RESEARCH ARTICLE

Comparative transcriptomic and physiological analyses of contrasting hybrid cultivars ND476 and ZX978 identify important differentially expressed genes and pathways regulating drought stress tolerance in maize

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Abstract

Background Drought is the major abiotic stress factor that negatively influences growth and yield in cereal grain crops such as maize (*Zea mays* L.). A multitude of genes and pathways tightly modulate plant growth, development and responses to environmental stresses including drought. Therefore, crop breeding efforts for enhanced drought resistance require improved knowledge of plant drought responses.

Objective Here, we sought to elucidate the molecular and physiological mechanisms underpinning maize drought stress tolerance.

Methods We therefore applied a 12-day water-deficit stress treatment to maize plants of two contrasting (drought tolerant ND476 and drought sensitive ZX978) hybrid cultivars at the late vegetative (V12) growth stage and performed a large-scale RNA sequencing (RNA-seq) transcriptome analysis of the leaf tissues.

Results A comparative analysis of the two genotypes leaf transcriptomes and physiological parameters revealed the key differentially expressed genes (DEGs) and metabolic pathways that respond to drought in a genotype-specific manner. A total of 3114 DEGs were identified, with 21 DEGs being specifically expressed in tolerant genotype ND476 in response to drought stress. Of these, genes involved in secondary metabolites biosynthesis, transcription factor regulation, detoxification and stress defense were highly expressed in ND476. Physiological analysis results substantiated our RNA-seq data, with ND476 exhibiting better cell water retention, higher soluble protein content and guaiacol peroxidase activity, along with low lipid peroxidation extent than the sensitive cultivar ZX978 under drought conditions.

Conclusion Our findings enrich the maize genetic resources and enhance our further understanding of the molecular mechanisms regulating drought stress tolerance in maize. Additionally, the DEGs screened in this study may provide a foundational basis for our future targeted cloning studies.

Keywords Differentially expressed genes (DEGs) · Drought stress · Secondary metabolites biosynthesis · RNA sequencing (RNA-seq) · Transcriptome profiles · *Zea mays* L

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Introduction

Despite the sharp rise in global demand for crop products, driven by population and income growth (Schils et al. 2018), agricultural productivity is challenged by numerous abiotic stress factors, often exacerbated by the changing global climate (Fahad et al. 2017). Additionally, the emerging role of crop products as raw material inputs in the transition from fossil fuels towards climate-smart low-fossil-carbon economies is putting further strain on agricultural systems (Schils et al. 2018). Among all the abiotic stress factors, drought is the most devastating (Basu et al. 2016), and the frequency of extreme drought events is estimated to increase under the changing climate scenario, further worsening damage to plants and crop yields (Zhao et al. 2016a). Therefore, to maintain sustainably higher crop yields, crop scientists need to understand how plants respond to drought stress and employ the generated knowledge in modern breeding programs.

Being an economically important cereal crop, ranked third after rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L., Thirunavukkarasu et al. 2017), maize (*Zea mays* L.) continue to play a critical role in evading the production gap of human food calories, animal feeds and bio-fuels in the world (Shiferaw et al. 2011; Opitz et al. 2016. However, the overall production of maize is adversely affected by drought stress (Singh et al. 2019). Drought inhibits growth by negatively impacting plant physiological processes (Prasad et al. 2008; Bhargava and Sawant, 2013; Anjum et al. 2011). It decreases plant cell turgor, inhibits CO_2 exchange, decreases photosynthetic efficiency, increases post-pollination embryo abortion rate, ultimately leading to reduced yield (Farooq et al. 2009; Zhang et al. 2018).

In response to drought and other stressors, plants institute numerous adaptive responses activated at the physiological, biochemical and molecular levels. At the biochemical and physiological levels, plants activate metabolic and osmotic adjustments, antioxidant scavenging defense and phytohormones (Fang and Xiong 2015; Basu et al. 2016; Liu et al. 2019; Singh et al. 2019). The role of stress phytohormones such as abscisic acid (ABA), jasmonic acid and salicylic acid in conferring plant drought tolerance has been acknowledged (Aimar et al. 2011; Aslam et al. 2015; Fahad et al. 2017). Moreover, several researchers have identified key regulators and pathways underlying molecular mechanisms of maize drought stress response. These include calcium (Ca^{2+}) signal sensors, calcium-dependant protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) responsible for signal transduction, stress response proteins such as the heat shock proteins (HSPs), dehydrins, late embryogenic

abundant (LEA) proteins and aquaporins (Al-Whaibi 2011; Bhargava and Sawant 2013; Harb 2016; Tai et al. 2016; Priya et al. 2019). Additionally, several TFs also regulate drought stress response including myeloblastosis (MYB), dehydration responsive element binding (DREB), abscisic acid responsive elements binding factor (ABF), [NAM, $ATAF_1/_2$, and CUC_2 containing proteins] (NAC) and WRKY, among others (Singh et al. 2015; Wang et al. 2018; Priya et al. 2019). Further, epigenetic regulation mechanisms at the DNA and histone levels, as well as regulation by small non-coding RNAs in drought stress tolerance have become important (Banerjee and Roychoudhury 2017). Despite all this, however, the elucidation of drought stress response mechanisms in crop plants still remains elusive.

Previous researchers have monitored the global gene expression profiles in response to water deficit stress in different maize parts including roots, leaves, and kernels by microarray analysis (Zheng et al. 2010; Opitz et al. 2014). With the revolutionisation of the next generation sequencing (NGS) approaches came RNA sequencing (RNA-seq) method to help us decipher plants` abiotic stress responses. RNA-Seq accords for fully quantitative gene expression analysis with absolute values and capturing of very subtle expression changes. Moreover, it provides for low cost, high throughput and high sensitivity analysis of data (Kakumanu et al. 2012; Opitz et al. 2016). RNA-seq technology has been employed in drought stress response studies in maize (Kakumanu et al. 2012; Shan et al. 2013; Bhanu et al. 2016; Min et al. 2016; Zenda et al. 2019). Although these studies provided global insight into the maize drought stress responses, most of them used inbred lines as experimental materials, did not target the late vegetative (V12) crop developmental stage, and were conducted under greenhouse environments. Yet, every year, most farmers exploit the advantage of hybrid vigour by planting hybrids under field conditions (Danilevskaya et al. 2019), and moisture deficit stress at the V12 stage drastically impact yield. This is so because the V12 stage is the most vigorous growth and development period in the whole maize life cycle (Wang et al. 2019a). At this phase, both number of ears per plant and number of kernel rows on an ear are being determined (Darby and Lauer 2006), so moisture deficit will have serious consequences on yield (Aslam et al. 2015). Additionally, more controlled environments are devoid of fluctuations in meteorological conditions (heat, solar radiation, wind and vapour pressure) which play vital roles in modulating plant growth and development (Danilevskaya et al. 2019). Therefore, it becomes imperative that we dissect maize hybrid cultivars` V12-stage-specific drought stress responses under conditions that closely mimic field growing environments.

In the current study, therefore, we have employed the use of the RNA-seq method, integrated with the

physiological analysis approach, to understand the molecular and physiological mechanisms associated with drought stress responses in maize at the V12 stage, by way of comparative analysis of two diverse (drought-tolerant Nongdan 476 (ND476) and drought-sensitive Zhongxin 978 (ZX978) hybrid cultivars grown under rain-proof shelters. We hope that the results generated in the current paper will provide knowledge that could be useful in future crop breeding programs aimed at engineering drought tolerant crop cultivars.

Materials and methods

Plant materials and drought treatment procedure

The two contrasting (comparably drought tolerant ND476 and sensitive ZX978) maize hybrids used in this study were provided the North China Key Laboratory for Crop Germplasm Resources of Education Ministry, Hebei Agricultural University, China. Maize hybrid ND476 is a comparably drought-resistant cultivar bred by the Dryland Research Institute of Hebei Academy of Agricultural and Forestry Sciences (Hengshui, China), and ZX978, a comparably drought sensitive cultivar, was developed by the Hebei Zhongxin Seed Technology Company Limited (Handan, Hebei Province, China). The experiment was conducted between May and July 2018 in a fully automated rain-proof shelter at Qing Yuan Experimental Station, Baoding, China (115.5602790 E; 38.7950930 N; 118 m). Each experimental plot measured 25 m^2 (5 m × 5 m), with 60 cm * 30 cm plant spacings. The soil water content was kept between 70 and 80% in the well-watered plots (control) and 15–20% in water-stressed plots (treatment) (Hsiao 1973). The relative soil water content of one meter underground was monitored by TZS-1 soil moisture measurement instrument (Zhejiang Tuopu Technology Co. Ltd, Zhejiang, China). To prevent the transverse infiltration of soil moisture, building waterproof membranes of one-meter depth were put between control and treatments units.

For the drought treatment group, plants were water deprived for 12 days from eight fully-expanded-leaves (FEL) (V8) to twelve FEL (V12) stage. Leaf tissues for both RNA-seq and qRT-PCR experiments were collected from the flag leaves of ND476 and ZX978 cultivars under both control and drought treatment conditions after twelve days of drought stress exposure; each sample had three technical replicates. All the leaf samples were immediately frozen in liquid nitrogen and then stored at − 80 °C for further analysis (Jin et al. 2019).

Phenotypic and physiological characterization

Phenotypic and physiological characterizations were measured for ND476 and ZX978 cultivars at the V12 stage under well-watered (control) and water-deficit (drought) treatment conditions as previously described (Wang et al. 2019b). In brief, we estimated the leaves` relative water content (RWC) by the method of Galmés et al (2007). Leaf guaiacol peroxidase (POD) activity was determined using Han (2008) method. The leaf cell membrane stability index, estimated as the level of lipid peroxidation (MDA content) was measured by the thiobarbituric acid method (Dhindsa, et al. 1981). Further, we estimated the soluble leaf protein content (SPC) using the Coomassie Brilliant Blue G250 method (Bradford, 1976).

Total RNA extraction, cDNA library construction and RNA sequencing

Maize hybrids ND476 (drought-tolerant) and ZX978 (drought-sensitive) were grown according to the method described (Jin et al. 2019). Total RNA of the control and drought-exposed flag-leaf samples which had been stored at −80 °C was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer`s instructions. For the removal of genomic DNA, RNA was purified and concentrated using a RNeasy column (QIAGEN, Pudong, Shanghai, China). RNA degradation and contamination (integrity) were monitored on 1% agarose gels, whereas RNA purity and concentration were checked using the NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The cDNA library construction and sequencing (on an Illumina Hiseq Xten platform, San Diego, CA, USA) were conducted by Shanghai Majorbio Bio-pharm Technology Co. Ltd. (Shanghai, China).

Data quality control, genome mapping, transcript assembly and gene expression quantification

Raw data (raw reads) of FASTQ format were trimmed and quality controlled by using SeqPrep software (https://github.com/jstjohn/SeqPrep) and Sickle software (https://github.com/najoshi/sickle), which discarded reads containing adaptor sequences, ploy-N sequences and poor quality from raw data. Meanwhile, Q20, Q30, GC-content and sequence duplication level of the clean data (clean reads) were calculated. Post-processed reads (clean reads) were then mapped to the maize reference genome sequence (B73 RefGen_v4) with orientation mode using TopHat (https://tophat.cbcb.umd.edu/, version 2.1.1) software. The Tophat parameters were as follows: sequencing reads that uniquely matched to the reference genome, allowing up to 2 mismatches, without insertions or deletions. The relative abundance of transcripts

in drought versus control conditions were estimated by reads per kilobase of transcript per million mapped reads (RPKM = total exon reads/mapped reads in million X exon length in kb) for each gene and log2 transformed to meet the assumptions of linear models (Mortazavi et al. 2008).

Functional annotation of unigenes

For functional annotation, the quality reads were used for BLAST (basic local alignment search) search, with the E value cut-off set to 1E-5 (Alstchul et al. 1997), against the following public databases: non-redundant protein sequence database (Nr) (ftp://ftp.ncbi.nlm.nih.gov/blast/db/), Swiss-Port (https://web.expasy.org/docs/swiss-prot_guideline.html), KOG/COG (Clusters of Orthologous Groups of proteins), Gene Ontology (GO) (https://www.geneontology.org) and the Kyoto Encyclopedia of Genes and Genomics (KEGG) (https://www.genome.jp/kegg).

Differentially expressed genes (DEGs) analysis and functional enrichment analysis

Differentially expressed genes (DEGs) sequencing libraries (for drought versus control conditions) were constructed with fragments per kilobase of exon model per million mapped reads (FPKM) as per the anticipated standards for transcriptome sequencing libraries (Mortazavi et al. 2008). DESeq2 package (version 1.12.3, Love et al. 2014) was employed for the differential expression analysis of the two groups. The analysis model uses student t test to calculate the p-values between the two groups (Wang et al. 2010); p value threshold was set at < 0.05. The raw p values were then corrected for multiple tests using the false discovery rate (FDR) according to the method of Benjamini and Hochberg (1995). Finally, the genes with an FDR < 0.05 and a fold change > 1.5 were considered to be significantly differentially expressed between the two samples. Moreover, functional-enrichment analysis including GO and KEGG were performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways, respectively, at Bonferroni-corrected P-value < 0.01 compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/Goatools) and KOBAS 2.1.1 (https://kobas.cbi.pku.edu.cn/download.php) (Xie et al. 2011).

Identification of key drought responsive genes

The expression abundances of each gene appearing in the two libraries (control versus drought treatment) were used to determine the expression changes of the genes in response to drought stress. The total DEGs identified to respond to drought stress (those that fell within the selection criteria specified above) were analysed by way of Venn diagram analysis. After filtering, the key/important drought responsive genes were specified by meeting the following criteria: the DEGs that specifically expressed in the tolerant genotype ND476 after drought treatment; DEGs shared between the drought-sensitive and drought-tolerant hybrids after drought treatment (SD_TD); DEGs of the tolerant cultivar that were also differentially expressed in SD_TD; and the common DEGs shared by the two hybrids under drought stress (TC_TD and SC_SD). This was substantiated by revealing their functional annotation and roles through GO and KEGG analyses, as well as refereeing to previously published works.

Quantitative real time-PCR (qRT-PCR) analysis

To validate the assembled sequences and the expression profiles obtained by Illumina RNA-seq, quantitative real-time PCR (qRT-PCR) was performed. Twenty (20) representative DEGs were randomly selected and gene-specific primers designed for qRT-PCR using Primer Premier 5 Designer (Premier Biosoft International, Palo Alto, CA, USA). Total RNA was isolated from V12-stage leaves as already described above in Sect. 2.3. Independent RNA from ND476 and ZX978 hybrids leaf samples, and from control and drought-stress conditions was prepared for qRT-PCR analysis. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed in a total volume of 25 μL, using HiFiscript cDNA Synthesis Kit (CWBIO, Beijing, China). The qRT-PCR analysis was carried out using Bestar ® SYBR Green qPCR Mastermix (DBI ® Bioscience, Germany) in a Bio-Rad iQ5 thermo cycler (Bio-RAD, Hercules, CA, USA) (Zenda et al. 2019). A steady and constitutively expressed maize gene *GAPDH* (accession no. X07156) was used for the housekeeping function. The qRT-PCR program was performed with 1 μl of template cDNA, 1 μl of each prim n er (100 μmol), and 10 μl of SYBR Green mix (TOYOBO, Japan) in a total reaction volume of 20 μl. Each sample had three technical replicates. The method of Livak and Schmittgen (2001) was used to calculate the relative mRNA abundance of samples. Correlation between RNA-Seq and qRT-PCR was analyzed using SPSS 22.0 software (SPSS Institute Ltd., Armonk, NY, USA).

Statistical analysis of physiological data

All statistical analyses were performed by using SPSS statistical software (version 22.0; SPSS Institute Ltd., Armonk, NY, USA), and the data was presented as mean ± standard error of mean. A two-way analysis of variance (ANOVA) and least significant difference (LSD) tests were used to compare physiological parameters between treatments and across genotypes, whereas qRT-PCR data underwent

one-way ANOVA and Duncan`s multiple range comparison tests. The significance level was set at $p < 0.05$.

Results

RNA-seq analysis results

RNA isolated from maize leaves that had been subjected to drought treatment for 12 days from V8 to V12 stages (as described in Sect. 2.1 above) was used for RNA-seq transcriptome analysis. Six samples for tolerant cultivar ND476 (NDC1, NDC2, NDC3, NDD1, NDD2 and NDD3) and six for sensitive cultivar ZX978 (ZXC1, ZXC2, ZXC3, ZXD1, ZXD2 and ZXD3) were categorized into four groups, viz; (NDC1, NDC2 and NDC3), (ZXC1, ZXC2 and ZXC3)— under water-abundant conditions; and (NDD1, NDD2 and NDD3), (ZXD1, ZXD2 and ZXD3)—under water-limited conditions. These four groups were named as TC, SC, TD, and SD, respectively. Then, the transcriptomes of the two hybrids in response to drought stress was analysed. The cDNA libraries were prepared from these two hybrids, before (control, C) and after drought treatment (D), and then subjected to RNA-seq profiling on the Illumina Hiseq Xten platform (San Diego, CA, USA).

After filtering, a total of 81.03 GB clean data was generated from ten samples (two unfit samples, NDC2 and NDD3, were retrieved). From the ten samples, we obtained 541.27 million total reads, with an average of 46.51 million clean reads each sample (Table 1). The clean reads were

then mapped to the maize reference genome B73. The Q30 base percentage, an indicator of the overall reproducibility and quality of the assay, was above 96.0%, which met the demands for further analysis. The mapping rates ranged from 89.62 to 92.91% (Table 1). Notably, the percentage of mapped reads was lower in the ZX978 samples than in the ND476 samples (Table 1), showing that there were greater transcriptomic perturbations in ZX978 than in ND476.

We performed the principal component analysis (PCA) of all twelve samples in order to analyse the similarities and differences between the samples, using FPKM according to Mortazavi et al. (2008). The PCA results showed that a technical fault may have caused low similarity amongst the three replications of the two (one drought-stress and one control) samples (Fig. S1). Therefore, we retrieved samples NDC2 (ND476 water-sufficient) and NDD3 (ND476 water deficit) before analyzing our sequencing results. The PCA results of the remaining 10 samples showed clear separation between the drought tolerant and drought sensitive hybrids. Moreover, the replicates of each treatment clustered together (Fig. S1). These results exhibited the reproducibility and reliability of our experiment.

Analysis of transcriptomic responses and functional annotation of unigenes

The FPKM values > 1 were used to determine genes expressed. Using Cufflinks software (Trapnell et al. 2010), we identified a total of 21 566 annotated transcripts in the four treatments. The number of genes specifically expressed

Table 1 Summary details of the RNA sequencing results for the twelve maize leaf samples

Group	Sample	Raw reads	Raw bases	Clean reads	Clean bases	Q20 (%)	Q30 (%)	Total mapped reads	Uniq. mapped reads
TC	NDC1	59,925,726	9,048,784,626	59,580,188	8,921,923,203	99.03	96.69	55,088,277(92.46%)	51,854,517(87.03%)
	NDC2	73,048,054	11,030,256,154	72,636,578	10,862,479,043	99.05	96.71	67,511,922(92.94%)	64,399,057(88.66%)
	NDC3	51,946,220	7,843,879,220	51,558,264	7,718,862,776	98.91	96.3	47,579,146(92.28%)	45,066,907(87.41%)
TD	NDD1	56,338,684	8,507,141,284	55,977,752	8,384,484,695	98.85	96.14	52,008,434(92.91%)	49,705,362(88.79%)
	NDD2	59,760,736	9,023,871,136	59,414,606	8,892,180,573	99.02	96.63	55,036,131(92.63%)	52,216,093(87.88%)
	NDD3	65,312,034	9,862,117,134	64,915,948	9,716,374,730	99.03	96.68	60,089,140(92.56%)	56,803,851(87.5%)
SC	ZXC1	46,852,950	7,074,795,450	46,519,954	6,961,974,576	98.91	96.31	42,255,379(90.83%)	39,599,584(85.12%)
	ZXC2	51,697,494	7,806,321,594	51,260,278	7,673,250,782	98.89	96.28	45,971,916(89.68%)	42,128,357(82.19%)
	ZXC3	56,773,660	8,572,822,660	56,378,394	8,441,485,433	98.99	96.58	50,528,784(89.62%)	46,385,524(82.28%)
SD	ZXD1	51,562,776	7,785,979,176	51,253,718	7,669,200,467	99.08	96.84	47,314,519(92.31%)	45,403,590(88.59%)
	ZXD2	60,047,950	9,067,240,450	59,668,804	8,924,728,621	98.99	96.52	54,785,106(91.82%)	52,135,588(87.37%)
	ZXD3	49,967,050	7,545,024,550	49,659,618	7,442,964,548	98.92	96.32	45,584,893(91.79%)	43,284,394(87.16%)

NDC1-3 and NDD1-3 are three replicate samples of tolerant cultivar ND476 under control (TC) and drought treatment (TD) conditions, respectively; ZXC1-3 and ZXD1-3 are three replicate samples of sensitive cultivar ZX978 under control (SC) and drought treatment (SD) conditions, respectively; Total reads, total clean reads counted by single end; Clean reads, total number of pair-end reads in clean data; GC content, clean data GC content, that is, the percentage of G and C bases out of total bases in the data; % ≥ Q20 and ≥ Q30, the percentage of the bases with a data mass value greater than or equal to 20 and 30, respectively; Mapped reads, the number of reads in the reference genome and the percentage in clean reads; Uniq. map reads, the number of reads compared in one location to the reference genome and the percentage in the clean reads; Multiple map reads, the number of reads compared in multiple locations to the reference genome and the percentage in clean reads
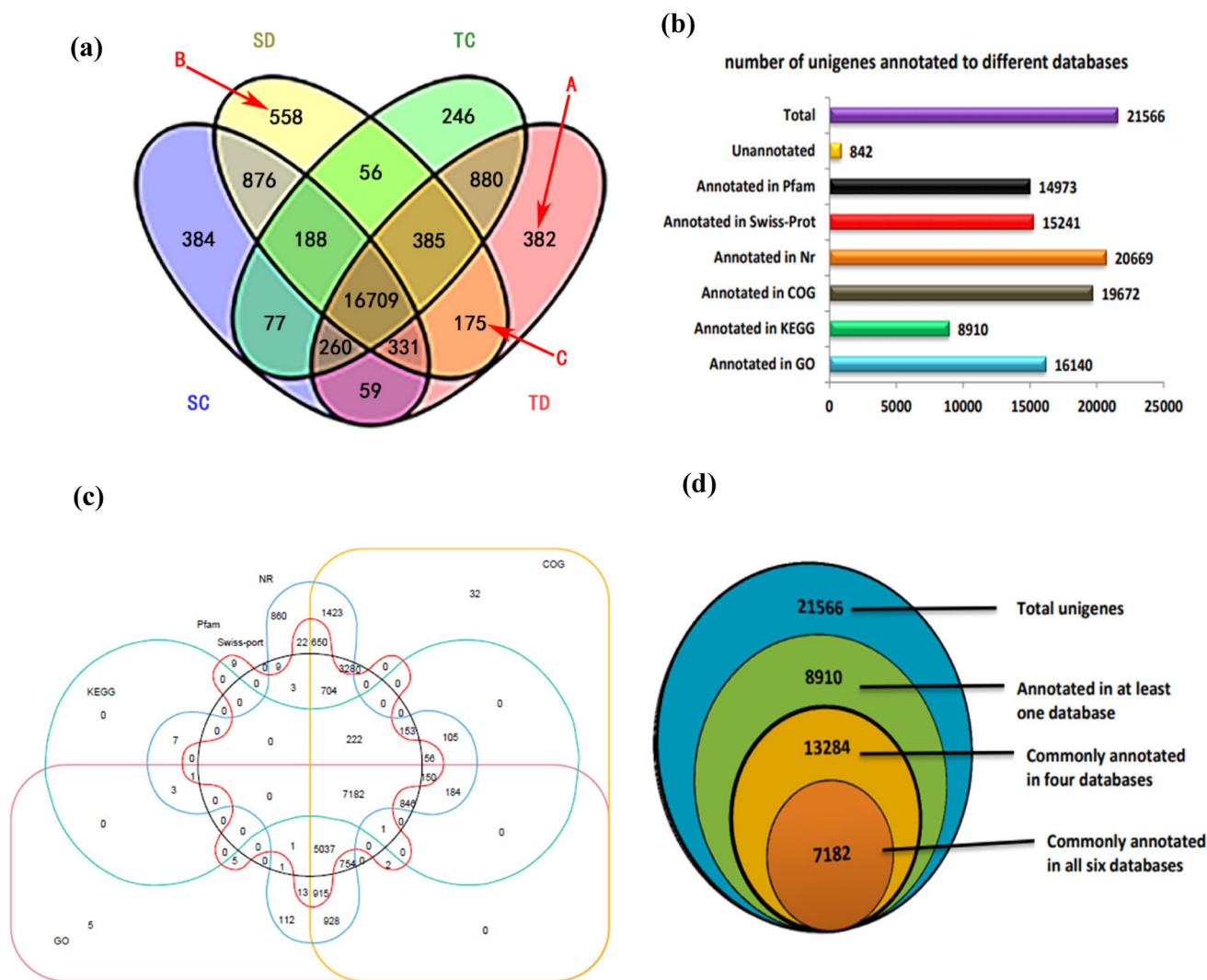
Fig. 1 Summary analysis of gene expression profile and functional annotation of unigenes. **a** Venn diagram analysis of transcriptomic responses by cultivar (tolerant ND476, T; and sensitive ZX978, S) and treatment (control, C; and drought, D). The biological samples of four combinations are TC, TD, SC, and SD, respectively. The area labeled 'A' represents the genes exclusively expressed in TD, area labeled 'B' represents the genes specifically expressed in SD, and labeled 'C' represents the drought responsive genes shared by the tolerant and sensitive lines; **b** number of unigenes annotated to different sequence databases; **c** Venn diagram analysis of unigenes annotated to different databases; and **d** number of unigenes commonly annotated among databases

in each treatment, genes shared between each treatment, and genes shared among all combinations of treatments are shown in Fig. 1a. Of these 21,566 gene transcripts, 77.48% (16 709) were represented in all treatments. Before drought stress, 87.56% (18 884) and 87.18% (18 801) of the genes were expressed in the sensitive cultivar (ZX978, SC) and the tolerant cultivar (ND476, TC), respectively. After drought stress, 89.39% (19,278) and 88.94% (19 181) were expressed in ZX978 (SD) and ND476 (TD) respectively (Fig. 1a). A total of 382 genes (Group A in Fig. 1a) were specifically expressed in tolerant cultivar ND476 after drought treatment (TD). Group B, containing 558 genes, represents the genes specifically expressed in sensitive cultivar ZX978

after drought treatment (SD). Group C represents the 175 expressed genes that were shared by the tolerant and sensitive hybrids after drought treatment.

The unigenes were aligned against sequences in the Nr, Swiss-Prot, COG, Pfam, GO and KEGG databases to assign functional annotations. Resultantly, a total of 21,566 unigenes got annotated, 7182 (33.3%) unigenes found matches in all the six databases and 8910 (41.32%) unigenes matched sequences in at least one of the databases. However, 842 (3.90%) unigenes did not align to any of the known sequences in the public databases (Table 2; Fig. 1b). Meanwhile, 20,699 (95.84%) unigenes were found to have significant similarity with protein sequences in the Nr database at

Table 2 Summary of functional annotation of unigenes

	Number of unigenes	Percentage (%)
Annotated in Nr	20,669	95.84
Annotated in Swiss-Prot	15,241	70.67
Annotated in Pfam	14,973	69.42
Annotated in GO	16,140	74.84
Annotated in KEGG	8910	41.31
Annotated in COG	19,672	91.21
Annotated in all databases	7182	33.30
Unannotated	842	3.90
Total	21,566	

a cutoff of $E \leq 1e-05$, whereas 15,241 (70.67%) unigenes were annotated by Swiss-Prot database (Tables 2; S1). Our Venn diagram analysis showed that most of the unigenes were annotated by more than one database. For instance, only 860, 32, 9 and 5 unigenes were specifically annotated in Nr, COG, Pfam and GO databases, respectively, but no unigene was specifically annotated by the KEGG and Swiss-Prot databases (Fig. 1c). At the same time, 7182 unigenes were annotated in all the six searched databases, whereas 13,284 unigenes were commonly annotated in the four (Nr, COG, Pfam and GO) databases (Fig. 1d). Overall, the results indicated that unigenes were much more easily annotated by Nr and Swiss-Prot than other databases (Fig. 1b, c). The number and proportions of unigenes assembled and annotated varied minimally between the present study and other drought stress related transcriptomic studies in maize (Li et al. 2017; Lu et al. 2017; Zenda et al. 2019), which may be as a result of the novel genes specially expressed in different organs, or probably due to technical or biological biases.

Gene differential expression analysis

The software Cuffdiff (Trapnell et al. 2012) was used to explore differentially expressed genes (DEGs) between different treatments. At a standard fold change of < 1.5 and FDR < 0.05, we identified a total of 3114 (1484 up- and 1630 down-regulated) genes to be differentially expressed between the tolerant and sensitive hybrids before drought treatment (SC_TC in Fig. 2a). Under drought conditions, 4140 (1972 up- and 2168 down-regulated) genes were identified between the tolerant and sensitive lines (SD_TD in Fig. 2a). By further comparing the differences in DEGs between the tolerant line ND476 and sensitive line ZX978, we identified 70 (29 up- and 41 down-regulated) DEGs in the tolerant line (TC_TD), and 1 199 (507 up-and 692 down-regulated) DEGs in the sensitive line (SC_SD) to be differentially expressed before and after drought treatment (Fig. 2a; Tables S2, S3).

Some of the Venn combinations shown in Fig. 2b are more essential than others in respect of drought tolerance. Area I represents 21 specific DEGs of TC_TD, that is, the specific drought responsive DEGs of the tolerant cultivar ND476. Of these DEGs, 9 were up-regulated and 12 were down-regulated (Table 3). Area II represents 1693 specific DEGs of SD_TD, that is, specific DEGs shared between the drought sensitive and drought tolerant hybrids after drought treatment. Of these, 811 were up-regulated whilst 882 were down-regulated (Table S4). Area III represents the 10 specifically shared DEGs between TC_TD and SD_TD, that is, drought responsive DEGs of the tolerant cultivar that were also differentially expressed between the tolerant and sensitive hybrids after drought treatment. Of these, two were up-regulated and eight were down-regulated in TC_TD comparison but showed differential expression in SD_TD comparison (Table S5). Area IV represents the 14 DEGs shared by TC_TD and SC_SD, that is, the common drought responsive DEGs of the two cultivars. These DEGs showed differential expression in the two hybrids after drought treatment, with 9 DEGs being up-regulated and 5 down-regulated in TC_TD, but showing differential expression is in SC_SD (Table S6). In total, we found 5805 DEGs to be differentially expressed among the four comparison groups, which reflect the impact of cultivars or treatment (Fig. 2a, b). Clustering analysis of the 21 DEGs specific to TC_TD comparison showed that, after drought stress exposure, DEGs were grouped into five clusters, with most of these DEGs being down-regulated than up-regulated (Fig. 2c).

DEGs annotation and functional categorization

We performed GO functional annotation and categorization of the DEGs from the four critical areas (labelled I–IV in Fig. 2b) using Blast2GO website (https://www.blast2go.com/) in order to determine their broad biological functions. The GO analysis results showed that a great number of DEGs were involved in BP functions (Fig. S3). Particularly, GO: 0009987 (cellular process); GO: 0008152 (metabolic process), GO: 0065007 (biological regulation), GO: 0050789 (regulation of biological process) and GO: 0050896 (response to stimuli) were common and top most significantly enriched level 2 GO terms in the BP category (Fig. S3a–c). Within the MF category, GO: 005488 (binding) and GO: 0003824 (catalytic activity) were most enriched among others (Fig. S3a–c). Further analysis of the Area I specific DEGs showed that GO terms related to photosynthesis (light harvesting) were the most significantly enriched under BP category whilst those related to chlorophyll binding and pigment binding were prominent under MF category (Fig. 3a). For the SD_TD specific DEGs, GO terms related to glutathione metabolic process and oxidation–reduction process were most significantly enriched under BP category
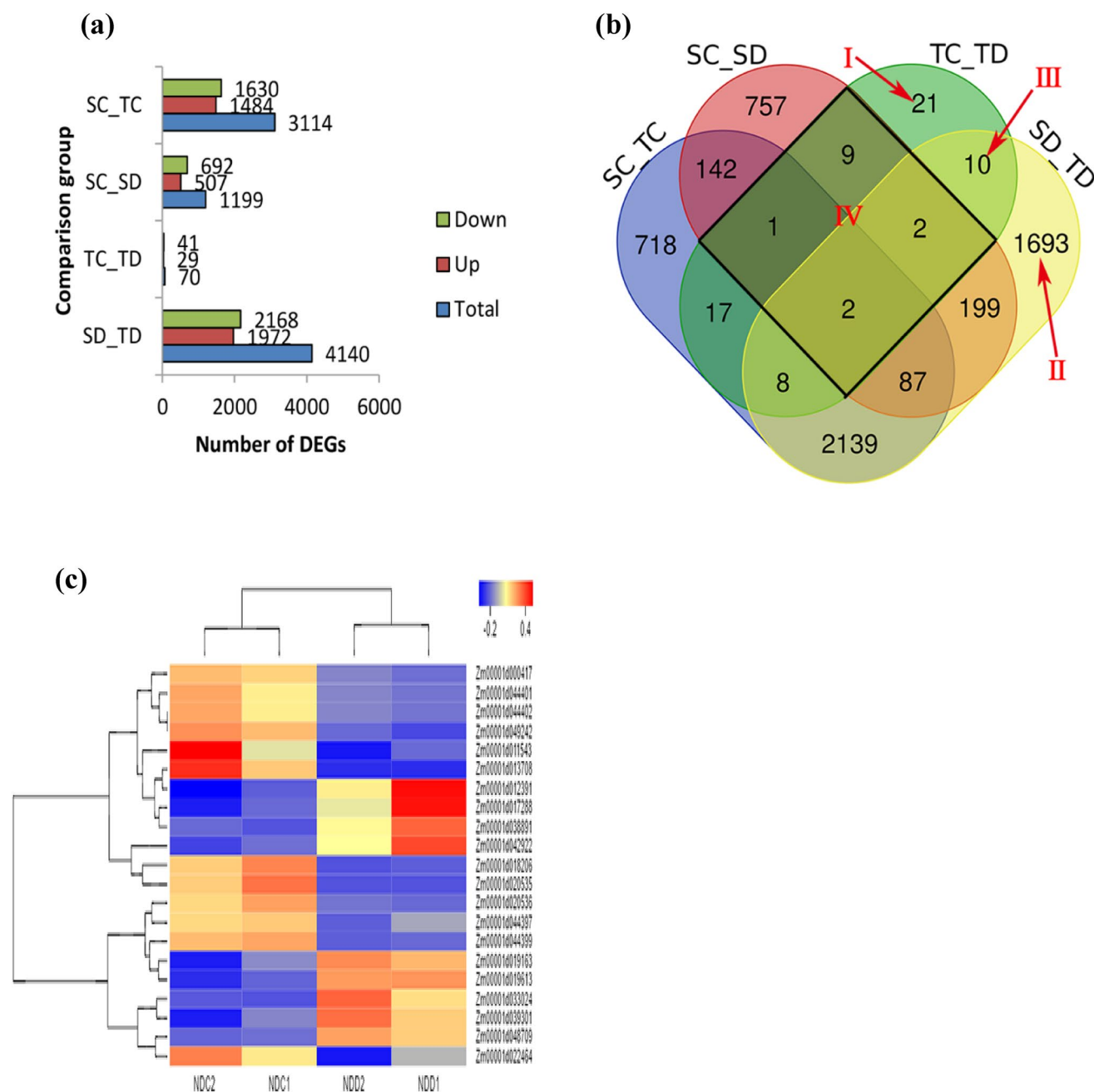
(a)

(b)

(c)

Fig. 2 Analysis of differentially expressed genes (DEGs). **a** Number of DEGs in each of the four experimental comparisons. Each compared combination is separated by an underscore (eg. TC_TD); **b** Venn diagram analysis of DEGs. Four critical areas, labeled I, II, III and IV, totally contain 1738 DEGs. Area I contains the tolerant treatment response DEGs, excluding others. Area II contains the line response under drought DEGs, excluding others. Area III contains both tolerance treatment response and line response under drought DEGs, excluding others. Area IV contains the treatment response DEGs within line; **c** clustering analysis of the tolerant line ND476 specific (Area I) DEGS. Each row indicates a gene differentially expressed (up-regulated—red, and down-regulated—blue). Columns show different samples. NDC1-2 and NDD1-2 refer to two replicates each for tolerant genotype ND476 under water sufficient (control) and drought stress conditions, respectively

(Fig. 3b). These results may suggest that the DEGs with these identified biological processes and molecular functions may be the key contributors to the drought stress responses in tolerant line ND476.

DEGs encoding transcription factors

Our analysis of the tolerant cultivar ND476 (TC_TD) DEGs identified four TF genes that were altered in

Table 3 Drought responsive differentially expressed genes specific to drought tolerant hybrid ND476

| No	Gene ID	Gene description	Log2FC	Express	P value	FDR	KEGG pathway
1	Zm00001d019613	Probable O-methyltransferase 2	1.31	Up	3.42E−08	2.96E−05	Gingerol biosynthesis
2	Zm00001d012391	Delta 1-pyrroline-5-carboxylate synthetase	1.27	Up	3.41E−06	2.00E−03	Arginine and proline metabolism
3	Zm00001d019163	Stachyose synthase	1.15	Up	1.29E−06	8.86E−04	Galactose metabolism
4	Zm00001d033024	Tyrosine decarboxylase 1	1.09	Up	3.17E−05	1.24E−02	Isoquinoline alkaloid biosynthesis
5	Zm00001d039301	Vegetative storage protein PNI288	1.09	Up	1.21E−05	6.04E−03	
6	Zm00001d017288	Aquaporin PIP2-4	1.06	Up	1.61E−04	4.66E−02	
7	Zm00001d042922	Probable mediator of RNA polymerase II transcription subunit 37c	1.05	Up	1.10E−04	3.71E−02	Protein processing in endoplasmic reticulum
8	Zm00001d048709	Benzoxazinless1	1.02	Up	1.13E−04	3.76E−02	Benzoxazinoid biosynthesis
9	Zm00001d038891	Phosphoethanolamine N-methyltransferase 3	0.98	Up	1.37E−04	4.19E−02	Glycerophospholipid metabolism
10	Zm00001d044397	Small RNA 2′-O-methyltransferase	− 0.97	Down	2.13E−05	8.48E−03	
11	Zm00001d022464	Ultraviolet-B-repressible protein	− 0.98	Down	1.55E−04	4.61E−02	
12	Zm00001d044401	Photosystem II light harvesting complex gene B1B2	− 1.02	Down	1.37E−05	6.26E−03	Photosynthesis-antenna proteins
13	Zm00001d044402	Chlorophyll a-b binding protein 2	− 1.02	Down	1.37E−05	6.26E−03	Photosynthesis-antenna proteins
14	Zm00001d011543	Grx_I1-glutaredoxin subgroup III	− 1.06	Down	1.52E−04	4.59E−02	
15	Zm00001d049242	–	− 1.07	Down	1.32E−04	4.19E−02	
16	Zm00001d000417	Photosystem Q(B) protein	− 1.08	Down	1.37E−06	9.08E−04	Photosynthesis
17	Zm00001d013708	Probable prefoldin subunit 4	− 1.10	Down	1.31E−05	6.26E−03	
18	Zm00001d020536	Eukaryotic aspartyl protease family protein	− 1.24	Down	6.09E−06	3.28E−03	
19	Zm00001d044399	photosystem II light harvesting complex gene B1B2	− 1.30	Down	4.18E−10	4.17E−07	Photosynthesis—antenna proteins
20	Zm00001d018206	Nitrate reductase [NADH] 2	− 1.46	Down	7.34E−11	9.14E−08	Nitrogen metabolism
21	Zm00001d020535	Probable folate-biopterin transporter 6	− 1.74	Down	5.30E−10	5.03E−07	

Gene ID, unique gene identifying number in the Maize Genetics and Genomics Database (Maize GDB); Gene description, description of the identified gene by gene ontology (GO) annotation; Log2 FC, log twofold change, expressed as the ratio of intensities of up-regulated or down-regulated genes between drought treatments and control (water-sufficient conditions); Express, gene relative expression, up- for up-regulation and down- for down-regulation; p-value, statistical level (using Student's t test) < 0.05; FDR, false discovery rate, the corrected p-value at < 0.01, the level of significance for the differentially expressed genes; KEGG pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways in which the identified gene was found to be significantly enriched

response to drought stress. Three genes; *Zm00001d008869* (Homeobox-TF52), *Zm00001d027900* (ZIM-TF27) and *Zm00001d037769* (C3H-TF339) were up-regulated whereas one gene *Zm00001d041886* (*NAC-TF100*) was down-regulated in response to drought stress (Table S2). Interestingly, *Homeobox-TF52* and *C3H-TF339* were also up-regulated in response to drought stress in sensitive line ZX978 (Table S3). Additionally, there were 98 drought responsive TF genes observed in the SD_TD specific DEGs (Table 4). These TFs were categorized into several classes, including MYB (9), NAC (2), WRKY (3), AP2-EREBP (5), bZIP (5), GARP (2), HSF (4), HAP (3) and bHLH (2) (Table 4). These TF genes are suggested to play vital roles in modulating drought stress tolerance in the tolerant maize hybrid cultivar.

'Response to stimuli' and 'catalytic activity' related DEGs

Our analysis of the DEGs that were enriched in each level 2 GO term showed that, among the tolerant cultivar ND476 specific DEGs (listed in Table 3), four were significantly enriched in GO term 'response to stimuli' (GO: 0050896). These genes were *Zm00001d044402* (chlorophyll a-b binding protein 2), *Zm00001d044401* (photosystem II light harvesting complex gene *B1B2*), *Zm00001d000417* (photosystem Q (B) protein) and *Zm00001d044399* (photosystem II light harvesting complex gene *B1B2*). All these four genes are involved in photosynthesis. Gene *Zm00001d000417* was also significantly enriched in GO term 'catalytic activity' (GO: 0003824) which had 12 genes
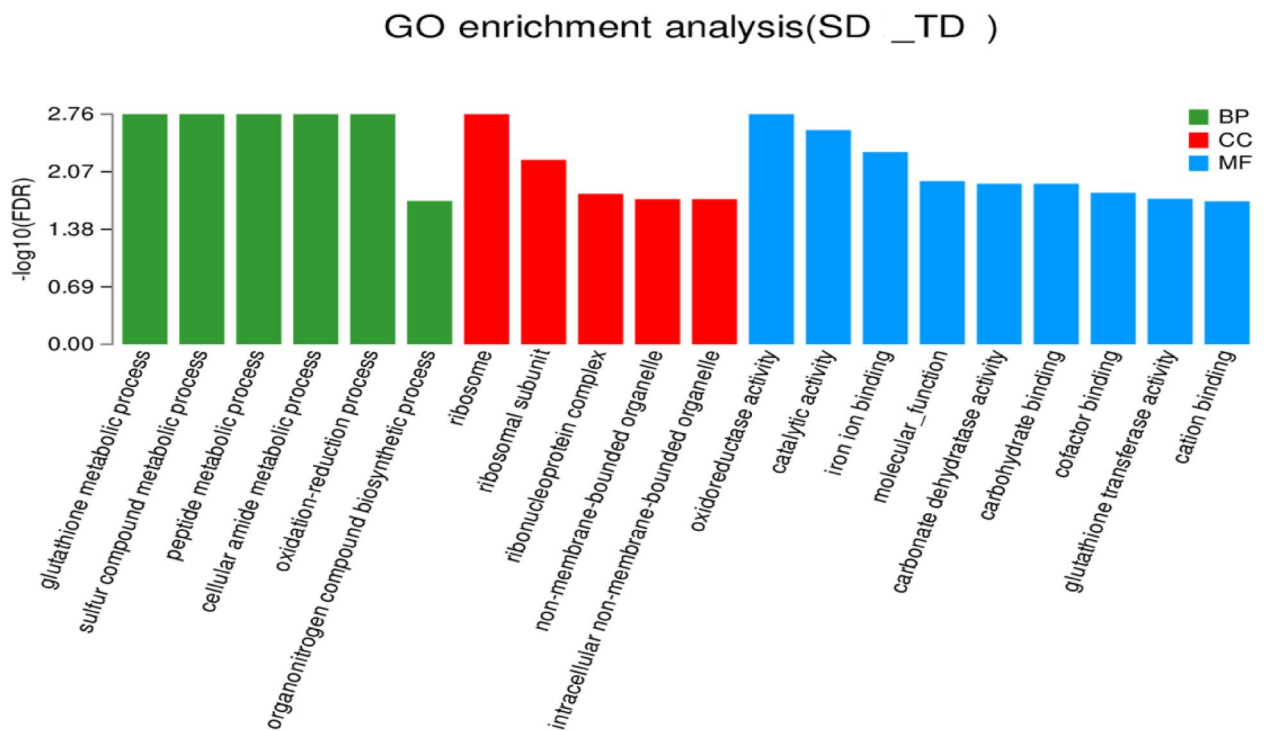
(a)
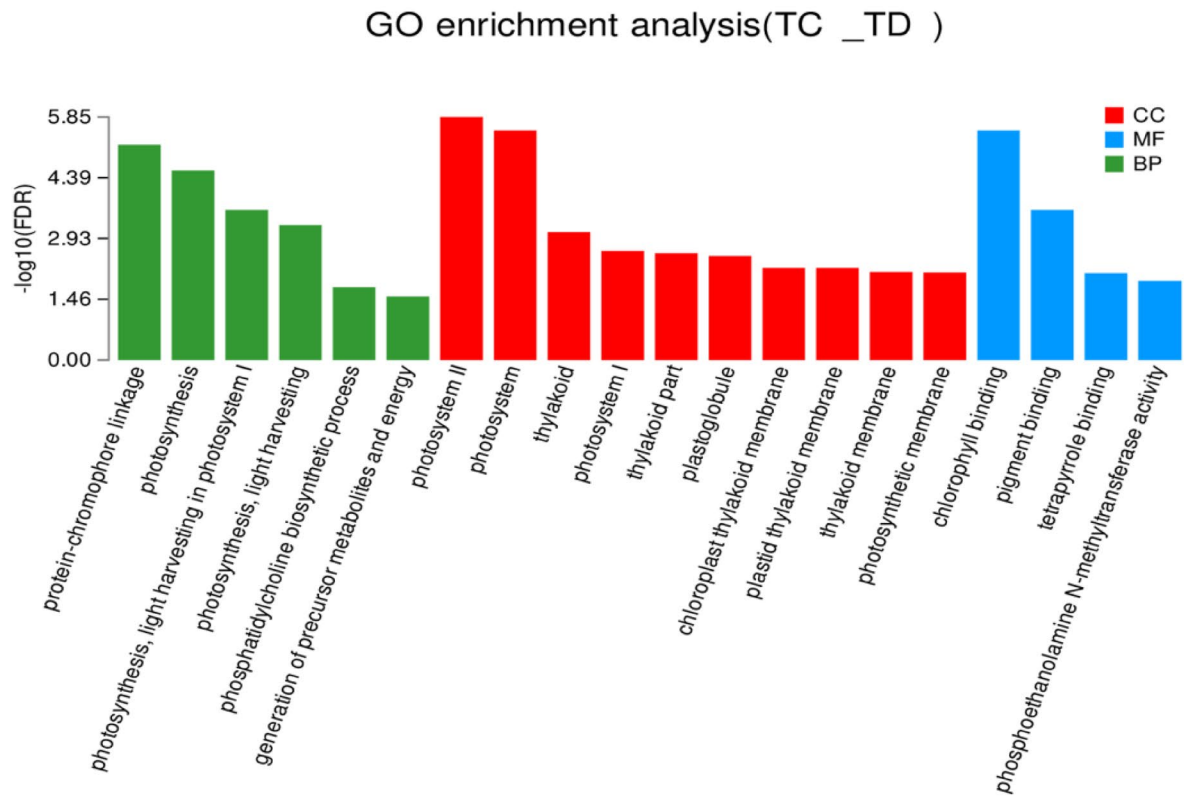
(b)

Fig. 3 Gene ontology (GO) enrichment analysis of DEGs. Results show GO enrichment analysis of genes corresponding to **a** tolerant line treatment response (TC_TD) specific; and **b** line response under drought (SD_TD) specific. DEGs were enriched in different functional categories, viz., biological processes (BF), molecular functions (MF) and cellular component (CC)

Table 4 Drought responsive transcriptional factors identified in the SD_TD comparison

SN	Transcriptional factor family	Gene number	SN	Transcriptional factor family	Gene number
1	Alfin-like	1	18	HDA	1
2	AP2/EREBP	5	19	Histone acetyltransferases	1
3	ARF	1	20	HSF	4
4	AS2	2	21	JUMONJI family	1
5	bHLH	3	22	MYB domain	9
6	bZIP	5	23	MYB-related	1
7	NAC domain	2	24	C2C2 (Zn) CO-like	4
8	C2C2 (Zn) DOF	1	25	PHD finger	1
9	C2C2 (Zn) GATA	1	26	PHOR1	2
10	C2H2	5	27	Psudo ARR	1
11	EIN3-like (EIL)	1	28	putative transcription regulator	10
12	GARP	2	29	SET-domain	1
13	SNF7	2	30	General transcription	1
14	GRAS	2	31	Trihelix	1
15	HAP3	1	32	unclassified	18
16	HAP5	1	33	WRKY domain	3
17	HB	4			

AP2/EREBP APETALA2/ethylene-responsive element binding protein, *ARF* auxin response factor, *AS2 ASYMMETRIC LEAVES 2* gene family, *bHLH* basic helix-loop-helix, *bZIP* basic leucine zipper, *C2C2(Zn) GATA* zinc protein family that binds the consensus DNA sequence (T/A) GATA (A/G), *CO-like* constans-like zinc finger, *C2H2* cystine (2) and histidine (2) residues, *EIN3-like (EIL)* ethylene-insensitive3-like, *GARP* made of ARRB-B and G2-like members, *HAP* heme activator protein, *GRAS* group composed of three members, viz., *GIBBERELLIC-ACID INSENSITIVE* (GAI), *REPRESSOR of GAI* (RGA) and *SCARE-CROW* (SCR), *SNF* sucrose non-fermentable, *HAD* histone deacetylase, *HB* homeobox, *HSF* heat-shock factor, *MYB* myeloblastosis oncogenes, *NAC domain* [NAM, ATAF₁/₂, and CUC2] containing proteins, *PHD finger* plant homedomain finger, *PHOR1 PHOTOPERIOD RESPONSE 1*, *Psudo ARR* pseudo AS response regulator, *SET-domain* conserved domain made of three proteins SuVar (3–9), E (z), and Tritho-rax, *WRKY* TF family denoted by protein domain composed of a conserved WRKYGQK motif and a zinc-finger domain

enriched in it. In common overlapping DEGs between ND476 and ZX978 (area IV in Fig. 2b), there were four genes enriched in GO term 'response to stimuli' (GO: 0,050,896), including *Zm00001d011183* (thiamine biosynthesis1), *Zm00001d037769* (C3H-transcription factor 339), *Zm00001d052213* and *Zm00001d024839* (glutathione *S*-transferase 2), and seven genes were enriched in GO term 'catalytic activity' (GO: 0003824), including *Zm00001d020627*, *Zm00001d002940*, *Zm00001d011183* (thiamine biosynthesis1), *Zm00001d033872*, *Zm00001d052213*, *Zm00001d009631* and *Zm00001d024839* (glutathione *S*-transferase2). These genes are involved in stress signal perception and transduction, nutrient and water uptake, and cell elongation under drought stress conditions during late vegetative (V12) growth stage, and thus, are considered key actors modulating drought tolerance in maize.

KEGG metabolic pathways enrichment analysis of the DEGs

Functional involvement of the DEGs in various metabolic pathways was further investigated by mapping them to the KEGG database (available online: https://www.genome.jp/kegg/; accessed on 15 March 2019). By analysing the top twenty pathways in which most DEGs were enriched, we discovered that photosynthesis-antenna proteins (4 DEGs), ribosome (3), nitrogen metabolism (2) and photosynthesis (2) were dominant in ND476 (TC_TD) (Fig. S4a). In SD_TD comparison, ribosome (99), starch and sucrose metabolism (39), and plant hormone signal transduction (35) pathways had the greatest number of genes enriched (Fig. S4b). Comparatively, the composition of the enriched KEGG pathways in sensitive cultivar ZX978 (SC_SD) differed significantly, with carbon fixation in photosynthetic organisms (25), ribosome (51), oxidative phosphorylation (29) and plant hormone signal transduction (25) being the top most enriched (Fig. S4c). Further, we performed significant pathway enrichment analysis of these DEGs by hypergeometric test, with pathways of q value < 0.01 considered to be significantly affected by drought stress. We then observed that photosynthesis-antenna proteins and nitrogen metabolism pathways were highly enriched in TC_TD comparison (Fig. 4a). Meanwhile, ribosome, starch and sucrose metabolism, and nitrogen metabolism pathways were highly
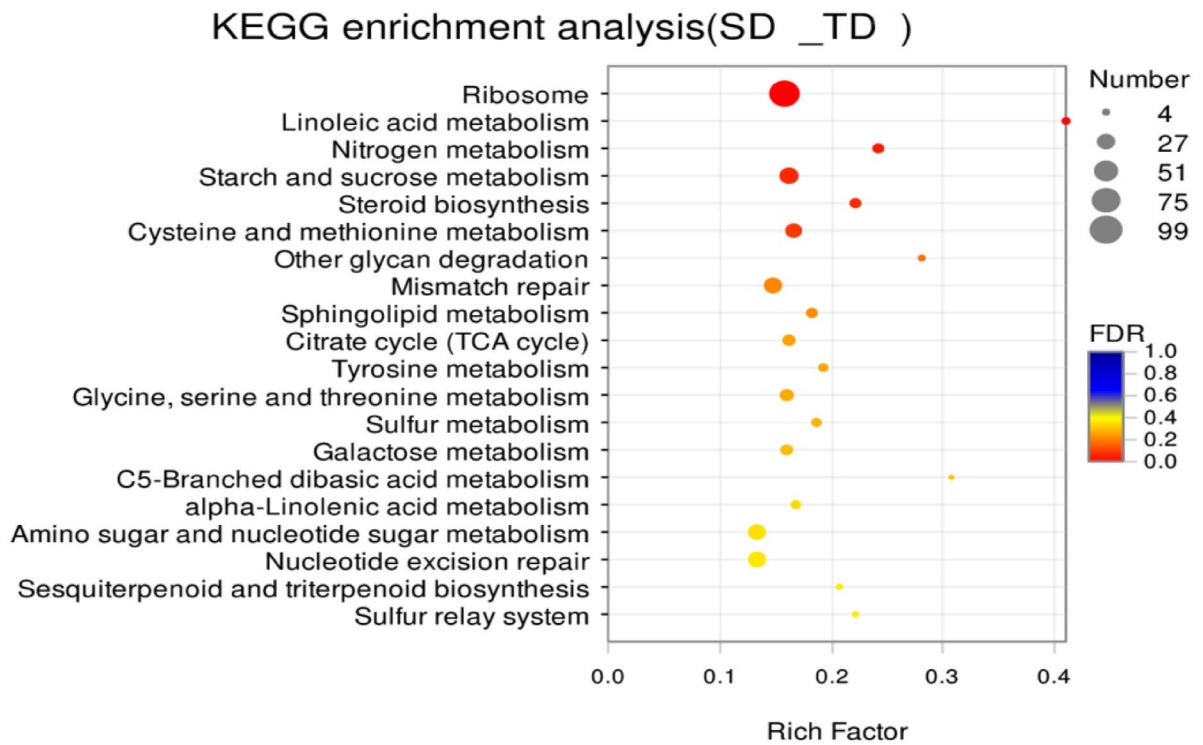
(a)
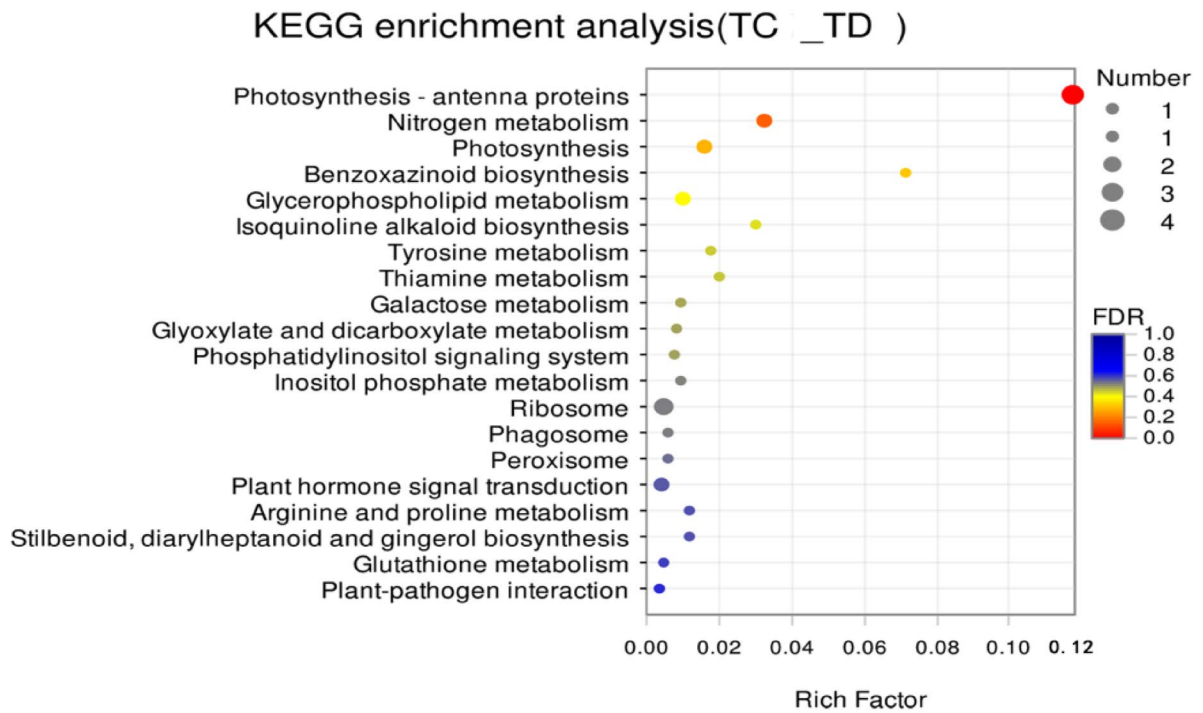
(b)

◄**Fig. 4** KEGG (Kyoto Encyclopedia of Genes and Genomics) pathway enrichment analysis of the DEGs. Results show the most significantly enriched pathways in **a** TC_TD; and **b** SD_TD experimental comparisons, based on the hypergeometric test. The significance of the enrichment of the KEGG pathway is based on the Student's t test, $q < 0.01$. The color gradient represents the size of the q value; the color is from red to blue, and the nearer to red, the smaller the q value, the higher the significant level of enrichment. The 'rich factor' shows the ratio of the number of the DEGs to the total gene number in certain pathways

enriched in the SD_TD (Fig. 4b). In contrast, carbon fixation in photosynthetic organisms, ribosome and oxidative phosphorylation were the most significantly enriched in sensitive cultivar ZX978 (Fig. S5).

Validation of RNA-seq by quantitative real-time PCR (qRT-PCR)

In order to validate the accuracy of the RNA-Seq results, we randomly chose a sample of 20 DEGs for qRT-PCR analysis. We designed gene specific primers (Table S7) for qRT-PCR analysis using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Our qRT-PCR analysis results were congruent to our RNA-seq based findings. Notably, RNA-seq expression patterns of all the sampled DEGs were closely replicated by the qRT-PCR approach, with an R^2 (correlation coefficient of qRT-PCR log2FC versus RNA-seq log2FC) of 92.98% (Fig. 5; Table S8; Fig. S6). This confirmed that our RNA-seq data was reliable.

Phenotypic and physiological responses of two maize hybrids to drought stress

Expectedly, no apparent phenotypic differences were observed between ND476 and ZX978 maize plants under water-sufficient conditions. They both exhibited vigorous and intact plant structures (Fig. S7a). However, post drought treatment exposure, considerable variations were noted between the two hybrids. Whilst the tolerant cultivar ND476 maintained erect green leaves and considerably intact plant stature, the sensitive line ZX978 exhibited pendulous leaves and a drooping plant stature (Fig. S7b). Meanwhile, in both maize lines, the RWC significantly ($p < 0.05$) decreased with increasing number of days post water deprivation exposure. Evidently, the rate of decline of RWC was greater in ZX978 than in ND476 under drought stress conditions (Fig. 6a). The SPC showed gradual increase in both cultivars, from day 1 until day 6 under drought conditions. Thereafter, SPC started to drop steadily. Notably, the tolerant genotype ND476 maintained significantly higher SPC values than the sensitive genotype ZX978 at any particular time point (Fig. 6b). The POD activity showed an almost similar trend to SPC in both maize lines, increasing from day 3 to 7 in drought-stressed

ND476 and day 3–6 in drought-stressed ZX978, and gradually dropping thereafter (Fig. 6c). Under drought treatment, MDA content gradually increased in both maize genotypes, starting from day 3 and becoming more significantly different between genotypes from day 6 onwards. Of note, the MDA content in ZX978 was significantly greater than in ND476 (Fig. 6d).

Discussion

In face of global climate change, the major goal for global agriculture is to develop drought-tolerant crops (Edmeades 2013; Min et al. 2016). To this end, a full understanding of physiological, biochemical, and gene regulatory networks relating to drought tolerance in plants becomes imperative. Here, using an integration of RNA-seq based technology and physiological parameters in comparative analysis of transcriptomes of two contrasting maize (drought-tolerant ND476 and drought-sensitive ZX978) hybrid cultivars, we report key differentially expressed genes and regulatory mechanisms involved in maize drought stress tolerance. In addition, we have conducted some physiological analyses to support the RNA-seq data. Our results enhance our further understanding of the mechanisms modulating drought tolerance in maize, in addition to aiding as foundational base to our future targeted cloning studies.

Maize hybrid cultivars differential drought stress responses at the physiological level

Cultivars within crop species are known to differ in their responses to drought and other stresses. These responses can occur at different levels, including physiological and molecular, as well as at different plant growth phases (Prasad et al. 2008). To avoid these deleterious effects of oxidative stress emanating from ROS, plants maintain cellular structures, including membrane and protein stabilities by several strategies such as cell turgor maintenance and osmotic adjustment (Oliver et al. 2007; Jogaiah et al. 2013). Here, our results revealed that tolerant genotype ND476 maintained comparably higher leaf RWC and SPC than sensitive genotype ZX978 under drought stress conditions (Fig. 6a, b). This resulted in limited loss of cell turgor and integrity, and less stress at the cellular level. Contrastingly, sensitive genotype ZX978 experienced significant decline in RWC, SPC and POD from day 6 onwards (Fig. 6a–c), causing a rapid loss of cell turgor and cell integrity. This possibly explains why, at the transcriptome level, fewer (70) genes were differentially expressed in ND476 than 1199 genes in ZX978 in response to drought stress (Fig. 2a). Thus, there was limited transcriptome perturbation in the tolerant cultivar as compared to
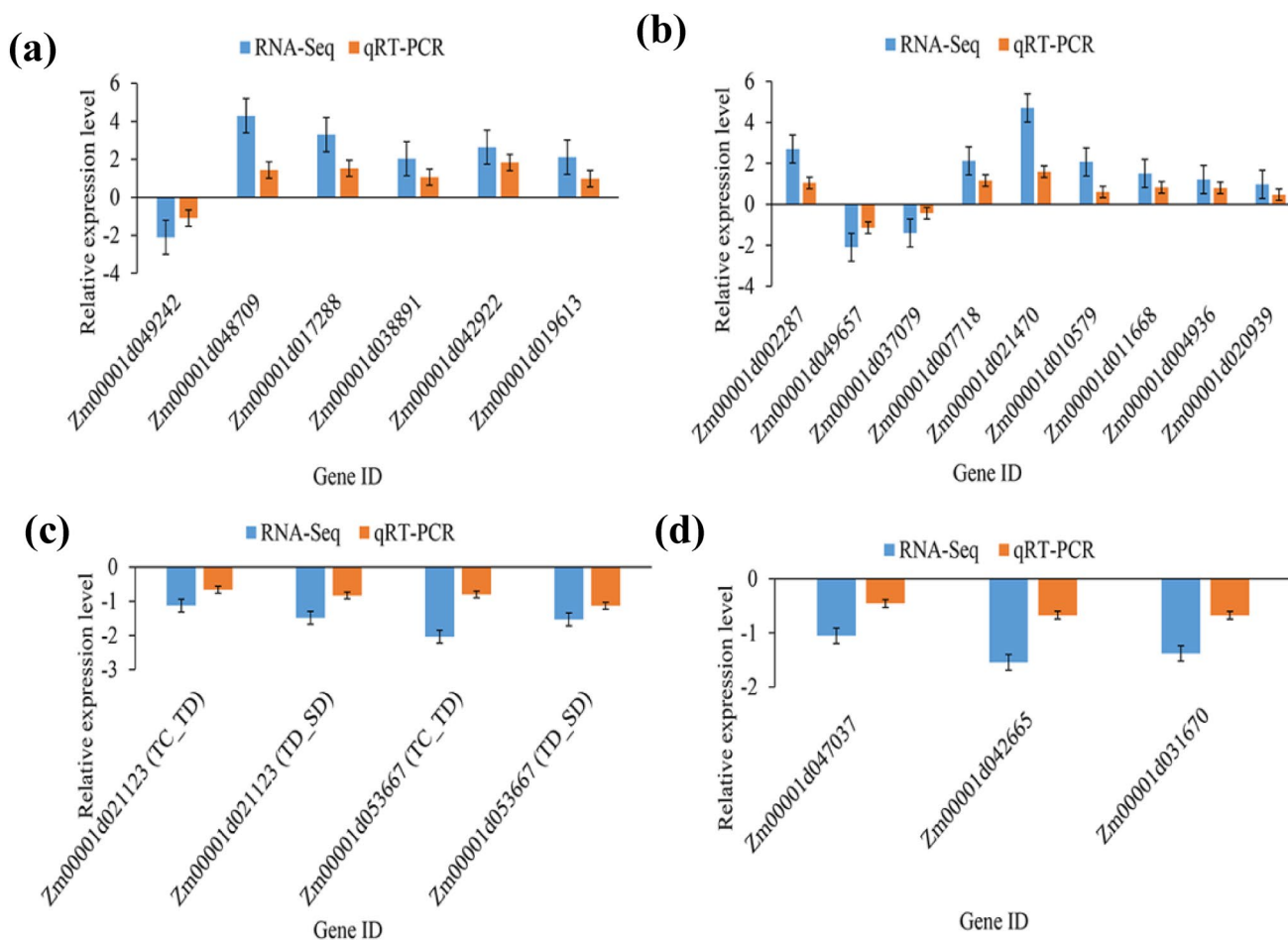
(a)

(b)

(c)

(d)

Fig. 5 qRT-PCR validation of the RNA-seq data of the 20 randomly selected maize V12-stage leaf DEGs. **a** TC_TD specific DEGs; **b** SD_TD specific DEGs; **c** DEGs shared between TC_TD and SD_TD; and (**d**) DEGs specific to SC_SD. The y axis represents the gene relative expression levels (Log2 fold changes) in the real-time PCR analysis and Log2 fold changes in the RNA-Seq data. All the genes with negative values of expression level means that they were down-regulated in response to drought stress. Maize gene GAPDH (Accession No. X07156) was used as the internal reference. Error bars represent the SE ($n = 3$)

wider transcriptome perturbation in the sensitive line at the same drought stress intensity. The role of POD in ROS scavenging and detoxification is well documented (Sharma et al. 2012). Here, we speculate that the sustained increase of POD in ND476 hybrid cultivar enhanced ROS quenching than in ZX978 under drought stress. Consequently, ND476 had better cell membrane stability and drought stress tolerance than ZX978, in conformity to previous studies (Moussa and Abdel-Aziz 2008; Zenda et al. 2018). Additionally, MDA content, which reflects the extent of lipid peroxidation and cell membrane stability (Sharma et al. 2012), was more significantly increased in ZX978 than in ND476 as the drought stress exposure period progresses, particularly from day 6 onwards (Fig. 6d). This may suggest that ND476 cells had better ROS quenching capacity than ZX978 cells, providing for enhanced cell membrane stability in ND476 than in ZX978 (Min et al. 2016). Taken together, our results reveal some differential

drought stress responses between the two hybrid cultivars at the physiological level.

Secondary metabolites biosynthesis and carbohydrates metabolism related enzymes under drought

Among the top most up-regulated drought responsive genes specific to the tolerant line ND476 (TC_TD) were secondary metabolite biosynthesis (SMB) related enzymes, including probable O-methyltransfarase 2 (OMT2; *Zm00001d019613*), tyrosine decarboxylase 1(*Zm00001d033024*) and benzoxazinless1 (*Zm00001d048709*), as well as one carbohydrate (CHO) metabolism related enzyme stachyose synthetase (SSG*; Zm00001d019163*) (Table 3). OMT2 is involved in SMB in *Sorghum bicolor* L (https://www.uniprot.org/uniprot/A8QW51). Tyrosine decarboxylase 1 constitutes isoquinoline alkaloids, one of the quite divergent natural chemicals
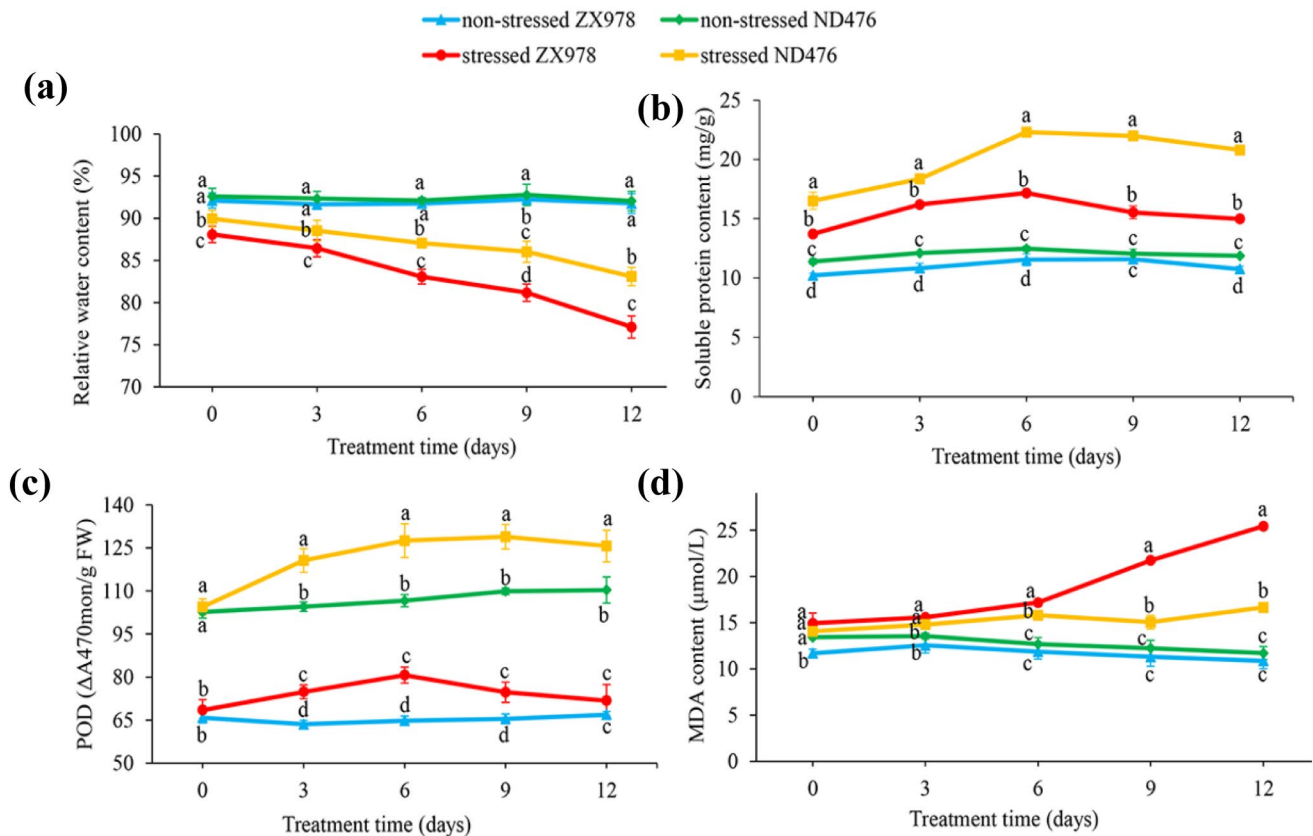
Fig. 6 Physiological responses of two contrasting maize hybrid cultivars to drought stress after 12 days of exposure to water-sufficient (control) or water-limited (drought) conditions. **a** RWC; **b** SPC; **c** POD activity; and **d** MDA content. Data are presented as mean ± SE of mean (n = 3). Different letters above line graphs show significant difference (p < 0.01) among treatments at a particular treatment time point

produced by higher plants, including alkaloids, terpenoids and phenylpropanoids. These compounds are biogenetically derived from phenylalanine and tyrosine, and are essential in conferring combined pathogenic and abiotic stress resistance in plants (Edreva et al. 2007). Benzoxazinless1, together with other enzymes such as cytochrome P450 *(Zm00001d040764* in Table S2*)* and OMT, is functionally recruited in the benzoxazinoid biosynthesis pathway (Glawischnig et al. 1999). These genes participate in phenylalanine metabolism, a stage in the phenylpropanoid metabolism, which is the first step of the SMB (Shinde et al. 2018); coordinated activation of these genes is considered vital to improved stress tolerance in plants (Ma et al. 2014). CHO metabolism is essential in bio-molecular metabolism, via CHO breakdown providing important saccharides and energy that are critical for cell growth and survival under stress conditions (Min et al. 2016; Yang et al. 2019). SSG enzyme participates in galactose metabolism processes. Stachyose, as a tetrasaccharide, is recognized as an important transport CHO in a large number of woody plants, cucurbits and legumes (Peterbauer et al. 1999). Therefore, SSG is a key player in CHO metabolism and energy provision in response to drought stress. Taken collectively, the SMB and CHO related genes are suggested to balance cell growth, defense and drought stress response in maize.

Transcription factor related genes are critical in regulating drought stress response

The role of TFs in various stress tolerances in different crop plants including maize is well-documented (Wang et al. 2016). In maize, different TF families have been identified as responsible for modulating the gene regulation in response to drought stress (Joshi et al. 2016; Kimotho et al. 2019). TFs such as MYB, NAC, WRKY, bZIP, bHLH, HD-zip via ABA-dependent or ABA-independent pathways play a significant role in drought tolerance (Mittal et al. 2018). They achieve this by regulating numerous physiological and molecular processes, including stomatal opening and closure, hormone signalling and osmoregulation (Mittal et al. 2017; Jin et al. 2019). In the current report, more than 33 drought-responsive TF families were differentially expressed under drought conditions (Tables 4, S9). Among these were nine MYB (4 up- and 5 down-regulated), two NAC (1

up- and 1- down), three WRKY (1 up- and 2 down-), five AP2-EREBP (all down-), five bZIP (3 up- and 2 down-), two GARP (both down-), and three bHLH (2 up- and 1 down-) that were regulated in response to drought stress (Tables S4 and S9). Previously, Shinde et al. (2018) identified several TF genes to be up-regulated in response to drought stress in foxtail millet (*Setaria italic* L.), including five NACs and three MYBs. On the other hand, Bianchi et al. (2015) revealed that WRKY TF genes were down-regulated under drought stress. Zhang et al. (2014) identified numerous drought responsive TF genes in *Medicago truncatula* L., including eight NACs, eight MYBs, six AP2/EREBPs, six bZIPs, five HDs, and four bHLHs, among others. Taken together, this discussion fortifies the important role TFs play in regulating drought stress tolerance in maize, with various TF families exhibiting differential responses, and interacting with each other in complex networks.

Aquaporins and glutathione S-transferases play crucial roles in drought tolerance

Detoxification and stress responsive proteins such as aquaporins, HSPs, glutathione S-transferases (GSTs), and LEA proteins play crucial roles in drought tolerance (Al-whaibi, 2011; Bhargava and Sawant, 2013; Harb, 2016; Jan et al. 2017). Here, our analysis of the TC_TD and the SD_TD specific DEGs showed that genes encoding aquaporins, GSTs and HSPs were differentially regulated in response to drought stress (Tables 3, S4). Aquaporins are membrane channels that facilitate the transport of water across biological membranes in most living organisms (Maurel et al. 2015). In plants, aquaporins are required for the facilitation of transport of water across cell membranes, and hence play an important role in water homeostasis by turgor regulation (Aslam et al. 2015; Min et al. 2016). Previously, aquaporins were also identified to be involved in drought tolerance in sheep grass [*Leymus chinensis* (Trin.) Tzvel] (Zhao et al. 2016a). Here, we suggest that aquaporin PIP2-4 (*Zm00001d017288*) functions to increase water absorption in tolerant genotype ND476 under drought stress, thus maintaining better cellular redox homeostasis under such conditions. GSTs play a crucial role in the plants` response to various types of abiotic stresses. GSTs quench reactive molecules with the addition of glutathione and protect the cell from ROS-induced oxidative damage (Kumar and Trivedi 2018). Their role in oxidation–reduction processes in plant cells in response to drought stress has been reported (Ahmad et al. 2016; Min et al. 2016). Taken together, this discussion reveals that detoxification and stress defense genes, including those involved in facilitating water transport across membranes and cellular oxidation–reduction processes constitute a vital drought stress response strategy in maize leaves.

Metabolic pathways significantly enriched under drought stress conditions

Photosynthesis antenna proteins pathway (PAPP) and nitrogen metabolism pathway (NMP) were the most significantly enriched in the tolerant genotype ND476 under drought stress (Fig. 4a). The PAPP is highly sensitive to drought stress (Ghannoum 2009; Zhao et al. 2016b). Photosynthesis antenna proteins, a part of the light harvesting complexes and the electron transport components of the photosystem II (PSII), act as peripheral antenna systems enabling more efficient absorption of light energy (Zhang et al. 2018). They are also involved in the intracellular non-photochemical quenching processes of the plant photosynthesis machinery (Murata et al. 2012). Previously, PAPP has been recognized as a critical pathway in drought stress response (Zhao et al. 2016b; Dudhate et al. 2018; Zenda et al. 2018). Here, the down-regulation of several photosynthesis-related genes in both genotypes implies the high sensitivity of the PSII to drought stress; with drought stress impeding photosynthesis process. The role of NMP in drought stress response has been acknowledged (Zenda et al. 2019). By directly influencing the formation of cellular components and regulation of cellular activities, NMP becomes the most basic and important physiological metabolic process during plant growth. Additionally, NMP is critical in the transformation of photosynthetic products, mineral nutrient absorption, and protein biosynthesis (Ahmadi et al. 2010). Previously, NMP has been implicated in salt stress response (Luo et al. 2018).

In the SD_TD experimental comparison, pathways related to 'ribosome', starch and sucrose' and 'cysteine and methionine metabolism' were the most significantly enriched in response to drought stress (Fig. 4b). The importance of starch and sucrose metabolism pathway in drought stress response in maize has been reported (Jin et al. 2019; Wang et al. 2019b). Starch and sucrose degradation are vital for the provision of cellular energy to help plants tolerate stressful conditions. Ribosomes are the site for protein synthesis, one of the fundamental biological processes that are influenced by drought stress (Shinde et al. 2018). Amino acids cysteine and methionine are essential in protein synthesis; hence the enrichment of these pathways is directly linked with protein biogenesis under drought stress conditions.

Conclusion

We have herein presented the results of our comprehensive comparative analysis of the transcriptomic and physiological responses of two contrasting maize hybrids to drought stress at the V12 growth stage. Phenotypically, the tolerant genotype ND476 had better cell water retention, higher metabolites, and low lipid peroxidation extent than the sensitive

genotype ZX978 under drought conditions. Our RNA-seq results identified total 3114 DEGs were expressed in response to drought, with 21 DEGs being uniquely found in ND476. We found out that genes associated with previously reported pathways involved in drought stress response were altered upon drought-stress exposure, including those associated with secondary metabolites biosynthesis, TF regulation, detoxification and stress defense. Our results enhance our further understanding of the mechanisms regulating drought tolerance in maize, in addition to aiding as foundational base to our future targeted cloning studies.

Author contributions GL, TZ, and HD conceived and designed the experiment; GL, TZ, SL, XW, HJ, AD and YY performed the investigations and collected data; GL, TZ, SL and XW analyzed the data; GL and TZ wrote the original manuscript; All authors made the revision of the manuscript and approved this submission.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. Furthermore, the founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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