Transcriptome differences between fiber-type and seed-type *Cannabis sativa* variety exposed to salinity

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Abstract The industrial hemp varieties ‘Yunma 5’ and ‘Bamahuoma,’ which demonstrate growth vigor and environmental adaptability, have been primarily cultivated in Yunnan and Guangxi, China, respectively, for fiber and seeds. The results of physiological measurements showed the phenotypic differences between the two varieties in response to salt stress. RNA-Seq analysis was first performed on leaves of both varieties sampled at four time intervals (0, 2, 4, 6 days) after treatment with salt (500 mM

NaCl). We identified 220 co-up-regulated differentially expressed genes (DEGs) in the two varieties, while 26 up-regulated DEGs and 24 down-regulated DEGs were identified exclusively in the single varieties after 2 days of salt stress. Among the 220 DEGs, we identified 22 transcription factors, including key transcription factors involved in salt stress, such as MYB, NAC, GATA, and HSF. We applied gene expression profile analysis and found that ‘Yunma 5’ and ‘Bamahuoma’ have variety-specific pathways for resisting salt stress. The DEGs of ‘Yunma 5’ were enriched in spliceosome and amino acid metabolism genes, while the DEGs of ‘Bamahuoma’ were enriched in fatty acid metabolism, amino acid metabolism, and endoplasmic reticulum protein processing pathway. Although there were common DEGs, such as genes encoding cysteine protease and alpha/beta-hydrolase superfamily, the two varieties’ responses to salt stress impacted different metabolic pathways. The DEGs that were co-expressed in both varieties under stress may provide useful insights into the tolerance of cultivated hemp and other bast fiber crops to saline soil conditions. These transcriptomes also represent reference sequences for industrial hemp.

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Abbreviations

REC	Relative electric conductivity
DEG	Differentially expressed gene
qRT-PCR	Quantitative real-time PCR
ABA	Absciscic acid
KEGG	The Kyoto Encyclopedia of Genes and Genomes database
FDR	False discovery rate

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Introduction

Soil salinization is a growing global problem due to environmental deterioration caused, especially in China, by a shortage of cultivated land (Li et al. 2014). Salt stress in plants causes cell dehydration, osmotic stress, the generation of reactive oxygen species (ROS), and lack of absorption of the nutrient K^+ due to Na^+ competition (Ren et al. 2005). Salt stress also influences electron transfer, carbon assimilation, and light absorption and conversion due to the closure of stomata, slowing down or even halting growth in crop plants (Mao et al. 2008). Although salt stress can cause great harm to plants, salt damage can be tolerated by plants to a certain extent through various mechanisms, such as hormone regulation, ion transport, induction of antioxidant enzymes, and K^+/Na^+ homeostasis regulation. Therefore, research on crop salt tolerance has important implications for agricultural production.

Hemp (*Cannabis sativa*) has been cultivated in China for thousands of years. *C. sativa* is an annual dioecious herbaceous species evolved from wild Cannabis (Cannabaceae) varieties (Yang 2003; Sun 1993). Industrial hemp contains less than 0.3 % of the psychoactive cannabinoid Δ^9 -tetrahydrocannabinol (THC) in the young leaves and flowers. Industrial hemp is grown for its fibers to be used as raw materials in making paper, textiles, and biocomposite products (Struik et al. 2000). Hemp seeds are rich in oleic acid, linoleic acid, and other polyunsaturated fatty acids essential to humans. Industrial hemp also has important medical value, including anti-inflammatory, analgesic and anti-seizure roles of cannabinol (CBN), which is extracted from hemp leaves (Dai 1989). Due to increased shortages of cultivated land and the vast amounts of salinated land in China, it is necessary to improve the growth of bast fiber crops in salinated soil. ‘Yunma No.5’ and ‘Bamahuoma’ are two distinct industrial hemp varieties for different uses in China. ‘Bamahuoma’ has cultivated in Guangxi Province for the seed production. ‘Yunma 5’ is one of the most important industrial hemp varieties and is grown in Yunnan Province for fiber production. We hypothesized that these two varieties have diverged from each other due to their adaptations to different growth environments, resulting in distinct regulatory and metabolic pathways under salt stress. In order to verify our hypothesis, RNA-Seq was first performed on the leaves from the seedlings of these two varieties exposed to high salinity during early developmental stage. All genes were compared with the available reference *C. sativa* sequence (Bakel et al. 2011). Our results would be helpful in further studies on salt resistance mechanisms in bast fiber crops. Differentially expressed salt-regulated genes in the two hemp varieties could also be used in breeding programs to improve the salt resistance of these crops.

Materials and methods

Plant materials and NaCl treatment

The plant materials of ‘Yunma 5’ and ‘Bamahuoma’ were provided by Yunnan Academy of Agricultural Sciences and Guangxi Academy of Agricultural Sciences respectively. The seeds of two varieties were planted in the pots of 19 cm in height and 16 cm with an equivalent weight matrix (peat and perlite mixed in 1:1 ratio). Every variety has 18 pots: 9 pots for control; 9 pots for salt treatment. The salt treatment was applied when seedlings had 3–4 pairs of true leaves (about 15–20 cm high). The pots for salt treatment were watered with 400 ml of 500 mM NaCl once, while the pots for control watered with 400 ml plain water. All pots were watered with 400 ml plain water every two days after salt treatment to supply evaporated water. The leaves in three pots per treatment of one variety were randomly sampled at four time intervals (treated for 0, 2, 4 and 6 days) and stored at -80°C for measurement of physiological parameters and total RNA extraction.

Measurement of physiological parameters

In this study, we assessed physiological parameters to evaluate the tolerance of the hemp varieties ‘Yunma 5’ and ‘Bamahuoma’ to a high concentration of NaCl (500 mM). After harvesting 3–4 pairs of true leaves from seedlings irrigated with 500 mM NaCl, the degree of salt tolerance of the two varieties was evaluated through measurements of the relative electrical conductivity (REC) and the free proline content.

Relative electrical conductivity (REC)

Sampled leaves (0.1 g) were cut into similar sized pieces and were soaked in capped tubes with 10 ml distilled water for 12 h at room temperature. The initial electrical conductivity of the extracting solution (R1) was tested using a DDS-306 electrical conductivity meter (Fangzhou Company, Chengdu, China). The solutions were then heated to 100°C for 30 min and subsequently cooled to room temperature; the final electrical conductivity was measured (R2). The REC was calculated as follows:

$$\text{REC (\%)} = R1/R2 \times 100 \text{ (Chen et al. 2010).}$$

Free proline content

The concentration of proline in control and salt-treated seedlings were measured following the method of Bates (Bates et al. 1973). The proline agent, extracted with toluene, was measured using a UV-1600

spectrophotometer (Ruili Company, Shanghai, China) at 520 nm. The concentration of proline was estimated using the standard curve prepared from L-proline (0–100 µg/ml). Proline concentration was measured as µg/g fresh weight of sample.

Total RNA extraction

In order to avoid variability between plant samples, we pooled leaves of three plants randomly selected from one pot to extract total RNA. The total RNA from three pots were mixed together as a single biological replicate. 8 total RNA samples obtained from two varieties for four time intervals. Total RNA was extracted using the TriZol up kit (TransGen Biotech, China) and dissolving into RNase-free water (TransGen Biotech, China). RNA degradation and contamination were inspected on 1 % agarose gels. The RNA was checked for quantity and quality spectrophotometrically at OD₂₆₀/OD₂₈₀ ratio. (NanoDrop 2000/2000C Spectrophotometer, Thermo Scientific, USA).

cDNA library construction for RNA-Seq

A total of 8 libraries were constructed and sequenced using the Illumina sequencing platform (Illumina HiSeq™ 2000) by Genedenovo Co.Ltd, Guangzhou, China. Raw reads were filtered by removing low quality sequences to obtain clean reads, on which all subsequent analyses were based.

Identification of DEGs in the two varieties under salt stress

Sequencing reads were mapped to the *C. sativa* Purple Kush reference genome with BWA (Burrows-Wheeler Alignment Tool, <http://bio-bwa.sourceforge.net/bwa.shtml>) to obtain the expression levels of all genes. Read counts were analyzed using the bioconductor software package *edgeR* for analysis of DGE in the statistical environment R (Robinson et al. 2010).

Gene expression level was measured using the number of uniquely mapped reads per kilo base of exon sequence per million mapped reads (RPKM). The false discovery rate (FDR) was used to determine the *P* value threshold taking into account the multiple tests. Gene expression differences were considered significant with an $FDR \leq 0.001$ and an absolute value of the log₂ ratio ≥ 1 . Hierarchical cluster analysis was conducted using software MeV (MultiExperiment Viewer) based on log₂FC ratio value for each DEG.

GO annotation and KEGG pathway of DEGs

To determine biological functions and functional classifications, DEGs were annotated with the Gene Ontology (GO) database (<http://www.geneontology.org/>) using Blast2GO (Conesa et al. 2005) followed by WEGO software (Ye et al. 2006) to obtain GO functional classifications for all the DEGs. All DGEs were mapped to the Kyoto Encyclopedia of Genes and Genome (KEGG) Pathways database using BLASTX.

Gene expression pattern analysis in two varieties under salt stress

The expression patterns of all DEGs were discovered using STEM (Short Time-series Expression Miner, v1.3.8). Genes were clustered according to their different expression patterns according to variations in time. DE genes belong to the same cluster had similar expression pattern with each other. Clusters with specific expression patterns were selected and verified.

Verification of DEG expression with quantitative real-time PCR

In order to verify the reliability of RNA-Seq, 13 selected candidate DEGs involved in salt stress in both ‘Yunma 5’ (Y5) and ‘Bamahuoma’ (BM) were verified by qRT-PCR. These 13 genes contained 2 genes that were co-up-regulated in the two varieties and 6 and 5 genes up-regulated or down-regulated exclusively in single variety. The functions of the 13 DEGs are shown in Appendix E. First-strand cDNA was synthesized from 0.5 µg of total RNA treated with genomic DNA remover using TransScrip All-in-One First-Strand cDNA Synthesis SuperMix (TransGen, China). Eighteen pairs of primers were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>). Primer sequences for the qRT-PCR assay are listed in Appendix A. Real-time PCR was performed on ABI Prism7500 (Applied Biosystems, USA) with 1 µL of the first-strand cDNA template that was diluted tenfold. PCR was performed in triplicate to exclude sampling errors using SYBR Green Master Mix under the following conditions: 30 s at 94 °C, followed by 40 cycles of 94 °C for 5 s and 58 °C for 30 s. A quantification method ($2^{-\Delta\Delta CT}$) was used to determine the relative expression levels of 13 DEGs from the two varieties.

Results

Effects of salt stress on physiological parameters

The effects of high salinity on electrical conductivity ‘Yunma 5’ and ‘Bamahuoma’ is shown in Fig. 1a. Before

Fig. 1 Determination of physiological indexes. The relative electrical conductivity (a) and proline contents (b) of ‘Yunma 5’ (Y5) and ‘Bamahuoma’ (BM)

Table 1 Statistics for Illumina reads mapped to the *Cannabis sativa* genome

Sample	Total clean reads	Unique matches (%)	Non-unique matches (%)	Unmapped reads (%)
Y5C (0 day)	28815872	18187183 (63.12)	1120488 (3.89)	9508201 (33.00)
Y5 (2 days)	34507230	23446718 (67.95)	1780043 (5.16)	9280469 (26.89)
Y5 (4 days)	34998280	21626809 (61.79)	1511467 (4.32)	11860004 (33.89)
Y5 (6 days)	34573380	22510844 (65.11)	1385184 (4.01)	10677280 (30.88)
BMC (0 day)	33652134	18597157 (55.26)	1165299 (3.46)	13889678 (41.27)
BM (2 days)	32591506	18327830 (56.23)	1468316 (4.51)	12795360 (39.26)
BM (4 days)	31423636	19192253 (61.08)	1422049 (4.53)	10809334 (34.40)
BM (6 days)	34276778	19846970 (57.90)	1492242 (4.35)	12937566 (37.74)

Y5C, ‘Yunma 5’ control plants; Y5(2, 4, 6 days), ‘Yunma 5’ salt-stressed plants for 2, 4, 6 days; BMC, ‘Bamahuoma’ control plants; BM(2, 4, 6 days), ‘Bamahuoma’ salt-stressed plants for 2, 4, 6 days

salt stress (0 day, there was a slight difference in REC between the two varieties. After 2 days, the REC of the ‘Bamahuoma’ variety (75 %) was significantly higher than that of ‘Yunma 5’ (60 %). After 6 days, the RECs of the two varieties had increased to 85 % and 80 %, respectively. The variation in REC was greater for ‘Bamahuoma’ than for ‘Yunma 5’ throughout the duration of salt stress.

The results of the proline content assessment revealed a significant effect of high salinity on the free proline content in the cells of the two varieties (Fig. 1b). Salt stress caused a clear increase in proline content in the first 2 days in both varieties. For example, the proline content of ‘Yunma 5’ increased from 375 µg/g at 0 day to 715 µg/g at 2 days. The two varieties, however, showed some significant differences. The proline content of both varieties decreased from 2 to 4 days, but there was a greater reduction in ‘Bamahuoma’, which demonstrated a lower level of proline over days 4–6.

Mapping of reads

After removing low-quality sequences (Q-value <20), adaptor sequences, and reads with more than 50 % N bases, we obtained more than 28 million clean reads from each of the eight cDNA libraries (Table 1) and mapped gene expression profiles to the *C. sativa* genome with match rates of 55.26–67.95 %. Most of these represented unique matches, with the remainder (3.46–5.16 %) being non-unique, multiple-position matches. For further analyses, we only used unique matches.

Scatter plots of the abundances of transcripts in control and salt-treated libraries showed that ‘Bamahuoma’ exhibited more DEGs at 2 and 4 days than ‘Yunma 5’ (Fig. 2). The scatter plots also showed that the most up-regulated DEGs occurred 2 days after exposure to salt stress that was consistent with the results of physiological

Fig. 2 Scatter plots of the abundances of transcripts in control and salt-treated libraries. **a–c** Control versus salt-treated samples of ‘Yunma 5’ (Y5) at 2, 4, and 6 days. **d–f** Control versus salt-treated samples of ‘Bamahuoma’ (BM) at 2, 4, and 6 days

Fig. 3 Venn diagrams for the number of differentially expressed genes (**a**) in ‘Yunma 5’ (Y5) and (**b**) in ‘Bamahuoma’ (BM) under salt stress for 2, 4, and 6 days, compared to controls (Y5C and BMC)

parameters. These results were consistent with the results of physiological parameters measurements.

DEGs in both varieties under salt stress

To compare DEGs between the salt-stressed and control samples, we selected DEGs whose expression met the following criteria: $\text{RPKM} > 1$, $\log_2\text{FC} > 2$ and P value < 0.05 . Numbers of DEGs that occurred in response to stress at 2, 4, and 6 days in the two varieties are shown in Fig. 3.

The DEGs that occurred at 2 days could be classified into eight clusters according to their expression patterns (Fig. 4). As these clusters show, 1883 and 282 DEGs were up-regulated in ‘Bamahuoma’ and ‘Yunma 5’, respectively, and among these, 220 DEGs were co-up-regulated and 249 were co-down-regulated in the two varieties. Similarly, 2991 and 528 DEGs were down-regulated in ‘Bamahuoma’ and ‘Yunma 5’, respectively, including 24 DEGs that were co-down-regulated in the two varieties. There were also 26 DEGs that were up-regulated in

Fig. 4 Number of DEGs in ‘Yunma 5’ (Y5) and ‘Bamahuoma’ (BM) at 2 days. DEGs that were up-regulated or down-regulated exclusively in single variety are shown in *each independent circle*. DEGs with the same or opposite expression patterns between the two varieties are shown in the *overlapping regions*

‘Yunma 5’ but down-regulated in ‘Bamahuoma’. In contrast, 24 were up-regulated in ‘Bamahuoma’ but down-regulated in ‘Yunma 5’. The functions of the 220 co-up-regulated genes, the 249 co-down-regulated genes, the 26 genes up-regulated in ‘Yunma 5’ but down-regulated in ‘Bamahuoma’, and the 24 genes up-regulated in ‘Bamahuoma’ but down-regulated in ‘Yunma 5’ are listed in Appendix B.

118 salt-responsive DEGs were selected from the four clusters (220 co-up regulated, 249 co-down regulated, 24 and 26 up-regulated and down-regulated exclusively in single variety) in Fig. 4 showed differences between ‘Yunma 5’ and ‘Bamahuoma’. These 118 DEGs and their expression patterns are listed in Appendix I. The 118 DEGs, including 47 co-up-regulated, 60 co-up-regulated, 5 and 8 up-regulated and down-regulated exclusively in a single variety, compared to control in two varieties were classified using hierarchical cluster (Fig. 5).

According to KEGG classification, the common clusters in ‘Yunma 5’ (Table 2) and ‘Bamahuoma’ (Table 3) include ‘Photosynthesis’ and “Photosynthesis - antenna proteins”, the variety-specific clusters are: “Inositol phosphate metabolism”, “Spliceosome” and “Other types of O-glycan biosynthesis” in ‘Yunma 5’, “Porphyrin and chlorophyll metabolism”, “Fructose and mannose metabolism” and “Plant hormone signal transduction” in ‘Bamahuoma’ were significantly enriched metabolic pathways ($P < 0.01$).

Co-expressed transporter-encoding genes and transcription factors in the two variety under salt stress

On the basis of annotation on the Plant Transcription Factor Database (<http://planttfdb.cbi.pku.edu.cn/>) (Saier et al. 2006, 2009), a number of co-expressed genes differentially regulated in ‘Yunma 5’ and ‘Bamahuoma’ during salt stress were categorized as transporters. The majority of the transporter genes belonged to ion and amino acid transmembrane transporter activity, anion channel activity and transferase (Fig. 6a, b). A sodium transporter hkt1-like protein homolog, PK17182.1 was found only in ‘Yunma 5’ while another DEG, PK06358.1, a homolog of K⁺ uptake transporter 3 isoform 1 in *Theobroma cacao*, was found only in ‘Bamahuoma’.

By searching on the Plant Transcription Factor Database v3.0 (PlantTFDB 3.0) (Jin et al. 2014), 84 and 130 co-expressed genes were differentially regulated in ‘Yunma 5’ and ‘Bamahuoma’ respectively during salt stress. The 84 transcription factors in ‘Yunma 5’ were categorized to 25 compared to the 130 transcription factors in ‘Bamahuoma’ were categorized to 39 transcription factor families (Fig. 6c, d). Differentially expressed TFs mostly classified to NAC, MYB, WRKY families in these two varieties during salt stress. The transcripts present in each transcription factor family are presented in supplementary data J and K (Fig. 7).

Fig. 5 Hierarchical clustering of 118 DEGs related to salt stress at 2 days. Y5-2d, ‘Yunma 5’; BM-2d, ‘Bamahuoma’

Table 2 KEGG classification for ‘Yunma 5’ at 2 days under salt stress

Pathway	DEGs with pathway annotation (1927)	All genes with pathway annotation (10351)	P value
Y			
Photosynthesis	53 (2.75 %)	86 (0.83 %)	1.10497E–18
Photosynthesis - antenna proteins	21 (1.09 %)	30 (0.29 %)	1.06844E–09
Inositol phosphate metabolism	31 (1.61 %)	95 (0.92 %)	0.000734443
Spliceosome	80 (4.15 %)	310 (2.99 %)	0.000929217
Other types of O-glycan biosynthesis	4 (0.21 %)	5 (0.05 %)	0.005099866

Table 3 KEGG classification for ‘Bamahuoma’ at 2 days under salt stress

Pathway	DEGs with pathway annotation (1927)	All genes with pathway annotation (10351)	P value
B			
Photosynthesis	66 (2.08 %)	86 (0.83 %)	1.27395E–18
Photosynthesis - antenna proteins	24 (0.76 %)	30 (0.29 %)	3.35059E–08
Porphyrin and chlorophyll metabolism	34 (1.07 %)	72 (0.7 %)	0.00225854
Fructose and mannose metabolism	29 (0.91 %)	62 (0.6 %)	0.005457017
Plant hormone signal transduction	106 (3.34 %)	282 (2.72 %)	0.007034509

Fig. 6 Classification of transcription factor and transporter gene. Distribution of transcription factor gene families in ‘Yunma 5’ (a) and ‘Bamahuoma’ (b). Distribution of transporter gene families in ‘Yunma 5’ (c) and ‘Bamahuoma’ (d)

The co-up-regulated DEGs at 2 days in the two variety under salt stress

Among the 220 DEGs at 2 days that were co-up-regulated in the two varieties, a few important ones are listed in Table 2, such as ERF (ethylene response factor) (Zhai et al. 2013; Li et al. 2013; Dong et al. 2012), UDP-glucosyl-transferase activity (Lin et al. 2008), alpha/beta-hydrolase

superfamily protein (Lenfant et al. 2013), ABC transporter B family member, calcium-dependent lipid-binding family protein isoform 2, cysteine protease, heat shock protein 70, proline-rich cell wall protein-like precursor (Stein et al. 2011), and galactinol synthase 2-like (Sun et al. 2013) (Table 4). Four genes, namely the gibberellin receptor 1b, SAUR-like auxin-responsive protein family, histidine kinase 4-like, and GATA domain class transcription factor,

Fig. 7 Hierarchical clustering of 22 transcription factors among the co-up-regulated DEGs classified into 14 families

are involved in plant hormone signal transduction (Legay et al. 2009).

Transcription factors co-expressed in both varieties under salt stress

22 transcription factors at 2 days were identified and classified into 14 families as MYB, NAC, GATA, and HSF based on the Plant Transcription Factor Database (<http://plantfdb.cbi.pku.edu.cn/>) and their annotations. Among the 22 transcription factors, 7 were co-up-regulated, 7 were co-down-regulated in the two varieties, and 2 were down-regulated in ‘Yunma 5’ but up-regulated in ‘Bamahuoma’. A heat shock factor gene (PK02428.1) exhibited the most significant up-regulated expression profile in ‘Bamahuoma’ (Fig. 8). A MYB gene (PK24260.1) exhibited opposite expression patterns in the two varieties, while two other MYB genes (PK06396.1 and PK05123.1) demonstrated similar expression patterns (Fig. 9).

Gene expression pattern analysis in two varieties under salt stress

Genes were clustered by software STEM according to their different expressions among sequential variations of time (Ernst et al. 2005; Lu et al. 2014) (Fig. 8). The four break points in each curve represent the four salt-stress time intervals (0, 2, 4 and 6 days). Based on this analysis, the profiles of genes that were up-regulated and then down-regulated ($P \leq 0.05$) were grouped into profile 16 for ‘Yunma 5’ and profile 14 for ‘Bamahuoma’ (Fig. 8A–B), including 1714 DEGs in Y5 profile 16 and 2509 DEGs in BM profile 14. The DEGs in profile 16 were highly expressed at 2–4 days, while the DEGs in profile 14 were highly expressed only at 2 days.

Genes in these two profiles were subjected to GO-term analysis (Fig. 8), which identified electron carrier activity only in ‘Bamahuoma’. Further, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.kegg.>

Table 4 Salt-regulated DEGs that are co-up-regulated in the two varieties

Gene ID	Gene annotation	RPKM				log2 Ratio (Y5S/ Y5C)	log2 Ratio (BMS/ BMC)
		Y5C	Y5S	BMC	BMS		
PK21644.1	Alpha/beta-Hydrolases superfamily protein (Arabidopsis thaliana)	2.7	21.87	1.72	4.07	2.77	5.98
PK28100.1	Alpha/beta-Hydrolases superfamily protein (Arabidopsis thaliana)	4.85	20.38	3.28	83	2.91	4.67
PK01237.6	Multidrug resistance protein ABC transporter family	17.95	122.41	9.79	257.23	3.02	4.72
PK00363.1	ABC transporter B family member 19-like	2.78	20.67	4.7	66.08	2.07	3.81
PK23459.1	UDP-glucosyltransferase activity	12	56.09	8.39	41.4	2.23	2.3
PK25200.1	UDP-D-glucose/UDP-D-galactose 4-epimerase 5 isoform 1	54.42	241.26	25.89	681.61	2.15	4.72
PK13470.1	Ethylene response factor ERF1	11.12	84	5.81	73.48	2.92	3.67
PK25561.1	Tyrosine metabolism	47.33	211.83	44.89	656.2	2.16	3.87
PK14938.1	Amino acid permease 6	12.3	125.26	26.5	432.76	3.35	4.03
PK25561.1	Aspartate aminotransferase	47.335	211.83	44.89	656.21	2.16	3.87
PK26287.1	Beta-galactosidase 3 isoform 4	2.32	18.24	44.89	656.2	2.98	3.87
PK05306.1	Calcium-binding EF-hand family protein	9.73	86.86	21.8	125.71	3.16	2.53
PK09339.1	Calcium-dependent lipid-binding family protein isoform 2, partial	5.71	26.48	6.37	28.75	2.21	2.18
PK16090.1	Calcium-dependent lipid-binding family protein isoform 4	2.8	22.87	3.63	23.36	3.03	2.69
PK13478.1	Calcium-dependent lipid-binding family protein isoform 4	3.59	24.04	5.18	23.98	2.75	2.21
PK16995.1	Calcium-dependent lipid-binding family protein isoform 4	2.7	14.96	3.2	17.6	2.47	2.46
PK16932.3	Cysteine protease	42.47	558.6	107.54	652.97	3.72	2.6
PK16932.2	Cysteine protease	43.07	378.16	83.35	410.58	3.13	2.3
PK09922.1	E3 ubiquitin-protein ligase RNF25	38.02	155.93	19.84	80.09	2.04	2.01
PK00197.1	Glutamate dehydrogenase 2	8.93	77.95	4.98	230.55	3.13	5.54
PK19568.2	Heat shock cognate 70 kDa protein 1	4.26	20.17	5	178.91	2.24	5.16
PK02428.1	Heat shock factor 4	5.57	26.98	1.92	55.59	2.28	4.85
PK06896.7	Heat shock protein 70	2.12	11.16	1.66	604.38	2.4	8.51
PK28308.1	Heat shock protein 81.4	10.47	121.17	19.54	788.9	3.53	5.34
PK04256.2	Leucine-rich repeat protein kinase family protein isoform 1	1.84	9.578	1.46	67.01	2.39	5.52
PK08070.1	Proline-rich cell wall protein-like precursor	3.62	58.973	17.48	86.67	4.03	2.31
PK15022.1	Aldehyde dehydrogenase family 7 member A1-like Fatty acid metabolism	43.31	387.36	51.81	788.55	3.16	3.93
PK12124.1	E3 ubiquitin-protein ligase RMA1H1-like	6.93	50.36	4.06	142.94	2.86	5.14
PK20686.1	E3 ubiquitin-protein ligase UPL1-like	4.91	38.58	4.29	17.68	2.98	2.04
PK07006.1	Gibberellin receptor 1b	13.45	128.84	3.35	411.16	3.26	6.94
PK17887.1	SAUR-like auxin-responsive protein family	1.02	5.01	1.29	14.55	2.30	3.50
PK02363.1	Histidine kinase 4-like	3.38	17.14	1.57	48.38	2.34	4.95
PK07593.1	GATA domain class transcription factor	20.34	107.90	15.68	79.19	2.41	2.34

Y5C, 'Yunma 5' control plants; Y5S, 'Yunma 5' salt-stressed plants; BMC, 'Bamahuoma' control plants; BMS, 'Bamahuoma' salt-stressed plants

RPKM reads per kilobase of exon model per million mapped reads, which was used to represent the gene's expression level in one tissue

jp/kegg/pathway.html) was used to assess the biological functions of the DEGs. Most genes clustered in the two profiles were significantly enriched in metabolic pathways according to the KEGG annotation ($P \leq 0.01$). The DEGs in profile 16 of 'Yunma 5' were enriched in the spliceosome and cysteine and methionine metabolism pathways (Appendix C). The profile also included six genes denoted

HSP70-like protein (PK19568.2, PK06896.7, PK06896.9, PK06896.1, PK06896.2, PK06896.6) that are involved in the MAPK signaling pathway (Zeng and Zhang 2006). Zinc finger family genes (PK07293.1, PK00562.1), which are involved in making rice plants more resistant to salt stress, were also identified (Wang et al. 2015). A 1-aminocyclopropane-1-carboxylate oxidase gene

Fig. 8 Gene expression patterns and GO enrichment analysis of DEGs in profile 16 of ‘Yunma 5’ (a) and profile 14 of ‘Bamahuoma’ (b)

Fig. 9 qRT-PCR validation of 13 DEGs. Expression changes of 13 genes in the two varieties at 2 days under salt stress compared to controls was measured by qRT-PCR and compared to RNA-Seq data. Y5C/Y5S = Y5 control sample/ Y5 salt-stressed sample; BMC/ BMS = BM control sample/ BM salt-stressed sample

(PK26770.1) involved in cysteine and methionine metabolism also changed its expression in response to salt stress (Pan and Lou 2008).

The DEGs in profile 14 of ‘Bamahuoma’ were enriched in fatty acid metabolism, amino acid metabolism, protein processing, peroxisome, and plant-pathogen interactions (Appendix D). Several genes were involved in fatty acid metabolism, such as lipoxygenase genes (PK00878.1, PK20949.1, PK08197.4, PK12808.1, PK08197.1, PK08197.2, and PK08197.3), aldehyde dehydrogenase family genes (PK00013.1 and PK15022.1), acyl-CoA oxidase genes (PK18899.1, PK09506.1, PK26277.1, PK10458.1, and PK10458.2) and peroxisomal 3-ketoacyl-CoA thiolase genes (PK02646.1 and PK05121.3). The seven lipoxygenase genes are involved, in particular, in alpha-linolenic acid metabolism, while the two peroxisomal 3-ketoacyl-CoA thiolase genes are also involved in leucine and isoleucine metabolism. The KEGG analysis of stage-specific genes suggests that the two varieties’ adaptation to salinity was controlled by different molecular regulatory mechanisms.

Confirmation of candidate genes expression patterns by qRT-PCR analysis

Results of the qRT-PCR showed that the main trends in the expression of the 13 genes were identical to those identified with RNA-Seq (Fig. 8), supporting the reliability of the RNA-Seq data. Moreover, the fold-changes of the 13 genes obtained by RNA-Seq were generally higher than those obtained by qRT-PCR.

Discussion

Few studies have been performed on transcriptional profiling of *Cannabis sativa* under salt stress. In this study, using a combination of physiological parameters, we investigated the differential expression genes and related pathways of two *Cannabis sativa* varieties from different habitats exposed to high salinity through RNA-Seq analysis. Some candidate genes were further validated by qRT-PCR analysis. The observed increase in REC indicated that salinity caused harm to the seedlings throughout the duration of treatment. Proline content is an important indicator of physiological parameters in plants under stress (Mittal et al. 2012). Therefore, the content of free proline and REC were determined to investigate the effects of salt stress in the two varieties. The two varieties exhibited differences in proline content under salt stress, though the highest proline content was seen at 2 days for both. The change of proline content increased initially and then showed decrease, a trend which is generally observed in

other species such as *Gossypium hirsutum* L. under stress (He et al. 2007).

RNA-Seq was performed to reveal changes in the transcriptomes of ‘Yunma 5’ and ‘Bamahuoma’ exposed to NaCl. As the scatter plots showed, ‘Bamahuoma’ exhibited more up-regulated and down-regulated genes than ‘Yunma 5’ during the salt stress stage. Both varieties had more up-regulated genes at 2 days. 4836 genes and 469 genes were differentially expressed at 2 days in ‘Bamahuoma’ and ‘Yunma 5’, respectively (Fig. 3). The trend of proline was the result of the regulation of these genes. A total of 220 genes were co-up-regulated in ‘Yunma 5’ and ‘Bamahuoma’. Among these, several salt-related genes were identified, indicating that there are overlaps at the transcriptional level between the two varieties in response to salt stress. These genes are involved in several mechanisms in plants under stress. In other studies, the genes of ABC transporter family members, UDP-glycosyltransferase proteins, ethylene response factors, heat shock proteins, alpha/beta-hydrolase superfamily proteins, and cysteine proteases were revealed to be salt-regulated in other species. These genes were found to have altered expression in two poplar species treated with NaCl (Zhang et al. 2014). We found that isoforms of heat shock protein were up-regulated in the two varieties at 2 days, a finding which was also reported previously in case of *Petunia hybrida* (Villarino et al. 2014). The alpha/beta-hydrolase superfamily of proteins catalyze proteolysis and play an essential role in allowing cells to survive under various stresses, including salt stress (Hilt and Wolf 1992). The papain family is the most well studied of the cysteine proteases that can be regulated by the plant hormone abscisic acid (ABA) induced by hydropenia. The expression of CYP15a, whose sequence is similar to cysteine proteases, was increased by more than two-fold in pea seedlings treated with NaCl and KCl (Jones and Mullet 1995). UDP-glucosyltransferase genes were up-regulated in both varieties. The stress-regulated UDP-glucosyltransferase gene UGT85U1 was shown to improve the salt tolerance of transgenic *Arabidopsis* plants (Ahrazem et al. 2015).

Based on searching in PlantTFDB 2.0, the DEGs co-expressed during salt stress in the two varieties had been clustered to different families, including MYB, GATA, NAC and HSF, that play important roles in the response to various stresses in plants (Singh et al. 2002; Shameer et al. 2009). MYB transcription factors have important functions in the stress response in industrial hemp. MYB transcription factors such as TaPIMPI positively regulate salt and disease resistance in wheat by coordinating regulation of stress-related genes involved in ABA and SA signaling pathways (Zhang et al. 2012). Two NAC genes (PK25206.1 and PK26157.1) were up-regulated in the two hemp varieties. NAC transcription factors were found to be

involved in several signal transduction pathways in potato. Moreover, salt- and drought-related genes in *Arabidopsis thaliana* and rice are members of this family (Li et al. 2015; Hu et al. 2006). A GATA gene (PK07593.1) was up-regulated in the two hemp varieties and significantly up-regulated in ‘Bamahuoma’. ENA1, encoding a lithium and sodium ion transporter, is important to salt tolerance in yeast, and its expression is regulated by the rapamycin (TOR) pathway through the GATA transcription factors GLN3 and GAT1 (Crespo et al. 2001). A heat shock transcription factor (PK02428.1) was up-regulated in the two varieties and was the most significantly up-regulated expression profile in ‘Bamahuoma’ under salt stress. Twenty-five heat shock protein genes (OsHsf1–OsHsf25) were identified in rice and their expression is regulated by the abiotic stresses of salt and drought (Wan et al. 2010). Nine heat shock protein genes were identified from a proteomic analysis of rice, and 6 genes of them (*OsHsp93.04*, *OsHsp71.10*, *OsHsp71.18*, *OsHsp72.57*, *OsHsp24.15* and *OsHsp18.03*) were up-regulated by salt stress (Ye et al. 2012). Moreover, the expression of the heat shock protein gene *OsHsfB2b* was strongly induced by heat, salt, abscisic acid (ABA), and polyethylene glycol (PEG) treatment and negatively regulated the response to salt and drought stress in rice (Xiang et al. 2013).

Different transporter genes were identified by searching against the transporter classification database (TCDB) and mostly clustered to ion, amino acid and glucose transmembrane transporters.

We applied STEM v1.3.8 to the genes with same expression patterns, and selected genes with expression pattern of first increase and then decrease after salt treatment for a more detailed functional analysis. These profiles (profile 16 in ‘Yunma 5’ and profile 14 in ‘Bamahuoma’) included 1714 ‘Yunma 5’ and 2509 ‘Bamahuoma’ differentially expressed genes. GO classification and KEGG enrichment were performed on these genes, revealing 13 DEGs in profile 16 and 15 DEGs in profile 14 that were enriched in amino acid metabolism. In addition, 17 DEGs in profile 14 in ‘Bamahuoma’ were enriched in the alpha-linolenic acid metabolism pathway and 12 in fatty acid metabolism. The accumulation of organic molecules is an example of an adaptation to stress. Small molecules such as amino acids and fatty acids can synthesize substances to keep the osmotic pressure in balance in plant cells. Salinity is a major stimulant for lipid production in microalgae, and an increase in NaCl content increased the production of lipids and unsaturated fatty acids in *Chlamydomonas mexicana* and *Scenedesmus obliquus* (Salama et al. 2013; Kaewkannetra et al. 2012). Fatty acid β -oxidation is a key step in lipid metabolism and hormone biosynthesis in plants, a key role that is played by acyl-CoA oxidase (Arent et al. 2008). Expression of five fatty acid genes in an

Antarctic ice alga enhanced its adaption to high salinity (An et al. 2013). Furthermore, we identified aldehyde dehydrogenase family members, members of the fatty acid metabolism pathway. Stress can lead to the accumulation of toxic degradation products, including aldehydes (Srivastava et al. 2002). The overexpression of aldehyde dehydrogenase genes (ALDHs) from *A. thaliana* catalyzed the dehydrogenation of aldehydes and enhanced the salt tolerance of transgenic plants (Sunkar et al. 2003). Thus it is clear that in terms of the response of industrial hemp to salt stress, small molecule metabolism pathways deserve our attention. Finally, 33 DEGs in profile 16 were enriched in the spliceosome pathway. Under stress, plants can begin expressing stress-regulated genes through alternative splicing. Alternative splicing is an important method of gene expression regulation, directly determining the structure and function of diverse proteins (Kazan 2003).

Conclusions

We have presented here the first comprehensive transcriptome profiling analysis of *Cannabis sativa* under salt stress combined physiological parameters measurement. In this study, numerous genes encoding transcription factors and transporters showed different expression patterns between two varieties under stress. These results revealed that two industrial hemp varieties might have developed variety-specific adaptive mechanisms to salinity in their different habitats. To develop cultivars with high salt tolerance, especially bast fiber crops, the DEGs obtained in these two varieties can be further manipulated. Few studies have been performed on transcriptional profiling of *Cannabis sativa* under salt stress. The use of RNA-Seq and gene expression profiling to investigate the differences between two varieties of industrial hemp represent important novel aspects of our study. Our findings provide useful insights into the mechanisms of salt tolerance in fiber crops, particularly those grown in high-salinity soil.

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References

- Ahrazem O, Moraga AR, Mozos AT, Climen MFL, Cadenas AG, Gómez LG (2015) Ectopic expression of a stress-inducible glycosyltransferase from saffron enhances salt and oxidative stress tolerance in *Arabidopsis* while alters anchor root formation. *Plant Sci* 234:60–73
- An ML, Mou SL, Zhang XW, Zheng Z, Ye NH, Wang DS, Zhang W, Miao JL (2013) Expression of fatty acid desaturase genes and

- fatty acid accumulation in *Chlamydomonas* sp. ICE-L under salt stress. *Bioresour Technol* 149:77–83
- Arent S, Pye VE, Henriksen A (2008) Structure and function of plant acyl-CoA oxidases. *Plant Physiol Biochem* 46:292–301
- Bakel HV, Stout JM, Cote AG, Tallon CM, Sharpe AG, Hughes TR, Page JE (2011) The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol* 12:R102
- Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–207
- Chen AK, Han RH, Li DY, Ling LL, Luo HX, Tang SJ (2010) A comparison of two methods for electrical conductivity about plant leaves. *J Guangdong Educ Inst* 30:87–91
- Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21:3674–3676
- Crespo JL, Daicho K, Ushimaru T, Hall MN (2001) The GATA transcription factors GLN3 and GAT1 link TOR to salt stress in *Saccharomyces cerevisiae*. *J Biol Chem* 276:34441–34444
- Dai F (1989) Origin, use and geographical distribution of hemp (*Cannabis sativa* L.) in China. *J Southwest Teach Univ* 3:114–119
- Dong W, Ai XH, Xu F, Quan TY, Liu SW, Xia GM (2012) Isolation and characterization of a bread wheat salinity responsive ERF transcription factor. *Gene* 511:38–45
- Ernst J, Nau GJ, Joseph ZB (2005) Clustering short time series gene expression data. *Bioinformatics* 21:i159–i168
- He C, Shen G, Pasapula V, Luo J, Venkataramani S, Qiu X, Kuppu S, Korniyev D (2007) Ectopic expression of AtNHX1 in cotton (*Gossypium hirsutum* L.) increases proline content and enhances photosynthesis under salt stress conditions. *J Cotton Sci* 11(4):266–274
- Hilt W, Wolf DH (1992) Stress-induced proteolysis in yeast. *Mol Microbiol* 6:2437–2442
- Hu HH, Dai MQ, Yao JL, Xiao BZ, Li XH, Zhang QF, Xiong LZ (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *PNAS* 103:12987–12992
- Jin JP, Zhang H, Kong L, Gao G, Luo JC (2014) PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors. *Nucleic Acids Res* 42(D1):D1182–D1187
- Jones JT, Mullet JE (1995) A salt- and dehydration-inducible pea gene, Cyp15a, encodes a cell-wall protein with sequence similarity to cysteine protease. *Plant Mol Biol* 28:1005–1065
- Kaewkannetra P, Enmak P, Chiu TY (2012) The effect of CO₂ and salinity on the cultivation of *Scenedesmus obliquus* for bio-diesel production. *Bioproc Biosyst Eng* 1:591–597
- Kazan K (2003) Alternative splicing and proteome diversity in plants: the tip of the iceberg has just emerged. *Trend Plant Sci* 8:468–471
- Legay S, Lamoureux D, Hausman JF, Hoffmann L, Evers D (2009) Monitoring gene expression of potato under salinity using cDNA microarrays. *Plant Cell Rep* 28:1799–1816
- Lenfant N, Hotelier T, Bourne Y, Marchot P, Chatonnet A (2013) A proteins with an alpha/beta hydrolase fold: relationships between subfamilies in an ever-growing superfamily. *Chemico-Biol Interact* 203:266–268
- Li XP, Zhu XY, Mao J, Zou Y, Fu DW, Chen WX, Lu WJ (2013) Isolation and characterization of ethylene response factor family genes during development, ethylene regulation and stress treatments in papaya fruit. *Plant Physiol Biochem* 70:81–92
- Li JG, Pu LJ, Han MF, Zhu Zhang RS, Xiang Z (2014) Soil salinization research in China: advances and prospects. *J Geogr Sci* 24:943–960
- Li T, Sun JK, Liu JT (2015) Role of different transcription factor families in the regulatory networks of drought and salinity tolerance in plants. *Chin Bull Life Sci* 27:217–226
- Lin FY, Lu QX, Xu JH, Shi JR (2008) Cloning and expression analysis of two salt and *Fusarium graminearum* stress associated UDP-glucosyltransferases genes in wheat. *Hereditas* 30:1608–1614
- Lu XY, Kim HJ, Zhong SL, Chen HB, Hu ZQ, Zhou BY (2014) De novo transcriptome assembly for rudimentary leaves in *Litchi chinensis* Sonn. and identification of differentially expressed genes in response to reactive oxygen species. *BMC Genom* 15:809
- Mao XH, Liu CL, Yan LPS, Li L, Wang SG, Yang QS, Xia Y (2008) Salt damage mechanism to plants and the coping strategies to salt stress in plants. *J Shandong For Sci Technol* 4:128–130
- Mittal S, Kumari N, Sharma V (2012) Differential response of salt stress on *Brassica juncea*: photosynthetic performance, pigment, proline, D1 and antioxidant enzymes. *Plant Physiol Biochem* 54:17–26
- Pan G, Lou CF (2008) Isolation of an 1-aminocyclopropane-1-carboxylate oxidase gene from mulberry (*Morus alba* L.) and analysis of the function of this gene in plant development and stresses response. *J Plant Physiol* 165:1204–1213
- Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat Genet* 37:1141–1146
- Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–140
- Saier MH Jr, Tran CV, Barabote RD (2006) TCDB: the transporter classification database for membrane transport protein analyses and information. *Nucleic Acids Res* 34:D181–D186
- Saier MH Jr, Yen MR, Noto K, Tamang DG, Elkan C (2009) The transporter classification database: recent advances. *Nucleic Acids Res* 37(D1):D274–D278
- Salama ES, Kim HC, Abou-Shanab RAI, Ji MK, Oh YK, Kim S, Jeon BH (2013) Biomass, lipid content, and fatty acid composition of freshwater *Chlamydomonas mexicana* and *Scenedesmus obliquus* grown under salt stress. *Bioproc Biosyst Eng* 36:827–833
- Shameer K, Ambika S, Varghese SM, Karaba N, Dayakumar MU, Sowdhamini R (2009) STIFDB—arabidopsis stress responsive transcription factor database. *Int J Plant Genom* 2009:583429
- Singh KB, Foley RC, Oñate-Sánchez L (2002) Transcription factors in plant defense and stress responses. *Curr Opin Plant Biol* 5(5):430–436
- Srivastava S, Bysani C, Aruni B, Sumanth DP (2002) Lipid peroxidation-derived aldehydes and oxidative stress in the failing heart: role of aldose reductase. *Am J Physiol-Heart Circ Physiol* 283:H2612–H2619
- Stein H, Honig A, Miller G, Erster O, Eilenberg H, Csonka LN, Szabados L, Koncz C, Zilberstein A (2011) Elevation of free proline and proline-rich protein levels by simultaneous manipulations of proline biosynthesis and degradation in plants. *Plant Sci* 181:140–150
- Struik PC, Amaducci S, Bullard MJ, Stutterheim NC, Venturi G, Cromack HTH (2000) Agronomy of fibre hemp (*Cannabis sativa* L.) in Europe. *Ind Crop Prod* 11:107–118
- Sun AG (1993) China is the origin of *Cannabis sativa*. *Plant Fibers Prod* 3:45–48
- Sun ZB, Qi XY, Wang ZL, Li PH, Wu CX, Zhang H (2013) Overexpression of TsGOLS2, a galactinol synthase, in *Arabidopsis thaliana* enhances tolerance to high salinity and osmotic stresses. *Plant Physiol Biochem* 69:82–89
- Sunkar R, Bartels D, Kirch HH (2003) Overexpression of a stress-inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. *Plant J* 35:452–464
- Villarino GH, Bombarely A, Giovannoni JJ, Scanlon MJ, Mattson NS (2014) Transcriptomic analysis of *Petunia hybrida* in response to

- salt stress using high throughput RNA sequencing. PLoS One 9:e94651
- Wan BL, Zha ZP, Du XS (2010) Expression profile analysis of rice heat shock transcription factor (HSF) genes in response to plant hormones and abiotic stresses. China Biotechnol 30:22–32
- Wang WY, Lu BH, Xu MY, Jamil M, Wang G (2015) ABA-induced CCCH tandem zinc finger protein OsC3H47 decreases ABA sensitivity and promotes drought tolerance in *Oryza sativa*. Biochem Biophys Res Commun 464:33–37
- Xiang JH, Ran J, Zhou J, Zhou XY, Liu AL, Zhang XW, Peng Y, Tang N, Luo Y (2013) Heat shock factor OsHsfB2b negatively regulates drought and salt tolerance in rice. Plant Cell Rep 32:1795–1806
- Yang RL (2003) Cannabis cultivation and utilization. China Fiber Insp 3:41
- Ye J, Fang L, Zheng HK, Zhang Y, Chen J, Zhang ZJ, Wang J, Li ST, Li RQ, Bolund L, Wang J (2006) WEGO: a web tool for plotting GO annotations. Nucleic Acids Res 34:W293–W297
- Ye SF, Yu SW, Shu LB, Wu JH, Wu AZ, Luo LJ (2012) Expression profile analysis of 9 heat shock protein genes throughout the life cycle and under abiotic stress in rice. Chin Sci Bull 57:336–343
- Zeng JQ, Zhang MY (2006) The role of alternative splicing in the regulation of plant stress-associated gene expression. Plant Physiol Commun 42:1005–1014
- Zhai Y, Wang Y, Li YJ, Lei TT, Yan F, Su LT, Li XW, Zhao Y, Sun X, Li JW, Wang QY (2013) Isolation and molecular characterization of GmERF7, a soybean ethylene-response factor that increases salt stress tolerance in tobacco. Gene 513:174–183
- Zhang ZY, Liu X, Wang XD, Zhou MP, Zhou XY, Ye XG, Wei XN (2012) An R2R3 MYB transcription factor in wheat, TaPIMP1, mediates host resistance to *Bipolaris sorokiniana* and drought stresses through regulation of defense- and stress-related genes. New Phytol 196:1155–1170
- Zhang J, Feng JJ, Lu J, Yang YZ, Zhang X, Wan DS, Liu JQ (2014) Transcriptome differences between two sister desert poplar species under salt stress. BMC Genom 15:337