

Comparative transcriptome profiling of chilling tolerant rice chromosome segment substitution line in response to early chilling stress

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Abstract Rice (*Oryza sativa* L.) is thermophilic crop and all of its growth stages are affected by low temperature stress. However, some populations of common wild rice in Guangxi Province (China) (GXWR) can tolerate low temperature stress. To understand the molecular mechanisms underlying chilling tolerance in these wild rice resources, transcriptome sequencing was performed to study the differentially expressed genes (hereafter referred to as DEGs) between GXWR-derived chilling-tolerant chromosome segment substitution line (hereafter referred to as CSSL), DC90, and its chilling-sensitive recurrent parent 9311 under early chilling stress. Comparative transcriptomic analysis revealed 659 DEGs exclusively identified in DC90 in response to early chilling stress. Functional clustering by CARMO (comprehensive annotation platform for functional exploration of rice multi-omics data) showed that majority of the DEGs were

enriched in chloroplasts, suggesting a connection between chilling stress tolerance and photosynthesis regulation exists in rice. KEGG analysis of these DEGs presented a complicated chilling responsive regulatory network, including ‘phytohormone signaling’, ‘photosynthesis pathway’, ‘ribosome translation machinery’, ‘phenylpropanoid biosynthesis’, which is coordinately involved in early chilling response. Here, the identified DEGs and unveiled molecular regulatory network sheds light on deep understanding the mechanisms underlying rice chilling stress tolerance. As well, KEGG term-enriched DEGs, chilling tolerant QTLs (quantitative trait loci), and co-localized DEGs in introgression interval, will be focused for further functional investigation of the molecular mechanisms of chilling stress response in rice.

Keywords Common wild rice · Comparative transcriptomics · Early chilling stress tolerance · Chromosome substitution line · Photosynthesis pathway · Ribosomal pathway

Yongliang Wang and Qigui Jiang have contributed equally to this work.

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Introduction

Rice (*Oryza sativa* L.) is a major staple food crop of the world, feeding nearly half of the world population. However, rice, as a thermophilic crop, is highly sensitive to low temperature (Li et al. 2006). Chilling or cold stress (sub or non-optimal temperature), which frequently occurs in temperate or high altitude of subtropical regions, acts as one of the major adverse environmental factors to rice growth, yield, and distribution (Cho et al. 2012). In life cycle of rice, all the growth stages are affected by chilling stress. At vegetative growth stage, when exposed to low temperature, rice seedlings exhibit retard growth,

yellowing, withering, reduced tillering, and lead to significant yield loss ultimately. Chilling stress occurring at the reproductive stage of rice causes delayed heading date, pollen abortion, and panicle exertion inhibition (Cho et al. 2012). The yield of rice in many countries, including China, Korea, Japan, is severely affected by chilling injury (Jiang et al. 2006; Wang and Yang 2007). Therefore, comprehensive understanding the knowledge about the molecular mechanisms underlying rice in response to early chilling stress is of fundamental importance and is also crucial for development of rational strategies for rice chilling tolerant improvement breeding (Xiong et al. 2002).

The molecular mechanisms of chilling stress responsiveness in *Arabidopsis* have been studied extensively. For instance, the dehydration-responsive element binding proteins (DREB1)/C-repeat (CRT) binding factors (CBF) pathway plays pivotal roles in cold responsive regulation during cold acclimation in *Arabidopsis* (Chinnusamy et al. 2007; Zhou et al. 2011). Rice is chilling sensitive species relative to *Arabidopsis*, suggesting that rice might employ different chilling stress regulation mechanisms, although it was proposed that DREB/CBF pathway conserves in many plants, including rice (Ito et al. 2006; Maruyama et al. 2012). Recently, the study of a newly cloned quantitative trait locus, *COLD1*, showed that it acts as cold sensor, which interacts with alpha subunit of G-protein to activate the Ca^{2+} channel for sensing low temperature and enhance the GTPase activity of G-protein, and therefore, confer chilling tolerance in *japonica* rice (Ma et al. 2015). However, the mechanisms underlying rice chilling tolerance remain elusive.

Wild relatives of cultivated rice, common wild rice (*Oryza rufipogon* Griff.), widely colonizes in sub-tropical and tropical areas. This leads to wild rice evolving specific mechanisms to adapt to their growth habitats. Some populations of common wild rice in Guangxi (China) (hereafter referred to as GXWR) are perennial and their underground rhizomes can tolerate the temperature as low to -4 °C. Similar to Dongxiang wild rice (Jiangxi Province, China), their rhizomes enable the plants regenerating and continuing normal growth and development in early spring of next year (Xu et al. 2010). To unveil the molecular mechanisms underlying chilling tolerance regulation of GXWR, a chilling-tolerant GXWR DP15 (as donor) was introgressed into recurrent parent 9311 (chilling sensitive) by backcrossing for five times in our previous study (Zheng et al. 2011). In subsequent, a chilling tolerant CSSL, DC90, was developed by marker assisted selection based on obtained advanced backcrossing lines. Genome-wide genotyping using DNA markers showed that only two DP15 segments were left in genetic background of DC90 (Fig. S1A–C; Table S1). CSSL, which contained a few genomic segments from donor and largely eliminated

genetic background noise disturbance, is suitable for identifying differentially expressed genes associated with chilling tolerance by comparing with its recurrent parent in comparative transcriptome analysis (Li et al. 2005).

In this study, comparative transcriptome analysis of DC90 and 9311 under early chilling stress was performed using Illumina paired-end RNA sequencing. The results presented here provide a new insight into the cold responsive mechanisms of GXWR in early chilling stress and provide valuable wild rice gene resources for the development of chilling tolerant rice cultivars.

Materials and methods

Plant materials and chilling stress treatments

Three rice genotypes, DC90, NC and 9311, were used for characterizing chilling stress tolerant phenotype in this study. 9311 is a widely cultivated *indica* rice in the mid and lower reaches of the Yangtze River, as well as the southern part of China, with high yield and good quality. However, 9311 is sensitive to chilling stress, and chilling injury would be happened once the temperature low to 15 °C. DC90 is a chilling tolerant CSSL and is highly similar to genetic background of 9311. Only two DP15 introgression segments (10 and 20 Mb) were found located on the chromosome 3 and 12 of DC90, respectively. NC, a CSSL with one introgression segment, which is co-linear with the one on chromosome 3 of DC90, was used as a negative control to determine which segments harbored the favorable alleles for chilling stress tolerance in DC90 (Fig. S1; Table S1).

Rice seedlings were planted in a plastic container filled with paddy soil, and grew under natural condition with natural sunlight at summer of 2014 to three-leaf stage. Rice seedlings were then exposed to low temperature of 10/8 °C (day/night) (artificial light was supplemented with day—13 h/night—11 h circadian rhythm) for 5 days. After chilling treatment, the rice seedlings were recovered at natural condition for 7 days. Whole plants of DC90 and 9311 (three plants each sample) were collected before and at 36 h time point of chilling treatment for total RNA isolation. The samples were frozen in liquid nitrogen immediately and stored at -80 °C.

Physiologically phenotyping of two genotypes under early chilling stress

Relative electrolyte leakage (REL) was measured according to the method of Yan et al. (2006) with minor modifications. Leaf samples (0.2 g) were collected and put into a tube containing 20 mL distilled water for 12 h at 25 °C. The

initial electric conductivity (R1) of the solutions was measured. The solutions with samples were then heated to 100 °C for 30 min. The final electric conductivity (R2) was measured when solutions cooled to room temperature. The REL was calculated as follows: $REL (\%) = R1/R2 \times 100$.

RNA isolation, RNA-seq library construction, and sequencing

Total RNA of each sample was isolated by TRIZOL (Invitrogen, CA, USA), and its quality was monitored by 1 % agarose electrophoresis. The purity of RNA was measured by Nano Photometer[®] spectrophotometer (IMPLEN, CA, USA). The quantification of RNA was measured by Qubit[®] RNA Assay Kit in Qubit[®] 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Equal amount (3 µg) of total RNA of three replicates of each sample were mixed to get pooled samples. A total amount of 3 µg RNA of each pooled group was used as input material for generating sequencing libraries using NEB Next[®] Ultra[™] RNA Library Prep Kit for Illumina[®] (NEB, USA) following manufacturer's instruction. cBot Cluster Generation System was used to perform the clustering of the index-coded samples using TruSeqPE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instruction. After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2500 platform (Novogene, Beijing, China).

RNA-seq data processing, transcriptome profiles analysis

Raw data (raw reads) were deposited to gene expression omnibus (GEO) database of NCBI (accession number: GSE76415; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76415>) (Edgar et al. 2002), and then were processed through in-house Perl scripts to remove reads containing adapter and low-quality reads to obtain clean data (clean reads). Meanwhile, Q20, Q30 and GC content in the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Indica rice reference genome and gene model annotation files were downloaded from Ensembl FTP server (ftp://ensemblgenomes.org/pub/release-23/plants/fasta/oryza_sativa/dna/) directly. Paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 (Trapnell et al. 2009). HTSeq v0.6.1 (Anders et al. 2010) was used to count the number of reads mapped to each gene. Expected number of fragments per kilo base of transcript sequence per millions base pairs sequenced (FPKM) was used for estimating gene expression levels.

DEGseq R package (1.20.0) (Wang et al. 2010) was used to identify DEGs. DEGs were defined by fold change values of normalized FPKM ($\log_2(FC)$, FC designates fold change) among pair-wise sample groups. *p*-values were adjusted using the Benjamini and Hochberg method. Highly stringent thresholds (Corrected $p \leq 0.005$ and $|\log_2(FC)| = 1$) were set to filter the significantly differential expression of genes between each pair of sequenced library. Here, we converted *indica* rice gene ID to their counterpart ones in *japonica* rice (var. Nipponbare) because of less annotation of *indica* rice gene model can obtain from public rice genomic databases currently. Thus, functional annotation of identified DEGs was performed based on Rice Genome Annotation Project version 7 (Kawahara et al. 2013). GO enrichment analysis was performed using agriGO (Zhou et al. 2010). The Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment was performed using a Hochberg test (the threshold of FDR ≤ 0.01) by web-based plant gene-set enrichment analysis toolkit (PlantGSEA) (Kanehisa and Goto 2000; Xin et al. 2013; Kanehisa et al. 2014). CARMO was used to perform functional enrichment analysis of DEGs using default setting value according to the manual of the toolkit (Wang et al. 2015).

Quantitative real-time reverse transcription-PCR analysis

To validate the results of the Illumina sequencing, additional set of samples (three biological replicates per sample) were collected and isolated total RNA for real time PCR. cDNA synthesis was performed by reverse transcription (RT) using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Cat# K1622) according to the manufacturer's protocol. The sequences of a subset of DEGs were downloaded from RGAP (Kawahara et al. 2013). Primers were designed to perform qRT-PCR to detect the relative expression of selected DEGs via Roche Lightcycler 480 Real-Time PCR System in 10 µL reactions using the SYBR[®] Green PCR Master Mix kit (BIORAD, USA) following the manufacturer's protocol. The relative expression of each gene was calculated according to the method of $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001). The *Actin-1* gene (LOC_Os03g50890) was used as endogenous reference for qRT-PCR. All analyses were performed with three technical replicates.

Results and discussion

Chilling tolerant phenotyping of DC90 under low temperature treatment

After 5-day chilling stress treatment, there was no visible difference observed between DC90 and the other two

genotypes before and after chilling stress treatment, respectively (Fig. 1A, B). Chilling treated plants were allowed to recover for 7 days in natural condition. After recovery, the significant differences were observed among tested samples (Fig. 1C, D). 9311 and NC Seedlings were completely wilted, whereas, DC90 seedlings were able to survive from chilling stress.

To determine the suitable duration time of chilling treatment for getting early chilling stress condition for this study, relative electrolyte leakage (REL), an indicator of chilling stress response in plants, was measured using chilling-treated DC90 and 9311 rice plant materials, respectively. The results of REL measurement showed that REL of DC90 was significantly lower than that of 9311 at 36 h, whereas, there was no significant difference between two genotypes before chilling treatment ($p < 0.01$, Students *T* test) (Fig. 1E).

Taken these together, the results demonstrated that the genomic segments derived from GXWR could enhance DC90 chilling tolerance, and the allele(s) harbored in the introgression segment on the short arm of chromosome 12 contribute(s) to the enhanced chilling tolerance as well. Furthermore, the results of REL analysis indicated that tested plant materials are differential responding to chilling

at 36 h treatment, and the condition is suitable for preparing the samples for RNA sequencing.

Transcriptomic profiling analysis of DC90 and 9311 in response to early chilling stress by RNA sequencing

A total of about 118 million of raw reads with the length of 125 bp paired-end reads were generated from Illumina HiSeq™ 2500 platform sequencing (Table S2). Low-quality reads with adaptor and unknown or low quality bases were removed from raw data sets. A total of 114 million clean reads were obtained, representing 96.61 % of the total raw reads. The averages of Q20, Q30, and GC contents were 96.06, 90.82, and 54.95 %, respectively (Table S2; Fig. S2). The clean reads of each sample groups were then mapped to reference genome (*Oryza sativa* ssp. Indica group var.9311) using TopHat v2.0.12 (Table 1). In summary, the mapped clean reads of four data sets ranged from 85.18 to 87.07 %. Among these, 82.82–84.81 % were uniquely mapped, 2.26–2.63 % were multiple mapped (Table 1). Furthermore, in the total of mapped reads, 88.10–90.90 % were mapped to exon regions, 7.6–9.6 %

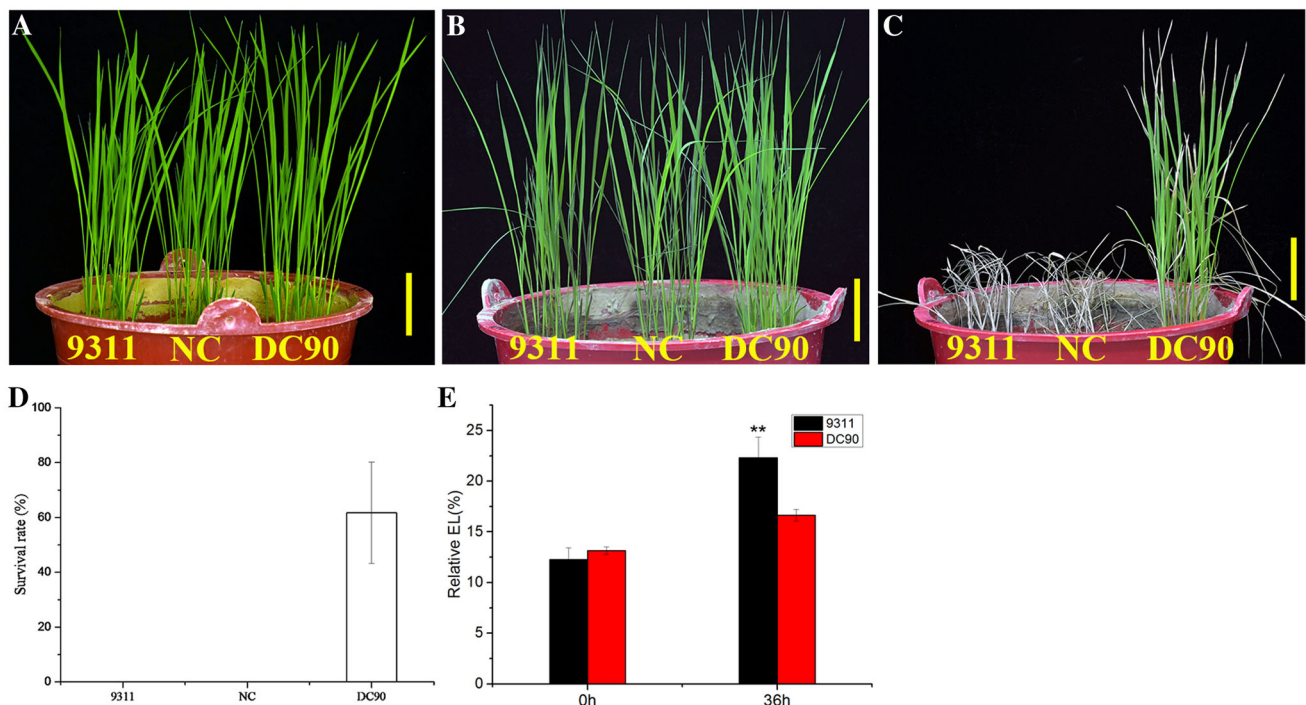


Fig. 1 Chilling stress phenotype of DC90 and 9311 at seedling stage. **A** The seedlings of DC90, NC, and 9311 before chilling stress treatment; **B** 5-day chilling stressed phenotypes of DC90, NC, and 9311; **C** 7-day recovery phenotypes of DC90, NC, and 9311 after chilling stress treatment; **D** The comparison of survival rates of DC90, NC, and 9311 after 7-day recovery from chilling treatment; **E** Relative

electrolyte leakages of DC90 and 9311 at 0 and 36 h time point, respectively. *NC*, designates that a line without wild rice introgression segment in the same position of chromosome 12 shown in the linkage map of S1 *EL* designates electrolyte leakage; *Scale bars* 10 cm **A–C**; data in **D–E** are shown as mean \pm SD ($n = 3$), $p < 0.01$. (Color figure online)

Table 1 Summary of RNA-seq data and mapped the clean reads to *indica* rice reference genome

Sample name	DC90_0 h	DC90_36 h	9311_0 h	9311_36 h
Total reads	29418358	27393550	29649930	27987288
Total mapped	25233606 (85.78 %)	23334541 (85.18 %)	25335016 (85.45 %)	24369079 (87.07 %)
Multiple mapped	763453 (2.6 %)	623614 (2.28 %)	778533 (2.63 %)	633409 (2.26 %)
Uniquely mapped	24470153 (83.18 %)	22710927 (82.91 %)	24556483 (82.82 %)	23735670 (84.81 %)
Reads map to '+'	12226880 (41.56 %)	11353539 (41.45 %)	12272367 (41.39 %)	11863199 (42.39 %)
Reads map to '-'	12243273 (41.62 %)	11357388 (41.46 %)	12284116 (41.43 %)	11872471 (42.42 %)
Non-splice reads	16308213 (55.44 %)	15688364 (57.27 %)	16541932 (55.79 %)	16562685 (59.18 %)
Splice reads	8161940 (27.74 %)	7022563 (25.64 %)	8014551 (27.03 %)	7172985 (25.63 %)

The numbers in parenthesis represent the percentage of corresponding types of mapped reads to clean reads

were mapped to intergenic regions, and 1.3–2.3 % were mapped to intron regions of reference genome (Fig. 2).

Identification of differentially expressed genes in response to early chilling stress and qRT-PCR validation

All the DEGs identified in our study were clustered by hierarchical cluster analysis. The results showed that DC90 and 9311 were clustered together at 0 and 36 h,

respectively (Fig. S3), indicating that they have highly similar genetic background and highly similar gene expression pattern in response to early chilling stress. The results obtained from both correlation analysis and FPKM density distribution plotting also reflected the similar genetic background between DC90 and 9311 (Fig. S4A–F).

To investigate the similarities and differences of gene expressions between DC90 and 9311 under chilling stress, two strategies were used to identify chilling-responsive DEGs under early chilling stress. First, the DEGs in

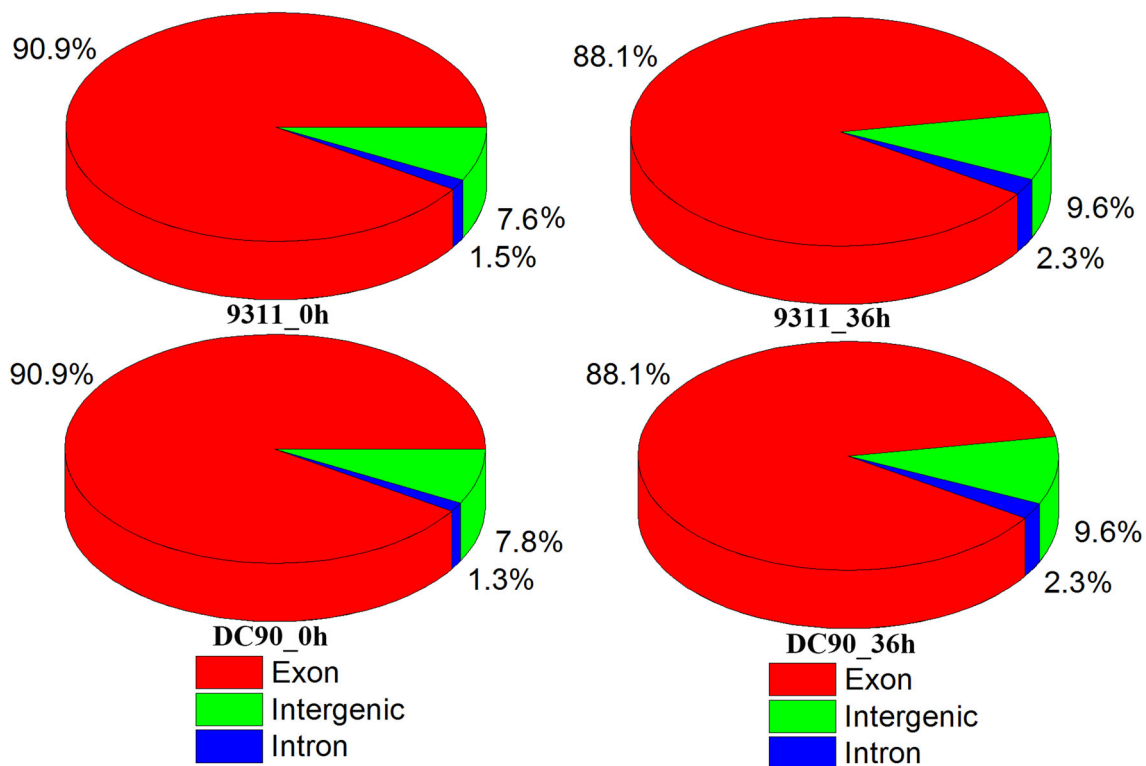


Fig. 2 Pie graph shows the distribution of mapped clean reads of RNA-seq in rice reference genome. Different colors represent the components. (Color figure online)

chilling tolerant and sensitive genotypes in response to chilling stress were obtained by comparing normalized FPKM of genes under chilling stress with those in their respective untreated control, respectively. By this comparison, a total of 2850 and 3587 significant DEGs were identified in DC90 and 9311 in response to chilling stress, respectively (q -value < 0.005 , $|\log_2(\text{FC})| \geq 1$) (Table S4-1,2). Among these, 2348 DEGs were common between two genotypes, including 1325 up-regulated and 1023 down-regulated genes (hereafter referred to as CRGs-1) (Fig. 3A; Table S4-3). It represented 82.39 and 65.46 % of total DEGs in DC90 and 9311, respectively. 502 DEGs, including 275 up-regulated and 227 down-regulated genes, were exclusively identified in DC90 in response to early chilling stress (hereafter referred to as CRGs-2) (Fig. 3A, Table S4-4). Second, genes differentially expressed between chilling tolerant and sensitive genotypes under chilling stress were obtained by comparing normalized FPKM of genes in chilling tolerant genotype with those in sensitive one after chilling. A total of 185 significant DEGs, including 31 up-regulated and 154 down-regulated genes, were obtained using second strategy (q -value < 0.005 , $|\log_2(\text{FC})| \geq 1$) (Table S4-5). Among these, 157 DEGs were exclusively identified in DC90 (hereafter referred to as CRGs-3) (Fig. 3B; Table S4-6).

To validate the results of RNA-seq sequencing, qRT-PCR was performed to test the alteration of the expression of DEGs identified in RNA-seq. The results of qRT-PCR for ten genes, which randomly selected from DEG datasets of DC90 in response to early chilling treatment, were consistent with what they were in RNA-seq analysis (Fig. S5; Table S3).

Functional enrichment analysis of chilling-responsive gene sets

Functional annotation of the common gene set CRGs-1 was conducted using GO enrichment analysis. The up-regulated DEGs of CRGs-1 were significantly enriched in 26 GO terms ($p < 0.05$, FDR as the cut-off) (Fig. 4A; Table S5-1). Down-regulated DEGs were significantly enriched in 34 GO terms ($p < 0.05$, FDR as the cut-off) (Fig. 4B; Table S5-2). The results showed that a large number of genes with diverse functional categories (GO terms) are widely involved in early chilling stress response.

To gain deep insight into the molecular mechanisms underlying DC90 chilling tolerant phenotype which were conferred by the effect of introgression, CARMO (Wang et al. 2015) was used to perform functional enrichment analysis of CRGs-2 and CRGs-3 gene sets. The DEGs were firstly subjected to GO enrichment analysis, and then the enriched GO terms were functional clustered via MCL (control the number of clusters) algorithms. For gene set CRGs-2, the analysis of up-regulated DEGs subset generated four clusters, including ‘ligase activity (cluster 1)’, ‘response to abiotic stimulus (cluster 2)’, ‘carbohydrate metabolic process (cluster 3)’, and ‘peptidase activity (cluster 4)’. Of which, cluster 2 was the most highly enriched (Fig. 5A; Tables S6-1, S7-1). The analysis of down-regulated subset, DEGs were also clustered into four functional clusters, of which, ‘chloroplast stroma (cluster 2)’ was the most highly enriched cluster (Fig. 5B; Tables S6-2, S7-2). For gene set CRGs-3, the subset of up-regulated DEGs was enriched in only one functional cluster ‘response to abiotic stimulus (cluster 1)’. The analysis of down-regulated DEGs subset

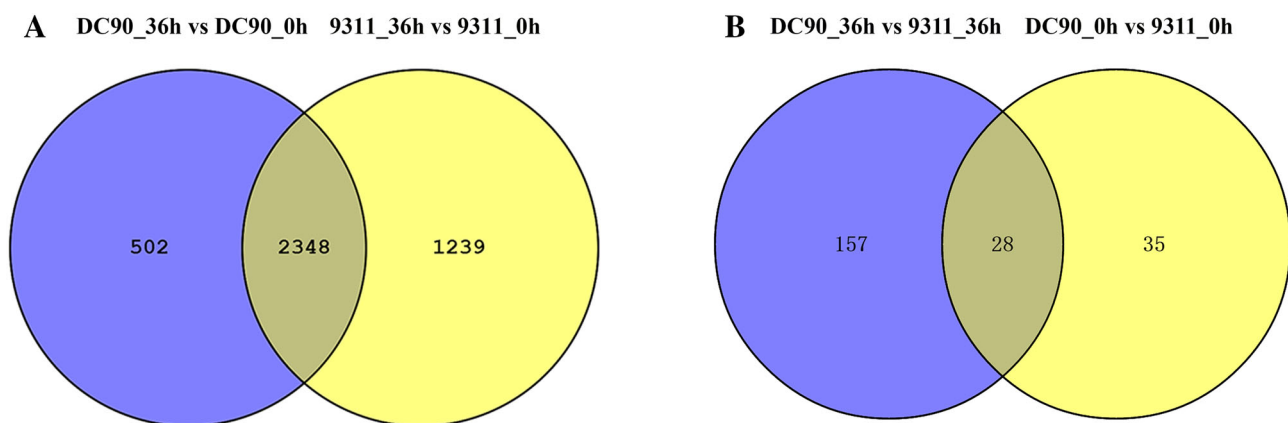


Fig. 3 Venn diagram analysis of DEGs obtained from chilling-stressed and untreated of DC90 and 9311 in RNA-seq analysis. **A** The number of DEGs identified in DC90, 9311, and the common DEGs in both genotypes; **B** The number of DEGs identified in 0, 36 h time point of experiment conditions, and the common DEGs between 0 and 36 h time points; *Blue* color represents number of DEGs exclusively identified in DC90 in **A** and number of DEGs exclusively identified

at 36 h in DC90 relative to 9311 in **B**; *yellow* color represents number of DEGs exclusively identified in 9311 in **A** and number of DEGs exclusively identified at 0 h in DC90 relative to 9311 in **B**; the overlaps indicate the number of common DEGs between DC90 and 9311 **A** and the number of common DEGs between 0 and 36 h time points **B**, respectively. (Color figure online)

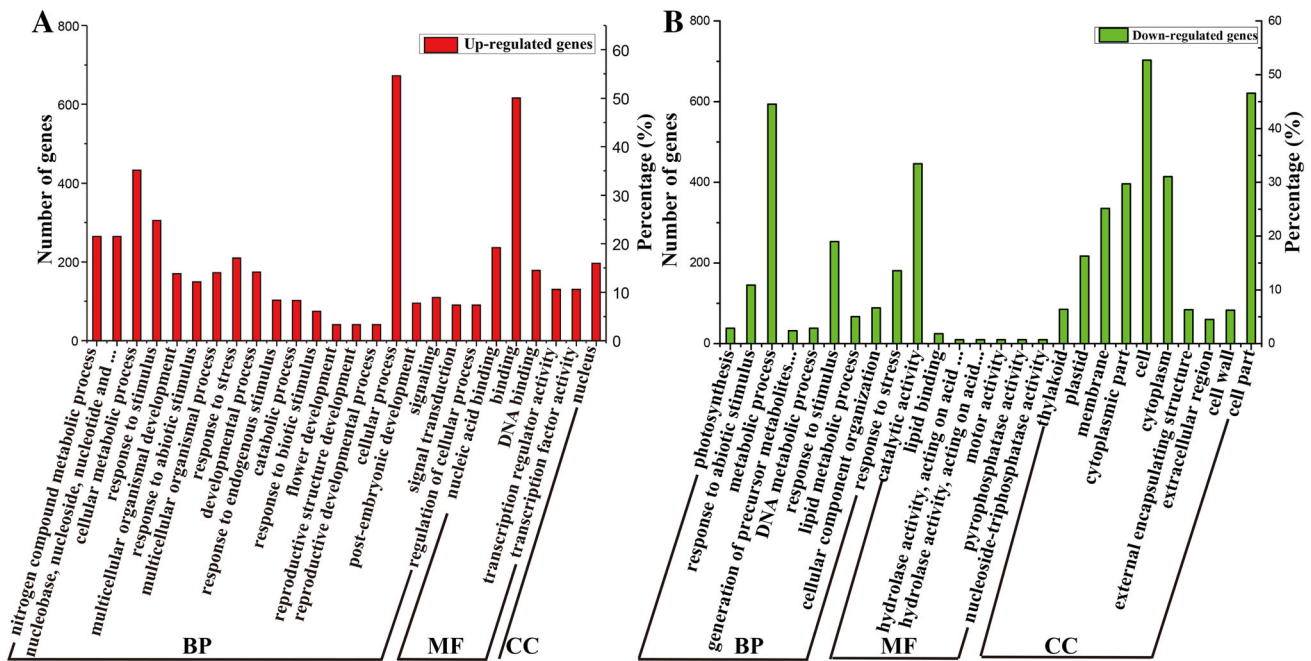


Fig. 4 GO enrichment analysis of common chilling responsive DEGs between DC90 and 9311 in response to early chilling stress. **A** GO enrichment of common up-regulated DEGs between DC90 and 9311; **B** GO enrichment of common down-regulated DEGs between DC90 and 9311; BP, MF and CC indicate the biological process, molecular

function and cellular component of Gene Ontology classification, respectively; the left Y-axis represents the number of genes in a GO term; the right Y-axis represents the percentage of genes in a GO term. (Color figure online)

was generated two clusters, ‘tryptophan metabolic process (cluster 1)’ and ‘catalytic activity (cluster 2)’ (Fig. 5C, D; Tables S6-1,2, S7-3,4). The clusters enriched here outlined the specific sub-localization and functional categories of DEGs in response to early chilling stress in DC90. Furthermore, gene module enrichment analysis was performed to organize the DEGs into high level modules. Gene module represents a gene set with related function and showing coordinated expression to carry out specific function. The up-regulated DEGs of gene sets CRGs 2 and 3 were enriched in ten gene module terms in total. Most of them were assigned to the following terms, ‘response to heat, high light intensity, hydrogen peroxide, and to endoplasmic reticulum stress (Module_40)’, ‘response to endogenous stimulus (Module_663)’, ‘GTPase activity; embryo development ending in seed dormancy; ligase activity (Module_489)’, ‘transcription, DNA-templated (Module_427)’, ‘catalytic activity; response to abiotic stimulus (Module_394)’, and ‘oxidoreductase activity; carbohydrate metabolic process (Module_859)’ (Fig. 6A, B; Table S6-3). The down-regulated DEGs were enriched in 28 terms. The annotations of these gene modules suggested that this subset of DEGs mainly involved in ‘plant photosynthesis pathway (Module_794, 282, 544, 875, 200, 784)’, ‘ribosome (Module_479)’, ‘response to stress and biotic stimulus (Module_504)’, ‘catalytic activity; response to abiotic stimulus (Module_394)’, and ‘oxidoreductase activity; carbohydrate metabolic process (Module_859)’ (Fig. 6C,

D; Table S6-4). It is noteworthy that ten DEGs were enriched in Gene Module_489 (GTPase activity; embryo development ending in seed dormancy; ligase activity) (Fig. 6A; Table S6-3). This consistent with previous report that GTPase activity, which regulated by *COLD1* (LOC_Os04g51180), involves in activating Ca^{2+} channel for sensing low temperature and confers chilling tolerance in rice (Ma et al. 2015).

Chilling-responsive pathway analysis in chilling tolerant genotype

To search for metabolic or signal transduction pathways that were significantly enriched, KEGG pathway enrichment analysis was performed for the exclusively identified DEGs in DC90 (gene set CRGs-2 and 3). The total of 24 KEGG pathways were significantly over-presented ($p < 0.01$, FDR as the cut-off) (Tables 2, S7-5,6). Among these, ‘metabolic pathways (osa01100)’ and ‘biosynthesis of secondary metabolites (osa01110)’ were most highly enriched, indicating that metabolism of DC90 is inhibited under early chilling stress condition. Besides, the following pathways caught our attention.

Plant hormone signal transduction

‘plant hormone signal transduction (osa04075)’ was the only one KEGG term which was significantly enriched in

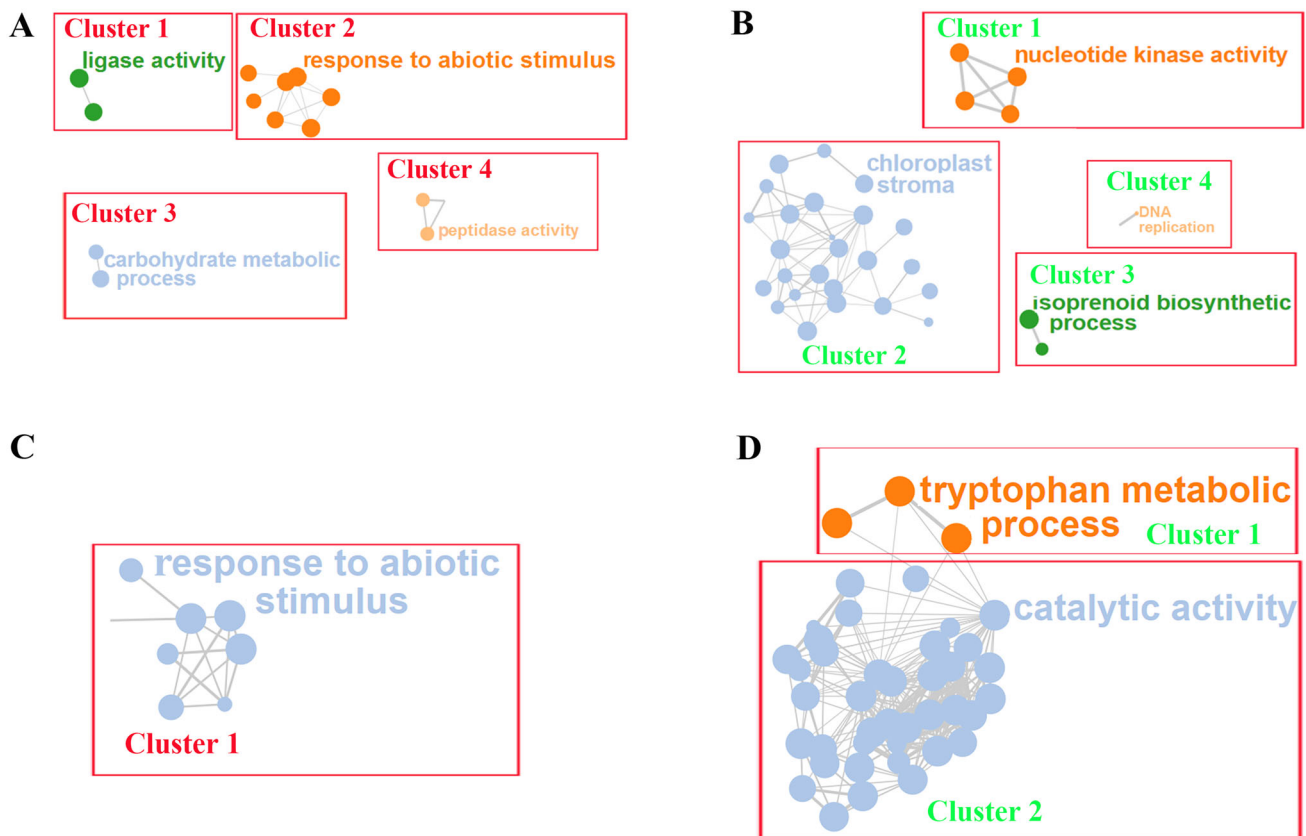


Fig. 5 Functional clustering of exclusive identified DEGs in DC90 in response to early chilling stress. **A** Functional cluster view of enriched up-regulated DEGs of gene set CRGs-2; **B** Functional cluster view of enriched down-regulated DEGs of gene set CRGs-2; **C** Functional cluster view of enriched up-regulated DEGs of gene set CRGs-3;

D Functional cluster view of enriched down-regulated DEGs of gene set CRGs-3; *Solid circles* with different colors represent different sub-clusters, and the length of an edge represents the number of genes shared by the two terms linked in the same cluster. (Color figure online)

analysis of the up-regulated DEGs in gene set CRGs-2 ($p < 0.01$, FDR as the cut-off). Four DEGs (LOC_Os01g55940.1, LOC_Os10g36703.1, LOC_Os05g06320.2, and LOC_Os07g06130.1), which were enriched in ‘response to abiotic stimulus (cluster 2)’ described above (Fig. 5A; Table S7-5), were also clustered to this term. LOC_Os01g55940.1 (*OsGH3.2*) (Du et al. 2012; Narsai et al. 2013) and LOC_Os10g36703.1 (*SAUR*) are downstream target genes of auxin signaling pathway. Auxin signaling regulates a large number of genes, including *Aux/IAA*, *GH3*, and *SAUR* gene families, by alteration of their expressions, and primarily involves in diverse aspect of growth and development of plants (Zhao 2011). Interestingly, *GH3*, *Aux/IAA*, and *SAUR* gene families have been shown in response to both biotic and abiotic stresses in rice as well (Hagen and Guilfoyle 2002). *OsGH3.2*, a DEGs identified in this study, is a member of GH3 family protein, which involved in modulating endogenous free IAA and ABA homeostasis and regulates drought and cold tolerance differentially in rice. Overexpression of *OsGH3.2* enhances rice cold tolerance (Du et al. 2012). Moreover, *OsGH3.2*,

OsSAUR22, *OsSAUR48*, and *OsSAUR54*, were found up-regulated under one or more abiotic stress condition(s) (Jain and Khurana 2009). They are consistent with our result. For the other two clustered DEGs, LOC_Os05g06320.2 (*ETR*) is receptor of ethylene. LOC_Os07g06130.1 (*OsEIN2*) acts downstream of *ETR*. Both of them are up-regulated in response to early chilling stress. In *Arabidopsis*, ethylene signal is perceived by ethylene receptors such as *ETR1* (Chen et al. 2002). Downstream of ethylene signaling cascade consists of *EIN2*, *EIN3*, and of members of AP2/ERF multi gene family (Steffens 2014). *ETR1*, *EIN2*, *EIN3*, and *ERF* proteins are shown to be involved in many stress responses (Zhou et al. 2006; Cao et al. 2007; Chen et al. 2009; Lei et al. 2011; Zhang et al. 2011; Shi et al. 2012). A rice gene, *MHZ7*, that is homologous to *EIN2* in *Arabidopsis*, encodes a membrane protein, involving in ethylene signaling and yield trait regulation (Ma et al. 2013). However, interestingly, our results also imply that *OsEIN2* plays a role in early chilling response in rice.

Taken together, our results show that auxin and ethylene may play crucial roles in plant stress responses, including

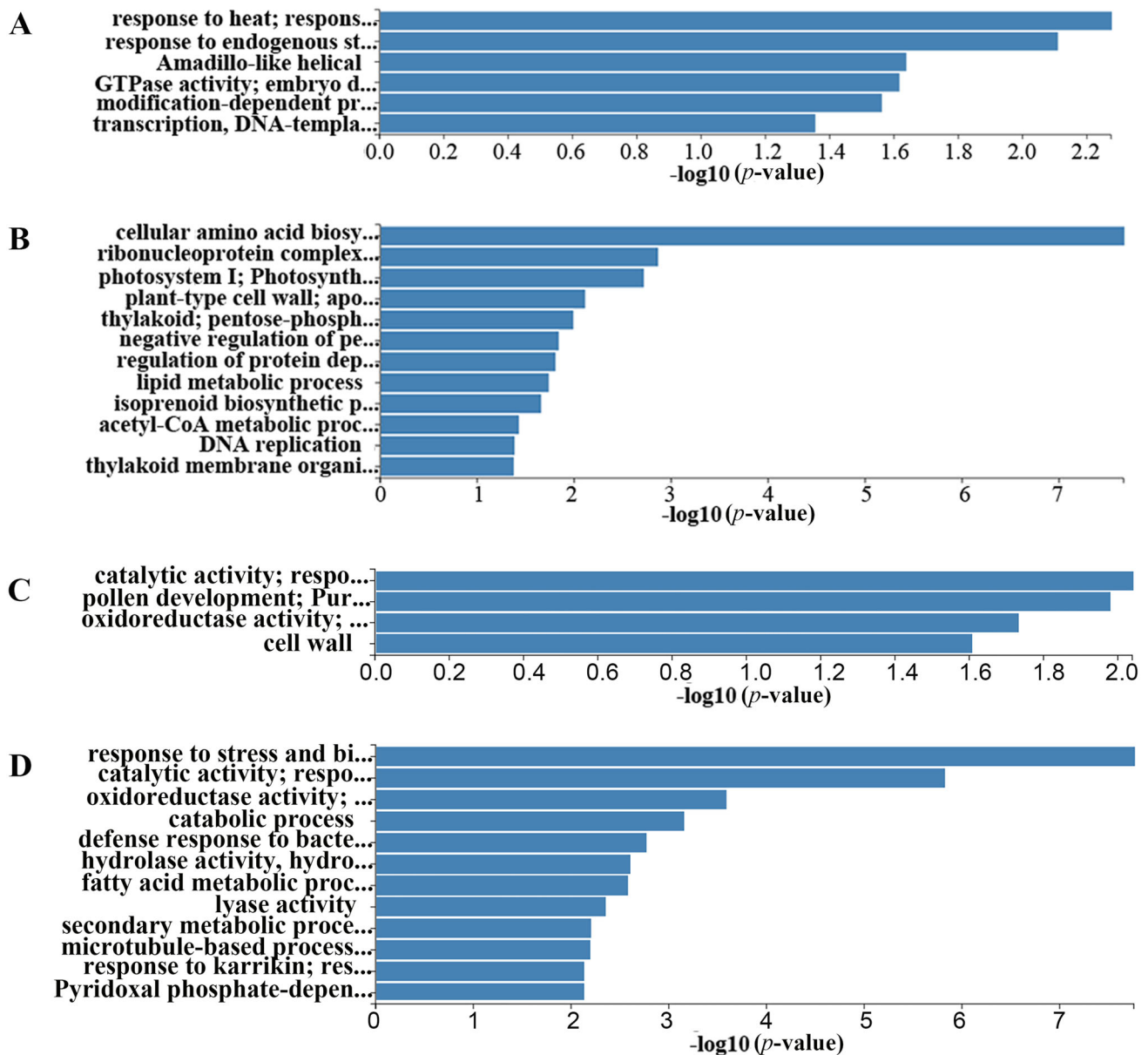


Fig. 6 Gene module enrichment analysis of exclusive identified DEGs in DC90 in response to early chilling stress. **A** up-regulated DEGs of gene set CRGs-2; **B** down-regulated DEGs of gene set

CRGs-2; **C** up-regulated DEGs of gene set CRGs-3; **D** down-regulated DEGs of gene set CRGs-3; X-axis represents the $-\log_{10}(p\text{-value})$; Y-axis represents the enriched gene module terms

chilling stress, through alteration of the expression of a large quantity of phytohormone responsive genes.

Aromatic amino acids biosynthesis and metabolism

Among the enriched pathways, ‘phenylalanine, tyrosine and tryptophan biosynthesis (osa00400)/phenylalanine metabolism (osa00360)’, ‘valine, leucine and isoleucine degradation (osa00280)’, ‘glycine, serine and threonine metabolism (osa00260)’, and ‘cysteine and methionine metabolism (osa00270)’ involve in biosynthesis and metabolism of amino acids, including aromatic amino

acids. Aromatic amino acids (phenylalanine, tyrosine and tryptophan) are synthesized through shikimate pathway and play important roles in plant growth, development, reproduction, defense, and environmental stimuli (Maeda and Dudareva 2012). Five enriched DEGs, *TSB1* (LOC_Os08g04180.1), *ASA2* (LOC_Os03g15780.1), *ASB1* (LOC_Os04g38950.1), *TSA* (LOC_Os07g08430.1), and *IGPS* (LOC_Os09g08130.2), which were enriched in ‘tryptophan metabolic process (cluster 1)’ and ‘catalytic activity (cluster 2)’ described above (Fig. 5D; Tables S6-2, S7-6), involve in five out of six reaction steps of tryptophan biosynthesis pathway. These genes are induced by rice

Table 2 KEGG enrichment of chilling responsive DEGs exclusive in DC90

Term	Type	Description	Query hits	Query total	Percentage (%)	<i>p</i> value	FDR
Gene set CRGs-2							
Up-regulated DEGs enrichment							
osa04075	KEGG	Plant hormone signal transduction	4	68	5.88	2.98E−05	5.36E−04
Down-regulated DEGs enrichment							
osa01100	KEGG	Metabolic pathways	20	103	19.42	3.15E−13	9.14E−12
osa00195	KEGG	Photosynthesis	5	103	4.85	2.12E−08	3.07E−07
osa01110	KEGG	Biosynthesis of secondary metabolites	11	103	10.68	6.46E−08	6.25E−07
osa03010	KEGG	Ribosome	7	103	6.79	3.65E−07	2.65E−06
osa00900	KEGG	Terpenoid backbone biosynthesis	4	103	3.88	1.75E−06	1.02E−05
osa00230	KEGG	Purine metabolism	4	103	3.88	7.14E−05	3.45E−04
osa00072	KEGG	Synthesis and degradation of ketone bodies	2	103	1.94	1.13E−04	4.66E−04
osa00480	KEGG	Glutathione metabolism	3	103	2.91	1.58E−04	5.11E−04
osa00630	KEGG	Glyoxylate and dicarboxylate metabolism	3	103	2.91	1.50E−04	5.11E−04
osa00650	KEGG	Butanoate metabolism	2	103	1.94	8.48E−04	2.46E−03
osa00920	KEGG	Sulfur metabolism	2	103	1.94	1.42E−03	3.74E−03
osa00280	KEGG	Valine, leucine and isoleucine degradation	2	103	1.94	2.35E−03	5.69E−03
Gene set CRGs-3							
Up-regulated DEGs enrichment							
None							
Down-regulated DEGs enrichment							
osa01100	KEGG	Metabolic pathways	25	140	17.86	2.50E−15	1.10E−13
osa01100	KEGG	Biosynthesis of secondary metabolites	17	140	12.14	2.15E−12	4.73E−11
osa00400	KEGG	Phenylalanine, tyrosine and tryptophan biosynthesis	5	140	3.57	8.49E−08	1.24E−06
osa00360	KEGG	Phenylalanine metabolism	4	140	2.86	4.19E−05	4.61E−04
osa00940	KEGG	Phenylpropanoid biosynthesis	4	140	2.86	6.99E−05	5.67E−04
osa00592	KEGG	Alpha-Linolenic acid metabolism	3	140	2.14	7.73E−05	5.67E−04
osa00591	KEGG	Linoleic acid metabolism	2	140	1.43	7.64E−04	3.73E−03
osa00280	KEGG	Valine, leucine and isoleucine degradation	3	140	2.14	1.36E−04	8.54E−04
osa00260	KEGG	Glycine, serine and threonine metabolism	3	140	2.14	2.61E−04	1.44E−03
osa00270	KEGG	Cysteine and methionine metabolism	3	140	2.14	8.76E−04	3.85E−03
osa04626	KEGG	Plant-pathogen interaction	3	140	2.14	1.17E−03	4.66E−03

Statistical test method Fisher, Multi-test adjustment method Hochberg (FDR) \leq 0.01 as threshold

bacterial and fungal pathogens, such as, *Agrobacterium tumefaciens*, *Xanthomonas oryzae*, and *Magnaporthe grisea*, respectively (Dharmawardhana et al. 2013), implying the positive roles of phenylpropanoid biosynthesis pathway in plant defense. ‘Phenylpropanoid biosynthesis (osa00940)’ pathway synthesizes phenylpropanoids and transferases to generate an enormous number of secondary metabolites (Vogt 2010). Phenylpropanoids play a wide range of important roles in plants, including protectants against biotic and abiotic stresses, for instance, anti-pathogenic phytoalexins, antioxidants and UV-absorbing compounds (Vogt 2010). This pathway has been found

over-presented both in the analysis of up and down-regulated DEGs sets in response to drought and bacterial stresses in rice (Shaik and Ramakrishna 2013), suggesting that differential regulation of the synthesis results in different end-products, therefore, has different stress responsiveness.

Ribosome

KEGG pathway ‘ribosome (osa03010)’ was one of the highly enriched terms in our data (Table 2), the similar result also found to be reported in comparative analysis of

another transcriptome profile of rice under chilling stress (Bai et al. 2015). Meanwhile, all the enriched DEGs were sub-localized to chloroplast stroma. Ribosomal proteins are essential for the establishment of translation apparatus. Six enriched DEGs, including *RPS10* (LOC_Os01g73160.1), *RPS11* (LOC_Os02g33140.1), *RPL21* (LOC_Os02g15900.1), *RPS23* (LOC_Os03g60400.1), *RPL35a-3* (LOC_Os05g48220.1), and a 60S acidic ribosomal protein (LOC_Os01g13080.1), which were enriched in ‘chloroplast stroma (cluster 2)’ described above, were supposed to be the components of subunits of ribosome in chloroplast stroma (Fig. 5B; Tables 2, S6-2, S7-5). However, to date, how chloroplast ribosomal proteins involve in abiotic stress remain unknown. Many ribosomal proteins have been reported to be up- or down-regulated under various stress conditions. In *Arabidopsis*, a 60S acidic ribosomal protein (P2-A) was found to be induced in response to cold stress in the nuclear proteomic analysis (Bae et al. 2003). *rpL33*, a chloroplast encoded ribosomal protein, is essential for plant survival under cold stress. The mutation of *rpL33* severely compromises the recovery of plants from low temperature stress (Rogalski et al. 2008). Interestingly, similar to our data, the expression of rice 60S ribosomal protein L32 (*rpL32*) encoded gene, *rpL32*, has been shown significant down-regulation under salt stress in rice (Mukhopadhyay et al. 2011). This implies that ribosomal proteins play important roles in early chilling stress regulation.

Photosynthesis pathway

Five chloroplast stroma located DEGs enriched in photosynthesis pathway (Tables 2, S6-2, S7-5). Three of them, LOC_Os07g04840.1 (*PsbP*), LOC_Os01g71190.1 (*Psb28*), and LOC_Os01g56680.1 (*PsbW*), were the components of photosystem II (PS II) in chloroplasts. The other two DEGs, LOC_Os01g64120.1 (*PetF*) and LOC_Os03g61960.2 (*PetF*), were involved in photosynthetic electron transport. In higher plants, six core intrinsic proteins, D1, D2, CP43, CP47, and two subunits of *cyt b559*, are required for oxygen evolution by PSII. *PsbP* and *Psb28* are extrinsic proteins of PSII, which is conserved among from cyanobacteria to higher plants (Thornton et al. 2004; Sakata et al. 2013). *PsbP* was found to interact with the coat protein (CP) of *Alfalfa mosaic virus* (AMV) and inhibit virus replication. Overexpression of *PsbP* significantly reduces virus replication in infected leaves (Balasubramaniam et al. 2014). *Psb28-1*, one of the two homologs of *Psb28* in *Synechocystis sp.* PCC 6803, plays an important role in PSII repair at high temperatures (Sakata et al. 2013). *PsbW* is exclusively identified in photosynthetic eukaryotes and associates with PS II protein complex. It is important for the contact and stability

between several PSII-LHCII supercomplexes (Garcia-Cerdan et al. 2011). In our results, the observations of repression of photosystem genes in response to abiotic stress also can be seen in other studies, including rice (Degenkolbe et al. 2009; Shen et al. 2014), *Arabidopsis* (Hannah et al. 2005), barley (Svensson et al. 2006), tomato (Liu et al. 2012), although, relative to cold-sensitive ones, the expression of those genes are less repressed in cold-tolerant genotypes. While plants exposed to environmental stimuli (such as salt, cold, moderate heat and oxidative stress etc.), an excess of light energy will damage photosynthetic machinery, including PS II, and therefore, cause photoinhibition (Takahashi and Murata 2008; Takahashi and Badger 2011). Photoinhibition results from the suppression of repair of damaged PS II, but not directly damage to PS II (Nishiyama et al. 2006; Murata et al. 2007). The increasing intracellular level of reactive oxygen species (ROS) inhibits translation of the factors (D1 protein), which required for the repair of PS II. In response to photoinhibition, plant evolves photoprotection mechanisms, such as ROS scavenging, to alleviate the inhibition of PS II repair by the consumption and dissipation of excess light energy (Nishiyama et al. 2004). Interestingly, *PsbS*, a component of PS II, was found to be involved in photoprotection. Mutated allele of *PsbS* has a pivotal role in excess light energy dissipation (Johnson and Ruban 2010). Therefore, we tend to speculate that *Psb28*, *PsbW*, and *PsbP*, three down-regulated DEGs identified in our results, similar to *PsbS*, also involve in photoprotection and confer GXWR chilling tolerance. In addition, sub-localization analysis showed 31.71 % of total DEGs located in chloroplasts (Fig. S7), suggesting that chloroplasts are one of the most important organelles in response to early chilling stress.

Taken these together, the chloroplast sub-localized ribosomal DEGs and their chilling stress responsiveness presented here suggest there is connection between PS II photoprotection mechanism and chloroplast translation machinery in response to early chilling stress. This arouse us to question what are their roles in plant chilling stress tolerance? However, recent literature does not adequately to address these observations. Therefore, this still needs to be further investigation.

Co-localization of chilling responsive DEGs in the introgression segments and QTL intervals

The exclusively identified DEGs in DC90 reflected the effect of the introgression of donor segments. For identifying the probable relationship among identified DEGs, chilling-resistant QTLs, and early chilling responsiveness, we co-localized previously reported chilling-resistant QTLs (<http://archive.gramene.org/qtl/>) (Table S8) (Yan

et al. 1999; Saito et al. 2001; Takeuchi et al. 2001; Abe et al. 2002; Cai and Morishima 2002; Andaya and Mackill 2003a, 2003b) and DEGs in gene sets CRGs-2 and CRGs-3 onto the introgression segment of DC90. Of which, only 3 QTLs and 13 DEGs (1.97 % of total) were co-localized onto this introgression segment (Fig. 7A; Table 3; Fig. S1B). Co-localized DEGs included translation initiation factor (LOC_Os12g31880.1), guanylate kinase (LOC_Os12g33100.1), calmodulin binding protein (LOC_Os12g36110.1), glycosyltransferase (LOC_Os12g36870.1), serine hydroxymethyltransferase (LOC_Os12g22030.1), carboxyvinyl-carboxyphosphonate phosphorylmutase (LOC_Os12g08760.1), serine/threonine-protein kinase AFC2 (LOC_Os12g27520.1), ATPase 3 (LOC_Os12g28177.1), transporter family protein (LOC_Os12g32760.1), pathogenesis-related Bet v I family protein (LOC_Os12g36850.1). The expressions of partial co-localized DEGs were validated by qRT-PCR (Fig. 7B; Table 3). Majority of these genes were differentially up-regulated in DC90 and 9311, respectively. Glycosyltransferase (LOC_Os12g36870.1) has been reported to conjugate ABA with UDP-D-glucose to regulate ABA activity in response to abiotic stress (Danquah et al. 2014). Calmodulin (CaM) is one of Ca²⁺-sensor protein, playing important roles in the control of plant growth and development, and in plant responding to environmental stimuli (Chantarachot et al. 2012). LOC_Os12g36110.1, a calmodulin binding protein, might involve in chilling stress response by binding to CaM. Moreover, Pathogenesis-related Bet v I family protein (LOC_Os12g36850.1), is a member of the ubiquitous PR-10 family (Radauer et al. 2008). PR-10 genes are differentially expressed in the presence of signaling molecules, and a number of biotic stresses and abiotic stresses (Jain and Kumar 2015). Therefore, the co-localized QTLs and stress associated DEGs in introgression region suggested that the roles what do they play in the alteration of the transcriptomic profile of DC90 to adapt to the early chilling stress. However, 13 co-localized DEGs did not

belong to any of over-presented KEGG pathways. This suggests that the co-localized DEGs might play roles at upstream part of the genetic regulation network to mediate rice early chilling stress tolerance, or they are not the candidates for the chilling tolerant QTL at the locus at all. If it's the latter, the candidate of the QTL may not enhance chilling tolerance in DC90 through alteration of its expression level but through other unknown regulatory strategy. If so, it is interesting to isolate the candidate(s) for this key locus for early chilling stress tolerance in rice in the future.

Conclusion

In this study, RNA sequencing was performed to comparatively analyze transcriptome of two rice genotypes with contrasting chilling stress tolerance at seedling stage under chilling stress. Our results show that the introgression segment on the short arm of chromosome 12 in DC90 results in transcriptomic reprogramming in response to early chilling stress. The DEGs exclusively identified in DC90 under early chilling stress might contribute to enhanced chilling stress tolerance. CARMO analysis presented us a scenario that complicated regulatory networks, such as, 'phytohormone signaling', 'ribosome components', 'photosynthesis pathway', 'phenylpropanoid biosynthesis', 'phenylalanine, tyrosine and tryptophan biosynthesis', etc., coordinately involves in early chilling stress response. These results extend our understanding the molecular mechanisms of chilling stress tolerance in GXWR.

Based on the above analysis, we suggest that the compensation of the phytohormone signaling, reduction of functional photosynthesis and ribosomal proteins, and phenylpropanoid biosynthesis, might contribute to gain cold tolerance in rice cold sensitive genotypes. Although we could not find out the connection among chilling

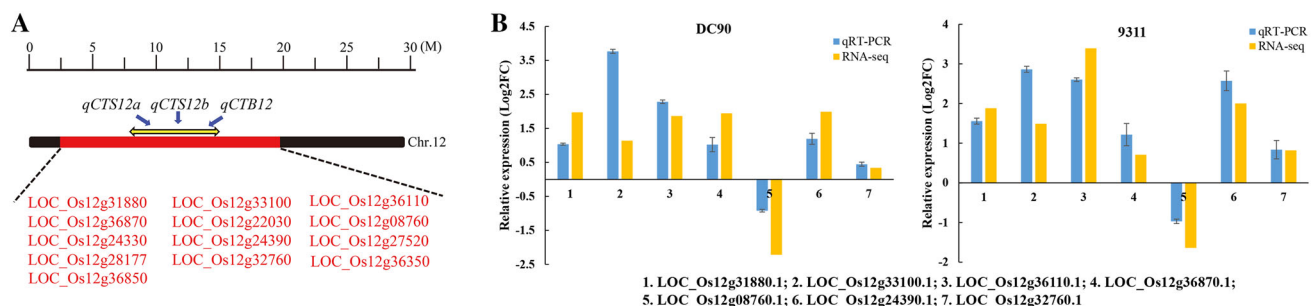


Fig. 7 The co-localization of DEGs and previously identified chilling-resistant QTLs onto introgression segment. **A** The diagram shows the DEGs co-localized with previous identified QTLs; **B** Validation of RNA-seq results of partial co-localized DEGs by qRT-PCR; Black bars represent genomic regions of chromosome 12

of 9311; red bar represents the introgression genomic segment derived from donor parent, DP15; yellow double headed arrow represents the region where previous reported QTLs located. LOC ids indicate the co-localized DEGs identified in this study. (Color figure online)

Table 3 List of co-localized DEGs on the introgression segment of DC90

BGI_ID ^a	LOC_ID ^b	Chr.	Relative expression level ^c		MSU putative function
			DC90	9311	
BGIOSGA037444	LOC_Os12g31880.1	12	1.9746	1.883	Translation initiation factor, putative, expressed
BGIOSGA036091	LOC_Os12g33100.1	12	1.1382	1.4906	Guanylate kinase, putative, expressed
BGIOSGA036047	LOC_Os12g36110.1	12	1.8661	3.3939	Calmodulin binding protein, putative, expressed
BGIOSGA037573	LOC_Os12g36870.1	12	1.9439	0.70949	Uncharacterized glycosyltransferase, putative, expressed
BGIOSGA036327	LOC_Os12g22030.1	12	0.5897	N	Serine hydroxymethyltransferase, mitochondrial precursor
BGIOSGA037112	LOC_Os12g08760.1	12	-2.2166	-1.6391	Carboxyvinyl-carboxyphosphonate phosphorylmutase
BGIOSGA036292	LOC_Os12g24330.1	12	1.6381	2.4154	Expressed protein
BGIOSGA037319	LOC_Os12g24390.1	12	1.9921	2.0042	Retrotransposon protein, putative, unclassified, expressed
BGIOSGA036231	LOC_Os12g27520.1	12	0.1289	0.6139	Serine/threonine-protein kinase AFC2, putative
BGIOSGA037368	LOC_Os12g28177.1	12	5.7009	4.4164	ATPase 3, putative, expressed
BGIOSGA036109	LOC_Os12g32760.1	12	0.3415	0.82011	Transporter family protein, putative, expressed
BGIOSGA037550	LOC_Os12g36350.1	12	1.4683	1.9938	Expressed protein
BGIOSGA037571	LOC_Os12g36850.1	12	0.56954	2.5731	Pathogenesis-related Bet v I family protein, putative

^a Indicates gene model ID of sequenced *indica* rice, 9311

^b Indicates gene model ID of *japonica* rice, Nipponbare;

^c Positive (up-regulated) and negative (down-regulated) values indicate Log₂ transformation of chilling stress/control ratio; 'N' represents FPKM of designated gene under treated and untreated conditions is zero, respectively

resistant QTL, co-localized DEGs, and over-presented KEGG pathways, all the genes mentioned here will provide valuable GXWR gene resources for further cold tolerant mechanism investigation and cold tolerant breeding application in rice. Next, we will perform functional verification of these genes in our subsequent research plan.

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Author's contribution Jijing Luo and Rongbai Li conceived and designed the experiments. Yongliang Wang and Qigui Jiang performed the experiments. Jijing Luo, Yongliang Wang, Qigui Jiang, Jianbin Liu, Wei Zeng, and Yindi Zeng did the field trials and collected samples for RNA-seq, and further data processing. Jijing Luo and Rongbai Li drafted the manuscript. Rongbai Li revised the manuscript. All authors approved the final version of manuscript.

Compliance with ethical standards

Conflict of interest Yongliang Wang declares no conflict of interest; Qigui Jiang declares no conflict of interest; Jianbin Liu declares no conflict of interest; Wei Zeng declares no conflict of interest; Yindi Zeng declares no conflict of interest; Rongbai Li declares no conflict of interest; and Jijing Luo declares no conflict of interest.

Studies with human or animal research This article does not contain any studies with human subjects or animals performed by any of the authors.

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