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Analysis of ABC1 protein family members in *Lepidium apetalum* seeds and the expression of *LaAbc1* in seedlings in response to abiotic stresses

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Abstract

To study the biological function of activity of bcl complex (ABC1) proteins in *Lepidium apetalum* Willd., genes encoding ABC1 family proteins were identified from the seed transcriptome. The sequence most closely related to germination at a low temperature was selected and gene expressions in response to low temperature stress further studied. The results show that 21 *ABC1* genes were expressed in seeds germinating at the low temperature: 4 genes were upregulated, 6 were downregulated, and 11 were not significantly different from controls. The results of fluorescence quantification of the low-temperature stress on the seedlings of 7-d-old *L. apetalum* showed that seven genes were up-regulated, six genes were down-regulated, and eight genes had no significant difference. Real-time quantitative PCR results show that under the low temperature stress, the expression of the *LaAbc1*-3 gene increased, but its expression decreased after some time. The expression of this gene increased again after removing the low temperature stress. The expression of *LaAbc1*-21 gene in *L. apetalum* seedlings showed a trend of decreasing first and then increasing. The *LaAbc1*-3 gene was insensitive to salt stress. Expression of the *LaAbc1*-21 gene was significantly up-regulated during the salt stress. Under osmotic stress, the expression of the *LaAbc1*-3 gene was down-regulated, and the expression was negatively correlated with polyethylene glycol (PEG-6000) concentration. Under the PEG-6000 treatment, the expression of the *LaAbc1*-21 gene was significantly up-regulated, and the expression was positively correlated with concentration. These results provide a basis for further analysis of the role of the *ABC1* genes in the stress resistance of *L. apetalum*.

Additional key words: LaAbc1-3 and *LaAbc1*-21 genes, low temperature, osmotic stress, salt stress.

Introduction

Lepidium apetalum Willd. is a cruciferous oleaginous plant, widely distributed, and with medicinal values. The ability to adapt to low temperatures in early spring is strong, and it can germinate when ice and snow begin to melt (Li *et al*. 2016). It is a pioneer plant in the process of ecosystem succession. This growth feature has gradually become the focus of research in many scientific research projects (Meng *et al*. 2008). The seeds are dormant at low temperature, but they can tolerate the low temperature for germination and seedling growth after high temperature treatment to break dormancy (Zhao *et al*. 2010). The ecotype in northern Xinjiang has been reported to grow at 0 - 5 °C in some cases (Mao and Zhang 1994). In a study of seed germination characteristics, it was found that the low-temperature germination of *L. apetalum* proceeds as follows, at temperatures of 0 - 5 °C, the initial germination of seeds can occur, but there is a stagnation period. Interestingly, brief exposure to a higher temperature (*e.g*., 25 °C for 50 min) allows the seeds to circumvent the stagnation period (Yang *et al*. 2015). When germinated seeds are again exposed to low temperatures, they can tolerate low temperatures and continue to germinate and grow (Zhao *et al*. 2010). Considering the response of seeds to temperature, the transcriptome of seeds under stagnant low temperature was compared with that of seeds after short-term high temperature treatment, and the differentially expressed genes were identified (Young *et al*. 2018, Smita *et al*. 2020). In addition to low temperature, plants also suffer from drought and salt stress at the stage of seed germination and seedling growth. They

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Abbreviations: ABC1 - activity of bcl complex; GO - gene ontology; KOG - eukaryotic ortholog groups; Nr - non-redundant; PEG - polyethylene glycol.
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can be used as ideal materials to study the mechanism of seedling tolerance to low temperature. Considering the harsh growth environment of *L. apetalum*, activity of bcl complex (ABC1) proteins have recently been investigated. ABC1 family proteins play an important role in abiotic stress tolerance in plants, including cold, osmotic, and salt stresses (Wang *et al*. 2004, Gao *et al*. 2011a,b, 2012, Yuan *et al*. 2014).

Activity of bcl complex (ABC1) is a member of the protein kinase superfamily and is involved in physiological regulation in prokaryotes and eukaryotes (Trumpower, 1981, Trumpower, 1990, Ernster and Forsmark 1993, Leonard *et al*. 1998, Villalba and Navas 2000, Michal *et al*. 2008). The prototype of this family was isolated from *Saccharomyces cerevisiae* and was named due to its role in the suppression of a defect in cytochrome *b* mRNA translation and maintainance of the activity of bcl complex in the mitochondrial respiratory chain (Bousquet *et al*. 1991). Evolutionary analysis shows that the ABCl protein located in the mitochondria and the chloroplast ABCl egg F1 have different origins (Leonard *et al*. 1998). Studies in yeast, *E. coli*, and other species including humans have shown that the mitochondrial and prokaryotic ABCl proteins regulate the biosynthesis of ubiquinone (David *et al*. 1998, Do *et al*. 2001, Hsieh *et al*. 2004, Mollet *et al*. 2008, Tauche *et al*. 2013) and participate in respiratory electron transport and anti-oxidative stress (Trumpower 1981, Ernster and Forsmark 1993, Villalba and Navas 2000). Michal *et al*. (2008) found that the *Arabidopsis* chloroplast ABCl protein AtOSAl is a cadmium and oxidative stress response factor, and the plants are subjected to oxidative stress after mutation of its coding gene.

In *Arabidopsis*, ABC1 gene family members *ATOSA1*, *AtSIA1*, and *AtACDO1* can be widely involved in response to abiotic stresses such as chromium, oxidation, and high salt (Michal *et al*. 2008, Yang *et al*. 2012a,b,c). Wheat *TaABC1L* gene has also been proved to be a response gene to various abiotic stresses such as high salt, high osmolality, low temperature and ABA (Wang *et al*. 2004, 2011). Of the 14 *ABC1* gene family members in rice, 7 were induced or suppressed by low temperature stress, 8 responded to salt stress, and 4 were suppressed by drought treatment (Gao *et al*. 2011b, Yang *et al*. 2012a).

In the previous study, we found that seedlings of *L. apetalum* can tolerate low temperature and high salinity. Moreover, the expressions of some functional genes, such as LaLEAs (Yang *et al.* 2020), show a significant correlation with stress. However, it is not clear whether ABC1 family transcription factors also respond to abiotic stresses. Therefore, we will screen and obtain the coding sequence of ABC1 family and analyze the relationship between the expression and stress.

Materials and methods

Plant, treatments, and related data sources: *Lepidium apetalum* Willd. ripe seeds were collected from Liyushan mountain, Urumqi, Xinjiang, northern China. After drying at room temperature, the seeds were stored at 4 °C. Seeds were treated with 98 % (m/v) sulfuric acid to remove the seed coat, rinsed with distilled water several times, placed on wet filter paper, and left at a temperature of 24 - 26 °C, a 16-h·photoperiod, an irradiance of 400 to 650 μmol m^{-2} s^{-1}, and a relative humidity of 60 ± 5 % to germinate for 1 week. The 7-d-old seedlings were obtained for cloning the cDNA of the *LaAbc1* gene, and to verify the response of the *LaAbc1* gene to abiotic stresses as was perviously described (Yang *et al*. 2020). The ABC1 sequence information on *L. apetalum* seeds was obtained from the transcriptome sequencing results (Zhou *et al*. 2016). Gene sequence information (taxonomy: *Lepidium apetalum.*) for other species was obtained from the *NCBI* database (https:// www. ncbi.nlm.nih.gov).

Screening for ABC1 protein family genes: *ABC1* gene related sequences were selected from the transcriptome data of *L. apetalum* seeds with ABC1 as the key word. Each sequence screened was analyzed in the *NCBI* database for *BLAST* analysis. Only those sequences that are highly homologous (consistency greater than 85 %) with the sequences annotated as ABC1 in *NCBI* and contain the conservative domain of ABC1 can be identified as members of the ABC1-related family.

Functional determination of ABC1 protein family genes was carried out using the *gene ontology* (*GO*; http:// geneontology.org/) and *eukaryotic ortholog groups* (*KOG*; https://blast.ncbi.nlm.nih.gov/Blast.cgi) databases, as well as *NCBI* non-redundant (*Nr*; ftp://ftp.ncbi.nlm.nih.gov/ blast/db) protein analyses.

Extraction of total RNA from seedlings of *Lepidium apetalum*: A *TRIzol* reagent was used to extract total RNA from 7-d-old seedlings, treatment with DNase I to remove DNA contamination in samples. The integrity and quality of the total RNA (1.2 % agarose gel electrophoresis), the purity of the RNA (UV spectrophotometer) were tested, and, finally, the *Agilent 2100* (*Applied Biosystems*, Beijing, China) was used to accurately detect the integrity of the RNA. The cDNA synthesis was carried out using a reverse transcription kit (a *RevertAid* First Strand cDNA synthesis kit) according to the manufacturer's instructions. The cDNAs were stored at -80 °C until further use.

Differential expression analysis of ABC1 protein family genes before and after low-temperature germination: Based on the fragments per kilobase of transcript per million fragments mapped method, the differential expression of ABC1 protein coding gene in transcriptome was analyzed. Up or down regulation of genes more than 0.5 - 1.5 times is considered to be differentially expressed. Target genes of *L. apetalum* seeds with significant differences in expression before *versus* after germination at low temperature were identified.

Cloning and sequence analysis of the full- length cDNA sequence of *LaAbc1*: Based on the transcriptome sequencing results of the seeds, specific primers for the amplification of *LaAbc1* were designed (Table 1 Suppl.). Using the cDNA of *L. apetalum* seedlings as a template,

the full-length sequence of the *LaAbc1* gene was amplified (commissioned by the Beijing *PMAD* company for sequencing). *ClustalX* (Thompson *et al*. 1999) software was used to compare these sequences with the transcriptome sequences. The *NCBI* database was used to analyze sequence homology. By using *MEGA 5* software (Tamura *et al.* 2011) to align amino acid sequences, and based on maximum likelihood (ML) method, the phylogenetic tree was constructed through 1 000 bootstrap tests. The properties of the protein were analyzed by *ProtParam* and the secondary structure was predicted using *ExPASy* (http://web.expasy.org/protparam/). The *Swiss-model* (https://swissmodel.expasy.org/) was used to predict the tertiary structure of the proteins.

Expression of *LaAbc1* in response to abiotic stresses: Analysis of the expression of 21 *LaAbc1* genes in *L. apetalum* seedlings was performed by fluorescence quantitative method (for detail see Yang *et al*. 2020). For low temperature stress, 7-d-old seedlings were placed at 0 ° C for 0, 1, 6, 12, 24, 36, and 48 h. For salt stress, using distilled water as the medium, seedlings were cultured in different NaCl concentrations (0, 150, and 300 mM) for 7 d. For osmotic stress, using distilled water as the medium, 7-d-old seedlings were cultured in medium containing 0, 20, and 30 % (m/v) polyethylene glycol (PEG 6000). Seedling recovery at 25° C, 0 mM NaCl, and 0 % PEG 6000 was performed for 12, 24, and 48 h after 48-h low temperature, salt, and osmotic stresses. The expression of the *LaAbc1* gene in response to cold, salt, and osmotic stress was analyzed by fluorescence quantitative method. The seedlings grown in media without any additions and at room temperature were used as control plants.

Statistical analysis: The data were analyzed by one-way *ANOVA* using *SPSS* 20 (*IBM*, Armonk, NY, USA). The significant differences among treatments were tested based on the least significant difference (LSD) at $P \leq 0.05$.

Results

Based on the transcriptome sequencing results of *L. apetalum* seeds, 29 sequences containing the keyword ABC1 were identified. Following removal of redundant sequences, 21 *ABC1* gene sequences were identified in *NCBI Nr* database for *L. apetalum*. A phylogenetic tree was constructed using 21 ABC1 protein enconding genes sequences, which show that all of them belong to the ABC1 protein family (Fig. 1). The longest and shortest sequences were 2 484 and 264 bp, respectively. Of the 21 *ABC1* sequences, five encoded complete open reading frames.

The predicted functions of the products encoded by the 21 *ABC1* genes of *L. apetalum* were obtained by *GO* analysis. In total, 15 *ABC1* genes were annotated to the biological process category, 14 to the molecular function category, and 5 to the cellular component category (Fig. 2*A*). Among the 21 *ABC1* genes, only 5 were annotated to the three ontologies. It is speculated that these five genes

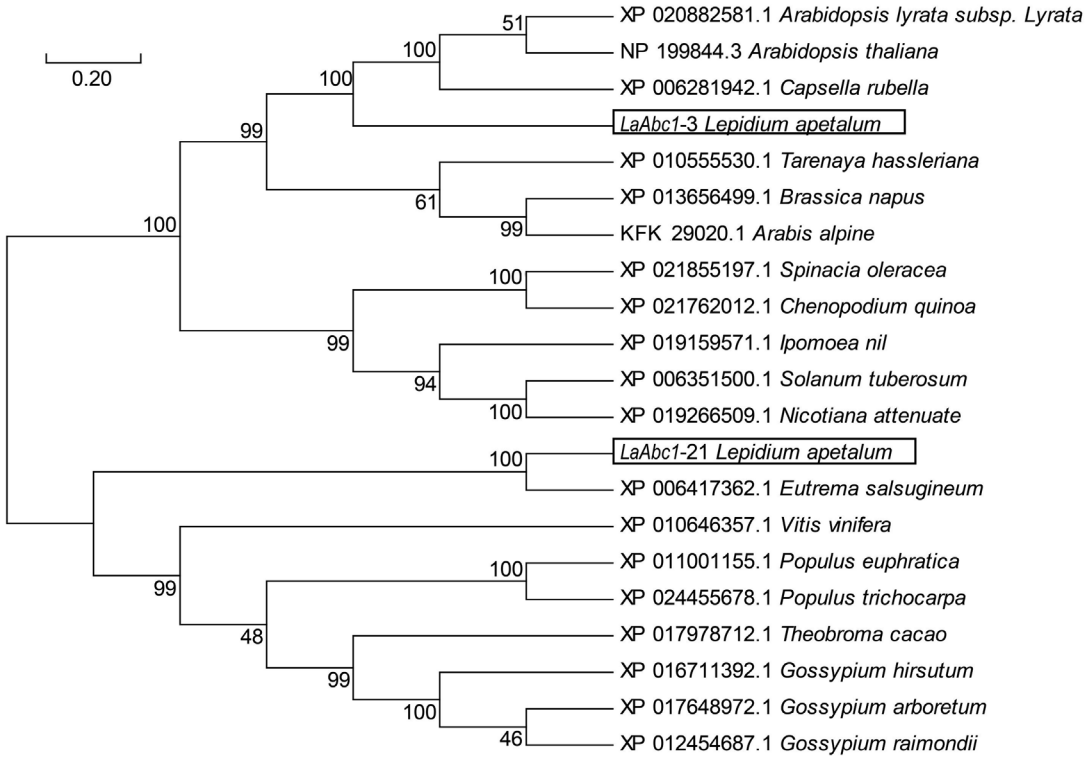

Fig. 1. A phylogenetic tree of *Lepidium apetalum* activity of bcl complex (ABC1) with the amino acid sequences of ABC1 from other species. The *numbers* at nodes represent bootstrap values based on 1 000 replicates. The *scale* represents genetic distance. NP - non-protein, XP - predicted model protein, KFK - hypothetical protein.

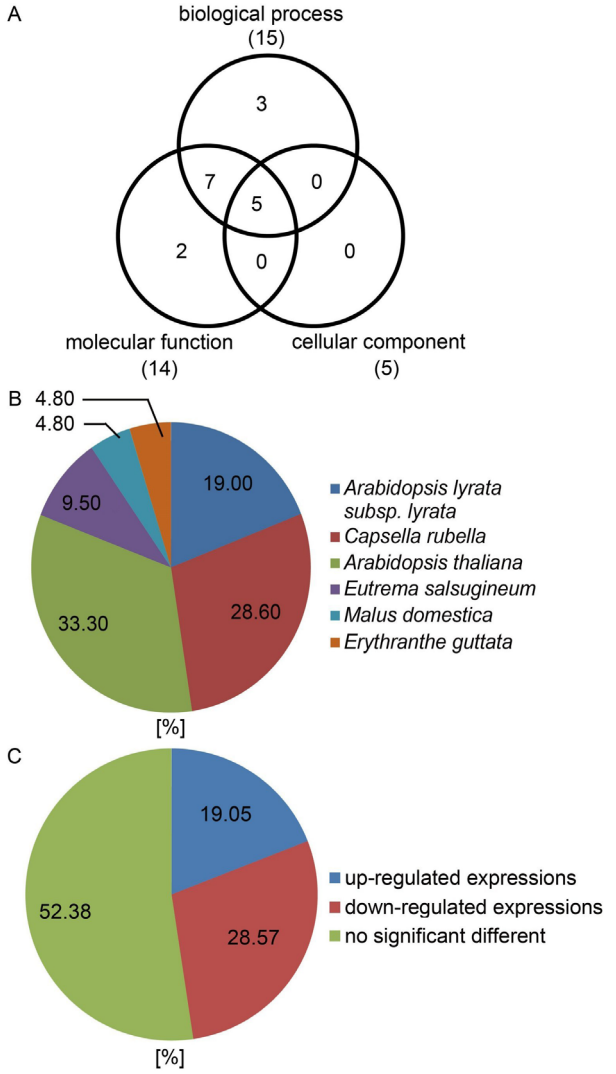

Fig. 2. *Lepidium apetalum* activity of bcl complex (ABC1) protein family unigene distribution based on the *gene ontology* analysis (*A*) and *NCBI Nr* database (*B*). Relative expression difference between controls and seedlings stressed by low temperature (4 °C for 9 d) (*C*).

may have multiple functions in biological development and molecular stress resistance. 80 % of the 21 ABC1 transcriptional factors were annotated to biological processes and molecular functions, indicating that these sequences are involved in the growth and development of *L. apetalum* and play an important molecular function in adaptation to stress.

Analysis of the *NCBI Nr* database indicates that the identified ABC1 protein family genes were homologous to varying degrees with the known genes of 6 related species (Fig. 2*B*), 33.3 % were homologous with the *Arabidopsis thaliana;* 28.6 % with the *Capsella rubella*, and 19 % with *Arabidopsis lyrata* subsp. *lyrata*.

Based on the conserved sites, the plant ABC1 protein sequences were grouped into 26 functional components using the *KOG* database. In this study, fourteen ABC1 protein family gene sequences isolated from the seeds of *L. apetalum* were functionally annotated in *KOG*. Only one sequence was reported, which was annotated as O (post-translational modification, protein turnover, chaperones); the other seven sequences were not annotated in the *KOG* database (Table 2 Suppl.).

The transcriptional data of ABC1 protein family genes of *L. apetalum* seeds were analyzed before and after germination stagnation at low temperature (Fig. 3). We conducted real-time fluorescence quantitative PCR to validate the transcriptome data and to analyze the gene expression changes of randomly selected genes. Although the expressions of selected genes were different between transcriptomics and quantitative PCR, the trends of expression were the same (Fig. 1 Suppl.). Four upregulated and six downregulated genes were identified, the remaining eleven genes showed no significant difference based on transcriptional data (Fig. 2*C*). The c13379_g1 and c21578_g1 sequences were selected for further analysis based on the relationship between their expression and abiotic stresses. The sequences were named *LaAbc1*-3 and *LaAbc1*-21 in this study.

Fluorescence quantitative analysis shows that *LaAbc1*-2, *LaAbc1*-3, *LaAbc1*-5, *LaAbc1*-6, *LaAbc1*-8, *LaAbc1*-18, and *LaAbc1*-21 were up-regulated in 7-d-old *L. apetalum* seedlings treated at 0° C for 0, 1, 2, 4, 8, 12, 24, and 48 h. After low temperature treatment, the expression of these genes increased significantly compared with the control group (0°C treatment for 0 h). The expression of the *LaAbc1*-3 and *LaAbc1*-5 genes increased first and then decreased. The *LaAbc1*-5 increased within 12 h when it reached its maximum, which was 14-times higher than in the control group. This result indicated that the increase of *LaAbc1*-5 expression might be related to the cold resistance of *L. apetalum* seedlings. After 12 h, the expression of the *LaAbc1*-5 gene showed a downward trend, but its expression was still higher than that of the control group. The rapid response of the *LaAbc1*-6 gene to low temperature stress made the seedlings adapt quickly to the adverse environment. With the prolongation of treatment time, the expression of the *LaAbc1*-6 gene was significantly up-regulated, indicating that the gene was closely related to low temperature stress tolerance of the *L. apetalum* seedlings. The expression of the *LaAbc1*-2, *LaAbc1*-8, and *LaAbc1*-21 genes increased rapidly after 1 - 2 h of low temperature treatment to enhance the adaptability of plants to low temperature, and then down-regulated expression of these genes may indicate that these genes have accumulated a large number of synthetic protein templates in a short time. The expression of the *LaAbc1*-21 gene decreased first and then increased, the decrease of expression may be due to the damage of *L. apetalum* seedlings by rapid low temperature treatment. After a short time of inhibition, the *LaAbc1*-21 gene expression increased (Fig. 2 Suppl.).

The results show that the expression of *LaAbc1*-7, *LaAbc1*-9, *LaAbc1*-12, *LaAbc1*-13, *LaAbc1*-15, *LaAbc1*-16, *LaAbc1*-19, and *LaAbc1*-20 in ABC1 transcription factors had no significant difference after low temperature treatment. Compared with the control group, the expression of genes decreased slightly with

GENE	SERIAL NUMBER	LENGTH	HOMOLOGOUS PROTEINENCODING GENE
Abc1-1	c6383_g1	1047	*Arabidopsis lyrata subsp. lyrata* serine/threonine-protein kinaseabkC
Abc1-2	c15065_g1	597	*Arabidopsis lyrata subsp. lyrata* UPF0051 protein ABCI8
Abc1-3	c13379_g1	1470	*Arabidopsis lyrata subsp. lyrata* aafF domain-containing protein kinase1
Abc1-4	c9338_g1	714	*Camelina sativea* aarF domain-containing protein kinase2
Abc1-5	c22062_g1	2442	*Arabidopsis lyrata* subsp. lyrata uncharacterized protein sll0005
Abc1-6	c21890_g1	2061	*Arabidopsis thaliana* protein kinase superfanmily protein (ACDO1)
Abc1-7	c12914_g1	534	*Eutrema salsugineum* aarF domain-containing protein kinasa1
Abc1-8	c22903_g2	2139	*Capsella rubella* ABC1-like kinase
Abc1-9	c22279_g1	2484	*Arabidopsis thaliana* protein kinase superfamily protein ATATH 8
Abc1-10	c22062_g2	264	*Camelina sativa* uncharacterized protein sll0005-like
Abc1-11	c8932_g2	318	*Capsella rubella* uncharacterized protein slr0889
Abc1-12	c10431_g1	588	*Camelina sativa* UPF0051 protein ABCI8
Abc1-13	c22596_g1	1146	*Camelina sativa* aarF domain-containing protein kinase At1g71810
Abc1-14	c22316_g1	996	*Arabidopsis lyrata subsp. lyrata* uncharacterized protein slr 1919
Abc1-15	c19216_g1	1689	*Arabidopsis thaliana* ATATH9
Abc1-16	c20945_g2	1152	*Arabidopsis thaliana* ABC1 family protein
Abc1-17	c23357_g1	1740	*Camelina sativa* aafF domain-containing protein kinase At5g05200
Abc1-18	c21106_g1	312	*Capsella rubella* aarF domain-containing protein kinaes1
Abc1-19	c8932_g1	327	*Arabidopsis taliana* At4g24810
Abc1-20	c18258_g1	2127	*Capsella rubella* protein ABC transporter1
Abc1-21	c21578_g1	1959	*Eutrema salsugineum* aarF domain-containing protein kinase2

Fig. 3. Relative expression analysis of activity of bcl complex (ABC1) protein family genes before (control mean) and after (experimental mean) seed germination at low temperature (4 °C for 9 d) in *Lepidium apetalum* based on transcriptome analysis.

the prolongation of low temperature treatment time, but the expression was similar, which indicated that the genes were not obvious in response to low temperature stress, and might be constitutive genes (Fig. 2 Suppl.).

The results show that *LaAbc1*-1, *LaAbc1*-4, *LaAbc1*-10, *LaAbc1*-11, *LaAbc1*-14, *LaAbc1*-17 of ABC1 transcription factors were down-regulated in the *L. apetalum* seedlings after low temperature treatment at 0 °C (Fig. 2 Suppl.). These down-regulated genes can reduce the decomposition of some proteins to reduce the hydrolysis of amino acids and protect plants from ammonia poisoning, thus increasing the tolerance of plants to low temperature stress.

Homologous cloning was used to amplify the bands with the expected gene size. The *LaAbc1*-3 encodes 478 amino acids (Fig. 3 Suppl.). The *LaAbc1*-3 *ProtParam* analysis shows that the gene had a length of 54063.82 kb, the protein a molecular mass of 54.07 kDa, a theoretical isoelectric point of 9.08, a molecular formula of $C_{2420}H_{3871}N_{671}O_{685}S_{23}$, and an instability index of 43.65. It belonged to unstable proteins with an average hydrophilic coefficient of -0.189, and it was a hydrophilic protein. The *LaAbc1*-21 encoded 630 amino acids (Fig. 4 Suppl.). The *ProtParam* analysis shows that a length of the gene was 71380.62 kb, a protein molecular mass of 71.39 kDa, a theoretical isoelectric point of 9.83, a molecular formula of $C_{3226}H_{5066}N_{912}O_{878}S_{22}$, an instability index of 41.44, (belonging to unstable proteins), a fat index of 81.94, and an average coefficient of hydrophilicity of -0.188; it was a hydrophilic protein.

The protein domain analysis of *LaAbc1*-3 using the *SMART* (http://smart.embl-heidelberg.de/) software shows that the protein encoded by this gene had a highly conserved protein kinase functional domain STYKc family from 132 to 299 (Fig. 5 Suppl.). The *LaAbc1*-21 protein domain analysis shows that the protein encoded by this gene had a transmembrane domain on peptide segments 171 to 193, which might be closely related to abiotic stress tolerance in the *L. apetalum* seedlings (Fig. 5 Suppl.). The protein sequence encoded by *LaAbc1*-3 had an ABCl characteristic domain between amino acids 122 and 240 and an AARF conserved domain between amino acids 58 and 430. The protein sequence encoded by *LaAbc1*-21 had an AABCl characteristic domain between amino acids 877 and 1200 and an AARF conserved domain between amino acids 625 and -1854.

Prediction of secondary structure of protein encoded

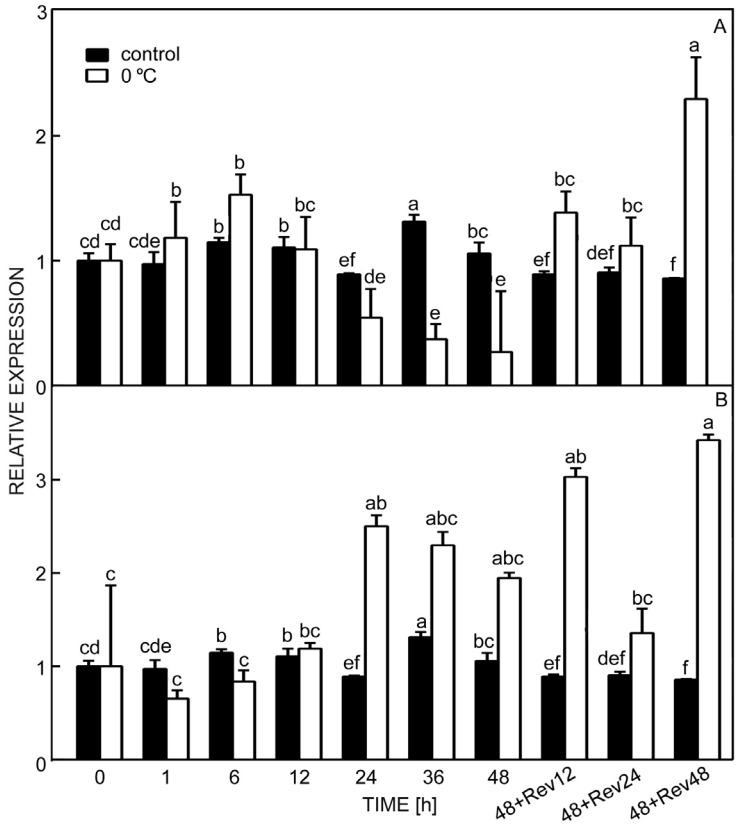

Fig. 4. *Lepidium apetalum activity of bcl complex* (*ABC1*) relative expression in seedlings under a short-term (48 h) low temperature stress (0 °C) and recovery at 25 °C (Rev). *A* - *LaAbc1*-3, *B* - *LaAbc1*-21. Means ± SEs, $n = 3$, different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

by the *L. apetalum LaAbc1*-3 gene showed that 60.25 % of the protein was α-helix and 26.36 % was irregular curl. Three-dimensional spatial structure prediction of *L. apetalum LaAbc1*-3 encoding proteins was carried out in univegetable by *Swiss-model* online software (Fig. 5 Suppl.). The results showed that the protein was mainly composed of α-helix and irregular curl, and the proportion of extended chain and β-angle was small, which was basically consistent with the secondary structure of the protein.

Prediction of secondary structure of protein encoded by the *L. apetalum LaAbc1*-21 gene showed that the protein was mainly α-helix (54.13 %) and irregular curl (32.54 %) (Fig. 5 Suppl.). The proportion of elongation chain and β-corner was small, which was basically consistent with the secondary structure of the protein.

Analysis of homology alignment of the *L. apetalum LaAbc1*-3 gene coding amino acid sequence with *Arabidopsis lyrata* subsp. *lyrata*, *Arabidopsis thaliana*, and *Capsella rubella* coding amino acid sequence showed low conservativeness at N and C ends of the sequence. The conservative genotype from 290 to 340 amino acids is higher, which may be related to the functional consistency of the gene in different species. The phylogenetic tree of *LaAbc1*-3 and its related species ABC1 amino acid sequence was constructed by *MEGA 5* (Fig. 3). *Lepidium apetalum LaAbc1*-3 is similar to *Arabidopsis lyrata subsp*, *Arabidopsis thaliana, Capsella rubella*, and *Brassica napus* and other *Brassicaceae* plants. The results showed that the gene was conservative, but it formed a single branch, indicating that the functions of homologous proteins were different.

The homologous alignment of the *L. apetalum LaAbc1*-21 gene coding amino acid sequence with *Eutrema salsugineum*, *Gossypium*, *Populus euphratica*, and *Vitis vinifera* coding amino acid sequence was analyzed. The low conservativeness at the near C end of the sequence may be related to the functional differences of the gene in different species. The N-terminal conservative type is higher, which may be related to the specific function of the gene in different species. The phylogenetic tree of *LaAbc1*-21 and its related species ABC1 amino acid sequence was constructed by *MEGA 5* (Fig. 3). The homology of *L. apetalum LaAbc1*-21 is only close to that of the genus *Eutrema salsugineum*, *Gossypium hirsutum*, *Populus euphratica*, and cocoa and grapes form another big branch. The signal peptides of *LaAbc1*-3 and *LaAbc1*-21 proteins were predicted and analyzed. The two proteins did not have signal peptides, indicating that they were not secretory proteins (Fig. 6 Suppl.).

Quantitative fluorescence analysis of different tissues of wild *L. apetalum* at the same growth period showed that the *LaAbc1*-3 gene was up-regulated in the old stem, and the *LaAbc1*-21 gene had no tissue specificity (Fig. 7 Suppl.).

The expression of *LaAbc1*-3 gene increased first, then

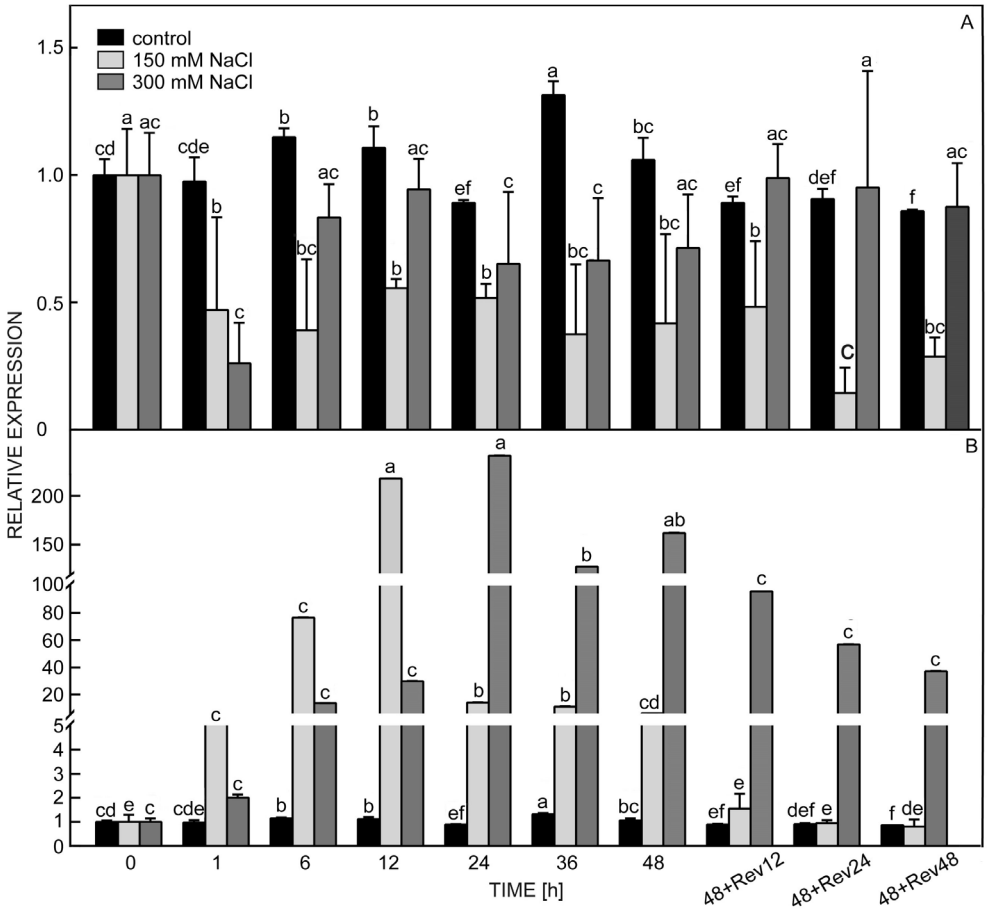

Fig. 5. *Lepidium apetalum activity of bcl complex* (*ABC1*) relative expression in seedlings treated with different concentrations of NaCl for 0 to 48 h and during recovery for 12 to 48 h (Rev). *A* - *LaAbc1*-3, *B* - *LaAbc1*-21. Means ± SEs, $n = 3$ samples from 40 - 50 plants; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

decreased, and then increased during low temperature treatment and recovery, and reached the maximum at 6 h of cold treatment. After 6 h, the expression of the gene decreased, and the expression of the gene during recovery was significantly higher than that in the control group, it indicated that low temperature stress had not been completely eliminated, and it would take some time for the seedlings to return to optimum conditions. The expression of the *LaAbc1*-21 gene decreased at 0 - 1 h after cold treatment, and increased after 1 h, after removing the stress. The expression was still up-regulated compared with the control, which indicated that the expression of the gene was closely related to the cold tolerance of the seedlings (Fig. 4).

The wilting rate of the seedlings was observed under different salt stress. The results showed that the wilting rate of the seedlings increased in 0 - 6 h. There was a positive correlation between wilting rate and salt concentration, the wilting rate of the seedlings treated with different salt concentration reached the maximum at 6 h. After 6 h, the wilting rate declined and returned to normal within 48 h. This result indicated that the salt tolerance of the *L. apetalum* seedlings was very strong (Table 3 Suppl.). Under 150 mM NaCl salt stress, the expression of the *LaAbc1*-3 gene decreased and tended to be relatively stable during salt stress, it was 0.5-fold of that of the control group, down-regulation of expression was found after seedling recovery. Under 300 mM NaCl salt stress, its expression decreased and stabilized, but there was no significant difference compared with the control group (Fig. 5*A*). The trend of the *LaAbc1*-21 gene expression under salt stress was consistent with that of wilting rate of single-headed *L. apetalum* seedlings. Under 150 mM NaCl, the gene expression reached its maximum at 12 h, and it was 217-fold higher than of the control group. With the prolongation of treatment time, the expression of the gene decreased, but still maintained high. There was no significant difference in the expression of the gene between the seedling recovery groups and the control group. The maximum expression of the gene was achieved at 24 h after 300 mM NaCl treatment, it was 247-fold of the control group, and with the increase of treatment time the gene still maintained a high expression. It was speculated that the gene played a role in salt stress tolerance of *L. apetalum* seedlings (Fig. 5*B*).

Different concentrations of PEG 6000 were used to treat the *L. apetalum* seedlings, and the wilting rate of the *L. apetalum* seedlings increased from 0 to 24 h, and it was concentration of PEG 6000 dependent. The wilting rate of seedlings treated with different concentrations of

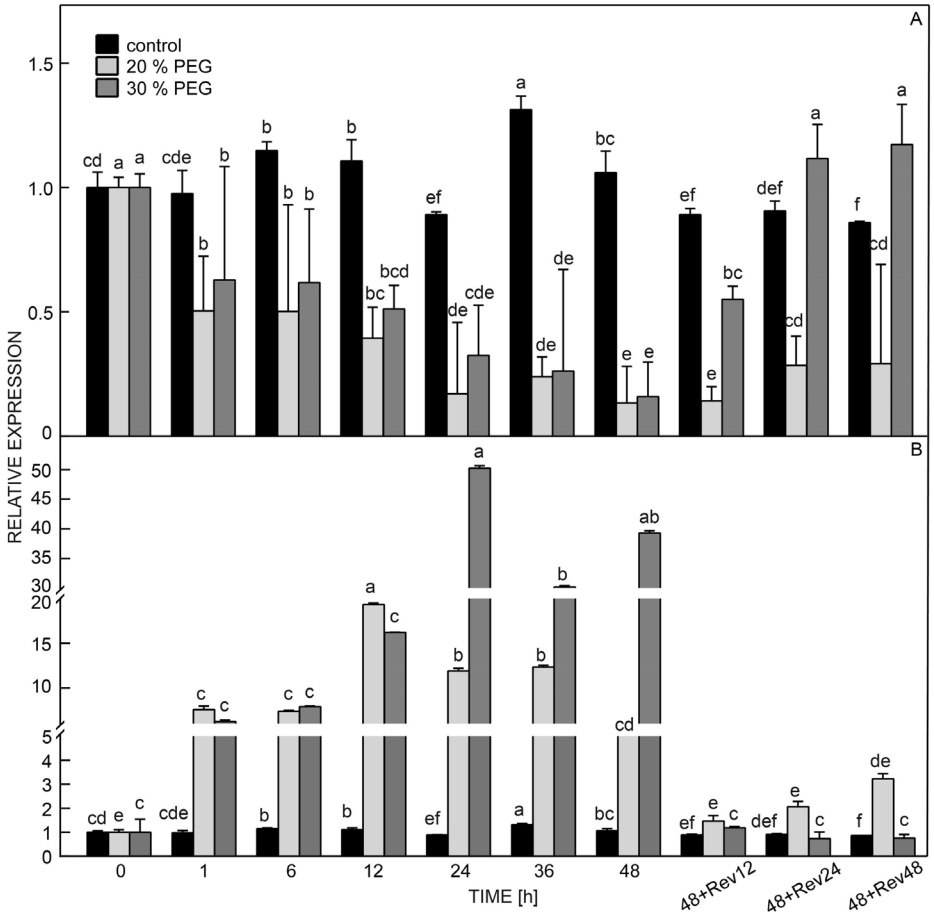

Fig. 6. *Lepidium apetalum* activity of *bcl complex* (*ABC1*) relative expression in seedlings treated with different concentrations of polyethylene glycol 6000 for 0 to 48 h and during recovery for 12 to 48 h (Rev). *A* - *LaAbc1*-3, *B* - *LaAbc1*-21. Means ± SEs, $n = 3$ samples from 40 - 50 plants; different letters indicate significant differences among treatments based on the least significant difference at $P \leq 0.05$.

PEG 6000 reached the maximum at 24 h, and decreased after 24 h, the seedlings returned to normal state after 48 h only for 10 and 15 % PEG 6000 treatment. The wilting rate of seedlings exposed to other concentrations was still high. Only a few of the seedlings treated with 30 % PEG 6000 failed to recover after PEG 6000 removal, which indicated that the *L. apetalum* seedlings had strong drought tolerance (Table 4 Suppl.). Under the stress conditions caused by 20 and 30 % PEG 6000, the expression of the *LaAbc1*-3 gene was down-regulated, the trend was more obvious under 30 % PEG 6000, and the expression increased after seedling recovery was resumed. There was no significant difference between the control group and the 30 % PEG 6000 treatment group (Fig. 6*A*). Under 20 and 30 % PEG 6000, the expression of the *LaAbc1*-21 gene increased rapidly together with the wilting rate of the seedlings. Under 20 % PEG 6000 treatment, the the *LaAbc1*-21 gene expression reached the maximum at 12 h, which was 20-times that of the control group, and then the expression level slightly decreased, but the expression was still maintained at a higher level. Under 30 % PEG 6000 treatment, the gene expression reached the maximum at 24 h which is 50-times higher than of the control group. After the recovery, the expression of the gene under the two treatments was not significantly different from that of the control group (Fig. 6*B*). It is speculated that the expression of this gene is related to osmotic stress tolerance of the *L. apetalum*. The low temperature (0 °C), salt (300 mM NaCl), and drought (30 % PEG 6000) treatments showed that *LaAbc1*-3 had no significant difference in low temperature and salt stress, and it was down-regulated under osmotic stress. It indicated that the expression of the *LaAbc1*-3 gene had a negative regulation effect on drought tolerance of *L. apetalum* seedlings. *LaAbc1*-21 was up-regulated under low temperature, salt, and osmotic stress, and its response to salt and osmotic stress was significant. It was speculated that the gene was related to abiotic stress tolerance of *L. apetalum.*

Discussion

Based on transcriptome sequencing data, researchers have screened members of *ABC1* gene family in many plants. For example, there are 15 members in *Oryza sativa L* (Gao *et al*. 2011a,b), 19 members in *Zea mays L.* (Gao

et al. 2010), and 17 members in *Arabidopsis thaliana* (Yang *et al*. 2012a,b,c). Our research team screened 21 *ABC1* gene members before and after the cessation of low temperature germination. Some *ABC1* genes are less variable in expression when *L. apetalum* is at a low-temperature during germination and may change as *L. apetalum* germinates and grows. Therefore, there are not only 21 genes members of *ABC1* family in *L. apetalum,* but the exact number needs to be further studied.

Quantitative analysis of *L. apetalum* seed fluorescence before and after releasing germination stagnation at low temperature showed that there were 4 up-regulated genes, 6 down-regulated genes, and 11 non-significantly differential genes. Comprehensive analysis found that the expressions of *LaAbc1*-7, *LaAbc1*-12, *LaAbc1*-13, and *LaAbc1*-19 genes were not significantly different in the low temperature germination of *L. apetalum* seedlings and the low temperature growth of *L. chinensis* seedlings. It was speculated that these genes may be insensitive to temperature or constitutively expressed genes. The *LaAbc1*-1, *LaAbc1*-4, *LaAbc1*-11, *LaAbc1*-14 and *LaAbc1*-17 genes were not significantly different in low temperature germination of *L. apetalum* seeds, but down-regulated in low temperature growth of *L. apetalum* seedlings. It is speculated that these genes may reduce the hydrolysis of amino acids by reducing the decomposition of certain proteins during low temperature growth of seedlings, thus reducing ammonia. The expressions of *LaAbc1* genes might alleviate plants damage which made *L. apetalum* tolerant to low temperature stress. The expression of the *LaAbc1*-18 gene was not significantly different during low-temperature germination, but up-regulated during low-temperature growth of *L. apetalum* seedlings. It is speculated that this gene was related to cold tolerance of *L. apetalum* seedlings (Li *et al*. 2017).

The *LaAbc1*-10 gene was down-regulated during low temperature germination and seedling growth of *L. apetalum*. It was speculated that the *LaAbc1*-10 gene might play a negative regulatory role in low temperature stress of *L. apetalum*. The *LaAbc1*-9 gene was down-regulated during low-temperature germination of *L. apetalum* seeds, but there was no significant difference in the expression of the *LaAbc1*-9 gene at the 0 °C treatment of *L. apetalum* seedlings. It is speculated that this gene was related to the release of low-temperature germination stagnation of *L. apetalum* seeds. The *LaAbc1*-2, *LaAbc1*-5, and *LaAbc1*-6 genes were slightly down-regulated during low-temperature germination but up-regulated during low-temperature growth of the *L. apetalum* seedlings, which may be related to low-temperature stress tolerance of the *L. apetalum* seeds and cold tolerance of the seedlings similarly as suggested Yuan *et al*. (2018).

The *LaAbc1*-16 and *LaAbc1*-20 genes were up-regulated during low-temperature germination of *L. apetalum* seeds, but there was no significant difference in the expression of *LaAbc1*-16 and *LaAbc1*-20 genes during low-temperature growth of *L. apetalum* seedlings. The *LaAbc1*-8 and *LaAbc1*-21 genes were up-regulated during low-temperature germination and seedling growth of *L. apetalum*. It was speculated that these genes might be closely related to low-temperature germination of *L. apetalum* seeds and cold tolerance of seedlings (Wang *et al*. 2004).

The expression of the *LaAbc1*-3 gene increased in order to enhance the adaptability of seedlings to low temperature. After removing low temperature stress, the expression of *LaAbc1*-3 decreased. It may be that the *L. apetalum* seedlings synthesized enough proteins to tolerate low temperature stress and resumed control culture. After removing low temperature stress, the expression of the *LaAbc1*-3 gene was up-regulated. It is speculated that in the *L. apetalum* seedlings under the influence of low temperature stress, physiological regulation did not return to normal. The expression of the *LaAbc1*-21 gene decreased first and then increased under low temperature stress. The decrease of *LaAbc1*-21 gene expression may be due to the damage of physiological characteristics of the seedlings, which inhibited the expression of the *LaAbc1*-21 gene. After a period of time, the *LaAbc1*-21 gene enhances plant tolerance to low temperature by increasing expression (Li *et al*. 2017, Yuan *et al*. 2018, Zhao *et al*. 2018).

The *LaAbc1*-3 gene was insensitive to salt stress. The expression of *LaAbc1*-21 gene was significantly up-regulated under salt stress, and the higher the salt concentration, the more obvious the up-regulated trend was. The expression trend of the *LaAbc1*-21 gene was consistent with the wilting rate of *L. apetalum* seedlings under salt treatment, which indicated that the expression of the *LaAbc1*-21 gene might be related to salt tolerance of *L. apetalum* seedlings (Yang *et al*. 2012a). Members of this family are involved in a wide range of abiotic stress responses and may be related to plant resistance to stress. At the same time, we noticed that the expression patterns of *LaAbc1*-3 and *LaAbc1*-21 genes were different under salt stress, indicating that homologous genes can perform different functions in different species.

Under osmotic stress, the expression of the *LaAbc1*-3 gene was down-regulated, the higher the concentration of PEG 6000, the more obvious was the down regulation trend of the gene. This result indicated that osmotic stress inhibited the expression of the *LaAbc1*-3 gene, and the expression was negatively correlated with the PEG 6000 concentration. The expression of the *LaAbc1*-21 gene was significantly up-regulated under osmotic stress, and the expression was positively correlated with the concentration of PEG. The trend of the *LaAbc1*-21 gene expression was consistent with the wilting rate of *L. apetalum* seedlings, suggesting that the gene expression might be related to drought tolerance of *L. apetalum* seedlings (Wang *et al*. 2011). Studies have shown that *ABC1* genes in *Lilium regale* do not respond to drought stress (Yuan *et al*. 2014), but four *OsABC1* genes in rice are inhibited by drought stress (Gao *et al*. 2011a,b). In this study, the expression patterns of *LaAbc1*-3 and *LaAbc1*-21 genes are different, indicating that the expression of *ABC1* gene can be induced by a variety of abiotic stresses and may perform different biological functions in different species.

The changes of *LaAbc1*-3 under the abiotic stresses of *L. apetalum* were not very obvious, but *LaAbc1*-3 had certain tissue specificity during the growth and

development of *L. apetalum*. The *LaAbc1*-3 was up-regulated in the old stem of *L. apetalum.*, but the expression in the other organs did not change significantly (Fig. 7 Suppl.). The ABC1 gene family has obvious tissue and organ expression specificity. Among them, the 14 *ABC1* gene family members of rice have the highest expression in leaves (Gao *et al*. 2011a,b). In wheat, the expression of this gene is mainly concentrated in green tissues such as leaves and stem tips (Gao *et al*. 2010). It is speculated that this gene may be related to plant growth and development. *LaAbc1-21* has obvious changes in *L. apetalum* under abiotic stresses. It is speculated that this gene plays an important role in *L. apetalum* abiotic stress tolerance, especially to salt stress and osmotic stress. Although both genes have the same aarF domain, they show different roles in biological functions, indicating that this domain may not be a key factor for the *ABC1* gene family to cope with abiotic stresses.

These results suggest that *LaAbc1* expression, especially the *LaAbc1*-21 gene, might play an important role in the tolerance of *L. apetalum* seedlings to a low temperature, salinity, and osmotic stress although additional experimental evidence is needed to determine whether *LaAbc1*-21 increases the ability of plants to retain water. The above analysis indicates that *L. apetalum* ABC1 family not only plays a role in stress response, but may also be related to specific physiological processes in *L. apetalum*.

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