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Research article

# Comparative transcriptome analysis provides insights into the distinct germination in sheepgrass (*Leymus chinensis*) during seed development

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ABSTRACT

Sheepgrass (*Leymus chinensis* ((Trin.) Tzvel)) is an important perennial forage grass that is widely distributed in the Eurasia steppe. The seed germination percentage show significant variation among the different germplasm in sheepgrass. However, the underlying molecular mechanisms of distinct germination during seed development are still mostly unknown. Here, we performed comparative transcriptomic analyses of high seed germination percentage (H) and low seed germination percentage (L) at 14, 28, and 42 days after pollination. After comparing 3 consecutive development stages, 9255, 5366, and 4306 genes were found to be significantly differently expressed between H and L. Pathway analysis indicated that transcripts related to starch and sucrose metabolism, phenylpropanoid biosynthesis, plant hormone signal transduction, amino sugar and nucleotide sugar metabolism, and photosynthesis were significantly changed between the two germplasm at three stages. ABA and GA metabolism- and signaling transduction-related genes were differentially expressed between two germplasm at development stages, suggesting that the reduced signaling of GA and ABA is likely to be related to seed germination and dormancy in sheepgrass. We also identified 81 transcription factor (TF) families, and some TFs genes such as *NAC48*, *NAC78*, *WRKY80*, *ZnFP*, *C3H14* and *ILR3* were significantly differential expressed in two germplasm. Our results provide insights into seed development, germination and dormancy in sheepgrass at the transcriptional level.

## 1. Introduction

Seed germination is one of the most important and critical developmental events in the life cycle of seed plants. Seed dormancy is an important agronomic trait in some gramineous plants that allows seeds to avoid germination under adverse conditions (Finkelstein et al., 2008). Under appropriate environmental conditions, seed germination occurs, and seed dormancy breaks. However, seed dormancy is influenced by both genetic and environmental factors; primary dormancy occurring during seed development includes two major phases: embryogenesis and seed maturation (Vaistij et al., 2013). During the stage of seed maturation, storage compounds are accumulated, tolerance to dehydration occurs, and metabolic activity stops (Graeber et al., 2012).

The ratio of two antagonistic phytohormones, abscisic acid (ABA, which induces seed maturation and promotes dormancy) and gibberellin (GA, which promotes seed germination), plays important roles in the regulation of seed germination and dormancy. ABA and GA synthesis and signaling are also important for seed dormancy and germination (Graeber et al., 2012; Nonogaki, 2014). In particular, hormone metabolism genes, such as the 9-cis-epoxycarotenoid dioxygenase (*NCED*) genes, are involved in ABA biosynthesis, and the *ABA-8′-hydroxylase* (*CYP707A*) genes are involved in ABA degradation. *GA2ox*, a

*Abbreviations:* bHLH, Basic helix-loop-helix; DAP, Days after pollination; DEGs, Different expressed genes; FDR, False discovery rate; FPKM, Fragments per kilobase per Million reads; GO, Gene Ontology; ILR, IAA-leucine-resistant; KEGG, Kyoto Encyclopedia of Genes and Genomes; MYB, Myeloblastosis, a transcription factor family; NAC, NAM, ATAF1,2, CUC2 transcription factor family; PCR, Polymerase chain reaction; qRT-PCR, Quantitative reverse transcription PCR; RNA-seq, Ribonucleic acid sequencing; TF, Transcription factor; ZnFP, zinc-finger protein

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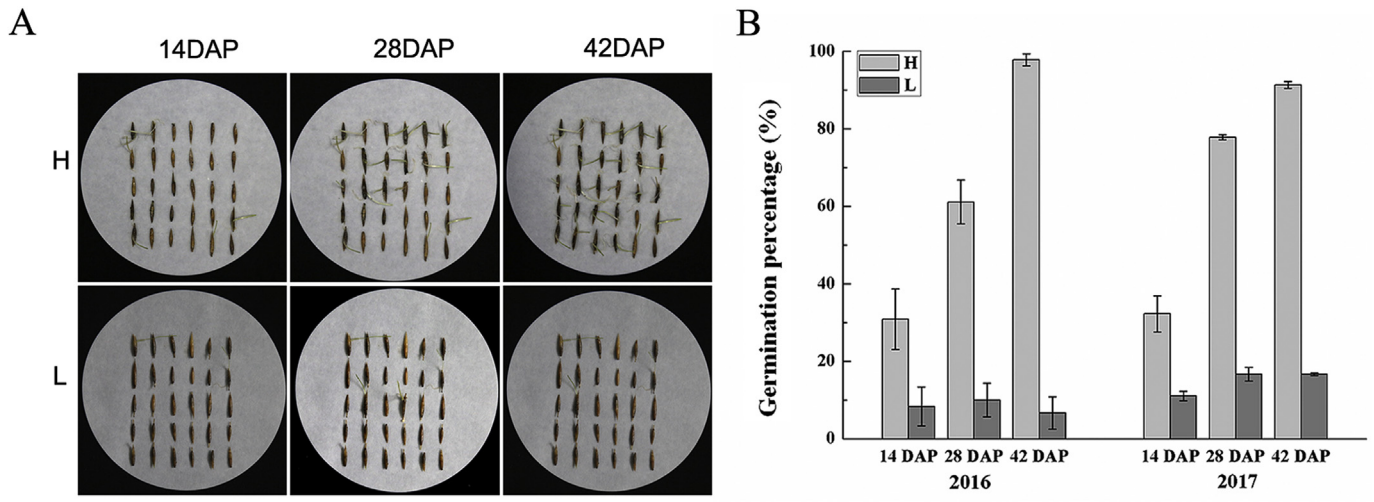


**Fig. 1.** Comprehensive analysis of seed germination percentage of two germplasm. A. The seed germination phenotype of H and L germplasm at 14, 28, and 42 DAP. B. Seed germination percentage at different developmental stages for two consecutive years. Approximately 120 seeds of each sample were used, and the seed germination percentages were calculated from the results of three independent experiments. Error bars represent ± standard errors (n = 3).

GA deactivation gene, and *GA3ox* and *GA20ox*, which are genes related to GA biosynthesis, have been shown to have universal roles in seed dormancy and germination mechanisms (Finkelstein et al., 2008). Furthermore, previous studies have indicated that the ABA-response transcription factors *ABA-INSENSITIVE3* (*ABI3*), *ABI4*, and *ABI5* affect seed dormancy and germination, whereas GA promotes germination by degradation of DELLA repressor proteins, which are negative regulators of GA signaling (Tyler et al., 2004; Vaistij et al., 2013).

Primary dormancy is induced during the seed maturation phase, and the mechanism for the establishment of primary dormancy is unclear, with only a few genes identified. DELAY OF GERMINATION 1 (*DOG1*) is a quantitative trait gene (QTG) that reveals the natural genetic variation of seed dormancy in *Arabidopsis thaliana* (Bentsink et al., 2006, 2010; Bai et al., 2018). Meanwhile, ectopic expression of wheat and barley *DOG1*-like genes promote seed dormancy in Arabidopsis (Ashikawa et al., 2010). In addition, mutations of the transcription factors FUSCA 3 (FUS3), LEAFY COTYLEDON 1 (LEC1) and LEC2 result in aberrant seed maturation and reduced dormancy levels (Graeber et al., 2012). Furthermore, abscisic acid-responsive kinase PKABA1 interacts with the wheat bZIP factor *TaABF* to influence the germination potential through repression of the transcription factor *GA-Myb* and activation of ABA-induced genes (Johnson et al., 2002).

Sheepgrass (*Leymus chinensis* (Trin.) Tzvel) is key specie in the Eurasia steppe and is widely distributed in North China. It belongs to *Leymus*, Poaceae family, BEP branch (Bambusoideae, Ehrhartoideae, and Pooideae) and is closely related to barley (*Hordeum vulgare* L) and wheat (*Triticum aestivum* L) (Buell, 2009; Chen et al., 2013). Sheepgrass has a high yield, high protein content, good palatability, and strong regeneration ability, and it also has strong cold resistance, saltine-alkali resistance, and tolerance to drought (Zhao et al., 2016; Gao et al., 2016; Li et al., 2013a, 2013b). Many germplasm show low seed germination, which restricts its production and utilization of sheepgrass. Previous studies have shown that the dormancy level of the seeds has decreased during the domestication process and that most of the seeds rapidly germinate after sowing compared with their wild ancestors (Graeber et al., 2012). Although our understanding of dormancy release in mature seeds is well advanced in some model plants and important crops (Gao et al., 2012; Du et al., 2015), the mechanisms involved in establishing dormancy during seed development are still unclear.

Our previous results showed that there were significant differences in seed germination among different germplasm during the seed development of sheepgrass, and a two-factor ANOVA analysis suggested that the germplasm has a large influence on seed germination (Yang et al., 2019). To better understand the potential mechanisms of seed germination and dormancy during seed development, transcriptome analyses were conducted in sheepgrass. Here, we produced the first transcriptional map of the seed developmental transcriptome of sheepgrass using Illumina paired-end sequencing technology. We sequenced three different seed development stages of two germplasm with high (H) and low (L) seed germination percentages to achieve the following objectives: (i) to catalog gene expression patterns during seed development across the three developmental stages, (ii) to characterize the major reasons for the variation in germination percentages of two sheepgrass germplasm during seed development, and (iii) to find the possible molecular mechanic for the difference of seed germination during seed development. Taken together, our data could serve as a valuable resource for transcriptomics studies related to seed germination characteristics for developmental biologists who are interested in studying seed development, dormancy, and germination.

## 2. Methods

### 2.1. Plant materials

Twenty germplasm of sheepgrass were selected for this study and grown in fields in the summer (May–July 2016 and 2017) at the experimental station of the Institute of Botany, the Chinese Academy of Sciences, Beijing. All seeds were harvested under strict hybridization conditions within each germplasm at the same development stage. The dry weight, fresh weight, and water content of the seeds were determined on the seventh, fourteenth, twenty-eighth, thirty-fifth, and 40-s day after pollination (DAP), and the different stages analyzed were based on our previous study (Yang et al., 2019). The seed germination percentage of each of the materials was also tested on different DAPs as a reference, and approximately 40 seeds were rinsed with running water and transferred into 9 cm Petri dishes with two layers of filter paper and 5 ml distilled water each in a growth chamber with fluorescent light ($\sim 500\ \mu mol\ m^{-2}s^{-1}$) under a 12-h light/12-h dark cycle at 28 °C/16 °C. A seed was considered to have germinated when the length of the germ is half of that of the seed, and the length of the radicle is about the same as that of the seed (International Seed Testing Association, 1996; Yang et al., 2019), and the final germination percentage was counted after 18 days of the incubation period. Approximately 120 seeds of each germplasm were used, and the seed germination percentages were calculated from the results of three independent experiments.

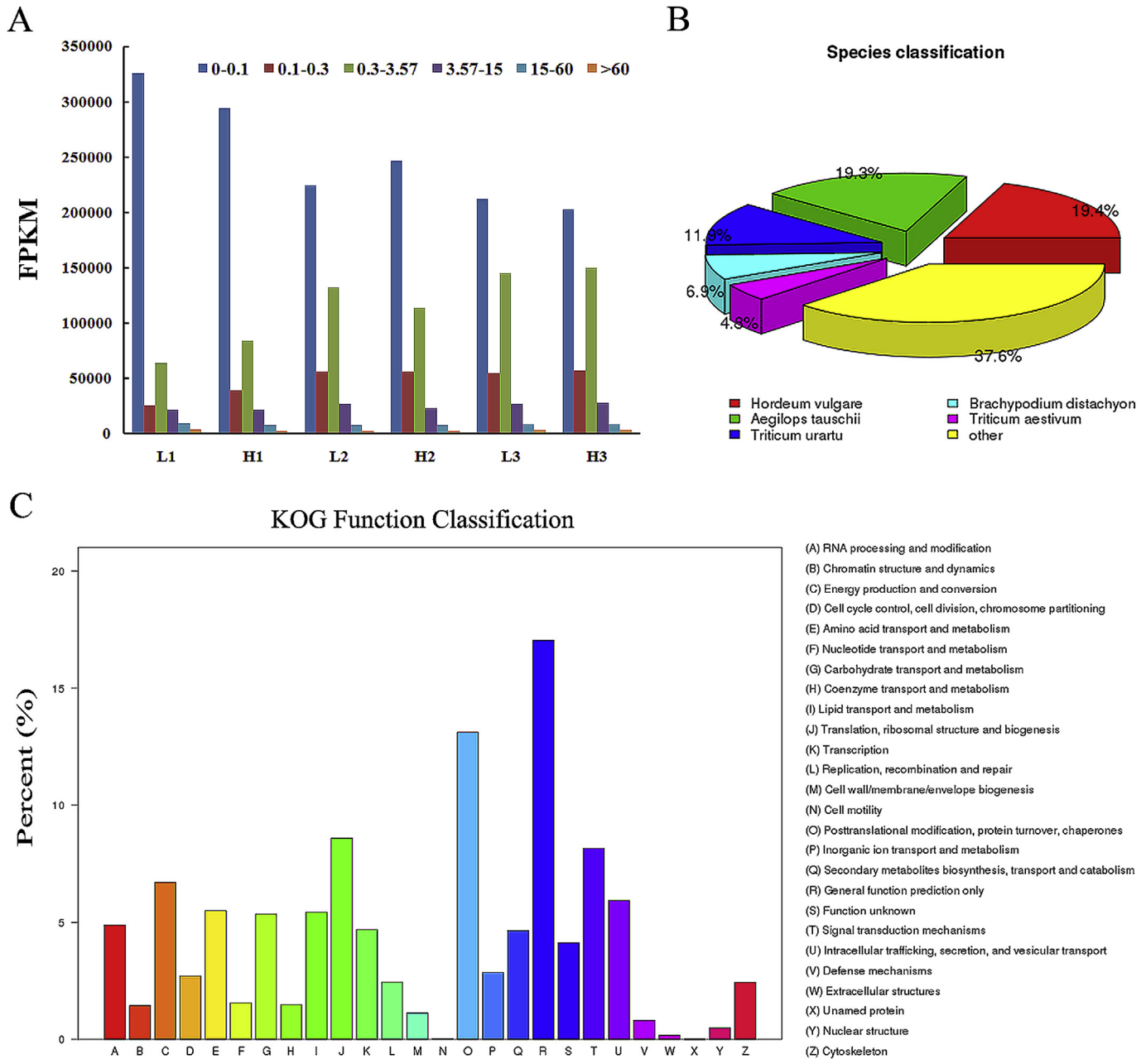


**Fig. 2.** Global analysis of transcriptome sequencing data. A. Proportion of FPKM values in different samples. Seeds of the H and L germplasm on the fourteenth (H1, L1), twenty-eighth (H2, L2), and 40-s (H3, L3) DAP were investigated, and each sample has two biological replicates. B. Species distribution of the BLASTX results of sheepgrass transcriptome. The numbers in the pie charts indicate the percentage of unique reads in each category. C. KOG function classification of assembled transcripts. Bars with numbers show the percentage of genes involved in a particular KOG category. The colors indicate different classifications. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### *2.2. RNA extraction, quantification and qualification*

For RNA preparation, the seeds of high germination percentages (H) and low germination percentages (L) germplasm on the fourteenth (H1, L1), twenty-eighth (H2, L2), and 40-s (H3, L3) DAP were separated from seeds pool of each germplasm. Two replicates of each sample were quickly frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using RNA Plant Plus (TaKaRa, Dalian, China). RNA degradation and contamination were monitored on 1% agarose gels. The quality and quantity of each RNA sample were assayed using a NanoDrop (Thermo Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

### *2.3. RNA-Seq and de novo transcriptome assembly*

Construction of the library and RNA-Seq was performed by Nobogene Corporation (Beijing, China). The RNA of the seeds was combined in equal quantity to construct a large pool. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations. The cDNA library was sequenced on the Illumina HiSeq™ X-ten platform using paired-end technology in a single run. Clean data were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads from raw data. The clean reads were assembled into contigs using the Trinity method, and the contigs were linked into transcripts according to the paired-end information of the sequences

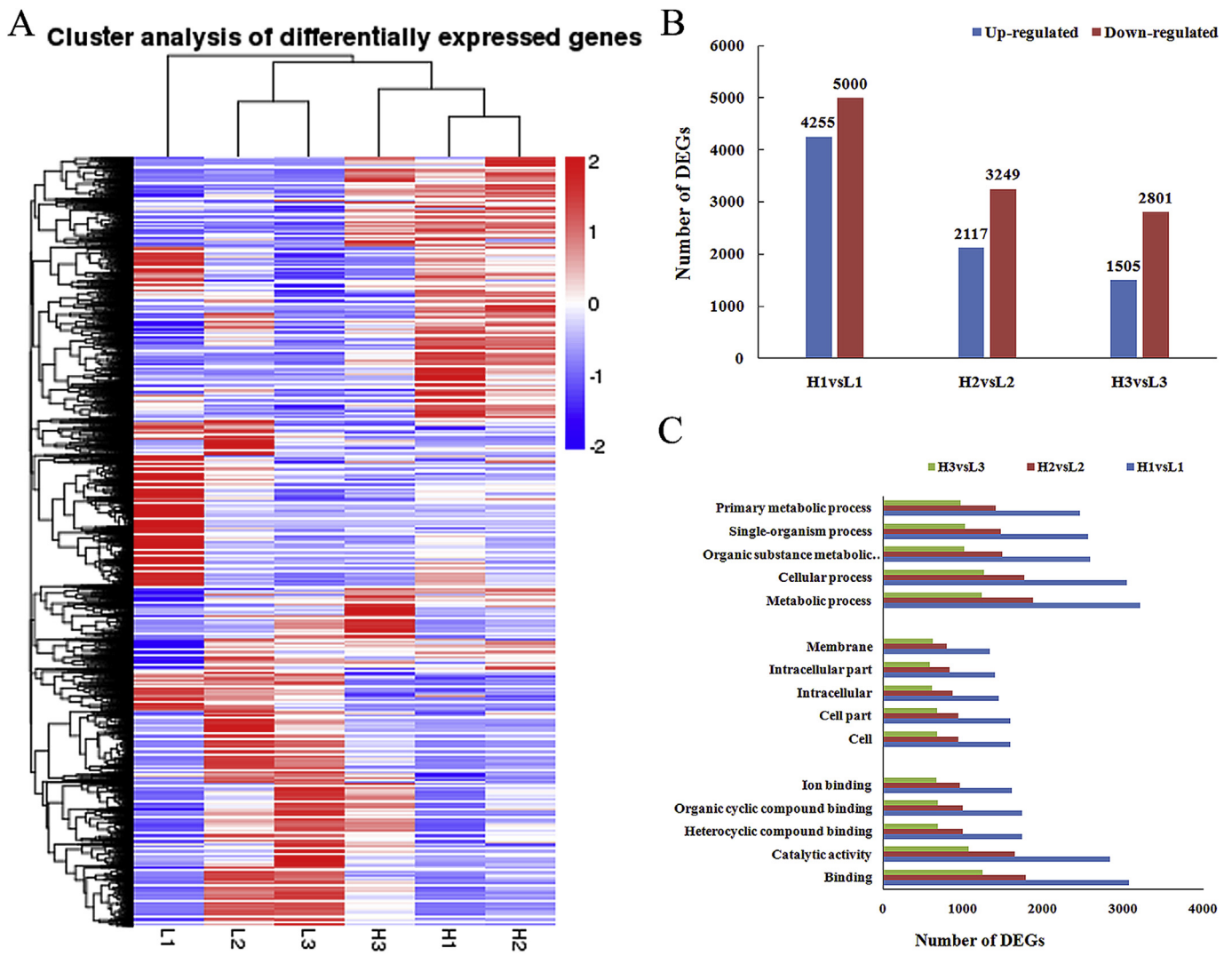


**Fig. 3.** Expression patterns of DEGs in two sheepgrass germplasm during the three seed development stages. A. Hierarchical clustering analysis of DEGs between two germplasm during the three development stages based on expression data. Red means up-regulated, and blue means down-regulated. B. Comparative analysis of differentially expressed genes (DEGs) between the two germplasm during the three development stages. C. Top 5 GO category annotation of differentially expressed genes between the seed development of the two germplasm at 14, 28, and 42 DAP. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Grabherr et al., 2011). Unigenes were combined to produce the final assembly used for annotation.

### 2.4. Functional annotation and classification

To predict the possible functions and biological pathways of the genes, all the assembled unigenes were searched against the following databases: the NCBI nonredundant protein database (NR), the SwissProt database, the Gene Ontology (GO) database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the Clusters of Orthologous Groups (COG) database. The specific methods used for soft annotations, pathways and GO function enrichment analyses were as described in previous studies (Zheng and Wang, 2008; Kanehisa and Goto, 2000). Furthermore, the NR and the SwissProt database with e-value 1e-5, the GO database with e-value 1e-6, the KEGG database with e-value 1e-10, and the COG database with e-value 1e-3.

### 2.5. Differential gene expression analysis

The reads from three seed development stages of two seed samples (H and L) were mapped onto the transcriptome assembly by BOWTIE 2 software according to the quantified default parameters (Langmead and Salzberg, 2012). Read counts per gene were expressed as the expected number of fragments per kilobase of transcripts per million mapped fragments (FPKM), and unigene abundance differences between the samples were calculated based on the ratio of the FPKM values and the false discovery rate (FDR). Genes with an adjusted P-value $< 0.05$ and an absolute value of "fold change $> 2$" were deemed to be significantly differentially expressed between the two samples. The FPKM values of related genes were used to plot heatmaps.

### 2.6. Mining transcription factor families

For mining transcription factor gene families (TFs), we downloaded the plant transcription factors database from the Plant Transcription Factor Database website (http://planttfdb.cbi.pku.edu.cn/) to construct a local database (Zhang et al., 2011).

### 2.7. Verification by qRT-PCR

To verify the validity of RNA-Seq, quantitative real-time RT-PCR (qRT-PCR) was conducted as previously described (Zhao et al., 2016).

**Table 1**
Top 10 KEGG enrichment analysis of DEGs in the three seed development stages: H1 vs L1, H2 vs L2, and H3 vs L3.

| Stage | Pathway term | Gene number |
|---|---|---|
| H1vsL1 | Starch and sucrose metabolism | 121 |
| | Phenylpropanoid biosynthesis | 96 |
| | Amino sugar and nucleotide sugar metabolism | 59 |
| | Galactose metabolism | 58 |
| | Plant hormone signal transduction | 58 |
| | Alanine, aspartate and glutamate metabolism | 55 |
| | Glycerophospholipid metabolism | 53 |
| | Cyanoamino acid metabolism | 43 |
| | Glycerolipid metabolism | 41 |
| | Arginine and proline metabolism | 34 |
| H2vsL2 | Phenylpropanoid biosynthesis | 47 |
| | Glutathione metabolism | 30 |
| | Cysteine and methionine metabolism | 30 |
| | Photosynthesis | 23 |
| | Flavonoid biosynthesis | 22 |
| | alpha-Linolenic acid metabolism | 21 |
| | Cyanoamino acid metabolism | 20 |
| | Diterpenoid biosynthesis | 17 |
| | Arginine biosynthesis | 17 |
| | Stilbenoid, diarylheptanoid and gingerol biosynthesis | 14 |
| H3vsL3 | Plant hormone signal transduction | 21 |
| | Galactose metabolism | 17 |
| | RNA degradation | 16 |
| | Alanine, aspartate and glutamate metabolism | 14 |
| | Terpenoid backbone biosynthesis | 12 |
| | Sphingolipid metabolism | 10 |
| | Circadian rhythm - plant | 8 |
| | Steroid biosynthesis | 8 |
| | Basal transcription factors | 8 |
| | Diterpenoid biosynthesis | 7 |

qRT-PCR of 6 differentially expressed TFs was conducted using the total RNA extracted from the seeds of each accession. Gene-specific primers were designed using Primer 5, and the actin gene was used as a reference to normalize the expression data. All the primer sequences are presented in Table S1. QRT-PCR was performed using the Roche LightCycler 480 system (Roche), and relative quantitative data were calculated using the $2-^{\Delta\Delta CT}$ method. qRT-PCR experiments were performed in three technical replicates and with at least two biological replicates.

### 2.8. Data analysis

The statistics analysis was conducted with SPSS software 22 (SPSS. Inc) for windows. One-Way ANOVA analysis was conducted to investigate the variance of seeds germination percentage between H and L germplasm during three development stage.

## 3. Results

### 3.1. Analysis of seed characteristics during seed development in sheepgrass

In our previous study, the seed germination of sheepgrass showed significant variation among the different germplasm during seed development stages, and many germplasm exhibited seed dormancy (Fig. S1). To investigate seed germination variations in sheepgrass, we selected two germplasm with high (H) and low (L) germination percentages during development stages for the additional studies. According to the difference of seed germination percentage between two germplasm, seed development stages were divided into three periods, 14 DAP, 28 DAP, and 42 DAP. The results of two consecutive years indicated that the differences in seed germination percentages in H and L appeared during the various seed development stages and became more evident at stages 28–42 DAP. At 28 DAP and 42 DAP, the seed germination percentages of the H germplasm were more than 60% and 90%, while the germination percentage of the L germplasm was less than 10% (Fig. 1A and B).

### 3.2. RNA-Seq analysis of developing seeds of two sheepgrass germplasm

For a comprehensive understanding of transcriptional mechanisms regulating seed dormancy establishment, we investigated three stages of seed development (14, 28, and 42 DAP) in two sheepgrass germplasm with high and low germination (H1, H2, and H3 and L1, L2, and L3, respectively) for RNA-Seq analysis. Using the Illumina HiSeq X-ten platform, a total of 824 432 994 raw and 791 755 010 clean reads were obtained with 93.1% Q30 bases (percentage of sequences with sequencing error percentages < 0.1%) in each library, and the number of raw reads and clean for each sample and replicate were supplied in Table S2. The transcriptome sequence was de novo assembled using Trinity software (Grabherr et al., 2011), and the sequence data were deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA507496.

The fragments per kilobase of transcript per million mapped fragments (FPKM) values of all genes were used to analyze the correlation between each of the samples (Trapnell et al., 2010). We estimated the expression levels of transcripts using FPKM, and the largest portion of transcripts showed medium expression $0 < FPKM < 3.57$ (Fig. 2A, Fig. S2). All the unigenes were annotated on the basis of similarity to the public NCBI nonredundant protein database (NR), NCBI nucleotide database (NT), Swiss-Prot protein database (Swiss-Prot, http://www.expasy.ch/sprot), Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/), Cluster of Orthologous Groups database (COG/KOG, http://www.ncbi.nlm.nih.gov/COG/), and the Gene Ontology database (GO). The results showed that NR has the highest proportion of successful annotations, while KEGG has the lowest proportion (Fig. S3). NR database queries revealed that sheepgrass unigenes have a significantly close match to *Hordeum vulgare* (19.4%), *Aegilops tauschii* (19.3%), *Tritcum urartu* (11.9%), *Brachypodium distachyon* (6.9%), *Triticum aestivum* (4.8%), and others (37.6%) (Fig. 2B). To further evaluate the completeness of the de novo transcriptome assembly and to predict the gene functions, all unigenes were annotated by the KOG database. The KOG classifications of all the unigenes were grouped into 26 functional categories, and significantly enriched functions were found, such as “General function prediction only” (17.04%), “Posttranslational modification, protein turnover, chaperones” (13.11%), and “Translation, ribosomal structure and biogenesis” (8.58%) (Fig. 2C).

### 3.3. Identification of differentially expressed genes between the two germplasm

We analyzed genes that were differentially expressed (DEGs) between the two germplasm during the three development stages as indicators of germination differentiation. Clustering analysis showed that the expression profiles of the DEGs varied significantly in two germplasm at 14, 28, and 42 DAP. Furthermore, principal component analysis revealed that the H samples from the three stages were clustered together, and L samples from the 28 DAP and 42 DAP were clustered together, suggesting that the overall transcriptome profiling is different for H and L at each developmental stage (Fig. 3A). To further reveal the molecular events occurring during seed development of the two sheepgrass germplasm, we filtered the DEGs with expression levels of FPKM > 5, log2-fold change > 2 or < −2, and FDR < 0.05 in at least one of the comparisons were considered DEGs for further analysis. We identified 9255 DEGs, namely, 4255 up-regulated genes and 5000 down-regulated genes, for the H germplasm compared with the L germplasm at 14 DAP; 5366 DEGs, namely, 2117 up-regulated genes and 3249 down-regulated genes, at 28 DAP; and 4306 DEGs, namely, 1505 up-regulated genes and 2801 down-regulated genes, at 42 DAP (Fig. 3B). These results indicate the dramatic changes in the

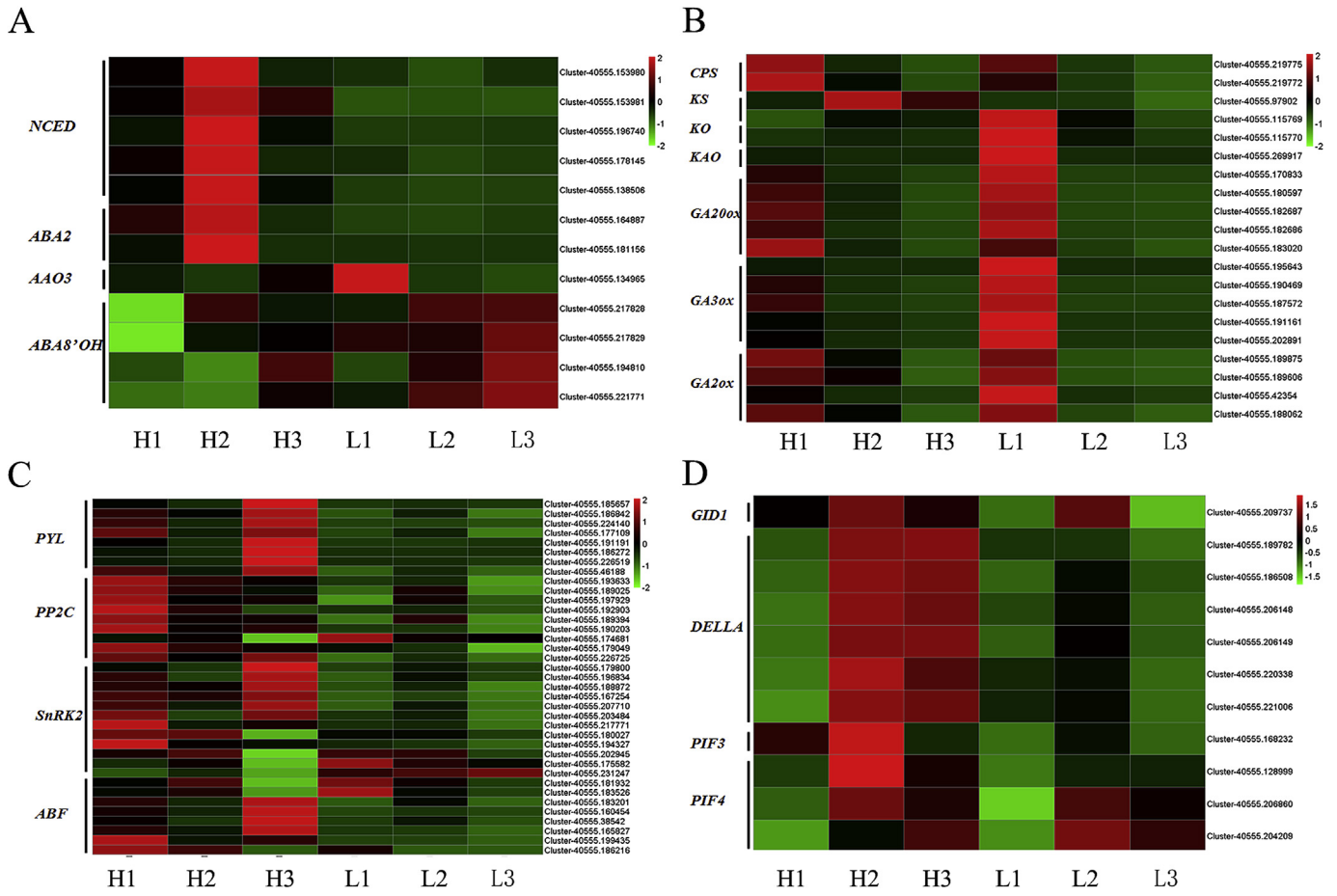


**Fig. 4.** Heatmap analyses of the genes involved in GA and ABA metabolic and signal transduction. A. ABA and B. GA metabolism-related genes. C. ABA and D. GA signaling pathway-related genes.

transcriptional programs of the two germplasm during the seed development stage (Table S3).

Furthermore, GO term analysis showed that genes coding for proteins involved in "metabolic process", "cellular process", "organic substance metabolic process", "single-organism process", and "primary metabolic process" were highly represented in the biological process category. Within the cellular component category, the terms "cell", "cell part", "intracellular", "intracellular part" and "membrane" were dominant. In the molecular function category, most unigenes were assigned to "binding", "catalytic activity", "heterocyclic compound binding", "organic cyclic compound binding" and "ion binding" (Fig. 3D and Table S4).

### 3.4. Pathway analysis of DEGs

We also identified the top 20 enriched KEGG pathways between the two germplasm during the three seed development stages, and the DEGs involved in starch and sucrose metabolism (121), phenylpropanoid biosynthesis (96), amino sugar and nucleotide sugar metabolism (59), galactose metabolism (58), and plant hormone signal transduction (58) all had significantly different expression between two germplasm at 14 DAP. However, genes involved in phenylpropanoid biosynthesis (47), glutathione metabolism (30), cysteine and methionine metabolism (30), photosynthesis (23), and flavonoid biosynthesis (22) were found to have uniformly different expression at 28 DAP. In contrast, DEGs in plant hormone signal transduction (21); galactose metabolism (17); RNA degradation (16); alanine, aspartate and glutamate metabolism (14); and terpenoid backbone biosynthesis (12) were presumed to be critical at 42 DAP (Table 1 and Table S5).

### 3.5. Expression patterns of gibberellic acid (GA) and abscisic acid (ABA) metabolic genes during seed development stages

Seed ABA and GA contents are regulated by the balance between their biosynthesis and catabolism (Graeber et al., 2012). Analysis of our data set showed genes encoding ABA metabolic enzymes, 9-cis-epoxycarotenoid dioxygenase (*NCED*), ABA deficient 2 (*ABA2*), and abscisic aldehyde oxidase 3 (*AAO3*) had different expression between high and low germination samples during the three stages. Specific members of the *NCED* family genes control the ABA level and dormancy in developing seeds, and the expression levels of the *NCED* and *ABA2* genes were higher in the L than the H germplasm at 14 DAP. The *AAO3* exhibited a higher FPKM in L seeds at 28 DAP. In contrast, the expression of the ABA catabolic genes *ABA8' OH* (*CYP707A*) was higher during stage 3 in both germplasm, although some gene expression was higher in L during the first stage (Fig. 4A). These results indicate that specific members of the *NCED*, *ABA2*, *AAO3* and *ABA8' OH* family are regulated by germplasm and development stages. Similarly, the GA metabolism genes *CPS* (*ent*-copalyl diphosphate synthase), *KS* (*ent*-kaurene synthase), *KO* (*ent*-kaurene oxidase), *KAO* (*ent*-kaurenoic acid oxidase), *GA20ox* (GA 20-oxidase), *GA3ox* (GA 3-oxidase), and *GA2ox* (GA 2-oxidase) are found among the sheepgrass transcripts. These gene families exhibited up-regulation in both H and L seeds at 14 DAP, suggested that the synthesis and metabolism of GA were more active after 14 days of pollination (Fig. 4B).

### 3.6. The genes involved in GA and ABA signal transduction

To identify specific ABA and GA signaling components that are

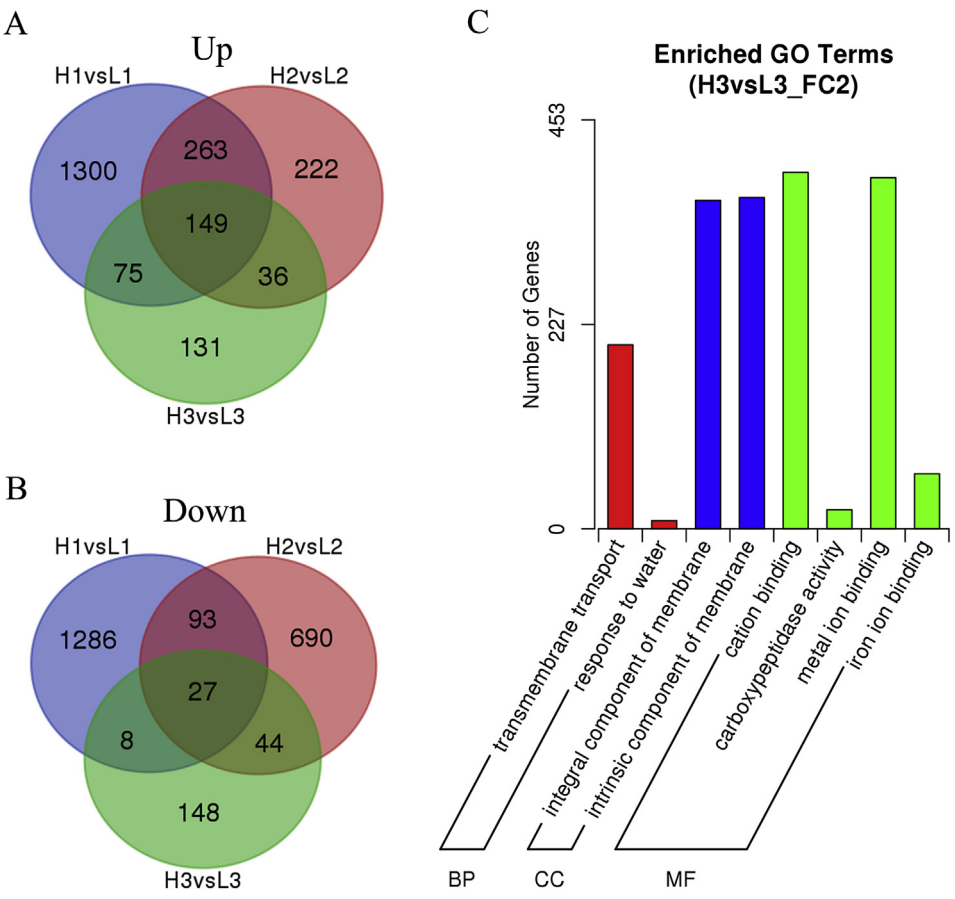


**Fig. 5.** The DEGs were significantly between two germplasm during the three stages. A. The numbers of up- or down-regulated genes B. between two germplasm at 14, 28, and 42 DAP. C. GO category annotation in the biological process, cellular component and molecular function of differentially expressed genes between H3 vs L3.

involved in the two sheepgrass germplasm, we compared the expression of genes related to ABA and signaling between high seed germination and low seed germination materials during the three seed development stages. ABA receptor pyrabactin resistance 1/PYR1-like/regulatory components of the ABA receptor (PYR1/PYL/RCAR), group A type 2C protein phosphatases (PP2Cs), and sucrose nonfermenting 1-related protein kinases (SnRKs) subfamily 2 (SnRK2s) have been proven to be key components of ABA signaling (Fujita et al., 2009; Park et al., 2009). Our results indicated that most PYL, PP2Cs, SnRK2s, and ABRE-binding factor (ABF) family genes displayed similar expression patterns between the H and L seeds and that the level of FPKM was higher in H than in L during the three stages (Fig. 4C). These results might suggest that specific members of the *PP2C* genes of sheepgrass are involved in the regulation of seed germination by induced loss of seed ABA sensitivity and dormancy during seed development stages. Furthermore, several GA signaling factors are involved in regulating seed germination. The action of GA takes place through GA-insensitive dwarf mutant 1 (*GID1*), a soluble GA receptor protein, that exhibits higher expression in H than in L during all three stages. However, the expression levels of *DEEAL* are higher in H2 and H3. Similarly, phytochrome-interacting factor 3 (PIF3) and PIF4 genes are also higher in the H germplasm at 28 DAP (Fig. 4D). In general, GA and ABA signal transduction-related genes are more active in H than in L.

### 3.7. Identification of significantly differently expressed genes between the two germplasm

To further identify the genes closely correlating with the two germplasm in the three-developmental stages, the DEGs were further filtered with expression levels of log2-fold change > 5 or < −5 in H vs L at the three developmental stages (Table S6). At each of the three different DAP stages, 1787, 670, and 391 genes were significantly up-regulated in H1vsL1, H2vsL2, and H3vsL3, respectively. The same analysis showed that 1414, 854, and 227 genes were down-regulated in H1vsL1, H2vsL2, and H3vsL3, respectively. Moreover, 149 and 27 genes were significantly up- or down regulated, respectively, in samples of the high germination germplasm H compared with L during all three stages (Fig. 5A and B). The representative genes for the up- or down-regulated DEGs in H are listed in Table 2 according to their functional description. Our results indicated that the expression of the zinc finger CCCH domain-containing protein, expansin-B4, dehydrin, cytochrome P450, and chitinase 5 genes were significantly up-regulated, while the NAC domain-containing protein 83, transmembrane proteins 14C, and protein-tyrosine phosphatase were down-regulated (Fig. 5C, Table 2 and Table S6).

### 3.8. The expression pattern of transcription factors

A total of 10,086 transcription factors (TFs) classified into 81 TFs families were identified. The top 5 TFs families were C2H2 (7.53%), Zn-clus (6.91%), bZIP (5.53%), Orphans (4.98%), and MYB (4.57%) (Table S7). The results indicated that some transcription factor families are expressed in specific germplasm or development periods. For example, most genes in the basic helix loop-helix (bHLH) and WRKY transcription factor family peaked in L at 14 DAP (L1) and were only expressed at low levels in other phases (Fig. 6A and B). The expression of some NAC and MYB family genes was up-regulated in both germplasm at 14 DAP (H1 and L1) (Fig. 6C and D); however, the Zn-clus and bZIP transcription factors were overrepresented in H3 and L3 (Fig. 6E and F). In addition to the above six transcription factor families, the C2H2 and C3H transcription factors were expressed differently during different phases (Fig. 6G and H).

**Table 2**
The DEGs were significantly up- or down-regulated in the H1 vs L1, H2vs L2, and H3vs L3.

| | gene_id | Description | H1vsL1 | H2vsL2 | H3vsL3 |
|---|---|---|---|---|---|
| **Up** | Cluster-40555.181103 | Zinc finger CCCH domain-containing protein 37 | 8.71 | 7.07 | 8.20 |
| | Cluster-40555.190917 | Zinc finger CCCH domain-containing protein 2 | Inf | 6.61 | Inf |
| | Cluster-40555.212569 | Zinc finger CCCH domain-containing protein 14 | 3.66 | 3.14 | 2.9125 |
| | Cluster-40555.187541 | NAC domain-containing protein 48 | 6.79 | 5.90 | 3.59 |
| | Cluster-40555.192486 | NAC domain-containing protein 78 | 5.43 | 5.02 | 4.47 |
| | Cluster-40555.181741 | putative Zn-finger transcription factor | 8.07 | 7.52 | 4.64 |
| | Cluster-40555.190381 | Uncharacterized protein C24B11.05 | 10.05 | 11.11 | 5.25 |
| | Cluster-40555.166523 | Ubiquitin domain-containing protein DSK2a | Inf | 6.47 | 5.65 |
| | Cluster-40555.207004 | Two pore potassium channel b | Inf | Inf | Inf |
| | Cluster-40555.189917 | Transcription elongation factor 1 homolog | 7.81 | 6.96 | Inf |
| | Cluster-40555.180045 | Sugar transport protein 10 | 6.78 | Inf | Inf |
| | Cluster-40555.187847 | Stem-specific protein TSJT1 | 8.38 | 8.25 | Inf |
| | Cluster-40555.190309 | Reticulon-like protein B1 | 8.88 | 5.52 | Inf |
| | Cluster-40555.232229 | Quinone oxidoreductase 1 | Inf | Inf | 5.86 |
| | Cluster-40555.187503 | Putative ribonuclease H protein | 8.03 | 9.66 | 8.20 |
| | Cluster-40555.205596 | Protein ZINC INDUCED FACILITATOR-LIKE 1 | 8.08 | Inf | Inf |
| | Cluster-40555.181905 | Protein YLS9 | 8.82 | Inf | 5.98 |
| | Cluster-40555.190851 | Protein translation factor SUI1 homolog | 7.33 | 9.63 | 6.77 |
| | Cluster-40555.200540 | Nucleobase-ascorbate transporter 6 | 7.67 | 7.57 | Inf |
| | Cluster-40555.183316 | Non-specific lipid-transfer protein Cw18 | 7.41 | 6.25 | 6.46 |
| | Cluster-40555.192333 | Kynurenine formamidase | Inf | Inf | Inf |
| | Cluster-40555.194563 | Horcolin | 10.40 | 7.91 | 8.49 |
| | Cluster-40555.198450 | F-box/kelch-repeat protein | Inf | 8.37 | 8.47 |
| | Cluster-40555.207652 | F-box protein SKP2A | Inf | Inf | Inf |
| | Cluster-40555.182269 | Expansin-B4 | 8.38 | 7.88 | 6.09 |
| | Cluster-40555.180182 | Eukaryotic translation initiation factor 3 subunit B | 6.59 | Inf | 6.70 |
| | Cluster-40555.189995 | Dehydrin DHN4 | 8.55 | 8.22 | 7.64 |
| | Cluster-40555.193614 | Cytochrome P450 | Inf | Inf | Inf |
| | Cluster-40555.124734 | Copper transport protein ATX1 | Inf | Inf | Inf |
| | Cluster-40555.171065 | Chitinase 5 | Inf | 7.16 | 6.05 |
| | Cluster-40555.188785 | Basic endochitinase A | 9.36 | 8.22 | 7.93 |
| | Cluster-40555.184939 | Anamorsin homolog 2 | Inf | 6.10 | Inf |
| | Cluster-40555.189872 | Allene oxide synthase 2 | 7.70 | 8.85 | 6.35 |
| | Cluster-40555.204237 | Agglutinin isolectin 2 | 7.07 | 7.04 | Inf |
| | Cluster-40555.186216 | ABSCISIC ACID-INSENSITIVE 5-like protein 5 | Inf | 6.60 | Inf |
| | Cluster-40555.190050 | 36.4 kDa proline-rich protein | 6.08 | Inf | Inf |
| **Down** | Cluster-40555.234751 | Transcription factor ILR3 | −5.49 | −3.13 | −4.41 |
| | Cluster-40555.192086 | WRKY80 transcription factor | −9.64 | −6.39 | −4.72 |
| | Cluster-40555.184480 | NAC domain-containing protein 83 | −9.40 | −7.59 | -Inf |
| | Cluster-40555.199319 | Transmembrane proteins 14C | −6.42 | -Inf | -Inf |
| | Cluster-40555.193802 | REV protein (anti-repression trans-activator protein) | −5.90 | -Inf | −5.71 |
| | Cluster-40555.130307 | Protein-tyrosine phosphatase | -Inf | −6.03 | -Inf |
| | Cluster-40555.148791 | mRNA cap methylation, RNMT-activating mini protein | −5.49 | −9.77 | −10.19 |
| | Cluster-40555.302720 | Domain of unknown function (DUF1939) | −8.51 | −10.48 | −5.54 |
| | Cluster-40555.99856 | AT hook motif | -Inf | −5.19 | −6.60 |

### *3.9. Validation of differentially expressed candidate TFs genes*

To confirm the accuracy and reproducibility of the RNA-Seq results, qRT-PCR was performed to examine the expression patterns of 6 transcription factors genes that were significantly expressed differently in the H and L germplasm during the seed development stages. Our qRT-PCR results showed that their relative expression based on qRT-PCR was consistent with the RNA-Seq data between the two germplasm (Fig. 7). The expression levels of *NAC48*, *NAC78*, *C3H14*, and *ZnFP* (a zinc-finger protein) had a higher abundance in H than in L during the three stages. In contrast, the transcription factors *WRKY80* and *ILR3* had a lower abundance in H than in L (Fig. 7).

## 4. Discussion

The seed dormancy process is an important component of plant fitness allowing plants to avoid adverse environment conditions (Graeber et al., 2012). Dormancy is a complex trait and is determined by genetic and environmental influences. Previous studies suggested that primary dormancy is induced during the seed maturation phase and reaches a high level in freshly harvested seeds and that dormancy slowly reduces during subsequent dry storage of seeds (Holdsworth et al., 2008; Graeber et al., 2012; Yan et al., 2018). Arabidopsis seed primary dormancy is promoted by low-temperature conditions during seed development, and a low temperature also decrease seed germination rate in rice (Wang et al., 2018). To better understand the mechanism of seed dormancy, germination and storability, the transcriptome and proteomic methods were conducted in some plants (Chen et al., 2018; Vishweshwaraiah et al., 2018). Sheepgrass is valuable for animal husbandry and ecological conservation in China, and low seed germination has become a bottleneck for its propagation and utilization (Chen et al., 2013). Previous studies found that many germplasm of sheepgrass undergo seed dormancy, and germination temperature, mechanical resistance of glumes and inhibition of endosperm are the main factors controlling dormancy and germination of sheepgrass seeds (Ma et al., 2010; Hu et al., 2012). Furthermore, the seed can reach its maximum germination percentage and energy harvested at 39 days (Lin et al., 2016). In our recent studies, the seed morphological and physical changes as well as the seed germination percentage of 20 sheepgrass germplasms during seed development stages were analyzed, and we found that the seed germination percentages of sheepgrass showed significant variation among the germplasm during seed development stages (Fig. 1, Fig. S1), suggesting that the seed dormancy of some germplasm is formed during development (Yang et al., 2019). In addition, two consecutive years of germination results showed that germplasm rather than environmental conditions is

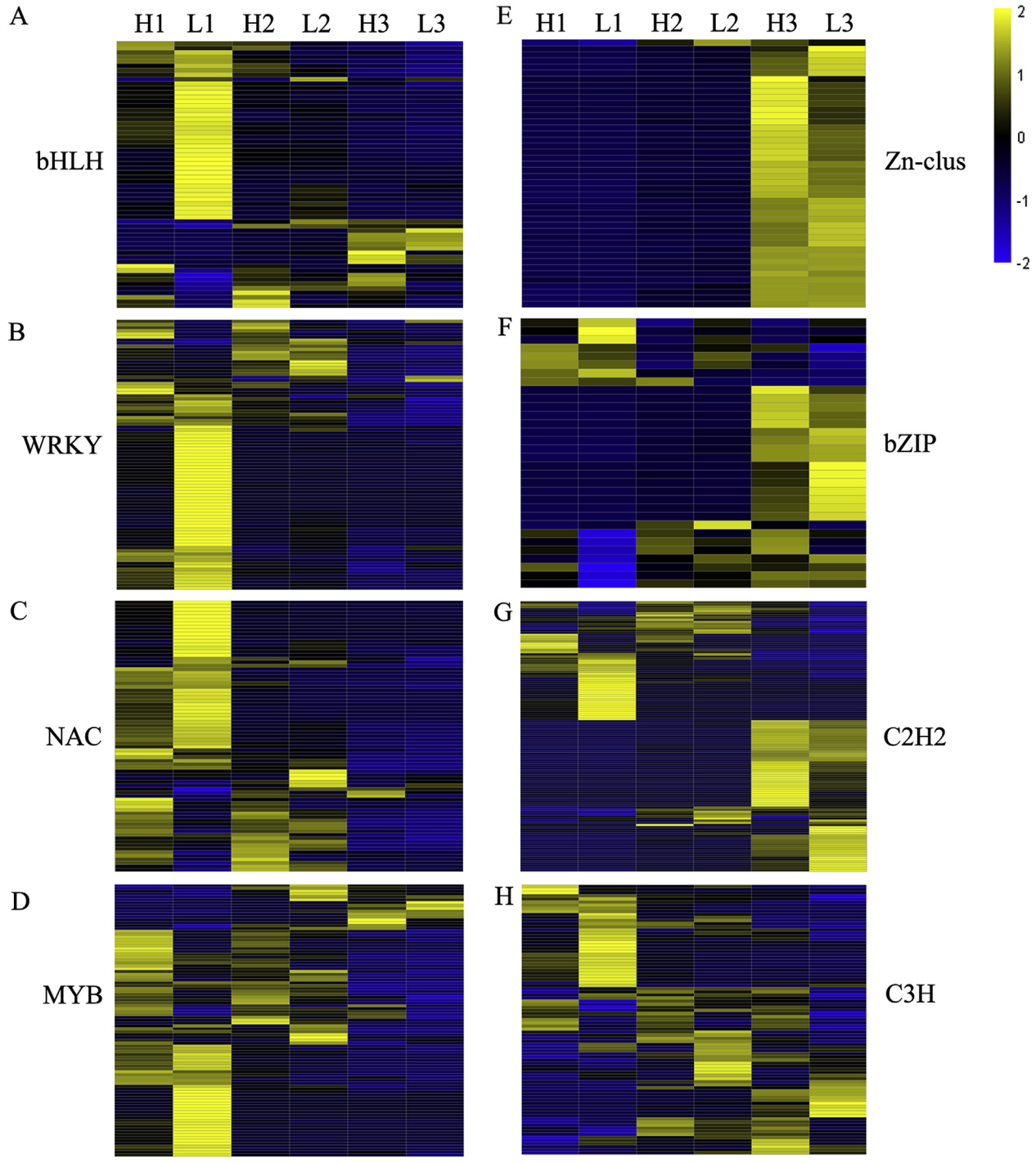


**Fig. 6.** Transcription factor profiling according to the RNA-Seq data. The heat map shows the expression of A. *bHLH*, B. *WRKY*, C. *NAC*, D. *MYB*, E. *Zn-clus*, F. *bZIP*, G. *C2H2*, and H. *C3H* transcription factor families.

the major factor in determining germination during seed development (Yang et al., 2019).

At present, there are few studies on the molecular and physiological mechanisms of seed germination and dormancy in sheepgrass. Moreover, there have been no studies conducted to investigate the molecular mechanisms of dormancy during seed development. Hence, a comparison of the gene expression profiling between the two sheepgrass germplasm is essential for the elucidation of molecular networks in sheepgrass seed germination and dormancy. This is the first ever report on the application of the RNA-Seq technique to classify a large number of transcripts from two sheepgrass germplasm with high and low seed germination percentage into three development stages. Previous studies have shown by phylogenetic analysis that sheepgrass is more closely related to barley and wheat than to *Brachypodium* (Chen et al., 2013; Zhao et al., 2016). In the present study, the BLASTX results of transcripts indicated sheepgrass has a significantly close match to *Hordeum vulgare* (19.4%), *Aegilops tauschii* (19.3%), *Tritcum urartu* (11.9%), *Brachypodium distachyon* (6.9%), *Triticum aestivum* (4.8%), and others (37.6%) (Fig. 2). Using a transcriptomic approach, we observed that the number and expression profiles of the DEGs differed between the two germplasm. A total of 9255, 5366, and 4306 transcripts were differentially expressed in the H1 vs L1, H2 vs L2 and H3 vs L3

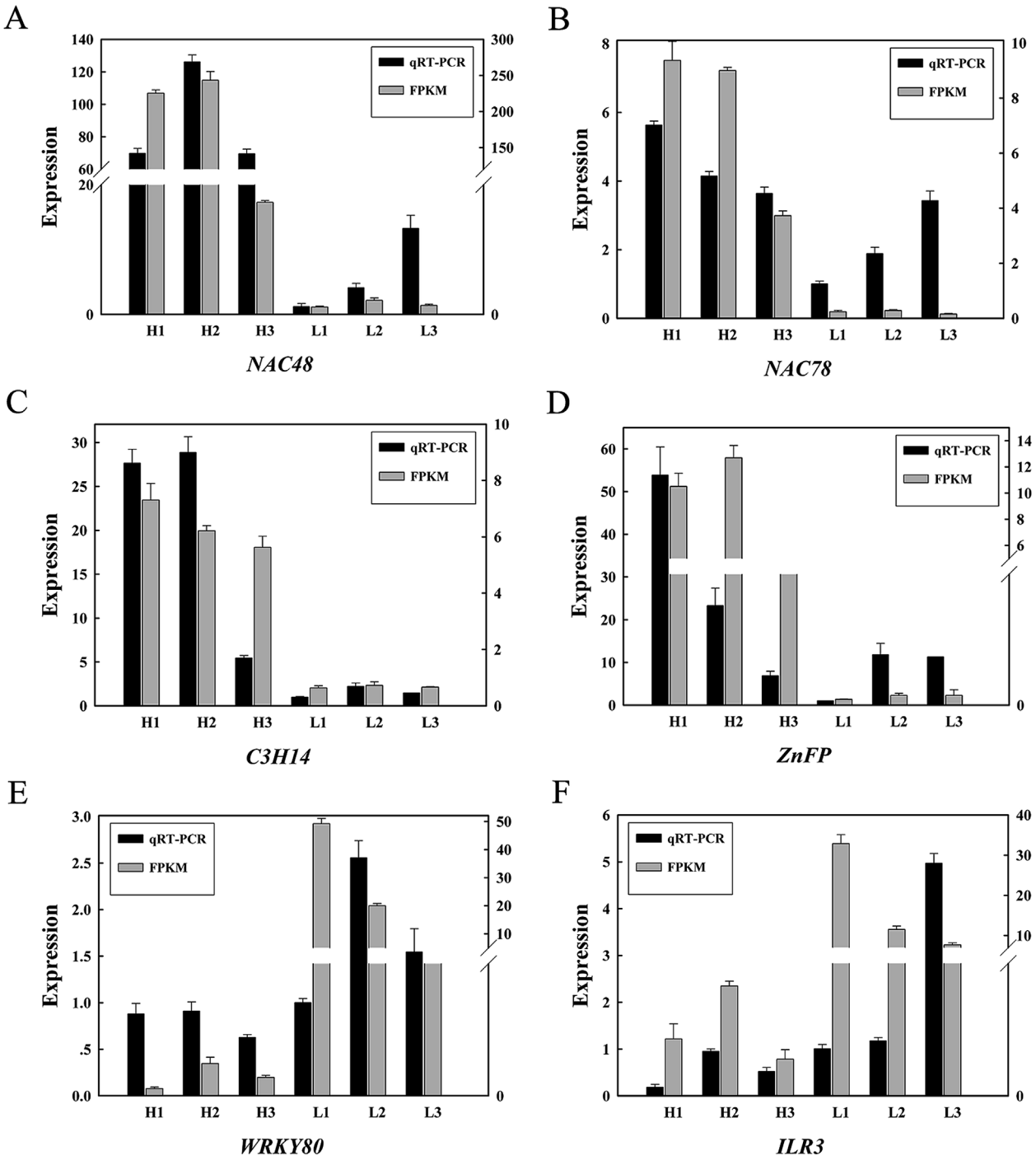


**Fig. 7.** qRT-PCR validations. The differentially expressed A. *NAC48*, B. *NAC78*, C. *C3H14*, D. *ZnFP*, E. *WRKY80*, and F. *ILR3* genes. The left y-axis represents the value of qRT-PCR, and the right y-axis represents the value of FPKM. The bars with black represent the relative expression based on qRT-PCR results, and the bars with gray are based on RNA-Seq results. The x-axis represents different development stages of the two germplasm. Each qRT-PCR experiments were performed in three technical replicates and with at least two biological replicates.

comparisons, respectively (Fig. 3A and B and Table S3). Furthermore, we found that DEGs involved in "metabolic process", "cellular process", "cell", "cell part", "binding", and "catalytic activity" were enriched during seed development (Fig. 3D and Table S4).

Additionally, KEGG analysis revealed that DEGs involved in starch and sucrose metabolism and phenylpropanoid biosynthesis were significantly different in the H1 vs L1; DEGs involved in phenylpropanoid biosynthesis and glutathione metabolism, in H2 vs L2; and DEGs involved in plant hormone signal transduction and galactose metabolism pathways, in H3 vs L3 (Table 1 and Table S5). Embryogenesis and seed maturation are two major phases of seed development, and seed dormancy is induced during the seed maturation phase (Graeber et al., 2012). The embryo must mobilize storage reserves used to germinate and grow, which is mainly the starch in grain seeds (Finkelstein et al., 2008). Our results indicated 121 and 56 transcripts related to starch and sucrose metabolism, and amino sugar and nucleotide sugar metabolism were specifically different in H1 vs L1 (Table 1). Previous studies found that most phenylpropanoid genes were highly expressed in the red fruits, leaves, flowers, seed, and seed coat (Zhao et al., 2013; MacGregor et al., 2015). In this study, we found that the

phenylpropanoid biosynthesis genes in the two germplasm were significantly different at 14 DAP and 28 DAP, speculating that the difference was mainly due to the difference in the color of the seeds between the two germplasm (Table 1).

Plant hormones are involved in regulating seed dormancy and germination, and ABA and GA are the two main hormones with essential and antagonistic roles in induction and maintenance of dormancy and promotion of germination (Finkelstein et al., 2008; Graeber et al., 2012). Meanwhile, the expression of GA and ABA metabolic genes has been shown to be correlated with seed germination and dormancy (Finkelstein et al., 2008; Nonogaki, 2014). From our results, the ABA metabolic enzyme genes *NCED* and *ABA2* had higher expression in L than in H at 14 DAP, while *AAO3* exhibited higher FPKM in L seeds at 28 DAP. However, the ABA catabolic gene *ABA8' OH* was found to be higher at 42 DAP in both germplasm (Fig. 4A), and it has been shown to regulate seed dormancy through modulating ABA content and seed germination potential (Nonogaki, 2014). Furthermore, the GA metabolism *KS*, *CPS KO*, *KAO*, *GA20ox*, *GA3ox*, and *GA2ox* gene families exhibited up-regulation in H and L seeds at 14 DAP, suggesting that the synthesis and metabolism of GA were more active after 14 days of pollination (Fig. 4B). In general, our results suggested that the ABA level in the mid to late maturation stages in combination with fast reduction of the GA biosynthesis contribute to the germination difference between H and L germplasm.

Previous studies showed that plant hormones, including ABA, gibberellin, ethylene, salicylic acid, and nitric oxide, were likely to be involved in germination and dormancy (Li et al., 2018; Zhong et al., 2015; Silva et al., 2018; Moravcova et al., 2018). Interestingly, auxin signaling or biosynthesis greatly enhances seed dormancy in Arabidopsis, and the roles of auxin and ABA in seed dormancy are interdependent (Liu et al., 2013; Zhou et al., 2018). PYR/PYL/RCAR are ABA receptors, and these PYR/PYL/RCAR proteins interact with type 2C protein phosphatase (PP2C) in mediating the ABA response (Park et al., 2009). Furthermore, three SnRK2 (SnRK2.2, SnRK2.3 and SnRK2.6) proteins have been shown to be involved in ABA signaling and to be essential for the control of seed development and dormancy in Arabidopsis (Nakashima et al., 2009). GA signaling is known to be regulated by DELLA proteins, and RGL2 (REPRESSOR OF ga1-3-LIKE1/2/3) seems to be the major DELLA factor, which mediates the interaction of GA and ABA during seed germination (Zhong et al., 2015). In the present study, we found that some genes related to two plant hormone signaling pathways, GA and ABA, had lower levels in L than in H, indicating that reduced signaling of GA and ABA is likely to be related to seed dormancy in sheepgrass (Fig. 4C and D).

Transcripts of many genes are synthesized during seed development, most of which likely take part in the regulation of seed dormancy in mature seeds (Gao et al., 2012). In our present study, 3201, 1524, and 618 genes were significantly differently expressed between the two germplasm during the three development stages. Moreover, 149 and 27 genes were significantly up- or downregulated in H compared with those in L among these developmental stages (Fig. 5A and B, and Table S6). Many candidate genes were screened through a comparison of the two germplasm, and we found that the transcript levels of transmembrane proteins 14C genes, and protein-tyrosine phosphatase genes were higher in the L germplasm, while dehydrin, cytochrome P450, and chitinase 5 genes had higher expression in H (Fig. 5C, Table 2 and Table S6). However, the question arises as to how these genes play a role in seed germination or dormancy and needs to be further verified.

Expression profiles of TFs during different developmental stages indicated that some transcription factor families are expressed in specific germplasm or development periods (Fig. 6 and Table S7). For example, bHLH and WRKY transcription factor families were found to preferentially accumulate in L at 14 DAP. NAC and the MYB transcription factor family were overrepresented in both the H and L germplasm at 14 DAP, while the *Zn-clus* and *bZIP* transcription factors were overrepresented in H3 and L3. Furthermore, the *C2H2* and *C3H* transcription factors have different levels of expression by stage (Fig. 6). Previous research demonstrated the Arabidopsis tandem CCCH zinc finger proteins AtTZF4, 5 and 6 are involved in light-, abscisic acid- and gibberellic acid-mediated regulation of seed germination (Bogamuwa and Jang, 2013). Meanwhile, the *AtC3H17*-overexpressing transgenic plants (OXs) showed an enhanced germination rate (Seok et al., 2016). A membrane-bound *NAC* transcription factor *NTL8* mediates the salt regulation of seed germination via the GA pathway, primarily independently of ABA (Kim et al., 2008). Auxin delays seed germination under high salinity through cross talk with the *NAC* transcription factor *NTM2*-mediated salt signaling in Arabidopsis (Park et al., 2011). A novel zinc-finger protein with a proline-rich N-terminus is an important downstream component of the ABA signaling pathway that mediates ABA-regulated seed dormancy in Arabidopsis (He and Gan, 2004). In our previous study, we found a pleiotropic gene *LcbHLH92* from sheepgrass, and our results suggested that *LcbHLH92* negatively regulates anthocyanins/proanthocyandins through analysis the transcriptome of H and L germplasm seeds at different developing stages. Furthermore, *LcbHLH92*- overexpressing Arabidopsis seeds with a yellow color showed a higher germination rate than did wild-type controls with a brown seed color, suggested that *LcbHLH92* negatively regulates anthocyanins/proanthocyandins and reduces seed dormancy in transgenic Arabidopsis (Zhao et al., 2019). In the present study, the transcript levels of the *NAC48*, *NAC78*, *C3H14*, and *ZnFP* genes were significantly enriched in H compared with those in L at all three stages; however, the transcription factors *WRKY80* and *ILR3* had lower expression in H than in L (Fig. 7). Therefore, we speculated that these TFs genes may be key candidate genes for regulating the seed germination and dormancy, but whether these genes participate in the seed germination will might be considered in future investigation.

## 5. Conclusions

In this study, transcriptome sequencing was firstly performed to investigate the patterns of gene expression in two sheepgrass germplasm. To understand the regulatory mechanism of germination and dormancy in sheepgrass, the differentially expressed genes between the two germplasm during seed developmental stages were analyzed, and 9255, 5366, and 4306 genes were significantly differently expression in the H germplasm than in the L germplasm during the three stages, respectively. Furthermore, biological processes such as starch and sucrose metabolism, phenylpropanoid biosynthesis, plant hormone signal transduction, amino sugar and nucleotide sugar metabolism, and photosynthesis changed significantly at different stages. Our results suggested that the ABA and GA pathways-related genes were critical for dormancy and germination regulation during seed development. Some transcription factors, dehydrin, and enzyme genes were identified as candidate regulators in seed germination and dormancy. Our study will provide a theoretical basis for utilizing these precious resources.

## Conflicts of interest

The authors have no conflict of interest to declare.

## Contributions

Xiaoxia Li and Gongshe Liu conceived and designed the experiments. Shu Liu, Guangxiao Yuan and Weiguang Yang performed most of the experiments. Xiaoxia Li and Pincang Zhao made substantial contributions to the data analysis and the manuscript writing. Shuangyan Chen revised and edited the manuscript. Weiguang Yang, Junting Jia, Liqin Cheng and Dongmei Qi were involved in performing the experiments. Gongshe Liu and Shuangyan Chen gave the final approval the manuscript. All authors read and approved the final manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.007.

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