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Identification of *Catalpa bungei* Aquaporin Gene Family Related to Low Temperature Stress

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Abstract: Low temperatures pose a significant threat to plant growth and development. Studies have shown that aquaporins (AQPs), as the main functional proteins on the cell membrane regulating water ingress and egress, play a vital role in maintaining dynamic water balance when plants face cold stress. *Catalpa bungei*, an important timber and ornamental tree species, has its cultivation range significantly limited by its poor cold tolerance. However, no study has been found aiming to identify its aquaporin gene family. This study aims to fill this gap using two *C. bungei* cultivars with differing cold tolerance as experimental material: "Qiuza 1", which is less cold-tolerant, and "Qiuza 2", which is more cold-tolerant. The plants were subjected to low-temperature stress at 4 °C for 24 h. Using high-throughput molecular sequencing technology, a transcriptome sequencing of the leaves was performed at 0, 6, 12, and 18 h of cold stress. Fifteen candidate aquaporin genes in *C. bungei* (CbAQP) were identified. Phylogenetic analysis showed that the CbAQP gene family is divided into five subfamilies: 5 PIPs, 4 TIPs, 3 NIPs, 2 SIPs, and 1 XIP. By analyzing AQPs related to cold stress in other plants and the expression patterns of CbAQP genes, 12 CbAQP genes related to cold stress were identified. The genes that responded positively include *CbPIP2;5*, *CbPIP1;2*, *CbTIP4;1*, and *CbNIP2;1*. The results provide a foundation for further analysis of the biological functions of candidate CbAQP genes related to cold tolerance and offer theoretical support for improving seedling quality, cold-resistant genetic breeding, and expanding its distribution range.

Keywords: *Catalpa bungei*; aquaporins; low-temperature stress; cold tolerance

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1. Introduction

Water is indispensable at every stage of plant growth and development, and is an essential component for the maintenance of normal physiological functions in cells. Plants constantly undergo water absorption, transmembrane transport, and inter-tissue transfer. Aquaporins (AQPs) are membrane proteins embedded in the biological membrane that efficiently transport water molecules and other small molecular compounds. AQPs can enhance the transmembrane transport efficiency of water molecules, increasing the permeability by more than tenfold [1]. They also regulate the flow of water within cells, with approximately 70%–90% of intracellular water movement facilitated through AQPs [2]. Additionally, AQPs are involved in various physiological and metabolic processes [3–5].

Aquaporin primary structures exhibit a high degree of homology between the amino (N) terminus and carboxyl (C) terminus sequences within the protein [6]. Aquaporins have small NPA motifs at both ends composed of highly conserved amino acid residues (Asn–Pro–Ala sequence). In higher plants, AQPs are divided into five subfamilies based on sequence homology, similarity, and subcellular localization: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs),

small basic intrinsic proteins (SIPs), and uncharacterized X intrinsic proteins (XIPs). Two additional subfamilies, GlpF-like intrinsic proteins (GIPs) and hybrid intrinsic proteins (HIPs) are found only in mosses. Studies have demonstrated that aquaporins play a crucial role not only as indispensable mediators in plant water transport but also in maintaining intracellular homeostasis and reducing external damage under abiotic stress [7–9].

Low-temperature stress is a common abiotic stress factor that severely limits plant growth and development. Numerous studies have demonstrated a close relationship between aquaporins and plant cold resistance. Low temperatures reduce the water absorption rate of plant roots and water transport within the plant body. Cold-tolerant plants can recover growth after exposure to low temperatures, while cold-sensitive plants may lose their ability to regulate water under cold stress and subsequently die from dehydration during recovery [10]. As one of the main pathways for transmembrane water transport, aquaporins play a crucial role when plants face low-temperature stress [11]. Research has shown that plant aquaporins exhibit a cold-stress response at the beginning of cold stress. For example, transgenic *Musa nana*s overexpressing *MusaPIP1;2* and *MusaPIP1;2* exhibits enhanced tolerance to low-temperature stress [12], and transgenic *Nicotiana tabacum* overexpresses the *Triticum aestivum* aquaporin gene TaAQP7 (PIP2), showing increased cold and drought tolerance [13,14]. When *Oryza sativa* is exposed to low temperatures over a long period, it increases the expression of *OsPIP2;5* to enhance root hydraulic conductivity (*Lpr*), thereby mitigating the impact of the cold stress on roots [15]. Studies indicate that plants such as *O. sativa* [16], *M. nana* [12,17,18], *T. aestivum* [13,19], and *Sorghum bicolor* [20] enhance their cold tolerance by increasing or suppressing the expression of related aquaporin genes under low-temperature stress, with many of the cold-stress-related aquaporin genes being PIP genes.

Catalpa bungei, a large deciduous tree in the Bignoniaceae family, is a traditional and precious native tree species unique to China, mainly used for wood processing and landscape greening, historically referred to as the "King of Woods" [21]. Due to its poor cold tolerance, low temperatures significantly limit its cultivation scope in China. This study aims to identify the aquaporin gene family of *C. bungei* (CbAQP), analyze their expression patterns, and investigate the changes in the expression of the CbAQP gene family during different cold stress periods, selecting candidate aquaporin genes responsive to low-temperature stress. The results of this study lay the foundation for further research into the biological functions of candidate aquaporin genes related to the cold resistance of *C. bungei*, provide a theoretical basis for improving the quality of *C. bungei* seedlings and cold-resistant breeding, and expanding its distribution range to the south and north.

2. Material and Method

2.1. Experimental Materials and Treatments

In this study, "Qiuza 1", with less cold tolerance, and "Qiuza 2", with more cold tolerance, were used as experimental materials. These were supplied by Henan Agricultural Science Garden Horticultural Technology Co., Ltd. (Zhengzhou, China) (34°79′ N, 113°68′ E). The plant materials were formally identified by Lou Changcheng, a senior agronomist at the Henan Academy of Agricultural Sciences, and subsequently authenticated by Professor Zhang Gang from Hebei Agricultural University. The experimental materials were seedlings that had been cultured in vitro for three months and then potted and normally managed for three months. The seedlings were maintained in the artificial climate room at the West Campus of Hebei Agricultural University (Baoding, China, 38°50′ N, 115°26′ E).

Uniformly growing seedlings of "Qiuza 1" and "Qiuza 2" were selected for acclimatization culture for 7 days under conditions of 25 °C (day)/17 °C (night) and 16 h (day)/8 h (night). Subsequently, they were moved to a 4 °C artificial climate chamber for 24 h, with the time point of 0 h serving as the control. Three replicates were set up for the experiment. Observations of the morphological changes in the plants at different periods of low-temperature stress were made, with photographs taken for records. Transcriptome

sequencing was performed on fully expanded leaves from the middle part of the plants at 0 h, 6 h, 12 h, and 18 h.

2.2. Transcriptome Sequencing and Analysis

2.2.1. RNA Extraction

The young leaves of "Qiuza 1" and "Qiuza 2" were selected. After washing the leaves, the samples were immediately placed into liquid nitrogen and stored in a −80 °C ultra-low temperature freezer in preparation for transcriptome sequencing. The RIN value of the samples used for sequencing was more than 7. Total RNA was extracted using the OminiPlant RNA Kit (DNase I) reagent kit, synthesized by Kangwei Century (Beijing, China).

2.2.2. Complementary DNA (cDNA) Library Construction

Eukaryotic mRNA was enriched using magnetic beads with Oligo (dT). Subsequently, the mRNA was fragmented using a fragmentation buffer. Using mRNA as a template, single-stranded cDNA was synthesized with random hexamers. Next, buffer, deoxyribonucleoside triphosphate (dNTPs), DNA polymerase I, and Ribonuclease (RNase) H were added to synthesize double-stranded cDNA, followed by purification of the double-stranded cDNA using AMPure XP beads. The purified double-stranded cDNA underwent end repair, A-tailing, and adapter ligation, followed by size selection using AMPure XP beads. Polymerase chain reaction (PCR) amplification was performed, and the PCR products were purified using AMPure XP beads to obtain the final library. Upon completion of library construction, preliminary quantification was performed using Qubit 2.0. Subsequently, the library was diluted, and the insert size of the library was checked. Once the insert size met expectations, the effective concentration of the library was quantified using Q-PCR (library effective concentration > 2 nM) to ensure library quality.

2.2.3. Transcriptome Data Analysis

Paired-end sequencing of the library was performed on the Illumina NovaSeq 6000 system at Tianjin Novogene Biotechnology Co., Ltd., (Tianjin, China); each read was 300 base pairs long.

2.2.4. Transcriptome Data Analysis

The raw image data generated by the sequencer was converted into sequence data, known as raw data, through base calling. Raw data underwent data processing, including removal of adaptor sequences, exclusion of reads with an N content greater than 10%, or removal of reads containing a substantial proportion of low-quality sequences (where bases with a quality value (Q) less than five accounted for more than 50% of the entire read), resulting in clean reads.

All transcriptome data were assembled using Trinity-v2.4.0 software with the following command and parameters: "Trinity --seqType fq --max_memory 300 G --left file_1.fq --right file_2.fq --CPU 50 --full_cleanup --KMER_SIZE 30 --min_kmer_cov 5". In genes with multiple transcripts, the longest transcript sequence was used as the basis for calculating expression levels. RNA-Seq by Expectation Maximization (RSEM) was employed for transcript abundance calculation, with Transient Multimon Manager (TMM) utilized as the method for inter-sample normalization.

2.3. Identification and Physicochemical Analysis of the Gene Family

Thirty-five aquaporin protein gene sequences from *Arabidopsis thaliana* and six X intrinsic proteins (XIP) aquaporin protein genes from *Populus* were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/ (accessed on 6 June 2020)) and used as query sequences [22]. These sequences were then subjected to blast homology analysis against the Chinese *C. bungei* transcriptome dataset to identify the CbAQP gene family. The screened CbAQP gene family protein sequences were further subjected to physicochemical analysis using the online software ExPASy (https://web.expasy.org/protparam/ (accessed on 7 July

2022)), including analysis of amino acid count, molecular weight, theoretical pI (isoelectric point), aliphatic index, and grand average of hydropathicity (GRAVY).

2.4. Construction of Phylogenetic Trees for the CbAQP Gene Family

Candidate genes were subjected to multiple sequence alignment using the E-INS-I mode of the online software MAFFT v7.487 (https://www.ebi.ac.uk/Tools/msa/mafft/ (accessed on 13 July 2022)), followed by necessary manual adjustments. The TBTOOLS 2.019 software [23] was utilized to construct phylogenetic trees for the CbAQP gene family and for both the CbAQP genes and cold-stress-related aquaporin protein genes. Default parameters were set, and the online software iTOL (https://itol.embl.de/ (accessed on 20 July 2022)) was employed for the beautification of the phylogenetic trees.

2.5. Conserved Motif Analysis

The conserved motifs of the CbAQP gene family were analyzed using the online analysis tool MEME V4.11.3 (https://meme-suite.org/meme/tools/meme (accessed on 30 July 2022)) with default parameters. The output includes motif sequences, positions, widths, and the E-value for each motif.

2.6. Analysis of Gene Expression Patterns

The gene expression patterns of the CbAQP gene family were constructed using the MeV software V4.9.0 [24]. Differential gene expression analysis was conducted on "Qiuza 1" and "Qiuza 2" under different durations of cold stress to preliminarily screen candidate genes.

2.7. Validation of CbAQP Gene Family via Real-Time Quantitative PCR

2.7.1. RNA Extraction and cDNA Synthesis

RNA extraction was performed following the method described in Section 2.2.1. Total RNA from *C. bungei* leaves treated at different durations of cold stress was extracted using the OmniPlant RNA Kit (DNase I) reagent kit. For RNA reverse transcription, the UEIris II RT-PCR System for First-Strand cDNA Synthesis (with dsDNase) reagent kit, synthesized by Suzhou Yuheng (Suzhou, China) Biotechnology Co., Ltd., was used. The protocol was followed as per the manufacturer's instructions.

2.7.2. Fluorescent Quantitative RT-qPCR Primer Design

Primers for RT-qPCR were designed using the online software Primer3 Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi (accessed on 8 August 2022)). The primers were synthesized by Shenggong (Shanghai, China) Biotechnology Co., Ltd. The *C. bungei Cbuactin* [25] gene was selected as the internal reference gene. Primer information is provided in Table 1.

Table 1. List of RT-qPCR primers.

Primer Name	Forward Primer Sequence	Reverse Primer Sequence
Cbuactin	F: GATGATGCTCCAAGGGCTGT	R: TCCATATCATCCCAGTTGCT
CbPIP2;6	F: TCCTGGTTTACACTGTCTTCTC	R: CTGCTCCGATGAATGGTC
CbPIP2;5	F: TACAGCGGAAAGGACTACCA	R: CAGAAACAGGCCGAACGT
CbPIP1;2	F: GGGGTGAACAGAGCACCTAA	R: GCATGAAACCCTTGACCACT
CbPIP1;4	F: GGAGAACAAAGAGGAGGATG	R: GCAGTAGACAAGGGCAAAG
CbTIP2;1	F: CTGCAATTGCTTTTGGAAGA	R: CCGGCAAAGACGAAAATTAAG
CbTIP4;1	F: GTCACCCTCGGACTATGC	R: CCAACCCAGTAAACCCAAT
CbNIP5;1	F: GCTGGCGGTGATGATAGT	R: GCGAATAGATAGCTGCTCC
CbNIP2;1	F: GCTTAGTGTTAGCGATGAA	R: CCTGTGGGTGTAGTTGTG
CbNIP6;1	F: CAAGAAAGGTGGGAGCTGAG	R: TTCCAAGGAAAATGCCTGAG
CbSIP2;2	F: GTTGCATGAATCCAGCCTCT	R: TCATTCTTCGGTCGGGATAG

2.7.3. RT-qPCR Reaction System and Reaction Conditions

According to the instructions for AugeGreenTM qPCR Master Mix, detection was performed using the QuantStudio 5 Real-Time PCR System (Sourced by Thermo Fisher Scientific, Waltham, MA, USA). The configuration of the reaction mixture and the reaction program are shown in Table 2.

Table 2. RT-qPCR reaction solution configuration and reaction procedure.

	Composition/Temperature and Time	Content/Circulation
Reaction solution configuration	cDNA	1 μL
	2× AugeGreen Master Mix	10 μL
	primer F	1 μL
	primer R	1 μL
	ddH2O	7 μL
Reaction procedure	95 °C 120 s	--
	95 °C 15 s 58 °C 60 s	40 cycles
	95 °C 10 s	--
	65 °C 60 s	--
	97 °C 1 s	--

Each sample was set up with 3 technical replicates and 3 biological replicates. The results were calculated using the $2^{-\Delta\Delta CT}$ method to determine gene expression levels.

3. Results

3.1. External Morphological Changes in Two C. bungei Varieties during Cold Stress

Prior to cold stress (0 h), the leaves of both varieties were fully expanded (Figure 1(A1,A2)). However, after exposure to 4 °C cold stress, notable differences were observed. In "Qiuza 1", leaf margins began to curl upwards at 9 h of stress (Figure 1(D1)), which is a characteristic of injury, followed by a slight downward inclination at 12 h (Figure 1(E1)) is characterized by severe injury. On the other hand, "Qiuza 2" showed leaves curling upwards at 6 h of stress (Figure 1(C2)), with a slight downward bending observed at 24 h (Figure 1(G2)). These morphological alterations suggest varying responses to cold stress between the two *C. bungei* varieties.

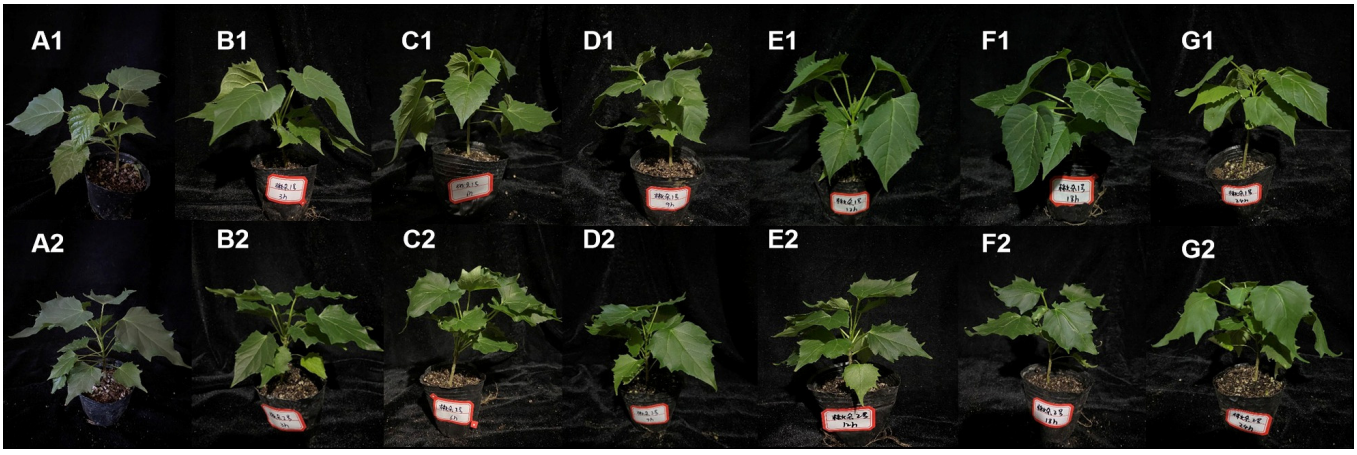

Figure 1. Morphological change of leaves of "Qiuza 1" and "Qiuza 2" during the low-temperature stress period ("1" and "2" in the figure denotes "Qiuza 1" and "Quiza 2", respectively; (**A**–**G**) represent the low-temperature stress at 4 °C for 0 h, 3 h, 6 h, 9 h, 12 h, 18 h, and 24 h, respectively) (The words on the label in the figure are chinese marks during the experimental processing).

3.2. Identification and Basic Information Analysis of the CbAQP Gene Family

Based on the analysis of the model plant *A. thaliana* aquaporin protein family and six XIP aquaporin protein genes from *Populus* (Table 3), fifteen candidate *C. bungei* aquaporin protein genes were identified (Table 4). The gene ID of *C. bungei* is renamed based on the homology with *A. thaliana* (*At*), *O. sativa* (*Os*), and *P. trichocarpa* (*Pt*) (Table 5). According to sequence alignment and characterization of related proteins (Table 4), the physicochemical properties of CbAQP family revealed differences. The amount of amino acid of CbAQP family sequences ranged from 144 to 340. The molecular weight of CbAQP family sequences ranged from 16,333.32 to 37,487.66 Da. The theoretical pI of CbAQP family sequences ranged from 5.06 to 10.53, with 11 out of 15 CbAQP having a theoretical pI greater than 7.5. The aliphatic index of CbAQP family sequences ranged from 90.04 to 125.00.

Table 3. 35 Aquaporins of *Arabidopsis thaliana* and 6 XIP aquaporins of *Populus trichocarpa*.

Subfamily	Name	Synonyms	NCBI Reference Sequence
PIP	*AtPIP1;1*	PIP1A	AT3G61430
	AtPIP1;2	PIP1B;TMPA	AT2G45960
	AtPIP1;3	PIP1C;TMPB	AT1G01620
	AtPIP1;4	TMPC	AT4G00430
	AtPIP1;5	PIP1D	AT4G23400
	AtPIP2;1	PIP2A	AT3G53420
	AtPIP2;2	PIP2B;TMB2B	AT2G37170
	AtPIP2;3	RD28;TMP2C	AT2G37180
	AtPIP2;4	PIP2F	AT5G60660
	AtPIP2;5	PIP2D	AT3G54820
	AtPIP2;6	PIP2E	AT2G39010
	AtPIP2;7	PIP3;SIMIP	AT4G35100
	AtPIP2;8	PIP3B	AT2G16850
AtTIP	*AtTIP1;1*	GAMMA-TIP	AT2G36830
	AtTIP1;2	TIP2	AT3G26520
	AtTIP1;3	GAMMA-TIP1	AT4G01470
	AtTIP2;1	DELTA-TIP	AT3G16240
	AtTIP2;2	DELTA-TIP2	AT4G17340
	AtTIP2;3	DELTA-TIP3	AT5G47450
	AtTIP3;1	α-TIP	AT1G73190
	AtTIP3;2	BETA-TIP	AT1G17810
	AtTIP4;1		AT2G25810
	AtTIP5;1		AT1G17820
NIP	*AtNIP1;1*	NLM1	AT4G19030
	AtNIP1;2	NLM2	AT4G18910
	AtNIP2;1		AT2G34390
	AtNIP3;1		AT1G31885
	AtNIP4;1		AT5G37810
	AtNIP4;2		AT5G37820
	AtNIP5;1		AT4G10380
	AtNIP6;1		AT1G80760
	AtNIP7;1		AT3G06100
SIP	*AtSIP1;1*	SIP1A	AT3G04090
	AtSIP1;2		AT5G18290
	AtSIP2;1		AT3G56950
XIP	*PtXIP1;1*		POPTR_0009s13090
	PtXIP1;2		POPTR_0009s13105
	PtXIP2;1		POPTR_0009s13110
	PtXIP3;1		POPTR_0009s13080
	PtXIP3;2		POPTR_0009s13070
	PtXIP3;3		POPTR_0004s17430

Table 4. Properties of the AQP family of proteins.

Subfamily	Gene Name	Number of Amino Acids (aa)	Molecular Weight (Da)	Theoretical pI	Aliphatic Index	Grand Average of Hydropathicity
PIP	*CbPIP1;3*	293	31,531.77	8.92	93.99	0.361
	CbPIP2;6	285	30,348.27	8.23	101.72	0.489
	CbPIP2;5	283	30,102.97	8.62	100.71	0.530
	CbPIP1;2	244	26,401.46	8.32	90.04	0.150
	CbPIP1;4	185	19,578.88	9.64	103.95	0.485
TIP	*CbTIP1;1*	267	27,667.00	5.80	106.10	0.691
	CbTIP1;2	225	23,240.03	5.59	112.36	0.952
	CbTIP2;1	212	21,541.24	5.06	121.42	0.986
	CbTIP4;1	260	27,268.91	5.63	114.42	0.837
NIP	*CbNIP5;1*	237	24,578.78	8.89	113.67	0.772
	CbNIP2;1	213	22,467.14	8.01	107.14	0.559
	CbNIP6;1	276	29,544.48	9.28	93.70	0.309
SIP	*CbSIP2;2*	178	20,170.98	9.48	104.04	0.384
	CbSIP2;1	144	16,333.32	10.53	102.85	0.637
XIP	*CbXIP3;1*	340	37,487.66	8.93	125.00	0.785

Table 5. The names of the identified CbAQPs genes.

Subfamily	Gene ID	Name	At/Os/Pt
SIP	*Cluser-8567.25081*	*CbSIP2;2*	*AtSIP2;2*
	Cluser-8567.34148	*CbSIP2;1*	*AtSIP2;1*
XIP	*Cluser-8567.37481*	*CbXIP3;1*	*PtXIP3;1*
PIP	*Cluser-8567.21102*	*CbPIP1;4*	*AtPIP1;4*
	Cluser-8567.21827	*CbPIP1;2*	*OsPIP1;2*
	Cluser-8567.21828	*CbPIP1;3*	*OsPIP1;3*
	Cluser-8567.27409	*CbPIP2;5*	*AtPIP2;5*
	Cluser-8567.31896	*CbPIP2;6*	*AtPIP2;6*
NIP	*Cluser-8567.3249*	*CbNIP2;1*	*OsNIP2;1*
	Cluser-8567.24887	*CbNIP5;1*	*AtNIP5;1*
	Cluser-8567.2348	*CbNIP6;1*	*AtNIP6;1*
TIP	*Cluser-8567.5190*	*CbTIP4;1*	*AtTIP4;1*
	Cluser-8567.23498	*CbTIP1;1*	*OsTIP1;1*
	Cluser-8567.23500	*CbTIP1;2*	*OsTIP1;2*
	Cluser-8567.31039	*CbTIP2;1*	*AtTIP2;1*

3.3. Phylogenetic Analysis of the CbAQP Gene Family

The CbAQP gene family comprises 15 members that form 5 subfamilies, namely PIPs, TIPs, NIPs, SIPs, and XIPs (Figure 2). The number of members in each subfamily is 5, 4, 3, 2, and 1, respectively. The distribution of subfamily members in the CbAQP gene family closely mirrors the proportions observed in other plant species, showing a gradual decrease in member numbers (Table 6).

Table 6. Distribution of subfamily members of AQP gene family in various plants.

Ref.	PIPs	TIPs	NIPs	SIPs	XIPs	Total Amount of AQP
A. thaliana [26]	13	10	9	3	0	35
O. Sativa [27]	11	10	10	2	0	33
Cucumis sativus [28]	19	8	9	2	1	39
S. bicolor [20]	14	13	11	3	0	41
Gossypium hirsutum [29]	28	23	12	7	1	47
P. Trichocarpa [30]	15	17	11	6	6	55
M. Nana [12]	18	17	9	3	0	47
C. bungei	5	4	3	2	1	15

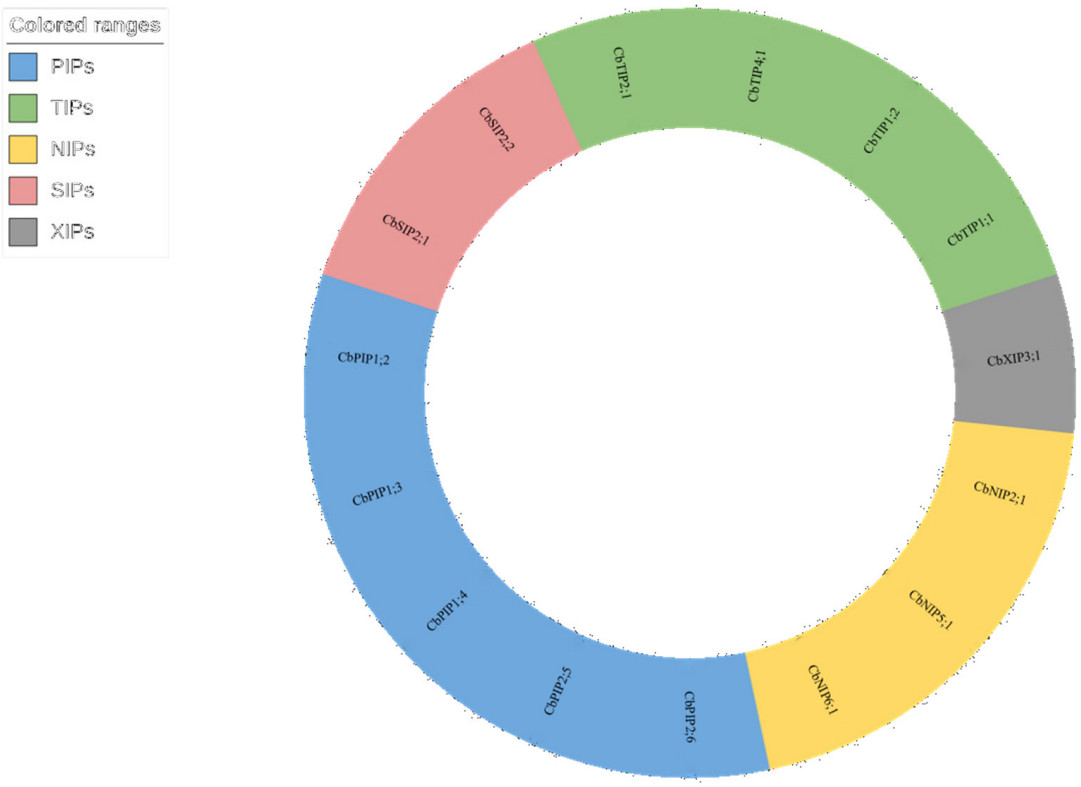

Figure 2. Phylogenetic analysis of the gene family of CbAQP.

In this study, a phylogenetic tree was constructed (Figure 3) based on 35 aquaporin protein genes from *A. thaliana* (*At*), 35 aquaporin protein genes from *O. sativa* (*Os*), 6 aquaporin protein genes from *P. trichocarpa* (*Pt*), 15 candidate CbAQP genes, and other plant aquaporin genes related to cold stress. According to the current findings, it was observed that cold-stress-related aquaporin protein genes in *M. nana and G. hirsutum* were predominantly distributed in the PIPs subfamily [12,29], while those in *A. thaliana* [11,31], *O. sativa* [16,32], *Hordeum vulgare*, and *Brassica rapa* [33] were all distributed in the PIPs subfamily.

3.4. Conserved Motif Analysis of the CbAQP Gene Family

Among the 15 candidate CbAQP genes, 12 major conserved motifs were identified (Table 7). The distribution of conserved motifs in the 15 candidate CbAQP genes is illustrated in Figure 4. The CbAQP gene family demonstrates homogeneity, with most subfamilies, such as PIPs, TIPs, NIPs, and XIPs containing motif 1 and motif 2. Additionally, motif 10 is present in members of the TIPs and SIPs subfamilies. Each subfamily also contains its unique yet similar conserved motifs: members of the PIPs subfamily contain motif 7, members of the TIPs subfamily contain motif 3 and motif 4, and members of the SIPs subfamily contain motif 8 and motif 10.

According to the research, it is known that currently, many AQP genes related to cold stress contain common gene sequence fragments (Appendices A and B), such as IAEFXXT, GIAW, GGMI, LVYCTAG, SGGHINPAVT, and GTFVLVYTVF. IAEFXXT exists in motif5, GIAW and GGMI in motif 6, VYCTAG and SGGHINPAVT in motif 1, and GTFVLVYTVF in motif 2. These motifs are distributed in various subfamilies of the AQP gene family, among which motif 6 only exists in the PIPs subfamily.

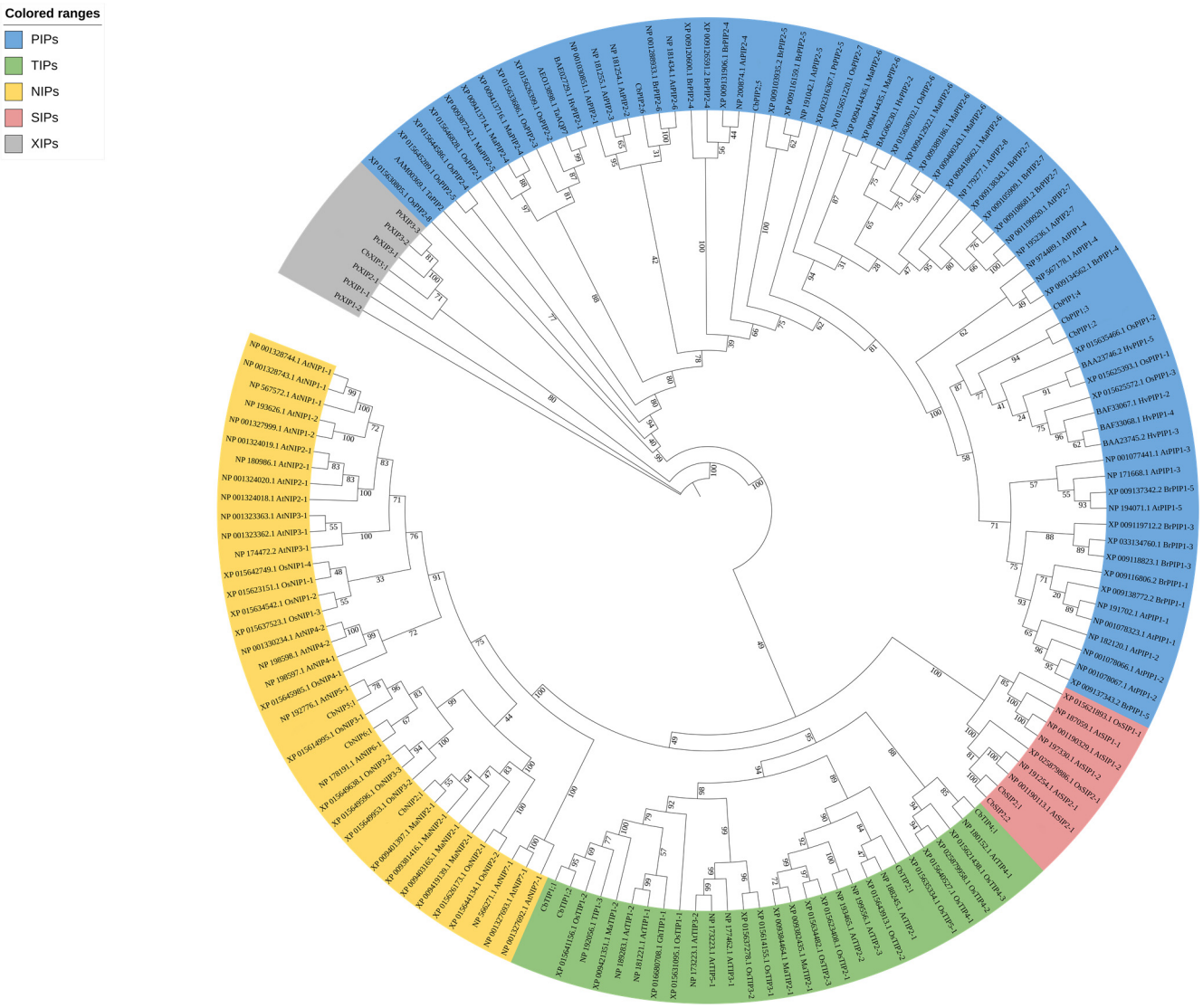

Figure 3. Phylogenetic analysis of CbAQP genes and cold-stress-related aquaporin genes.

Table 7. 12 Conserved motif information of CbAQP (note: motif sequences represent the protein motifs; sites indicate the number of occurrences of this motif in the 15 *C. bungei* aquaporin proteins; width indicates the width of the motif; E-value indicates the statistical significance of the motif. A smaller E-value indicates a more reliable result).

Motif Type	Motif Sequences	Sites	Width	E-Value
Motif 1	VYCTAGISGGHINPAVTFGLFLARHISLTRALFYMVAQLLGAICACGLLK	13	50	1.2×10^{-213}
Motif 2	TGQALVAEIIGTFVLVYTVYAAADDKRKA	13	29	7.3×10^{-94}
Motif 3	LAPLPIGFAVGANILATGPFTGTSMNPARSFGPAVI	9	36	1.2×10^{-87}
Motif 4	SHAWDDHWIFWVGPFIGAAJA	9	29	6.0×10^{-71}
Motif 5	YTDKDYKDPPPAPLFDPGELKSWSFYRAGIAEFIATFLFLYITILTVIG	4	49	1.4×10^{-60}
Motif 6	PDKCGGVGIQGIAWAFGGMIF	4	21	8.6×10^{-18}
Motif 7	FQKGPYQRYGGGANFVAHGYT	5	21	2.3×10^{-17}
Motif 8	WRLLVADFLMSFMWVWSSVLNKIFVHKILGYGAHZVEGEIVRYGVSILNM	2	50	7.6×10^{-13}
Motif 9	ESMAENKEEDVRLGANKFIEKQP	2	23	3.9×10^{-10}
Motif 10	AKLTNGGAYNPAGLIAAAIAHAFALF	5	26	4.7×10^{-8}
Motif 11	RDSHEP	8	6	2.1×10^{-7}
Motif 12	YHQFIJRAGPFK	4	12	1.1×10^{-6}

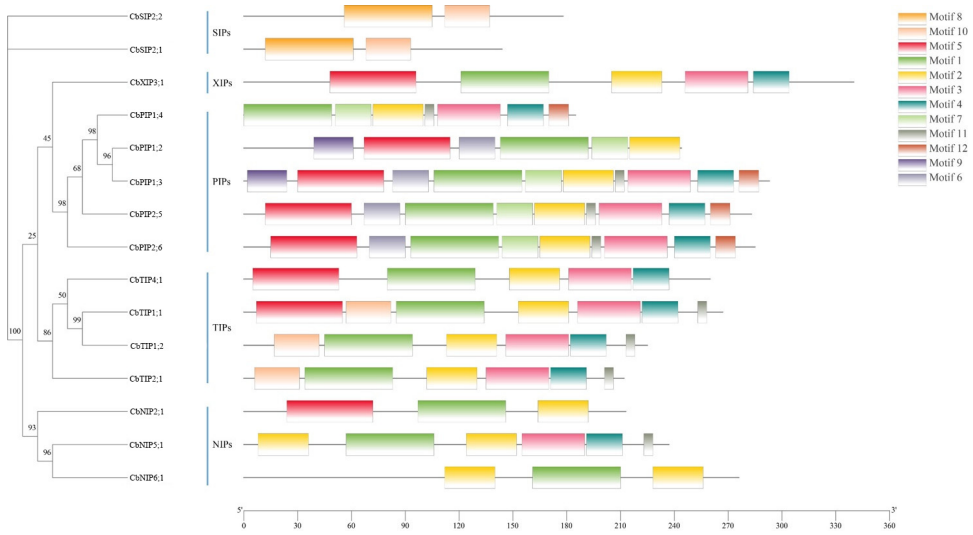

Figure 4. Conserved motif distribution map of CbAQP.

3.5. The Analysis of Expression Patterns of CbAQP Genes

The study examined the expression levels of 15 AQP genes in the two cultivars "Qiuza 1" and "Qiuza 2" under low-temperature stress at 0 h, 6 h, 12 h, and 18 h. It was found that three AQP genes, *CbTIP1;1*, *CbTIP1;2*, and *CbSIP2;1*, showed no expression in both cultivars. Therefore, further analysis was conducted on the expression levels of the remaining 12 aquaporin genes (Figure 5).

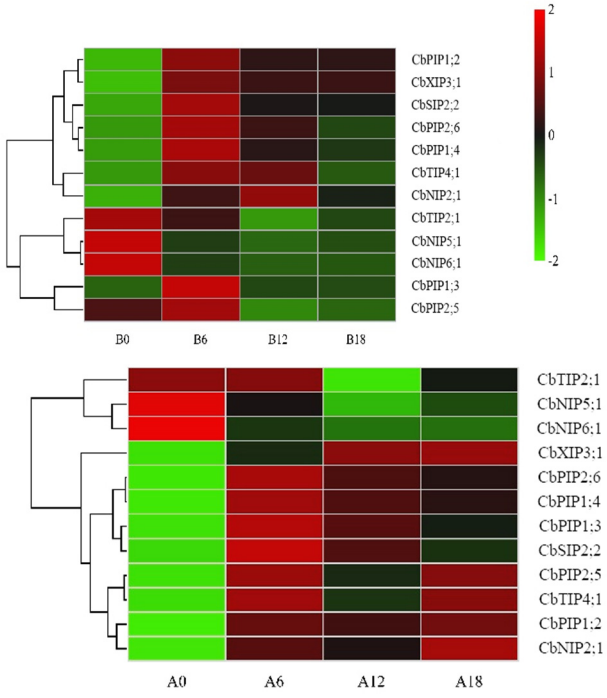

Figure 5. Relative transcript abundance profiles of CbAQP during natural overwintering period (A0, A6, A12, and A18 are the expression level of AQP genes of "Qiuza 1" at low-temperature stress 0 h, 6 h, 12 h, and 18 h, respectively; B0, B6, B12, and B18 are the expression level of AQP genes of "Qiuza 2" at low-temperature stress 0 h, 6 h, 12 h, and 18 h, respectively).

In "Qiuza 1" (Figure 5, A0, A6, A12 and A18), the expression of 12 genes was clustered into two branches. One branch consists of *CbNIP6;1*, *CbNIP5;1*, and *CbTIP2;1*, which overall show a trend of decreasing gene expression levels with the extension of cold stress duration. The other branch exhibits an overall increase in expression levels as the cold-stress duration

extends and is further divided into three sub-branches. The first sub-branch, represented by *CbXIP3;1*, shows a gradually increasing trend in expression levels, with a significant rise at 12 h and peaking at 18 h with a value of 3.44. The second sub-branch, including *CbPIP2;6*, *CbPIP1;4*, *CbPIP1;3*, and *CbSIP2;2*, overall reaches its highest expression level of 8.54 at 6 h of cold stress, followed by a gradual decrease in expression levels over time, yet the subsequent levels remain significantly higher than that of the control. The third sub-branch, containing *CbPIP2;5*, *CbTIP4;1*, *CbPIP1;2*, and *CbNIP2;1*, shows a trend where the expression levels peak at 6 h of cold stress, decrease at 12 h, and then increase again at 18 h. Genes *CbPIP1;2*, *CbNIP2;1*, and *CbXIP3;1*, among those with overall increasing expression levels, all have higher expression levels at 18 h compared to 6 h, with the high expression occurring later in time. Notably, *CbNIP2;1* shows a 27.93% increase in expression level at 18 h over 6 h, and gene *CbXIP3;1* is the only AQP gene exhibiting a continuous upward trend.

In "Qiuza 2" (Figure 5, B0, B6, B12 and B18), the expression of 12 Catalpa genes was clustered into two branches. One branch consists of *CbPIP2;5*, *CbPIP1;3*, *CbTIP2;1*, *CbNIP5;1*, and *CbNIP6;1*, which overall show a transition from high to low gene expression levels as the cold stress progresses. This branch is further divided into two sub-branches. The first sub-branch, including *CbTIP2;1*, *CbNIP5;1*, and *CbNIP6;1*, has a consistent expression pattern where each exhibits high expression prior to cold stress, with expression levels gradually decreasing as the cold stress duration extends. The second sub-branch, composed of *CbPIP2;5* and *CbPIP1;3*, shows a trend of initial increase followed by a decrease in expression levels. They maintain relatively low expression levels before the cold stress, experience an increase in expression at 6 h of the cold stress, and then rapidly decrease to levels below that of the control as the cold stress continues. At 18 h, there is a slight increase in expression, but levels remain below that of the control.

The other branch includes *CbTIP4;1*, *CbNIP2;1*, *CbXIP3;1*, *CbPIP2;6*, *CbPIP1;4*, *CbPIP1;2*, and *CbSIP2;2*. Overall, as the duration of cold stress extends, the expression levels show a trend of initially increasing and then decreasing, with peak expression levels for most occurring at 6 h. Exceptionally, *CbNIP2;1* exhibits a different expression pattern, with its peak expression level appearing at 12 h. This branch is further divided into two sub-branches. The first sub-branch comprises *CbTIP4;1* and *CbNIP2;1*. *CbTIP4;1* reaches its peak expression level at 6 h, whereas *CbNIP2;1* peaks at 12 h. The second sub-branch, consisting of *CbPIP2;6*, *CbPIP1;4*, *CbPIP1;2*, *CbSIP2;2*, and *CbXIP3;1*, all exhibit peak expression levels at 6 h of cold stress.

From the perspective of expression levels, *CbTIP4;1* exhibits the most significant difference between "Qiuza 1" and "Qiuza 2". At 0 h, the expression level in "Qiuza 2" was already seven times higher than in "Qiuza 1". In "Qiuza 2", the expression level at 6 h of cold stress increases by 1.85 times compared to 0 h, then slightly decreases at 12 h, and by 18 h, it is only 24.33% higher than the expression at 0 h. In contrast, in "Qiuza 1", the expression level of *CbTIP4;1* at 6 h of cold stress is 14.79 times that of 0 h. However, at 12 h, the expression level dramatically decreases to 4.95 times that of 0 h, and then at 18 h, it sharply rises again, reaching 13.8 times the expression level at 0 h.

Through the analysis of the relative expression patterns of aquaporin proteins in "Qiuza 1" and "Qiuza 2" (Figure 5), as well as the phylogenetic and cold-stress-related analysis of AQP (Figure 3), this study has identified 12 catalpa AQP genes that respond to cold stress. These genes are *CbPIP1;3*, *CbPIP2;6*, *CbPIP2;5*, *CbPIP1;2*, *CbPIP1;4*, *CbTIP2;1*, *CbTIP4;1*, *CbNIP5;1*, *CbNIP2;1*, *CbNIP6;1*, *CbSIP2;2*, and *CbXIP3;1*.

3.6. Validation of CbAQP Candidate Genes' Expression in Response to Cold

To verify the accuracy of RNA-seq data, RT-qPCR validation was conducted on 10 selected *C. bungei* candidate differentially expressed genes, with primer information provided in Table 1. As illustrated in Figure 6, although there were minor differences in the fold change of individual genes between RT-qPCR results and RNA-seq data, the overall trends of upregulation or downregulation in expression were consistent between the two methods.

Furthermore, correlation analysis between RT-qPCR results and RNA-seq data revealed a very high correlation in the relative expression trends of 9 genes with the results obtained from RNA-seq sequencing ("Qiuza 1": R^2 = 0.7156; "Qiuza 2": R^2 = 0.7825). This indicates that the transcriptome sequencing results are highly accurate. The comparison of results between the two methods is shown in Figure 7.

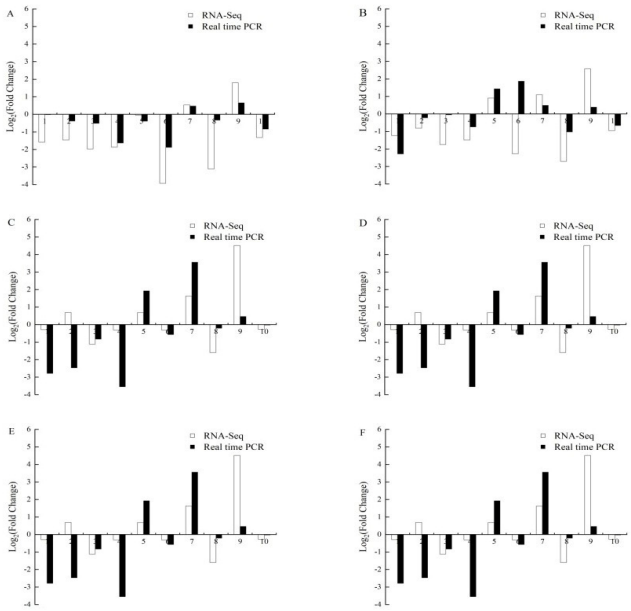

Figure 6. Comparison of differential expression fold changes between RNA-seq and RT-qPCR (1: *CbPIP2;6*; 2: *CbPIP2;5*; 3: *CbPIP1;2*; 4: *CbPIP1;4*; 5: *CbTIP2;1*; 6: *CbTIP4;1*; 7: *CbNIP5;1*; 8: *CbNIP2;1*; 9: *CbNIP6;1*; 10: *CbSIP2;2*; (**A**–**C**) "Qiuza 1" treated with 4 °C cold stress for 6 h, 12 h, and 18 h, respectively; (**D**–**F**): "Qiuza 2" treated with °C cold stress for 6 h, 12 h, and 18 h, respectively").

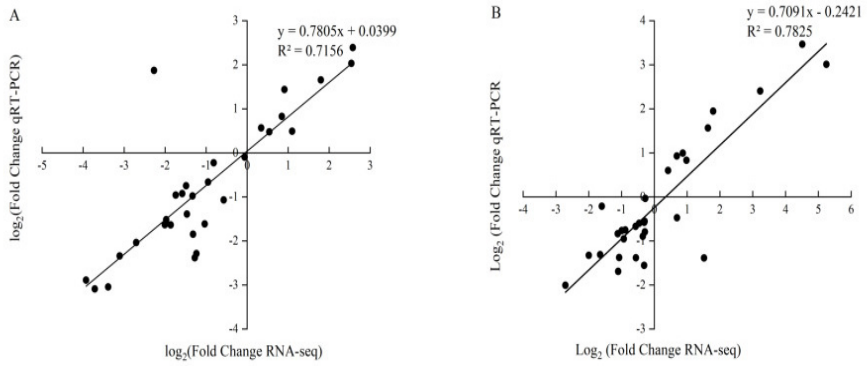

Figure 7. Correlation analysis between RNA-seq and RT-qPCR ((**A**): "Qiuza 1"; (**B**): "Qiuza 2").

4. Discussion

Leaves are highly sensitive and adaptable to environmental changes during plant evolution [34]. Their external morphology can intuitively and rapidly reflect the growth status of the plant and its sensitivity to adversity [35]. Low temperatures disrupt the normal physiological metabolism of plants, affecting the transport of water between cells, resulting in symptoms such as leaf curling and wilting due to dehydration. In this study, under cold stress, the external morphology of the leaves of the less cold-tolerant "Qiuza 1" exhibited damage symptoms later than the more cold-tolerant "Qiuza 2". However, as the duration of cold stress increased, "Qiuza 1" showed severe damage symptoms earlier than "Qiuza 2". This suggests that "Qiuza 1" has weaker cold tolerance compared to "Qiuza 2" under prolonged cold conditions. This characteristic of delayed initial damage but earlier severe damage under prolonged cold treatment is inconsistent with the weak cold tolerance

observed by Huang et al. [36] and Wei [37]. This may indicate that *C. bungei* has a unique mechanism in response to cold tolerance.

In vertebrates, there are approximately 11 to 13 AQPs, and the number of AQPs in most plants ranges from 30 to 50 [38]. Although the number of genes encoding aquaporin proteins in *C. bungei* is relatively small, the overall distribution ratio of aquaporin protein genes within subfamilies is consistent with *A. thaliana*. Through transcriptome sequencing, this study identified 15 aquaporin protein genes in *C. bungei*, which can be classified into 5 subfamilies: PIPs, TIPs, NIPs, SIPs, and XIPs. The specific number of CbAQP gene family members awaits further validation through genome sequencing.

In the evolution of plants, XIP genes are prone to events such as substitution of Ar/R selective filtering sites, insertion and loss of the C loop, and loss of introns [30]. Currently, XIP subfamilies are absent in monocotyledonous plants such as *O. sativa*, *Zea mays*, and *S. bicolor*, as well as in some dicotyledonous plants like *A. thaliana*. However, XIP subfamilies are present in dicotyledonous plants such as *P. trichocarpa*, *G. hirsutum*, and *C. sativus* (Table 3). This study found that a XIP gene also exists in dicotyledonous *C. bungei*, suggesting that XIP subfamilies may only exist in dicotyledonous plants in the plant kingdom. Evolutionary analysis indicates that CbAQPs have a closer relationship with AQPs in *A. thaliana* than with those in *O. sativa*, which is consistent with the current view of the differentiation between monocotyledons and dicotyledons in plant evolution [39].

The study found that PIPs exhibit high selectivity in transporting substrates and play an important role in maintaining the water balance in plant cells. Whether plant plasma membrane aquaporin proteins can accurately locate to the plasma membrane determines their ability to function as water channel proteins. Among the 12 cold-related genes screened in this study, 5 belong to the PIPs subfamily, indicating that the water balance in *C. bungei* under low-temperature stress mainly relies on the PIPs subfamily of aquaporin proteins. This result is consistent with previous research findings on aquaporin proteins responding to low-temperature stress [40]. What sets this study apart from other research is that genes in the NIPs and TIPs subfamilies of the CbAQP gene family also respond to low-temperature stress.

When plants are subjected to low-temperature stress, the water balance within the plant is disrupted. Aquaporin protein, as a key factor in transmembrane water transport, actively responds to low-temperature stress. However, the response pattern of plant aquaporin proteins may vary depending on the species, organ, and subfamily, indicating that aquaporin proteins may have different functions within plants. Seong et al. [11] found that overexpression of *PIP2;5* in *A. thaliana* resulted in increased tolerance to low temperatures in stems, leaves, and roots compared to wild-type plants. Matsumoto et al. [32] chemically treated *O. sativa* to abolish its cold resistance and found that PIP1 was closely associated with the plant's cold resistance.

Under 4 °C low-temperature treatment conditions, overexpression of Os*PIP1;3* can enhance the cold resistance of *O. sativa* [41]. Researchers have also found that although the water permeability of *PIP1;3* is lower than that of *OsPIP2;2* and *OsPIP2;4*, co-expression of *PIP1;3* with either *OsPIP2;2* or *OsPIP2;4* significantly enhances the water permeability of *OsPIP2;2* or *OsPIP2;4*. Interaction between PIP1 and PIP2 in *O. sativa* significantly enhances the plant's cold resistance. After low-temperature stress treatment, overexpression of the *MusaPIP1;2* gene in *M. nana*s improves resistance to various stresses, including low-temperature stress [17]. Overexpression of *MaPIP2;7* reduces the levels of malondialdehyde (MDA) and ion leakage in plants while increasing the levels of chlorophyll, proline, soluble sugars, and abscisic acid (ABA), thereby enhancing tolerance to various stresses such as cold [18]. Overexpression of genes such as TaAQP7 (PIP2), *MaSIP2;1*, and *OsPIP2;7* regulates osmotic balance in plants, reduces membrane damage and oxidation, and enhances cold tolerance by regulating levels of hormones such as ABA and GA.

Based on the CbAQP genes and cold-stress-related aquaporin genes (Figure 4), the CbAQP gene *CbPIP2;5* shows the highest similarity to the *A. thaliana* aquaporin *AtPIP2;5*. Jang et al. found that *AtPIP2;5* is the main aquaporin responding to low-temperature stress

when overexpressed in *A. thaliana* and *N. tabacum* [42]. In both *A. thaliana* and *N. tabacum* subjected to low-temperature stress, the expression of *PIP2;5* was highly induced on the first day of low-temperature stress (compared to days 1, 7, and 14), followed by a gradual decrease in expression during continued low-temperature stress. The expression pattern of the CbAQP gene *CbPIP2;5* is highly consistent with that of *AtPIP2;5*, suggesting that *CbPIP2;5* plays a crucial role in response to low-temperature stress in CbAQP. Comparing the expression levels of *CbPIP2;5* in the two CbAQP varieties at the same stage, it was found that the expression level of *CbPIP2;5* in the less cold-resistant variety "Qiuza 1" was significantly higher than that in the more cold-resistant variety "Qiuza 2" when facing low-temperature stress. This indicates that to maintain water homeostasis within the plant, CbAQP upregulates *PIP2;5* expression to maintain root water permeability and water transport within the plant, enabling rapid response to low-temperature stress. However, with prolonged exposure to low temperatures, overexpression of *PIP2;5* reduces the sensitivity of plant roots to low temperatures, which is not conducive to long-term adaptation of CbAQP to low-temperature environments. In this study, under cold stress, the time at which "Qiuza 1" exhibited upward curling at the leaf edges was later than "Qiuza 2". However, the time at which "Qiuza 1" showed slight downward leaf curling was earlier than "Qiuza 2". These changes in external morphology were consistent with the expression patterns and functions of *CbPIP2;5*. Overexpression of the *T. aestivum* aquaporin gene *TdPIP2;1* effectively enhances *T. aestivum*'s stress resistance [43], while increased expression of *OsPIP2;5*, *OsPIP2;8*, *OsPIP2;3*, and *OsPIP2;7* in *O. sativa* effectively enhances its cold resistance [15], which is consistent with the short-term cold stress response observed in CbAQP.

The CbAQP gene *CbPIP1;2* belongs to the PIPs subfamily and shows the highest similarity to the *A. thaliana* AQPs *AtPIP1;4* and *AtPIP1;5*. It is reported that *AtPIP1;4* exhibits a certain functional synergy with *AtPIP2;5* and affects root water permeability by upregulating expression [11]. *AtPIP1;4* also demonstrates higher sensitivity to low temperatures, and its overexpression can maintain the high water permeability of cells. *CbPIP1;2* exhibits peak expression at 18 h of low-temperature stress in the less cold-resistant "Qiuza 1", while in the more cold-resistant "Qiuza 2", peak expression occurs at 6 h of low-temperature stress. The delayed peak expression in "Qiuza 1" suggests that "Qiuza 2" responds more rapidly to low temperatures. Additionally, the expression level of *CbPIP1;2* in "Qiuza 1" shows a fluctuating upward trend, indicating that prolonged periods of high water permeability may lead to increased vulnerability to damage during the later stages of low-temperature stress.

The CbAQP gene *CbTIP4;1* belongs to the TIPs subfamily, which is an important subfamily of plant aquaporins. Overexpression of the ginseng PgTIP gene in *A. thaliana* significantly alters nutrient growth and reproductive development and reduces resistance to low-temperature stress. When facing low-temperature stress, the expression level of *CbTIP4;1* in "Qiuza 1" significantly increases, far exceeding that in "Qiuza 2". Therefore, we believe that the high expression of *CbTIP4;1* may reduce the cold resistance of *C. bungei*. In low-temperature environments, plant tissues usually freeze due to heterogeneous ice nucleation occurring extracellularly [44]. Because the water potential of ice is lower than that of water, the cell sap moves out of the cell along the gradient, leading to cell dehydration. It can be speculated that plant cold resistance should include mechanisms to resist cell dehydration induced by freezing. The downregulation of TIPs may be part of this strategy. Therefore, the overexpression of TIPs in plant cells may reduce the cold resistance of plants.

The CbAQP *CbNIP2;1* belongs to the NIPs subfamily, and there are few reports on the response of the NIPs subfamily to low-temperature environments in plants. Based on the family properties of Nodulin26 intrinsic membrane protein of NIPs, we speculate that the *CbNIP2;1* gene may affect the absorption and release of metal ions by plant cell ion channels, thereby affecting the concentration of solutes in plant cells and changing the ion concentration of plant cells in low-temperature environments. *CbNIP2;1* peaks in

expression at 6 h and 12 h in "Qiuza 1" and "Qiuza 2", respectively, with overall expression levels in "Qiuza 2" significantly higher than in "Qiuza 1" ($p < 0.05$). This result is consistent with Verma's study in *O. sativa* [45], indicating that the high expression level of *CbNIP2;1* helps to improve plant cold resistance. However, further research is needed to determine how *CbNIP2;1* specifically affects plant ion channels.

In this study, by comparing the CbAQP genes with other reported cold-related aquaporin genes and analyzing the changes in CbAQP during four periods of low-temperature stress, we identified the specific expression patterns of individual members of this gene family during low-temperature stress. Among the 15 CbAQP genes, we found 12 CbAQP genes responsive to low-temperature stress, including 5 in the CbPIPs subfamily, 2 in the CbTIPs subfamily, 3 in the CbNIPs subfamily, 1 in the CbSIPs subfamily, and 1 in the CbXIPs subfamily. Based on reported sequences related to cold stress, we found that the sequences CIAW and GGMI in motif 6 are unique to the CbAQP PIPs subfamily, suggesting that these two gene sequences may not be key sequences in the response of CbAQP to low-temperature stress. The motifs IAFEXXT, SGGHINPAVT, and GTFVLVYTVF are distributed in motifs 1 and 2, and motifs 1 and 2 are simultaneously present in the PIPs, TIPs, and NIPs subfamilies of CbAQP. We speculate that these gene sequences may play a role when *C. bungei* faces low-temperature stress. Upon analyzing the gene sequences of *CbTIP4;1*, *CbNIP2;1*, *CbPIP1;2*, and *CbPIP2;5*, we found that the sequences IXEXIAT and EIXXTF are highly conserved among AQPs in different subfamilies. Therefore, we speculate that these two gene sequences may play a key role when *C. bungei* faces low-temperature stress. The absorption and transportation of water by plant roots directly depend on the transcriptional regulation of aquaporins and other factors that change the permeability of cell membranes to water. Facing low-temperature stress, *C. bungei* regulates the expression levels of AQP genes and the corresponding protein activities to adjust the water permeability of roots, thereby maintaining water balance within the plant and ensuring normal physiological activities.

5. Conclusions

This study was the first to investigate the expression of aquaporin protein genes in two *C. bungei* varieties with different cold resistance under low-temperature stress. In total, 15 aquaporin protein genes were identified and classified into 5 subfamilies, including 5 PIPs, 4 TIPs, 3 NIPs, 2 SIPs, and 1 XIPs, based on phylogenetic analysis. Conservation analysis of conserved motifs revealed that the PIPs, TIPs, and NIPs subfamilies in the CbAQP gene family maintain high conservation during evolution. We identified 12 cold-responsive genes in the CbAQP gene family under low-temperature stress. Among these 12 genes, four were found to be actively related to low-temperature stress in *C. bungei*. These genes are *CbPIP2;5*, *CbPIP1;2*, *CbTIP4;1*, and *CbNIP2;1*. These four CbAQP genes may play crucial roles in *C. bungei* responses to low-temperature stress. The results of this study provide a foundation for future research on the functional validation and molecular regulatory mechanisms of candidate genes. Additionally, these findings offer a theoretical basis for improving the quality of *C. bungei* seedlings, enhancing cold-resistant genetic breeding, and expanding its distribution range to the south and north.

Author Contributions: T.L., J.Z., S.N., J.Q. and B.D. designed the experiments. T.L., J.Z., H.Z., Z.C., T.M. and Y.M. performed the experiments and collected the data. T.L., J.Z., S.N., J.Q. and Z.C. analyzed the data. T.L. wrote the manuscript. S.N., J.Q. and B.D. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original data presented in the study are openly available in (National Center for Biotechnology Information) at (PRJNA1110610).

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Fifty conserved motif data (note: "motif sequences" refer to the protein motifs characterized by specific amino acid sequences within the AQP library. "Sites" denote the frequency of these motifs within the library. "Width" indicates the span of the motif in terms of amino acids. The "E value" represents the statistical significance of the motif, with lower E values indicating higher reliability of the results).

Motif Type	Motif Sequences	Sites	Width	E-Value
Motif1	DKDYKDPPPAPLFDPGELKSWSFYRAGIAEFIATLLFLYVTVLTVIGYKR	68	50	3.4×10^{-2729}
Motif2	KGFQKSYYQRLGGGANTVADGYSKGTGLGAEIIGTFVLVYTVFSATDPKR	70	50	2.4×10^{-2726}
Motif3	PIGFAVFLVHLATIPITGTGINPA	141	24	1.8×10^{-1766}
Motif4	GISGGHINPAVTFGL	131	15	1.0×10^{-1304}
Motif5	WDDHWIFWVGPFIGA	135	15	1.9×10^{-1229}
Motif6	APNMCAGVGILGIAWAFGGMIFALVYCTA	65	29	6.7×10^{-1420}
Motif7	RAVLYIVAQCLGAIC	140	15	3.8×10^{-1036}
Motif8	QALVMEIIITFGLMFVVYAVATDPRAGGELAG	67	32	6.5×10^{-903}
Motif9	RSLGPAVIYNK	132	11	2.6×10^{-639}
Motif10	AEFYHTFVLRFAGCK	133	15	1.8×10^{-624}
Motif11	RDSHVPVLAPL	69	11	1.1×10^{-565}
Motif12	MEGKEEDVRVGANKFPERQPI	29	21	4.3×10^{-481}
Motif13	LTNGGAGGPVGLVGIAVAHGLAVFVMVYS	59	29	2.3×10^{-411}
Motif14	FLARKVSL	70	8	1.1×10^{-312}
Motif15	TLRLLFGLDNDVCSGKHDVFVGSSPSGSD	11	29	1.4×10^{-180}
Motif16	ACLLLKFATGGLAVP	27	15	3.5×10^{-119}
Motif17	MAKEVEEEGGG	48	11	5.9×10^{-97}
Motif18	SGAWVYNFIRFTDKPLREITK	18	21	1.5×10^{-91}
Motif19	LGSFRSNA	32	8	2.8×10^{-83}
Motif20	GLAGLIYEDVFIGSY	27	15	6.1×10^{-76}
Motif21	MRKIALGSPGEAFSPDSJKAY	22	21	4.3×10^{-94}
Motif22	PRPLKKQDSLPLVSVPFLQKL	10	21	5.5×10^{-73}
Motif23	ASSRRFPW	34	8	6.2×10^{-73}
Motif24	ASFALKGLLHPIMSGGVTVPS	14	21	3.4×10^{-69}
Motif25	GALALKAVVNSEIEQTFSLGGCTLTYYA	5	41	6.2×10^{-56}
Motif26	SFNPLGAAAFYVAGVFSDSJFSLAIRIPAQAIGAAGGAITIMEVIPEKYK	5	50	7.5×10^{-43}
Motif27	VGIVLGLLVFYSTTVTATKGYAAGLNP	7	29	1.4×10^{-42}
Motif28	KLSSFKLRRJQSQDMASPLNV	11	21	3.2×10^{-44}
Motif29	GTSAQS	26	6	6.6×10^{-30}
Motif30	SRSNTRPNYSNEIHDIDVVTAQT	5	23	1.2×10^{-26}
Motif31	KGNCKDSQGGMETAICSSPSIVCLTQKLI	3	29	4.5×10^{-26}
Motif32	HEQLPTTD	14	8	5.9×10^{-26}
Motif33	AVGGHITL	24	8	2.4×10^{-24}
Motif34	MGRIKLVVGDLVISFMWVWASALVGIQVH	7	29	4.1×10^{-20}
Motif35	AAWLFRVIFPPPPPEQKKQKK	6	21	2.6×10^{-19}
Motif36	IGVLTYRSISLKTRPCPSPVSPSVSSLLR	3	29	8.3×10^{-21}
Motif37	MDDISVSKSNHGNVVVLNIKASSLADTSL	3	29	2.7×10^{-24}
Motif38	LLHRKSLKELFPPFLLRKVY	7	20	2.5×10^{-19}
Motif39	ASLTLRLMFGGTPEAFFGTTP	7	21	3.3×10^{-17}
Motif40	IVISTIETQTKTPNL	9	15	1.1×10^{-13}
Motif41	GWAYAYGSHNT	6	11	3.9×10^{-11}
Motif42	DEESLYSGNKIQPFATTP	4	18	5.0×10^{-11}
Motif43	YGVNADIMATKPALSCVSAFF	3	21	2.6×10^{-12}
Motif44	NQNYFICSSPTDINGKCNVTC	2	21	6.5×10^{-11}
Motif45	PSLQVGVHHGAJSEGILSF	6	19	1.1×10^{-10}
Motif46	QSDPTVNT	7	8	3.0×10^{-9}
Motif47	YTKIIPRQLSHTIE	5	14	3.5×10^{-9}
Motif48	LHCGPHQNLGN	3	11	3.2×10^{-9}
Motif49	CCSCCSLPRDSHQSHPFQVQD	2	21	4.4×10^{-9}
Motif50	GFSRTDPSGEIVRYLFSIISMFIFAYLQQ	3	29	6.3×10^{-8}

Appendix B

Figure A1. Fifty conserved motif data.

References

1. Obroucheva, N.V.; Sin'kevich, I.A. Aquaporins and cell growth. *Russ. J. Plant Physiol.* **2010**, *57*, 153–165. [CrossRef]
2. Henzler, T.; Waterhouse, R.N.; Smyth, A.J.; Carvajal, M.; Cooke, D.T.; Schaeffner, A.R.; Steudle, E.; Clarkson, D.T. Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the roots of Lotus Japonicus. *Planta* **1999**, *210*, 50–60. [CrossRef]
3. Kaldenhoff, R.; Fischer, M. Aquaporins in plants. *Acta Physiol.* **2006**, *187*, 169–176. [CrossRef]
4. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *7*, 3022–3027. [CrossRef]
5. Saja-Garbarz, D.; Libik-Konieczny, M.; Fellner, M.; Jurczyk, B.; Janowiak, F. Silicon-induced alterations in the expression of aquaporins and antioxidant system activity in well-watered and drought-stressed oilseed rape. *Plant Physiol. Biochem.* **2022**, *174*, 73–86. [CrossRef]
6. Li, R. Characterization of Aquaporin Genes in Tomato during Drought Stress Ang Fruit Development Process. Ph.D. Thesis, China Agricultural University, Beijing, China, 2015.
7. Ayadi, M.; Cavez, D.; Miled, N.; Chaumont, F.; Masmoudi, K. Identification and characterization of two plasma membrane aquaporins in durum wheat (*Triticum turgidum* L. subsp. *durum*) and their role in abiotic stress tolerance. *Plant Physiol. Biochem.* **2011**, *49*, 1029–1039. [CrossRef]
8. Chen, J.Z.; Lin, L.R.; Lv, G.A. An index of soil drought intensity and degree: An application on corn and a comparison with CWSI. *Agric. Water Manag.* **2010**, *97*, 865–871. [CrossRef]
9. Rodrigues, O.; Reshetnyak, G.; Grondin, A.; Saijo, Y.; Leonhardt, N.; Maurel, C.; Verdoucq, L. Aquaporins facilitate hydrogen peroxide entry into guard cells to mediate ABA- and Pathogen-Triggered stomatal closure. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 201704754. [CrossRef]
10. Maurel, C.; Nacry, P. Root architecture and hydraulics converge for acclimation to changing water availability. *Nat. Plants* **2020**, *6*, 744–749. [CrossRef]
11. Lee, S.H.; Chung, G.C.; Jang, J.Y.; Ahn, S.J.; Zwiazek, J. Overexpression of PIP2;5 aquaporin alleviates effects of low root temperature on cell hydraulic conductivity and growth in Arabidopsis. *Plant Physiol.* **2012**, *159*, 479–488. [CrossRef]
12. Hu, W.; Hou, X.W.; Huang, C.; Yan, Y.; Tie, W.W.; Ding, Z.H.; Wei, Y.X.; Liu, J.H.; Miao, H.X.; Lu, Z.W.; et al. Genome-Wide identification and expression analyses of aquaporin gene family during development and abiotic stress in Banana. *Int. J. Mol. Sci.* **2015**, *16*, 19728–19751. [CrossRef]
13. Huang, C.; Zhou, S.; Hu, W.; Deng, X.; Wei, S.; Yang, G.; He, G. The wheat aquaporin gene *TaAQP7* confers tolerance to cold stress in transgenic tobacco. *Z. Für Naturforschung C* **2014**, *69*, 142–148. [CrossRef]
14. Zhou, S.; Hu, W.; Deng, X.; Ma, Z.; Chen, L.; Huang, C.; Wang, C.; Wang, J. Overexpression of the wheat aquaporin gene, *TaAQP7*, enhances drought tolerance in transgenic tobacco. *PLoS ONE* **2012**, *7*, e052439. [CrossRef]
15. Tsuneo, K.; Junko, I.; Hidehiro, H.; Kiyoshi, N.; Keiko, F.; Arifa, A.; Katsuko, T.; Maki, K.; Mari, M. Influence of low air humidity and low root temperature on water uptake, growth and aquaporin expression in rice plants. *Plant Cell Physiol.* **2012**, *53*, 1418–1431. [CrossRef]
16. Yu, X.; Peng, Y.H.; Zhang, M.H.; Shao, Y.J.; Su, W.A.; Tang, Z.C. Water relations and an expression analysis of plasma membrane intrinsic proteins in sensitive and tolerant rice during chilling and recovery. *Cell Res.* **2006**, *16*, 599–608. [CrossRef]
17. Xu, Y.; Li, J.; Song, S.; Liu, J.; Jin, Z. A novel aquaporin gene *MaSIP2-1* confers tolerance to drought and cold stresses in transgenic banana. *Mol. Breed.* **2020**, *40*, 62. [CrossRef]
18. Xu, Y.; Hu, W.; Liu, J.; Song, S.; Hou, X.; Jia, C.; Jin, Z. An aquaporin gene *MaPIP2-7* is involved in tolerance to drought, cold and salt stresses in transgenic banana (*Musa acuminata* L.). *Plant Physiol. Biochem.* **2020**, *147*, 66–76. [CrossRef]
19. Xu, C.; Wang, M.; Zhou, L.; Quan, T.; Xia, G. Heterologous expression of the wheat aquaporin gene *TaTIP2;2* compromises the abiotic stress tolerance of *Arabidopsis thaliana*. *PLoS ONE* **2013**, *8*, e79618. [CrossRef]
20. Reddy, P.S.; Rao, T.S.R.B.; Sharma, K.K.; Vadez, V. Genome-wide identification and characterization of the aquaporin gene family in *Sorghum bicolor* (L.). *Plant Gene* **2015**, *1*, 18–28. [CrossRef]
21. Wei, L.B.; Dong, J.H.; Shi, W.J.; Qian, J.; Di, B. Comprehensive Evaluation of Cold Resistance of Four *Catalpa* spp. Seedlings. *Mol. Plant Breed.* **2021**, *1*, 17. Available online: https://kns.cnki.net/kcms/detail/46.1068.S.20211009.1135.002.html (accessed on 2 July 2022).
22. Lopez, D.; Bronner, G.; Brunel, N.; Auguin, D.; Bourgerie, S.; Brignolas, F.; Venisse, J.S. Insights into Populus XIP aquaporins: Evolutionary expansion, protein functionality, and environmental regulation. *J. Exp. Bot.* **2012**, *63*, 2217–2230. [CrossRef]
23. Chen, C.; Chen, H.; He, Y.; Xia, R. TBtools, a Toolkit for Biologists integrating various HTS-data handling tools with a user-friendly interface. *BioRxiv* **2018**. [CrossRef]
24. Saeed, A.I.; Sharov, V.; White, J.; Li, J.; Liang, W.; Bhagabati, N.; Braisted, J.; Klapa, M.; Quackenbush, J. TM4: A free, open-source system for microarray data management and analysis. *Biotechniques* **2003**, *34*, 374–378. [CrossRef]
25. Zhao, J.M.; Fan, E.Q.; Liu, Y.; Wang, Z.; Wang, J.H.; Qv, G.Z. Cloning and bioinformatics analysis of *CbuATX1*, *CbuATX1-like* and *CbuATX2* genes from *Catalpa bungei*. *Bull. Bot. Res.* **2022**, *42*, 47–61. [CrossRef]
26. Quigley, F.; Rosenberg, J.M.; Shachar-Hill, Y.; Bohnert, H. From genome to function: The Arabidopsis aquaporins. *Genome Biol.* **2001**, *3*, 97–108. [CrossRef]
27. Sakurai, J.; Ishikawa, F.; Yamaguchi, T.; Uemura, M.; Maeshima, M. Identification of 33 rice aquaporin genes and analysis of their expression and function. *Plant Cell Physiol.* **2005**, *46*, 1568–1577. [CrossRef]

28. Zhu, Y.X.; Yang, L.; Liu, N.; Yang, J.; Zhou, X.K.; Xia, Y.C.; He, Y.; He, Y.Q. Genome-wide identification, structure characterization, and expression pattern profiling of aquaporin gene family in cucumber. *BMC Plant Biol.* **2019**, *19*, 1–23. [CrossRef] [PubMed]
29. Park, W.; Scheffler, B.E.; Bauer, P.J.; Campbell, B.T. Identification of the family of aquaporin genes and their expression in upland cotton (*Gossypium hirsutum* L.). *BMC Plant Biol.* **2010**, *10*, 142. [CrossRef] [PubMed]
30. Gupta, A.B.; Sankararamakrishnan, R. Genome-wide analysis of major intrinsic proteins in the tree plant *Populus trichocarpa*: Characterization of XIP subfamily of aquaporins from evolutionary perspective. *BMC Plant Biol.* **2009**, *9*, 134. [CrossRef] [PubMed]
31. Rahman, A.; Kawamura, Y.; Maeshima, M.; Rahman, A.; Uemura, M. Plasma membrane aquaporin members PIPs act in concert to regulate cold acclimation and freezing tolerance responses in *Arabidopsis thaliana*. *Plant Cell Physiol.* **2020**, *61*, 787–802. [CrossRef]
32. Matsumoto, T.; Lian, H.L.; Su, W.A.; Tanaka, D.; Liu, C.W.; Iwasaki, I.; Kitagawa, Y. Role of the aquaporin PIP1 subfamily in the chilling tolerance of rice. *Plant Cell Physiol.* **2009**, *50*, 216–229. [CrossRef]
33. Kayum, M.A.; Park, J.I.; Nath, U.K.; Biswas, M.K.; Kim, H.T.; Nou, I.S. Genome-wide expression profiling of aquaporin genes confer responses to abiotic and biotic stresses in *Brassica rapa*. *BMC Plant Biol.* **2017**, *17*, 23. [CrossRef]
34. Fei, X.; Guo, W.H.; Xu, W.H.; Wei, Y.H.; Wang, R.Q. Leaf morphology correlates with water and light availability: What consequences for simple and compound leaves? *Prog. Nat. Sci.* **2009**, *19*, 1789–1798. [CrossRef]
35. Farooqi, M.Q.U.; Zahra, Z.; Lee, J.K. Molecular genetic approaches for the identification of candidate cold stress tolerance genes. In *Cold Tolerance in Plants: Physiological, Molecular and Genetic Perspectives*; Wani, S.H., Herath, V., Eds.; Springer International Publishing: Cham, Switzerland, 2018; pp. 37–51. [CrossRef]
36. Huang, Z.; He, J.; Xia, D.; Zhong, X.J.; Li, X.; Sun, L.X.; Cai, S.Z. Evaluation of physiological responses and tolerance to low-temperature stress of four Iceland poppy (*Papaver nudicaule*) varieties. *Plant Interact.* **2016**, *11*, 117–123. [CrossRef]
37. Wei, W.J. Identification of Low Temperature Tolerance of Different Spring Cabbage Cultivars. Ph.D. Thesis, Northwest A&F University, Xi'an, China, 2023.
38. Li, Q.Y. Genome-Wide Identification and Transcrriptome Analysis of Aquaporin Gene Family in *Nicotiana tabacum*. Ph.D. Thesis, Shandong Agricultural University, Taian, China, 2020.
39. Parkin, J. The families of flowering plants. *Nature* **1926**, *117*, 478–479. [CrossRef]
40. Kumar, K.; Mosa, K.A.; Meselhy, A.G.; Dhankher, O.P. Molecular insights into the plasma membrane intrinsic proteins roles for abiotic stress and metalloids tolerance and transport in plants. *Indian J. Plant Physiol.* **2018**, *23*, 721–730. [CrossRef]
41. Tao, L.; Wang, B.; Xin, S.; Li, W.; Huang, S.; Liu, L.; Cui, J.; Zhang, Q.; Cheng, X. A cluster of mutagenesis revealed an osmotic regulatory role of the *OsPIP1* genes in enhancing rice salt tolerance. *Crop J.* **2023**, *11*, 1204–1217. [CrossRef]
42. Jang, J.Y.; Lee, S.H.; Rhee, J.Y.; Chung, G.C.; Ahn, S.J.; Kang, H. Transgenic *Arabidopsis* and tobacco plants overexpressing an aquaporin respond differently to various abiotic stresses. *Plant Mol. Biol.* **2007**, *64*, 621–632. [CrossRef]
43. Ayadi, M.; Brini, F.; Masmoudi, K. Overexpression of a Wheat Aquaporin Gene, *TdPIP2;1*, Enhances Salt and Drought Tolerance in Transgenic Durum Wheat cv. *Maali. J. Mol. Sci.* **2019**, *20*, 2389. [CrossRef]
44. Peng, Y.; Lin, W.; Arora, C.R. Overexpression of a Panax ginseng tonoplast aquaporin alters salt tolerance, drought tolerance and cold acclimation ability in transgenic *Arabidopsis* plants. *Planta* **2007**, *226*, 729–740. [CrossRef]
45. Verma, H.; Devi, K.; Baruah, A.R.; Sarma, R.N. Relationship of root aquaporin genes, *OsPIP1;3*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP2;1* and *OsNIP2;1* expression with drought tolerance in rice. *Indian J. Genet. Plant Breed.* **2020**, *1*, 50–57. [CrossRef]