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DNA-AFLP ANALYSIS REVEALS DIFFERENTIAL GENE EXPRESSION IN RESPONSE TO SEED AGING IN SIBERIAN WILDRYE

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ABSTRACT

Siberian wildrye (*Elymus sibiricus* L.) is widely cultivated in the central and western regions of China. It is an important agricultural produce and utilized in animal feed and is also important for ecological environment protection. It is of great significance for the preservation and production of germplasm resource to elucidate the seed aging mechanism, such as delaying seed aging, prolonging seed life as well as repairing seed vigor. Information on seed aging including aging conditions, physiological and biochemical characteristics, damage to genomic DNA and genetic integrity of germplasm resource is limited but necessary for germplasm collection, conservation and effective commercial use. However, it is rarely reported on the transcriptome analysis of seed aging in Siberian wildrye. In this study, we analyzed the differentially accumulated TDF (Transcript-Derived Fragments) by cDNA-AFLP technique using 136 pairs primers, a total of 653 TDFs were identified. These up-regulated TDFs were picked for sequence alignment and functional analysis. The results showed that the sequences of TDFs are highly homologous in plants, which are pyruvate kinase, metal transport protein, DNA damage repair protein, lectin receptor kinase, and leucine zip transcription factor. Additionally, chloroplast structure was destroyed and energy consumption was increased in seed aging, at the same time, it could resist aging by increasing transcripts of relevant defense genes transporter genes and corresponding DNA damage repair genes when Siberian wildrye is aged. The purpose of this study was to provide a theoretical basis for revealing the potential aging mechanism of Siberian wildrye from the transcriptional level.

Keywords: Siberian wildrye, seed aging, cDNA-AFLP, transcript-derived fragments (TDFs).

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INTRODUCTION

Siberian wildrye (*Elymus sibiricus* L.) is a perennial, self-pollinating, allotetraploid grass with the StStHH genome constitution (2n = 28) native to northern Asia with the significant tolerance to abiotic stresses including cold and drought tolerance, as well as good forage quality (Dewey, 1974; Dou *et al*. 2011; Ma *et al*. 2012; Xie *et al*. 2015). It is widely planted in the central and western regions of China and plays important roles in ecological environment protection (Yan *et al*. 2007). The research on Siberian wildrye involves many aspects such as classification, herbage production, cultivation and utilization, seed production, resistance analysis, karyotype analysis, genetic diversity of germplasm resource, seed aging and so on. For the storage of germplasm resources, seed plays an essential role in both facilitating the preservation of rare species and genetic innovation. However, seed aging negatively affects the germination rate, seed vigor and viability, embryo growth, and seedling emergence of future generations, which has become a cause of increasing concern (Liu *et al*. 2016). Therefore, it is important to study seed aging for protecting germplasm resources and maintaining crop yield and quality. Seed aging is an irreversible and gradual change process which involves many physiological and biochemical reactions, such as membrane lipid peroxidation, changes in protective enzymatic activity, accumulation of toxic and harmful substances, protein and nucleic acid metabolism, and endogenous hormones (Murthy *et al.* 2000; Liu *et al*. 2012; Parkhey *et al*. 2012; Ratajczak *et al*. 2015). Until now, various academic viewpoints have already explained the aging mechanism from different perspectives (Kalemba *et al*. 2014; Spanò *et al*. 2007; Kranner *et al*. 2011; Bachem *et al*. 1996), while the transcription level on seed aging mechanism is unclear. Thus, our research further indicated that the process of seed aging is not only caused by a single factor, and the aging mechanism is still need to explain.

Hearteningly, more and more new biological technologies, such as molecular markers, transcriptome, proteomics and genetic engineering, are applied to research on seed aging mechanism in many plants. Currently, transcriptome sequencing is an efficient and rapid method to generate large EST sequences, and next-generation sequencing also has been successfully and increasingly used in most plants. The genome sequences

of *E. sibiricus* or other species belonging to the *Elymus* genus are not intact. So, cDNA-AFLP (cDNA-amplified fragment length polymorphism) is a better way to analyze the expression differences of mRNA by mRNA fingerprint technology (Bachem *et al*. 1996). It retains the reliability and high efficiency of AFLP technology and is widely applied to the research on the expression characteristics of differential genes during plant growth and development (Pak *et al*. 2017; Yang *et al*. 2017). cDNA-AFLP becomes widely available because of low cost and celerity, even if there is few information about EST sequences. Due to the advantages of sensitivity and specificity, we could detect poorly expressed genes these rare transcripts, and even distinguishing homologous sequences. Additionally, this is an extremely efficient and less labor-intensive mRNA fingerprinting method to analyze the differential expression gene in stressed conditions (Fukumura *et al*. 2003). Many researchers had used cDNA-AFLP technology to understand the molecular basis in other plants under abiotic stress. It was reported that 28 TDFs were identified representing different groups of genes involved in ion transporting and compartmentalization, cell division, metabolism, and protein synthesis in a halophyte *Spartina alterniflora* L. (smooth cordgrass) under salt stress (Baisakh *et al*. 2006). Futhermore, 27 non-redundant TDFs are unique response to salt tolerant in foxtail millet which belong to different groups of genes involved in metabolism, cellular transport, cell signaling, transcriptional regulation, mRNA splicing, seed development and storage (Jayaraman *et al*. 2008). Under water deficit, TDFs present homologies chiefly function in protein degradation, protein synthesis and ROS scavenging pathway (Yang *et al*. 2003).

The previous reports on seed aging were focused on aging conditions, physiological and biochemical characteristics, damage to genomic DNA and genetic integrity of germplasm resources (Dargahi *et al*. 2014; Chen *et al*. 2013).Transcription level on seed aging have not been reported in *E. sibiricus* so far. Our study focused on revealing the seed aging mechanism on transcription level using cDNA-AFLP technology in *E. sibiricus*. We gained the sequences of TDFs for sequence alignment and functional analysis, aiming to find the critical genes that affect seed aging, and try to explain the mechanism in *E. sibiricus*. Finally, it will be used to provide theoretical basis for extending the life of seeds and repairing seed vigor in *E. sibiricus*.

MATERIALS AND METHODS

Plant material and seed aging treatment: Seeds of *E. sibiricus* (*Elymus sibiricus* L. CV. Nongmu) from Grassland Research Institute, Chinese academy of agricultural sciences, harvested in July 2015, and saved at 4 ℃. Uniformly sized and rich plump seeds were treated by continuous 40 ℃ at constant temperature incubator. The aging gradient of seeds is determined by the relative germination rate, and the normal seeds were used as control. Four aging gradients were selected depending on time duration of treatment under 40 ℃ as follow, 100% (0 h), 75% (24 h), 50% (72 h) and 25% (144 h). Treated seeds were sowed in plots in the artificial light incubator, growth condition was 14 h light (25 ℃)/10 h dark (15 ℃) with 80% relative humidity. When the seedlings grew to 8-10 cm, 30 plants were randomly selected for the extraction of total RNA using RNA prep Pure Plant Kit (Tiangen, China) according to the manufacturer's instruction.

cDNA preparation and AFLP analysis: cDNA was synthesized using Prime Script TM Double Strand cDNA Synthesis Ki (Takara, Japan) following the manufacturer's instruction. *Mse* I and *Pst* I enzymes (New England Biolabs) were used for digestion by one step method, primers and adapters were listed in Table 1.

Transcript-derived fragments (TDFs) isolation and re-amplification: PCR reaction system of Pre and selective amplification are 20 μL, including 4 μL template, 1 μL primers, 2 μL 10×Buffer (Mg^{2+}), 2 μL dNTP, 0.2 μL and 0.3μL Taq-polymerase, finally added ddH_2O to 20 μL. Both reaction amplifications were used the same PCR conditions as follows: 94 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 1 min for 25 cycles and final extension for 10 min at 72 ℃. Pre amplifications were detected in 1.2% agarose gel electrophoresis, while 8% modified polyacrylamide gel electrophoresis was used to detect the selective amplification products. Finally, the fragments were recycled to make secondary amplification and purification.

Analysis of sequences: Fragments were sequenced by BGI (The Beijing Genomics Institute). The nucleotides and translated sequences were analyzed for their homology using the BLASTX and BLASTN algorithms, respectively, in the database (http.//www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Identification of seed aging related transcripts: In this study, extracted total RNA was identified by 1.2% agarose gel electrophoresis (Figure 1). The total RNA bands were neat and clear without protein contamination and degradation. Quantity and quality of total RNA were detected by ultraviolet spectrophotometer, concentration of all RNAs were 0.5 μg/μL at least, while absorb rations of A260/A280 were all in the range of 1.9-2.0, these data indicated that the purity and integrity of total RNA were good enough for next step. Then cDNA expression patterns were identified by selective amplification using 136 pairs of primers, the results showed that a total of

2720 bands were amplified, and 653 accumulated TDFs from genes were identified under different seed aging level (Figure 2).

Expression patterns of the TDFs: In order to validate the changes in mRNA abundance as detected by cDNA-AFLP and to quantitatively evaluate the relative abundance of transcripts in seed aging, we analyzed the expression patterns of the TDFs. When the degree of aging stress was lower, larger TDFs were appeared. With the increasing of stress intensity, some large TDFs disappeared, while the number of small TDFs was increased. Based on the above results, there are four types of expression patterns of 653 TDFs in response to seed aging in *Elymus sibiricus* L. cv. Nongmu seeds (Table 2). 154 TDFs belongs to group Ⅰ type, and the transcription level was none or lower under normal condition, while the transcription level was significantly up-regulated under aging stress. In contrast to group Ⅰ type, the expression level of genes in group Ⅱ type (106 TDFs) was significantly down-regulated under seed aging stress. There are another 274 TDFs belonging to group Ⅲ type, transcripts of genes were increased first, and then decreased which meant some gene expression was silenced during the seed aging process. The last type (119 TDFs) is contrary to group Ⅲ type, and the expression patter of differential expression genes was decreased firstly, and then increased, gene expression suddenly silenced and appeared again during the seed aging process.

Sequencing and functional analysis: To further analyze the up-regulated genes in seed aging, we gained the sequences of the partial up-regulated TDFs by Sanger sequencing (Table 3). Then we made sequence alignment with 19 TDFs in NCBI, the results showed that ten TDFs of 19 TDFs had homologous genes with functional annotation, three TDFs were unknown proteins without functional annotation, and the other six TDFs had no homologous sequence information which might be unreported new genes (Table 4). To further analyze the sequence homology, 19 TDFs were divided into four types. Sequence homology is over 90% belonging to group Ⅰ type involving seven TDFs, which are P13M62, P13M65, P4M57, P2M47, P13M50, P15M95 and P13M57. Five TDFs (P2M51, P13M47, P2M96, P10M56 and P13M96) belong to group Ⅱ type, and the sequence homology is 80% - 90%. Moreover, only sequence homology of P13M61 TDF was less than 80% belonging to group Ⅲ type. The last type was involving P6M57, P13M54, P13M60, P2M52, P2M57 and P9M56 that had no homologous gene in the NCBI database. Next, we divided 13 TDFs into seven functional categories according to the functional annotation. (1) Genes from four TDFs (P2M51, P13M62, P13M47 and P13M65) were located in chloroplast and involved in the energy transformation. (2) Gene from P13M61 TDF was the homologous gene of pyruvate kinase. (3) The homologous genes of metal transporter protein included differential expression of genes from P2M96 and P4M57 TDFs. (4) Gene from P2M47 TDF was the homologous gene of DNA damage-repair/toleration protein. (5) TDF P10M56 showed similarity with Lectin receptor kinases. (6) TDF P13M50 was similar to Leucine zipper transcription factor. (7) The other TDFs (P15M95, P13M96 and P13M57) encoded proteins with unknown function. Among those homologous genes, eleven genes were from Poaceae plants including *Aegilops tauschii*, *Hordeum bogdanii*, *Oryza brachyantha*, *Triticum aestivum* and *Setaria viridis*, besides, only one gene annotation of TDF from *Pharbitis nil*, indicating that gene similarities were higher among plants of the same family.

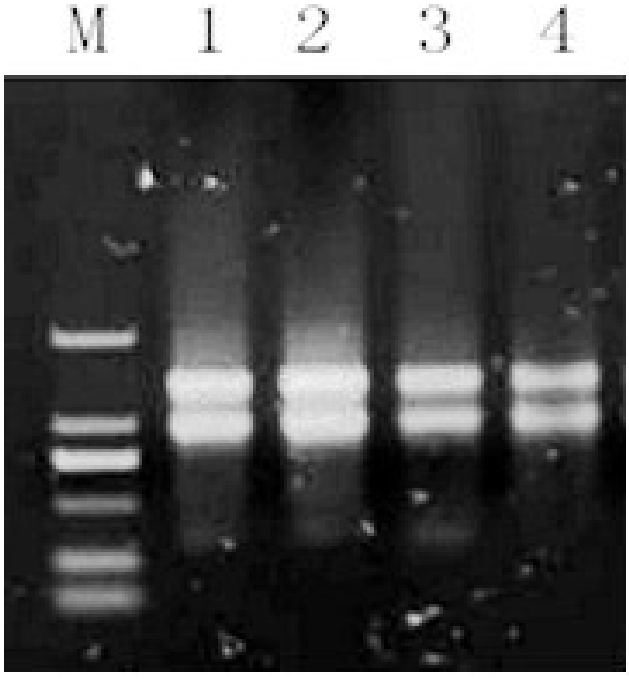


Figure 1. Total RNA. Lane M: 2000 bp ladder; Lane 1: control; Lane 2-4: seed aging 24 h, 72 h and 144 h.

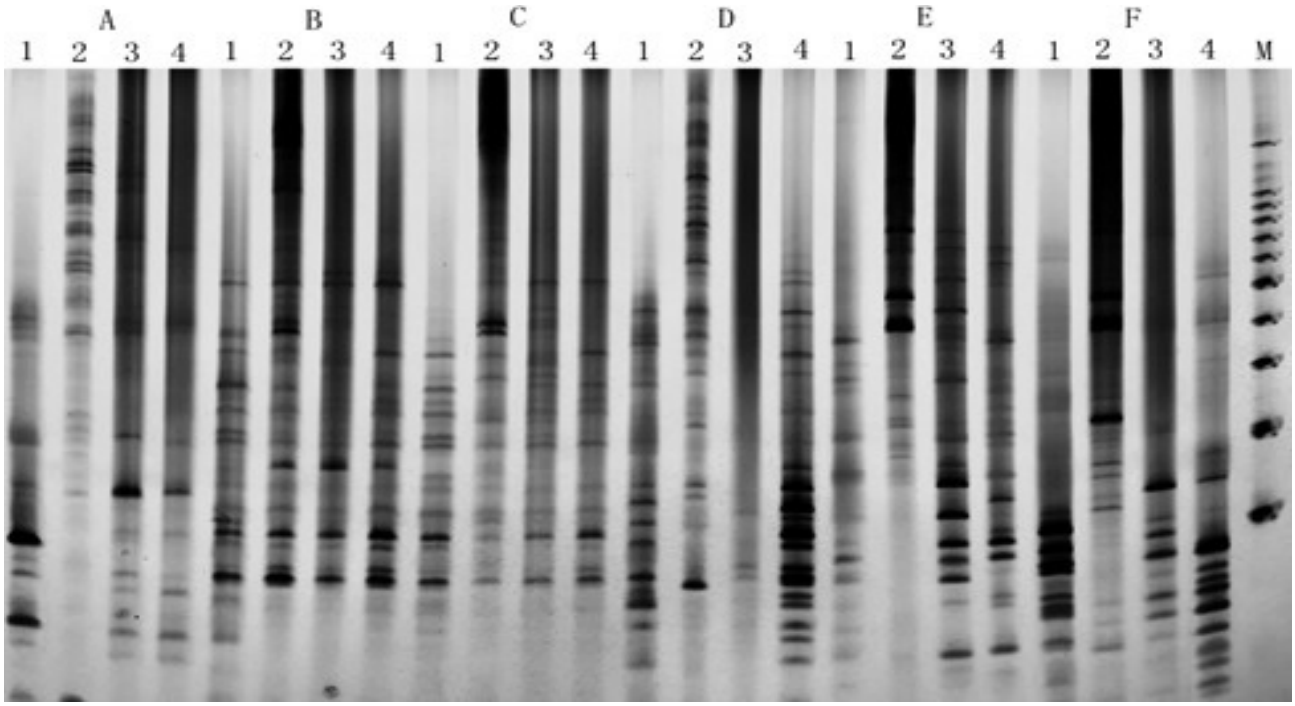


Figure 2. Gene differential expression of cDNA-AFLP display (a part of results). Lane M: 100 bp ladder; Lane 1: control; Lane 2-4: seed aging 24 h, 72 h and 144 h. A-F: selective amplification primers combination (A：P2/M64; B: P2/M51; C: P2/M94; D: P13/M54; E: P13/M61; F: P13/M62).

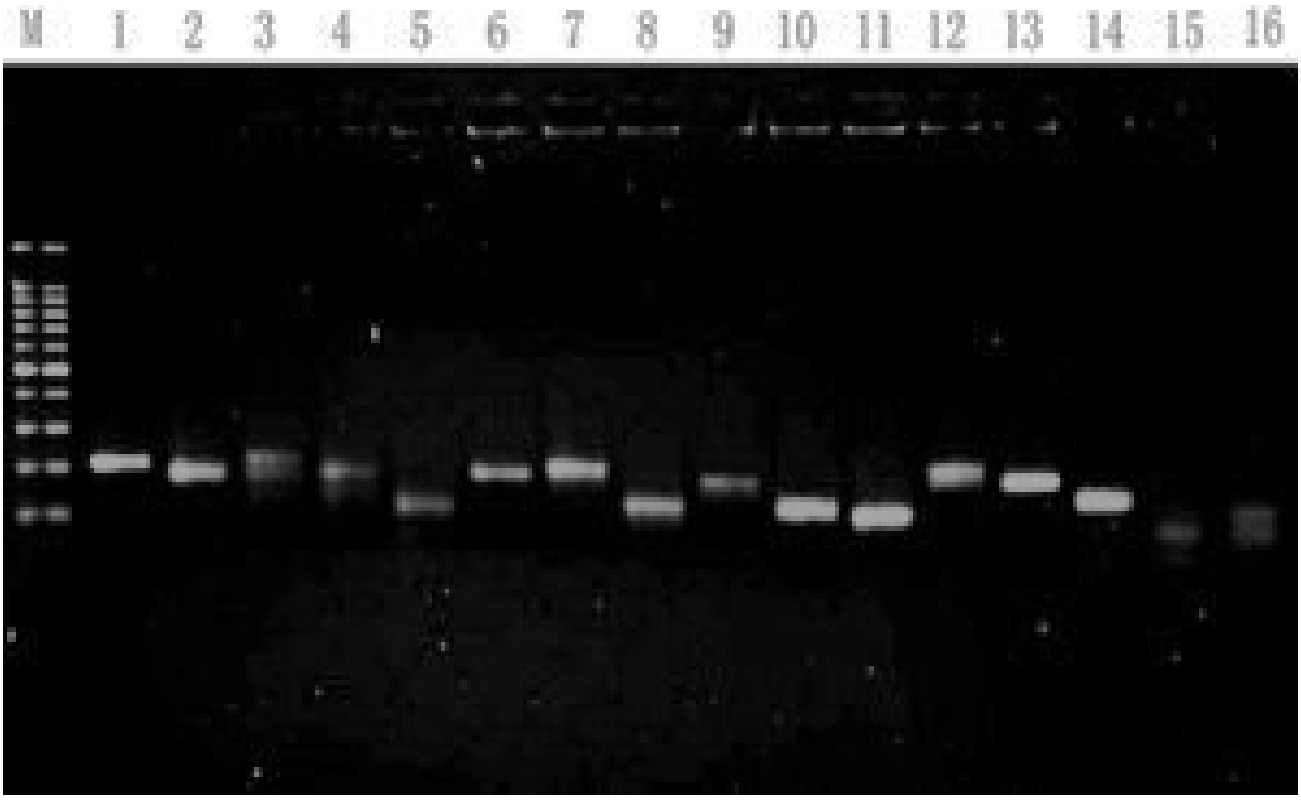


Figure 3. The second PCR amplification products. Lane M:100 bp ladder.

Table 1. Sequences of adaptors and primers

Name	*Pst* I *adapter and primer sequences*	*Name*	MseI *adapter and primer sequences*
Adapter1	*5'-GAC GAT GAG TCC TGA G-3'*	*Adapter1*	*5'-CTC GTA GAC TGC GTA CA-3'*
Adapter2	*3'-TA CTC AGG ACT CAT-5'*	*Adapter2*	*3'-TGC ACA TCT GAC GCA TGT-5'*
P-00	*5'-GAC TGC GTA CAT GCA G-3'*	*M-00*	*5'-GAT GAG TCC TGA GTA A-3'*
P-1	*5'-GAC TGC GTA CAT GCA GGA A-3'*	*M-47*	*5'-GAT GAG TCC TGA GTA ACA A-3'*
P-2	*5'-GAC TGC GTA CAT GCA GAA T-3'*	*M-48*	*5'-GAT GAG TCC TGA GTA ACA C-3'*
P-3	*5'-GAC TGC GTA CAT GCA GAC A-3'*	*M-51*	*5'-GAT GAG TCC TGA GTA ACC A-3'*
P-4	*5'-GAC TGC GTA CAT GCA GAC T-3'*	*M-52*	*5'-GAT GAG TCC TGA GTA ACC C-3'*
P-5	*5'-GAC TGC GTA CAT GCA GGA G-3'*	*M-54*	*5'-GAT GAG TCC TGA GTA ACC T-3'*
P-6	*5'-GAC TGC GTA CAT GCA GCA C-3'*	*M-56*	*5'-GAT GAG TCC TGA GTA ACG C-3'*
P-7	*5'-GAC TGC GTA CAT GCA GGA A-3'*	*M-57*	*5'-GAT GAG TCC TGA GTA ACG G-3'*
P-8	*5'-GAC TGC GTA CAT GCA GGA C-3'*	*M-58*	*5'-GAT GAG TCC TGA GTA ACG T-3'*
P-9	*5'-GAC TGC GTA CAT GCA GCA A-3'*	*M-60*	*5'-GAT GAG TCC TGA GTA ACT C-3'*
P-10	*5'-GAC TGC GTA CAT GCA GGT C-3'*	*M-61*	*5'-GAT GAG TCC TGA GTA ACT G-3'*
P-12	*5'-GAC TGC GTA CAT GCA GAG T-3'*	*M-62*	*5'-GAT GAG TCC TGA GTA ACT T-3'*
P-13	*5'-GAC TGC GTA CAT GCA GAT A-3'*	*M-64*	*5'-GAT GAG TCC TGA GTA AGA C-3'*
P-14	*5'-GAC TGC GTA CAT GCA GAT C-3'*	*M-65*	*5'-GAT GAG TCC TGA GTA AGA G-3'*
P-15	*5'-GAC TGC GTA CAT GCA GAT G-3'*	*M-94*	*5'-GAT GAG TCC TGA GTA AGG A-3'*
P-42	*5'-GAC TGC GTA CAT GCA GTA G-3'*	*M-95*	*5'-GAT GAG TCC TGA GTA AGG C-3'*
P-44	*5'-GAC TGC GTA CAT GCA GTC T-3'*	*M-96*	*5'-GAT GAG TCC TGA GTA AGT A-3'*

Table 2. Four types of expression patterns of TDFs in response to seed aging in *Elymus sibiricus* L. cv. Nongmu seeds.

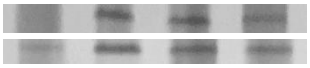
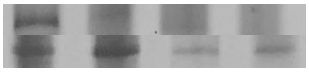
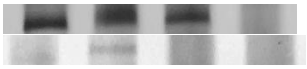
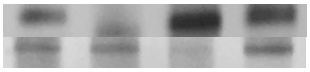
Expression type	Belt type 0 h 24 h 72 h 144 h	Percentage
Up-regulation (Ⅰ type)		154 (23.58%)
Down-regulation (Ⅱ type)		106 (16.23%)
Up-regulation to Down-regulation (Ⅲ type)		274 (41.96%)
Down-regulation to Up-regulation (Ⅳ type)		119 (18.30%)
Total		653 (100%)

Table 3. Sequence of TDFs.

Name	Sequence
P2M51 (170bp)	GGGAACTTCAGAAGCAACCGGCGTCTTCTACACTTGCGCAGCCTGTCTTGGTTCTTAGCTTGGG ACCACGCAGCACTTCTTCATTCATCCGTTCTGCCTGTCCATTACTTGTCAGCGCCGCCTTTTACT ATGCTCTGATTTCTGAGGCCTGATTTAGCATGGGTACGAAT
P13M62 (160bp)	GAGTTTAAGATAGGCCAAGAAAATGGCGGCTTGTGCTGCGCCCCCCTACGTCCGCATCTCCTGC TCCACACCTTCTGCCCTTCGGACACGATGGCTTTGGCTTCAACGCACGGACCCCTCTCGGCCCT GGAGCCACCTTCATATCTGATGTACGCAGTCA
P13M47 (127bp)	CCGGGCAACAACCAAATCGTTCGATTGTGTCTGGAGCTCACTAGCAAGGGTAACGTCGTCCTG GGTAATCTCCGTTTGCTGCGTGTATTCACTCAGTTTTTATGGACAATCTGCATGTACCCAGCCA
P13M65 (162bp)	TACGCCCCAAAGGGACCGAGCACGCCTGTGCTGGCCTCTCTCTTGGATAAGCTTCCTACCTCCT TACGGAACTTGTTCTTATGGTCATTTGGAATCGACTTGATGTTGCTCGGCGTCTACTCTCCTCCT

	CCTTCCACCATGTCTGCATGTACGCACTCACAA
P13M61	GGACCCAACACCCCACAGAATAAACGATTTTGATACTTTTCTCTGAATCCTGACGAAACTGTTT
(200bp)	TGATTCTCCTGTATTTCCTTGATGTCCTTCTGCATGCCTTGCCATCAGCAAGCATTGGAAAGAG
	GCCTCTAACTATGATTCAATTGATTTGTTTTCAGACGAGGAATGACAACGGAAGCCCGTCCGGA
	TGAGGTGA
P2M96	CCGGGGGAAATTGCTAAATAGCATGTCTACATCGCTACTCAGCAGGACAGACGCTCAACGTCT
(112bp)	CAGCCGCGCTCATCGCCTTCGTCGTCCTCCGCGGGCATGTACCAAGTCA
P4M57	CAGAAGGAACAGGACAAAAATCTCCGATCATATGATTCGTGCAGCCACTGTCCAAGACCCATT
(253bp)	CAGTGGCACAAGGTGTATCATCCTGTAGAAGCATTAGTCTGTCTTACAAATTCATATGTGTAAT
	AAGAGAGCAAAATCGGTGCCCCGCAGAATGAGGTACAATACGAATGAGGCATAGGCAAGTAC
	TGCCACGCAGCCGTTGATTGTGATCAATAGTGCAGCCACTGTCCAAGTTACAGCTTGCATTTTA
P2M47	CAAAGAAATTTAAAGAGTCATCAGATCACCTTGGTCCACCAATTTCTCTACGGCTGGTTACGCC
(170bp)	TTCGGAGGCGTCATCCCGGACGCGCTCTCGCGGCTGGTGCGCCTCCAGCAGGCGTCATCCCGG
	ACGCGCTCTCGCGGCTGGTGCGCCTCCACGTCCTCATGGAGCT
P10M56	TGGAGGACCTTTCAACCGATGTGCCGGCTGGCGGATGGCCCATGTCCGCTAGAAATCCCTCGT
(212bp)	AATAAAGTGACCGGAGCACGACGCGAGGATCACCGACGGCAGGATTGACCTCCTCGGCGACA
	AGATTGGTTGGTGCACCGCTGTTGATCTGCGCCAAATTCGATAAGCACGCACGTTTGCCAAGCG
	AGTCACGCTGCAGGTACGCAGTC
P13M50	CCCGGTATAAAAGTAAAAAGATTTGATGTCGACGTCACTAACCAAGGTTCGCTCGTGACCCAT
(178bp)	GATGCCGATGCCGGCATCCGTGTAGAAGATGCATCAACCATGCCGCTCAGTTTTAGGGTACGC
	ATGCATCGCGCCGACGGCCTCGACCTGGGCCTCGGGCTGGGGCTCGGCCTCC
P15M95	CCAACAAAAGATCGTACCTACTCTGAGATCCCTGTATCGAAACCTGCTAGCCCATCCCTCGACT
(189bp)	TCTTCGCTGACCTTTCTGCATCGGGTGGTGATTTCCGCAGGGCATCCGTGGATCGCGAGCTGCG
	GCGGGAATGCCGTGGCGGGCTGAAGAAATCGATCGACCGACATCTGCATGTACACAGTCAA
P13M96	CACGGGGCCGGCCATCCAAGGTTTGATCCATGTTAACCCTACTAAACCTTTATTGTCACCAGAG
(151bp)	CCCCGCGTGTCGAGGCGGAAGGAGGCGGCGGCGGAGTTGCCTGATTCCTTTGATGATGATCCT
	GGGTATCTGCATGTACGCAGTCAG
P13M57	GCGGAGGGCGGGCGGCTGAGACTTGGGTGGGGTACCGCGCTGACGATAATCTTCATGATTCGG
(160bp)	CGGGCCACTCCCGTCGACCACCGCCGGCGCGCACACCGTCGCGCCGTTGTAATCGACGATCTC
	TTTTTGGGAACATGCTCTGCGTGTCCACCAACA
P6M57	CCAAATCATTAAGCAGATGTGGATTCGATTCCGCATGGGAGCGAATTTCAAGGCTGGTTACAA
(117bp)	AACCTCCCTCACTGGCAACCCTTATATCTGCATGAACGCAGTCATCGCCGTCAA
P13M54	GGGGGAGATCCAATAAACCACACTGGCACTGATATGGGCTTTTAAACAGGCTTGGAAGCTGTG
(110bp)	CCGTATCTGTAGGGCTAGAGTATGCGCCGGACAATCACAACACAACA
P13M60	GCGGGCGGGAAGTAAAAAAGTCGCGCGGCCTCGTGCACATTGACCGCGCACGTCTTTATG
(100bp)	CGGTTGTTCTGTGGTGTCTGTCTGAGTCACCCAGCAATCA
P2M52	TCGGGGGGGGGAGGGTCCCGTTTGAAAGAGTTGCATGCAGACGAATCTCCTATCGCCCGCTCC
(121bp)	TCGAGGCCTCCTGTGTTGTTTTTTGTCCGTATCCGCATGTAGGCAGTCACACGCCACA
P2M57	CACCGGTCCCTCCCCCCCCAGGGATGGCTCGGTGCACTTTTGCAGTTGAGACCAGAGAAGTCGT
(303bp)	ACTTCGGCGTGTGATTTCTTGCTGTGAACCATCCCCTTTCCACGCAGGTTTGCATCCACGGCTTC
	CATGCCTGTCCGGCGGCCCTGAGAAGGACCGTCCTGCTTGTACACAATCATTCGTTTCACGATG
	CGGGATAACAATCAATTGCTAGCAGATACTTACTGGTGAAATCATGACATAAATACGGTATAT
	CCTTTTTGAATATAATAAATAAACAGTTATAATTATTTATAAATATTT
P9M56	CACGGGCCGGACAGCTGCAAGTACAACACGAAATCATGCTGCACAACCATCCAGCTAAAACTT
(118bp)	ACCTGTAATTTTTGAATGGTAACATGTTTTTTCCCATTCTGCAAGTACGCAGTCA

Table 4. Differential expression gene.

Name	Database	GenBank ID	Description	Similarity	Expect value
P2M51	blast	KP211145.1	Hordeum bogdanii voucher BOP022829 psaJ-rpl33 intergenic spacer region, partial sequence; chloroplast	88%	8e-14
P13M62	blast	XM_020339963.1	PREDICTED: Aegilops tauschii subsp. tauschii probable plastid-lipid-associated protein 8,	99%	3e-17

			chloroplastic (LOC109781357), mRNA		
P13M47	blast	XM_019307796.1	PREDICTED: Ipomoea nil inorganic phosphate transporter 2-1, chloroplastic (LOC109159701), mRNA	84%	7e-13
P13M65	blast	XM_004962119.4	PREDICTED: Setaria italica protein PALE CRESS, chloroplastic (LOC101779970), mRNA	97%	1e-16
P13M61	blast	XM_006663753.2	PREDICTED: Oryza brachyantha pyruvate kinase 1, cytosolic (LOC102702985), mRNA	73%	3e-09
P2M96	blast	XM_015781583.1	PREDICTED: Oryza sativa Japonica Group probable metal-nicotianamine transporter YSL9 (LOC4336545), transcript variant X5, mRNA	88%	5e-14
P4M57	blast	XM_020311197.1	PREDICTED: Aegilops tauschii subsp. tauschii metal transporter Nramp6-like (LOC109752301), mRNA	100%	2e-17
P2M47	blast	XM_020315503.1	PREDICTED: Aegilops tauschii subsp. tauschii DNA damage-repair/toleration protein DRT100-like (LOC109756656), mRNA	94%	2e-15
P10M56	blast	XM_020311637.1	PREDICTED: Aegilops tauschii subsp. tauschii L-type lectin-domain containing receptor kinase IX.1-like (LOC109752741), mRNA	84%	1e-12
P13M50	blast	DQ353857.1	Triticum aestivum homeodomain-leucine zipper transcription factor TaHDZipII-1 mRNA, complete cds	95%	5e-16
P15M95	blast	AK366656.1	Hordeum vulgare subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv2045G14	97%	2e-16
P13M96	blast	AK368952.1	Hordeum vulgare subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv2082M11	82%	3e-12
P13M57	blast	AK376085.1	Hordeum vulgare subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv3114F16	95%	4e-16

DISCUSSION

Seed aging has adverse impact on crop yield and quality, which negatively affects the germination rate, seed vigor and viability, and seeding emergence, and has become a problem causing great concern (Liu *et al*. 2016). To preliminarily understand the mechanism of seed aging in transcription level, we identified the seed aging related genes in *E. sibiricus*. Since there is no available database to gain genome sequences and very little is known about the genome structure. cDNA-AFLP technology is the best option for transcripts analysis because there is no requirement for sequence information and it enables to identify novel changed genes (Moustafa and Cross, 2016). cDNA-AFLP technology had been used to identify the differentially expression genes in seed aging of the other plants. It was reported that there were 717 TDFs in pea seeds with different aging time, 330 TDFs of them up-regulated while 387 TDFs were down-regulated. The altered genes were mostly related to protein post-translation processing and ribosome structure in seed aging of pea (Chen *et al*. 2013). Additionally, compared treated corn seeds (45 ℃, 100% relative humidity for 72 h) with the untreated seeds, the differential expression genes were mainly involved in energy metabolism, signal transduction, stimulus response and aging in the artificially degraded corn seeds (Yang *et al*. 2014). The main reason for change of expression of genes is glycolytic pathway which is inhibited leading to the accumulation of ROS and decrease of seed vigor (Zhang *et al*. 2014). In our results, we screened a total of 653 TDFs in different aging seeds, up-regulated TDFs were 154, accounting for 23.58%; 106 TDFs were down-regulated, accounting for 16.23%. Besides, transcripts of 274 TDFs were increased first and then decreased, accounting for 41.96%; 119 TDFs had exactly opposite changes compared to group III, accounting for 18.30%. Partial up-regulated TDFs include chloroplast protein, pyruvate kinase, metal transport protein, DNA damage repair protein, lectin receptor kinase, and leucine zip transcription factor, and these genes will help plants survived in establishing homeostatic environment.

Chloroplast proteins and pyruvate kinase are critical for energy metabolism. The latest research reported that signal exchange between chloroplasts and

mitochondria regulated programmed cell death in plants. Chloroplast is the organelle for plant photosynthesis, and also the main place for reactive oxygen species (ROS) production and degradation under adversity stress (Van and Van 2015; Van and Dat 2006; Gorlach *et al*. 2015; Xu *et al*. 2006). Pyruvate kinase is one of the rate-limiting enzymes for the energy supply of sugar oxidation, involving in the final step of glycolysis to generate pyruvate, which is a key intermediate substance in cell metabolism involved in lipid metabolism and amino acid metabolism (Huang and Luo 2009). The researchers analyzed the different expression genes in maize embryos after seeds were treated with artificial aging, and found that the gene expression level of pyruvate kinase involved in the process of energy metabolism is significantly up-regulated (Yang *et al*. 2014). Similarly, the gene expression level of pyruvate kinase was significantly up-regulated under water stress in wheat (Li 2010). In our study, four groups of up-regulated genes were related to chloroplast function, and the other changed gene is pyruvate kinase. These results indicate that the chloroplast membrane structure of aging seedlings was likely damaged, which caused in increased reactive oxygen species, weakened photosynthesis and increased energy consumption, resulting in decreased seed vitality and even death. Metal transporters are responsible for metal absorption and translocation to keep metal homeostasis, which can defend against biotic and abiotic stress to reduce the damage to plant (Zhang *et al*. 2017). Both of lectin-like receptor kinase and leucine zipper transcription factor belong to defense-related genes, which are involved in plant stress resistance and hormone signaling pathways (Choi *et al*. 2000; Singh *et al*. 2002). It was reported that transcription level of lectin-like receptor kinases LecRK-b2 is significantly increased under salt stress (Dong 2009). Our results showed that the two metals transporters belong to YSL protein family and the natural resistant metallotropic protein family (NRAMP), respectively, which were mainly located in the cytoplasm membrane. Two proteins function in transferring heavy metals to cytoplasm to provide nutrients for the early growth of plants, maintaining the normal concentration of metal ions of cell area, and minimizing the damage caused by metal ions (Lanquar *et al*. 2005). Thus, we speculated DNA damage repair genes and stress tolerance genes were used to resist seed aging in *E. sibiricus*. However, none of the TDFs observed during the present study show homology compare with any other known genes, indicating that there is a need to elucidate the mechanism of these genes in seed aging of *E. sibiricus* in the further work.

To the best of our knowledge, this study identified the seed aging related genes of *E. sibiricus* using cDNA-AFLP technology. We analyzed the differentially accumulated TDFs using 136 pairs of primers, and identified a total of 653 TDFs. The sequences of TDFs had high homology with the plants in the same family or genus, and these genes were including pyruvate kinase, metal transport protein, DNA damage repair protein, lectin receptor kinase, and leucine zip transcription factor. In conclusion, this study provides a theoretical basis for revealing the aging mechanism of *E. sibiricus* from the transcriptome level, and a valuable sequence resource for analysis on seed aging in *E. sibiricus*.

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