Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2022.020515 Published Online: 27 January 2022

ARTICLE

De novo Transcriptome Analysis in *Leymus mollis* to Unveil Genes Involved in Salt Stress Response

Wenting Wu[1], Yajing Zhang[1], Yu Gao[1], Kai Zhang[2,3], Luying Zhu[1,3,*] and Hongxia Zhang[2,3]

[1]College of Agriculture, Ludong University, Yantai, 264025, China

[2]Engineering Research Institute of Agriculture and Forestry, Ludong University, Yantai, 264025, China

[3]Key Laboratory of Molecular Module-Based Breeding of High Yield and Abiotic Resistant Plants in Universities of Shandong, Ludong University, Yantai, 264025, China

[*]Corresponding Author: Luying Zhu. Email: 1742@ldu.edu.cn

Received: 28 November 2021 Accepted: 13 December 2021

ABSTRACT

Leymus mollis, a wild relative of wheat, is very tolerant to salt stress, and has been considered as a valuable genetic resource for wheat breeding. However, the genetic basis for salt tolerance of this species is still largely unknown. In this study, *de novo* sequencing, assembly and analysis of *L. mollis* transcriptome in response to salt stress was performed. A total of 110,323 and 112,846 unigenes were generated for the NaCl-free (CK) and 180 mM NaCl-treated (CT) library, respectively. For the two libraries, 73,414 unigenes were successfully annotated in five common protein databases, and 7521 differentially expressed genes (DEGs) between CK and CT libraries were identified. GO enrichment analysis of the DEGs showed that the significantly enriched GO terms were predominantly involved in environmental adaptation (including "response to abiotic stimulus", "response to water deprivation"), regulation of signaling pathway (such as "regulation of abscisic acid mediated signaling pathway", "regulation of cell communication"), and photosynthesis (including "response to light stimulus", "photosynthesis, light harvesting" and "chlorophyll metabolic process"). KEGG pathway enrichment analysis showed that "mRNA surveillance pathway", "RNA transport" and "plant hormone signal transduction" were predominantly enriched pathways, followed by several secondary metabolic pathways, photosynthesis, carbohydrate metabolism and lipid metabolism. In addition, DEGs related to osmotic stress, ion homeostasis and oxidative stress, including four dehydrins, five aquaporins, an LmNHX2 and several antioxidant enzymes or proteins genes, were found to be up-regulated in response to salt stress. These results will be helpful for further studies on the molecular mechanisms of salt responses in *L. mollis*.

KEYWORDS

Leymus mollis; transcriptome; salt stress

Abbreviations

CK	NaCl-free sample
CT	NaCl-treated sample
DEG	Differentially expressed gene
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes

NR	NCBI non-redundant protein
COG	Clusters of Orthologous Groups
SRA	NCBI Sequence Read Archive
FPKM	Fragments per kilobase per million fragments
FDR	False discovery rate
NHX	Vacuolar Na^+/H^+ antiporter
DHN	Dehydrin
AVP	Vacuolar proton-inorganic pyrophosphatase
HAK	K^+ transporter
ROS	Reactive oxygen species
POD	Peroxidase
TRX	Thioredoxin
APX	L-ascorbate peroxidase
GST	Glutathione-S-transferase
CAT	Catalase
qRT-PCR	Real-time quantitative PCR

1 Introduction

Soil salinity can severely affect plant growth and development, and consequently reduce crop yield and food production. Currently, more than 6% of lands throughout the world are affected by salinity [1]. Therefore, improving the resistance of crops to salt stress and the utilization efficiency of salty land have become one of the most important objectives for crop breeders.

Generally, increased soil salt concentration first causes osmotic stress which instantly affects plant growth. Then ion toxicity occurs when the salt taken up by roots reaches a threshold level, beyond which the plant cannot maintain ion homeostasis [2]. Osmotic stress and ion toxicity often cause oxidative stress and a series of secondary stresses. These harmful effects caused by salt stress inhibit plant growth and productivity in various ways, including impairing physiological and biochemical metabolic processes, decreasing photosynthetic efficiency and causing nutritional disorders in plants [3,4].

In order to survive under adverse environmental conditions, plants have developed a serie of mechanisms involving developmental, morphological, physiological and biochemical strategies. Osmotic adjustment and maintaining ion balance are two important mechanisms at a molecular level for plant resistance to salinity stress [1,5]. In addition, some antioxidant enzymes and transcription factors also respond to and regulate plant tolerance to salt stress [6]. In recent years, more and more gene expression information involved in signal transduction, regulation of transcription and metabolism, redox reaction and other processes in response to salt stress have been reported [7–11].

Wheat (*Triticum aestivum* L.) is one of the most important grain crops moderately tolerant to saline conditions and its production is often limited by soil salinization. Therefore, it is necessary to seek a salt-tolerance genetic resource among various genotypes or wild relatives for wheat resistance breeding. *Leymus mollis* (Triticeae; Poaceae), a dune grass growing mainly along sea coasts and in inland dry areas, is a wild relative of wheat [12]. This species is tolerant to salt and drought, and resistant to various diseases [13–15]. However, the genetic basis for its biochemical and physiological responses to salt and drought stresses still remains largely unknown, despite the high tolerance of this species to multiple environmental stresses.

Transcriptome sequencing using next-generation sequencing technology is a fast and effective approach to generate genome-scale sequence resources, and provides an efficient way to investigate the mechanisms of

plant response to various stresses [16–20]. To better understand the salt-stress response of *L. mollis* at transcriptional level, a comparative transcriptome analysis was performed based on the Illumina sequencing in this study. Numerous differentially expressed genes in *L. mollis* responsive to salt stress were identified and classified.

2 Materials and Methods

2.1 Plant Growth and Salt Stress Treatments

Leymus mollis seeds were immersed in water for 12 h and then planted into vermiculite in a chamber at a day/night temperature of 25/20°C with a 14 h light/10 h dark photoperiod. After germination, seedlings of the same size with the first fully expanded true leaf were transferred into a 1/2 hoagland nutrient solution and grew under the above temperature and light conditions. For comparative transcriptome analysis, three-week-old uniform plants with three true leaves were treated with 180 mM NaCl and the first true leaves were collected after treated with NaCl for 0, 6, 12, 24 and 48 h. All leaf samples were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

2.2 RNA Extraction, cDNA Library Construction and Sequencing

Total RNA was extracted from the frozen leaf samples using Trizol reagent (Invitrogen, USA) according to the manufacture's protocol, and then digested with RNase-free DNase I (Takara, Japan) to remove the residual genomic DNA. The quantity and quality of total RNAs were determined with spectrophotometry and electrophoresis assays. To obtain a global and high-quality overview of the *L. mollis* leaf transcriptome, individual RNA obtained from different samples collected at different time points (6, 12, 24 and 48 h after the initiation of NaCl treatment) was equally pooled for library construction, and RNA from NaCl-untreated samples was used as the control.

cDNA libraries were constructed following the Illumina manufacturer's instructions. Briefly, the poly (A) mRNA was purified from pooled total RNA using Oligo (dT) magnetic beads, and fragmented into short fragments. The first-strand was synthesized using the short fragments as templates with random hexamer-primer, and then the second-strand was amplified. The short cDNA fragments were purified and ligated to sequencing adapters. Then suitable fragments were chosen for PCR amplification to create final cDNA libraries. The resultant cDNA libraries were sequenced on the Illumina HiSeq 2000 platform at Beijing Genomic Institute (BGI) Life Tech Co., Ltd. (China).

2.3 De novo Assembly and Functional Annotation

After the clean reads were obtained by trimming adapters and removing low-quality sequencing, *de novo* transcriptome assembly was performed using the Trinity program [21]. The generated transcript sequences were subjected to BLASTx against the protein databases, including NCBI non-redundant protein (NR) database, Swiss-Prot protein database, Clusters of Orthologous Groups (COG), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, using an E-value $< 10^{-5}$, retrieving proteins with the highest sequence similarity along with their protein functional annotations. The sequencing raw data have been deposited to the NCBI Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/Traces/sra) with an accession number SRP109343.

2.4 Identification of Differentially Expressed Genes (DEGs)

Transcriptional expression levels were calculated using the FPKM (fragments per kilobase per million fragments) method [22]. Genes that were differentially expressed between the control and NaCl-treated groups were considered to be significantly different at an FDR (false discovery rate) ≤ 0.001 and an absolute value of log2 ratio ≥ 1, according to the method described previously [23]. GO functional and

KEGG pathway enrichment analysis of the DEGs were performed with the threshold of P value ≤ 0.01 to isolate significantly enriched functional classification and metabolic pathways.

2.5 Quantitative Real-Time PCR (qRT-PCR) Validation

For the validation of differentially expressed genes, qRT-PCR analyses were performed. Total RNAs isolated from the control and NaCl-treated samples were used to conduct qRT-PCR according to the manufacturer's protocols of PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan) and TransStart Tip Green qPCR SuperMix (TransGen Biotech, China), respectively. The primer sequences used in qRT-PCR analysis were designed based on the assembled transcript sequences and shown in Table S1. PCR reaction was carried out on an ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, USA) with a condition of 94°C for 30 s, 40 cycles of 94°C for 5 s, 55°C for 15 s, and 72°C for 34 s. Each experiment was performed in three biological replicates and actin gene was used as the reference gene. Expression levels of the selected genes were calculated using the $2^{-\Delta\Delta CT}$ method.

3 Results

3.1 RNA-Seq and De novo Assembly

In order to obtain a global view of gene expression response to salt stress in *L. mollis*, we constructed two cDNA libraries from NaCl-free (CK) and 180 mM NaCl-treated (CT) *L. mollis* leaves and performed Illumina HiSeqTM 2000 platform sequencing. A total number of more than 46.91 and 47.30 Mb raw reads were respectively generated for the CK and CT libraries (Table 1). After the removal of low-quality reads and adapter sequences, 46.15 Mb clean reads were obtained for the CK library and 46.41 Mb clean reads were obtained for the CT library. The Q20 and GC percentages obtained from the CK and CT libraries were 96.44% and 55.18%, and 96.96% and 55.90%, respectively. All these results suggested that the quantity and quality of the sequencing data were good enough for accurate sequence assembly and adequate transcriptome coverage.

Table 1: An overview of the sequencing and *de novo* assembly for the two libraries

Item	Number (CK)	Number (CT)
Total raw reads	46,910,902	47,302,274
Total clean reads	46,154,210	46,406,704
Q20 percentage of clean reads	96.44%	96.96%
GC percentage of clean reads	55.18%	55.90%
Total contigs	218,966	228,637
Mean length (bp) of contigs	382	376
N50 (bp) of contigs	766	738
Total unigenes	110,323	112,846
Mean length (bp) of unigenes	825	795
N50 (bp) of unigenes	1379	1319

We then used the clean reads to perform *de novo* assembly analyses with the Trinity software. A total number of 218,966 and 228,637 contigs with a mean length of 382 and 376 bp for CK and CT libraries were generated, respectively. Then these contigs were connected into unigenes to generate 110,323 unigenes with

a mean length of 825 bp for the CK library, and 112,846 unigenes with a mean length of 795 bp for the CT library. The N50 of all unigenes were 1379 and 1319 bp for the CK and CT libraries, respectively (Table 1).

3.2 Functional Annotation and Classification

We further performed functional annotation using all the assembled unigenes with NR, Swiss-Prot, COG, GO and KEGG functional databases. In total, 73,414 unigenes for the two libraries were successfully annotated in at least one of the above databases. Among them, 49,225 (67.05%) unigenes showed significant matches in the NR database, and 36,630 (49.90%) unigenes showed significant matches in the GO database (Table 2). In addition, some unigenes (47.53%) showed no significant similarity to any known functional proteins. These unigenes might be long noncoding RNAs or novel genes, and might also play important roles in the *L. mollis* response to salt stress.

Table 2: An overview of functional annotation of assembled unigenes for the two libraries

Annotated database	Number of annotated unigenes	Percentage of total annotated unigenes
NR	49,225	67.05%
GO	36,630	49.90%
Swiss-Prot	35,349	48.15%
KEGG	33,390	45.48%
COG	21,311	29.03%
All databases	73,414	–

3.3 Identification of Differentially Expressed Genes (DEGs) in Response to Salt Stress

To reveal the transcriptional changes in *L. mollis* leaves in response to salt stress, the transcription levels based on the RPKM values of the unigenes from the NaCl treated library were compared with those of the control library. A total number of 7521 DEGs were identified using the combined criteria of at least a two-fold change and an FDR ≤ 0.001. Among them, 4112 were up-regulated and 3409 were down-regulated (Fig. 1). Therefore, a number of genes were involved into salt stress responses, implying the complexity of salt stress response in *L. mollis*.

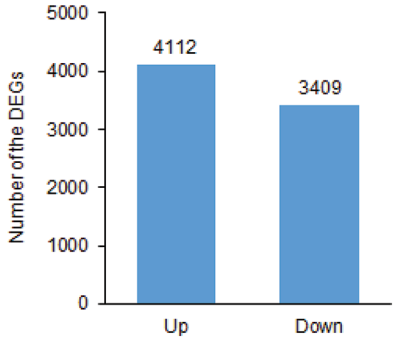

Figure 1: Number of up- and down-regulated differentially expressed genes (DEGs) between NaCl-free (CK) and NaCl-treated (CT) *L. mollis* leaves

3.4 GO Functional Analysis of the DEGs

To understand the possible functions of these DEGs in *L. mollis* leaves in response to salt stress, we conducted GO functional enrichment analysis. All the DEGs including both up- and down-regulated were

classified into three categories: biological progress, molecular function and cellular component. Based on the results of GO enrichment analyses, the significantly enriched GO terms in the biological process category were closely associated with environmental adaptation (including "response to abiotic stimulus" and "response to water deprivation"), regulation of signaling pathway (such as "regulation of abscisic acid mediated signaling pathway", "regulation of cell communication", "regulation of signal transduction" and "hormone-mediated signaling pathway") and photosynthesis (including "response to light stimulus", "photosynthesis, light harvesting" and "chlorophyll metabolic process"). The significantly enriched GO terms in the molecular function and cellular component categories were respectively associated with "oxidoreductase activity", "heme binding", "monooxygenase activity" and "abscisic acid 8'-hydroxylase activity", and "nucleosome", "intrinsic to membrane", "thylakoid" and "membrane" (Fig. 2). These results suggested that under short term salt stress conditions, *L. mollis* has been subjected to osmotic and oxidative stresses, which negatively affected a wide spectrum of biochemical responses, especially photosynthesis and metabolic process. In addition, the significantly enriched GO terms in the biological process category were also closely associated with "cuticle development", "wax biosynthetic process" and "fatty acid biosynthetic process", suggesting that the cuticularization in *L. mollis* leaves may be a protective way for its adaptation to salt stress.

3.5 KEGG Pathway Analysis of DEGs

We further performed a KEGG pathway enrichment analysis to help interpret the possible functions of the DEGs. According to the KEGG pathway analysis results, the basic pathways "mRNA surveillance pathway" and "RNA transport", followed by "biosynthesis of secondary metabolites", "plant hormone signal transduction", and several secondary metabolic (such as "carotenoid biosynthesis", "stilbenoid, diarylheptanoid and gingerol biosynthesis", "limonene and pinene degradation" and "flavonoid biosynthesis") and photosynthesis pathways were predominantly enriched in response to salt stress in *L. mollis*. Furthermore, pathways involved in carbohydrate metabolism (including "galactose metabolism", "ascorbate and aldarate metabolism", "fructose and mannose metabolism" and "pentose phosphate pathway") and lipid metabolism (including "cutin, suberine and wax biosynthesis", "glycerolipid metabolism" and "fatty acid elongation") were also significantly enriched. These results imply that salt stress could make a great impact on these pathways in *L. mollis* (Fig. 3).

3.6 DEGs Related to Osmotic Stress and Ion Homeostasis

Under salt stress conditions, a high concentration of salt ions around the plant roots reduces the water potential on the root surface and inhibits the absorption of water by plants, leading to osmotic stress [24]. We found that among the DEGs involved in osmotic stress and water deprivation, 4 dehydrin genes and 5 aquaporin genes were up-regulated in response to salt stress in *L. mollis* leaves (Table 3). Among them, compared with CK, the unigenes annotated as "dehydrin Rab15", "aquaporin TIP1-1" and "aquaporin PIP1-5" in CT were respectively up-regulated by 3.03, 3.15 and 3.11 times, suggesting that these genes could play important roles in the adaptation of *L. mollis* to salt stress.

In saline environments, Na^+ is the main harmful ion. High concentrations of Na^+ in the cytoplasm of leaf cells hinder the normal absorption of K^+, resulting in a K^+/Na^+ imbalance and metabolic disorders in plants [25]. We also analyzed DEGs related to ion homeostasis in this study. We found that the DEG annotated as "vacuolar Na^+/H^+ antiporter 2 (NHX2)" was up-regulated, whereas the DEG annotated as "vacuolar proton-inorganic pyrophosphatase 1 (AVP1)", which was considered to provide energy for the activity of the *NHXs*, was down-regulated, although no significant alterations were observed on most *NHXs* and proton pumps

genes at the transcript level after a salt treatment. In addition to *NHX2* and *AVP1*, four *HAK* genes encoding K^+ transporters *HAK24* and *HAK25*, and *HAK27* and *HAK7*, were respectively up- and down-regulated (Table 4).

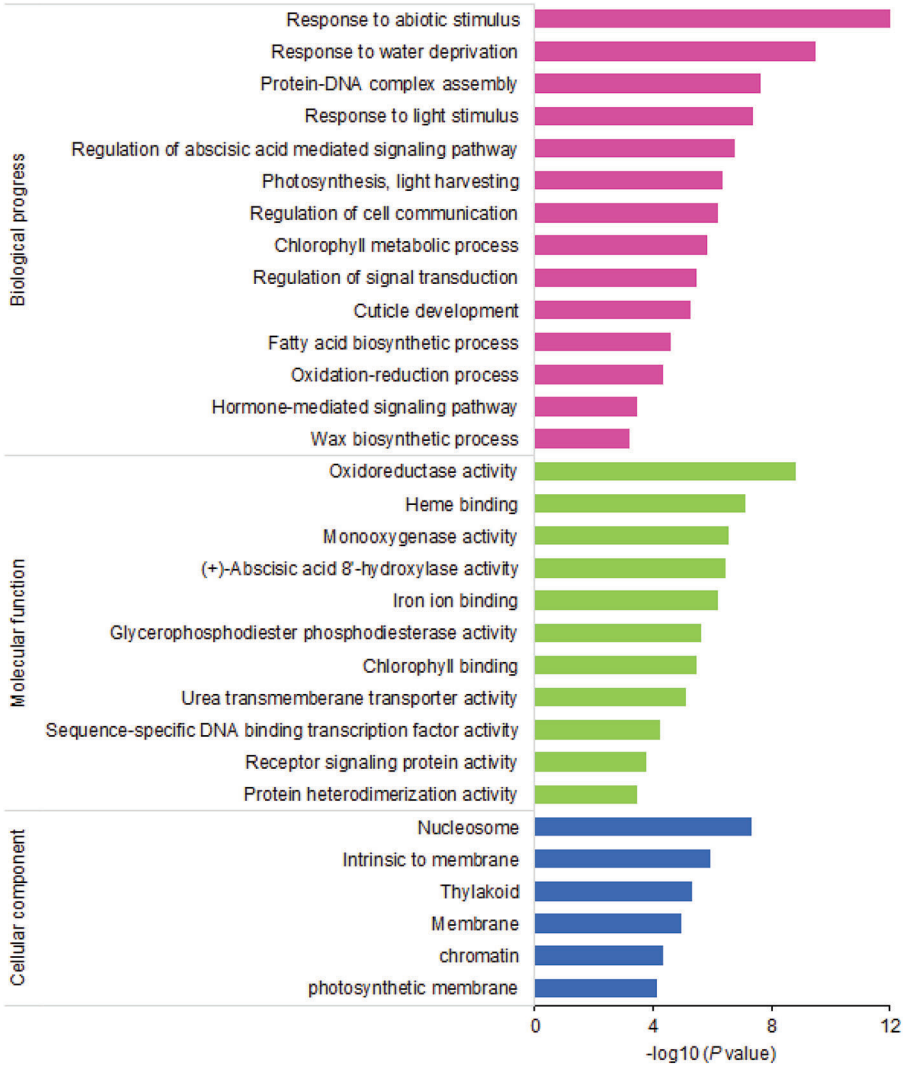

Figure 2: GO functional enrichment analysis of DEGs in response to salt stress in *L. mollis* leaves. Significantly enriched GO terms in the biological progress, molecular function and cellular component categories are shown

3.7 DEGs Related to Oxidative Stress

Osmotic stress, ion toxicity and the secondary effects caused by salt stress, induce the production of reactive oxygen species (ROS) which leads to oxidative stress in plants [26,27]. To alleviate the peroxidation damage, the reactive oxygen scavenging mechanism is activated in plants [28,29]. We further analyzed the DEGs involved in the reactive oxygen species scavenging system. We observed that DEGs encoding peroxidases (POD) showed different inducible expression patterns, where two genes (*POD21*, *POD1*) were up-regulated and three genes (*POD70*, *POD16* and *PODP7*) were down-regulated in response to salt stress. Three DEGs encoding thioredoxin (*CDSP32*, *TRXM2* and *TRXH*) and one gene

encoding L-ascorbate peroxidase 2 (APX2) exhibited a significant up-regulated expression in response to the salt treatment, while the DEG annotated as "probable L-ascorbate peroxidase 8" was down-regulated. The DEGs encoding for the glutathione-S-transferase (GST) and Catalase 1 (CAT1) were also up-regulated by salt stress (Table 5).

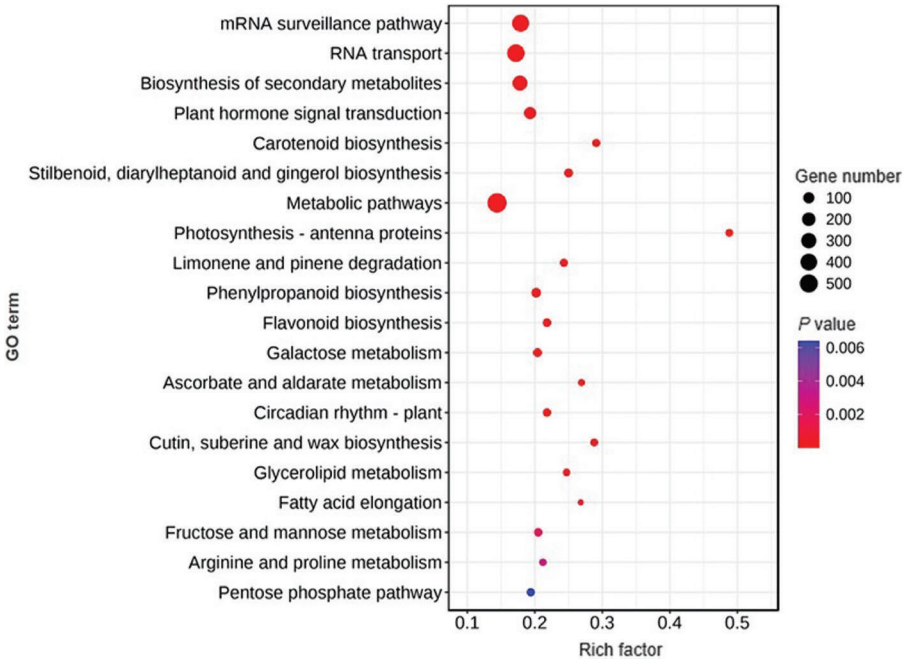

Figure 3: The scatter plot of KEGG pathway enrichment assay in response to salt stress in *L. mollis* leaves. Each circle represents a KEGG pathway. The size of circle indicates the number of enriched transcripts in the pathway

Table 3: DEGs related to osmotic stress

Gene ID	Gene name	Description	Regulation	log2 (fold change)
CL9012. Contig3	*Rab15*	Dehydrin Rab15	Up	3.03
CL27613. Contig22	*DHN2*	Salt-induced YSK2 dehydrin 2	Up	1.86
CL14301. Contig4	*COR410*	Dehydrin COR410	Up	1.53
CL330. Contig1	*DHN3*	Dehydrin 3	Up	1.50
CL4625. Contig3	*TIP1-1*	Aquaporin TIP1-1	Up	3.15
CL26324. Contig13	*PIP1-5*	Aquaporin PIP1-5	Up	3.11
Unigene4479	*TIP-like*	Aquaporin γ-TIP protein	Up	2.80
Unigene51004	*TIP1-3*	Aquaporin TIP1-3	Up	2.06
Unigene12554	*PIP2-5*	Aquaporin PIP2-5	Up	1.62

Table 4: DEGs related to ion homeostasis

Gene ID	Gene name	Description	Regulation	log2 (fold change)
CL26585. Contig9	*NHX2*	Vacuolar Na^+/H^+ antiporter 2	Up	1.22
CL2024. Contig4	*AVP1*	Vacuolar proton-inorganic pyrophosphatase 1	Down	1.38
CL22108. Contig1	*HAK24*	Potassium transporter 24	Up	2.23
Unigene25323	*HAK25*	Potassium transporter 25	Up	1.53
Unigene33743	*HAK27*	Potassium transporter 27	Down	1.60
Unigene13254	*HAK7*	Potassium transporter 7	Down	1.01

Table 5: DEGs response to oxidative stress

Gene ID	Gene name	Description	Regulation	log2 (fold change)
CL22792. Contig1	*POD21*	Peroxidase 21	Up	4.02
CL5100. Contig3	*POD1*	Peroxidase 1	Up	1.96
CL11222. Contig2	*POD70*	Peroxidase 70	Down	1.94
CL3543. Contig2	*POD16*	Peroxidase 16	Down	1.70
Unigene16823	*PODP7*	Peroxidase P7	Down	1.69
Unigene32600	*CDSP32*	Thioredoxin-like protein CDSP32	Up	3.01
CL1228. Contig3	*TRXM2*	Thioredoxin M2, chloroplastic	Up	2.19
Unigene23971	*TRXH1*	Thioredoxin H1	Up	1.68
CL18214. Contig1	*APX2*	L-ascorbate peroxidase 2	Up	2.28
CL14430. Contig5	*APX8*	Probable L-ascorbate peroxidase 8	Down	1.16
CL6020. Contig7	*GST*	Glutathione-S-transferase	Up	1.88
CL18580. Contig1	*CAT1*	Catalase 1	Up	1.25

3.8 The Expression of DEGs is Validated by qRT-PCR

To confirm the accuracy and reproducibility of the Illumina sequencing results, a total number of 13 up-regulated and 9 down-regulated DEGs in response to salt stress were randomly selected for qRT-PCR analyses. The result showed that the expression trends of these selected DEGs were consistent with those concluded by Illumina sequencing analyses, indicating that the transcriptome data in this study are reliable (Fig. 4).

4 Discussion

Salt stress generally causes osmotic stress, ion toxicity, oxidative damage and even nutrient imbalance on plants, which adversely affect plant growth and development [24]. To survive salt stress, plants respond and adapt to the saline environment at physiological, biochemical, morphological and developmental levels through regulating massive gene expressions [1,3,4]. In this study, a total number of 7521 DEGs between the control and the salt treatment groups (Fig. 1), involving a wide range of GO categories and KEGG pathways,

were obtained. Among them, GO categories and KEGG pathways involved in responses to abiotic stimulus, water deprivation, plant hormone signaling, photosynthesis, secondary product metabolism, lipid metabolism, carbohydrate metabolism and amino acid metabolism were significantly enriched (Fig. 2 and Fig. 3). These results reflected the complexity of salt signal transduction and its influence on photosynthesis and metabolism in *L. mollis*.

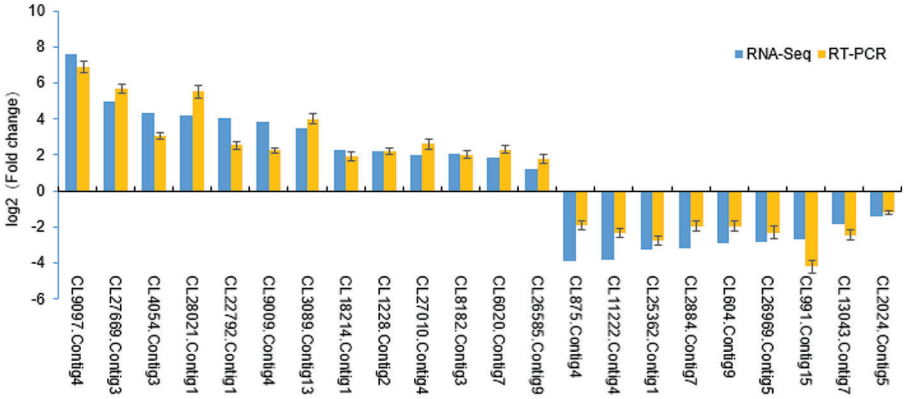

Figure 4: qRT-PCR validation of the differentially expressed genes (DEGs) between NaCl-free (CK) and NaCl-treated (CT) *L. mollis*. The expression levels of DEGs randomly selected from RNA-Seq data were verified by qRT-PCR analyses

Plant responses to salinity stress generally occur in two phases: a rapid response largely due to osmotic stress, and a slower response resulted by the accumulation of Na^+ in the shoot [1]. For the DEGs responsive to salt stress in *L. mollis*, the significantly enriched GO terms were predominantly associated with "response to abiotic stimulus", "response to water deprivation" and "oxidoreductase activity", suggesting that *L. mollis* was subjected to severe osmotic stress and oxidative stress under short-term salt stress (Fig. 2). However, genes related to "response to salt stress" did not exhibit the same enrichment possibly because of the Na^+ absorbed by the samples collected (in the first 48 h after salt treatment) did not accumulate to the level that causes serious ion toxicity.

Dehydrins are a kind of hydrophilic proteins belonging to the late embryogenesis abundant protein family. They protect biomacromolecules in plant cells from being damaged during dehydration [30,31]. Once plants are in a state of cellular dehydration, such as osmotic, temperature and salt stresses, dehydrins will accumulate in the growing tissues [32–34]. Aquaporins are a class of water transport proteins belonging to the major intrinsic protein (MIP) family and regulate cell water potential [35]. Thereinto, PIP and TIP are aquaporins located on the plasma membrane and vacuolar membrane, respectively, and they work together to maintain the normal metabolic process and improve the stress tolerance of plants [36]. In this study, four dehydrin genes and five aquaporin genes exhibited up-regulated expression in response to salt stress, indicating that *L. mollis* changed the membrane permeability to water by regulating aquaporin, and on the other hand, reduced the damage from salt stress by dehydrin to adapt to the saline environment (Table 3).

Cuticular wax is an important hydrophobic structure outside the epidermal cell wall of plants. It can reduce the non-stomatal dissipation of water and enhance the resistance of plants to drought, salt and high temperature [37]. In barley, it was found that leaf wax content and cuticular transpiration were significantly negatively correlated; when leaf wax content increased, cuticular transpiration decreased under salt stress [38]. Similar results were also observed in this study (Figs. 2 and 3). The DEGs related

to synthesis of cuticular wax were significantly enriched, suggesting that *L. mollis* possibly regulates cuticular transpiration by accumulating cuticular wax on leaves to improve the salt tolerance.

In saline environments, excessive Na^+ absorbed by roots is transported to the shoots over long distances, and eventually is accumulated in the leaves. High concentrations of Na^+ in the cytoplasm of leaves hinder the normal absorption of K^+, leading to K^+/Na^+ imbalance and metabolic disorders [25]. To prevent cytosolic Na^+ toxicity and maintain ion homeostasis inside the cell, plants generally exclude Na^+ to the apoplast and compartmentalize Na^+ into the vacuole by operating the Na^+/H^+ exchangers (NHXs) [39,40]. The vacuolar membrane Na^+/H^+ antiporters (NHXs) compartmentalize excess Na^+ from the cytoplasmic matrix into the vacuole to reduce the concentration of Na^+ in the cytoplasm. This process is energized by the H^+-ATPase or H^+-pyrophosphorylase located in the vacuolar membrane [5,41]. We found that the transcriptional level of *LmNHX2* was significantly increased in response to salt stress, suggesting that it may play an important role in ion compartmentation (Table 4). *LmAVP1* exhibited a decreased expression after salt treatment, indicating that *LmNHX2* was possibly driven by the proton pumps, other than AVP1, in *L. mollis* leaf cells (Table 4).

Salt stress can induce plants to produce reactive oxygen species (ROS) [26]. Low concentrations of ROS can act as signal molecules to regulate many biological processes in plants, including plant growth and responses to a variety of stresses [42,43]. However, excessive accumulation of ROS under abiotic stress conditions would result in oxidative stress, including lipid peroxidation in cellular membranes, DNA damage, protein denaturation and impairment of enzymatic activities [42]. To reduce the oxidative damage under high salinity conditions, plants rely on the activation of antioxidant enzymes and nonenzymatic antioxidant metabolites to scavenge excessive ROS [28]. We found that the transcriptional levels of genes encoding peroxidase, thioredoxin, L-ascorbate peroxidase, glutathione-S-transferase and catalase all exhibited a significant altered expression in response to NaCl treatment (Table 5). In addition, the DEGs involved in the biosynthesis of secondary metabolites (including carotenoid and flavonoid) showed significant enrichment (Fig. 3). All these results suggest that these antioxidant enzymes and secondary metabolites may take part in protecting *L. mollis* from oxidative damage caused by salt stress.

5 Conclusions

In this study, we sequenced and *de novo* assembled the transcriptome, and investigated the transcriptomic changes of *L. mollis* leaves in response to salt stress. A total of 110,323 and 112,846 unigenes were assembled for the NaCl-free (CK) and NaCl-treated (CT) libraries, respectively. 73,414 unigenes were successfully annotated for two libraries, and 7521 DEGs were identified between the CK and CT libraries. Numerous DEGs involved in environmental adaptation, signal pathway, photosynthesis and metabolism were identified. In addition, DEGs related to osmotic stress, ion homeostasis and oxidative stress were also analyzed. Our findings in this study will provide helpful cues for further research on the molecular mechanisms of salt responses in *L. mollis*.

Author Contributions: Luying Zhu designed the experiments. Wenting Wu, Yajing Zhang, Yu Gao and Kai Zhang conducted the experiments and analyzed the data. Luying Zhu and Hongxia Zhang wrote the manuscript.

Availability of Data and Materials: The raw sequencing data in this study were deposited at the NCBI Sequence Read Archive (SRA) database (Accession No. SRP109343).

Funding Statement: This work was supported by the National Natural Science Foundation of China (31400226), the Key Research and Development Program of Shandong Province (2019GSF110015), the Project of Shandong Province Higher Educational Science and Technology Program (J14LE07) and the Key Research and Development Program of Yantai, Shandong Province (2018XSCC043).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

1. Munns, R., Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology, 59 (1),* 651–681. DOI 10.1146/annurev.arplant.59.032607.092911.
2. Horie, T., Schroeder, J. I. (2004). Sodium transporters in plants. Diverse genes and physiological functions. *Plant Physiology, 136(1),* 2457–2462. DOI 10.1104/pp.104.046664.
3. Zhang, J. L., Shi, H. Z. (2013). Physiological and molecular mechanisms of plant salt tolerance. *Photosynthesis Research, 115(1),* 1–22. DOI 10.1007/s11120-013-9813-6.
4. Deinlein, U., Stephan, A. B., Horie, T., Luo, W., Xu, G. et al. (2014). Plant salt-tolerance mechanisms. *Trends in Plant Science, 19(6),* 371–379. DOI 10.1016/j.tplants.2014.02.001.
5. Zhu, J. K. (2003). Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology, 6(5),* 441–445. DOI 10.1016/s1369-5266(03)00085-2.
6. Zhou, C., Busso, C. A., Yang, Y. G., Zhang, Z., Wang, Z. W. et al. (2017). Effect of mixed salt stress on malondialdehyde, proteins and antioxidant enzymes of *Leymus chinensis* in three leaf colors. *Phyton-International Journal of Experimental Botany, 86(1),* 205–213, DOI 10.32604/phyton.2017.86.205.
7. Puranik, S., Jha, S., Srivastava, P. S., Sreenivasulu, N., Prasad, M. (2011). Comparative transcriptome analysis of contrasting foxtail millet cultivars in response to short-term salinity stress. *Journal of Plant Physiology, 168(3),* 280–287. DOI 10.1016/j.jplph.2010.07.005.
8. Wang, H., Zhang, M., Guo, R., Shi, D., Liu, B. et al. (2012). Effects of salt stress on ion balance and nitrogen metabolism of old and young leaves in rice (*Oryza sativa* L.). *BMC Plant Biology, 12,* 194. DOI 10.1186/1471-2229-12-194.
9. Yao, D., Zhang, X., Zhao, X., Liu, C., Wang, C. et al. (2011). Transcriptome analysis reveals salt-stress-regulated biological processes and key pathways in roots of cotton (*Gossypium hirsutum* L.). *Genomics, 98(1),* 47–55. DOI 10.1016/j.ygeno.2011.04.007.
10. Çiçek, N., Oukarroum, A., Strasser, R. J., Schansker, G. (2018). Salt stress effects on the photosynthetic electron transport chain in two chickpea lines differing in their salt stress tolerance. *Photosynthesis Research, 136(9),* 291–301. DOI 10.1007/s11120-017-0463-y.
11. Zhang, H., Yang, Z., You, X., Heng, Y., Wang, Y. (2021). The potassium transporter AtKUP12 enhances tolerance to salt stress through the maintenance of the K^+/Na^+ ratio in *Arabidopsis*. *Phyton-International Journal of Experimental Botany, 90(2),* 389–402. DOI 10.32604/phyton.2021.014156.
12. Fan, X., Sha, L. N., Yang, R. W., Zhang, H. Q., Kang, H. Y. et al. (2009). Phylogeny and evolutionary history of *Leymus* (Triticeae; Poaceae) based on a single copy nuclear gene encoding plastid acetyl-CoA carboxylase. *BMC Evolutionary Biology, 9,* 247. DOI 10.1186/1471-2148-9-247.
13. McGuire, P. E., Dvorak, J. (1981). High salt-tolerance potential in wheatgrasses. *Crop Science, 21(5),* 702–705. DOI 10.2135/cropsci1981.0011183x002100050018x.
14. Fatih, A. M. B. (1983). Analysis of the breeding potential of wheat-*Agropyron* and wheat-*Elymus* derivatives. *Hereditas, 98(2),* 287–295. DOI 10.1111/j.1601-5223.1983.tb00607.x.
15. Habora, M. E. E., Eltayeb, A. E., Tsujimoto, H., Tanaka, K. (2012). Identification of osmotic stress-responsive genes from *Leymus mollis*, a wild relative of wheat (*Triticum aestivum* L.). *Breeding Science, 62,* 78–86. DOI 10.1270/jsbbs.62.78.
16. Wang, J., Li, B., Meng, Y., Ma, X., Lai, Y. et al. (2015). Transcriptomic profiling of the salt-stress response in the halophyte *Halogeton glomeratus*. *BMC Genomics, 16,* 169. DOI 10.1186/s12864-015-1373-z.
17. Cui, J., Ren, G., Qiao, H., Xiang, X., Huang, L. et al. (2018). Comparative transcriptome analysis of seedling stage of two sorghum cultivars under salt stress. *Journal of Plant Growth Regulation, 37(3),* 986–998. DOI 10.1007/s00344-018-9796-9.

18. Yuenyong, W., Chinpongpanich, A., Comai, L., Chadchawan, S., Buaboocha, T. (2018). Downstream components of the calmodulin signaling pathway in the rice salt stress response revealed by transcriptome profiling and target identification. *BMC Plant Biology, 18,* 335. DOI 10.1186/s12870-018-1538-4.
19. Arisha, M. H., Aboelnasr, H., Ahmad, M. Q., Liu, Y., Tang, W. et al. (2020). Transcriptome sequencing and whole genome expression profiling of hexaploid sweetpotato under salt stress. *BMC Genomics, 21,* 197. DOI 10.1186/s12864-020-6524-1.
20. Zhang, P., Duo, T., Wang, F., Zhang, X., Yang, Z. et al. (2021). *De novo* transcriptome in roots of switchgrass (Panicum virgatum L.) reveals gene expression dynamic and act network under alkaline salt stress. *BMC Genomics, 22,* 82. DOI 10.1186/s12864-021-07368-w.
21. Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. et al. (2011). Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nature Biotechnology, 29(7),* 644–652. DOI 10.1038/nbt.1883.
22. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L., Wold, B. (2008). Mapping and quantifying mammalia transcriptome by RNA-seq. *Nature Methods, 5(7),* 621–628. DOI 10.1038/nmeth.1226.
23. Audic, S., Claverie, J. M. (1997). The significance of digital gene expression profiles. *Genome Research, 7(10),* 986–995. DOI 10.1101/gr.7.10.986.
24. Hasegawa, P. M., Bressan, R. A., Zhu, J. K., Bohnert, H. J. (2000). Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Molecular Biology, 51(1),* 463–499. DOI 10.1146/annurev.arplant.51.1.463.
25. Kronzucker, H. J., Coskun, D., Schulze, L. M., Wong, J. R., Britto, D. T. (2013). Sodium as nutrient and toxicant. *Plant and Soil, 369(1–2),* 1–23. DOI 10.1007/s11104-013-1801-2.
26. Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology, 141(2),* 391–396. DOI 10.1104/pp.106.082040.
27. Choudhury, F. K., Rivero, R. M., Blumwald, E., Mittler, R. (2017). Reactive oxygen species, abiotic stress and stress combination. *The Plant Journal, 90(5),* 856–867. DOI 10.1111/tpj.13299.
28. Hanin, M., Ebel, C., Ngom, M., Laplaze, L., Masmoudi, K. (2016). New insights on plant salt tolerance mechanisms and their potential use for breeding. *Frontiers in Plant Science, 7,* 1787. DOI 10.3389/fpls.2016.01787.
29. Kao, C. H. (2017). Mechanisms of salt tolerance in rice plants: Reactive oxygen species scavenging-systems. *Journal of Taiwan Agricultural Research, 66(1),* 1–8. DOI 10.6156/JTAR/2017.06601.01.
30. Brini, F., Saibi, W., Hanin, M., Amara, I., Gargouri, A. et al. (2010). The wheat dehydrin DHN-5 exerts a heat-protective effect on β-glucosidase and glucose oxidase activities. *Bioscience Biotechnology and Biochemistry, 74(5),* 1050–1054. DOI 10.1271/bbb.90949.
31. Hanin, M., Brini, F., Ebel, C., Toda, Y., Takeda, S. et al. (2011). Plant dehydrins and stress tolerance: Versatile proteins for complex mechanisms. *Plant Signaling and Behavior, 6(10),* 1503–1509. DOI 10.4161/psb.6.10.17088.
32. Danyluk, J., Perron, A., Houde, M., Limin, A., Fowler, B. et al. (1998). Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell, 10(4),* 623–638. DOI 10.2307/3870737.
33. Saavedra, L., Svensson, J., Carballo, V., Izmendi, D., Welin, B. et al. (2006). A dehydrin gene in *Physcomitrella patens* is required for salt and osmotic stress tolerance. *The Plant Journal, 45(2),* 237–249. DOI 10.1111/j.1365-313x.2005.02603.x.
34. Haimi, P., Vinskienė, J., Stepulaitienė, I., Baniulis, D., Stanienė, G. et al. (2017). Patterns of low temperature induced accumulation of dehydrins in *Rosaceae* crops-evidence for post-translational modification in apple. *Journal of Plant Physiology, 218,* 175–181. DOI 10.1016/j.jplph.2017.08.008.
35. Maurel, C., Verdoucq, L., Luu, D. T., Santoni, V. (2008). Plant aquaporins: Membrane channels with multiple integrated functions. *Annual Review of Plant Biology, 59(1),* 595–562. DOI 10.1146/annurev.arplant.59.032607.092734.
36. Maurel, C., Boursiac, Y., Luu, D. T., Santoni, V., Shahzad, Z. et al. (2015). Aquaporins in plants. *Physiological Reviews, 95,* 1321–1358. DOI 10.1152/physrev.00008.2015.

37. Carvalho, H. D. R., Heilman, J. L., McInnes, K. J., Rooney, W. L., Lewis, K. L. (2020). Epicuticular wax and its effect on canopy temperature and water use of *Sorghum*. *Agricultural and Forest Meteorology, 284,* 107893. DOI 10.1016/j.agrformet.2019.107893.
38. Hasanuzzaman, M., Davies, N. W., Shabala, L., Zhou, M., Brodribb, T. J. et al. (2017). Residual transpiration as a component of salinity stress tolerance mechanism: A case study for barley. *BMC Plant Biology, 17(1),* 107. DOI 10.1186/s12870-017-1054-y.
39. Ward, J. M., Hirschi, K. D., Sze, H. (2003). Plants pass the salt. *Trends in Plant Science, 8(5),* 200–201. DOI 10.1016/s1360-1385(03)00059-1.
40. Bassil, E., Blumwald, E. (2014). The ins and outs of intracellular ion homeostasis: NHX-type cation/H^+ transporters. *Current Opinion in Plant Biology, 22,* 1–6. DOI 10.1016/j.pbi.2014.08.002.
41. Maeshima, M. (2001). Tonoplast transporters: Organization and function. *Annual Review of Plant Physiology and Plant Molecular Biology, 52,* 469–497. DOI 10.1146/annurev.arplant.52.1.469.
42. Apel, K., Hirt, H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology, 55(1),* 373–399. DOI 10.1146/annurev.arplant.55.031903.141701.
43. Baxter, A., Mittler, R., Suzuki, N. (2013). ROS as key players in plant stress signaling. *Journal of Experimental Botany, 65(5),* 1229–1240. DOI 10.1093/jxb/ert375.

Supporting Information

Table S1: Primer sequences used for qRT-PCR in this study

Gene ID	Forward primer	Reverse primer
CL9097. Contig4	TTGCAGTCGTCCATGTGTGA	ATCATCGGTTGGTGGCACTT
CL27669. Contig3	GCTATCTGAAGCCGTTCGTG	GTCCGAGCTGTTCTTCGTCA
CL4054. Contig3	TGACGGCTGTGATGGATCTG	GGTCTTGTTGAGGTCGACGT
CL28021. Contig1	CCGTCAACACCTACAAACCC	GTAGATCCGGCAAAGCACC
CL22792. Contig1	GAGCAGATCGTCAAGGAGCA	GCCGACGTACTTGAAGTTGC
CL9009. Contig4	GTGCCGTTCCCGTTCTCTAA	TCATAGCAACAGTGCGAGGG
CL3089. Contig13	CCGTTTGCTGGTAGCCCATA	TGCACGCATCAACAAACACA
CL18214. Contig1	TCCTACGCCGACTTCTACCA	GGGTAGCATCAGGAAGACGG
CL1228. Contig2	AGCAGCAAAAACAGCCATCC	GCGTGTTTGTTGTGATGCGA
CL27010. Contig3	AAGCTCCAGGCTATCTCCCA	TGAAACTCTCCGACACGTCC
CL8182. Contig3	TGGGGTTGGTGTTGATCTCG	CATGCCGTGGAATACCCTCA
CL6020. Contig7	TACGAGAGGTACGGCGAGTT	TTGAGCAGGCCGATGAAGTC
CL26585. Contig9	TTGTCTTCCCGCTCTCGTTC	TTACGACTTGCTGCCTCCAG
CL875. Contig4	AAGTGCCTCGTGACGAAGAG	AAGCGTGTCAATGTGGGGAT
CL11222. Contig4	TACCAGCCACGTCATCCAAG	TTCTACGACAAGAGCTGCCC
CL25362. Contig1	CGACTCCCAAATCCACACGA	GGCCACCACTTATTTGCAGC
CL2884. Contig7	GACAAAAGGTCGCCTGCAAT	GTGGATGATTGGCTCTGGCT
CL604. Contig9	CCTTGCCTGCGTACTTGTTG	TCCTTGCTACGGCCATACTG
CL26969. Contig5	ATGGCTCTCAGGGTGATTGC	GTAGTCGATCGTGCCACTGT
CL991. Contig15	CGCAGCTCCACGAATACTCT	TCGATGCCCCTAAACCGATG
CL13043. Contig7	CTTGGTCAAAGTGCCTCTGC	GGTGTGGTTCCTCTTGCTGT
CL2024. Contig5	CTTGGTCAAAGTGCCTCTGC	GGTGTGGTTCCTCTTGCTGT
Actin	ATGAGTACGACGAGTCTGGC	TAGATGATAACGGCGGCGGA