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BULK SEGREGANT TRANSCRIPTOME ANALYSIS BASED DIFFERENTIAL EXPRESSION OF DROUGHT RESPONSE GENES IN MAIZE

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Drought stress is the major threat to maize development and production. Here the bulked segregant transcriptome analysis (BSTA) was applied to discover the molecular mechanisms of drought stress responses in maize. The evaluation of drought stress tolerance was contacted for 200 maize inbred lines. Two bulk pools were constructed by selecting 10 inbred lines that were tolerant to drought (TD) and other 10that were sensitive to drought (SD). BSTA was performed to identify the differentially expressed genes (DEGs) within two kinds of maize under drought treatment. A total of 4886 and 5274 DEGs were detected in TD and SD bulk pools, respectively. The functional annotations showed that most of the DEGs are involved in the hormone metabolism, transcription regulation and alternative splicing pathways. Comparisons of the DEGs between two bulk pools revealed that the number of up-regulated genes related to alternative splicing in TD bulk pool was significantly higher than that in SD bulk pool. Besides, 928 up-regulated genes and 346 down-regulated genes among 1274 DEGs were identified including protein kinase andtranscription factors only in TD bulk pool. This result indicates that many common mechanisms are involved in drought regulation, moreover alternative splicing may play dominant role in response to drought stress in maize. Our results contribute to understand the mechanisms of drought stress tolerance of maize.
Keywords: maize (*Zea mays* L.), drought, abiotic stress, bulked segregant transcriptome analysis

INTRODUCTION

Plants must overcome various environmental challenges to survive during growth and development. Abiotic stresses like drought, cold, heat and salinity are the major factors having adverse effects on crop production and threat to food security (Zhu, 2016). As a important cereal crop in the world, maize plays crucial role in consolidating world food security (Strable and Scanlon, 2009). However, drought stress is the main limiting factor for production of maize (Gong *et al.,* 2014). During developmental phases, the seedling stage is much more sensitive to dehydration. Severe drought stress causes stunted growth leading to death of maize seedling (Peleg and Blumwald, 2011). Understanding of strategies involved in drought stress responses in maize seedlings, especially the cultivars with excellent drought tolerance, will provide considerable clues to improve the drought tolerance of maize.

Phytohormones including abscisic acid (ABA), cytokinin (CK), auxin (IAA), brassinosteriods (BR), salicylic acid (SA) and ethylene (ET) were found involved in drought stress response in plants. Among them, ABA plays central roles in drought stress responses by regulating water loss and adjusting the gene expression (Golldack *et al.,* 2011). The synthesis of ABA rely on catalytic enzymes such as NCED and ABA3/LOS5 (Nambara and Marion-Poll, 2005; Peleg and Blumwald, 2011). In tomato, ABA accumulation and the ability of drought tolerance were significantly increased by overexpressing of *LeNCED1* in transgenic plants (Thompson *et al.,* 2007). Similarly, the overexpression of *SgNCED1* could enhance the accumulation of ABA in leaves and improve the tolerance to drought stress in tobacco (Zhang *et al.,* 2008). *ABA3/LOS5*has been modulated for reducing drought damage by overexpressing in transgenic rice (Xiong *et al.,* 2001).

During drought stress, induction of transcription factors (TFs) such as AP2/EREBP (APETALA2/ethylene-responsive element binding proteins*)*, bZIP (basic region/leucine zipper motif*)*, NAC (NAM, ATAF and CUC) and MYB (v-myb avian myeloblastosis viral oncogene homolog) regulates the expression of down stream genes and enhances tolerance to abiotic stresses in plants. For example, the expression levels of transcription factors *MYBJ7*, *BZIP50*, *C2H2*and *NAC2*increasedafter imposition of drought stress in soybean roots (Pereira *et al.,* 2011). Overexpression of the gene *SNAC2* enhanced the ability of drought tolerance in rice (Hu *et al.,* 2008).Tolerance to drought was enhanced by overexpressing the maize transcription factor *ZmMYB3R* in transgenic Arabidopsis (Wu *et al.,* 2019).Transcription factors are an important molecular mechanism for plants to

cope with stresses, so the study of TFs may provide important information for the improvement of drought resistance.

Alternative splicing (AS) is one kind of the post-transcriptional regulation which affects the diversity of proteomics. As is pervasive in the regulation of plant development and stress response by increasing in functional proteins and regulating of gene expression (Reddy *et al.*, 2013). The genome-wide analysis of maize indicated that alternative splicing responds to abiotic stresses and increases the ability of stress tolerance (Thatcher *et al.*, 2014).

Bulked sample analysis (BSA) belongs to trait-based sampling which used to select representative samples by separating extreme individuals from sample populations. It can reduce the differences between individuals and enhance the trait of sampled groups (Zou *et al.*, 2016).With the development of bioinformatics technology, RNA-seq technology was widely used for analyzing gene expressions and regulatory networks (Mcgettigan, 2013).In our study, BSA was employed in selecting two pools of extreme individuals from 200 samples including 10 drought-tolerance inbred lines (TD) and 10 drought-sensitive inbred lines (SD). Then RNA-seq was used to discover the differences of drought response between drought-tolerance and drought-sensitive individuals in maize at seedling stage. The differentially expressed genes (DGEs) were identified both in TD and SD. The comparison of DEGs between two bulks was conducted to find the character of TD at transcriptome level. The credibility of identified DEGs was confirmed by qRT-PCR.

MATERIALS AND METHODS

***Plant material and drought stress treatment*:** A total of 200 maize inbred lines were grown in plastics pots (14 cm×12 cm) filed with the same soil (uniformly mixed and cleaned from trashes by using sieve) at the rate of 1.5 kg per pot and placed in an identical environment. Each pot was given 200 ml irrigation water after sowing. Three replicates for each treatment were applied. After germination, the seedlings were cultured in glass greenhouse (28/22 ℃, day/night) under a photoperiod of 10-hlight/14-h dark and a humidity of 60% at 25 ℃. Drought treatment was applied at three-leaf stage by stopping irrigation. The seedlings were re-irrigated after 5-days of drought treatment. With the application of full treatment including drought and re-irrigation, all of the maize inbred lines were divided into five groups according to the survival rate (L5: 0% - 20%, L4: 20% -40 %, L3: 40% -60%, L2: 60% -80%, L1: 80% -100%). The typical phenotypes of five groups showed different degrees of wilting (Figure S1) after drought treatment. A total of 20 inbred lines (10 inbred lines with excellent drought tolerance and another 10 sensitive to drought) were selected according to the wilting degree statistics.

Each tolerant to drought (DT) and sensitive to drought (SD) groups of inbred lines were further classified into two subgroups according to the different treatments (Table 1). After 4-days of treatment, the third leaf of each TD and SD inbred line with and without drought treatments was sampled and thoroughly mixed according to the subgroup. The samples were frozen in liquid nitrogen and stored at -80℃.

***RNA isolation and RNA sequencing*:** Total RNA was extracted from the bulked samples using RNAiso Plus Total RNA extraction reagent (Cat#9109, Takara). The Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US) was used to check the RNA integrity. Total RNA was further purified by clearing the DNA. The mRNA was selected from purified total RNA and fragmented into short pieces. The first strand and the second strand cDNA were synthesized before the end-repair, 3'dA addition and adapter ligation. The cDNA libraries were created for RNA-seq analysis which was performed on an Illumina HiSeq 2500 sequencerat shanghai biotechnology corporation.

Table 1. Four groups of abbreviations.

Tolerant to Drought (DT)		**Sensitive to drought (SD)**	
Tolerant to drought with drought treatment (TD-DT)	Tolerant to drought without drought treatment (TD-NDT)	Sensitive to drought with drought treatment (SD-DT)	Sensitive to drought without drought treatment (SD-NDT)

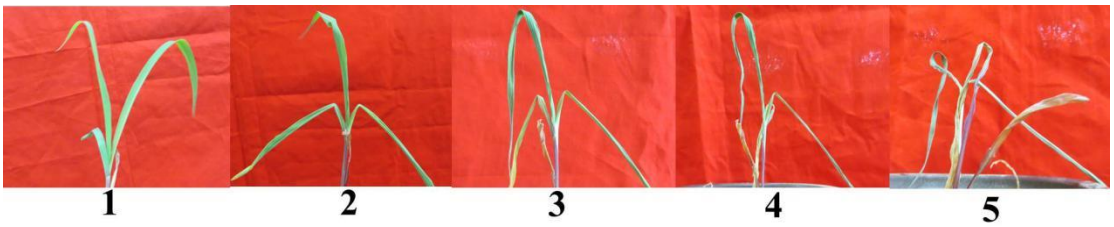

Figure S1. Wilting level reference. Plant wilting is divided into five levels (L1-5) after drought treatment. L1: Wilting begins to appear; L2: All leaves appear wilted; L3: Leaves withered and sinking; L4: Yellow leaves and dried leaves appear; L5: Plant death.

Table S1. Primer pairs for quantitative real-time PCR verification.

Gene	F primer	R primer
GRMZM2G064096	GCGGTTAACTTGTTTTTCGAGA	TAGACCACGAGTACTTTGACAC
GRMZM2G047732	ATCATGTTGTTATGGGTCCTGT	ATCGATTGCACACAGTAATTCG
GRMZM2G109812	CACTGTGTCATCTCTGTGTTTG	TTAGCACAATAATCGCCAAGTG
GRMZM2G011932	GTACTCCAGTTTAGGCACTGAT	TACGTTACAACTCTGGGTACAC
GRMZM5G898290	CTTCTGGGGCTTCTGAACTTC	GGCACAATTACAATCCCAGTTT
GRMZM2G082520	CATCATCCAGTACTAGCTCTCG	GCAGCAATACAATAGCACTACC
GRMZM2G176595	GGCTTTGCTTGTACTTGTACAG	CCTACAGCTACTACATACAGCG
GRMZM2G176998	ATATGGGTTTGTACGGTTCCAT	GAGTCGGAGGTAGAATACGAAG
GRMZM2G131266	CAGCAACCTACTGAATTCATCG	GAAGCTCATGTCGTTATCGTTC
GAPDH	CCATCACTGCCACACAGAAAAC	AGGAACACGGAAGGACATACCAG

Using the Seqtk tool (https://github.com/lh3/seqtk), the clean reads were achieved by removing the adapters, low-quality reads (Q values < Q20, Q=-10logerror_ratio), ribosome RNA reads and the short reads (the length < 25) from the raw reads. Hisat2 (version:2.0.4) (Kim *et al.*, 2015) was used to map the clean reads to the reference genome B73 (AGPv3 release24, ftp://ftp.ensemblgenomes.org/pub/plants/release-24/fasta/zea_mays/dna/).Theexpression of each genewas evaluated by Fragments Per Kilobases of exon model per Million mapped reads (FPKM) (Mortazavi *et al.*, 2008).

***Analysis of differentially expressed genes (DEGs)*:** The R package was used for differential expression analysis between control and drought treatments. Firstly, genes were filtered based on FPKM values (both below 5 in control and treated samples were removed). Then, genes with p-value ≤ 0.05 and |fold changes (FC)| ≥ 2 were identified as differential expressed genes (DEGs). The p-value was corrected by the false discovery rate (FDR).GO (Gene Ontology) term enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis of DEGs was performed by using the R package. The analysis of plant transcription factor was relying on the plant TF database Plant TFDB (version 4.0, http://planttfdb.cbi.pku.edu.cn/index.php?sp=Zma).

***Quantitative real-Time PCR (qRT-PCR) analysis*:** Quantitative real-time PCR was performed to validate the gene expression profiles of RNA-seq. Using gene primers (Table S1), nine genes were selected for the validation of the RNA-seq results by qRT-PCRin the BIO-RAD CFX Connect. RNA extraction, cDNA synthesis and qRT-PCR were performed with RNAiso Plus (Code No.: 9109) (Takara), EasyScript One-Step gDNA Removal (TransGen), cDNA Synthesis SuperMix (TransGen) and RealUniversal Color PreMix (SYBR Green) (TIANGEN), respectively.Each PCR reaction (10μL) contained 5 μL 2 ×RealUniversal Color PreMix, 0.3 μM of each primer and appropriately diluted cDNA. The thermal cycling conditions were 95°C for 15 min, followed by 40 cycles of 10s at 95 and 30s at62°C. At the melt curve stage, 65 °C to 95 °C with increment of 0.5 °C for 0.05s were used. The relative expression levels of eleven genes was calculated with the $2^{-\Delta\Delta Ct}$method (Pfaffl, 2001; Schmittgen and Livak, 2008).

RESULTS

***Evaluation of drought tolerance of maize inbred lines*:** Drought and re-irrigation is a continuous process in natural agricultural production. Drought tolerance is considered as a

Table S2. List of the TD and SD maize inbred lines.

	TD			SD	
No.	**Maize #**	**Name**	**No.**	**Maize #**	**Name**
21	13HF2351	La Posta Seq C7-F180-3-1-1-1	71	13HF0933	CML96
38	13HF1849	(CML165/GEM0005)-1-1	73	13HF0823	CML103
46	13HF2126	CML489/CML444//ZM521B-66-4-1-1-1-BB]-7-3-1-B	75	13HF0826	CML115
55	13HF2202	[G16SeqC1F47-2-1-2-1-BBBB-B-xP84c1 F27-4-1-6-B-5-B] F23-2-1-2-3 x P43C9-1-1-1-1-1-BBBB-1-xP84c1 F26-2-2-6-B-3-B]-2-1-B/CML395]-1-1	80	13HF0831	CML130
85	13HF1595	CML154	86	13HF0838	CML162
127	13HF0904	CML431	104	13HF0865	CML304
154	13HF0792	[CML312/[TUXPSEQ]C1F2/P49-SR]F2-45-3-2-1-BB//INTA-F2-192-2-1-1-1-BBBB]-1-5-1-1-1-BBB-B-B-B	106	13HF0876	CML312SR
155	13HF2119	[CML312/[TUXPSEQ]C1F2/P49-SR]F2-45-3-2-1-BB//INTA-F2-192-2-1-1-1-BBBB]-1-5-1-1-2-BB-B	107	13HF0877	CML312SRQ=[[(CLQ-RCWQ83xCML312SR)xCML312SR]xCML312SR)]-15-1-BBB
207	13HF2351	La Posta Seq C7-F180-3-1-1-1-B-B-B-B-B	111	13HF0882	CML325
217	JH13A-501-57	((CML451/OFP9)-B)-1-2-1-1	167	13HF1480	90[SPMATC4/P500 (SELY)]#-B-4-2-B-B

Table S3. Summary of the illumine sequencing data.

Sample	Replicate	Raw reads	Clean reads	Mapped reads	Unique reads	Mapping ratio
TD-DT	1	39,385,562	37,274,417	30,606,822	29,970,242	85.61%
TD-DT	2	46,529,578	42,959,362	34,406,363	33,688,122	85.41%
TD-DT	3	57,484,006	54,595,713	45,292,831	44,361,400	86.06%
TD-NDT	1	48,019,986	45,600,513	37,892,891	37,112,438	86.19%
TD-NDT	2	48,349,506	45,627,775	37,825,421	37,089,970	86.46%
TD-NDT	3	47,317,620	44,322,809	36,722,493	36,000,525	86.42%
SD-DT	1	57,302,710	55,215,851	45,775,385	44,763,873	85.10%
SD-DT	2	50,199,246	47,610,522	39,171,293	38,330,376	85.41%
SD-DT	3	45,646,176	43,481,331	35,879,876	35,125,393	85.40%
SD-NDT	1	47,236,450	42,253,140	33,082,098	32,415,920	86.50%
SD-NDT	2	49,098,876	43,664,302	34,016,993	33,319,040	86.63%
SD-NDT	3	50,039,644	47,207,868	39,189,490	38,415,838	86.76%

Mapping ratio=Mapped reads/All reads.

complex mechanism based on the ability of plants to maintain vigor during drought and to recover after drought. Here the wilting degree under drought treatment and the recovery rate after drought were used to evaluate the drought tolerance of maize inbred lines in this study. In present study, 200 maize inbred lines were selected from 400 tropical maize inbred lines of CIMMYT (Yunbi Xu, personal communication).A total of 10 lines, tolerant to drought (TD) and other 10 lines, sensitive to drought (SD) (Table S2) were selected by giving different degrees of wilting after comparing to reference (Figure S1). The TD lines (No. 21, 38, 46, 55, 85, 127, 154, 155, 207 and 217) have higher life vigor than others under drought stress and have a higher recovery rate after drought period.

***Overview the transcriptome of maize leaves under drought treatment*:** Seedling of two extreme phenotypic pools including 10 tolerant and 10 sensitive inbred lines were subjected to drought stress for four days and grown without treatment. The total RNA was extracted from maize leaves of these pools. A total of 88.7Gb raw data were obtained from 12 cDNA libraries. With filtering out the adapter, low-quality reads and the short reads (< 25 bases), clean reads were obtained and used for future analysis. Most of the clean reads were mapped to the reference genome sequences (FigureS2). The mapping rate is higher than 85% for each sample and is shown in Table S3. The FPKM was used to calculate the expression level of genes. The Pearson correlation was applied to quantify the gene expression level between samples. As shown in the heat map (Figure S3), the R^2between replicates of each sample is around 0.95, which indicated the excellent repeatability of transcriptome data.

***Identification of differentially expressed genes (DEGs)*:** A total of 31771 and 31828 genes were identified in TD and SD, respectively. The DEGs in each bulked pools were identified by comparing the gene expression levels before and after drought treatment (TD-DT vs TD-NDT, SD-DT vs SD-NDT; FPKM both higher than 5, $|\log_2 FC| \geq 1$ and p-value ≤ 0.05). The number of DEGs with at least two or five-fold change is shown in Table 2. For TD bulked pool, 4886 DEGs were obtained with at least two folds change including 3124 up-regulated and 1762 down-regulated genes. In SD, 5274 DEGs including 3359 up-regulated and 1915 down-regulated genes were found with at least two fold change. With at least 5-fold change, 1450 and 1709 DEGs were identified in TD and SD pool, respectively (Table 2).

As shown in the Figure S4a, the number of up-regulated genes was higher than that of down-regulated DEGs under drought treatment. A total of 3612 DEGs overlapped between TD and SD, of which 3593 genes (2193 up- and 1400 down-regulated genes) have the same expression pattern (Figure S4b). Three genes were down-regulated in SD pool whereas up-regulated in TD pool. While 16 genes were up-regulated in SD pool whereas down-regulated in TD pool. With drought treatment, 1274DEGs, including 928 up-regulated and 346 down-regulated genes, were uniquely identified in the TD pool, 1662 DEGs (1150 up-regulated and 512 down-regulated) were uniquely identified in the SD pool.

Table 2. Differential expressed genes (DEG) during drought treatment.

		TD	SD	Common in both
DEG	Total DEG	4886	5274	3612
(at least two fold)	Up-regulated	3124	3359	2193
	Down-regulated	1762	1915	1400
DEG	Total DEG	1450	1709	1067
(at least five fold)	Up-regulated	870	1061	653
	Down-regulated	580	648	412

Table S4. List of ABA-related DEGs in TD and SD.

Gene ID	Categorization	Arabidopsis homologs	TD		SD	
			Fold	Qvalue	Fold	Qvalue
GRMZM2G014392	NCED（vo14）	AT1G78390	5.77	1.08E-38	5.31	3.66E-150
GRMZM5G838285	NCED（vo14）	AT1G78390	4.66	1.28E-23	6.09	1.53E-89
GRMZM2G417954	NCED（vo14）	AT1G78390	6.86	7.48E-68	6.28	1.81E-145
GRMZM2G179147	CYP707As	AT4G19230	-2.03	1.31E-16	-2.36	2.83E-44
GRMZM2G126505	CYP707As	AT5G45340	-2.17	8.11E-14	N/A	N/A
GRMZM2G002142	CYP707As	AT3G19270	3.68	6.65E-10	3.02	4.02E-18
GRMZM2G168016	CYP707As	AT1G19630	3.09	7.88E-13	N/A	N/A
GRMZM2G103773	CYP707As	AT3G30180	N/A	N/A	1.35	2.77E-07
GRMZM2G012391	CYP707As	AT5G05690	-1.81	3.83E-14	-2.02	7.12E-45
GRMZM2G167336	CYP707As	AT5G06900	-4.00	4.16E-29	-3.56	9.73E-74
GRMZM2G070508	CYP707As	AT2G26170	-2.11	1.63E-21	-2.16	2.17E-43
GRMZM2G014580	CYP707As	AT3G16100	-1.13	1.13E-08	N/A	N/A
GRMZM2G104783	CYP707As	AT2G46660	1.42	1.15E-03	N/A	N/A
GRMZM2G139874	CYP707As	AT2G30490	1.60	3.12E-06	2.15	7.62E-32
GRMZM2G106468	CYP707As	AT2G46950	1.77	3.47E-05	1.52	4.66E-06
GRMZM2G161472	CYP707As	AT5G25900	1.84	8.25E-06	2.11	8.57E-11
GRMZM2G102318	CYP707As	AT3G14630	2.21	1.17E-06	N/A	N/A
GRMZM2G020761	CYP707As	AT2G46950	2.31	6.59E-07	2.26	3.01E-25
GRMZM2G067225	CYP707As	AT5G42650	2.57	2.26E-04	3.86	5.64E-104
GRMZM2G010468	CYP707As	AT2G30490	3.11	7.43E-09	4.58	2.73E-119
GRMZM2G161169	CYP707As	AT5G36110	N/A	N/A	-4.64	2.01E-85
GRMZM2G138248	CYP707As	AT4G39950	N/A	N/A	-3.29	4.36E-47
GRMZM2G140817	CYP707As	AT2G40890	N/A	N/A	1.42	4.02E-11
GRMZM2G123309	CYP707As	AT3G14690	N/A	N/A	1.44	1.08E-07
GRMZM5G875732	CYP707As	AT3G48280	N/A	N/A	1.67	4.77E-13
GRMZM2G087875	CYP707As	AT4G37370	N/A	N/A	4.54	1.05E-47
GRMZM2G059453	PP2Cs	AT2G29380	3.28	8.11E-26	2.97	3.54E-58
GRMZM2G383807	PP2Cs	AT1G17550	1.59	1.85E-04	2.20	1.52E-19
GRMZM2G122228	PP2Cs	AT2G29380	8.21	6.42E-45	6.87	3.77E-103
GRMZM2G308615	PP2Cs	AT2G29380	4.71	8.35E-09	6.06	8.65E-113
GRMZM2G134628	PP2Cs	AT1G72770	2.88	2.39E-21	2.90	3.60E-53
GRMZM2G059453	PP2Cs	AT2G29380	3.28	8.11E-26	2.97	3.54E-58
GRMZM2G300125	PP2Cs	AT2G29380	6.38	3.52E-31	6.86	2.56E-115
GRMZM2G177386	PP2Cs	AT1G72770	3.83	2.47E-25	3.46	1.48E-84
GRMZM5G818101	PP2Cs	AT2G29380	4.86	4.80E-47	3.73	8.84E-77
GRMZM2G001243	PP2Cs	AT1G72770	3.68	8.24E-31	3.00	1.44E-66
GRMZM2G437575	PP2Cs	AT4G26080	3.69	1.53E-16	3.63	1.25E-37
GRMZM2G166297	PP2Cs	AT2G29380	2.50	6.58E-15	2.65	1.81E-44
GRMZM2G159811	PP2Cs	AT5G51760	4.83	1.30E-32	3.94	6.67E-83
GRMZM2G019819	PP2Cs	AT5G51760	5.82	2.13E-50	4.21	1.47E-63
GRMZM2G082487	PP2Cs	AT2G29380	5.01	2.75E-22	5.83	9.21E-133
GRMZM2G035809	SnRK	AT1G78290	N/A	N/A	1.02	4.84E-05
GRMZM2G110922	SnRK	AT1G10940	3.17	8.42E-19	3.24	1.10E-62
GRMZM2G110908	SnRK	AT1G10940	N/A	N/A	-1.78	9.71E-38
GRMZM2G171435	SnRK	AT4G33950	1.52	4.55E-05	1.58	2.17E-14
GRMZM2G035809	SnRK	AT1G78290	N/A	N/A	1.02	4.84E-05
GRMZM2G110922	SnRK	AT1G10940	3.17	8.42E-19	3.24	1.10E-62
GRMZM2G334791	SnRK	AT1G10940	2.64	9.16E-16	1.63	2.14E-13
GRMZM2G000278	SnRK	AT1G60940	2.33	7.10E-07	2.08	6.69E-17
GRMZM2G157722	ABF	AT1G49720	2.49	7.74E-15	2.33	1.76E-30
GRMZM5G858197	ABF	AT1G03970	2.30	3.19E-12	2.14	1.75E-26
GRMZM2G132868	ABF	N/A	1.37	1.10E-02	1.44	7.26E-07
GRMZM2G479760	ABF	AT3G56850	2.70	3.34E-19	2.31	2.81E-27
GRMZM2G168079	ABF	AT2G36270	2.12	2.95E-03	4.51	3.12E-46
GRMZM2G033413	ABF	AT3G56850	1.79	8.13E-06	1.86	9.27E-14
GRMZM2G478417	ABF	AT1G45249	1.69	2.86E-07	1.84	2.31E-23

Table S5. DEGs involved in spliceosome pathway and RNA splicing tremin TD and SD.

Gene ID	Categorization	TD		SD	
		Fold	Q value	Fold	Q value
GRMZM2G057450	Spliceosome pathway	1.046961	0.017415	N/A	N/A
GRMZM2G107896	Spliceosome pathway	1.093161	0.015406	N/A	N/A
GRMZM2G037698	Spliceosome pathway	1.113433	0.006992	N/A	N/A
GRMZM2G080930	Spliceosome pathway	1.018466	0.029868	N/A	N/A
GRMZM2G178227	Spliceosome pathway	1.021863	0.025182	N/A	N/A
GRMZM2G099317	Spliceosome pathway	1.186474	0.004019	1.066361	7.81E-06
GRMZM2G356894	Spliceosome pathway	1.272105	0.001498	N/A	N/A
GRMZM2G331811	Spliceosome pathway	3.213836	1.23E-22	3.033561	1.8E-56
GRMZM2G090869	Spliceosome pathway	1.011425	0.044216	N/A	N/A
GRMZM2G006673	Spliceosome pathway	1.022263	0.02949	N/A	N/A
GRMZM2G088218	Spliceosome pathway	1.027936	0.021834	N/A	N/A
GRMZM2G083783	Spliceosome pathway	1.040721	0.021065	N/A	N/A
GRMZM2G477694	Spliceosome pathway	1.05021	0.017284	N/A	N/A
GRMZM5G892645	Spliceosome pathway	1.065953	0.015154	N/A	N/A
GRMZM5G803433	Spliceosome pathway	1.152493	0.008862	1.110636	1.69E-07
GRMZM2G027571	Spliceosome pathway	1.211483	0.048242	1.158324	0.000499
GRMZM2G022041	Spliceosome pathway	1.234114	0.019234	N/A	N/A
GRMZM2G055682	Spliceosome pathway	1.255859	0.00289	N/A	N/A
GRMZM2G138572	Spliceosome pathway	1.528438	0.004516	1.416365	0.000213
GRMZM2G072671	Spliceosome pathway	2.549727	4.98E-08	N/A	N/A
GRMZM2G310431	Spliceosome pathway	3.836957	4.57E-16	3.507933	1.84E-79
GRMZM2G366532	Spliceosome pathway	5.881996	1.85E-36	5.978763	2.1E-116
GRMZM2G358311	RNA splicing trem	-2.23587	1.02E-16	-2.184960	8.53E-29
GRMZM2G124047	RNA splicing trem	1.019991	0.043123	N/A	N/A
GRMZM2G130034	RNA splicing trem	1.095588	0.011231	N/A	N/A
GRMZM2G040995	RNA splicing trem	1.115849	0.012567	N/A	N/A
GRMZM2G047949	RNA splicing trem	1.150336	0.004996	N/A	N/A
GRMZM2G433801	RNA splicing trem	1.166313	0.004097	N/A	N/A
GRMZM2G104375	RNA splicing trem	1.240205	0.023142	N/A	N/A
GRMZM2G012262	RNA splicing trem	1.244003	0.028452	N/A	N/A
GRMZM2G052926	RNA splicing trem	1.303051	0.001538	1.20365	3.7E-07
GRMZM2G083689	RNA splicing trem	1.347599	0.00082	N/A	N/A
GRMZM2G057646	RNA splicing trem	1.392945	0.000148	N/A	N/A
GRMZM2G087712	RNA splicing trem	1.445622	0.001249	N/A	N/A
GRMZM2G032409	RNA splicing trem	1.465593	5.31E-05	1.020564	6.09E-05
GRMZM2G139533	RNA splicing trem	2.818727	7.41E-17	2.151375	8.08E-26
GRMZM2G132021	RNA splicing trem	N/A	N/A	-1.1384	1.82E-20
GRMZM2G061783	RNA splicing trem	N/A	N/A	-1.01122	7.46E-14
GRMZM2G078412	RNA splicing trem	N/A	N/A	-1.00511	1.13E-19
GRMZM2G139837	RNA splicing trem	N/A	N/A	1.039465	0.002413
GRMZM2G169871	RNA splicing trem	N/A	N/A	1.071772	8.94E-06
GRMZM2G026490	RNA splicing trem	N/A	N/A	1.21885	1.98E-05
GRMZM2G386608	RNA splicing trem	N/A	N/A	1.362581	1.82E-07

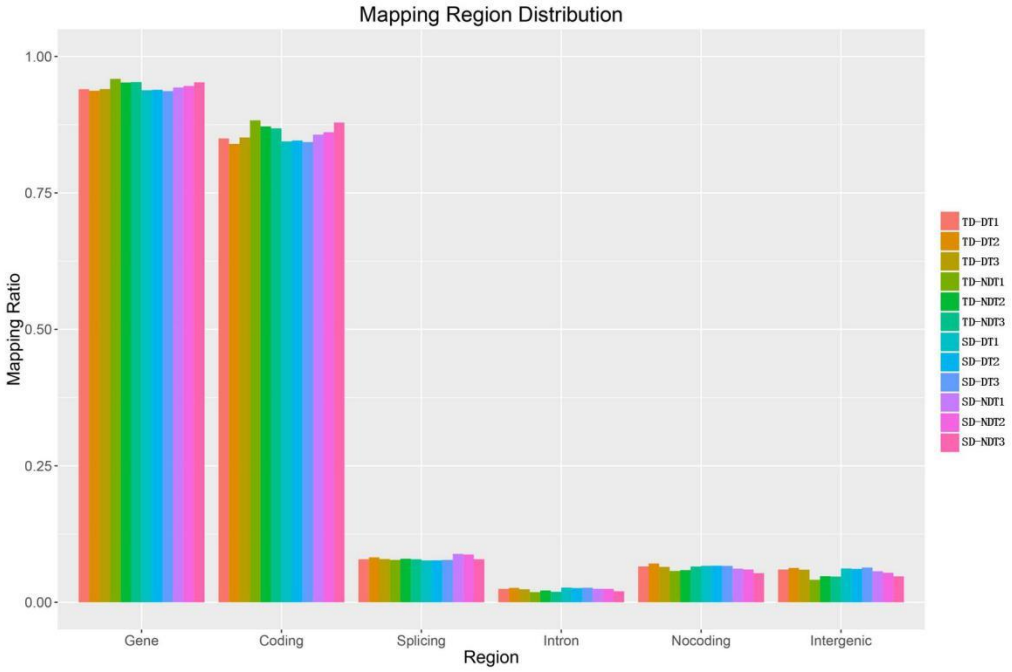

Figure S2. Mapping region distribution of reads. Reads were mapped to gene regions, coding regions, splice sites, introns, and non-coding regions. Non-coding region includes 5UTR, 3UTR, non-coding RNA regions. Samples are represented by different colors, and the samples are as following: TD-DT, tolerance to drought-drought treatment; TD-NDT, tolerance to drought-non drought treatment; SD-DT, sensitive to drought-drought treatment; SD-NDT, sensitive to drought-non drought treatment.

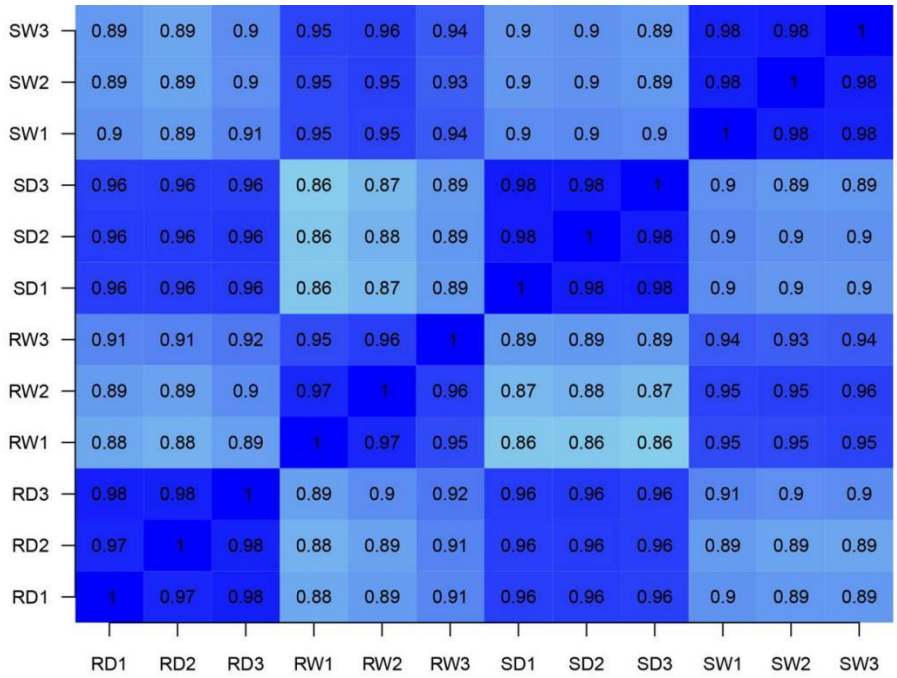

Figure S3. Sample correlation coefficient. The correlation coefficient is close to 1, indicating that the similarity between samples is higher. Correlation coefficient is calculated based on FPKM results. The samples are as following: TD-DT, tolerance to drought-drought treatment; TD-NDT, tolerance to drought-non drought treatment; SD-DT, sensitive to drought-drought treatment; SD-NDT, sensitive to drought-non drought treatment.

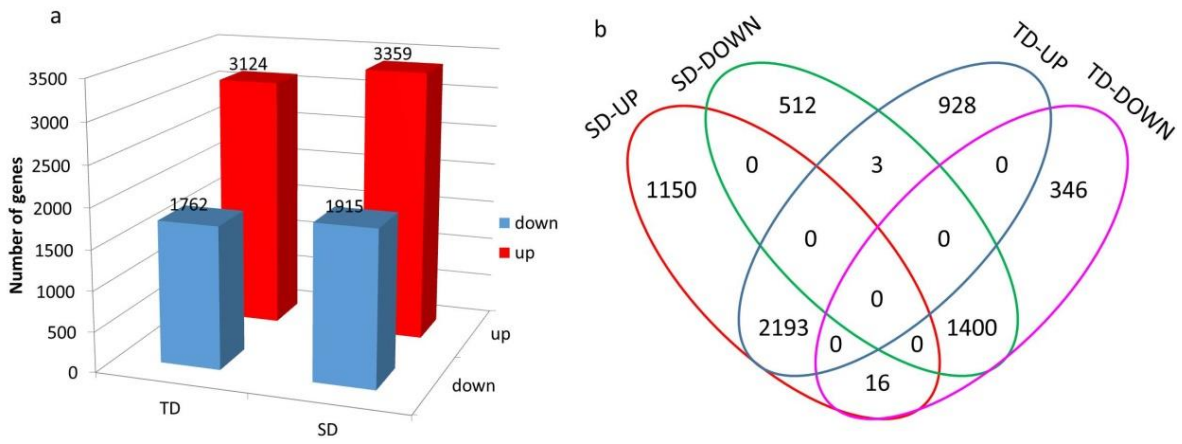

Figure S4. Number of differentially expressed genes in TD and SD pool (a)and overlap of DEGs under drought stress (b). Red and blue fonts indicate up-regulated and down-regulated DEGs at least two fold, respectively.

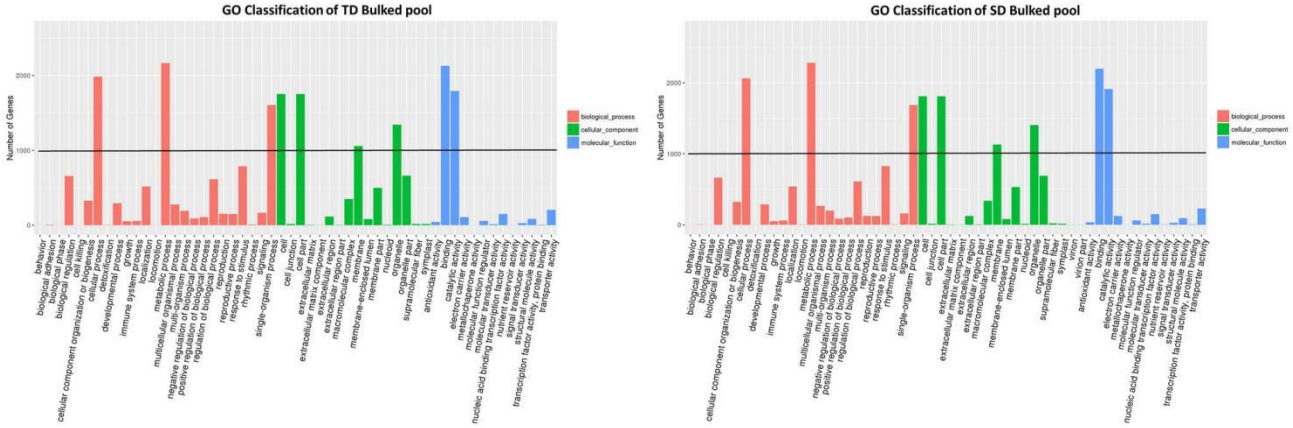

Figure S5. Functional classification of DEGs in TD and SD bulked pool. Functional classification based on Gene Ontology (GO) knowledgebase. The Y-axis represents the number of DEGs in GO terms. The black line indicates that the number of DEGs reaches to 1000. Red, green and blue fonts indicate biological process, cellular component and molecular function, respectively.

***GO enrichment of DEGs under drought stress*:** A GO enrichment analysis was performed to discover the function of the DEGs. In TD bulked pool, 4886 DEGs were enriched in 54 GO terms that include16 cellular component terms, 13 molecular function terms and 25 biological process terms (Figure S5). For SD bulked pool, 5274 DEGs were enriched in 56 GO terms that included 18 cellular component terms, 13 molecular function terms and 25 biological process terms (Figure S5).Total nine GO terms i.e. binding (GO:0005488), catalytic activity (GO:0003824), cell (GO:0005623), cell part (GO:0044464), cellular process (GO:0009987), membrane (GO:0016020), metabolic process (GO:0008152), organelle (GO:0043226), single-organism and process (GO:0044699), were significantly enriched and included by more than 1000 DEGs.

Under drought treatment, DEGs were enriched in response to abiotic stimulus (GO:0009628) and transporter activity (GO:0005215), significantly. Apparently, 173 up-regulated DEGs such as calmodulin gene *CALM1* (*GRMZM2G004703*), catalase isozyme 1 gene *CAT1* (*GRMZM2G088212*), heat shock protein (*GRMZM2G024718*, *GRMZM2G360681*, *GRMZM2G179802* and *GRMZM2G333635*), ABA-responsive protein (*GRMZM2G106622*),nuclear transcription factor Y subunit B gene*NFY2* (*GRMZM5G804893*), chaperonin gene *CPN60II* (*GRMZM2G416120*) and dehydrin gene *DHN1* (*GRMZM2G079440*) were identified for the response to abiotic stimulus in two bulked inbred lines. A total of 58 uni genes including nucleotide binding protein *GRMZM2G058345* and two calmodulin genes (*GRMZM2G149923* and *GRMZM2G044963*) were enriched in TD. Genes for transporter activity (GO:0005215) were up-regulated under treatment. These genes include many aquaporin genes. Aquaporin genes play an important role in

drought resistance by regulating water transport in many plant species (Matsunami *et al.*, 2016).In present study, genes for aquaporin such as *SIP1-1* (*GRMZM2G113470*), *PIP2-1* (*GRMZM2G014914*), *PIP2-3* (*GRMZM2G081192*), *PIP1-3* (*GRMZM2G392975*), *PIP1-1* (*GRMZM2G174807*), *PIP2-4* (*GRMZM2G154628*), *PIP1-5* (*GRMZM2G081843*), *TIP4-2* (*GRMZM2G108273*), *PIP1-2* (*AC209208.3_FG002*) and *TIP3-1* (*GRMZM2G305446*) were up-regulated commonly in TD and SD under drought stress. *NIP2-2* (*GRMZM2G137108*) and *TIP4-1* (*GRMZM2G103945*) were up-regulated in TD whereas, *NIP2-3* (*GRMZM2G081239*) and*NIP2-1* (*GRMZM2G028325*) were down-regulated in SD. Additionally, three genes for dehydrin (*GRMZM2G169372, GRMZM2G373522 and GRMZM2G079440)* were up-regulated in TD and SD.

Plant hormone is an important class of regulators during drought stress. The expression levels of phytohormones were different between TD and SD, but one thing in common was that most of these DEGs were up-regulated in response to drought stress (Figure S6). A total of 289 DEGs in TD lines and 318 DEGs in SD lines related to plant hormones like abscisic acid (ABA), auxin (IAA), brassinosteroids (BRs), cytokinins (CKs), ethylene (ETH), gibberellins (GAs), jasmonic acid (JA) and salicylic acid (SA) were obtained. Moreover, information presented in Table S4 described that 76 DEGs involved in ABA biosynthesis, catabolism and signal transduction were identified, including NECD (9-cis-epoxycarotenoid dioxygenase), CYP707A, PP2C (protein phosphatase 2C), SnRK (serine/threonine-protein kinase SRK2) and ABF (ABA responsive element binding

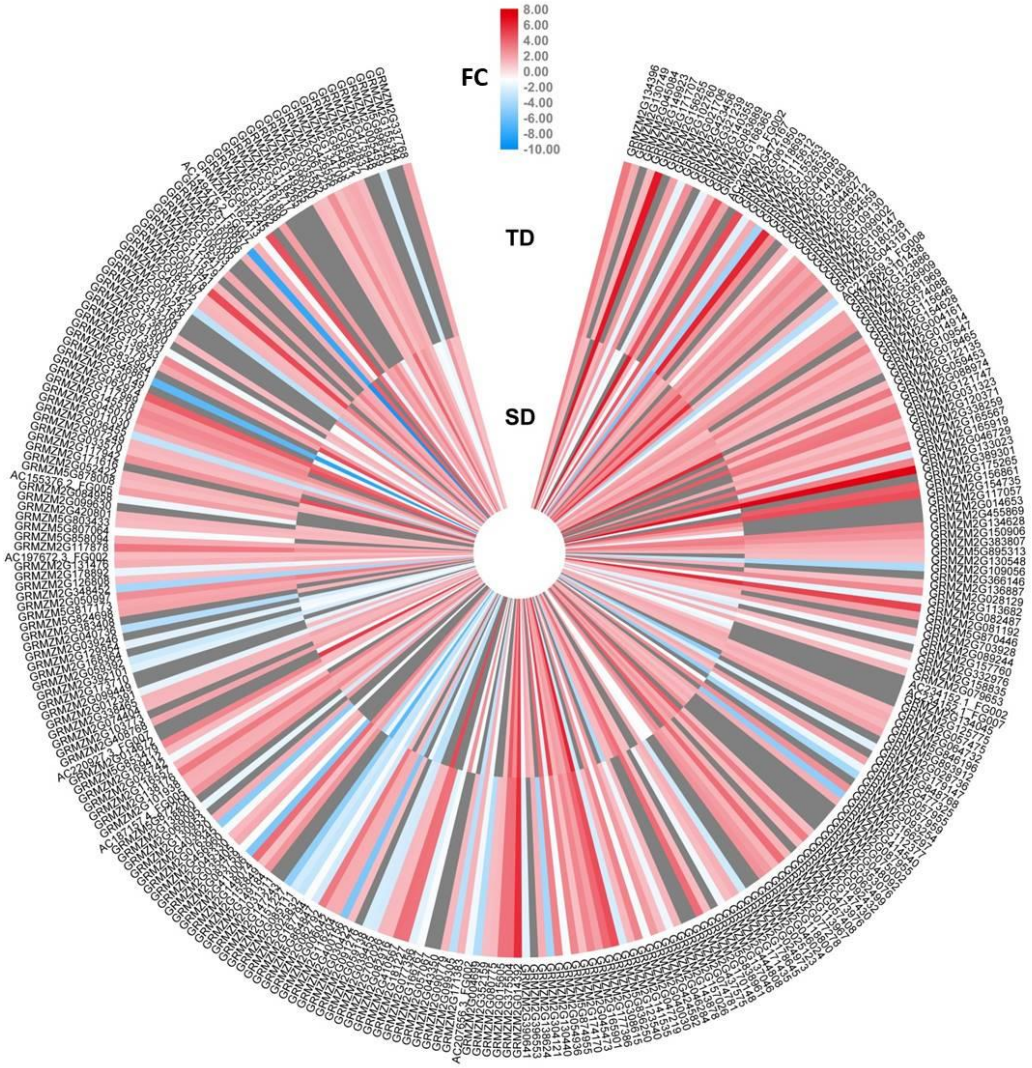

Figure S6. Heatmap of DEGs involved in the phytohormone in TD and SD bulked pool. The expression values of genes are presented as fold-change (FC) based on FPKM calculation. Red and blue colors indicate up-regulated and down-regulated DEGs, respectively.

factor).Three NECDs, *GRMZM5G838285*, *GRMZM2G417954* and *GRMZM2G014392* (*vp14*, the first cloned NECD gene in maize), were up-regulated both in TD and SD. The expression level ofCYP707As was a slightly different, 16 DEGs (10 up- and 6 down-regulated) were identified in TD, and 18 DEGs (12 up- and 6 down-regulated) were identified in SD. Total 15 up-regulated PP2Cs,common in TD and SD, were significantly identified in maize seedling leaves under drought stress. However, five up-regulated SnRKs were obtained in TD and SD, while *GRMZM2G110908* was down-regulated only in SD. Genes for ABA responsive element binding factor (ABF), such as *GRMZM2G157722*, *GRMZM5G858197*, *GRMZM2G132868*, *GRMZM2G479760*, *GRMZM2G168079*, *GRMZM2G033413 and GRMZM2G478417*, were up-regulated in both TD and SD. Additionally, 57 IAAs (36 up- and 21 down-regulated), 29 ETHs (22 up- and 7 down-regulated), 39 JAs (29 up- and 10 down-regulated), 17 SAs (10 up- and 7 down-regulated) and 6 GAs (4 up- and 2 down-regulated) were detected in TD lines. And in SD, we identified 49 DEGs (28 up- and 21 down-regulated) in IAA, 28 DEGs (19 up- and 9 down-regulated) in ETH, 50 DEGs (33 up- and 17 down-regulated) in JA, 30 DEGs (16 up- and 14 down-regulated) in SA and 5 DEGs (3 up- and 2 down-regulated) in GA.

***Expression of transcription factors in response to drought stress*:** An analysis involved transcription factors was performed by mapping of plant transcription factor database (http://planttfdb.cbi.pku.edu.cn/index.php?sp=Zma). In this transcriptome study,412 DEGs belonging to 46 TF families, ERF, bZIP, bHLH, NAC, MYB, MYB-related and WRKY TF families (Figure 1a),were identified in response to drought stress.A total of 45 genes were enriched in ERF family, including 35 (28 up- and 7down-regulated) and 40 (33 up- and 7down-regulated) DEGs in TD and SD lines, respectively. In addition, 33, 28 and 21 DEGs belonging to NAC, MYB and WRKY TF family, respectively, were activated under drought treatment. Most of the ERFs and bZIPs were up-regulated under drought stress (Figure 1b). The bZIP TF family, with 40 (33 up- and 7down-regulated) and 29 (25 up- and 4 down-regulated) DEGs in TD and SD, respectively, wereidentified in maize seedling leaves. Conversely, the down-regulated genes predominated in bHLH and MYB-related TF

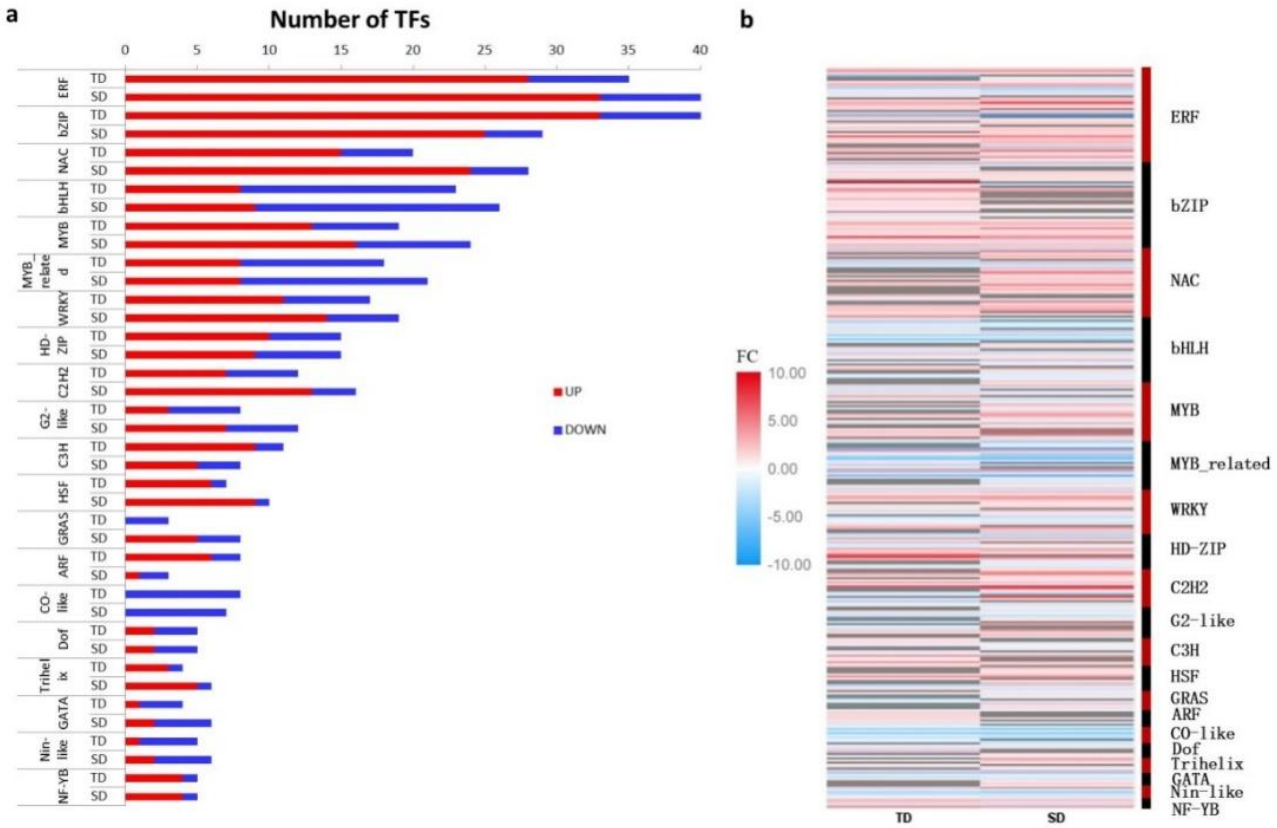

Figure 1. Overview of DEGs into 20 major transcription factor families in TD and SD bulked pool (a). Heatmap of differentially expressed transcription factors under drought treatment (b). Red and blue colors indicate up-regulated and down-regulated TFs, respectively. The expression values of genes are presented as fold-change based on FPKM calculation.

families;15bHLHsand 10 MYB-related were down-regulated in TD, 17 bHLHs and 13 MYB-related were down-regulated in SD.

***Regulation of alternative splicing under drought treatment*:** Molecular regulation of plants under abiotic stresses is a complex process in which transcriptional regulation of stress related genes plays a major role (Mazzucotelli *et al.*, 2008).The alternative splicing plays critical roles in the process of biological development and stress responses by controlling gene transcription and producing more functional proteins. Herea total of 22 DEGs were enriched in spliceosome pathway (zma03040) under drought stress (Figure 2A, Table S5). Among 22 DEGs, 22 up-regulated genes and 7 up-regulated genes were identified in TD and SD, respectively. As shown in Figure 2A and Table S5, 14 DEGs and 11 DEGs, 4 genes overlapped, were identified to RNA splicing term (GO:0008380) in TD and SD lines, respectively. Furthermore, the expression levels of DEGs were significantly different between TD and SD; only one gene was down-regulated in TD whilefour genes were down-regulated in SD.

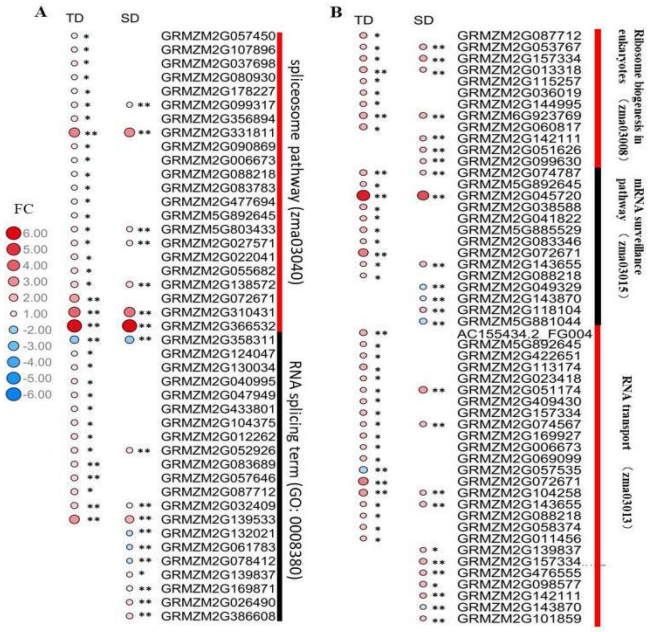

Figure 2. Circle heatmap of DEGs involved in spliceosome pathway, RNA splicing trem and RNA-related pathway in TD and SD bulked pool. The expression values of genes are presented as fold-change based on FPKM calculation. Red and blue colors indicate up-regulated and down-regulated DEGs, respectively. *Q<0.05; **Q<0.001.

Between two bulked pools, the number of DEGs involved in spliceosome pathway and the expression patterns of these DEGs involved in RNA splicing term were significantly different in maize seedling leaves under the drought stress, which suggested that alternative splicing may play a critical role in response to drought stress.

In addition to alternative splicing affecting the transcription of genes, post-transcriptional mechanisms also significantly work on gene expression by controlling the formation of mature ribosomal particles, the quality of RNA quality and RNA transport (Mazzucotelli *et al.*, 2008).The results of post-transcriptional mechanisms analysis were shown in Figure 2B. Nine up-regulated (in TD) and seven up-regulated (in SD) DEGs were identified in ribosome biogenesis pathway of eukaryotes (zma03008). In mRNA surveillance pathway (zma03015), 10 up-regulated DEGs were identified in TD, 4 up-regulated and 3 down-regulated DEGs were identified in SD. Moreover, 19 DEGs (18 up- and 1 down-regulated) werefound involved in the RNA transport pathway (zma03013) in TD, and 11 DEGs (10 up-regulated and 1 down-regulated) were detected in SD.

***DEGs uniquely identified in TD lines*:** In comparison with SD, 1274 genes involving 928 up-regulated and 346 down-regulated were uniquely identified in TD pool (Figure S4b). Among them, 23 up-regulated genes were related to protein kinase, including mitogen activated protein kinase kinase kinase (MAPKKK) *GRMZM6G513881* and fructokinase genes *GRMZM2G072091*which are homologous genes of*AT3G52390* and *AT5G02540*in Arabidopsis, respectively. The analysis of protein protein interaction network (PPI network) was performed based on the string database. As shown in the Figure S7, the protein ofAT3G52390 interacts with SCE1 (AT3G57870.1), KU80 (AT1G48050.1) and AT1G49250.SCE1with reduced levels show higher sensitivity to ABA in plant root growth, AT1G49250is related to DNA repair and nucleic acid-binding.

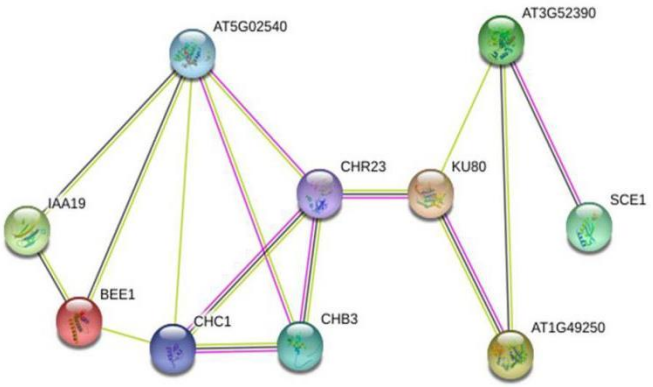

Figure S7. Protein-protein interaction network in Arabidopsis. Nodes represent genes and edges represent interactions between the two genes.*AT3G52390* is a homologous gene of *GRMZM6G513881*; *AT5G02540*is a homologous gene of *GRMZM2G072091*.

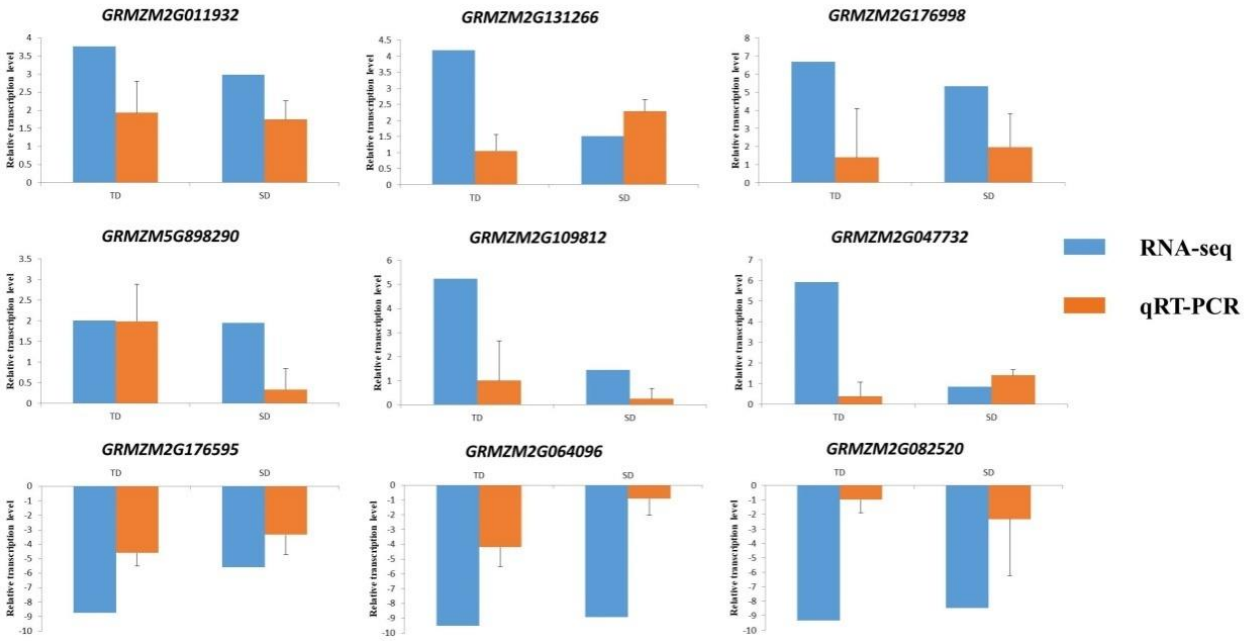

Figure 3. Validation of 9 selected genes by quantitative real-time PCR. Transcription values were normalized to *GAPDH*(glyceraldehyde-3-phosphate dehydrogenase). The calculation of relative transcription levels was performed by $\log 2^{-\Delta\Delta Ct}$ method.

The protein of AT5G02540 interacts with CHB3 (AT4G34430.4), CHC1 (AT5G19310.1), BEE1 (AT1G18400.1) and IAA19 (AT3G15540.1) (Figure S7). The function of CHB3 is facilitating or repressing thebinding of gene-specific transcription factors. CHC1 protein may affect root morphology or growth rate.BEE1 is a transcription factor in regulating brassinosteroid signaling. IAA19 is the abbreviation of indole-3-acetic acid inducible 19. As regulatory proteins, transcription factors can improve drought tolerance of plants (Liu *et al.,* 2010). Among ofthe 13 TFs, especially *GRMZM2G031323* (MYB) and *GRMZM2G066734* (bZIP), wereup-regulated with drought treatment in TD. And two aquaporin genes, *NIP2-2* (*GRMZM2G137108*) and *TIP4-1* (*GRMZM2G103945*), were up-regulated in TD. Transmembrane protein genes such as *GRMZM2G164821* and *GRMZM2G146951* were only induced by drought stress in TD. Importantly, three up-regulated genes (*GRMZM2G057450*, *GRMZM2G107896* and *GRMZM2G037698*) were identified as splicing factor, which were involved in responses to abiotic stresses (Shen *et al.,* 2014).A total of 10 genes were enriched in zinc finger protein according to the description of gene annotation. The result indicated that these unique genes may be key genes for improving drought tolerance in maize seedlings.

***Verification of expression by qRT-PCR*:** For validating the results of RNA-seq analysis, nine DEGs with different expression patterns were selected for qRT-PCR analysis. According to the RNA-seq result, 6 up-regulated DEGs were induced by drought stress both in TD and SD pools, and 3 DEGs were down-regulated in TD and SD pools. With the verification of qRT-PCR (Figure 3), *GRMZM2G011932*, *GRMZM2G131266* and *GRMZM2G176998* were significantly up-regulated in tolerant and sensitive pools. With drought treatment, *GRMZM5G898290* and *GRMZM2G109812* were up-regulated in TD, but *GRMZM2G176595* and *GRMZM2G064096* were significantly down-regulated in TD. In SD pool, two down-regulated genes (*GRMZM2G176595* and *GRMZM2G082520*) were identified. Finally, the expression pattern of nine genes was consistent between RNA-seq data and qRT-PCR results.

DISCUSSION

Phenotypic traits based onscreening plays an important role in cultivating varieties with excellent resistance. No doubt genes affect phenotypic traits of the cultivars but genetic differences among cultivars with similar phenotypic traits are complex. Bulked segregant analysis (BSA) is usefulto analyze bulk samples by building two pools of extreme phenotypic samples. However, the traits of plants are the result of a consented interaction of many genes and the environment, and the responses are complex under abiotic stress. RNA sequencing, as a powerful analysis tool, has been widely used in analyzing gene expression levels and regulatory networks. BSR-Seq (bulked segregant RNA-seq), is a combination of bulked segregant analysis and RNA sequencing technology.

In present study, BSR-Seq was applied to analyze the gene

expression of the maize seedling leaf under drought treatment. Two extreme phenotypic sample from bulked pools (TD and SD) were constructed by selecting 10 tolerant to drought (TD) maize inbred lines and 10 sensitive to drought (SD) maize inbred lines. RNA-seq was used in transcriptomic profiling of two extreme phenotypic sample pools (TD and SD), 4886 DEGs were identified in the tolerance pool (TD)by comparing drought treatment with normal conditions, and 5274 DEGs were obtained in SD.

***ABA biosynthesis and signaling pathways response to drought stress*:** Abscisic acid (ABA), as one of stress hormones, has been reported to be involved in the plant regulatory networks while responding to environmental abiotic stresses. For example, ABA is associated with the regulation of cellular transcription factor and signal transduction under drought and salt damage (Golldack *et al.,* 2011).ABA regulation can be affected significantly by water stress (Wan and Li, 2006).Under drought stress condition, the transcriptome level of *GRMZM2G417954*and*Vp14* (*GRMZM2G014392*)increased in maize seedling leaf (Kakumanu *et al.,* 2012; Li *et al.,* 2017).As the research conclusions of predecessors, ABA-related genes, especially ABA biosynthesis, were up-regulated under drought treatment in present study. In TD and SD pools, 3 NECD genes (*GRMZM5G838285, GRMZM2G417954* and*GRMZM2G014392*) , including the first cloned NECDs *Vp14* (*GRMZM2G014392*) in maize (Tan *et al.,* 1997),were significantly up-regulated. Meanwhile, others ABA biosynthesis-related genes were identified, such as CYP707As, PP2Cs and SnRKs. Three CYP707As differed between TD and SD; *GRMZM2G168016* and *GRMZM2G102318* were up-regulated and identified in TD only; down-regulated gene *GRMZM2G1161169* was identified in SD. A total of 15 PP2Cs were up-regulated in TD and SD pools. These results showed that ABA biosynthesis and signaling pathways play vital roles in response to drought stress.

***Transcription factors involved in drought stress*:** As an important regulator, transcription factors (TF) are closely related to gene expression and signal transduction. Many transcription factor families such as bZIP, NAC, MYB and WRKY have been shown to be involved in regulated networks and respond to abiotic stresses (Golldack *et al.,* 2011; Miao *et al.,* 2015; Shankar *et al.,* 2016; Wang *et al.,* 2016). A previous study found that *ABP9* belongs to bZIP family improved the resistance to drought in Arabidopsis (Zhang *et al.,* 2011). *OsMYB35* encodes a MYB TF, and can enhance resistance to drought by overexpression in maize (Casaretto *et al.,* 2016).

In present study, 46 transcription factors families e.g.were identified in412 DEGs. ERF, bZIP, NAC, MYB and WRKY, and most of them were up-regulated in TD and SD. Consistent with our transcriptional analysis, some scholars have found that G*RMZM2G479760* (*bzip4*), *GRMZM2G061487* (*dbf1*), *GRMZM2G127379*（*ZmNAC111*）, *GRMZM5G846057* and *GRMZM2G347043* can be induced by drought stress in maize (Ma *et al.,* 2018; Shiriga *et al.,* 2014; Voitsik *et al.,* 2013; Yang *et al.,* 2014; Zhang *et al.,* 2017). Moreover, 9 up-regulated bZIPs (*GRMZM2G019446, GRMZM2G066734, GRMZM2G088140, GRMZM2G094352, GRMZM2G095078, GRMZM2G103647, GRMZM2G120167, GRMZM2G131961* and *GRMZM2G138340*), 3 up-regulated ERFs (*GRMZM2G081892, GRMZM2G129777* and *GRMZM2G138396*), 3 up-regulated MYBs (*GRMZM2G031323, GRMZM2G149958* and *GRMZM2G403620*), 4 up-regulated NACs (*GRMZM2G163251, GRMZM2G166721, GRMZM2G894234*and *GRMZM2G257110*) and one up-regulated WRKY (*GRMZM2G475984*) were identified only in TD under drought stress. The analysis indicated that TFs are a key regulator for resistance to drought stress.

***DEGs involved in alternative splicing*:** The complexity of living things is not entirely determined by genes. Some species have great similarities in their genomes, but their biological properties may be completely different because of the complexity of RNA generated by alternative splicing (Reddy *et al.,* 2013). Alternative splicing, including exon skipping, intron retention, mutually exclusive exons, alternative 5' splice site, and alternative 3' splice site, is an important transcriptional regulatory mechanism that can cause the diversity of transcripts and produce abundant functional proteins. In present study, 22 differentially expressed genes were found to be involved inspliceosome pathway (zma03040), which were up-regulated during drought treatment. Among these DEGs, all the 22 DEGs were identified in TD bulked pool; 7 DEGs including heat shock protein HSP70 (*GRMAM2G310431*) overlapped between TD and SD. Furthermore,14 DEGs (14 DEGs in TD, 11 DEGs in SD) including IDI2 (*GRMZM2G139533*) were enriched in RNA splicing term (GO: 0008380). For RNA transport pathway (zma03013), 19 and 11 DEGs were identified in TD and SD bulked pool, respectively. Total 10 and seven DEGs belonging to mRNA surveillance pathway (zma03015) were enriched in TD and SD, respectively.

Conclusion: Having potential to select extreme numbers of samples, BSA can be used to make bulked sample pools and pool representative sample genomes. RNA sequencing was a powerful tool for detecting the response of genes to abiotic stresses. In present study, BSA and RNA sequencing were applied to pool drought-extreme maize inbred lines and identify differentially expressed genes in maize seedling leaves under drought stress treatment. With drought treatment, two bulks were built by selecting 10 tolerant to drought (TD) inbred lines and 10 sensitive to drought (SD) inbred lines from 200 maize inbred lines. Using RNA-seq, 4886 (TD) and 5274 (SD) DEGs were determined by comparing drought stress to control samples. According to the annotate results,

DEGs related to TFs, plant hormone and alternative splicing were significantly up-regulated under water treatment in maize seedling leaf. Importantly, the number of DEGs enriched in alternative splicing-related pathway in the TD pool was higher than SD, including spliceosome, ribosome biogenesis in eukaryotes, mRNA surveillance pathway and RNA transport pathway. The present study was a supplement to understand the molecular mechanisms of maize response to drought stress in the seedling leaf. Besides, the analysis of the transcriptome can be helpful in identifying stress-resistance candidate genes and provide a reference of tolerance mechanisms in future studies.

***Acknowledgements*:** The funding of this work was supported by National Natural Science Foundation of China (No. 31971839, 31471510) and Technical Innovation Project of Hubei Province (No. 2017AHB056).

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