Comprehensive comparative analysis and expression profiles and effects on physiological response of DEAD-box RNA helicase genes in *Lumnitzera littorea* (Jack) Voigt under cold stress

LuLu Hao\(^{ab}\), Ying Zhang\(^b\), Yin Li\(^b\), Linxia Bai\(^b\), Danfei Yue\(^a\), Huiyu Zhang\(^a\) and Chunfang Zheng\(^a\)

\(^a\)National and Local Joint Engineering Research Center of Ecological Treatment Technology for Urban Water Pollution, College of Life and Environmental Science, Wenzhou University, Wenzhou, People’s Republic of China; \(^b\)School of Life Sciences and Technology, Lingnan Normal University, Zhanjiang, People’s Republic of China

**ABSTRACT**

The DEAD-box family has been shown to play an important role in a variety of abiotic stresses, but little is known in studies of mangrove plants. Here, the effects of cold stress on various physiological changes and the role of the DEAD-box RNA helicase family in response to cold stress were determined. First, we identified 73 DEAD-box RNA helicase family members in *L. littorea*. Second, the evolutionary relationships between the DEAD family in *L. littorea* and the model species *Arabidopsis thaliana* were investigated by evolutionary phylogenetic analysis. Finally, qRT-PCR study of representative DEAD-box genes showed that DEAD-box genes played a major role in the low-temperature stress response of *L. littorea*. Furthermore, we found that LIDEAD48, LIDEAD36, and LIDEAD47 might be involved in the maintenance of chlorophyll function, and LIDEAD43 might play a role in the maintenance of mitochondrial function in *L. littorea* under cold stress.

1. Introduction

*Lumnitzera littorea* (Jack) Voigt is a typical mangrove plant found in tropical and subtropical coastal areas (Su et al. 2019) with various roles (Siikamäki et al. 2012; Martin et al. 2019; Wilda et al. 2020). *L. littorea* is listed as a national class I Key protected plant, with only nine wild individuals existing in Sanya Tielu harbor (Guo et al. 2018). There are many reasons for the low number of *L. littorea*, including low pollen viability, low seed fertility, and low seedling survival rate (Zhang et al. 2016; Zhang et al. 2021). The natural range of *L. littorea* is in the southernmost part of the country, which is considered as the least cold-tolerant actual mangrove plant. *L. littorea* is highly sensitive to low temperature, and shows heavy damage under low temperature and is difficult to recover (Chen et al. 2010). In addition, due to the increased frequency of extreme cold weather, improving the cold resistance of *L. littorea* is an urgent task to protect this species safely through the winter.

Among abiotic stresses, low temperature is one of the most critical environmental factors, which plants need to address during their evolution and development since it limits the species, growth, and distribution of plants (Stuart et al. 2007; Osland et al. 2017). Cold stress may cause structural rupture of plant cell membranes (Wolfe 1978; Palta 1990; Mahajan and Tuteja 2005; Vigh et al. 2007), reduce the activity of various enzymes involved in plant metabolic synthesis, water loss in plant cells, plant wilting (Ashworth and Pearce 2002; Jin et al. 2022), and impede photosynthesis (Allen and Ort 2001; Asada 2006). Low-temperature stress leads to increase in reactive oxygen species (ROS), which causes damage to organelles and macromolecules, including DNA, proteins, lipids, and ultimately leads to cell death (Foyer and Noctor 2005; Mahajan and Tuteja 2005; Das and Roychoudhury 2014). At the same time, under low temperature stress, plants altered a series of biological processes such as accelerating ROS scavenging, enhancing antioxidant mechanisms and protective protein synthesis (Guy et al. 1987; Barrero-Gil et al. 2016; Chang et al. 2021), increasing osmotic substances (Sun et al. 2021), and altering endogenous cellular phytohormones (Karimi et al. 2016).

The mechanisms of photosynthesis involve numerous components including photosynthetic pigments and photosystems, electron transport systems, and CO\(_2\) reduction pathways, damage to each part caused by low-temperature stress may decrease the overall photosynthetic capacity of mangrove plants (Ashraf and Harris 2013). Photosynthesis is considered as one of the criteria for healthy plant growth (Sharma et al. 2020), and several studies have reported the mechanism of low-temperature stress damage to mangrove plants. Low-temperature stress inhibits photosynthesis in mangrove plants by blocking photosynthetic pigment synthesis, inhibiting anti-oxidant metabolism (e.g. CAT, POD, and APX), suppressing sucrose transport, reducing the maximum photochemical efficiency of PSII, and accelerating nucleic acid endonuclease senescence. It is complicated to recover from impaired photosynthesis due to constant low temperature (Peng et al. 2015; Zheng et al. 2016). There were several cases of widespread mangrove plant mortality due to cold stress caused by...
extremely cold weather in North America and southern China (Li et al. 2013; Chen et al. 2017).

The DEAD-box family is named DEAD because motif II, which makes up the majority of the SF2 helicase subfamily, contains the amino acid sequence Asp-Glu-Ala-Asp (D-E-A-D) feature (Ali 2021). DEAD-box RNA helicases contain nine conserved motifs (Q, I, Ia, Ib, and II-VI) (Ali 2021), each of which plays its role (Pause et al. 1993; Tanner and Linder 2001; Shen et al. 2007). Although their sequence and structure are similar, each DEAD-box gene has a different function. For instance, AtRH47/ISE1 is associated with embryonic development (Stonebloom et al. 2009). AtCAF plays a vital role in meristematic tissue determination in Arabidopsis thaliana (Jacobsen et al. 1999), PRH75 is involved in accelerated cell growth and division (Lorković et al. 1997), OsRH34 and OsRH2 are involved in the regulation of rice plant height and seed development (Huang et al. 2016). With the first confirmation of the function of FL25A4, an abiotic stress-related gene, it was found to limit the plant growth and development under low-temperature stress (Seki et al. 2001). PMH1 and PMH2, which localized on mitochondria, also played essential roles during low-temperature stress in Arabidopsis thaliana (Matthes et al. 2007). LOS4 participates in the regulation of plant cold response by mediating the expression of CBF family transcription factors (Gong et al. 2002; Gong et al. 2005; Braud et al. 2012). AtRH3 localized on chloroplasts and involved in low-temperature stress and salt stress in Arabidopsis thaliana (Asakura et al. 2012; Gu et al. 2014), and OsRh58 in rice improved tolerance to drought and salt in Arabidopsis thaliana by regulating the translation level of chloroplasts (Asakura et al. 2012). The continuous exploration of the functions of these genes in the DEAD box provided the new insights into the mechanism of cold resistance in L. littorea.

In this study, we identified the DEAD-box gene family and predicted its physicochemical properties, performed a phylogenetic analysis of DEAD protein sequences in Arabidopsis thaliana and L. littorea, and then analysed the conserved structural domains of DEAD protein sequences in L. littorea. In addition, we investigated the physiological characteristics of L. littorea and the response of DEAD family genes under low temperature to lay the preliminary foundation for improving the cold resistance of L. littorea.

2. Materials and methods

2.1 Identification of the DEAD-box RNA helicase family in L. littorea

The transcriptome data of L. littorea was obtained from the college of life science and technology, Lingnan Normal College, and submitted to NCBI with the accession number (PRJNA791944). The DEAD-box sequence of the model plant Arabidopsis thaliana was downloaded from the TAIR (https://www.arabidopsis.org/) and used as a probe to perform the blast search in L. littorea transcriptome database. Meanwhile, a direct search was performed by using the keyword ‘DEAD-box’. The conserved sequences were identified by InterPro (https://www.ebi.ac.uk/interpro/), SMART (https://smart.embl-heidelberg.de/smart/set_mode.cgi?) and CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) software. The genes and repetitive sequences without no complete DEAD-box structural domain were excluded, 73 DEAD-box gene sequences were obtained.

2.2 Characteristics, structure and motif analysis of DEAD genes

The protein characteristics, including isoelectric point, lengths, and molecular weight of DEAD-box proteins, were analyzed by ExPASy (https://web.expasy.org/compute_pi/). The conserved motifs were detected by using the online MEME tool (https://meme-suite.org/meme/doc/meme.html). Parameters were set as follows: number of repetitions; optimum motif width set to ≥ 6 and ≤ 200; the maximum number of motifs set to 8. The conserved structural domains were predicted by the Batch CD-Search function on the NCBI website (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The conserved motifs and their structural domains were visualized against the evolutionary tree of the L. littorea DEAD-box gene family using by TBtools software. Subcellular localization analysis was done by using PSORT (https://www.genscript.com/psort.html?src=leftbar) software.

2.3 Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignments of homologous proteins in L. littorea were performed by using the ClustalW program with the default parameters together with 54 Arabidopsis thaliana DEAD-box protein sequences. The DEAD-box protein sequences of Arabidopsis thaliana and L. littorea were downloaded from UniProt (https://www.uniprot.org/) and Arabidopsis Information Resource (https://www.arabidopsis.org/), respectively. The phylogenetic trees for DEAD-box RNA helicase case genes were constructed by using the Nearest-Neighbor-Interchange (NNI) method in MEGA7 software and assessed by bootstrap analysis with 1000 resampling replicates. Trees were visualized by TBtools and drawn by using the Interactive Tree of Life (iTOL: https://itol.embl.de)/.

2.4 Plant materials and growth conditions

One-year-old L. littorea seedlings with 5–6 true leaves and a height of 30 ± 2 cm were chosen as experimental materials, and then divided into four groups: (1) Seedlings were treated at a temperature of 8°C day/5°C night (SLS); (2) Seedlings treated under temperature 15°C day/12°C night (MLS); (3) Seedlings treated under temperature 25°C day/23°C night (LLS); (4) Seedlings handled in temperature 34°C day/30°C night (HLS). All plants were cultured for 48 h in a cold light plant incubator (LRG-450-LED, LV BO, China) under controlled climatic conditions: day/night photoperiod, 12/12 h; 50% relative humidity and zero relative light intensity during the night. All harvested leaves, stems, and roots were quickly washed and wrapped in aluminum foil and immediately placed in liquid nitrogen, and stored at ~80°C until use. Three biological replicates were analysed for each treatment.

2.5 Determination of physiological indicators at different temperatures

2.5.1 Measurements of antioxidant enzymes, osmoregulatory substances, hydrogen peroxide (H₂O₂) and chlorophyll content

After chilling stress, leaves from each treatment were collected for physiological analysis. The contents of antioxidant...
enzymes, osmoregulatory substances, hydrogen peroxide (H₂O₂) and Chlorophyll were determined with commercial assay kits (Jiancheng, China).

One unit of CAT enzyme activity is defined as 1 umol of H₂O₂ being decomposed by 1 mg histone per second. One unit of POD activity is defined as the amount of enzyme per mg of tissue catalyzing 1ug of substrate per minute at 37°C. The enzyme activity was expressed as U/g fresh weight.

Proline content was determined by the Proline Assay Kit (Jiancheng, China) according to the operating instructions. Soluble sugars were determined with plant soluble sugar content Assay Kit (Jiancheng, China) by adding ten times the volume of distilled water and grinding into a homogeneous solution, heating in a boiling water bath for 10 min, and then centrifuging at 4000 rpm for 10 min, followed by determination of their content by anthrone colorimetry. The concentration of total soluble phenols was determined by using commercial assay kits (Nanjing, China).

The content of H₂O₂ was determined by using a commercially available kit (Nanjing, China). Chlorophyll a, and chlorophyll b were determined by the Chlorophyll assay kit (Jiancheng, China) according to the operating instructions.

2.5.2 Measurement of photosynthetic characteristics
Photosynthetic characteristics were measured by using a portable photosynthesis instrument (LI-6800, LICOR, USA). The following variables were measured: net photosynthetic rate (Pn, umolCO₂·m⁻²·s⁻¹), intercellular CO₂ concentration (Ci, µmol mol⁻¹), stomatal conductance (Gs, molH₂O·Om⁻²·s⁻¹), and transpiration (Tr, mmolH₂O·Om⁻²·s⁻¹). Measurements were performed from 9 to 11 am at 20°C–25°C and CO₂ concentration ranging around 500 umol·mol⁻¹. The flow rate was 400 umols⁻¹, and the artificial light was set as 2000 umolm⁻²·s⁻¹ provided by a light-emitting diode (LED) light source (model 6800-02 Red-Blue, Li-Cor Inc.) with 10% blue light and 90% red light to maximize the stomatal aperture and photosystem responses. All photosynthetic data were collected from 9:00 to 11:00 am.

Chlorophyll fluorescence was measured simultaneously with CO₂ gas exchange on the third leaves using an OS55p Portable Pulse Modulated Chlorophyll Fluorometer. The seedling leaves were dark-adapted for 20 min before measurements. The fluorescence parameters for non-photochemical (NPQ) and photochemical (qP) quenching, the maximum fluorescence (Fm), the maximal efficiency of PSII photochemistry (Fv/Fm), and the actual quantum yield of PSII photochemistry (ΦPSII) were obtained by the method described in the previous study (Livak and Schmittgen 2001; Leckie and Neal Stewart 2011).

2.6 RNA isolation and quantitative real-time RT–PCR analysis
Total RNA was extracted from L. littorea using TRI reagent according to the manufacturer’s protocol. RNA quantity and integrity were estimated at 260 nm using a Nano-Drop ND-8000 UV-Vis spectrophotometer, and gel electrophoresis was performed in a 1% agarose gel stained with ethidium bromide in 1x TAE (Tris-Acetate-EDTA) buffer. Then, first-strand cDNAs were synthesized using the HiScript QRT SuperMix for qPCR (+gDNA wiper) with Oligo (dT) primers. Twenty DEAD-box genes were chosen for quantitative real-time RT–PCR analysis including those localized to different organelles and had high similarity to the DEAD-box amino acid sequences of the other plant species. The primers are listed in Table S1. The Real-time PCR experiments were performed three times.

The expression of selected L. littorea DEAD-box family genes in five tissues (root, stem, leaf, flower and fruit) at different temperatures was researched. The average threshold cycle (Ct) was calculated for each sample, AthU6 was used as the internal control. The relative expression levels of individual genes were calculated using the 2^−ΔΔCt method described by Livak and Schmittgen (Livak and Schmittgen 2001). The average expression folds of three biological replicates were calculated. The heatmap of DEAD gene expression in five tissues of L. littorea was plotted by TBtools software.

3. Results
3.1 Identification of the DEAD-box family proteins in L. littorea
We identified 73 non-redundant DEAD-box genes (LIDEAD1 to LIDEAD73) from the transcriptome analysis databases of L. littorea. Bioinformatic analysis of the DEAD-box gene family of L. littorea revealed 73 newly identified LIDEAD proteins ranging from 406 to 2084 aa in length, with LIDEAD18 having the longest amino acids sequences. Molecular weight analysis showed that the molecular weight of this family member was between 9546.8 and 198608.51 Da, which positively correlated with the number of amino acids. Isoelectric point analysis shows that LIDEAD52 has the lowest isoelectric point, with strong sedimentation ability and weak solubility. LIDEAD72 has the highest isoelectric point with more vital intermolecular forces. The lipid coefficient ranged from 41.25 to 99.07. According to the predicted analysis of subcellular localization, a total of 50.68% of 73 DEAD-box genes localized in the nucleus, 17.81% in the mitochondria, 27.40% in the cytoplasm and 4.11% in the endoplasmic reticulum (Table S3).

3.2 Conserved structural domain and motif analysis
A total of 9 conserved motifs were identified among 73 LIDEAD proteins (Figure 1(A)). Figure 1(B) shows the details of those conserved amino acid sequences and lengths of the nine motifs. Most of the closely related members have the same motif compositions, indicating functional similarity between the evolutionarily closely related LIDEAD proteins.

We use Batch CD-search software to analyze the conserved domains of LIDEAD proteins (Figure S1). The results showed that, except for LIDEAD10, LIDEAD3, LIDEAD9, LIDEAD8, LIDEAD6, LIDEAD7, LIDEAD12, LIDEAD11, LIDEAD13, LIDEAD14, LIDEAD16, all other LIDEAD family members contained DEAD domains, and LIDEAD17 and LIDEAD18 had DEAD domain overlaps.

3.3 Multiple Sequence Alignment and Phylogenetic Analysis
To investigate the phylogenetic relationship of DEAD-box proteins between L. littorea and Arabidopsis thaliana, we constructed an unrooted phylogenetic tree (Figure 2). The
**L. littorea** DEAD-box members exhibit interspersed distribution among different subgroups.

### 3.4 Physiological responses caused by chilling stress in **L. littorea**

As shown in Figure 3, the phenology of **L. littorea** changed significantly under different culture temperature conditions. No significant changes were observed in LLS and HLS culture conditions. Under MLS conditions, the leaves of **L. littorea** appeared to wilt. Under SLS culture conditions, the leaves of **L. littorea** appeared to dry up and turn yellow.

As shown in Figure 4(A–G), after treatment at different temperatures, the activities of CAT and POD as well as the contents of soluble sugars, total phenols, proline, soluble protein and hydrogen peroxide were measured in **L. littorea** seedlings. In the process of low-temperature stress, the activity of POD, soluble sugar, total phenol, proline, and soluble protein content increased significantly compared with the control. Still, the range of CAT and H₂O₂ decreased.

As shown in Figure 5, there was no significant difference in chlorophyll content under LLS and HLS treatments. Under MLS treatment, the content of chlorophyll increased, while chlorophyll a/b decreased, indicating that chlorophyll b increased more than chlorophyll a. Under SLS treatment, the content of chlorophyll decreased, while the amount of...
chlorophyll a/b increased, indicating that chlorophyll b decreased more than chlorophyll a and it shows that chlorophyll b is more susceptible to the influence of environmental temperature.

Furthermore, as shown in Figure 6(A–I), photosynthetic characteristics and chlorophyll fluorescence characteristics of L. littorea treated at different temperatures revealed that the photosynthesis of L. littorea seedings was severely hindered under low-temperature stress. After 48 h of treatment, all measured indicators in SLS changed significantly, with the net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration (Tr), the fluorescence parameters for non-photochemical (NPQ) and photochemical (qP) quenching, the maximum fluorescence (Fm), the maximal efficiency of PSII photochemistry (Fv/Fm), and the actual quantum yield of PSII photochemistry (ΦPSII) decreased to 114.45%, 85.79%, 94.83%, 73.6%, 30.17%, 62.82%, 87.54% and 29.34% in the HLS group, respectively. And the intercellular CO₂ concentration (Ci) increased to 66.28% in the HLS group.

3.5 Quantitative real-time PCR analysis

3.5.1 Expression profiling of DEAD genes

To study the expression characteristics of the DEAD-box gene in the growth and development of L. littorea, the expression characteristics of 20 representative DEAD-box genes were studied in different tissues and organs (Figure 7). LlDEAD13 are highly expressed in roots; LlDEAD68 expressed explicitly in stems; a total of 15 genes are highly expressed in leaves; LlDEAD46 highly expressed in flowers; LlDEAD1 showed highly in fruits. A total of 19 genes of those LlDEAD genes were not defined in the roots of L. littorea and most genes were highly expressed in leaves, followed by stems and flowers.

3.5.2 Quantitative real-time PCR analysis under cold stress

As shown in Figure 8, quantitative real-time PCR analysis was performed on 20 representative genes of the DEAD-box family. These 20 genes were all significantly down-regulated under low-temperature stress.

4. Discussion

Based on previous studies of DEAD-box genes in rice (Tuteja et al. 2013; Nawaz, Lee, et al. 2018), wheat (Zhang et al. 2014), Arabidopsis thaliana (Gong et al. 2002; Western et al. 2002; Matthes et al. 2007; Nishimura et al. 2010; Guan et al. 2013), kale (Nawaz, Sai, et al. 2018), maize (Li et al. 2001), and tomato (Zhu et al. 2015; Cai et al. 2018; Pandey et al. 2019), DEAD-box genes are found to be structurally similar, high functional similarity and versatility so that they can be used as candidate genes to study cold resistance in plants. 73 LlDEAD genes obtained in L. littorea
Figure 3. Phenotypic characteristics of under L. littorea different treatment conditions. SLS indicates seedlings treated at a temperature of 8°C day/5°C night for 48 h; MLS indicates seedlings treated at a temperature of 15°C day/12°C night for 48 h; LLS indicates seedlings treated at a temperature of 25°C day/23°C night for 48 h; and HLS indicates seedlings treated at a temperature of 34°C day/30°C night for 48 h.

Figure 4. Physiological variations due to different treatment temperatures. (A) CAT enzyme activity; (B) POD enzyme activity; (C) soluble sugar content; (D) total phenol content; (E) proline content; (F) soluble protein content; (G) \( \text{H}_2\text{O}_2 \) content. SLS indicates seedlings treated at a temperature of 8°C day/5°C night for 48 h; MLS indicates seedlings treated at a temperature of 15°C day/12°C night for 48 h; LLS indicates seedlings treated at a temperature of 25°C day/23°C night for 48 h; and HLS indicates seedlings treated at a temperature of 34°C day/30°C night for 48 h. Statistical analysis was performed comparing different treatments, with \( p \)-values is 0.05.
were compared with *Arabidopsis thaliana* indicate the possible function responding cold stress.

In this paper, the Pn, Gs, Tr, Fm, Fv/Fm, ΦPSII of the leaves in *L. littorea* showed a trend of reduction under low-temperature stress, which was same with the studies of *Sonneratia apetala* and *Kandelia candel* (Chen et al. 2012; Wang et al. 2018). The hindrance of photosynthesis in plants includes two reasons: stomatal limitation and non-stomatal limitation (Farquhar and Sharkey 1982). Under MLS, Pn, Gs and Tr of *L. littorea* decreased while Ci increased, indicating that stomatal limitation was the main factor in the decrease of photosynthesis. Moreover, at SLS, *L. littorea* was severely stressed by low temperature, and the Pn of the plant is less than 0, indicating that only respiration, without photosynthesis is present in the seedlings and non-stomatal limitation plays a major role in the reduction of photosynthesis (Allen and Ort 2001; Ploschuk et al. 2014). Chlorophyll fluorescence parameters, which can reflect the state of the PSII reaction center, can reveal the intrinsic characteristics of the plant photosynthetic system better than photosynthetic parameters (Murchie and Lawson 2013). Fv/Fm and ΦPSII showed a decreasing trend with decreasing temperature, reflecting that the activity of PSII decreased (Jiang et al. 2002; Krause et al. 2010; Krause et al. 2013). Under SLS conditions, NPQ decreased, indicating a deepening of plant exposure to low temperature stress, demonstrating that non-stomatal limitation is the main cause of reduced photosynthesis.

Moreover, chlorophyll a, chlorophyll b, and total chlorophyll of *L. littorea* showed a trend of increasing and then decreasing with the enhanced intensity of low-temperature stress, indicating that the chlorophyll synthesis system was disturbed, which was also a reason for the decrease of photosynthesis. The osmoregulatory substances such as soluble protein, proline, and total phenols increased significantly under low-temperature stress and were used to maintain osmotic pressure homeostasis, which was similar to the results of the study in *Kandelia candel* (Yong et al. 2011), but a further drop in temperature, proline, and total phenols...
showed a decrease, indicating that their synthesis mechanism was blocked and the defense function of the organism was weakened. The results of CAT were similar to the results of the study on *Cerops tagal*, both with the drop in temperature and the inhibition of enzyme activity, probably due to the impairment of the physiological metabolic sites of CAT by low temperature (Zhong et al. 2012). The activity of POD showed an increasing trend with decreasing temperature, the same as in the study of *Kandelia candel* (Yong et al. 2011), probably due to the dual role of POD in both scavenging H$_2$O$_2$ and mediating the conversion of H$_2$O$_2$ to hydroxyl radicals, so the decrease in H$_2$O$_2$ content may due to the scavenging effect of POD.

As the primary site of photosynthesis, the maintenance of chloroplast function is vital for plant growth and development under abiotic stresses (Zhao et al. 2020). The ability of plants to survive in adversity is positively correlated with the expression of genes regulating the chloroplast stress response (Zhao et al. 2020). DEAD-box gene is the majority of the second subfamily of RNA helicases whose mechanism of function involves every step of RNA metabolism, including nuclear transcription, precursor mRNA splicing, ribosome biogenesis, nucleoplasmic translocation, translation, RNA decay, and organelle gene expression (de la Cruz et al. 1999; Lorsch 2002).

According to previous studies in model species, several DEAD-box genes were found to be associated with cold stress response in plants, such as AtRH42 (AT1g20920) (Guan et al. 2013), AtRH25 (AT5G08620) (Kim et al. 2008), AtRH9 (AT3G22310.1) (Cordin et al. 2006; Kim et al. 2008; Köhler et al. 2010), AtRH7 (AT5G62190.1) (Lorković et al. 1997), and AtRH3 (AT5G26742.2) (Asakura et al. 2012; Lee et al. 2013; Gu et al. 2014). The genes LlDEAD36, LlDEAD43, LlDEAD47, LlDEAD44 and LlDEAD45, which have a close evolutionary relationship with the cold-responsive genes of the model plants, provide the foundation for our study of DEAD-box genes in *L. littorea* responding to low-temperature stress.

AtRH3 is a chloroplast-localized RNA helicase, which plays a role in the splicing of introns and the assembly of 5OS ribosomal particles (Asakura et al. 2012). And AtRH3 plays an essential role in the processing of rRNA precursors
during the conversion of plastids to chloroplasts, which is vital for the maintaining normal chloroplast function (Lee et al. 2013). Furthermore, the correct splicing of introns controlled by the RNA chaperone activity of AtRH3 has an essential role in the low-temperature response of plants, as proven by the severely inhibited growth of AtRH3 knockout mutant plants under low-temperature stress (Gu et al. 2014). By evolutionary analysis, LlDEAD44 and LlDEAD45 in *L. littorea* have a close evolutionary relationship with AtRH3, and it can be guessed that LlDEAD44 and LlDEAD45 may play a similar function. Validation by real-time fluorescence quantitative PCR revealed that these two genes were significantly down-regulated under low-temperature stress. RH42 is an essential gene in the low-temperature response of plants. It is required for the precise regulation of cold-induced precursor mRNA splicing in rice during mRNA maturation at low temperatures (Guan et al. 2013). Combined with evolutionary analysis and real-time fluorescence quantitative PCR verification, it is hypothesized that LlDEAD36 has a possible role in mRNA splicing under low temperature stress. It has been shown that AtRH22 can play a role in the assembly of 50S ribosomal subunits in chloroplasts and the maintenance of plastid mRNA levels through its RNA chaperone activity (Chi et al. 2012; Chi et al. 2012; Nawaz, Lee, et al. 2018), and play a positive role in abiotic stresses such as low-temperature stress by affecting the translation of chloroplast genes (Tripurani et al. 2011). AtRH38/LOS4 is a cold stress response gene that plays a positive role in plant response to cold stress by regulating the export of RNA molecules from the nucleus to the cytoplasm (Gong et al. 2002; Gong et al. 2005). In addition, AtRH7 governs plant growth and development and responds to cold temperatures by participating in ribosome assembly (Lorković et al. 1997). It can interact with a molecular chaperone, *Arabidopsis thaliana* cold-excited structural domain protein 3 (AtCSP3) improved plant tolerance to low temperatures (Liu et al. 2016). Through evolutionary analysis, it can be inferred that LlDEAD48 and LlDEAD47, which are more closely related to the evolution of AtRH38 and AtRH7, may have the function of maintaining chloroplast function under low temperature stress, and the specific functions need to be verified later.

**Figure 7.** Heat map representation of DEAD-box genes in five tissues of *L. littorea*. The heat map from blue to red represents the expression of DEAD-box gene from low to high respectively.
Under low-temperature stress, reactive oxygen species accumulate rapidly in plants, resulting in oxidative damage to the organism. Existing studies have shown that OsBIRH1 is a DEAD-box gene and transgenic plants with overexpression of OsBIRH1 exhibit increased tolerance to oxidative stress. They increased expression levels of oxidative defense genes \textit{AtApx1}, \textit{AtApx2}, and \textit{AtFSD1}, which are essential for scavenging the accumulation of reactive oxygen species under low-temperature stress (Li et al. 2008). LlDEAD34 had a close evolutionary relationship with RH1. Combined with the decrease in H$_2$O$_2$ content and the increase in the activities of antioxidant enzymes such as CAT and POD, it can indirectly prove that LlDEAD34 may reduce the reactive oxygen species damage due to low temperature by increasing the activities of antioxidant enzymes.

Mitochondrial energy metabolism is an essential cellular process for plant growth and survival under low-temperature stress and normal growth conditions (Zsigmond et al. 2008). DEAD-box RNA helicases (RHS) can aid in the formation of mature RNA function in mitochondria (Cordin et al. 2006). For instance, PMH1 (AtRH9) is a mitochondrial localization protein with the potential to function as an RNA chaperone required for the formation or maintenance of intron complex RNA secondary structures, aiding the formation of functionally mature RNAs in mitochondria (Cordin et al. 2006). Both AtRH9 and AtRH25 were up-regulated under cold stress with different nucleic acid binding properties between them (Kim et al. 2008). The evolutionary relationship analysis showed that LlDEAD43 have a high similarity in structure to AtRH25 that has been demonstrated to be...
functional in cold stress in *Arabidopsis thaliana*. These predicted genes are the priority genes for our subsequent studies to improve cold resistance in *L. littorea*.

5. Conclusions

In this study, we investigated the response mechanism of *L. littorea* under cold stress from two aspects: physiological changes and DEAD-box gene family analysis. Our physiological data showed that cold stress severely impaired photosynthesis in *L. littorea*, which radically reduced net photosynthetic rate, stomatal conductance, and transpiration rate. We identified 73 DEAD-box genes in *L. littorea* by transcriptome data analysis. After evolutionary relationship analysis, we found that LI DEAD44, LI DEAD45, LI DEAD36, LI DEAD47, and LI DEAD48 might play a role in the maintenance of chloroplast function under low-temperature stress, LI DEAD34 might be associated with the scavenging of excess ROS caused by low-temperature stress, and LI DEAD43 might be involved in the maintenance of mitochondrial function. These potential cold-responsive genes provide the basis for our subsequent studies to improve cold resistance in *L. littorea*.

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Notes on contributors

Lulu Hao, a master student, focuses on resistance research of mangrove plants.

Ying Zhang, a professor of phytoecology, researches the endangered mechanisms of mangrove species. She recently published Comparative Transcriptome Reveals the Genes’Adaptation to Herbogamy of Lunmizera littorea(Jack) Voigt.

Yin Li, an undergraduate student.

Chunfang Zheng, a professor of physiology, researches the chilling resistance mechanism of mangrove plants. She has recently published academic papers such as Transcriptome analysis of Sonneratia caseolaris seedlings under chilling stress.

Linxia Bai, an undergraduate student.

Danfei Yue, a master’s student, studied the cold resistance of mangrove plants.

Huiyu Zhang, a master’s student, studied the cold resistance of mangrove plants.

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