacetamide (IAM) in the dark at room temperature for 45min. Five volumes of cold acetone were added to samples, to precipitate the proteins at -20°C for 2 hours, or overnight. Lysis buffer was added to dissolve the proteins using sonication, on ice for 5 minutes (2sec/3sec).

**iTRAQ labeling and peptide fractionation**

The peptides were dissolved in 30µl 0.5M TEAB. Peptide labelling was performed by iTRAQ Reagent 8-plex Kit, according to the manufacturer’s instructions. The labelled peptides
with different reagents were pooled and desalted with a Strata X C18 column (Phenomenex), and dried by vacuum centrifugation, according to the manufacturer's protocol.

The peptides were separated on a Shimadzu LC-20AB HPLC Pump system with a high pH RP column. The peptides were resuspended with buffer A (5% ACN, 95% H2O, pH 9.8) to 2 ml and loaded onto a column containing 5-μm particles (Phenomenex). The peptides were separated at a flow rate of 1 mL/min with a gradient of 5% buffer B (5% H2O, 95% ACN, pH 9.8) for 10 min, 5-35% buffer B for 40 min, and 35-95% buffer B for 1 min. Then, the system was maintained in 95% buffer B for 3 min and changed to 5% within 1 min, and then equilibrated with 5% buffer B for 10 min. By measuring the elution absorbance at 214 nm, fractions are collected every 1 min. The eluted peptides were pooled into 20 fractions and then vacuum-dried.

**LC-MS/MS analyses using the Q Exactive**

The dried eluted peptides fraction was resuspended in buffer A (2% ACN and 0.1% FA) and centrifuged at 20,000 g for 10 min. The supernatant was loaded onto a C18 trap column 5 μL/min for 8 min using a LC-20AD nano-HPLC instrument (Shimadzu, Kyoto, Japan). After that, the peptides were eluted from the trap column and separated by an analytical C18 column (inner diameter 75 μm) packed in-house. The gradient was run at 300 nL/min from 8 to 35% of buffer B (2% H2O and 0.1% FA in ACN) for 35 minutes, then changed up to 60% for 5 minutes, then maintained at 80% buffer B for 5 minutes, and then decreased to 5% over 1 min and equilibrated for 10 min.

The peptides separated from the nano HPLC were subjected into the tandem mass spectrometry Q EXACTIVE (Thermo Fisher Scientific, San Jose, CA) for DDA (data-dependent acquisition) detection by nano-electrospray ionization. The parameters for MS analysis were as follows: electrospray voltage was 1.6 kV; precursor scan ranged from 350
to 1600 m/z at a resolution of 70,000; MS/MS fragment scan range was >100 m/z at a resolution of 17,500 in HCD mode; normalized collision energy was 27%; dynamic exclusion time was 15 s; automatic gain control (AGC) for the full MS target and the MS2 target was respectively 3e6 and 1e5; the number of MS/MS scans following one MS scan: the 20 most abundant precursor ions were above a threshold ion count of 20000.

Database search and quantification

Protein identification and quantification were performed with Proteome Discoverer Software (Thermo Fisher Scientific), using two included algorithms, Mascot and SEQUEST. Searches were constructed against the genome database. Peptide and MS/MS tolerance was set to 10 ppm and 0.6 Da, respectively. Enzyme specificity was set to trypsin, with two missed cleavages. Each confident protein identification and quantification required at least one unique peptide. The false discovery rate (FDR) of the identified proteins was ≤0.01. For each of the three biological repeats, spectra were combined into one file and searched. A peptide confidence level of 95%, or an unused confidence score > 1.3, were used as the qualification criteria. The relative quantification of proteins was calculated based on the ratio of peak areas from the MS/MS spectra. Differentially abundant proteins were determined using a Student's t-test. Proteins with a < 0.05 P-value and a fold change ≥1.5 were considered up-regulated and a fold change ≤0.67 was considered to be down-regulated.

Proteome bioinformatic analysis

The R package top GO version 2.28.0 was used for the GO enrichment analysis, with the classic algorithm option (each GO term was tested independently). The pathway enrichment analysis of the identified proteins was performed using the KOBAS software (KEGG ortholog-based annotation system, http://kobas.cbi.pku.edu.cn), and the analysis was conducted by a hypergeometric statistics test. The Benjamin-Hochberg correction was
used to correct the probability values, and only corrected P-value ≤ 0.05 were considered significantly enriched pathways.

Abbreviations

iTRAQ: isobaric tags for relative and absolute quantification; DEP: differentially expressed protein; DEG: differentially expressed gene; KOBAS: KEGG Orthology -Based Annotation System; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Declarations

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Availability of data and materials

The raw Illumina sequencing data from this study has been submitted to the NCBI Sequence Read Archive (SRA) under the accession number PRJNA557063.

Authors’ contributions

Zhao Hongwei and Zou Detang designed the experiment, Jia Yan, Liu Hualong and Qu Zhaojun wrote the manuscript. Wang Xinpeng, Wang Zhuoqian and Zhang Dong conducted the metabolite analyses and analysed the data. Jia Yan carried out the experiments, Wang
Jin and Yang Liang prepared the experimental materials. All authors read and approved the final manuscript.

**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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Supplementary Information

Table S1: Summary of RNA-Seq paired-end data produced by Illumina sequencing.

Table S2. Significant KEGG pathways of DEGs (P-value ≤0.05) involved in DN and SJ under low -T_{w} treatment.

Table S3. Significant KEGG pathways of DEPs (P-value ≤0.05) involved in DN and SJ under low -T_{w} treatment.

Table S4. A list of significantly enriched GO terms (P-value ≤0.05) having DEGs in DN and SJ under low -T_{w} treatment.

Table S5. A list of significantly enriched GO terms (P-value ≤0.05) having DEPs in DN and SJ under low -T_{w} treatment.
**Table S6.** A detailed list of TFs expressed differentially in CKDN vs. D15DN under low $-T_w$ treatment.

**Table S7.** A detailed list of TFs expressed differentially in CKSJ vs. D15SJ under low $-T_w$ treatment.

**Figures**
Figure 1
Phenotypic and physiological responses of the two rice cultivars with low-Tw treatment during reproductive growth. a, Plants of the DN and SJ cultivars with low-Tw treatments at the full heading stage. b, Nitrogen (N) concentration, and NR, GOGAT, GS, GDH, GOT, and GPT activities in the control (CK) and after 15d of low-Tw treatment of the DN and SJ cultivars. c, Differences in the amino acid contents of the CK and 15d low-Tw treatments of DN and SJ.

Figure 2
Comparison of the protein and transcript abundances in roots of two rice cultivars. a, Congruency between the detected transcripts and the proteins of the rice endosperm. b, Number of differentially expressed proteins in the roots (1.2-fold change with P-value <0.05). c, Number of differentially expressed genes in the roots (absolute value of log2 (FC≥1) with FDR≤0.01).
Figure 3

Enrichment analysis of the cold responsive transcriptome and proteome changes in two rice cultivars. a, KEGG enrichment analysis of the differentially expressed genes and proteins presented in a bubble chart. b, GO enrichment analysis of the differentially expressed genes and proteins presented as a heat map.
Comparative transcriptome analysis of the two cultivars revealed cold tolerance mechanisms. a, Venn diagram of the down-regulated differentially expressed genes in CKDN vs. D15DN and CKSJ vs. D15SJ. b, Venn diagram of the up-regulated differentially expressed genes in the CKDN vs. D15DN and CKSJ vs. D15SJ. c, GO enrichment analysis of the specific CKSJ vs. D15SJ differentially expressed genes.
Figure 5

Comparative proteome analysis of the rice cultivars revealed cold tolerance mechanisms. a, Venn diagram of the down-regulated differentially expressed proteins in CKDN vs. D15DN and CKSJ vs. D15SJ. b, Venn diagram of the up-regulated differentially expressed proteins in CKDN vs. D15DN and CKSJ vs. D15SJ. c, Protein-protein interaction analysis of the specific CKSJ vs. D15SJ differentially expressed genes, performed using Cytoscape.
Figure 6

Identification of the transcription factors (TFs) responding to cold responses.
Figure 7

Expression differences in the amino acid metabolism pathways of the transcriptome and proteome. a, Amino acid metabolism pathway in KEGG pathway database. b, Differentially expressed genes in the amino acid metabolism pathway of the transcriptome. c, Differentially expressed proteins in the amino acid metabolism pathway of the proteome.

Supplementary Files

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