Identification of Aquaporin Gene Family in Response to Natural Cold Stress in *Ligustrum* × *Vicaryi* Rehd.

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Research Article

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Abstract

Background: Maintaining water balance in various adversities is a difficult and critical challenge for plants. Studies have shown that aquaporins located on cytomembrane play an important role in maintaining water homeostasis under various environmental stresses. Some studies have shown that aquaporins are involved in the tolerance mechanism of plant cells under cold stress, and the aquaporin gene family is closely related to the cold resistance of plants. *Ligustrum × vicaryi* Rehd. plays a significant role in urban landscaping with poor cold resistance at the seedling stage and early planting stage. Screening the target aquaporin genes of *Ligustrum × vicaryi* related to cold resistance during natural cold stress will provide a scientific theoretical basis for cold resistance breeding of *Ligustrum × vicaryi*.

Results: In this study, the genome-wide identification of the aquaporin gene family was performed at four different overwintering periods in September, November, January and April, and finally 58 candidate *Ligustrum × vicaryi* aquaporin (LvAQP) genes were identified. The phylogenetic analysis revealed that four subfamilies of the LvAQP gene family: 32 PIPs, 11 TIPs, 11 NIPs, and 4 SIPs, among which there were more genes in the PIPs subfamily than that in other plants. The key LvAQP genes were found through analyzing aquaporin genes related to cold stress in other plants and LvAQP genes expression profiles. The up-regulated key LvAQP genes were *Cluster-9981.114831*, *Cluster-9981.104986*, and *Cluster-9981.120365*, and the down-regulated key LvAQP genes were *Cluster-9981.112839*, *Cluster-9981.109034*, *Cluster-9981.89369*, *Cluster-9981.110451*, *Cluster-9981.107281*, *Cluster-9981.112777*, *Cluster-9981.112789*, *Cluster-9981.122691* and *Cluster-9981.88037*. These genes play a key role related to cold tolerance in the nature low temperature growth stage of *Ligustrum × vicaryi*.

Conclusions: This study systematically identified the AQP gene family in *Ligustrum × vicaryi* and screened for 20 differential expression LvAQP genes related to cold stress, among which 11 genes belonged to PIPs subfamily. The results of this research will lay the foundation for further biological function verification of cold resistance-related aquaporin candidate genes in *Ligustrum × vicaryi*, especially PIPs subfamily, and provide theoretical basis and technical support for improving seedling quality and breeding.

1. Background

Aquaporin is a protein located on the cytomembrane that controls the entry and exit of water in cells. Water uptake, transport across membranes and tissues are essential for plants growth and development, and the transmembrane transport of water molecules is mainly regulated by aquaporins. In biological membrane, plant aquaporins have a highly conserved Asn-Pro-Ala (NPA) motif structure, which plays a crucial role in the formation of water-selective channels [1]. Aquaporins have been found to constitute a huge gene family in plants by genomic sequencing, and these aquaporins are divided into five subfamilies according to their amino acid sequences [2]: plasma membrane intrinsic proteins (PIPs) located mainly on the cytomembrane, tonoplast intrinsic proteins (TIPs) located mainly on the tonoplast, nodulin26-like intrinsic proteins (NIPs) identified on legume root nodules, small basic intrinsic proteins (SIPs) and uncharacterized X intrinsic proteins (XIPs). Numerous studies have confirmed that it is a difficult but critical challenge for plants to maintain water balance under various adversities. Therefore, aquaporins have important effect on

maintaining water homeostasis under different environmental stress [3]. Studies have revealed the correlation between PIP transcription abundance and hydraulic conduction efficiency, including PIP1 and PIP2. The down-regulation of some genes of PIP1 and PIP2 lead to the decrease of hydraulic conductivity in leaves and roots [4, 5, 6]. It is reported that there is a strong correlation between root specific aquaporin expression and transpiration in *Arabidopsis thaliana* [7]and *Oryza sativa* [8]. Studies have shown that aquaporins are expressed at higher levels in stress recovery stage due to aquaporin is of importance to the restoration of restoring water homeostasis after cessation of stress [9, 10]. Ayadi showed that the overexpression of the wheat aquaporin gene *TdPIP2;1* could enhance salt tolerance and drought tolerance in transgenic durum wheat [11]. Braz also found that *GhPIP1;1*, *GhTIP2;1* and *GhSIP1;3* could improve salt tolerance in *Gossypium hirsutum* [12]. Under gradual drought stress, Alexandersson found that PIP transcription abundance was generally down-regulated in *Arabidopsis thaliana* except for *AtPIP1;4* and *AtPIP2;5*, which were up-regulated [13]. Feng found the change of *GmTIP2;6* expression under heat stress enhanced the response of *Glycine max* to heat stress [14].

Plants will strengthen cold resistance by overexpressing or repressing expression of some related genes during cold acclimation. Some evidences are observed that the overexpression of aquaporin genes can enhance cold resistance in various plants. For instance, under cold stress, *OsPIP2;4* and *OsPIP2;5* were abundantly expressed in the root system in order to enhance the cold resistance in rice [15]. *OsPIP2;5* and *OsPIP2;7* of *Oryza sativa* were involved in rapid water transport and maintaining water balance during cold stress stage, which played a major role in regulating water channel opening under cold stress [16]. The overexpression of *AtPIP1;4* or *AtPIP2;5* in transgenic plants of *Arabidopsis thaliana* could enhance water conductivity and promote germination [17]. The overexpression of *TaTIP2;2* in transgenic plants of wheat could make plants grow normally under cold conditions too [18]. Overexpressing or repressing expression of related aquaporin genes to enhance cold resistance of *Musa acuminata* [19], *Populus trichocarpa* [20], *Hordeum vulgare* [21] and *Brassica rapa* [22] have been studied under cold stress. Numerous studies have shown that the aquaporin gene family is closely related to the cold resistance of plants.

Ligustrum × vicaryi has poor cold resistance at the seedling stage and early planting stage, so it is prone to suffer from cold in chill winter in open field cultivation [23]. In this study we aimed to identify the *Ligustrum × vicaryi* aquaporin (LvAQP) gene family, and screen the target aquaporin genes related to cold resistance during natural low temperature stress. The results provide insight into stress-related biological functions of LvAQP gene family, which will provide a scientific theoretical basis for cold resistance breeding of *Ligustrum × Vicaryi*.

2. Results

2.1. Overview of stress related-genes of aquaporin gene family

In the present study, there have been studies on identification and expression analysis of the whole aquaporin gene family in more than 20 plants, such as *Arabidopsis thaliana* [24], *Oryza sativa* [25], *Zea mays* [26], *Hordeum vulgare* [27], *Glycine max* [28], *Gossypium hirsutum* [29], *Citrullus lanatus* [30], and so on.

Transcriptome analysis showed some aquaporin genes were responsive to abiotic stress, and some genes were up-regulated or down-regulated during drought, salt, and exogenous abscisic acid stress. Hove analyzed the mRNA-seq data of barley leaves to determine significantly different expression of *HvPIP1;2* and *HvTIP4;1* under salt stress [27]. Kayum found that among 59 aquaporin genes of *Brassica rapa*, 12, 7 and 17 genes were up-regulated under cold stress, drought stress and salt stress, respectively. In addition, 18 *BrPIP* genes were up-regulated under abscisic acid treatment [22]. Transgenic *Arabidopsis thaliana* enhanced drought tolerance by overexpression of *PIP1* and *PIP2* genes of *Jatropha curcas* [31]. Aquaporins are also involved in the response of plants to cold stress and play an important role in plant resistance to cold stress. There are 11 and 13 PIP genes in *Oryza sativa* and *Arabidopsis thaliana* that respond to cold stress [13, 17, 32], and 11, 8, 6, 9, 2, 8 and 1 AQP genes in *Hordeum vulgare* [21], *Musa acuminata* [33, 34], *Populus trichocarpa* [20], *Sorghum bicolor* [35], *Triticum aestivum* [36, 37], *Brassica rapa* [22], and *Gossypium hirsutum* [38], respectively, which showed significant correlation to cold stress (Table 1).

Table 1
Plants and cold stress-related aquaporin genes

Plants	Number	Gene name
Oryza sativa	11	*OsPIP1;1、OsPIP1;2、OsPIP1;3、OsPIP2;1、OsPIP2;2、OsPIP2;3、OsPIP2;4、OsPIP2;5、OsPIP2;6、OsPIP2;7、OsPIP2;8*
Arabidopsis thaliana	13	*AtPIP1;1、AtPIP1;2、AtPIP1;3、AtPIP1;4、AtPIP1;5、AtPIP2;1、AtPIP2;2、AtPIP2;3、AtPIP2;4、AtPIP2;5、AtPIP2;6、AtPIP2;7、AtPIP2;8*
Hordeum vulgare	11	*HvPIP1;1、HvPIP1;2、HvPIP1;3、HvPIP1;4、HvPIP1;5、HvPIP2;2、HvPIP2;3、HvPIP2;5、HvTIP1;2、HvTIP2;2、HvTIP2;3*
Musa acuminata	8	*MaPIP2;4、MaPIP2;5、MaPIP2;6、MaPIP2;7、MaTIP1;2、MaTIP2;1、MaNIP2;1、MaSIP2;1*
Populus trichocarpa	6	*PtPIP1;2、PtPIP1;3、PtPIP1;4、PtPIP1;5、PtPIP2;3、PtPIP2;5*
Sorghum bicolor	9	*SbPIP1;1、SbPIP1;2、SbPIP2;2、SbPIP2;5、SbPIP2;7、SbPIP2;8、SbPIP2;9、SbTIP1;1、SbTIP3;1*
Triticum aestivum	2	*TaAQP7、TaPIP2*
Brassica rapa	8	*BrPIP1;1、BrPIP1;3、BrPIP1;4、BrPIP1;5、BrPIP2;4、BrPIP2;5、BrPIP2;6、BrPIP2;7*
Gossypium hirsutum	1	*GhTIP1;1*

2.2. LvAQP gene family identification

Based on the *Arabidopsis thaliana* aquaporin gene family (Table 2), 58 candidate LvAQP genes were identified (Table 3). According to sequence alignment, the correlation of characteristic proteins and

phylogenetic relationship, the 58 LvAQP genes were divided into four subfamilies: PIPs, TIPs, NIPs and SIPs, which contained 32, 11, 11 and 4 genes, respectively.

Table 2
35 aquaporin genes of *Arabidopsis thaliana*

Subfamily	Name	Synonyms	NCBI Reference Sequence
PIPs	*PIP1;1*	PIP1A	AT3G61430
	PIP1;2	PIP1B;TMPA	AT2G45960
	PIP1;3	PIP1C;TMPB	AT1G01620
	PIP1;4	TMPC	AT4G00430
	PIP1;5	PIP1D	AT4G23400
	PIP2;1	PIP2A	AT3G53420
	PIP2;2	PIP2B;TMB2B	AT2G37170
	PIP2;3	RD28;TMP2C	AT2G37180
	PIP2;4	PIP2F	AT5G60660
	PIP2;5	PIP2D	AT3G54820
	PIP2;6	PIP2E	AT2G39010
	PIP2;7	PIP3;SIMIP	AT4G35100
	PIP2;8	PIP3B	AT2G16850
TIPs	*TIP1;1*	GAMMA-TIP	AT2G36830
	TIP1;2	TIP2	AT3G26520
	TIP1;3	GAMMA-TIP1	AT4G01470
	TIP2;1	DELTA-TIP	AT3G16240
	TIP2;2	DELTA-TIP2	AT4G17340
	TIP2;3	DELTA-TIP3	AT5G47450
	TIP3;1	α-TIP	AT1G73190
	TIP3;2	BETA-TIP	AT1G17810
	TIP4;1		AT2G25810
	TIP5;1		AT1G17820
NIPs	*NIP1;1*	NLM1	AT4G19030
	NIP1;2	NLM2	AT4G18910
	NIP2;1		AT2G34390
	NIP3;1		AT1G31885
	NIP4;1		AT5G37810

Subfamily	Name	Synonyms	NCBI Reference Sequence
	NIP4;2		AT5G37820
	NIP5;1		AT4G10380
	NIP6;1		AT1G80760
	NIP7;1		AT3G06100
SIPs	*SIP1;1*	SIP1A	AT3G04090
	SIP1;2		AT5G18290
	SIP2;1		AT3G56950

Table 3
58 aquaporin genes of *Ligustrum × vicaryi*

Subfamily	Name	characteristic
PIPs	*Cluster-9981.115068*	Predicted: probable aquaporin PIP-type
	Cluster-9981.109600	Plasma membrane intrinsic protein
	Cluster-9981.29850	Aquaporin PIP1-2
	Cluster-19966.0	Aquaporin PIP1-2
	Cluster-19036.0	Aquaporin PIP1
	Cluster-9981.112839	Predicted: probable aquaporin PIP-type
	Cluster-9981.117133	Predicted: probable aquaporin PIP-type
	Cluster-9981.112265	Plasma membrane intrinsic protein PIP1-1
	Cluster-9981.29849	Plasma membrane intrinsic protein
	Cluster-9981.21661	Aquaporin
	Cluster-9981.200292	Aquaporin
	Cluster-9981.111171	Plasma membrane intrinsic protein
	Cluster-9981.109034	Predicted: aquaporin PIP2-1-like
	Cluster-9981.89369	Predicted: probable aquaporin PIP2-5
	Cluster-9981.170229	Aquaporin
	Cluster-9981.689	Plasma membrane intrinsic protein
	Cluster-9981.690	Plasma membrane intrinsic protein
	Cluster-9981.110451	Predicted: aquaporin PIP2-7
	Cluster-9981.198491	Plasma membrane intrinsic protein
	Cluster-9981.691	Plasma membrane intrinsic protein
	Cluster-9981.154931	Aquaporin
	Cluster-9981.154932	Aquaporin
	Cluster-9981.114832	Predicted: aquaporin PIP2-7
	Cluster-9981.114831	Predicted: aquaporin PIP2-7
	Cluster-9981.47893	Predicted: aquaporin PIP2-1-like
	Cluster-9981.47892	Hypothetical protein
	Cluster-9981.111170	Plasma membrane intrinsic protein
	Cluster-9981.118516	Plasma membrane intrinsic protein 2;1

Subfamily	Name	characteristic
	Cluster-9981.21660	Aquaporin
	Cluster-9981.107281	Predicted: aquaporin PIP2-7
	Cluster-48310.0	Aquaporin PIP2
	Cluster-9981.86061	Predicted: aquaporin PIP2-4-like
TIPs	*Cluster-9981.112777*	Predicted: aquaporin TIP1-3-like
	Cluster-9981.35612	Predicted: aquaporin TIP1-1-like
	Cluster-9981.111753	Tonoplast intrinsic protein
	Cluster-9981.115801	Predicted: aquaporin TIP1-3-like
	Cluster-24993.0	Gamma-type tonoplast intrinsic protein
	Cluster-9981.112790	Tonoplast intrinsic protein, putative
	Cluster-9981.112789	Putative delta TIP
	Cluster-20432.0	Delta tonoplast intrinsic protein TIP2;3
	Cluster-9981.122691	Predicted: probable aquaporin TIP-type
	Cluster-9981.172823	Predicted: aquaporin TIP4-1
	Cluster-9981.111196	Predicted: low quality protein: aquaporin TIP2-1-like
NIPs	*Cluster-9981.78169*	Predicted: aquaporin NIP1-1-like
	Cluster-9981.133629	Predicted: aquaporin NIP1-1-like
	Cluster-9981.133630	Predicted: aquaporin NIP1-1-like
	Cluster-51933.0	Predicted: aquaporin NIP1-1-like
	Cluster-9981.178700	Predicted: aquaporin NIP1-1-like
	Cluster-36924.0	Predicted: aquaporin NIP1-1-like
	Cluster-9981.104986	Predicted: probable aquaporin NIP5-1
	Cluster-9981.123071	Predicted: probable aquaporin NIP5-1
	Cluster-9981.97911	Predicted: aquaporin NIP2-1-like
	Cluster-28512.0	Aquaporin, MIP family, NIP subfamily isoform 1
	Cluster-9981.54345	Predicted: aquaporin NIP1-1-like
SIPs	*Cluster-9981.120365*	Small basic intrinsic protein 1-2
	Cluster-9981.105938	Small basic intrinsic protein 1-2
	Cluster-9981.88037	Predicted: probable aquaporin SIP2-1

Subfamily	Name	characteristic
	Cluster-9981.88036	Predicted: probable aquaporin SIP2-1

2.2.1. Phylogenetic analysis of LvAQP gene family

By constructing phylogenetic tree, the distribution and development of the 58 candidate aquaporin genes of four subfamilies of LvAQP gene family could be seen clearly (Fig. 1). The internal genes of PIPs subfamily were more similar than that of TIPs subfamily, NIPs subfamily, and SIPs subfamily. The PIPs subfamily had the largest genes, which was due to tandem repeats of some genes with similar structures on the chromosome. Among the four subfamilies, the PIPs subfamily with the most genes contained 7 pairs of tandem repeats genes, while just 1 tandem repeats gene in the NIPs subfamily and SIPs subfamily (Table 4).

Table 4
Tandem repeats genes in LvAQP gene family

Subfamily	Number of subfamily tandem repeats	Tandem repeats gene name	
PIPs	7	*Cluster-9981.114831*	*Cluster-9981.114832*
		Cluster-9981.689 Cluster-9981.690 Cluster-9981.691	
		Cluster-9981.47892	*Cluster-9981.47893*
		Cluster-9981.154931	*Cluster-9981.154932*
		Cluster-9981.21660	*Cluster-9981.21661*
		Cluster-9981.111170	*Cluster-9981.111171*
		Cluster-9981.29849	*Cluster-9981.29850*
NIPs	1	*Cluster-9981.133629*	*Cluster-9981.133630*
SIPs	1	*Cluster-9981.88036*	*Cluster-9981.88037*

Compared the aquaporin gene family of Monocotyledonous *Oryza sativa*, *Zea mays* and *Musa acuminata*, and dicotyledonous *Arabidopsis thaliana* and *Brassica rapa* with that of *Ligustrum × vicary*, the results showed that the number of PIPs gene subfamily was the largest, the number of SIPs gene subfamily was the smallest (Table 5). In addition, the distribution of four gene subfamilies of the LvAQP gene family was generally the same as that of subfamily members of other plants. *Ligustrum × vicaryi* was a dicotyledonous plant. Unlike other plants, in *Ligustrum × vicaryi*, the number of aquaporin gene in PIPs subfamily was nearly 2 times higher than that of TIPs subfamily and NIPs subfamily, while it was similar to that of TIPs subfamily and NIPs subfamily in other plants. PIPs located on the cytomembrane was highly selective to transport matrix, and was critical for maintaining the water balance of cells in plants [39]. Thus, it was speculated that

the *Ligustrum × vicaryi* PIPs subfamily (LvPIPs) may play a major role in maintaining its own water balance of cells.

Table 5
Distribution of subfamily members of AQP gene family in various plants

Plants	PIPs	TIPs	NIPs	SIPs	Total
Arabidopsis thaliana	13	10	9	3	35
Oryza sativa	11	10	10	2	33
Zea mays	13	11	4	3	31
Brassica rapa	22	16	15	6	59
Musa acuminata	18	17	9	3	47
Ligustrum × vicaryi	32	11	11	4	58

In this study, a phylogenetic tree was constructed based on 35 *Arabidopsis thaliana* aquaporin genes, 35 *Oryza sativa* aquaporin genes, 58 candidate LvAQP genes and aquaporin genes related to cold stress in other plants (Fig. 2). Most of the aquaporin genes related to cold resistance were distributed in the PIPs subfamily (Fig. 2), while the gene number of PIPs subfamily in *Arabidopsis thaliana* and *Oryza sativa* were relatively low (Table 5). There was only a pair of tandem repeats gene (At2G37170 and At2G37180) in the PIPs subfamily of *Arabidopsis thaliana* (Table 2), while there were 7 pairs of tandem repeats genes in the LvPIPs subfamily. Therefore, the reason for the large number of LvPIPs might be that genes were relatively tightly distributed on chromosomes, and tandem duplication led to gene amplification.

2.2.2. LvAQP sequence characteristics

The identified LvAQP genes all contained conserved domains. Table 6 showed that the LvAQP gene family contained 19 main conserved motifs. The distribution of 58 LvAQP conservative motifs was shown in Figure 3, and the four subfamilies shared common conservative motifs, such as motif1. Each subfamily had similar conserved sites, and the members of each subfamily contained very similar conserved motifs, even the same, such as the *Cluster-9981.115068* and *Cluster-9981.109600* of the PIPs subfamily. There was sequence diversity among motifs. For example, relatively few conserved motifs in the SIPs subfamily, motif 3, motif 4, motif 5, and motif 11 in the PIPs subfamily, motif 7 and motif 19 in the TIPs subfamily, motif 12, motif 14, and motif 15 in the NIPs subfamily and connected motif 17 and motif 1 in the SIPs subfamily. Each LvAQP subfamily was highly conserved during the process of evolution, which was beneficial to the phylogeny of LvAQP gene family.

Table 6
19 Conserved motif information of LvAQP genes

Motif type	Motif sequences	Sites	Width	E-value
Motif1	KRSARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSFGAAVIYNK	54	50	7.7e-1602
Motif2	VYCTAGISGGHINPAVTFGLFLARKVSLIRAIMYIVAQCLGAICGVGLVK	50	50	2.5e-1531
Motif3	KDYKDPPPAPLFDAGELKKWSFYRALIAEFIATLLFLYITVLTVIGYKSQ	28	50	9.2e-1070
Motif4	YQKYGGGANELADGYSKGTGLGAEIIGTFVLVYTVFSATDP	32	41	6.3e-911
Motif5	KAWDDHWIFWVGPFIGAAIAAFYHQYILR	26	29	4.3e-568
Motif6	DKCGGVGILGIAWAFGGMIFV	35	21	2.1e-374
Motif7	KAALAEFISTLIFVFAGEGSGMAYNKLTGBAPLTPAGLVAAAVAHAFALF	9	50	2.8e-167
Motif8	KGIWVYWVGPLIGAGLAAWVY	25	21	5.9e-162
Motif9	SDWZALVVEIIITFGLVFTVY	22	21	3.0e-168
Motif10	AMENKEEDVRLGANKYSERQPJGTAAQSD	8	29	3.4e-125
Motif11	AIKALGSFRSS	19	11	2.1e-090
Motif12	KHGNSSGCSLLTLSFIQKIIAEILGTYFLIFAGCAAVVVNA	8	41	5.2e-079
Motif13	MAKDVEEEPEG	19	11	6.4e-049
Motif14	NIIRFTDKPLREITKS	6	16	2.2e-045
Motif15	LLFTGKHDHFSGTLP	7	15	5.0e-037
Motif16	AFQKSY	24	6	2.3e-035
Motif17	TPVIPAPYPDILRGPSLNVDLKSGALAEGLLTFAITF	6	37	2.3e-027

Note: Motif sequences represent the motif consensus in this experiment; Sites stands for the number of occurrences of this motif in 58 LvAQP genes; Width represents the width of the motif, E-value represents the statistical significance of the motif; the smaller E-value, the more reliability of the result.

Motif type	Motif sequences	Sites	Width	E-value
Motif18	LRQQGHIFNPSJPKPSHKAPNAFLLNRSRPPKSRFLFDSVQ	4	47	1.2e-025
Motif19	FFINHSHEPLPSSEY	7	15	1.7e-019

Note: Motif sequences represent the motif consensus in this experiment; Sites stands for the number of occurrences of this motif in 58 LvAQP genes; Width represents the width of the motif, E-value represents the statistical significance of the motif; the smaller E-value, the more reliability of the result.

All the known aquaporin genes related to cold stress, they all contained several common gene sequence fragments (Fig. 4), namely IAEF, GIAW, GGMI, LVYCTAG, GTFVLVYTVF and ATD, which might play a key role in resisting cold. The above fragments in LvAQP came from motif 2, motif 3, motif 4, motif 6. Most of these Motifs were distributed in the PIPs subfamily of LvAQP. Therefore, the PIPs subfamily might be important for *Ligustrum × vicaryi* under cold stress.

2.2.3. Analysis of LvAQP gene expression pattern

The transcript abundance of LvAQP was analyzed in four sampling times, and combining the phylogenetic relationship between cold stress aquaporin genes of various plants and LvAQP genes (Fig. 2), which was helpful to identify the specific expression patterns of individual genes of LvAQP gene family.

There was a change of expression of the 58 LvAQP genes in September, November, January and April (Fig. 5). Transcriptional analysis showed that the PIPs subfamily and TIPs subfamily contained relatively high expression in four samping times. Compared to September, 8% LvAQP genes expression increased in November and January, and decreased in April. 21% LvAQP genes expression decreased in November and January, and increased in April. 24% LvAQP genes expression increased in November, decreased in January, and increased in April. 17% LvAQP genes expression decreased in November, increased in January, and decreased in April. 21% LvAQP genes expression increased in November, and decreased in January and April. 3.4% LvAQP genes expression decreased in November, and increased in January and April. 5% LvAQP genes expression decreased consecutively in November, January, and April. According to relevant researches, the overexpression of *MusaPIP1;2* in *Musa acuminata* enhanced plant chilling resistance [40]; in *Arabidopsis thaliana*, the overexpression of *AtPIP1;4* and *AtPIP2;5*, along with repressed expression of other PIPs family members to enhance plant cold resistance [17]; in *Oryza sativa*, increased expression of *OsPIP2;5* and *OsPIP2;7* and decreased expression of *OsPIP1;3* helped to improve cold resistance [32, 41]. Studies had shown that plants enhance cold resistance by overexpressing or inhibiting the expression of aquaporin genes under cold stress. Therefore, in this study, the researchers selected LvAQP genes, whose gene expression increased in November and January, and decreased in April, and whose gene expression decreased in November and January, and increased in April in four sampling times, as the target genes.

By analyzing the relative transcript abundance profile of LvAQP genes (Fig. 5) and the phylogenetic relationship between cold stress aquaporin genes of various plants and LvAQP genes (Fig. 2), 20 LvAQP

genes related to cold stress were determined: *Cluster-9981.109600, Cluster-9981.112839, Cluster-9981.112265, Cluster-9981.111171, Cluster-9981.109034, Cluster-9981.89369, Cluster-9981.110451, Cluster-9981.114832, Cluster-9981.114831, Cluster-9981.107281, Cluster-9981.86061, Cluster-9981.112777, Cluster-9981.111753, Cluster-9981.115801, Cluster-9981.112789, Cluster-9981.122691, Cluster-9981.104986, Cluster-9981.123071, Cluster-9981.120365* and *Cluster-9981.8803*. Among the determined 20 LvAQP genes, 11 genes were PIPs subfamily, 5 genes were TIPs subfamily, and 2 genes were NIPs subfamily and SIPs subfamily, respectively. The result of 11 genes belonged to the PIPs subfamily was in accordance with previous studies on aquaporins in response to cold stress, suggesting that the PIPs subfamily of aquaporin might play a major role in resistance to cold stress in *Ligustrum × vicaryi* [13, 20, 21, 22, 32, 33, 34]. Different from previous studies, 2 genes of SIPs subfamily in LvAQP gene family were also responded to cold stress.

Among the 20 LvAQP genes identified in response to cold stress, the expression of three genes, *Cluster-9981.114831*, *Cluster-9981.104986*, and *Cluster-9981.120365*, were significantly up-regulated during the two periods of lowest natural temperature in November or January, while the expression of nine genes were significantly down-regulated, namely, *Cluster-9981.112839*, *Cluster-9981.109034*, *Cluster-9981.89369*, *Cluster-9981.110451*, *Cluster-9981.107281*, *Cluster-9981.112777*, *Cluster-9981.112789*, *Cluster-9981.122691* and *Cluster-9981.88037*. All the significantly up-regulated genes contained motif 6, and all the significantly down-regulated genes contained motif 1 and motif 2, which was basically consistent with the common special motifs reported in aquaporin genes related to cold stress. It was speculated that the key role of some AQP genes in *Ligustrum × vicaryi* for cold resistance might be related to the presence of these specific modular motifs.

2.3. KEGG enrichment analysis of differentially expressed genes

KEGG PATHWAY enrichment analysis of differentially expressed genes was conducted under natural cold stress in *Ligustrum × vicaryi* (Table 7). A total of 12,872 differentially expressed genes were distributed in 338 pathways, and 10 of them showed significant differences (P<0.05). The differentially expressed genes were significantly enriched in Ribosome (ko03010), Starch and sucrose metabolism (ko00500), Plant hormone signal transduction (ko04075).

Table 7
KEGG Pathway enrichment analysis in DEGs of *Ligustrum × vicaryi*

Pathway ID	Pathway	*P* value	Gene number
ko03010	Ribosome	1.51E-07	448
ko00940	Phenylpropanoid biosynthesis	1.18E-06	131
ko00460	Cyanoamino acid metabolism	5.77E-06	85
ko00500	Starch and sucrose metabolism	8.38E-05	208
ko04075	Plant hormone signal transduction	2.11E-03	170
ko05322	Systemic lupus erythematosus	7.93E-03	48
ko05034	Alcoholism	8.15E-03	87
ko04915	Estrogen signaling pathway	1.16E-02	68
ko04612	Antigen processing and presentation	2.38E-02	72
ko00520	Amino sugar and nucleotide sugar metabolism	3.49E-02	122

2.4. Expression verification of cold-responsive LvAQP target genes

The nine screened LvAQP target differentially expressed genes expression were verified by Real-time PCR. The qRT-PCR results were first calculated by the $2^{-\triangle\triangle CT}$ method followed by log calculation based 2. The change of $\log_2$ multiples for the real-time fluorescence quantification of the nine target genes were shown in Table 8.

Table 8
Fluorescence quantification of nine cold-responsive LvAQP target genes

Gene name	Types of Aquaporin genes	log_2 Fold Change	
		RNA-seq	Real-time
Cluster-9981.109600	PIPs	-1.8929	-0.76
Cluster-9981.112839	PIPs	-1.5391	-1.18
Cluster-9981.111171	PIPs	-2.0145	-0.63
Cluster-9981.109034	PIPs	-2.2498	-2.82
Cluster-9981.114831	PIPs	2.5535	2.86
Cluster-9981.107281	PIPs	-3.7174	-0.79
Cluster-9981.112777	TIPs	-3.4918	-4.25
Cluster-9981.115801	TIPs	-1.9979	-0.38
Cluster-9981.122691	TIPs	-4.1174	-3.16

Although the Real-time PCR results of individual genes deviated from the RNA-Seq results in terms of differential fold, the up-regulated and down-regulated expression trends between them were consistent (Fig. 6). In addition, the correlation analysis between the results of qRT-PCR analysis and RNA-seq sequencing results showed that the correlation coefficient R^2 reached 0.70 (Fig. 7), indicating that the transcriptome sequencing results of *Ligustrum vicaryi* cold resistance were reliable.

3. Discussion

The number of genes encoding aquaporin of *Ligustrum vicaryi* was more than that in *Arabidopsis thaliana*, especially in the PIPs subfamily due to gene amplification. In this study, 58 candidate LvAQP genes were found. Phylogenetic analysis showed that these 58 LvAQP genes can be divided into four subfamilies: PIPs, TIPs, NIPs, and SIPs. And the fifth subfamily that has been reported: XIPs is a class of atypical non-specific intrinsic aquaporins. It was absent in *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays* and *Ligustrum vicaryi*. Plasma membrane intrinsic proteins, located on the cytomembrane, are highly selective for transporting matrix, and plays an important role in maintaining cell water balance under various adversities [42]. Studies have shown that plants resist abiotic stress by regulating the density and activity of PIPs at cytomembrane [39, 43, 44, 45]. Plants mainly regulate their response to stress through the expression or inhibition of PIPs genes of aquaporin family (Table 1). Under the natural low temperature adversity, maintaining water balance in the body is a huge challenge to *Ligustrum × vicaryi*. At this time, the transmembrane transport of water in *Ligustrum × vicaryi* mainly depend on the PIPs subfamily of the aquaporin family. This study found that the number of PIPs subfamily was the largest in the LvAQP gene family, which was consistent with the results of previous studies. The difference from previous studies is that there are 13 and 11 PIPs genes in *Arabidopsis thaliana* and *Oryza sativa*, respectively, while this study found that there were 32 PIPs subfamily genes in

Ligustrum × vicaryi, which were far more numerous than other plants. When a certain gene family has obvious gene clusters on the chromosome, it is often accompanied by the gene expansion mechanism of tandem replication [46]. The large number of PIPs subfamilies of LvAQP gene family was caused by the expansion and tandem duplication of some genes with similar structure in the gene cluster. In this study, the number of LvPIPs genes was higher than that of other plants. 11 of the 20 aquaporin genes screened that were related to low temperature stress belonged to the PIPs subfamily, indicating that these PIPs genes in *Ligustrum × vicaryi* also acted a regulatory role under adversity.

In the face of cold stress, plants generally respond to stress by regulating water homeostasis in the body, in which aquaporin proteins are one of the key pathways of water transport [47, 48, 49]. The expression patterns of aquaporins in various plant tissues are different, which indicat that aquaporins may have different functions in plants [50]. After freezing treatment, the low temperature tolerant *Zea mays* variety z7 maintained root hydraulic conductivity and water transport by expressing a large amount of aquaporins to reduce freezing damage [51]. The aquaporins PIP1 and PIP2 of *Arabidopsis thaliana* cooperated synergistically in the roots under cold stress to affect root hydraulic conductivity and regulate plant cold resistance [52]. Overexpression of *PtPIP2;5, PtPIP2;1* and *PtPIP2;3* in *Populus trichocarpa* affected its response to cold stress and osmotic stress [53]. Under cold stress, the overexpression of banana *MaPIP2;7* lowered the MDA content and electrolyte leakage in the plant, while the content of chlorophyll, proline, soluble sugar and ABA was higher, thereby enhancing the tolerance to various stresses such as cold [54]. The overexpression of *MaSIP2;1*, *OsPIP2;7*, *TaAQP7 (PIP2)* regulated the osmotic balance in plants, reduced membrane damage and oxidation, and adjusted the levels of hormones such as ABA and GA to improve the cold tolerance of plants [19, 37, 44].

In this study, the phylogenetic comparison between LvAQP genes and reported aquaporin genes related to cold stress in other plants as well as the changes of aquaporin genes transcription abundance in four sampling times were conducted to identify the specific expression patterns of individual gene of the gene family under natural cold stress. 20 aquaporin genes related to cold stress were screened from the 58 LvAQP genes, 11 belonged to LvPIPs subfamily, 5 belonged to LvTIPs subfamily, 2 belonged to LvNIPs subfamily and LvSIPs subfamily, respectively, which indicated that genes of PIPs subfamily played a major role in response to natural cold stress. In this study, 75% of the LvAQP genes that were significantly related to cold stress decreased in November and January, and their expression increased in April which is consistent with the results of transcriptome analysis of *Arabidopsis thaliana*, *Oryza sativa*, and the roots and leaves of *Zea mays* [17, 44, 51]. In the process of natural cold stress, *Ligustrum × vicaryi* regulated the decrease or increase of the expression of aquaporin genes and the corresponding protein activity, and adjusted root hydraulic conductivity, thus maintaining the water balance in the plant, resisting the effects of natural low-temperature stress, and ensuring normal life activities.

Aquaporins are important membrane functional proteins in many physiological reactions, which play a key role mainly through transcriptional regulation, post-translational modification and subcellular localization [55, 56]. Plasma membrane intrinsic proteins and tonoplast intrinsic proteins are located on the inner chloroplast membrane and thylakoid membrane [2]. KEGG enrichment analysis of *Ligustrum × vicaryi* genes showed that they responded to cold stress mainly through sucrose metabolism pathway and plant hormone

signal transduction pathway. It was speculated that some genes of PIPs and TIPs subfamily on cytomembrane and chloroplast were up-regulated or down-regulated, which would enhance the cold resistance of *Ligustrum × vicaryi* by regulating the synthesis and transformation of soluble sugar or starch. After the nature low temperature is experienced of *Ligustrum × vicaryi*, the differentially expressed genes related to hormone signaling were enriched under cold stress, and ABA signaling and other pathways were opened under cold stress to induce the expression of downstream related regulatory genes, thus inducing changes in aquaporin genes, regulating the synthesis of corresponding proteins and other macromolecular substances, stabilizing the cytomembrane structure and reducing the water translocation rate to enable *Ligustrum × vicaryi* to avoid low temperature injury.

4. Conclusion

In this study, the expression of LvAQP under natural cold stress was studied. 58 candidate LvAQP genes were identified by *Arabidopsis thaliana* aquaporin genes blast comparison. Based on phylogenetic analysis, the 58 candidate LvAQP genes were divided into four subfamilies: 32 belonged to PIPs subfamily, 11 belonged to TIPs subfamily, 11 belonged to NIPs subfamily and 4 belonged to SIPs subfamily. The number of genes in the PIPs subfamily was nearly twice as large as that in other plants. The LvAQP gene family contained 9 pairs of tandem repeats genes, which had high conservatism in the process of evolution by searching of conserved motifs. Through analyzing aquaporin gene related to cold stress in *Arabidopsis thaliana*, *Oryza sativa*, *Hordeum vulgare*, *Musa acuminata*, *Populus trichocarpa*, *Sorghum bicolor*, *Triticum aestivum*, *Brassica rapa*, *Gossypium hirsutum* and LvAQP genes expression profile, 20 differentially expressed genes of LvAQP gene family under natural cold stress were obtained: *Cluster-9981.109600, Cluster-9981.112839, Cluster-9981.112265, Cluster-9981.111171, Cluster-9981.109034, Cluster-9981.89369, Cluster-9981.110451, Cluster-9981.114832, Cluster-9981.114831, Cluster-9981.107281, Cluster-9981.86061, Cluster-9981.112777, Cluster-9981.111753, Cluster-9981.115801, Cluster-9981.112789, Cluster-9981.122691, Cluster-9981.104986, Cluster-9981.123071, Cluster-9981.120365* and *Cluster-9981.88037*. Among the 20 differentially expressed genes, 11 belonged to LvPIPs subfamily. Among the 20 differentially expressed genes, the significantly up-regulated key genes were: *Cluster-9981.114831, Cluster-9981.104986, Cluster-9981.120365*; the significantly down-regulated key genes were: *Cluster-9981.112839, Cluster-9981.109034, Cluster-9981.89369, Cluster-9981.110451, Cluster-9981.107281, Cluster-9981.112777, Cluster-9981.112789, Cluster-9981.122691* and *Cluster-9981.88037*.

5. Methods

5.1. Plant materials

One-year-old container seedlings of *Ligustrum × vicaryi* (txid1133299), were obtained from the Beijing Florascape Co., Ltd. located in Beijing City (40°11'N, 116°48'E). We got the permission to collect the plant samples from the Beijing Florascape Co., Ltd., and the plant materials were formally identified by senior engineer Ju Chen of the company, and later identified by professor Gang Zhang of Hebei Agricultural University. The *Ligustrum × vicaryi* were cultivated in Specimen Park (38°50'N, 115°26' E) of Hebei Agricultural University, Baoding City, Hebei Province, in September 2019. In the experimental set-up, the

container seedlings were divided in three replicates for the measurements at each sampling time, 25 plants in each replicate. the spacing in the rows and spacing between rows was 25cm × 50cm, consistent cultivation conditions and conventional maintenance management.

5.2. Treatment

The seedlings were sampled on the 24th of each month in September, November 2019, January and April 2020. There were three parallel test in each replicate. The roots of the plants were washed by tap water to remove the soil, and the fine roots were washed with tap water, distilled water and ultrapure water in turn, and then were frozen in liquid nitrogen and stored in an ultra-low temperature freezer at -80°C.

5.3. RNA-seq

5.3.1. RNA extraction and detection

Total RNA was extracted by Omniplant RNA Kit (DNase I).

The RNA integrity was detected by agarose gel electrophoresis with 2% concentration, 150V, 150mA. The concentration of each RNA sample and its optical density in the wavelength range of 260 nm and 280 nm were measured by Nano Drop one spectrophotometer, and OD_{260}/OD_{280} value was calculated to detect the purity of RNA, then the RNA was stored in ultra-low temperature freezer at -80°C.

5.3.2. cDNA library construction

Firstly, magnetic beads with Oligo(dT) were used to enrich eukaryotic mRNA. Secondly, mRNA was broken into short fragments by adding fragmentation buffer. One-stranded cDNA was synthesized with six-base random hexamers using mRNA as template. Thirdly, double-stranded cDNA was formed by adding buffer, dNTPs, DNA polymerase I, RNase H, which was purified by AMPure XP beads. The purified double-stranded cDNA was end-repaired and dA-tailed to ligate to sequencing connectors, and then fragment size selection was performed with AMPure XP beads. Finally, Polymerase Chain Reaction (PCR) amplification was conducted, and the PCR products were purified with AMPure XP beads to obtain the final cDNA library. After the completion of cDNA library construction, the initial quantification was performed by using Qubit 2.0, then the library was diluted. Subsequnetly, the insert size of the library was tested. When the insert size met the expectation, the effective concentration of the library was accurately quantified by Q-PCR method (effective library concentration > 2nM) to ensure the quality of cDNA library.

5.3.3. RNA data analysis

The raw image data generated by the sequencer was transformed into raw data or raw reads by base calling. The results were stored in fastq format, which was original file, including the sequence of reads and the sequencing quality of reads. Raw reads were processed to obtain clean reads by removing reads containing adaptor, reads containing more than 10% of N and reads containing a small amount of low-quality sequences (the number of bases with quality value Q<5 accounts for more than 50% of the entire reads).

The transcriptome data were assembled by Trinity-v2.4.0 software with the following commands and parameters: Trinity –seq Type fq –max_memory 300G –left file_1.fq –right file_2.fq –CPU 50 –full_ clean up –

KMER_SIZE 30 –min_kmer_cov 5. Among genes containing multiple transcripts, the sequence with the longest transcript was used as the basis for calculating expression, and RSEM worked as the method for transcript abundance calculation, and trimmed mean of M-values (TMM) was used as the method for normalization between samples.

KEGG PATHWAY enrichment analysis on the results of variance analysis was performed by kobas software.

5.4. Construction of phylogenetic tree

Multiple sequence alignment of candidate genes was performed by MAFFT software's E-INS-I strategy with necessary manual corrections. PhyML 3.0 software and iTOL online software were used to construct the phylogenetic tree, and the constructed phylogenetic tree was analyzed by Alrt detection method and WAG model.

5.5. Screening of target genes

According to the protein sequences of 35 aquaporin genes of *Arabidopsis thaliana*, the transcriptome database of *Ligustrum × vicaryi* was searched by blast homology retrieval method, and the LvAQP gene family was identified. The LvAQP genes were screened by MAFFT comparison software and manual correction process, and then the candidate genes were initially selected by differential gene expression analysis between natural low temperature treatment and non-low temperature treatment. A phylogenetic tree of LvAQP genes and the known cold resistance aquaporin genes of other plants was constructed to find homologs of known cold resistance genes of other plants within *Ligustrum × vicaryi.*

5.6. Quantitative real-time PCR (qRT-PCR)

5.6.1. RNA extraction

Refer to 5.3.1. for RNA extraction.

5.6.2. Reverse transcription of RNA into cDNA

The UElris II RT-PCR System for First-Strand cDNA Synthesis (with dsDNase) reverse transcription kit was used as follows: The RNA was denatured thermally at 65°C for 5 minutes, immediately iced for more than 3 minutes, and then the reaction system was prepared as 20μL of reverse transcription system: Total RNA 2μL, UElris RT MasterMix(5X) 4μL, RNase-free Water 13μL, dsDNase 1μL.

Reaction conditions: reverse transcription 37°C for 2 minutes; 55°C for 10 minutes; 85°C for 10 seconds. After the reaction, stored at -20°C.

5.6.3. Design of primers for Quantitative real-time qRT-PCR

Nine LvAQP genes related to cold stress were selected for qRT-PCR. Primers were designed by Primer3 Plus and synthesized by Sangon Biotech (Shanghai) and *Ligustrum × vicaryi LvEF-1α* was selected as an internal reference gene, the primer information is shown in Table 9.

Table 9
The genes and primers used for qRT-PCR analysis

Gene	Primer name	Sequence (5′-3′)
Plasma membrane intrinsic protein *Cluster-9981.109600*	LvPIPa-F	GCATGATCTTTGCCCTTGTT
	LvPIPa-R	ACCCTTCGTGTAACCGTGAG
Predicted: probable aquaporin PIP-type pTOM75 *Cluster-9981.112839*	LvPIPb-F	TGCACTGCTGGTATCTCAGG
	LvPIPb-R	ATGAAGCCCTTGACAACACC
Plasma membrane intrinsic protein *Cluster-9981.111171*	LvPIPc-F	GCGGCATGATTTTCATTCTT
	LvPIPc-R	CAGATTGCACCCAAACATTG
Predicted: aquaporin PIP2-1-like *Cluster-9981.109034*	LvPIP2-1-F	GCCACCAAGGACTACCAAGA
	LvPIP2-1-R	GCGCTCTGACTTTTGTACCC
Predicted: aquaporin PIP2-7 *Cluster-9981.114831*	LvPIP2-7a-F	GGACAAGTGCACAATGATGG
	LvPIP2-7a-R	TCGGCGTTGTTTAGCTTCTT
Predicted: aquaporin PIP2-7 *Cluster-9981.107281*	LvPIP2-7c-F	ATTGCCACCCTTCTCTTCCT
	LvPIP2-7c-R	AAATGGTGGTGGAACCTGAG
Predicted: aquaporin TIP1-3-like *Cluster-9981.112777*	LvTIP1-3a-F	TGACAGTTTGGAACGCAGTC
	LvTIP1-3a-R	CCATGTCCAACTGACCACAG
Predicted: aquaporin TIP1-3-like *Cluster-9981.115801*	LvTIP1-3b-F	TCATGCTTTCGCACTTTTTG
	LvTIP1-3b-R	CAACCCACCAGTGGAGAACT
Predicted: probable aquaporin TIP-type *Cluster-9981.122691*	LvTIPa-F	TCGGAGGCAAAATAACCATC
	LvTIPa-R	TCTGCAGCAGTGGCATAGAC
Cluster-9981.111777	EF-1α-F	TGGTTTTGAGGGTGACAACA
	EF-1α-R	TCAATCCAGAGGGACCAAAG

5.6.4. qRT-PCR reaction system and reaction conditions

According to the instructions of AugeGreen^{TM} qPCR Master Mix reagent, Roche fluorescence quantitative PCR instrument lightcycl96 was used to detect the expression of target genes. Preparation of reaction solution for 20μL reaction system: 2×AugeGreen^{TM} Master Mix 10μL, ddH_2O 7μL, Forward Primer 1μL, Reverse Primer 1μL, cDNA template 1μL. qRT-PCR reaction procedure is as follows: 95℃ for 2 minutes; 95℃ 15 for seconds and 58℃ for 60 seconds run 40 cycles; 95℃ for 10 seconds; 65℃ for 60 seconds; 97℃ for 1 seconds.

The last step was to analyze the solubility curve of the amplification products to determine the specificity of the primers. There were 3 technical replicates and 3 biological replicates for each sample during qRT-PCR reactions. qRT-PCR results were calculated by the $2^{-\Delta\Delta CT}$ method to get gene expression.

Abbreviations

LvAQP: *Ligustrum × vicaryi* aquaporins; PIPs: Plasma membrane intrinsic proteins; TIPs: Tonoplast intrinsic proteins; NIPs: Nodulin26-like intrinsic proteins; SIPs: Small basic intrinsic proteins; XIPs: Uncharacterized X intrinsic proteins; TMM: Trimmed mean of M-values; qRT-PCR: Quantitative real-time PCR; LvPIPs: *Ligustrum × vicaryi* plasma membrane intrinsic proteins; LvTIPs: *Ligustrum × vicaryi* tonoplast intrinsic proteins; LvNIPs: *Ligustrum × vicaryi* nodulin26-like intrinsic proteins; LvSIPs: *Ligustrum × vicaryi* small basic intrinsic proteins;

Declarations

6.1. Ethics approval and consent to participate

Not applicable.

6.2. Consent for publication

Not applicable.

6.3. Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files]. The analysis websites used in this study are as follows: Gene sequence retrieval (https://www.ncbi.nlm.nih.gov/gene/), MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/), PhyML (http://www.atgc-montpellier.fr/phyml/), iTOL (https://itol.embl.de/index.shtml), MeMe (http://meme-suite.org/tools/meme), MeV (https://sourceforge.net/projects/mev-tm4/files/mev-tm4/).

6.4. Competing interests

The authors declare no conflicts of interest.

6.5. Funding

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6.6. Authors' contributions

J.D., S.N., J.Q. and B.D. designed the experiments, J.D., S.N., J.Q., J.Z., M.Z. and Y.M. performed the experiments and collected the data. J.D., S.N., J.Q., J.Z. and M.Z. analyzed the data. J.D. wrote the manuscript. S.N., J.Q. and B.D. revised the manuscript. All the authors reviewed and approved the final manuscript.

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Figures

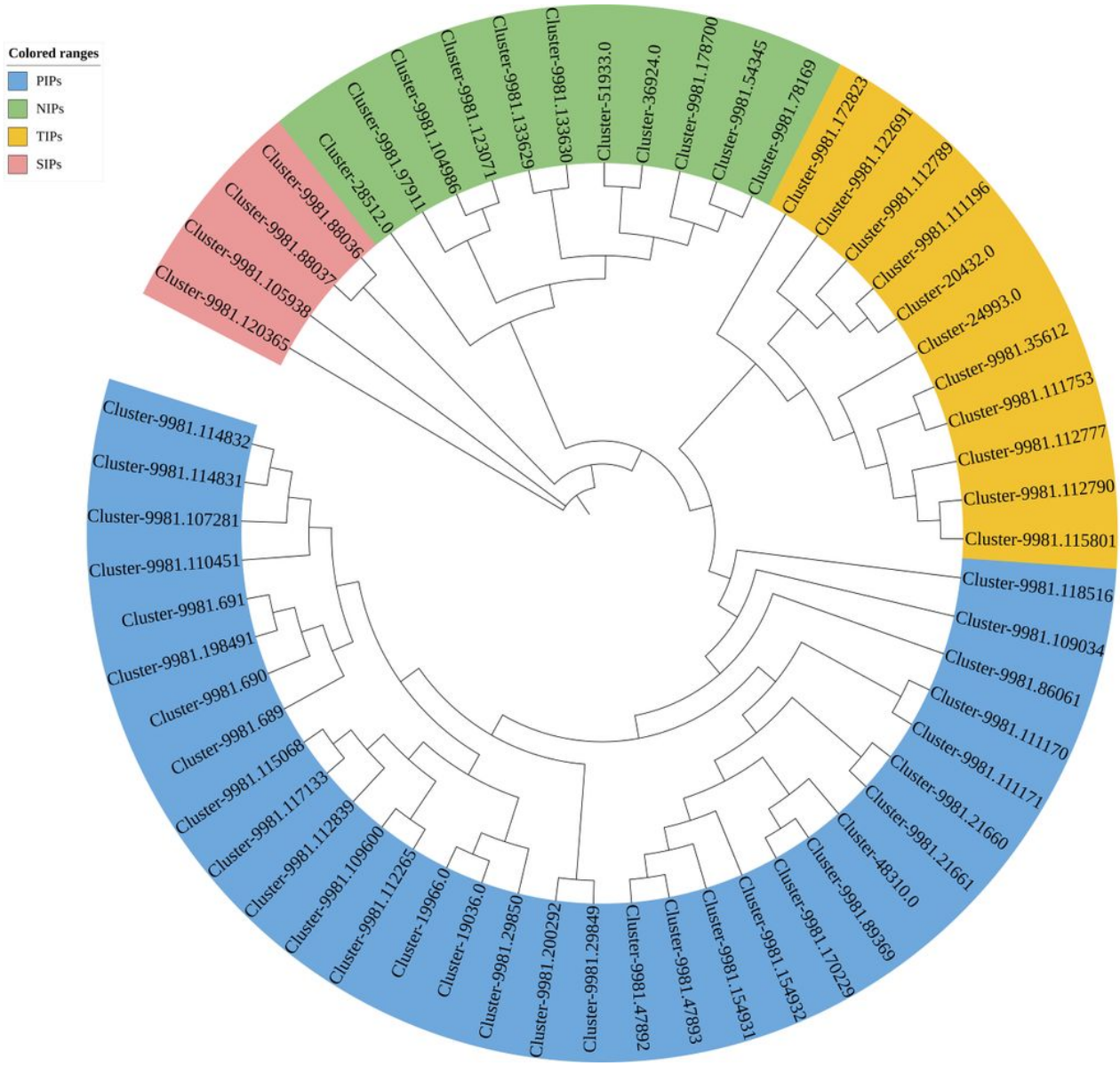

Figure 1

Phylogenetic analysis of LvAQP gene family.

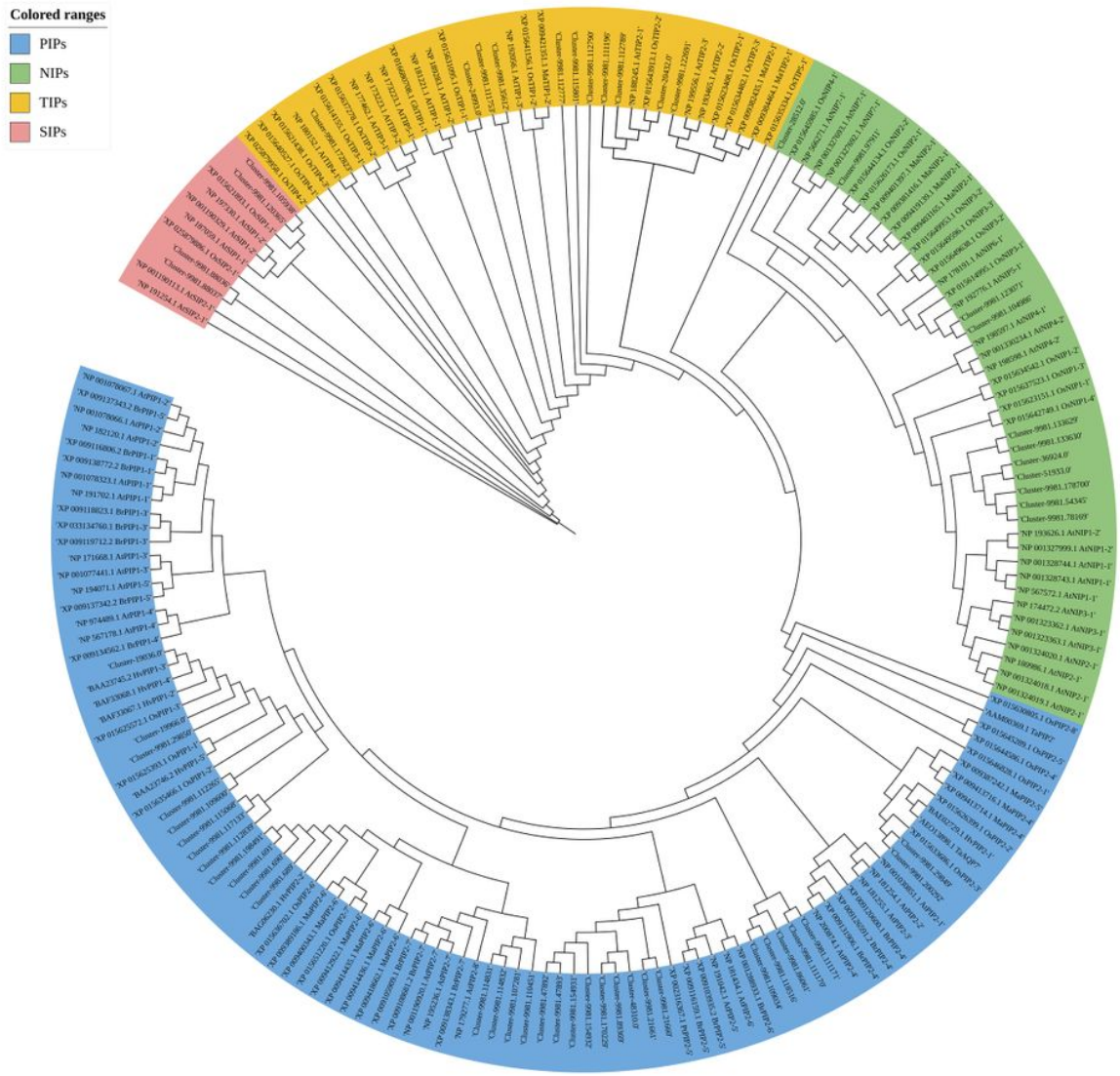

Figure 2

Phylogenetic analysis of LvAQP genes and cold stress-related aquaporin genes in other plants.

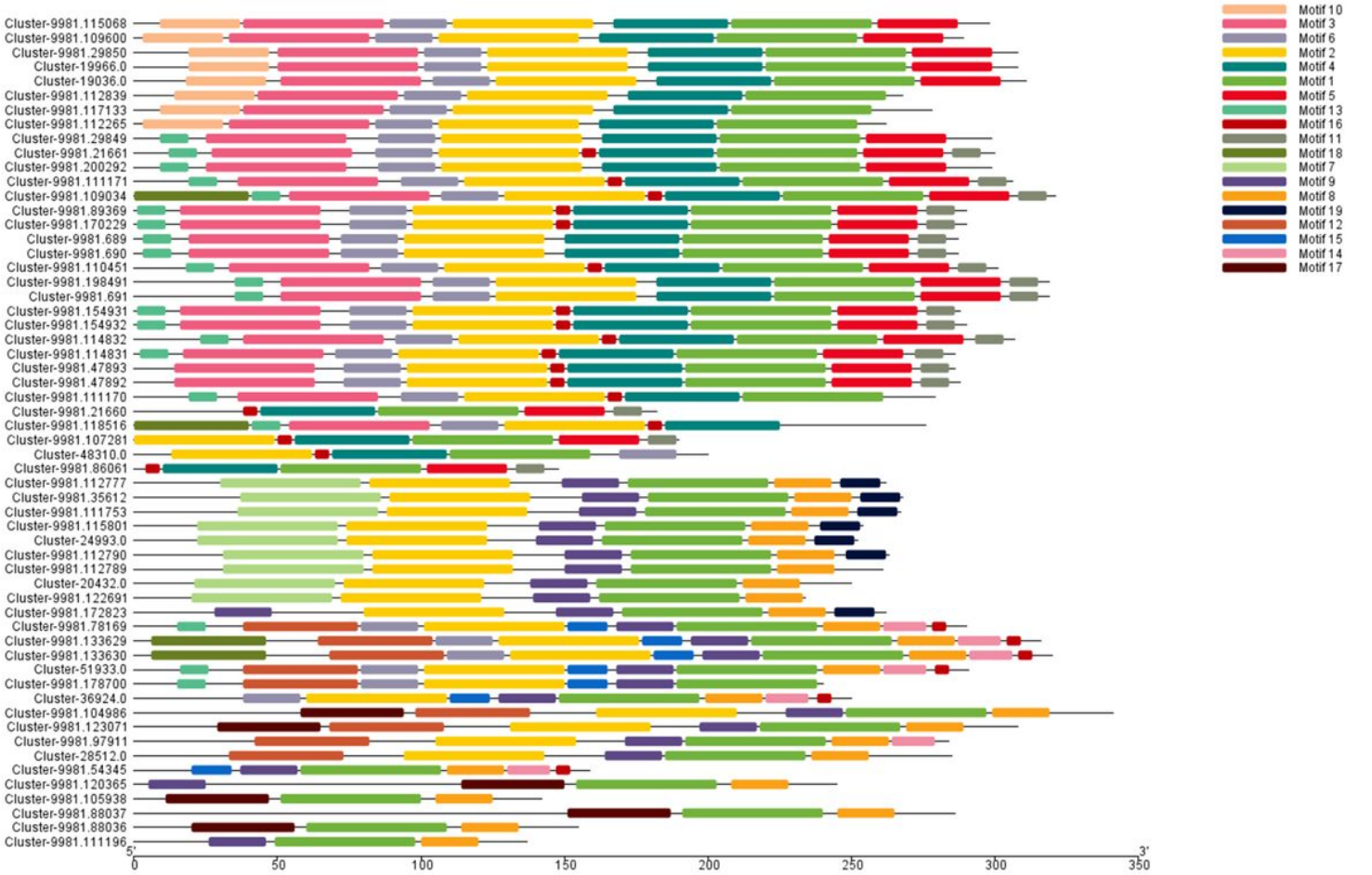

Figure 3

Conserved motif distribution map of LvAQP genes.

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AtPIP2-5 KWSFYRALIAEFIATLLFLYVTIMTVIGYKSQTDPALNPD--QCTGVGVLGIAWAFGGMIFILVVCTAGISGGHINPAVTFGLLLARKVTLVRAVMYMVAQCLGAICGVALVKAFQS-AYFTRYGGGANGLSDGYSIGTGVAAEIIGTFVLVYTVFS-ATDPKRSARDSHVPV-
OsPIP1-3 SWSFYRAGIAEFVATFLFLYISILTVMGVN--KSASK------CATVGIQGIAWSFGGMIFALVVCTAGISGGHINPAVTFGLFLARKLSLTRAVFYMAMQCLGAICGAGVVKGFQR-GLYMGSGGGANAVNPGYTKGDGLGAEIVGTFVLVYTVFS-ATDAKRNARDSHVPI-
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OsPIP2-5 RWSLYRAVIAEFVATLLFLYVTVATVIGYKHQTDASASGDDAACGGVGVLGIAWAFGGMIFILVVCTAGISGGHINPAVTFGLFLARKVSLVRAILYIVAQCLGAVCGVALVKGFQS-SFYDRYGGGANELAAGYSKGTGLAAEIIGTFVLVYTVFS-ATDPKRNARDSHVPV-
HvPIP1-2 SWSFYRAGIAEFLATFLFLYISVLTVMGVVGNPSGSK------CGTVGIQGIAWSFGGMIFVLVYCTAGISGGHINPAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAGVVKGFQT-TLYMGNGGGANSVAPGYTKGDGLGAEIVGTFVLVYTVFS-ATDAKRSARDSHVPI-
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HvPIP1-5 SWSFYRAGIAEFIATFLFLYVTVLTVMGVS--KAPSK------CATVGVQGIAWSFGGMIFALVVCTAGISGGHINPAVTFGLFLARKLSLTRAIFYIIMQCLGAICGAGVVKGFQQ-GLYMGNGGGANVVASGYTKGDGLGAEIIGTFVLVYTVFS-ATDAKRNARDSHVPI-
HvPIP2-1 KWSLYRAVIAEFVATLLFLYVTVATVIGYKHQSDPTVNTTDAACSGVGILGIAWAFGGMIFVLVYCTAGVSGGHINPAVTFGLFLARKVSLIRALLYIIAQCLGAICGVGLVKGFQS-SYYVRYGGGANELSAGYSKGTGLAAEIIGTFVLVYTVFS-ATDPKRNARDSHIPV-
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Figure 4

Special gene sequence fragments of aquaporins related to cold stress in plants.

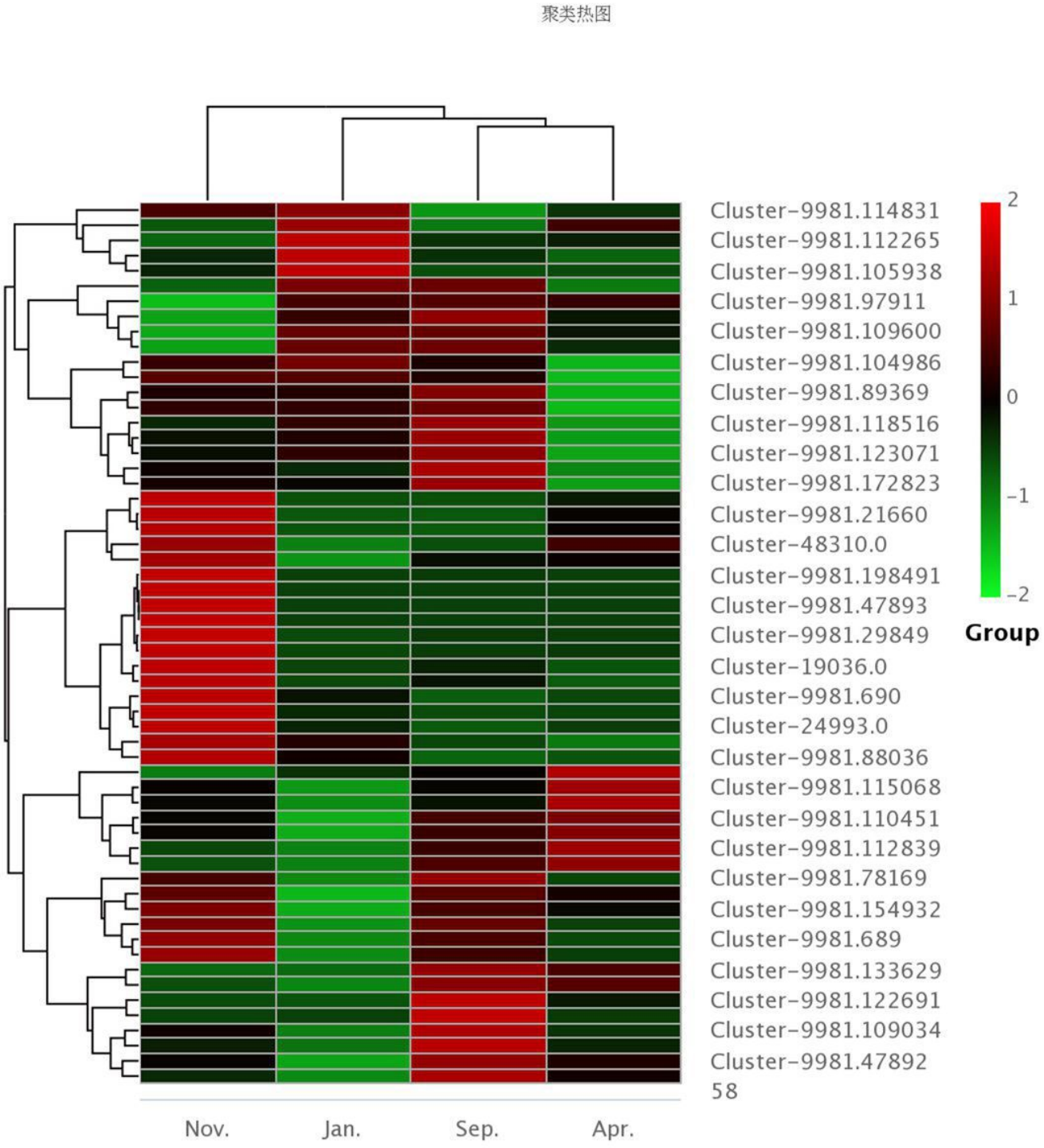

Figure 5

Relative transcript abundance profiles of LvAQP genes during natural cold stress period.

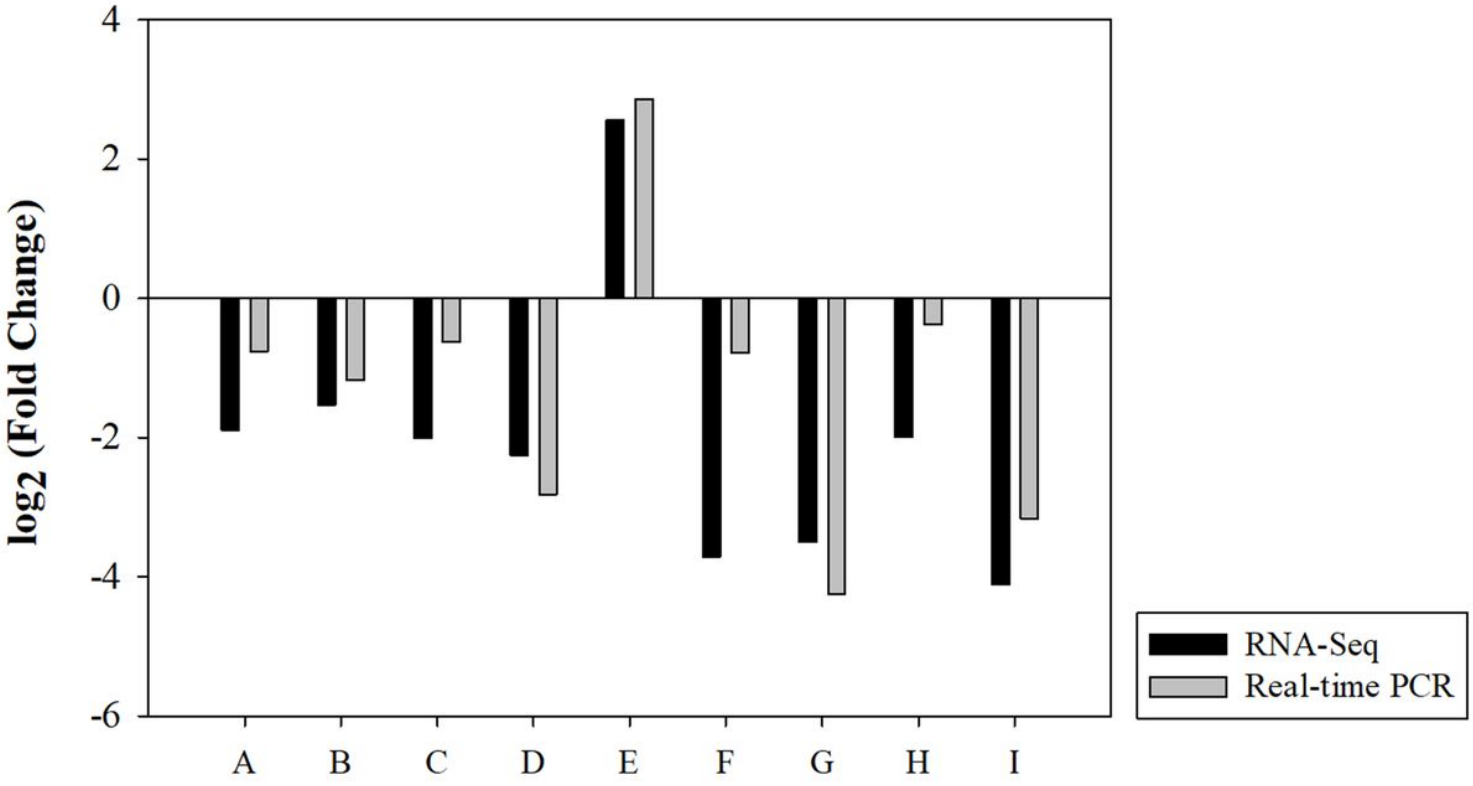

Figure 6

Comparison between RNA-seq and real-time PCR results. A: Cluster-9981.109600; B: Cluster-9981.112839; C: Cluster-9981.111171; D: Cluster-9981.109034; E: Cluster-9981.114831; F: Cluster-9981.107281; G: Cluster-9981.112777; H: Cluster-9981.115801; I: Cluster-9981.122691.

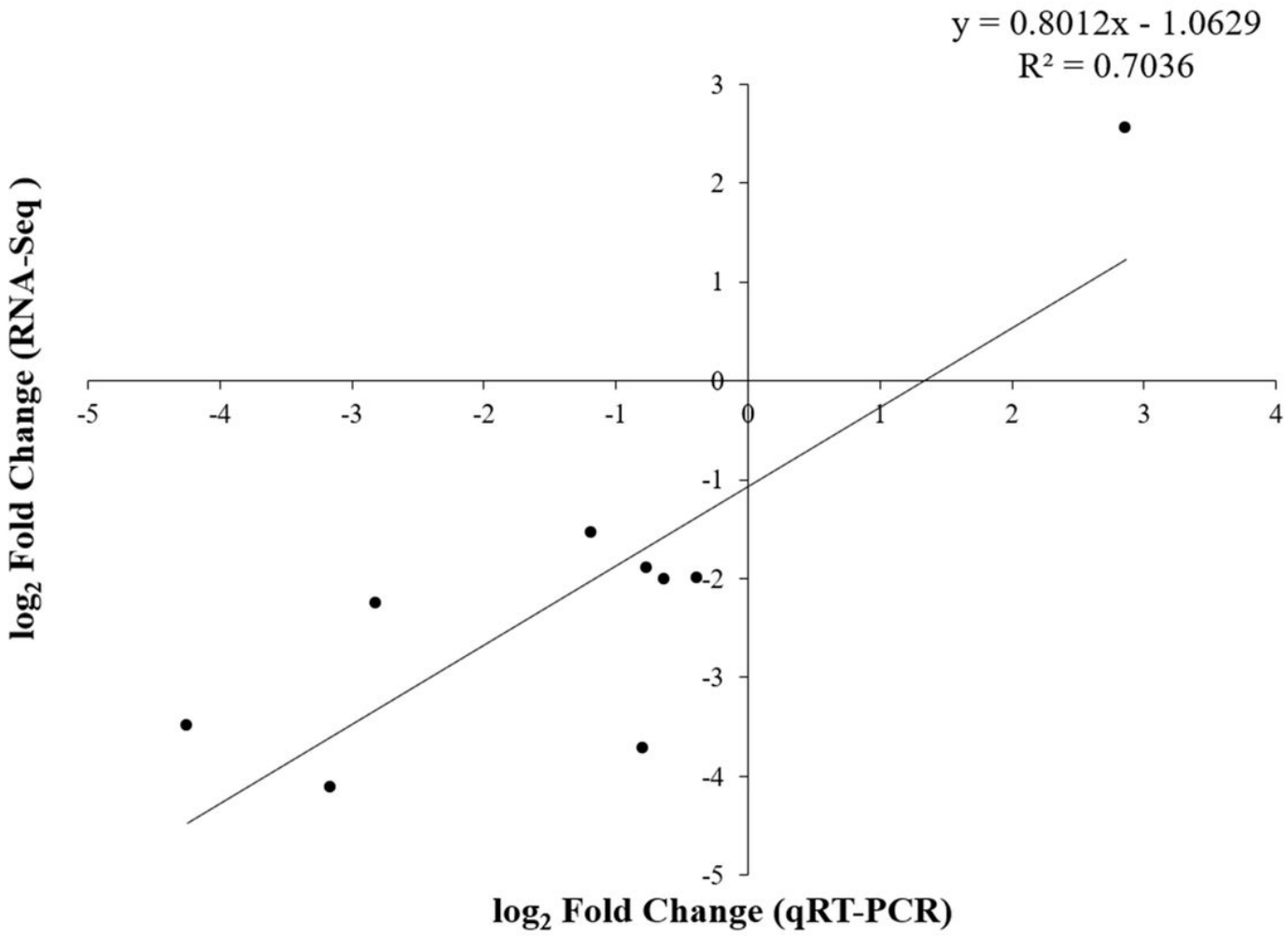

Figure 7

Correlation analysis between RNA-seq and qRT-PCR.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Aquaporingenesassociatedwithcoldstressinotherplants.txt
- SequencesoftheArabidopsisthalianaaquaporingenes.txt
- SequencesoftheLvAQPgenes.txt
- SequencesoftheOryzasativaaquaporingenes.txt