



# De novo transcriptome sequencing and gene expression profiling of sweetpotato leaves during low temperature stress

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## Abstract

Sweetpotato [*Ipomoea batatas* (L.) Lam] is one of the most important crops for food, feed, industrial material, and bio-energy resource. However, since it originated in tropical regions, sweetpotato exhibits limited cold tolerance, hindering its cultivation in low-temperature areas. To comprehend the sweetpotato response to chilling stress, de novo transcriptome assembly was conducted on leaves from two sweetpotato cultivars: the cold-tolerant Liaohanshu 21 and the cold-sensitive Shenshu 28, exposed to low-temperature stress for 3 h and 24 h. 787.24 million raw reads and 763.44 million clean reads were obtained, 60.60–66.06% of clean reads successfully mapped to the reference database across all 18 libraries. 2524 unigenes were identified as transcript factors and classified into 55 families in Plant Transcription Factor Database (PlantTFDB) and 4671 unigenes were matched to 24 groups in Plant Resistance Gene Database (PRGdb). In Liaohanshu 21, 3618 genes and 8391 genes exhibited significant differential expression 3 h and 24 h of low-temperature exposure, respectively. In Shenshu 28, 13,394 genes and 11,627 genes displayed substantial differential expression after 3 h and 24 h of low-temperature exposure, respectively. The differentially expressed genes (DEGs) were further analyzed using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genome (KEGG) database. DEGs related to “active oxygen scavenging systems”, “lipid metabolism”, and “plant hormone signal transduction” were potentially associated with sweetpotato’s cold resistance. This research offers insights into the mechanisms and genes involved in cold stress in sweetpotato’s response to cold stress and will contribute valuable information for cold-tolerant breeding of sweetpotato.

**Keywords** Sweetpotato · Low temperature · Transcriptome · Differentially expressed genes

## Introduction

Low temperature stress, categorized into chilling (0–15 °C) and freezing (<0 °C), constitutes a major abiotic challenge affecting physiological activity, biochemical metabolism, growth, development, crop yield, and quality, particularly

for tropical and subtropical plants (Sanghera et al. 2011; Rinalducci et al. 2011). Numerous plants enhance their cold tolerance through exposure to chilling temperatures, known as cold acclimation (Thomashow et al. 1999). However, many tropical and subtropical crops like tomato, maize, and rice lack cold acclimation capacity. When faced with cold stress, plants undergo a series of signal transduction events, triggering or suppressing the expression of genes related to cold stress. The C-repeat-binding factor/dehydration responsive element-binding factor (CBF/DREB) signaling pathway has been extensively studied in plants, with CBFs primarily functioning in the early stages of cold stress to enable rapid plant responses (Kurepin et al. 2013). The ICE1 (Inducer of CBF expression 1) transcription factor directly binds to the CBF promoter region, activating CBF transformation (Zuo et al. 2019; Liu et al. 2009; Miura et al. 2007; Chinusamy et al. 2003). Subsequently, CBF binds directly to the promoters of downstream target genes, such as *COR* genes, and contributes to regulating plant cold tolerance through

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target gene transcription modulation (Liu et al. 2018). When exposed to stress, reactive oxygen species was rapidly accumulated leading to metabolic disorders in plants and efficient defense mechanisms including the expression and activation of genes related to intracellular and extracellular ROS scavengers have developed to cope with oxidative stress (Kim et al. 2013). Biological membranes participate in various physiological activities such as signal transduction, energy conversion, metabolic regulation, and transport, which are thought to be the primary sites for the development of chilling injury (Rui et al. 2010). The membranes of chilled tissues undergo a phase transition from a flexible liquid-crystalline to a solid-gel structure, which significantly increases the risk of loss of cell membrane semi-permeability (Xie et al. 2019). Meanwhile, cold transiently activates calcium-permeable channels in *Arabidopsis* mesophyll cells (Carpabeto et al. 2007). Phytohormones work as chemical messengers to regulate a variety of cellular processes in plants in very low concentrations and play key roles in abiotic stress (Peleg and Blumwald 2011). ABA, SA, and JA trigger phosphoprotein cascade pathways, leading to expression of genes associated with cold stress tolerance (Kolaksazov et al. 2013).

Sweetpotato is a vital crop for food, feed, and energy production, offering high yields and nutritional value. However, as a typical tropical plant, it is highly sensitive to low temperatures (Ji et al. 2017, 2019; Xie et al. 2019). Prolonged exposure to temperatures below 10 °C can lead to the death of stems and leaves, and storage roots can decay if kept below 9 °C for more than ten days, increasing susceptibility to fungal infections (Xie et al. 2019). Cold stress is a significant challenge for sweetpotato cultivation in China, particularly in the northeastern region. A deeper understanding of the molecular mechanisms underlying sweetpotato's response to low temperatures could provide valuable genetic information for enhancing cold tolerance through genetic improvement and molecular breeding. Sweetpotato's autohexaploid nature ( $2n = 6 \times = 90$ ) and high heterozygosity across six chromosome groups result in limited genomic data sources (Zhang et al. 2017). Consequently, transcriptome sequencing (RNA-seq) has been employed to investigate specific biological processes in sweetpotato. RNA-seq studies have focused on various molecular regulatory mechanisms in sweetpotato under low temperatures, with two studies examining tuberous roots and one analyzing leaves (Ji et al. 2017, 2019; Xie et al. 2019). Transcriptome sequencing comparing tuberous roots stored at optimal (13 °C) and low temperature (4 °C) for six weeks revealed that up-regulated differentially expressed genes (DEGs) are involved in the phenylalanine metabolism, pathogen defense, and biosynthesis of unsaturated fatty acids, while downregulated DEGs are associated with glycerophospholipid, carbohydrate, and energy metabolism (Ji et al. 2017). Another transcriptome study on Xushu 18

cultivar tuberous roots indicated that numerous DEGs are related to hormone metabolism, carbohydrate metabolism, antioxidant enzymes, and cell membrane systems under low temperatures (Xie et al. 2019). An investigation into leaf transcriptomes during exposure to low temperature and subsequent recovery revealed an exploration into genes associated with antioxidant enzymatic processes. These genes play a significant role in managing reactive oxygen species (ROS) reactions, particularly under the influence of cold stress conditions (Ji et al. 2019).

To comprehend the cold response in sweetpotato leaves, de novo transcriptome assembly was conducted under cold stress, comparing the distinct low-temperature responses of two sweetpotato cultivars: cold-tolerant variety Liaohanshu 21 and cold-sensitive variety Shenshu 28. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) analyses suggested that DEGs related to antioxidant enzymes, lipid metabolism, and hormone signal transduction might play crucial roles in combating low temperatures in sweetpotato. This study offers insights into the molecular mechanisms of low-temperature response in sweetpotato leaves, contributing to the development of enhanced cold tolerance in sweetpotato cultivation.

## Materials and methods

### Plant materials and stress treatment.

For the RNA-seq analysis, two sweetpotato varieties were chosen: Liaohanshu 21, which exhibits cold tolerance, and Shenshu 28, a cold-sensitive variant, both developed by the Liaoning Academy of Agricultural Sciences. These plants, cultivated in pots, were housed in a light incubator for a fortnight at 25 °C, under a light–dark cycle of 16 h light and 8 h dark, with a cool-white fluorescent light intensity of 150  $\mu\text{mol m}^{-2}\text{s}^{-1}$  and 75% humidity. Subsequently, these plants were relocated to a 4 °C environment for periods of 3–24 h, respectively. The third fully expanded leaf from the top was harvested, promptly immersed in liquid nitrogen, and then stored at -80 °C for future analysis. Control plants were those unexposed to the low-temperature condition. Each treatment was replicated in three separate biological instances.

### Antioxidant enzyme assays

Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were determined as previously described (Wang et al. 2016).

For the SOD activity determination, 1 g sweetpotato leaves were homogenized on ice with a mortar in 5 mL 0.05 M sodium phosphate buffer containing 2% PVP (pH 7.8), centrifuged for 15 min at 12,000 rpm at 4 °C. The

supernatant was collected and stored before use. Then the SOD activity was assayed using the photochemical nitro blue tetrazolium (NBT) method. Sodium phosphate buffer was instead of SOD extract as a control, incubated for 20 min at 25 °C, and then SOD activities were determined at 560 nm.

For the CAT activity determination, 1 g sweetpotato leaves were homogenized on ice with a mortar in 10 mL 0.1 M sodium phosphate buffer containing 5 mmol/L dithiothreitol and 5% PVP (pH 7.8), centrifuged for 30 min at 12,000 rpm at 4 °C. The supernatant was collected and stored before use. The enzyme catalysis system was composed of 2.9 mL H<sub>2</sub>O<sub>2</sub> and 100 µL enzyme extract, and ddH<sub>2</sub>O was as the control. The absorbance at 15 s and 2 min at 240 nm were recorded, respectively.

For the POD activity determination, 1 g sweetpotato leaves were homogenized on ice with a mortar in 20 mL phosphoric acid buffer, centrifuged for 15 min at 4000 rpm at 4 °C. The supernatant was collected and stored before use. The reaction mixture was consisted of 30% H<sub>2</sub>O<sub>2</sub> solution, 20 mmol/L KH<sub>2</sub>PO<sub>4</sub> solution and 100 mmol/L phosphoric acid buffer. phosphoric acid buffer was instead of POD extract as a control. The absorbance at 1 min and 2 min at 470 nm were recorded, respectively.

SOD activity, CAT activity, and POD activity were all determined by Molecular Devices SpectraMax ABS plus Multiskan Spectrum (Molecular Devices, CA, USA) according to the operating manual.

## Biochemical assays

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content, malondialdehyde (MDA) content, and the production rate of superoxide anion (•O<sup>2-</sup>) (Zhang et al. 2019) were determined as previously described.

For the H<sub>2</sub>O<sub>2</sub> content determination, 1 g sweetpotato leaves were homogenized on ice with a mortar in 5 mL pre-cooled acetone, centrifuged for 10 min at 3000 rpm at 4 °C. The supernatant was collected and stored before use. The reaction mixture was consisted of 100 µmol/L H<sub>2</sub>O<sub>2</sub>-acetone solution, acetone at – 20 °C, 10% HCl-Titanium tetrachloride solution, and stronger ammonia water. 1 mL H<sub>2</sub>O<sub>2</sub> extract was added into the reaction mixture, centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatant was abandoned, 3 mL 2 mol/L H<sub>2</sub>SO<sub>4</sub> was added to dissolve the precipitate. Then H<sub>2</sub>O<sub>2</sub> content were determined at 412 nm.

For the MDA content determination, 1 g sweetpotato leaves were homogenized on ice with a mortar in 10 mL 5% trichloroacetic acid, centrifuged for 10 min at 3000 rpm at 4 °C. The supernatant was collected and stored before use. 2 mL MDA extract was mixed with 2 mL 6% 2-thiobarbituric acid solution, incubated for 15 min at 100 °C, then absorbance at 450 nm, 532 nm, and 600 nm were recorded.

For the production rate of •O<sup>2-</sup> determination, 1 g sweetpotato leaves were homogenized on ice with a mortar in 3 mL 65 mmol/L potassium phosphate buffer (pH 7.8), centrifuged for 20 min at 12,000 rpm at 4 °C. The supernatant was collected and stored before use. 0.5 mL of •O<sup>2-</sup> extract was mixed with 0.1 mL of hydroxylamine hydrochloride, incubated for 20 min at 25 °C. 1 mL sulfanilic acid solution and 1 mL α-naphthylamine were then added, incubated for 20 min at 25 °C. At last, 3 mL chloroform was added and then centrifuged for 3 min at 10,000 rpm at 4 °C. The supernatant was collected and stored before use.

H<sub>2</sub>O<sub>2</sub> content, MDA content, and the production rate of •O<sup>2-</sup> were all detected by Molecular Devices SpectraMax ABS plus Multiskan Spectrum (Molecular Devices, CA, USA) according to the operating manual.

## RNA extraction, library construction, and RNA-Seq

Total RNA extraction from the leaves was performed using the RNAPrep Pure Plant Kit (Tiangen Biotech, Beijing, China). The extracted RNA's purity was assessed using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was verified via an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Poly-A mRNA was subsequently enriched from these total RNA samples using poly-T oligo-attached magnetic beads. A DNA probe was utilized to hybridize with rRNA, after which RNaseH was deployed to selectively digest the DNA/RNA hybridization chain, followed by the use of DNaseI to digest the DNA probe, yielding purified RNA. This RNA was then fragmented using an interrupt buffer, and a random N6 primer was used for reverse transcription. This process was followed by synthesizing the cDNA second strand to form double-stranded DNA. The 5' end of this synthesized double-stranded DNA was phosphorylated, and the 3' end was adenylated, after which a bubble-like adaptor with a 3' thymine overhang was ligated. The ligated products were then amplified using PCR with specific primers. The PCR product underwent thermal denaturation into a single strand, then circularized with a bridge primer to create a single-stranded circular cDNA library. These constructed cDNA libraries were sequenced using a DNBSEQ platform. The raw sequence reads were submitted to the National Genomics Data Center (NGDC) Biological Project Library (BioProject) database.

## Preprocessing and de novo transcriptome assembly

The filtration of total raw sequencing data was accomplished using SOAPnuke (v1.4.0), a software developed by Huada (Huada, Beijing, China), using a Phred quality score threshold (Q ≥ 20). The filtered reads were aligned to the assembled unigenes using HISAT (Hierarchical Indexing

for Spliced Alignment of Transcripts) software (Kim et al. 2015a, b). Moreover, Bowtie2 (Langmead and Salzberg 2012) was utilized to map the clean reads to the assembled unigenes. Following the quantification of the mapped reads for each unigene, the expression value was calculated using RSEM (Li and Dewey 2011). The determination of DEGs was based on a Qvalue (Adjusted Pvalue)  $\leq 0.05$  as per the method outlined by (Love Michael et al. 2014), implemented using DEseq2 (Anders and Huber 2010).

## Functional annotation

The open reading frame (ORF) of the unigenes was pinpointed by employing getorf (<http://emboss.sourceforge.net/apps/cvs/emboss/apps/getorf.html>), after which hmmsearch (<http://hmmer.org>) was utilized for the alignment of the ORFs with the transcription factor protein domain. Subsequently, the unigenes were categorized based on the transcription factor family traits outlined by Plant Transcription Factor Database (PlantTFDB). This allowed for determining the transcription factor families to which the unigenes belonged.

For annotation, the unigenes were aligned with Plant Resistance Gene Database (PRGdb) using DIAMOND software (Buchfink et al. 2015; Sanseverino et al. 2010) (<https://github.com/bbuchfink/diamond>). The parameters set for this alignment included a query coverage of  $\geq 50\%$  and an identity of  $\geq 40\%$ . The annotation results were further refined based on the query coverage and identity.

## Quantitative real-time PCR (qRT-PCR) analysis

The first-strand cDNA for qRT-PCR was synthesized utilizing Fastking gDNA Dispelling RT SuperMix FastKing (Tiangen Biotech, Beijing, China). The qRT-PCR was conducted on an ABI Step One Plus Real-time PCR system, employing an SYBR green Realtime PCR Master (Toyobo, Beijing, Shanghai). The primer pairs used for qRT-PCR amplification were specifically designed with the Primerblast online tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The design parameters included amplification products ranging from 80 to 150 bp, an optimal primer melting temperature of 58–62 °C, and GC contents between 40 and 60%. The PCR followed a thermal cycling program of 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s, after an initial denaturation stage for 1 min at 94 °C. The  $2^{-\Delta\Delta CT}$  method was employed for gene expression calculations (Livak and Schmittgen 2001). The  $\beta$ -actin gene of sweetpotato was used as an internal control. The primers,

designed according to the sequencing data, are provided in Table S1.

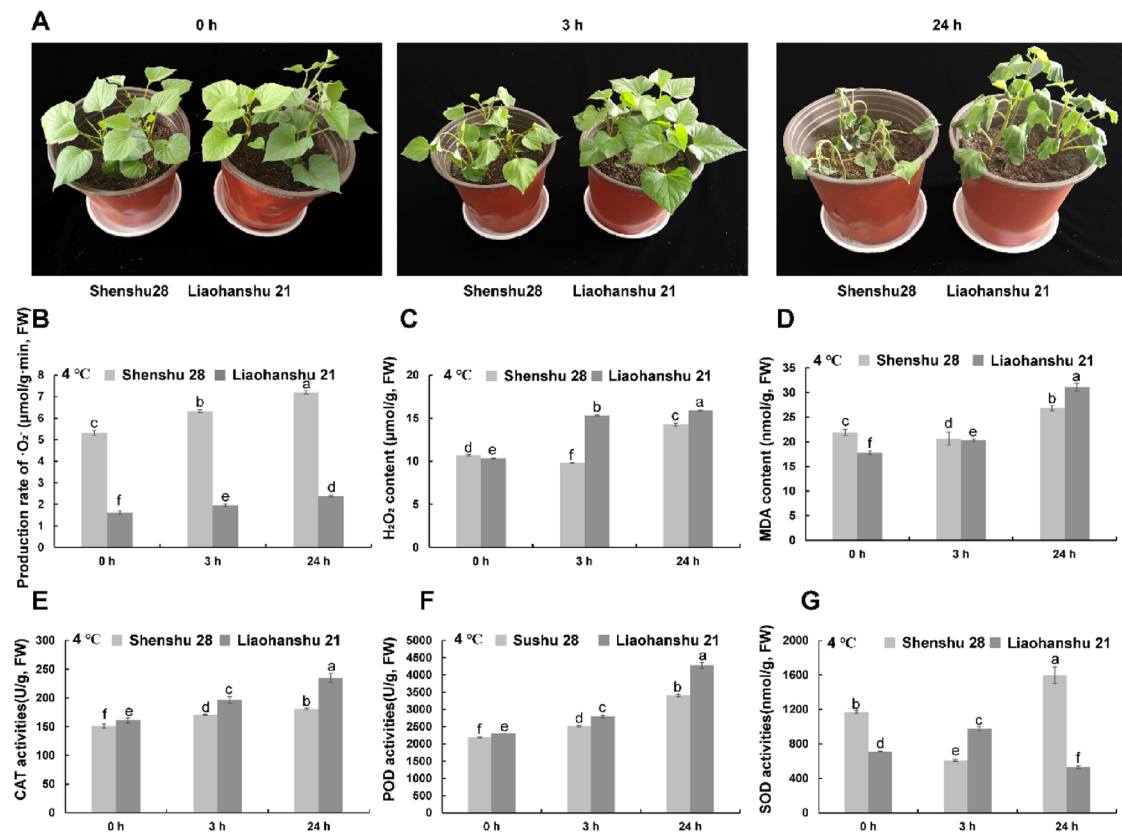
## Results

### Different physiological responses of sweetpotato leaves between Shenshu 28 and Liaohanshu 21 under cold stress

To identify the effect of low temperature on sweetpotato, cold-tolerant sweetpotato variety Liaohanshu 21 and cold-sensitive sweetpotato variety Shenshu 28 were treated at low-temperature (4 °C) for 3 h and 24 h, respectively. Shenshu 28 and Liaohanshu 21 grew normally at room temperature as control (Fig. S1). After 3 h of low-temperature treatment, the leaves of Shenshu 28 are slightly wilted, while there is no significant change on Liaohanshu 21. After 24 h, the leaves of Shenshu 28 were all wilted, while the leaves of Liaohanshu 21 were moderately wilted (Fig. 1A).

To investigate the physiological responses of leaves under low temperature conditions, the production rate of  $\bullet O_2^-$ ,  $H_2O_2$  content, and MDA content were measured. When exposed to low-temperature, the production rate of  $\bullet O_2^-$ ,  $H_2O_2$  content, and MDA content continued to accumulate in Liaohanshu 21, while in Shenshu 28, the production rate of  $\bullet O_2^-$  continued to accumulate over time, but MDA and  $H_2O_2$  decreased firstly in 3 h and then increased in 24 h (Fig. 1B–D). The production rate of  $\bullet O_2^-$ ,  $H_2O_2$  content, and MDA content in Liaohanshu 21 were significantly lower than that in Liaohanshu 21 under normal condition (Fig. 1B–D). Under low temperature conditions, the production rate of  $\bullet O_2^-$  in Shenshu 28 was continuous lower than that in Shenshu 28 (Fig. 1B), while the  $H_2O_2$  content in Liaohanshu 21 was high than that in Shenshu 28 (Fig. 1C). The MDA content in Liaohanshu 21 was lower than that in Shenshu 28 at 3 h but higher in 24 h under low temperature conditions (Fig. 1D).

CAT, POD, and SOD activity were measured to investigate the antioxidant capacity of leaves under low temperature conditions. The activity of CAT and POD continued to increase over time both in Liaohanshu 21 and Shenshu 28 (Fig. 1E–F). SOD in Liaohanshu 21 came to its highest at 3 h under low temperature and then decreased at 24 h, while in Shenshu 28, SOD activity decreased firstly at 3 h and then increased at 24 h (Fig. 1G). CAT and POD activity in Liaohanshu 21 were higher than that in Shenshu 28 both in normal and low temperature conditions (Fig. 1E–F). However, the SOD activity in Liaohanshu 21 was firstly lower than that in Shenshu 28 under normal conditions, but then higher than that in Liaoshu 28 after



**Fig. 1** Phenotypic and biochemical analysis of changes in sweetpotato leaves under low temperature. **A** Sweetpotato leaves under low temperature. **B** production rate of  $\bullet\text{O}_2^-$  in leaves **C**  $\text{H}_2\text{O}_2$  levels in leaves. **D** MDA levels in leaves. **E** CAT activities in leaves. **F** POD

activities in leaves. **D** SOD activities in leaves. Each experiment included three independent replicates. The error bars indicate  $\pm$ SD ( $n=3$ ). Different lowercase letters indicate a significant difference at  $P < 0.05$  based on Student's  $t$  test

exposed to low temperature for 3 h, and finally became lower than that in Liaoshu 28 again at 24 h (Fig. 1G).

### Transcriptome sequencing and de novo transcriptome assembly

The DNBSEQ platform was deployed to investigate the transcriptome in three biological replicates of various samples. These included samples under normal temperature (ss\_ck and lhs\_ck), those exposed to a low temperature for durations of 3 h (L\_ss\_3h and L\_lhs\_3h), and 24 h (L\_ss\_24h and L\_lhs\_24h). On average, each sample generated 6.36 Gb of data. The study gathered a total of 787.24 million raw reads, and after filtering out reads with adaptors, unknown base N content  $\geq 5\%$ , and low-quality reads, 763.44 million clean reads were retained (Table 1). From all 18 libraries, 60.60% to 66.06%, with an average of 65%, of the clean reads successfully aligned with the reference database (Table 1). The process identified 82,513 unigenes, comprising 71,157 known genes and 11,356 predicted new genes. Moreover, 38,207 new

**Table 1** Overview of transcriptome sequencing and mapping in sweetpotato leaves

Sample	Total Raw Reads (Millions)	Total Clean Reads (Millions)	Total Mapping (%)	Uniquely Mapping (%)
L_lhs_24h1	133.21	128.49	65.14	38.11
L_lhs_3h	128.84	124.49	65.76	39.19
L_ss_24h	131.2	127.06	63.14	36.74
L_ss_3h	131.46	128.47	65.28	38.71
lhs_ck	131.46	127.64	65.51	37.88
ss_ck	131.07	127.29	65.17	38.69
Total/average	787.24	763.44	65.00	38.22

transcripts were detected, 26,784 of which were new variable splicing subtypes of known protein-coding genes, and 11,423 were transcripts of newly predicted protein-coding genes. All clean reads were deposited in the NGDC and can be accessed under BioProject accession number PRJNA987163.

### Annotation and classification of sweetpotato unigenes

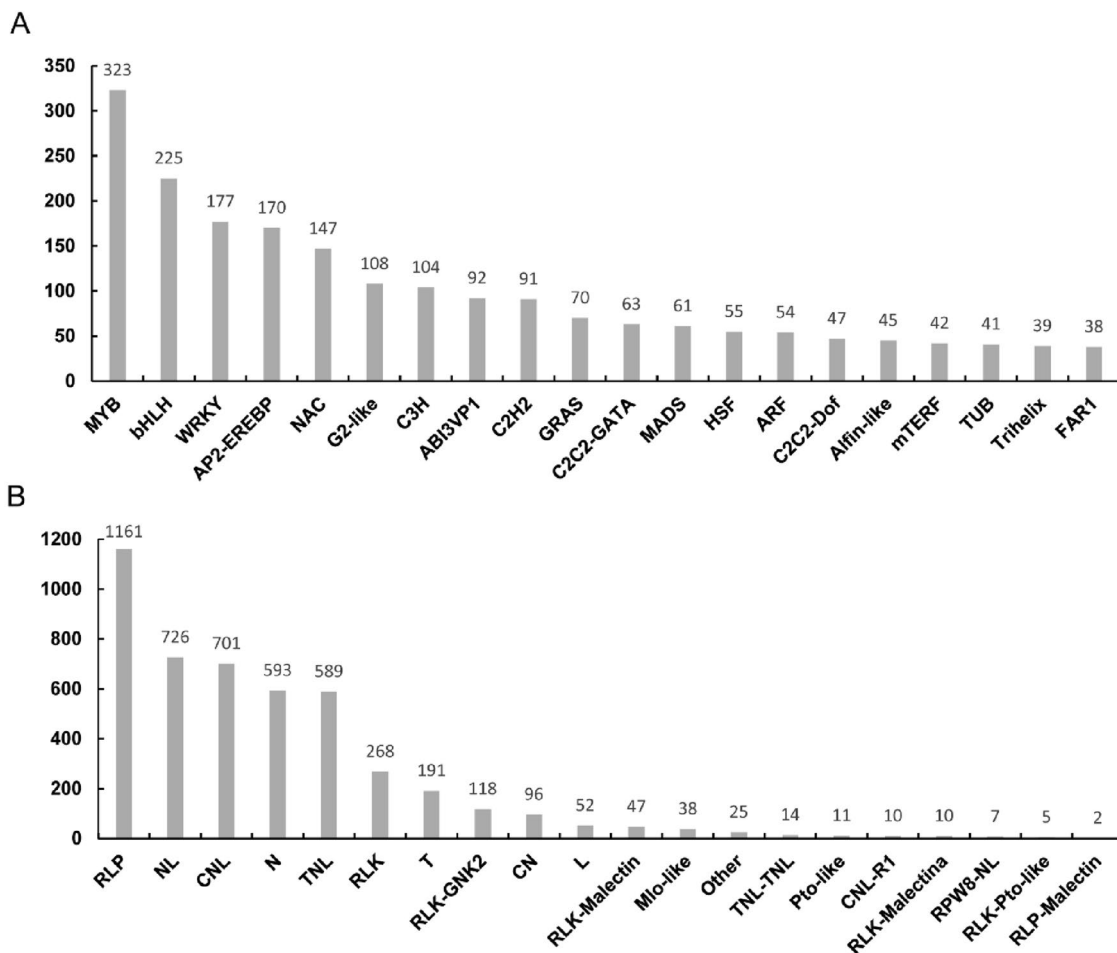
To identify the function of the unigenes, functional annotation was carried out using two databases, PlantTFDB and PRGdb. PlantTFDB is a plant transcription factor database that provides a web link to access the transcription factor collection of several plants, such as *Arabidopsis*, poplar, and rice, providing protein sequences, coding regions, genomic sequences, and expression sequence tags for each transcription factor family. A total of 2524 unigenes were identified as transcript factors and classified into 55 families. The MYB family (323 genes, 12.80%), bHLH family (225 genes, 8.91%), WRKY family (177 genes, 7.01%), and AP2-ERF family (170 genes, 6.73%) formed the four largest clusters (Fig. 2A).

PRGdb is a comprehensive and regularly updated database that contains known and predicted disease-resistant genes in many plants, providing information such as nucleic acid and protein sequences, structural domains, Genbank

numbers, disease-resistant hosts, pathogens, and disease descriptions of disease-resistant genes. A total of 4671 unigenes were matched to 24 groups. Genes containing RLP (1161 genes, 24.86%), NL (726 genes, 15.54%), and CNL (701 genes, 15.01%) were the three largest groups (Fig. 2B).

**Table 2** Grouping in pairwise comparison

Number	Control	Treatment
1	lhs_ck	L_lhs_3h
2	lhs_ck	L_lhs_24h
3	L_lhs_3h	L_lhs_24h
4	ss_ck	L_ss_3h
5	ss_ck	L_ss_24h
6	L_ss_3h	L_ss_24h
7	lhs_ck	ss_ck
8	L_lhs_3h	L_ss_3h
9	L_lhs_24h	L_ss_24h



**Fig. 2** Top 20 of transcription factor family (A) and plant disease resistance gene domains (B)

## Analysis of differentially expressed unigenes

Nine comparison groups obtained from the samples were identified using DEseq2 with Qvalue (Adjusted Pvalue)  $\leq 0.05$  for the filter parameter (Table 2).

In the Liaohanshu 21 samples, significant expression differences were observed in 3618 genes in the L\_lhs\_3h samples compared to the lhs\_ck samples. Among these, 1883 genes were up-regulated, and 1735 genes were down-regulated. In the L\_lhs\_24h samples, 8391 genes exhibited notable expression deviations from lhs\_ck, with 4035 genes up-regulated and 4356 genes down-regulated. Additionally, 2781 genes in the L-lhs\_24h samples demonstrated substantial expression differences from the L-lhs\_3h samples, with 1234 genes up-regulated and 1547 genes down-regulated (Fig. 3) (Table 3).

In the Shenshu 28 samples, the L-ss-3h sample set demonstrated significant expression changes in 13,394 genes compared to ss-ck, with 6737 genes up-regulated and 6657 genes down-regulated. In the L-ss-24h samples, 11,627 genes showed notable expression variations from L\_ss\_3h, with 6032 genes up-regulated and 5595 genes down-regulated. Also, 211 genes in the L-ss\_24h samples revealed significant expression differences from the L\_ss\_3h samples, with 154 genes up-regulated and 57 genes down-regulated (Fig. 3).

When comparing the gene expression of Liaohanshu 21 and Shenshu 28 at the same sampling time, DEGs were analyzed between the two. In the L\_lhs\_3h vs. L\_ss\_3h comparison, 1030 and 1145 genes were concurrently up-regulated

and down-regulated, respectively; 20 genes were up-regulated in L\_lhs\_3h but down-regulated in L\_ss\_3h, and conversely, 7 genes were up-regulated in L\_ss\_3h but down-regulated in L\_lhs\_3h. In the L\_lhs\_24h vs. L\_ss\_24h comparison, 1805 and 1890 genes were simultaneously up-regulated and down-regulated, respectively; 21 genes were up-regulated in L\_lhs\_24h but down-regulated in L\_ss\_24h, while 36 genes were up-regulated in L\_ss\_24h, but down-regulated in L\_lhs\_24h.

Relative to lhs\_ck samples, 13,547 genes showed significant expression differences in ss\_ck samples, with 6460 genes up-regulated and 7087 genes down-regulated. Relative to L-lhs\_3h samples, 16,385 genes showed significant expression differences in L-ss\_3h samples, with 8118 genes up-regulated and 8267 genes down-regulated. Compared to L-lhs\_24h samples, 13,556 genes showed significant expression differences in L-ss\_24h samples, with 6826 genes up-regulated and 6728 genes down-regulated (Fig. 3).

## GO annotation and KEGG classification of DEGs

The GO analyses were conducted on the DEGs in nine datasets to understand their biological functions better (Table S3). The DEGs were categorized into three main groups: biological process (BP), cellular component (CC), and molecular function (MF). Within the BP category, the two largest subclasses across all nine datasets were "cellular process" and "metabolic process." In the CC category, only "cellular anatomical entity" and "protein-containing complex" were identified across all groups. Within the MF

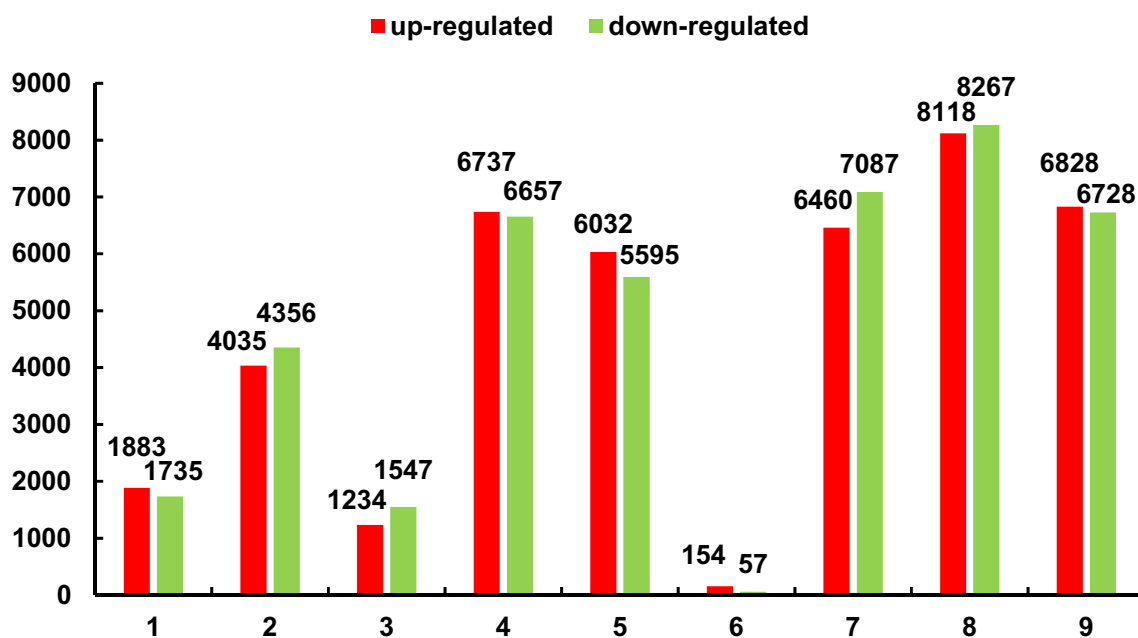


Fig. 3 Numbers of DEGs identified by pairwise comparisons between the six datasets

**Table 3** Top 20 KEGG pathways of Liaohanshu 21 after exposure to low temperature for 24 h

Pathway	Description	KEGG-B	KEGG-A	Num
ko01100	Metabolic pathway	GOM	M	1692
ko01110	Biosynthesis of secondary metabolites	GOM	M	1042
ko04626	Plant-pathogen interaction	EA	M	427
ko04075	Plant hormone signal transduction	ST	EIS	258
ko00500	Starch and sucrose metabolism	CM	M	252
ko01200	Carbon metabolism	GOM	M	199
ko01230	Biosynthesis of amino acids	GOM	M	170
ko01240	Biosynthesis of cofactors	GOM	M	160
ko00520	Amino sugar and nucleotide sugar metabolism	CM	M	131
ko00040	Pentose and glucuronate interconversions	CM	M	116
ko00010	Glycolysis / Gluconeogenesis	CM	M	114
ko00052	Galactose metabolism	CM	M	113
ko01250	Biosynthesis of nucleotide sugars	GOM	M	103
ko00940	Phenylpropanoid biosynthesis	BOSM	M	101
ko00564	Glycerophospholipid metabolism	LM	M	82
ko00999	Biosynthesis of various plant secondary metabolites	BOSM	M	78
ko00620	Pyruvate metabolism	CM	M	72
ko00561	Glycerolipid metabolism	LM	M	71
ko00030	Pentose phosphate pathway	CM	M	69
ko00270	Cysteine and methionine metabolism	AAM	M	68

*AAM* Amino acid metabolism, *BOSM* Biosynthesis of other secondary metabolites, *CM* Carbohydrate metabolism, *EA* Environmental adaptation, *GOM* Global and overview maps, *LM* Lipid metabolism, *ST* Signal transduction, *EIS* Environmental Information Processing, *OS* Organismal Systems, *M* Metabolism

category, "catalytic activity" was the largest subclass in groups 1, 2, 3, 5, and 9, followed by "binding." However, in groups 4, 6, 7, and 8, "binding" was the largest subclass, with "catalytic activity" as the second largest.

To further understand the significant biological pathways involved in the sweetpotato's response to cold stress, the DEGs from the nine datasets were annotated to the reference pathways in the KEGG (Table S4). Across all nine datasets, the two most abundantly represented pathways were "global and overview maps" and "carbohydrate metabolism." The third most represented pathway varied: it was "environmental adaptation" in groups 1, 2, 3, and 8; "translation" in groups 4, 7, and 9; and "signal transduction" in groups 5 and 6. These findings may indicate the diverse functional and metabolic adaptations of sweetpotato under cold stress conditions.

### Validation of RNA-seq results

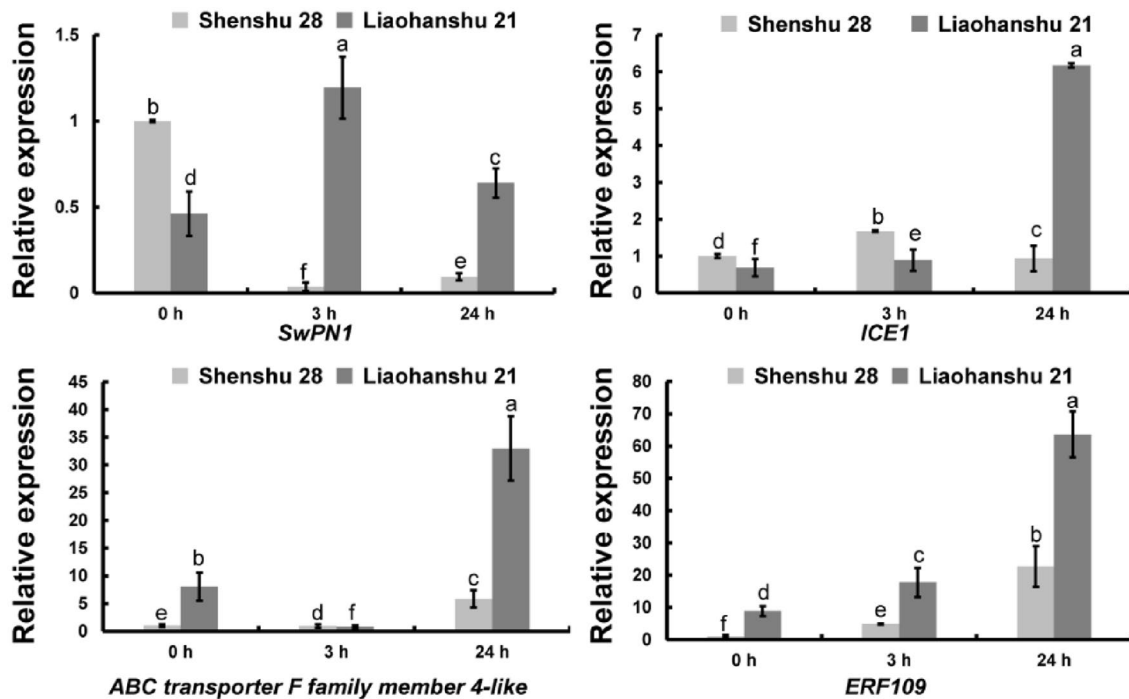
To validate the RNA-seq results, 4 genes from the sequencing data (encoding SwPN1, ICE1-like, ABC transporter F family member 4-like, and ERF109-like) were selected for qRT-PCR analysis. All the 4 genes showed the same expression profiles as those in the transcriptome profiling, although the fold changes were inconsistent with those in sequencing data (Fig. 4).

## Discussion

### Different responses to cold between Shenshu 28 and Liaohanshu 21

Our transcriptomic investigation revealed divergent patterns in the number of DEGs between the sweetpotato varieties Liaohanshu 21 and Shenshu 28 as the duration of low-temperature treatment extended. Shenshu 28 exhibited a considerably higher tally of DEGs across all phases of the cold stress, with 13,394 DEGs emerging after a mere three hours of low-temperature exposure, contrasted by only 3,618 DEGs in Liaohanshu 21. These figures underscore Shenshu 28's rapid responsiveness to cold stress, while Liaohanshu 21 demonstrated a more gradual adaptation. Further analysis revealed a minor count of 211 DEGs in *L\_lhs\_24h* compared to *L\_lhs\_3h*, as opposed to the substantial 2,781 DEGs in *L\_ss\_24h* versus *L\_ss\_3h*, hinting at Shenshu 28's stability in gene expression during the latter phase of cold stress. This underpins the notion that the cold-sensitive variety, Shenshu 28, experiences pronounced early-stage reactions that persist, whereas the cold-tolerant Liaohanshu 21 undergoes less severe changes. Examining the gene expression of both varieties at identical sample times revealed significant disparities between Liaohanshu 21 and Shenshu 28 under normal conditions. The quantity of DEGs escalated at the 3-h mark





**Fig. 4** Expression patterns of selected mRNAs obtained by qRT-PCR and sequencing. mRNA expression levels during qRT-PCR analysis were normalized to the level of  $\beta$ -actin. All qRT-PCRs were repeated

three times for each sample. The error bars indicate  $\pm$ SD ( $n=3$ ). Different lowercase letters indicate a significant difference at  $P<0.05$  based on Student's  $t$  test

and subsequently declined at 24 h, possibly reflecting their distinct cold response mechanisms.

Tables 3 and 4 elucidate the principal 20 KEGG pathways enriched among the DEGs in L\_lhs\_24h versus lhs\_ck and L\_ss\_24h versus ss\_ck. Shared pathways across both varieties, encompassing "Metabolic pathway", "Biosynthesis of secondary metabolites", "Plant-pathogen interaction", "Plant hormone signal transduction", "Starch and sucrose metabolism", "Carbon metabolism", "Biosynthesis of cofactors", "Amino sugar and nucleotide sugar metabolism", "Pentose and glucuronate interconversions", "Glycolysis/Gluconeogenesis", "Galactose metabolism", and "Biosynthesis of nucleotide sugars", suggest a common cold stress response. Notably, 19 out of 20 pathways in Liaohanshu 21 were implicated in "Metabolism". In contrast, Shenshu 28's DEGs were not only implicated in "Metabolism" but also in "Organic Systems", "Mental Information Processing" and "Genetic Information Processing" pathways, indicate a more substantial response from Shenshu 28 to the cold stress.

### CBF cold response pathway

The CBF transcriptional cascade plays a crucial role in responses to low temperature in diverse plant species. CBF can directly bind to the promoters of downstream target genes (such as *COR*) and regulate plant cold tolerance by

regulating the transcription of target genes. At the same time, the transcriptional activity of *CBF* genes is regulated by upstream regulatory factors (Liu et al. 2018). Under cold stress, *ICE1* can bind to the MYC binding site of the *CBF1/2/3* promoter, competing with MYB15 (negatively regulated CBF transcription) for this site, inhibiting the negative regulatory effect of MYB15, thereby promoting the expression of *CBF* and downstream target genes (Kim et al. 2015a, b). Cold tolerance could be enhanced by over-expressing *ICE1* to regulate *CBF* genes transcriptional activity in crops such as rice, maize, and tomato (Deng et al. 2017; Lu et al. 2017; Nosenko et al. 2016). *ICE1* genes (G11642\_TU19184 in Liaohanshu 21, G11642\_TU19184 and G12016\_TU19749 in Shenshu 28) were up-regulated in plants exposed to low-temperature stress. However, no other transcription factors positively regulate *CBF* genes, such as CAMTAs and CESTAs (Doherty et al. 2009), were identified in our analysis.

Our transcriptomic data revealed numerous factors associated with negative regulation. Plant Intracellular Factor (PIF) proteins, known for their specific binding to the G-box in gene promoters, regulate target gene expression (Leivar and Monte 2014). PIF4 and PIF7 have been identified as inhibitors of the CBF signaling pathway under warm temperatures, thereby conserving energy and nutrients to expedite plant growth and development. However,

**Table 4** Top 20 KEGG pathways of Shenshu 28 after exposed to low temperature for 24 h

Pathway	Description	KEGG-B	KEGG-A	Num
ko01100	Metabolic pathway	GOM	M	2537
ko01110	Biosynthesis of secondary metabolites	GOM	M	1349
ko04626	Plant-pathogen interaction	EA	OS	458
ko04075	Plant hormone signal transduction	ST	EIP	342
ko04016	MAPK signaling pathway—plant	ST	EIP	334
ko00500	Starch and sucrose metabolism	CM	M	289
ko03040	Base excision repair	Transcription	GIP	248
ko01200	Carbon metabolism	GOM	M	246
ko04141	Protein processing in endoplasmic reticulum	FSD	GIP	245
ko01240	Biosynthesis of cofactors	GOM	M	227
ko01230	Biosynthesis of amino acids	GOM	M	222
ko00520	Amino sugar and nucleotide sugar metabolism	CM	M	202
ko03010	Ribosome	Translation	GIP	196
ko04144	Endocytosis	Transport and catabolism	CP	186
ko01250	Biosynthesis of nucleotide sugars	GOM	M	168
ko00052	Galactose metabolism	CM	M	152
ko00040	Pentose and glucuronate interconversions	CM	M	148
ko00010	Glycolysis / Gluconeogenesis	CM	M	144
ko03018	RNA degradation	FSD	CP	143
ko04120	Ubiquitin mediated proteolysis	FSD	GIP	130

CM Carbohydrate metabolism, EA Environmental adaptation, GOM Global and overview maps, ST Signal transduction, EIS Environmental Information Processing, GIP Genetic Information Processing, OS Organismal Systems, M Metabolism, CP Cellular Processes, MP Metabolic pathway, FSD Folding, sorting and degradation

at lower temperatures, the repression of *CBF* transcription by PIF4 and PIF7 diminishes, enabling plants to adapt more effectively to the forthcoming cold conditions (Lee and Thmashow 2012). In Liaohanshu 21, four PIF4-encoding genes (G23568\_TU38558, G24019\_TU39314, G24019\_TU39315, and G24019\_TU39317) were found up-regulated under low temperature, while only two such genes (G23568\_TU38558 and G24019\_TU39314) were up-regulated in Shenshu 28. Three PIF3-encoding genes (G14363\_TU23442, G15876\_TU25939, and G46233\_TU74501) showed increased expression in Liaohanshu 21, whereas no differential expression was observed in Shenshu 28. The role of bZIP transcription factors in plant development, environmental signaling, and stress response has been well-established (Wolfgang et al. 2018). Specifically, bZIP68 inhibits cold tolerance in maize by binding to the A-box element of the *DREB1s* (*CBFs*) gene promoter (Li et al. 2022). An up-regulation of a bZIP68-encoding gene (G12592\_TU20673) was observed in Shenshu 28 under low-temperature stress, whereas no change was detected in Liaohanshu 21. Besides, *PIF16* (G1516\_TU2558), *PIF17* (G3557\_TU5832) and *PIF60* (G13483\_TU22051) exhibited the same expression pattern as bZIP68. EIN3 (ethylene insensitive 3) can negatively regulate the expression of *CBF* by binding to the promoters of *CBFs*. The cold tolerance was reduced when *EIN3* overexpressed in *Arabidopsis*, while in

*ein3-1* mutants, *CBF* gene expression was significantly up-regulated (Shi et al. 2012). Three genes (G26606\_TU43719, G27348\_TU4485, and G27348\_TU44856) in Liaohanshu 21 and 2 genes in Shenshu 28 (G27348\_TU44853 and G24019\_TU39314) encoding EIN3 were up-regulated under low-temperature stress.

*CBF* genes, known for their rapid induction in response to low temperatures, bind to the CCGAC sequence found in the promoters of *COR* genes. This interaction results in forming a CBF-COR regulatory network, which boosts plant resilience to low temperatures (Park et al. 2015). In *Arabidopsis*, *COR* gene expression has been higher in cold-tolerant leaves than in cold-sensitive pollen (Lee and Lee 2003). Upon exposure to low-temperature stress, six *COR*-encoding genes (G14164\_TU23132, G14640\_TU23898, G17721\_TU28981, G17751\_TU29024, G26475\_TU43482, and G8901\_TU146600) in Liaohanshu 21 and another six genes (G14640\_TU23898, G17721\_TU28981, G17751\_TU29024, G19198\_TU31379, G26475\_TU43482, and G8901\_TU14660) in Shenshu 28 displayed down-regulation. Notably, our DEG analysis did not identify any putative *CBF* genes. This suggests that the CBF cold response pathway may have a limited function in sweetpotato under low-temperature stress. Therefore, enhancing cold tolerance in sweetpotato might be achievable by activating the plant's CBF pathway.

## Antioxidant genes

During exposure to environmental stressors, plants rapidly accumulate ROS such as  $\bullet\text{O}^{2-}$  and  $\text{H}_2\text{O}_2$ , disrupting membrane structures and nucleic acid integrity (Waszczak et al. 2018). To counteract this, plants have evolved systems to scavenge active oxygen species, including enzymes like SOD, CAT, POD, ascorbate peroxidase (APX), and glutathione peroxidase (GPX) (Gill and Tuteja 2010). Following 24 h of low-temperature exposure, the expression of 20 and 28 antioxidant enzyme-encoding genes differed in Shenshu 28 and Liaohanshu 21, respectively.

SOD, the primary enzymatic antioxidant system in plants combatting ROS, catalyzes the conversion of  $\bullet\text{O}^{2-}$  into  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  (Finkel 2011). Given that more  $\bullet\text{O}^{2-}$  was generated in Shenshu 28 than in Liaohanshu 21 during cold stress (Fig. 1B), the greater SOD activity in Shenshu 28 (Fig. 1G) is justifiable. However, our findings deviate from previous studies (Ji et al. 2019), wherein *FeSOD* (G46877\_TU75463) and *CuZnSOD* (G33204\_TU54407) were found to be down-regulated in both Shenshu 28 and Liaohanshu 21, and in Shenshu 28, respectively, after 24 h of cold stress. CAT can keep  $\text{H}_2\text{O}_2$  at a low level by catalyzing the decomposition of  $\text{H}_2\text{O}_2$  into  $\text{H}_2\text{O}$  and  $\text{O}_2$ . Previous studies showed that catalase-related genes were downregulated during cold stress in sweetpotato (Ji et al. 2017), while in our study, expression of *CAT1* (G27995\_TU45897) was up-regulated concurrently in Shenshu 28 and Liaohanshu 21. Another *CAT1* coding uni-gene (G27995\_TU45897) was also up-regulated in Shenshu 28, but *CAT3* (G29753\_TU48752, G29753\_TU48753) and putative *CAT* (G33052\_TU54145, G33052\_TU54148) were down-regulated in Liaohanshu 21. The relationship between *SOD* and *CAT* expression may vary with varieties by the type, cellular location, and mechanisms of action of SODs and CATs. In terms of *POD* genes, 21 and 14 showed different expression patterns in Liaohanshu 21 and Shenshu 28, respectively. Notably, cationic peroxidase 2-like (G30887\_TU50643) and *POD21* (G8404\_TU13835) demonstrated an increased expression in both Shenshu 28 and Liaohanshu 21, whereas the expression of *POD47*-like (G6059\_TU10000, G6057\_TU9998), *POD4*-like (G37128\_TU60871, G37121\_TU60862), and *POD3*-like (G23304\_TU38123) decreased in both plants. A particularly interesting finding was that *SwPNI* (G31194\_TU51163 and G31292\_TU51304 in Shenshu 28, G31292\_TU51304 in Liaohanshu 21) was less expressed in Shenshu 28 but more expressed in Liaohanshu 21, suggesting a potential role in cold stress defense.

APX serves as a key enzyme in converting  $\text{H}_2\text{O}_2$  into  $\text{H}_2\text{O}$ . Past studies found that overexpression of *OsAPXa* in rice increased the plant's cold tolerance at the booting stage (Sato et al. 2011), and overexpression of *LeAPX* in tomato resulted in enhanced chilling resistance (Duan et al. 2012). GPX, the main enzyme responsible for cellular lipid

peroxidation repair (Kühn and Borchert 2002), showed that overexpression led to increased cold tolerance in *Arabidopsis thaliana* and tomato (Gaber et al. 2004, 2010). The present study found that *APX* (G46795\_TU75338) and *GPX* (G9028\_TU14900) were more expressed in Shenshu 28, while only one *APX* (G46795\_TU75338) exhibited increased expression in Liaohanshu 21.

Upon comparing the gene expression levels of antioxidant enzymes in Shenshu 28 and Liaohanshu 21 post-24-h exposure to low temperature, no variation in the expression of *SOD* genes was observed. While the expression of *CAT3* (G29753\_TU48752) and *CAT* (G21007\_TU34349) was elevated in Shenshu 28, higher expression levels of *CAT1* (G27995\_TU45897) and *CAT3* (G29753\_TU48753) were noted in Liaohanshu 21. Out of 12 differentially expressed *POD* orthologs in Shenshu 28 and Liaohanshu 21, *POD15* (G30329\_TU49715) and *POD15*-like (G30329\_TU49714, G30329\_TU49716) showed enhanced expression in Shenshu 28, while the expression of *POD* P7-like (G25807\_TU42358, G25835\_TU42401) was reduced in the same. Interestingly, *SwPNI*, being more expressed in Liaohanshu 21 and less expressed in Shenshu 28, exhibited higher expression levels in Liaohanshu 21 than in Shenshu 28 (G31198\_TU51167). This finding suggests a potential role of *SwPNI* in cold stress defense. Furthermore, *APX* (G3714\_TU6097) demonstrated higher expression in Liaohanshu 21, while *GPX* (G9025\_TU14894, G9028\_TU14900) showed enhanced expression in Shenshu 28.

These gene expressions were consistent with the changes of  $\text{H}_2\text{O}_2$  content (Fig. 2C), CAT enzyme activity (Fig. 2E), and POD enzyme activity (Fig. 2F) in Liaohanshu 21 and Shenshu 28.

## Lipid metabolism

Biological membranes, which play vital roles in transportation, signal transduction, and metabolic regulation, are regarded as primary sites susceptible to chilling injury (Rui et al. 2010). During exposure to low temperatures, plants generally increase the content of unsaturated fatty acids to enhance their cold stress resistance (Hazel 1995; Wongsheree et al. 2009). In Liaohanshu 21, differential expression was observed in 199 genes associated with glyceride metabolism and 170 genes linked with glycerophosphate metabolism.

Lysophospholipid acyltransferase (LPEAT) is critical for acyl modification processes such as fatty acid desaturation and contributes to membrane biosynthesis (Shindou et al. 2013). Phosphatidic phosphatase (PAH) catalyzes the conversion of phospholipid into diacylglycerol (DAG), serving as a key enzyme in controlling the levels of PA and DAG in organisms. This enzymatic activity can regulate lipid metabolism and signal transduction, significantly influencing

plant stress responses (Eastmond et al. 2010; Csaki et al. 2013). Phospholipase D (PLD) hydrolyzes phospholipids into inositol triphosphate (IP<sub>3</sub>), DAG, phosphatidic acid (PA), and acetylcholine. DAG, IP<sub>3</sub>, PA, and acetylcholine molecules function as secondary messengers in intracellular signal transduction and alter cell structure and physiological function. Unigenes encoding LPEAT2 (G38146\_TU62498 and G38146\_TU62500), PAH4-like (G8614\_TU14174), and PLD-like (G19676\_TU32200) were found to be over-expressed in Liaohanshu 21, but no differential expression was observed in Shenshu 28. These genes may be crucial in maintaining membrane integrity and protecting Liaohanshu 21 from the deleterious effects of low temperatures.

### Plant hormone signal transduction

In the plant varieties, Shenshu 28 and Liaohanshu 21, 342 and 258 DEGs were found to participate in the plant hormone signal transmission pathway. Plant hormones, acting as chemical messengers, enable the efficient adaptation of plants to abiotic stress (Peleg and Blumwald 2011; Shabir et al. 2016).

Auxin, a crucial plant hormone, regulates the growth and development of many plants (Shivani et al. 2013). Among the DEGs identified in the two varieties, a significant number of auxin-responsive genes were found, primarily consisting of auxin transporters and auxin-responsive proteins. Most of these genes were down-regulated, although a few were up-regulated. OsSAUR45, which influences auxin synthesis and transport, has been found to be dependent on PIF4 under heat-stress conditions in rice (Xu et al. 2017). However, in the present study, the unigenes encoding PIF4-like proteins (G23568\_TU38558, G24019\_TU39314, G24019\_TU39315, and G24019\_TU39317 in Liaohanshu 21; G23568\_TU38558 and G24019\_TU39314 in Shenshu 28) were up-regulated when exposed to low temperature. This suggests that the interaction between PIF4 and auxin-related genes might be an adaptive mechanism for plants to cope with low-temperature stress.

Abscisic acid (ABA) is an important plant hormone whose endogenous level can be increased by salinity, drought, and low temperature (Roychoudhury et al. 2013). As ABA receptors, oyrabactin resistance-like (PYL) proteins were the upstream regulators of the ABA signal pathway and then activated SnRK2 by inhibiting PP2C phosphatase activity in activating the ABA signal pathway (Hao et al. 2011). Overexpressing of a rice *PYL3* enhanced cold and drought tolerance in *Arabidopsis thaliana* (Lenka et al. 2018). In our study, unigenes encoding *PYL3*-like (G18318\_TU29932 and G18361\_TU29998 in Shenshu 28 and Liaohanshu 21, G18385\_TU30035 in Liaohanshu 21) and *PYL4*-like (G3894\_TU6426 in Liaohanshu 21) were up-regulated when exposed to low temperature.

As a pivotal regulator of plant–microbe interactions under abiotic stress conditions, ethylene plays a crucial role (Shekawat et al. 2022). Ethylene response factors containing AP2 domains are transcription factors that orchestrate many processes. These include the regulation of gene expression related to stress responses, developmental pathways, growth processes, and mechanisms associated with hormone interactions (Cheng et al. 2013). Unigenes encoding EIN3-LIKE (G26606\_TU43719, G27348\_TU44853 and G27348\_TU44856 in Liaohanshu 21, G35436\_TU58103, G27348\_TU44853 and G27348\_TU44856 in Shenshu 28) were up-regulated; Unigenes encoding ethylene response factor ERF109 (G14664\_TU23931, G16250\_TU26531, G16257\_TU26539, G16257\_TU26541, G24812\_TU40712 in Shenshu 28, G14664\_TU23931, G16257\_TU26539, G16257\_TU26541, G16250\_TU26531 in Liaohanshu 21) were up-regulated.

### Conclusion

The molecular mechanisms governing sweetpotato's response to low temperatures were investigated by acquiring transcriptomic data from cold-tolerant and cold-sensitive sweetpotato leaf samples, resulting in the identification of 82,513 unigenes. The DEGs observed when exposed to low temperatures for 3 and 24 h were subjected to GO and KEGG analysis. The cold-tolerant variant, Liaohanshu 21, exhibited fewer DEGs than the cold-sensitive variant, suggesting its greater resilience against cold stress. Upon exposure to low temperatures, an ICE1-encoding gene was found to be up-regulated, while three COR-encoding genes were downregulated in both variants. The absence of DEGs encoding CBF indicated a potentially limited role of the CBF cold response pathway in sweetpotato under cold stress. Greater importance was attributed to genes associated with antioxidant enzymes, lipid metabolism, and plant hormone signal transduction in enhancing sweetpotato's resistance to low temperatures. This study shed light on the molecular dynamics of sweetpotato under cold stress, offering valuable insights for the breeding of cold-tolerant sweetpotato varieties.

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