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Physiological and genome-wide gene expression analyses of cold-induced leaf rolling at the seedling stage in rice (*Oryza sativa* L.)☆

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ABSTRACT

Leaf rolling and discoloration are two chilling-injury symptoms that are widely used as indicators for the evaluation of cold tolerance at the seedling stage in rice. However, the difference in cold-response mechanisms underlying these two traits remains unknown. In the present study, a cold-tolerant rice cultivar, Lijiangxintuanheigu, and a cold-sensitive cultivar, Sanhuangzhan-2, were subjected to low-temperature treatments and physiological and genome-wide gene expression analyses were conducted. Leaf rolling occurred at temperatures lower than 11 °C, whereas discoloration appeared at moderately low temperatures such as 13 °C. Chlorophyll contents in both cultivars were significantly decreased at 13 °C, but not altered at 11 °C. In contrast, the relative water content and relative electrolyte leakage of both cultivars decreased significantly at 11 °C, but did not change at 13 °C. Expression of genes associated with calcium signaling and abscisic acid (ABA) degradation was significantly altered at 11 °C in comparison with 25 °C and 13 °C. Numerous genes in the *DREB*, *MYB*, *bZIP*, *NAC*, *Zinc finger*, *bHLH*, and *WRKY* gene families were differentially expressed. Many aquaporin genes and the key genes in trehalose and starch synthesis were down regulated at 11 °C in comparison with 25 °C and 13 °C. These results suggest that the two chilling injury symptoms are temperature-specific and are controlled by different mechanisms. Cold-induced leaf rolling is associated with calcium and ABA signaling pathways and is regulated by multiple transcriptional regulators. The suppression of aquaporin genes and reduced accumulation of soluble sugars under cold stress results in a reduction in cellular water potential and consequently leaf rolling.

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1. Introduction

Cold stress is one of the main abiotic stresses affecting the growth and distribution of rice globally. More than 15 Mha of rice worldwide suffer from cold damage at various growth stages, and about 7 Mha of land are unsuitable for rice cultivation in south and southeast Asia owing to low-temperature stress [1]. Cold stress at the seedling stage in rice can lead to slowed growth, delayed maturation, and poor establishment, ultimately leading to decreases in yield. Accordingly, cold tolerance at the seedling stage is considered to be an important target in rice breeding.

The response of rice to cold stress is complex. Compared with cold-sensitive rice cultivars, cold-tolerant cultivars are more vigorous [2], show lower reductions in chlorophyll content [3,4], have higher leaf water contents [4,5], and have higher levels of free polyamine [6] under cold stress. Genetic studies suggest that the cold tolerance in rice is a quantitative trait, and many cold-tolerant quantitative trait loci (QTL) at the seedling stage in rice have been identified and mapped [7–11]. The diversity of reactions and numerous QTL identified for cold tolerance in rice suggest the existence of diverse response mechanisms in cold stress tolerance. It is thus critical to identify the cold stress resistance mechanisms so that effective strategies can be developed to alleviate cold damage in rice.

With the rapid development of genome sequencing and microarray technologies in the past decade, RNA sequencing and microarray analysis have emerged as powerful tools for dissecting the mechanisms of the cold stress response in plants. Genome-wide gene expression profiling of cold stress has been performed in rice [12–18]. Gene expression profiling of rice under cold, drought, high-salinity, and abscisic acid (ABA) treatment revealed [12] that some cold-induced genes overlapped with ABA-responsive genes, and these cold-induced genes contained ABA-responsive elements (*ABREs*) in their promoter regions, suggesting the importance of ABA in the cold stress response in rice. Our previous study [16] also showed that genes associated with auxin, ABA, and salicylic acid (SA) metabolism or signal transduction were differentially expressed, implying the involvement of these phytohormones in the regulation of response to cold stress in rice. Moreover, many ABA-responsive genes were significantly upregulated, and six of the 10 enriched binding sites of the upregulated genes ABADESI1, ABREOSRAB21, ACGTABOX, ACGTOSGLUB1, ABREZMRAB28, and ACGTABREMOTIFA2OSEM are ABRE motifs, suggesting the active involvement of ABA signal transduction in cold response [16]. Owing to the pivotal role of transcription factors (TFs) in the regulation of gene expression in response to stress, TFs have become the primary focus in genome-wide gene expression analyses in many studies. Comparative transcriptome profiling of cold stress in two contrasting rice genotypes showed that *OsDREB1A*, *OsDREB1B*, and *OsDREB2B* were upregulated during cold stress [15]. Similar results were observed in rice by Dubouz et al. [19] as well as in our previous study [16]. These results suggest that the *DREB1/CBF* regulatory pathway plays a key role in rice cold response. In addition to *DREBs*, many members of the *WRKY*, *NAC*, *MYB* gene families have been observed to be differentially expressed under cold stress [15,16], indicating the existence of multiple regulatory pathways in rice under cold stress. These studies identified numerous genes that are potentially involved in the response to cold stress in rice, and provide valuable information for understanding the mechanisms of cold stress response in rice.

However, the response of rice to cold stress is complex, and the cited studies have not focused on elucidating cold stress phenotypes. Different reactions or phenotypes are observed when the rice plant is subjected to different cold environments, and may represent different underlying response mechanisms. Associating the observed cold stress phenotypes with genes showing altered expression would help to elucidate the mechanisms underlying the cold stress response.

Generally, rice seedlings show leaf rolling or leaf discoloration when subjected to cold stress, depending on the cold environment imposed. These two chilling injury symptoms have been widely adopted as indicators for the evaluation of cold tolerance at the seedling stage in rice. Identifying the response mechanisms that cause leaf rolling and leaf discoloration at the genome-wide level would thus enhance our understanding of cold stress tolerance in rice and facilitate molecular breeding for rice cold tolerance. Current genome-wide gene expression profiling studies have not focused on explaining these two chilling-injury phenotypes. Leaf discoloration under cold stress is associated mainly with reduction in chlorophyll content [3,4]. In contrast, the mechanisms of cold-induced leaf rolling are more complex. Although it is known that leaf rolling is associated with water balance, it is not known how low temperatures affect water balance and how leaf rolling occurs at a genome-wide level.

In the present study, a cold-tolerant *japonica* and a cold-sensitive *indica* cultivar were subjected to low-temperature treatments and physiological and genome-wide gene expression analyses were conducted. To identify the genes associated with leaf rolling and elucidate the causal mechanisms of leaf rolling under cold stress, the gene expression profiles at the low temperatures that induced leaf rolling and leaf discoloration were compared with those at 25 °C. Differentially expressed genes were associated with water metabolism based on gene annotations and the literature. Our results demonstrated that the two chilling injury symptoms were controlled by different mechanisms. Based on a comparison of the genome-wide gene expression profiling of rice at 11 °C, 13 °C, and 25 °C, a model to describe the mechanisms of cold-induced leaf rolling is proposed. Our study provides new insights into the response of rice to cold stress. Since leaf rolling and discoloration constitute two common chilling injury symptoms and indicators of cold tolerance, the results should inform the discovery of genes associated with cold tolerance and facilitate molecular breeding for cold tolerance at the seedling stage in rice.

2. Materials and methods

2.1. Plant material, cold treatment, and sample preparation

Lijiangxintuanheigu (LTH), a cold-tolerant *japonica* cultivar, and Sanhuangzhan-2 (SHZ-2), a cold-sensitive *indica* cultivar, were used. After incubation at 49 °C for 96 h to break dormancy, seeds were soaked in water for 36 h and then incubated at 30 °C for 48 h for pre-germination. Germinated seeds were sown in plastic trays (41 cm × 26.5 cm × 7.5 cm) filled with fine field soil. The

germinated seeds of the two cultivars were planted in a tray with six rows, with 11 germinated seeds per row for each cultivar. Six replicates were included in each temperature treatment. The seedlings were allowed to grow for two weeks in a Conviron PGV36 growth chamber (Controlled Environments Ltd., Winnipeg, Canada) maintained at a constant temperature of 25 °C, 13 h daylight at a light intensity of 237 μmol m^{-2} s^{-1}, and relative humidity of 75% ± 5%.

For the cold treatment, six trays of seedlings representing three replicates were subjected to cold treatment for each of the selected low temperatures. Although the temperature was varied, the day length, light intensity, and humidity remained the same in the growth chambers for each experiment.

For RNA sequencing (RNA-seq), the second and third leaves of two-week-old seedlings were harvested 24 h after cold treatment. The samples were rapidly frozen in liquid nitrogen and stored at –80 °C until use. Three biological experiments and three replicates in an experiment were used for each cold treatment and control. The samples extracted from two of the three biological experiments at 25 °C, 13 °C, and 11 °C were used for RNA-seq analyses.

2.2. Measurement of leaf chlorophyll content, relative water content, and electrolyte leakage

After the two-week-old seedlings were subjected to cold treatment for 8 days, the middle parts of the second expanded leaves from the top of the tested seedlings were used for chlorophyll content measurement using a SPAD-502 chlorophyll meter (Konica Minolta Sensing, Osaka, Japan). The chlorophyll content was calculated as $Y = 0.0996X - 0.152$ (Y: chlorophyll content; X: the reading from the chlorophyll meter). Rolled leaves were unrolled prior to measurement. Three replicates were performed in each experiment.

Leaf water content was measured following Wang et al. [20]. After seedlings were subjected to cold treatment for 5 days, the leaves were cut into pieces. The fresh weight of the cut leaves was measured, and then the leaves were oven-dried at 90 °C for 72 h and weighed to determine their dry weight. Relative leaf water content was calculated as follows:

$$\text{Relative leaf water content } (\%) = [(\text{fresh weight} - \text{dry weight})/\text{fresh weight}] \times 100\%.$$

Three replicates were performed in each experiment.

Electrolyte leakage was measured following Song et al. [21]. After chilling injury symptoms (discoloration or wilting) were observed in the growth chambers (13 °C for 8 days and 11 °C for 5 days), 0.1 g of leaf material was collected and washed several times in ultrapure water and then immersed in 20 mL ultrapure water for 24 h. The electrical conductivity of the resulting solution was measured and defined as R1. This solution was placed in boiling water for 20–30 min. After cooling, the electrical conductivity was measured and defined as R2. The relative electrolyte leakage was expressed as follows:

$$\text{Relative electrolyte leakage } (\%) = (R1/R2) \times 100\%.$$

Three replicates were performed in each experiment.

2.3. Total RNA extraction

The second and third leaves of two-week-old seedlings were harvested 24 h after cold treatment and total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified with NucleoSpin RNA Clean-up (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instructions. RNA quality and quantity were assessed by formaldehyde denaturing agarose gel electrophoresis and spectrophotometry (Nanodrop-1000, Thermo Fisher Scientific, Waltham, MA, USA), respectively.

2.4. Real-time quantification of mRNAs

The purified total RNA was reverse-transcribed using the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan) to generate cDNA. Real-time PCR was performed using SYBR Ex*Taq* (Takara Bio Inc., Shiga, Japan). The *EF1 alpha* gene was chosen as a reference control. Gene expression was quantified by the comparative CT method. Experiments were performed in triplicate, and the results were represented as mean ± standard derivation (SD). The primers used are listed in Table S1.

2.5. Data analysis

Differentially expressed genes (DEGs) were identified using the combined criteria of threefold change and a cutoff *P*-value of ≤0.05 based on two biological replicates. Hierarchical cluster analysis of the DEGs was performed using software GENE CLUSTER 3.0 [22]. Gene Ontology (GO) analysis was performed using the Singular Enrichment Analysis tool in agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) using the default settings of the Fisher's *t*-test ($P < 0.05$) and false discovery rate (FDR) correction by the Hochberg method. A minimum of five mapping entries against the species-specific pre-computed background reference of the DEGs were analyzed with OSIRIS (http://www.bioinformatics2.wsu.edu/cgibin/Osiris/cgi/home.pl [23]).

The original RNA sequencing data of this study have been deposited in NCBI's Gene Expression Omnibus (http://www.ncbi.nih.gov/geo/) under GEO series number GSE112547.

3. Results

3.1. Relationship between chilling injury symptoms and low-temperature treatments

SHZ-2 seedlings began yellowing after 14 °C treatment for 8 days or 13 °C for 6 days, whereas LTH seedlings began yellowing after 14 °C treatment for 9 days or 13 °C treatment for 7 days (Fig. 1-B). In contrast, SHZ-2 seedlings began leaf rolling after 11 °C treatment for 4 days or 8 °C treatment for 3 days, whereas leaves of LTH seedlings began rolling after 11 °C treatment for 5 days (Fig. 1-C) or 8 °C treatment for 4 days. Under the given light intensity and humidity, the chilling symptom of rice seedlings was yellowing under moderately low temperatures, including 14 °C or 13 °C, whereas leaf rolling occurred under lower temperatures (11 °C and 8 °C). Accordingly, the two closest temperatures, 13 and 11 °C, were selected to represent the cold

environments associated with leaf discoloration and leaf rolling for the given light intensity and humidity and were used for investigating the associated physiological and molecular mechanisms.

3.2. *Leaf chlorophyll content, relative water content, and relative electrolyte leakage of the two cultivars under 13 °C and 11 °C cold stress*

The leaf chlorophyll contents of the two cultivars grown at 13 °C were significantly lower than those at 25 °C and 11 °C ($P < 0.01$), and no significant difference was observed between 25 °C and 11 °C ($P > 0.05$) (Fig. 1-D). The relative leaf water contents of the two cultivars significantly decreased after 13 °C or 11 °C treatment for 5 days compared to those grown at 25 °C ($P < 0.05$), but a lower water content was observed at 11 °C than at 13 °C (Fig. 1-E). No significant change in leaf relative electrolyte leakage of the two cultivars was observed after 13 °C cold stress for 8 days compared to that at 25 °C ($P > 0.05$). In contrast, the leaf relative electrolyte leakage of the two cultivars increased significantly after cold treatment at 11 °C for 5 days ($P < 0.01$), and the increase in SHZ-2 was much greater than the increase in LTH (Fig. 1-F).

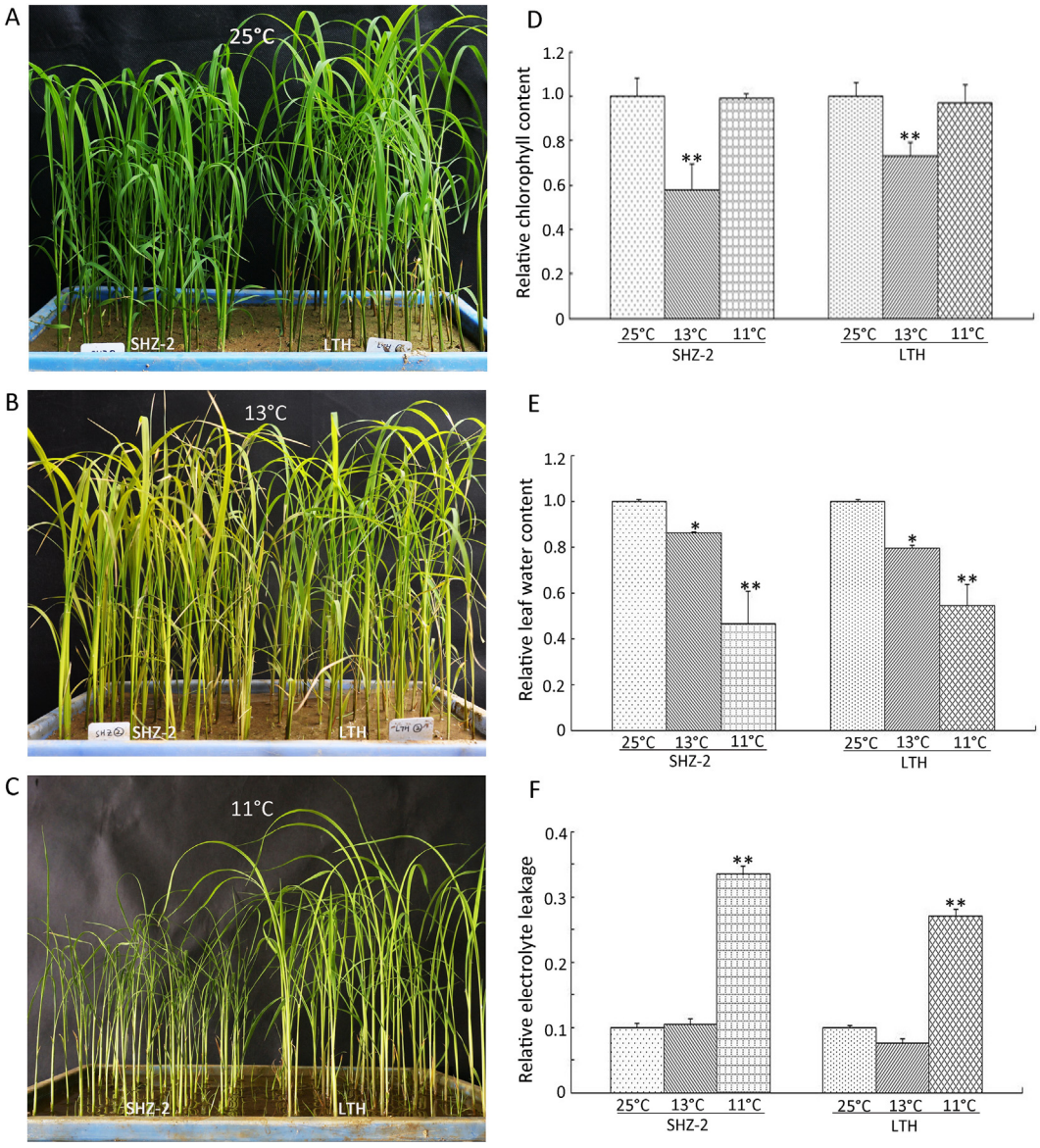

Fig. 1 – Leaf phenotypes, chlorophyll contents, relative water contents, and relative electrolyte leakages of LTH and SHZ-2 at 25 °C, 13 °C, and 11 °C. (A) Seedlings of SHZ-2 and LTH grown at 25 °C (CK). (B) Seedlings of SHZ-2 and LTH grown at 13 °C for 9 days, showing leaf discoloration symptoms. (C) Seedlings of SHZ-2 and LTH grown at 11 °C for 5 days, showing leaf rolling symptoms. (D) Relative chlorophyll contents of the two rice cultivars grown at 25 °C, 13 °C, and 11 °C. (E) Relative leaf water contents of SHZ-2 and LTH seedlings at 25 °C, 13 °C and 11 °C. (F) The relative electrolyte leakage of the SHZ-2 and LTH seedlings at 25 °C, 13 °C and 11 °C. * indicates a significant difference at $P < 0.05$ compared with that at 25 °C; ** indicates a significant difference at $P < 0.01$ compared with that at 25 °C.

3.3. DEGs under 13 °C and 11 °C cold stress

Overall, 5103 and 4906 genes were upregulated, whereas 3650 and 2780 genes were downregulated under 11 °C and 13 °C cold stress, respectively. Of these, respectively 3027 and 1758 were commonly regulated at both temperatures (Fig. 2-A).

To confirm the results of RNA-Seq, 21 genes showing different expression levels under 13 °C and 11 °C cold stress in the RNA-Seq analyses were selected for quantitative real-time (qRT) PCR assays. Strong linear relationships were observed between the results from RNA-Seq and qRT-PCR results (R^2 = 0.855 for 11 °C cold stress, R^2 = 0.895 for 13 °C cold stress), suggesting that the gene expression values obtained from RNA-Seq were reliable (Fig. 2-B, C). Furthermore, the hierarchical clustering of the DEGs indicated similar gene expression patterns between the two biological replicates (designated as I and II) for a given low-temperature stress, further supporting the reproducibility and reliability of the genome-wide expression profiling (Fig. 2-D).

3.4. GO enrichment analyses of DEGs identified at 11 °C and 13 °C

Among the significant GO terms at 11 °C compared to 13 °C, "transcription (GO: 0006350)", "regulation of transcription (GO: 0045449)", and "regulation of gene expression (GO: 0010468)" were enriched only in upregulated genes, while "carbohydrate metabolic process (GO: 0005975)", "polysaccharide metabolic process (GO: 0005976)", and "carbohydrate biosynthetic process (GO: 0016051)" were enriched only in downregulated genes (Table S2).

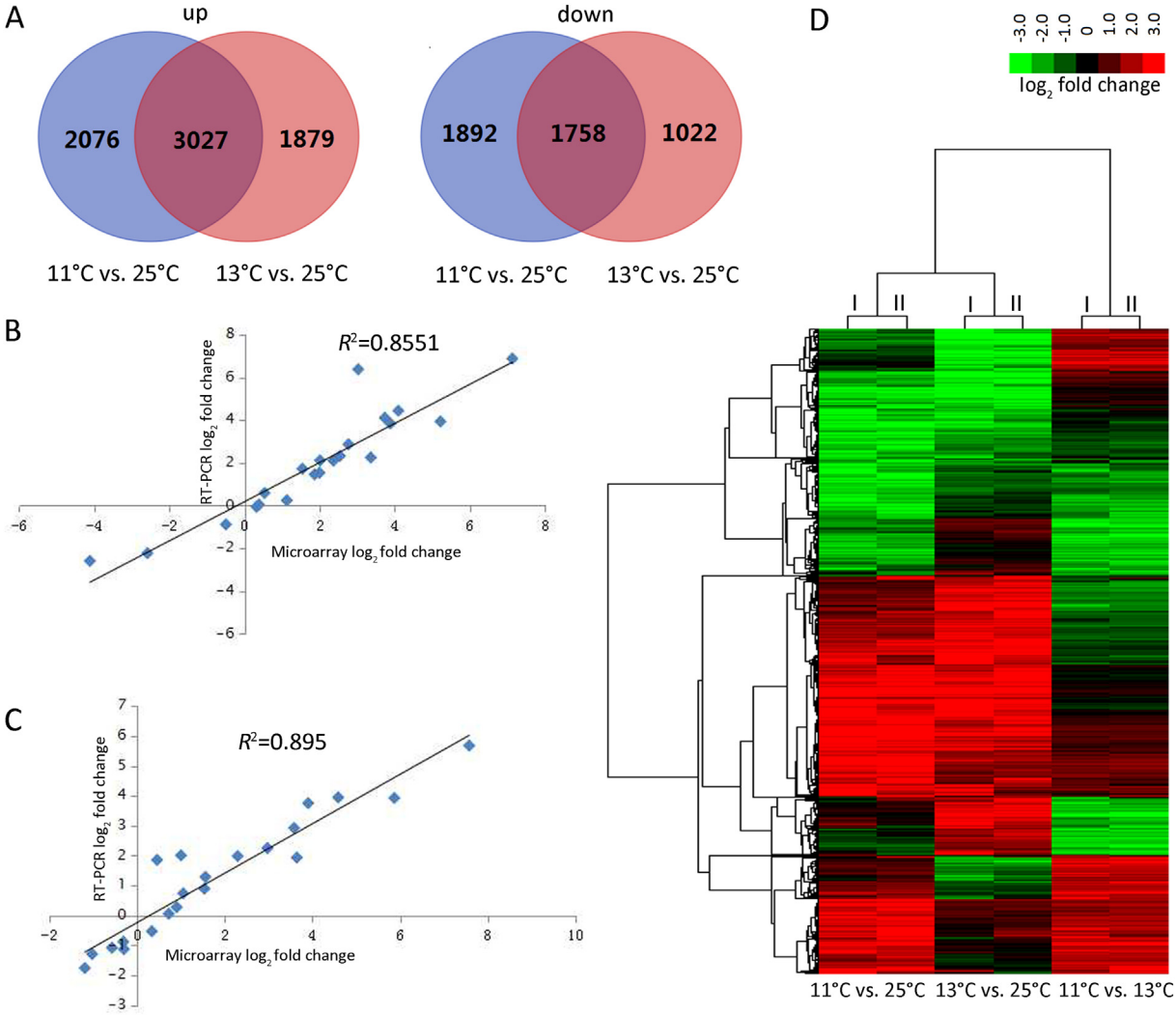

Fig. 2 – Gnome-wide gene expression profiling of LTH seedlings under 11 °C and 13 °C cold stresses. (A) Venn diagram of DEGs under 11 °C and 13 °C cold stresses. (B) Regression of qRT-PCR on RNA-Seq DEGs under 11 °C cold stress. (C) Regression of qRT-PCR on RNA-Seq DEGs under 13 °C cold stress. (D) Hierarchical cluster analysis of DEGs. DEGs were identified using the combined criteria of threefold change and a P-value cutoff of ≤0.05. Upregulated genes are shown in red, downregulated genes are shown in green, and no change is indicated in black. "13 °C vs. 25 °C" indicates relative gene expression at 13 °C in comparison to 25 °C. "11 °C vs. 25 °C" and "11 °C vs. 13 °C" indicate relative gene expression at 11 °C in comparison to 25 °C and 13 °C, respectively.

Table 1 – Expression patterns of 17 calmodulin genes in LTH during 11 °C cold stress.

MSUID[b]	11 °C vs. 25 °C	11 °C vs. 13 °C	Annotation[b]
	Log_2 fold change[a]	Log_2 fold change	
LOC_Os11g11340	4.36	0.85	Calmodulin-interacting protein 111
LOC_Os03g05470	3.32	1.60	Calmodulin-binding receptor-like cytoplasmic kinase 2
LOC_Os02g57560	3.18	1.36	Calmodulin-binding receptor-like cytoplasmic kinase 2
LOC_Os03g27080	2.87	0.62	Calmodulin-binding transcription activator 1
LOC_Os09g03620	2.55	1.47	Calmodulin-binding receptor-like cytoplasmic kinase 3
LOC_Os03g61060	1.98	1.34	Calmodulin-binding receptor-like cytoplasmic kinase 2
LOC_Os05g41090	1.95	1.66	Calcium and calcium/calmodulin-dependent serine/threonine-protein kinase
LOC_Os01g59530	1.77	0.29	Calmodulin-like protein 1
LOC_Os04g42480	1.76	0.94	Calmodulin-binding receptor-like cytoplasmic kinase 3
LOC_Os09g28480	1.54	0.64	Calmodulin-like protein 1
LOC_Os04g57140	1.29	0.79	Kinesin-like calmodulin-binding protein homolog
LOC_Os03g25070	1.11	0.27	Calcium/calmodulin-dependent serine/threonine-protein kinase 1
LOC_Os04g31900	0.95	1.21	Calmodulin-binding transcription activator 4
LOC_Os03g53200	0.41	2.43	Calmodulin-like protein 4
LOC_Os03g14020	−0.86	−1.79	Calmodulin-lysine *N*-methyltransferase
LOC_Os07g48780	−1.32	0.76	Calmodulin
LOC_Os09g28500	−3.13	3.17	Calmodulin

[a] log_2 fold change: the fold change of gene expression transformed to its log (base 2). "11 °C vs. 25 °C" and "11 °C vs. 13 °C" denote gene expression at 11 °C relative to those at 25 °C and 13 °C, respectively.
[b] Rice Genome Annotation Project – MSU Rice Genome Annotation (Osa1) Release 7: http://rice.plantbiology.msu.edu/.

3.5. *Differential expression of genes in the calcium signaling pathway during cold stress*

Calcium ion (Ca^{2+}), as a major secondary-messenger signaling molecule, plays a critical role in regulating the abiotic stress response in plants. Calcium signals are transduced via calmodulin-like proteins (CML), calcium-dependent protein kinases (CDPKs), and other Ca^{2+}-controlled proteins to influence a wide array of downstream responses involved in plant protection and toward a number of abiotic stresses, including low temperature, osmotic stress, heat, oxidative stress, anoxia, and mechanical perturbation. The expression levels of 12 and eight calmodulin genes increased more than twofold at 11 °C relative to their respective expression levels at 25 °C and 13 °C (Tables 1, S3). Five calmodulin genes were common to the two comparisons (Table 1). The expression levels of eight and nine CDPK genes increased more than twofold at 11 °C relative to their respective expression levels at 25 °C and 13 °C (Table S3). Seven CDPK genes were common to the two comparisons (Table 2).

3.6. *Differential expression of ABA degradation-associated genes during cold stress*

Of the three ABA 8′-hydroxylase genes *OsABA8ox1* (LOC_Os02g47470), *OsABA8ox2* (LOC_Os08g36860), and *OsABA8ox3* (LOC_Os09g28390), *OsABA8ox1* was upregulated, while *OsABA8ox2* and *OsABA8ox3* were downregulated at 11 °C in comparison to 25 °C and 13 °C (Fig. 3-A).

Table 2 – Expression patterns of the 12 CDPK genes in LTH during 11 °C cold stress.

MSUID[b]	11 °C vs. 25 °C	11 °C vs. 13 °C	Annotation[b]
	Log_2 fold change[a]	Log_2 fold change[a]	
LOC_Os02g58520	5.25	3.67	Calcium-dependent protein kinase 4
LOC_Os02g03410	3.27	2.78	Calcium-dependent protein kinase 16
LOC_Os04g49510	2.79	1.96	Calcium-dependent protein kinase 4
LOC_Os05g39090	2.23	1.42	Calcium-dependent protein kinase 13
LOC_Os09g33910	2.20	−1.40	Calcium-dependent protein kinase 32
LOC_Os02g46090	1.52	2.42	Calcium-dependent protein kinase 5
LOC_Os03g62040	1.47	1.11	Calcium-dependent protein kinase isoform 11
LOC_Os01g43410	1.12	1.51	Calcium-dependent protein kinase 3
LOC_Os07g22710	0.97	1.10	Calcium-dependent protein kinase 16
LOC_Os07g33110	0.93	1.23	Calcium-dependent protein kinase isoform 2
LOC_Os07g06740	−2.32	−0.26	Calcium-dependent protein kinase 1
LOC_Os03g48270	−3.73	−1.62	Calcium-dependent protein kinase 30

[a] Log_2 fold change: the fold change of gene expression transformed to its log (base 2). "11 °C vs. 25 °C" and "11 °C vs. 13 °C" denote gene expression at 11 °C relative to those at 25 °C and 13 °C, respectively.
[b] Rice Genome Annotation Project - MSU Rice Genome Annotation (Osa1) Release 7: http://rice.plantbiology.msu.edu/.

3.7. Differential expression of TF genes during cold stress

In total, 296 TF genes were upregulated and 174 TF genes were downregulated at 11 °C compared to 25 °C, whereas 241 TF genes were upregulated and 161 TF genes were downregulated at 11 °C compared to 13 °C (Table S4).

Numerous genes in several important TF gene families, such as *AP2/ERF* (Fig. 3-B), *MYB* (Fig. 3-D), *bZIP* (Fig. 3-E), *NAC* (Fig. 3-F), *Zinc finger* (Fig. 3-G), *bHLH* (Fig. 3-H), and *WRKY* (Fig. 3-I), were differentially expressed at 11 °C relative to 25 °C and 13 °C. *OsDREB1D*, *OsDREB1H*, *OsDREB2B*, *OsDREB1A*, *OsDREB1B*, and *OsDREB1G* were upregulated, while *OsDREB1I* and *OsDREB3* were downregulated across the two comparisons (Fig. 3-C).

3.8. Differential expression of aquaporin genes during cold stress

Aquaporin genes have been reported to play distinct roles in facilitating water flux and maintaining the water potential in different tissues and cells. In the present study 17 and 11 aquaporin genes were downregulated at 11 °C in comparison to 25 °C and 13 °C, respectively (Table S5). The 11 aquaporin genes *OsPIP1-1* (LOC_Os02g44630), *OsPIP1-2* (LOC_Os04g47220), *OsNIP2-1* (LOC_Os02g51110), *OsPIP2-1* (LOC_Os07g26690), *OsPIP2-2* (LOC_Os02g41860), *OsTIP1-2* (LOC_Os01g74450), *OsPIP2-4* (LOC_Os07g26630), *OsPIP2-5* (LOC_Os07g26660), *OsTIP1-1* (LOC_Os03g05290), *OsPIP1-3* (LOC_Os02g57720), and

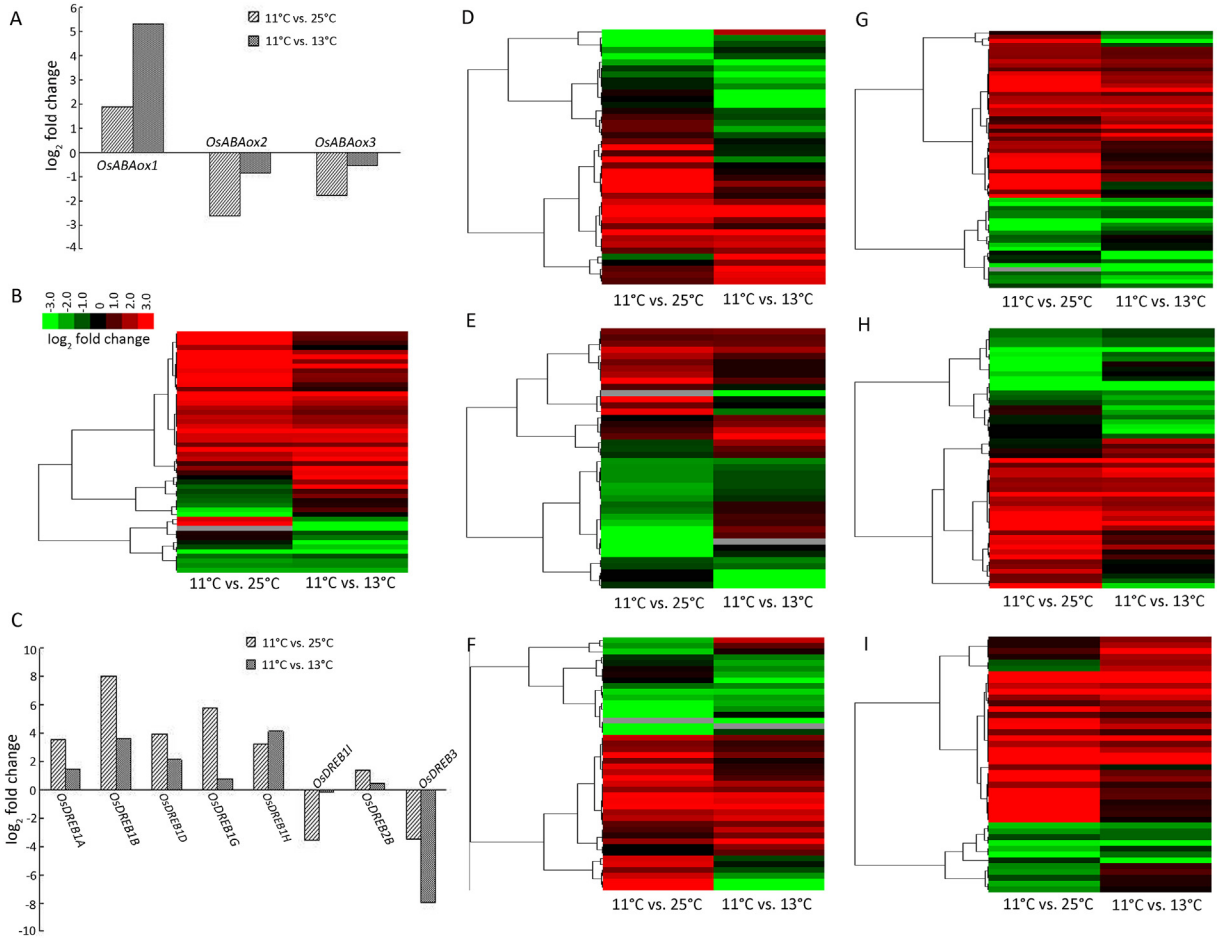

Fig. 3 – Expression levels of differentially expressed ABA degradation-associated genes and transcription factor genes at 11 °C relative to 25 °C and 13 °C. (A) Expression levels of three ABA degradation-associated genes at 11 °C relative to 25 °C and 13 °C. (B) Heat map view of the differential expression of *AP2/ERF* genes at 11 °C relative to 25 °C and 13 °C. (C) Expression levels of *OsDREB* genes at 11 °C relative to 25 °C and 13 °C. (D) Heat map view of the differential expression of *MYB* genes at 11 °C relative on to 25 °C and 13 °C. (E) Heat map view of the differential expression of *bZIP* genes at 11 °C relative to 25 °C and 13 °C. (F) Heat map view of the differential expression of *NAC* genes at 11 °C relative to 25 °C and 13 °C. (G) Heat map view of the differential expression of Zinc finger genes at 11 °C relative to 25 °C and 13 °C. (H) Heat map view of the differential expression of *bHLH* genes at 11 °C relative to 25 °C and 13 °C. (I) Heat map view of the differential expression of *WRKY* genes at 11 °C relative to 25 °C and 13 °C. The fold change of gene expression was log_2-transformed and subjected to hierarchical clustering analysis using GENE CLUSTER 3.0. Upregulated genes are shown in red, downregulated genes are shown in green, and no change is indicated in black. "11 °C vs. 25 °C" and "11 °C vs. 13 °C" indicate relative gene expression at 11 °C in comparison to 25 °C and 13 °C, respectively.

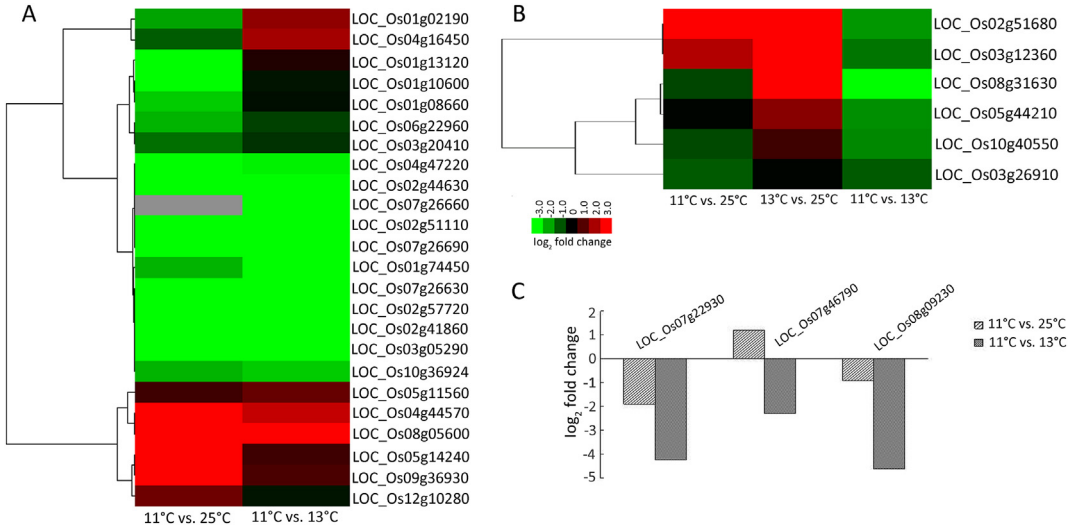

Fig. 4 – Relative expression levels of differentially expressed aquaporin genes and genes associated with carbohydrate metabolism at 11 °C in comparison to 25 °C and 13 °C. (A) Expression patterns of 24 aquaporin genes. (B) Heat map view of six differentially expressed trehalose-synthesis related genes in different low-temperature comparisons. (C) Relative expression levels of three DEGs in starch and sucrose metabolic pathways at 11 °C in comparison to 25 °C and 13 °C. The fold change of gene expression was log_2-transformed and subjected to hierarchical clustering analysis using GENE CLUSTER 3.0. Upregulated genes are shown in red, downregulated genes are shown in green, and no change is indicated in black. "13 °C vs. 25 °C" indicates relative gene expression at 13 °C in comparison to 25 °C. "11 °C vs. 25 °C" and "11 °C vs. 13 °C" indicate relative gene expression at 11 °C in comparison to 25 °C and 13 °C, respectively.

OsNIP3-1 (LOC_Os10g36924) were consistently downregulated in both comparisons (Fig. 4-A).

3.9. *Differential expression of genes associated with carbohydrate metabolic processes during cold stress*

"Metabolic process" (GO: 0008152) was the most common enriched GO category in both up- and downregulated genes at 11 °C in comparison to 25 °C. "Carbohydrate metabolic process" (GO: 0005975), "polysaccharide metabolic process" (GO: 0005976), and "carbohydrate biosynthetic process" (GO: 0016051) were enriched in the downregulated genes at 11 °C in comparison to 13 °C (Table S2). These results implied that carbohydrate metabolic processes were profoundly affected in rice under 11 °C cold stress. We further analyzed the expression patterns of genes associated with carbohydrate metabolism.

Trehalose has been reported to play an important role in metabolic regulation and abiotic stress tolerance in plants. Trehalose biosynthesis is catalyzed by two key enzymes: trehalose-6-phosphate phosphatase (TPP) and trehalose-6-phosphate synthase (TPS). In the present study, *OsTPP7* (LOC_Os02g51680) and *OsTPS11* (LOC_Os03g12360) were downregulated at 11 °C compared to 13 °C. The expression levels of *OsTPP6* (LOC_Os08g31630), *OsTPP3* (LOC_Os10g40550) and *OsTPS1* (LOC_Os05g44210) decreased more than twofold at 11 °C in comparison to 13 °C (Fig. 4-B, Table S6).

Among six DEGs in the starch and sucrose metabolism pathway, five genes (LOC_Os01g48200, LOC_Os01g34880, LOC_Os07g46790, LOC_Os07g22930, and LOC_Os08g09230) were upregulated at 13 °C in comparison to 25 °C. Among them, genes encoding glycogen/starch synthases (LOC_Os07g22930), 4-alpha-glucanotransferase (LOC_Os07g46790), and a soluble starch synthase (*SSIIIa*, LOC_Os08g09230) were all upregulated at 13 °C in comparison to 25 °C, but were downregulated at 11 °C in comparison to 13 °C (Fig. 4-C, Table S6).

4. Discussion

4.1. *There are different mechanisms of rice plant response to different low temperatures*

These results in this study indicate that leaf discoloration is associated mainly with reduction in chlorophyll content under moderate cold stress, whereas leaf rolling results primarily from water deficit under severe cold stress. Leaf rolling was not associated with reduction in chlorophyll content under cold stress, and the two chilling injury symptoms were temperature-specific. In our previous study, a weak (R^2 = 0.2174) linear relationship between leaf discoloration and leaf rolling was observed under two distinct cold environments in a recombinant inbred line population derived from SHZ-2 and LTH and different sets of QTL affecting leaf discoloration and leaf rolling were identified [10]. These results together suggest that rice plants respond differently to different low temperatures. Under moderate low temperature stress, rice seedlings show leaf discoloration due to reduction in chlorophyll content, whereas leaf rolling observed under severe cold stress (11 °C or less) is due to water deficit.

4.2. *Calcium signaling and ABA metabolic pathways are involved in cold stress-induced leaf rolling*

Calcium plays important roles in regulating the response of plants to environmental stresses, including cold stress [24–27]. Calcium constitutes a second messenger in stress sensing. Low temperature leads to changes in membrane fluidity and rearrangement of the cytoskeleton following the influx of calcium [28]. CML proteins and CDPKs/CPKs have been reported to act as calcium sensors [29]. *AtCML42* has been reported to serve as a Ca^{2+} sensor and has multiple functions in defense against insect herbivory and abiotic stress responses [30]. *AtCML8* was involved in *Pseudomonas syringae* plant immunity [31]. *AtCML9* mutant lines present a hypersensitive response to ABA that could be correlated with enhanced tolerance to salt stress and water deficit [32]. *AtCML24* may function to enable responses to ABA, daylength, and the presence of various salts [33]. Previous studies [34] showed that *OsCPK4* (LOC_Os02g03410) was induced by high salinity, drought, and ABA. Over-expression of *OsCPK4* significantly enhanced tolerance to salt and drought stress in rice [34], and *OsCDPK7* (LOC_Os04g49510) was induced by cold and salt stress [35]. Overexpression of *OsCDPK7* also increased the expression of other stress-responsive genes under salt/drought stress in rice [35]. Calcium influx and calcium signaling can lead to the activation of key TFs involved in stress tolerance in rice, such as *CBF/DREB* [36]. In the present study, calcium signaling pathway genes were differentially expressed under 11 °C cold stress. As shown in Tables 1 and 2, most of the genes encoding CMLs and CDPKs were upregulated under 11 °C cold stress in comparison to 25 °C and 13 °C. *OsCPK4* and *OsCDPK7* were also upregulated under 11 °C cold stress. These results suggest that the calcium signaling pathway is triggered under 11 °C cold stress.

The three ABA 8′-hydroxylases encoded by *OsABA8ox1*, *OsABA8ox2*, and *OsABA8ox3*, are responsible for ABA degradation in rice. *OsABA8ox1* (LOC_Os02g47470), but not *OsABA8ox2* (LOC_Os08g36860) and *OsABA8ox3* (LOC_Os09g28390), increased dramatically in rice shoots after submergence [37]. Zhu et al. [38] reported that *OsABA8ox2* and *OsABA8ox3* were suppressed in the presence of exogenously supplied glucose. Furthermore, *OsABA8ox3* was promptly induced by rehydration after polyethylene glycol (PEG)-induced dehydration, a tendency opposite to the changes in ABA levels. *OsABA8ox3* plays a role in controlling ABA level and drought stress resistance in rice [39]. Our previous study [16] also showed that ABA plays an important role in the response of rice to cold stress. In the present study, *OsABA8ox1* was upregulated significantly at both 11 °C and 13 °C in comparison to 25 °C, but higher expression levels were observed at 11 °C than at 13 °C. In contrast, *OsABA8ox2* and *OsABA8ox3* were upregulated at 13 °C instead of 11 °C. The differential expression of ABA metabolic pathway genes suggests that ABA is involved in the regulation of leaf rolling under cold stress.

4.3. *Multiple regulatory pathways are involved in cold stress-induced leaf rolling in rice*

TFs play central roles in gene expression by regulating the expression of downstream genes as *trans*-acting elements via specific binding to *cis*-acting elements in the promoters of target genes. The *DREB* subfamily has two major subgroups, *DREB1* and *DREB2*, which have been reported to be involved in abiotic stress responses in plants [40]. *DREB* activates the expression of abiotic stress-responsive genes via specific binding to the dehydration-responsive element/C-repeat (*DRE/CRT*) *cis*-acting element in their promoters. In *Arabidopsis*, *DREB1s* were induced by 4 °C cold stress, whereas *DREB2s* were induced by dehydration and salt stress [41]. However, the responses of *DREBs* to cold stress in rice are complex. In two studies [19,42], the expression of *OsDREB1A*, *OsDREB1B*, and *OsDREB2B* was induced by 4 °C cold stress. In contrast, in our previous study [16], *OsDREB1A*, *OsDREB1B*, and *OsDREB2B* were induced 6 h after cold treatment at 8 °C, while *OsDREB2A* was induced 24 h after cold treatment at 8 °C. Transgenic analysis [43] indicated that the overexpression of *OsDREB1D* increased both cold (4 °C) and high-salt tolerance. In the present study, *OsDREB1A*, *OsDREB1B* and *OsDREB1G* were induced under both 11 °C and 13 °C cold stress, but their expression levels were higher at 11 °C than at 13 °C. *OsDREB1D*, *OsDREB1H*, and *OsDREB2B* were induced only by 11 °C cold stress (Fig. 3-C). The present study reports the first association of *OsDREB1G*, *OsDREB1H*, and *OsDREB3* with cold tolerance in rice. Thus, based on results from the present and previous studies, we suggest that *OsDREB1A* and *OsDREB1B* act as general regulators of cold acclimation under different low temperature stresses, including 4 °C, 8 °C, 11 °C, and 13 °C, whereas *OsDREB2B* constitutes an important regulator of cold acclimation to severe cold stresses that cause leaf rolling, including 4 °C, 8 °C, and 11 °C.

In addition to DREB TF genes, many *MYB* (42 genes), *NAC* (44 genes), *bZIP* (42 genes), *Zinc finger* (63 genes), *bHLH* (55 genes), and *WRKY* (44 genes) genes were differentially expressed (Fig. 3-D–I). The finding that numerous genes are differentially expressed in these TF gene families suggests that multiple regulatory pathways are involved in cold stress-induced leaf rolling in rice.

4.4. *Leaf rolling under cold stress is associated with the suppression of aquaporin genes*

Aquaporins, a class of channel-forming integral membrane proteins, play a vital role in the transport of water and many small solutes within and between cells [44]. Plant aquaporins are classified mainly into five subfamilies based on phylogenetic relationships: plasma membrane intrinsic proteins (PIPs), nodulin 26-like intrinsic proteins (NIPs), tonoplast intrinsic proteins (TIPs), small intrinsic proteins (SIPs), and uncharacterized intrinsic proteins (XIPs) that are present in only a few dicots [45,46]. To date, 33 aquaporin genes have been identified in rice [47]. Water use efficiency in rice is affected by aquaporin gene expression [48]. In previous studies [49,50], antisense inhibition of aquaporins of *PIP1* and/or *PIP2* reduced hydraulic conductivity by approximately 50% in tobacco and *Arabidopsis*. The *OsPIP2* members *OsTIP1-2* and *OsTIP2-2* have been reported to possess water transportation activity in rice [51,52]. In the present study, 17 aquaporin genes, including *OsPIP1-1*, *OsPIP1-2*, *OsPIP1-3*, *OsPIP2-1*, *OsPIP2-2*, *OsPIP2-4*, *OsTIP1-2*, and *OsTIP2-2*, were downregulated during 11 °C cold stress in comparison to 25 °C. Although eight aquaporin genes were also differentially expressed during 13 °C cold stress, lower expression levels of these genes were observed at 11 °C than at 13 °C. These

results suggest that the downregulation of many aquaporin genes under 11 °C cold stress affects water transportation and causes water deficit and consequently leaf rolling.

4.5. *Carbohydrate metabolism plays pivotal roles in regulating leaf rolling under cold stress*

Soluble sugars typically accumulate in plants under stress [53]. Soluble sugars, as important osmotic regulators, can protect specific macromolecules or contribute to the stabilization of membrane structures, protect cells during desiccation, and interact with the polar head groups of phospholipids in membranes to prevent membrane fusion [54,55].

Trehalose, which may play a role in regulating carbohydrate allocation in plants during development [56,57], has often been proposed as an osmoprotectant during drought or water-deficit-induced stresses [58]. As a non-reducing α,α-1,1-disaccharide, trehalose is formed from UDP-glucose and glucose-6-phosphate in a reaction catalyzed by the enzyme TPS. The product is dephosphorylated into trehalose by the enzyme TPP [58–60]. Fourteen *OsTPS* and 13 *OsTPP* genes are present in the current rice sequence database [61]. In the present study, nine *OsTPS* genes and four *OsTPP* genes were differentially expressed (Table S6). A previous study suggested that *OsTPS1* (LOC_Os05g44210) may increase the abiotic stress tolerance of plants by increasing the amount of trehalose and proline and by regulating the expression of stress-related genes [72]. *OsTPP7* (LOC_Os02g51680) has been reported [63] to play an important role in enhancing starch mobilization to drive the growth kinetics of the germinating embryo and elongating coleoptile, consequently increasing anaerobic germination tolerance. In the present study, many *TPS* and *TPP* genes were downregulated under cold stress. Compared to at 13 °C, *OsTPS1* (LOC_Os05g44210), *OsTPS11* (LOC_Os03g12360), *OsTPP7* (LOC_Os02g51680), *OsTPP3* (LOC_Os10g40550), and *OsTPP6* (LOC_Os08g31630) were downregulated at 11 °C. The lower expression levels of the *TPS* and *TPP* genes at 11 °C than at 13 °C may result in lower trehalose accumulation and water-absorption capacity, possibly partly explaining the leaf rolling observed at 11 °C rather than 13 °C.

Starch accumulation has been observed under various stresses, including cold stress in different plants [64–67]. For instance, contents of glucose and starch increased in *Arabidopsis* leaves under mild osmotic stress conditions [65]. It has also been reported [68,69] that carbon allocation, including starch accumulation and degradation, is required for cold acclimation. A previous study [70] indicated that the genes encoding enzymes for starch biosynthesis, including AGPase and starch synthase, were upregulated. Furthermore, the starch content in leaves increased under mild drought conditions that did not cause leaf rolling. However, AGPase and starch synthase were downregulated, and the starch content decreased under severe drought conditions, causing leaf rolling in the rice seedlings [70]. In the present study, the genes encoding glycogen/starch synthases (LOC_Os07g22930), 4-alpha-glucanotransferase (LOC_Os07g46790), and a soluble starch synthase (*SSIIIa*, LOC_Os08g09230) were all upregulated at 13 °C, but downregulated at 11 °C. These results are in accord with the observation that drought stress [70] and the inhibition of starch synthesis cause leaf rolling under 11 °C cold stress. The low accumulation of trehalose and starch in rice seedlings under 11 °C cold stress results in water deficit and subsequent leaf rolling.

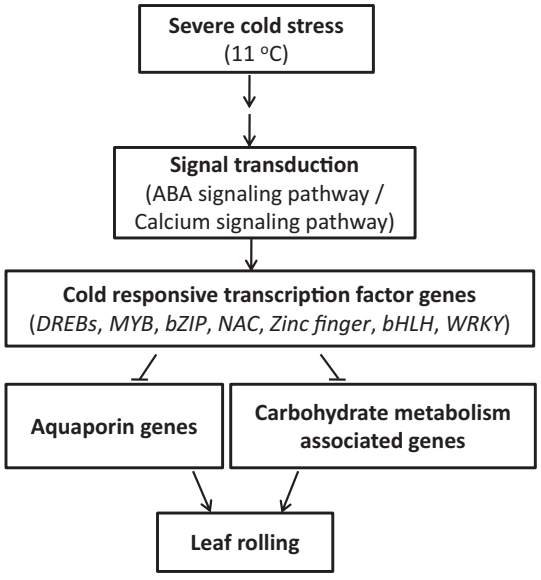

Fig. 5 – Proposed model of leaf rolling under severe cold stress. Under severe cold stress, such as at 11 °C, calcium- and ABA-signaling pathways are triggered with changes in the expression of genes encoding CMLs, CDPKs, and ABA8oxs. As messengers, calcium and ABA transduce cold signal to activate downstream TFs. In addition to *DREBs*, numerous genes in the *MYB*, *bZIP*, *NAC*, *Zinc finger*, *bHLH*, and *WRKY* gene families are differentially expressed. The effective TFs downregulate aquaporin genes and genes involved in trehalose and starch synthesis. Suppression of aquaporin genes and reduced accumulation of soluble sugars under cold stress results in a reduction in cellular water potential and consequently leaf rolling.

Based on the discussion above, a model describing the mechanism of leaf rolling under severe cold stress in rice is presented in Fig. 5.

5. Conclusions

Physiological analyses of leaf discoloration and leaf rolling and genome-wide gene expression analyses of leaf rolling under cold stress at the seedling stage in rice were performed. The results suggest that leaf discoloration and leaf rolling are not associated and are controlled by different mechanisms. Leaf rolling is associated with water deficit, whereas leaf discoloration is associated with reduction in chlorophyll content. Under severe cold stress, such as at 11 °C, calcium- and ABA-signaling pathways are triggered with changes in the expression of genes encoding CMLs, CDPKs, and ABA8oxs. As messengers, calcium and ABA transduce cold signals to activate downstream TFs. In addition to *DREB*s, numerous genes in the *MYB*, *bZIP*, *NAC*, *Zinc finger*, *bHLH*, and *WRKY* gene families are differentially expressed, suggesting the existence of multiple regulatory mechanisms in response to cold stress that result in leaf rolling. The functional TFs downregulate aquaporin genes and genes involved in trehalose and starch synthesis. The suppression of aquaporin genes and reduced accumulation of soluble sugars under cold stress result in a reduction in cellular water potential and consequently, leaf rolling.

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