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RESEARCH ARTICLE

Tolerance mechanisms in maize identified through phenotyping and transcriptome analysis in response to water deficit stress

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Abstract Water deficit is a key limiting factor for maize (*Zea mays* L.) productivity. Elucidating the molecular regulatory networks of stress tolerance is crucial for genetic enhancement of drought tolerance. Two genotypes of maize contrasting in their yield response to water deficit were evaluated for tolerance traits of water relations, net CO_2 assimilation rate, antioxidative metabolism and grain yield in relation to the expression levels, based on transcription profiling of genes involved in stress signaling, protein processing and energy metabolism to identify functional tolerance mechanisms. In the genotype SNJ201126 upregulation of calcium mediated signaling, plasma membrane and tonoplast intrinsic proteins and the membrane associated transporters contributed to better maintenance of water relations as evident from the higher relative water content and stomatal conductance at seedling and anthesis stages coupled with robust photosynthetic capacity and antioxidative metabolism. Further the protein folding machinery consisting of calnexin/calreticulin (CNX/CRT) cycle was significantly upregulated only in SNJ201126. While the down regulation of genes involved in photosystems and the enzymes of carbon fixation led to the relative susceptibility of genotype HKI161 in terms of reduced net CO_2 assimilation rate, biomass and grain yield. Our results provide new insight into intrinsic functional mechanisms related to tolerance in maize.

Keywords ABA and calcium mediated signaling · Lignin biosynthesis · Maize · Protein folding machinery · Transcriptome · Water deficit stress

Introduction

Drought is one of the most important abiotic stresses limiting maize (*Zea mays* L.) crop yields (Mir et al. 2012; Lobell et al. 2014). The adverse effects of drought on crop yields are likely to be exacerbated by the impending climate change (Feller and Vaseva 2014). Hence improvement of grain yield in maize under harsh environments is an urgent priority to meet the increasing demands for food of the ever increasing population. Although assessing drought tolerance on the basis of yield stability or drought susceptibility index (Sinha et al. 1986) has been a major approach to characterize tolerance, an in depth understanding of the associated metabolic and molecular reprogramming is fundamental for genetic enhancement of stress tolerance. In maize, although the seedling stage is sensitive, anthesis silking interval (ASI) is the most critical stage adversely affected by drought (Bolaòos and Edmeades 1993). Further, the final kernel number at harvest is highly controlled by the leaf area at anthesis in maize (Khanna and Maheswari 1998). Water deficit stress often leads to reduction in plant height, longer ASI and an eventual yield loss by adversely affecting realization of both source and sink potentials in maize. Intrinsic tolerance traits related to turgor homeostasis such as osmotic

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adjustment and leaf turgidity as well as stomatal conductance, transpiration, maintenance of leaf/canopy temperatures and photosynthesis are also negatively impacted by water deficit stress (Shanker et al. 2014; Thirunavukkarasu et al. 2014; Maheswari et al. 2016) in crops. On the other hand, anti-oxidative detoxification, secondary metabolism and senescence are known to be triggered in response to water deficit stress (Xie et al. 2019).

A multitude of signaling molecules such as intracellular Ca^{+2}, abscisic acid (ABA) and reactive oxygen species (ROS) are important for drought signal transduction (Xiong et al. 2002). ABA perception and signaling pathway includes three core components of receptors (PYR/PYL/RCAR), protein phosphatases (PP2C) and protein kinases (SNRK2/OST1) (Zhu 2016; Mega et al. 2019). Mitogen activated protein kinases (MAPKases) are also involved in the signaling of multiple stresses through phosphorylation of downstream signaling targets including enzymes, proteins and transcription factors (TFs) (Bigeard and Hirt 2018).

Endoplasmic Reticulum (ER) mediated protein processing is known to affect signaling response in plants (Beaugelin et al. 2020). Chloroplastic retrograde signaling also involves endoplasmic reticulum (Walley et al. 2015; de Souza et al. 2017). ER is also known to mediate Unfolded Protein Response (UPR). Signaling pathways during stress and changes in these UPR pathways is known to affect abiotic stress tolerance in plants (Fu et al. 2016).

The downstream transcriptional regulatory networks are activated by several TFs families, such as MYB, NAC, bZIP and ERF. Functional gene regulation involves a myriad of genes being up or down regulated resulting in the complex adaptive mechanisms which coupled with epigenetic plasticity manifests in ultimate phenotypic expression of tolerance or susceptibility. With the development of next generation sequencing tools, RNA sequencing has remarkably aided in transcriptome analysis of drought stress response in plants (Miao et al. 2017; Zenda et al. 2019).

In this context, the present study was aimed at deciphering the major mechanisms related to intrinsic tolerance traits of water relations, protein processing and energy metabolism in two contrasting genotypes of maize differing in their yield under water limited environment. The adaptive response to water deficit is intricate in plants and the rapidity and intensity of stress development are also influenced by the growth environment. In order to decipher the stress effects clearly as well as to effectively integrate the responses elicited at different levels, experiments were undertaken in field conditions as well as pot culture. Further, the expression levels of genes involved in major functional mechanisms were examined along with the corresponding phenotypic expressions to identify crucial metabolic functions to cope with water deficit stress.

Materials and methods

Plant material

Two contrasting genotypes of maize, SNJ201126, one of the high yielding and drought tolerant genotypes and HKI161, one of the drought sensitive but moderate yielder identified based on previous study (Maheswari et al. 2016) were used. The two genotypes also are from diverse sources in India viz., Indian Institute of Maize Research, Ludhiana (HKI161) and National Bureau of Plant Genetic Resources, Regional station, Hyderabad (SNJ201126).

Experiment 1: Phenotyping of the maize genotypes under field conditions

Location

Field experiments were conducted in the crossing block area, Central Research Institute for Dryland Agriculture (CRIDA), located between 17.20° N latitude and 78.30° E longitude, Hyderabad, Telangana, India, during post rainy seasons of 2015–16, 2016–17 and 2017–18. Physical and chemical properties of soil of experimental field were same as in Maheswari et al. 2016.

Experimental design and treatment details

The genotypes were sown in a randomized complete block design (RCBD) with three replications at a plot size of 4 rows of 2 m length having row to row spacing of 60 cm and plant to plant spacing of 25 cm for three seasons. In each season, the genotypes were grown under two different water regimes i.e., well-watered (WW) and water deficit stress (WD). Under well-watered conditions, plants were watered at regular intervals in order to maintain the plants in stress free condition and in water deficit stress treatment the plants were watered at regular intervals till the anthesis silking interval (ASI) stage and subsequently were exposed to stress by withdrawing water for a period of 7 days and were then allowed to grow normally till maturity. The recommended dose of fertilizers i.e. 60 kg N ha^{-1} and 60 kg P ha^{-1} as diammonium phosphate, 30 kg K ha^{-1} as muriate of potash (MOP) was applied as basal dose; second dose of 30 kg N ha^{-1} at vegetative stage i.e. 30 days after sowing (DAS) and third dose of 30 kg N ha^{-1} as urea and 30 kg K ha^{-1} as MOP was top dressed at 50 DAS. The crop was maintained pest and disease free with regular plant protection measures. The weekly average minimum

and maximum temperature recorded during the dry seasons ranged from 11.2 to 32.8 °C and the relative humidity varied from 32.9 to 79.6%. Total rainfall received during crop growth period of post rainy seasons of 2016–17 and 2017–18 was 9.2 and 6.0 mm respectively while there were no rain events in 2015–16 (Supplementary Fig. 1). Soil moisture content at the depth of 0–15 cm was measured gravimetrically for WW and WD stress plots at stress point in each season and mentioned in Table 1.

The various physiological traits such as relative water content (RWC), net CO_2 assimilation rate (AN), transpiration rate (TR), stomatal conductance to water vapour (g_s), leaf temperature (LT), canopy temperature (CT), SPAD chlorophyll meter reading (SCMR), normalized difference vegetation index (NDVI) were recorded in all the three seasons at stress point in both the treatments. All the traits were recorded in the flag leaf.

RWC was determined in the leaf tissue according to Bars and Weatherly (1962). The stomatal conductance to water vapour, transpiration, net CO_2 assimilation rate and leaf temperature were measured between 1000 and 1100 h by using LI-6400 portable photosynthesis system (LI-COR, Inc. Lincoln, USA). Canopy temperature was recorded by IR-Thermometer, Fluke/568. Leaf chlorophyll content was recorded using SPAD-502, Minolta, Tokyo, Japan. NDVI was recorded using Trimble Green Seeker handheld crop sensor and calculated in as NDVI = NIR − RED/NIR + RED where NIR = reflection in the near-infrared spectrum, RED—reflection in the red range of the spectrum. Yield parameters i.e., seed yield, hundred seed weight and total seed number per cob on three representative plants of each genotype were also recorded.

Experiment 2: Phenotyping under greenhouse conditions in pot culture at anthesis silking stage

The seeds of genotypes were sown one in each pot of upper diameter 30 cm lower diameter 23 cm and a height of 25 cm filled with 16 kg of sandy loam soil mix (soil, sand and farm yard manure in a ratio of 3:1:1) in green house. The plants were irrigated with normal tap water and grown under natural illumination ($\sim$ 1500 µmol m^{-2} s^{-1}) with a 14 h photoperiod. The mean air temperature during the experiment was 30 °C and the relative humidity ranged between 45 and 55% at 14.00 h. A basal dose of 0.54 g per pot, both N and P were supplied before sowing. There were three replications and two treatments, well-watered and water-deficit stress. Water-deficit was imposed at ASI stage (60DAS) by withholding water for a period of 5 days. Soil moisture content was recorded using gravimetric method. RWC, SCMR, AN, TR, g_s and LT were recorded in flag leaf as described in experiment 1. The experiments were repeated thrice.

Table 1 Soil moisture content of experimental field in different seasons

Season	Data availability	
	Well-watered	Water deficit stress
Post rainy season 2015–16	11.66 ± 0.38	7.89 ± 0.17
Post rainy season 2016–17	11.25 ± 0.39	8.22 ± 0.41
Post rainy season 2017–18	13.94 ± 0.19	8.13 ± 0.32

Experiment 3: Phenotyping under greenhouse conditions at seedling stage

Four seeds of each genotype were sown in plastic boxes of size 24 × 17 × 10 cm, filled with 4 kg sandy loam soil (soil, sand and farm yard manure in a ratio of 3:1:1) under controlled conditions for phenotyping at 3 leaf seedling stage. There were three replications and two treatments, well-watered and water-deficit stress. Plants were raised in the same conditions as described in experiment 2. Fifteen days old seedlings were subjected to water deficit stress by withdrawing water for 3 days. The relative water content and activities of antioxidative enzymes (AOX) such as superoxide dismutase (SOD), catalase (CAT), Guiacol peroxidase (GPOx) and glutathione reductase (GR) were measured under both treatments. The antioxidative enzymes SOD were analyzed according to Dhindsa et al. (1981), catalase by Claiborne (1985), GPOx by Chance and Machly (1955) and GR by Smith et al. (1988). The experiments were repeated thrice.

Leaf tissue from the above seedlings of both the genotypes and treatments were harvested for RNA isolation and transcriptome analysis. Each treatment is represented by a composite of three individual replicates per genotype resulting in four samples which are originally from 12 samples in total.

Statistical analysis

Analysis of variance (ANOVA) was carried out for the physiological and yield traits in the experiments as per Snedecor and Cochran (1967).

Total RNA extraction and quality check

Total RNA was extracted from leaves of HKI161 and SNJ201126 genotypes from both well-watered (1C = HKI161 well-watered, 2C = SNJ201126 well-watered) and water stressed (1S = HKI161 water deficit stress, 2S = SNJ201126 water deficit stress) samples using QIAGEN

RNeasy Plant Mini Kit according to manufacturer's instructions. Ribosomal RNA was removed from total RNA by using Ribo Zero Magnetic kit and Agencourt RNA clean XP kit. RNA was treated with RNase free DNase I to remove any possible DNA. Total RNA integrity was confirmed using 1% agarose gel electrophoresis and RNA concentration was determined using Nanodrop 1000 (Thermo Fisher Scientific Inc., USA). RNA samples with RNA integrity number (RIN) values > 7.5 (1C—7.6, 2C—8.0, 1S—7.9, 2S—8.2) was used for RNA-seq transcriptome analysis.

cDNA library construction and sequencing

PolyA-enriched cDNA library was prepared using TruSeq stranded total RNA sample preparation kit v2 according to manufacturer's instructions. The quality of cDNA libraries was tested using Agilent 2100 bioanalyzer and quantified cDNA was subjected to purification using AMPpure XP beads. Purified cDNA was end repaired using 3′ to 5′ exonuclease activity of end repair mix which removed 3′ overhangs and filled 5′ overhangs through its polymerase activity. cDNA was then polyadenylated and multiple indexing adapters were ligated to its end. The established cDNA libraries were sequenced using Illumina Hiseq2500 platform at Scigenom Private Ltd., Cochin, India to obtain paired-end reads of 100 base pairs.

Sequence analysis

Raw Fastq files obtained from the sequencer were checked for base quality score distribution (Phred quality score) average base content per read and GC distribution in the reads. The raw Fastq files were trimmed to remove low quality bases and adaptor sequences using AdapterRemoval-v2 (version 2.2.0). The ribosomal RNA sequences were removed by aligning reads with silva database using bowtie2 (version 2.2.6) and subsequent workflow using samtools (version 0.1.19), sambamba (version 0.6.5), BamUtil (version 1.0.13) tools and scripts developed at Scigenom Private Ltd., Cochin.

Differential expression analysis

The pre-processed and rRNA removed reads were aligned to maize genome and gene model downloaded from Ensembl Plants (ftp://ftp.ensemblgenomes.org/pub/plants/release-34/fasta/zea_mays/dna/Zea_mays.AGPv4.dna.toplevel.fa.gz). The alignment was performed using STAR program (version 2.5.2b). After aligning reads with reference genome, aligned reads were used for estimating expression of genes and transcripts using cufflinks program (version 2.2.1). Differential expression analysis was performed by cuffdiff program of cufflinks package using the FPKM (fragments per kilo base of transcript per million fragments mapped) of each gene. Genes with log2-fold change ≥ 2 and p value cutoff ≤ 0.05 were assigned as differentially expressed. Edward plots were plotted to elucidate comparison of contigs in all samples, both for up and down regulated genes. The sequencing data was deposited in NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) with accession number SRP133547 (https://www.ncbi.nlm.nih.gov/sra/?term=SRP133547).

Gene ontology (GO) enrichment and pathway analysis

GO term enrichment analysis of DEGs was performed by *AgriGo* using singular enrichment analysis (SEA) with *Zea mays AGP3.30* as the reference genome background with web server analysis tools (http://bioinfo.cau.edu.cn/agrigo). GO terms with p value ≤ 0.05 and FDR ≤ 0.1 were considered significantly enriched in our study. Transcription factor enrichment analysis was done using the plant transcriptional regulatory map tools (http://plantregmap.cbi.pku.edu.cn/tf_enrichment.php). KEGG pathway analysis of DEGs was performed using KEGG Automatic Annotation Server Ver. 2.1 web server (https://www.genome.jp/kaas-bin).

Validation of DEG expression profiles using quantitative real time PCR (qRT-PCR)

To further validate the results of our RNA-Seq data, quantitative real time PCR analysis was conducted using specific primers designed for the 8 DEGs of the major pathways (Supplementary table 1) that were identified by RNA-Seq. Gene-specific primers were designed using Primer-blast of NCBI. GAPDH was used as endogenous control. Total RNA was isolated from HKI161 and SNJ201126 genotypes from both well-watered and water stressed samples using the RNA isolation kit from GCC Biotech (Cat No: GR 1004A) according to the manufacturer's instructions. The RNA samples were subjected to DNase treatment and quantified using Biophotomer Plus, Eppendorf. About 1 µg of RNA was taken and used for the synthesis of cDNA using Takara Prime Script 1st strand cDNA synthesis kit (Cat No 6110A). qRT-PCR was carried out in the Qiagen Rotor-Gene Q 5 Plex HRM according to the manufacturer's instructions. Each PCR reaction contained 1 µl (25 ng) of cDNA, 1 µl each of 10 pm of forward and reverse primer, 10 µl TB Green Premix Ex Taq II (Takara) and the final volume made upto 20 µl with the reactions being performed in triplicate. The thermal cycling conditions were 95 °C for 1 min, followed by 45

cycles of 5 s at 95 °C and 30 s at 60 °C. Relative mRNA abundance in the samples was calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Results

Phenotypic and physiological assessment of two maize genotypes HKI161 and SNJ201126 in response to water deficit stress

The response mechanisms of the two contrasting genotypes were characterized under field conditions for three seasons at ASI stage (experiment1) and under greenhouse conditions in pots at ASI stage (experiment 2) as well as seedling stage (experiment 3).

Under field conditions, the relative water content decreased in response to water deficit stress at ASI stage in both the genotypes however the reduction in RWC was more severe in HKI161 compared to SNJ201126 over all seasons. Net CO_2 assimilation rate, transpiration and stomatal conductance to water vapour were also reduced more in HKI161 as compared to SNJ201126 in response to WD stress. The canopy and leaf temperatures under stress conditions were high in both genotypes and increase in temperatures was higher in HKI161 (Table 2). Genotype SNJ201126 maintained higher chlorophyll content and NDVI compared to HKI161 under water deficit stress condition. The seed yield, hundred seed weight and seed number per cob of both genotypes were affected under water deficit stress compared to well-watered conditions. However, the reduction in total seed yield as well as seed size in SNJ201126 was lower compared to HKI161.

The pooled analysis of variance of the physiological and yield traits under well-watered and water deficit stress conditions across seasons revealed significant variances due to treatment and season × treatment for all the traits. Significant variances due to season and genotype were found for all traits except SPAD and NDVI respectively. Variances due to season × genotypes were recorded for all traits except RWC and SPAD chlorophyll meter reading (SPAD), while variances due to genotype × treatment were recorded for all traits except canopy temperature, stomatal conductance and NDVI. Interactions due to season × genotype × treatment were significant for RWC, net CO_2 assimilation rate and SPAD (Table 2).

Also, significant variances due to season, genotype, treatment and season × genotype for seed yield, hundred seed weight and seed number per cob under well-watered and water deficit stress across seasons were observed. While significant season × treatment interaction was observed for seed yield and hundred seed weight and season × genotype × treatment interaction was observed for seed yield (Table 2).

Under pot culture conditions, the soil moisture percentage in WW treatment was 15.25 and 16.82% and WD stress treatment was 9.87 and 7.22% in HKI161 and SNJ201126 genotypes respectively. The percent reduction in RWC under WD stress as compared to WW conditions was less in SNJ201126 (14.9%) than HKI161 (23.9%) (Fig. 1a). Similarly, SCMR, AN, g_s and TR decreased under WD stress conditions in both the genotypes although the decrease was less in SNJ201126 (Fig. 1b–e). The increase in leaf temperature under WD stress was higher in HKI161 compared SNJ201126 (Fig. 1f).

The response mechanisms were also analyzed in another experiment under controlled conditions at three leaf seedling stage. Genotypic differences at seedling, ASI stage as well as in the field level yielded similar results. The soil moisture content in the WW pots of HKI161 and SNJ201126 was 16.83 and 17.13%, while in case of WD stress pots it was 11.12 and 11.43% respectively. Under WW conditions RWC in HKI161 and SNJ201126 was 91.96 and 94.17% while in case of WD stress conditions it was 73.55 and 77.42% respectively. The results on various antioxidative enzyme activities showed that the activity of SOD was higher in both SNJ201126 and HKI161 genotypes under WD stress conditions but the percentage increase in SNJ201126 (25.9%) was higher compared to HKI161 (16.9%) (Table 3). The levels of enzyme catalase were increased in both genotypes under stress conditions (Table 3). The percentage increase in CAT was significantly higher in SNJ201126 (133.5%) compared to HKI161 (51.4%). Although, there was increase in the activity of GPOx in both genotypes under stress conditions, the differences were not significant (Table 3). Glutathione reductase activity was higher in both genotypes but the percent increase in SNJ201126 (37.9%) was higher compared to HKI161 (29.3%) (Table 3).

Analysis of differentially expressed genes of water deficit stress transcriptome in maize

RNA seq analysis of the genotypes HKI161 and SNJ201126 resulted in a total of 299.18 million clean reads with an average of 74.8 million reads per sample after filtering, removing the adapter sequences and rRNA. The highest number of reads 8.94 and 7.83 million under water deficit stress and well-watered conditions respectively were obtained in SNJ201126 while, 7.69 and 5.46 million reads were obtained in HKI161 under well-watered and water deficit stress conditions respectively. The mapping statistics of both the genotypes under well-watered and water deficit stress conditions are given in Table 4. The average Q30 score of all libraries was 95.1% with a minimum of

Table 2 Physiological and yield related traits in two genotypes of maize evaluated under well-watered and water deficit stress conditions for three seasons

Genotype	RWC (%)	AN (μmol Co_2m^{-2} s^{-1})	TR (m mol H_2om^{-2} s^{-1})	g_s (μ mol m^{-2} s^{-1})	LT (°C)	CT (°C)	SCMR	NDVI (%)	SY (g)	100 seed weight (g)	Seed number
HKI161-WW	92.38	39.25	11.08	0.62	31.61	29.99	49.44	0.76	46.09	24.14	274
HKI161-WD	70.41	13.72	5.52	0.24	33.37	34.1	41.56	0.65	25.67	17.07	161
SNJ201126-WW	92.71	47.37	12.16	0.65	30.08	28.71	51.1	0.74	64.27	27.5	341
SNJ201126-WS	78.15	26.41	8.12	0.32	30.27	31.88	48.57	0.67	44.53	20.78	223
Source											
Season (S)	8.30**	101.24**	36.89**	0.013**	23.95**	21.65**	2.26	0.017**	20.79**	97.66**	2549.45*
Genotypes (G)	146.28**	973.64**	30.47**	0.02**	9.58**	27.56**	169.00**	0.0004	2983.75**	119.66**	33,024.83**
Treatment (T)	3002.85**	4862.27**	207.07**	1.12**	10.78**	119.17**	414.80**	0.076**	3594.10**	400.91**	124,486.78**
Season × genotype (S × G)	2.69	9.48**	3.76**	0.006**	8.71**	11.89**	1.01	0.0059**	17.96**	6.40**	6099.28**
Season × treatment (S × T)	18.71**	27.57**	17.36**	0.004*	1.80**	9.67**	16.06**	0.032**	151.15**	11.96**	878.85
Genotype × treatment (G × T)	123.17**	47.10**	5.24**	0.0038	4.09**	2.01	10.89*	0.002	0.42	0.91	293.19
Season × genotype × treatment (S × G × T)	10.15**	114.18**	0.41	0.0004	1.02	0.52	30.93**	0.002	67.47**	0.23	134.43

Values are means of three seasons

*, ** represent values significant at 5% and 1% level respectively

WW, well-watered; WD, water deficit; RWC, relative water content; AN, net CO2 assimilation rate; TR, transpiration; gs, stomatal conductance to water vapour; LT, leaf temperature; CT, canopy temperature; SCMR, SPAD chlorophyll meter reading; NDVI, normalized difference vegetative index; SY, seed yield

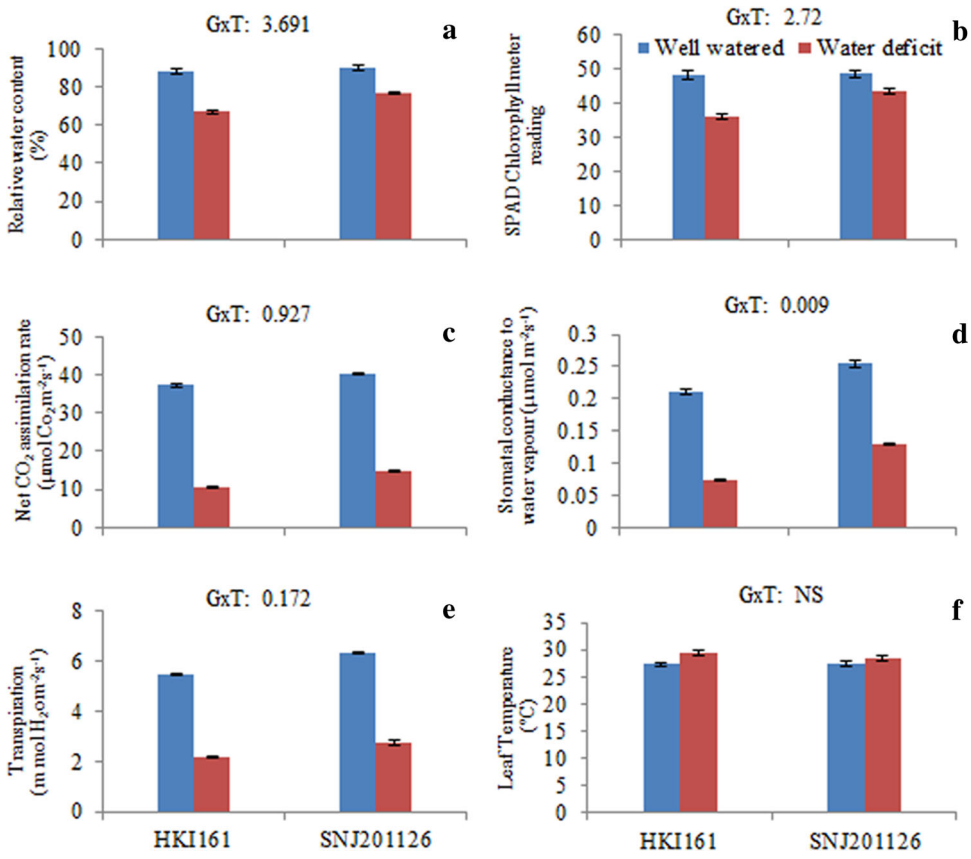

Fig. 1 Effect of water deficit stress on **a** relative water content, **b** SPAD chlorophyll meter reading, **c** Net CO_2 assimilation rate, **d** stomatal conductance, **e** transpiration and **f** leaf temperature in the two genotypes of maize at anthesis silking stage in pot culture results are means ± SE (n = 6); Values given in the chart are critical difference, (ANOVA), ($P \leq 0.05$); G × T indicate interaction between genotype and treatment.

Table 3 Effect of water deficit stress on antioxidative enzyme activities viz. superoxide dismutase (SOD), catalase (CAT), Glutahione peroxidase (GPOx) and Glutathione reductase (GR) activities in 15-day old seedlings of two genotypes of maize in pot culture

Genotype	Δ RWC_{leaf} at Water deficit	Treatment	SOD	CAT	GPOX	GR
HKI161	18.41	Well-watered	35.7 ± 0.4	24.2 ± 0.6	21 ± 0.3	29.7 ± 0.5
HKI161		Well deficit	41.7 ± 0.5	36.6 ± 0.6	32.7 ± 0.4	38.4 ± 0.5
SN201126	16.75	Well-watered	39 ± 0.4	28.1 ± 0.5	25.1 ± 0.4	31.7 ± 0.4
SN201126		Water deficit	49.1 ± 1.2	65.6 ± 0.9	37.1 ± 0.8	43.8 ± 0.9
CV%			4.35	4.35	4.04	4.04
CD G			1.565	1.462	1.019	1.262
CD T			1.565	1.462	1.019	1.262
CD GxT			2.214	2.067	NS	1.785

Results are means ± SE (n = 6)

Δ RWC_{leaf} at Water deficit represents the difference in RWC between well-watered and water deficit treatments

94.2% indicating reliable result of sequencing. Under water deficit stress 97.98% and 94.25% and well-watered conditions 94.36% and 96.93% reads were mapped to the reference genome (ftp://ftp.ensemblgenomes.org/pub/plants/release-34/fasta/zea_mays/dna/Zea_mays.AGPv4.dna.toplevel.fa.gz) in HKI161 and SNJ201126 respectively with STAR program (version 2.5.2b) having default parameters, indicating sufficient coverage of the genome. In order to understand the transcriptional variations occurring in response to water deficit stress in both the genotypes the differentially expressed genes (DEGs) were analysed. Comparisons were made in the following four groups viz. 1C vs 1S, 2C vs 2S, 1C vs 2C and 1S vs 2S for both upregulated and down regulated DEGs.

The dynamic analysis for functional gene ontology (GO) annotation in response to water deficit stress

There were 165 common water deficit stress responsive genes for the genotypes HKI161 and SNJ201126. The

Table 4 Mapping statistics of transcriptome sequencing data of genotypes HKI161 and SNJ201126 under well-watered (WW) and water deficit stress (WD) conditions

Particulars	HKI161		SNJ201126	
	WW	WD	WW	WD
No. of bases (Gb)	7.89	5.63	8.22	9.82
Number of paired end reads	3,94,43,152	2,81,29,327	4,10,96,681	4,90,75,723
Total reads	7,88,86,304	5,62,58,654	8,21,93,362	9,81,51,446
Trimmed reads	7,68,87,964	5,46,37,110	7,82,81,594	8,93,74,236
Aligned read count	7,25,52,000	5,29,59,306	7,67,03,193	8,42,31,181
Q30 percentage	96.4	94.7	95.2	94.2
Aligned percentage	94.36	96.93	97.98	94.25
GC content	43.56	44.89	43.93	46.09
Total no. of transcripts	139,122			
Gene number	44,474			
Transcripts with length $\geq$ 1000 bp	111,415			
Min transcript length (bp)	32			
Average transcript length (bp)	2544			
N50 transcript length (bp)	3612			
Longest transcript length(bp)	16,233			

GB, giga byte; bp, base pair

functions of these common genes were mostly related to stress response (Table 5). Out of these 125 were upregulated and 40 downregulated. The up regulated genes were mostly responsive to various kinds of stress stimuli, water deprivation, abscisic acid while in case of down regulation the responsive genes were related to carbohydrate metabolic process and cellular carbohydrate metabolic process. These results revealed the existence of conservative stress-induced regulation pathways between the two genotypes.

Apart from the commonly expressed genes, in the genotype HKI161, the stress responsive genes enriched in biological process GO terms were "metabolic process", "secondary metabolic process", "small molecule metabolic process", "oxidation reduction" and molecular function GO terms "catalytic activity", oxidoreductase activity (Fig. 2a). While in SNJ201126, the stress responsive genes enriched in biological processes were "response to biotic stimulus", "cell wall organization or biogenesis", "cell wall macromolecule metabolic process", "cellular cell wall organization or biogenesis", "multi-organism process", "response to other organism", "plant type cell wall organization or biogenesis" and cellular component GO term "extracellular region" (Fig. 2b).

Further analysis of the enriched GO terms for identification of regulation revealed that SNJ201126 specific responsive genes were involved in regulation systems, responsive to water deficit stress. In SNJ201126, 86.83%, 90.48%, 87.16%, 85.90%, 87.5%, 85.37%, 86.84%, 81.58% and 83.33% of the stress responsive genes showed up-regulation under water deficit in the GO terms "response to stimulus", "response to stress", "response to chemical stimulus", "response to abiotic stimulus", "response to biotic stimulus", "multi-organism process", "response to other organism", "response to endogenous stimulus" and "extra cellular region" respectively. However, this regulation was not observed in the HKI161 specific stress responsive genes, with approximately equal number of up-regulated and down-regulated genes under each GO term (Fig. 2c, d).

Based on the overlapping of DEGs, water deficit stress responsive genes could be classified into two groups: (I) genotype-specific unique responsive genes and (II)

Table 5 Gene ontology (GO) enrichment analysis of common water deficit stress responsive genes in maize genotypes HKI161 and SNJ201126

GO term	Biological process description	Number of genes	*p* value	FDR
GO:0050896	Response to stimulus	50	3.20E−13	1.20E−10
GO:0042221	Response to chemical stimulus	38	2.30E−13	1.20E−10
GO:0006950	Response to stress	35	2.80E−10	7.00E−08
GO:0009628	Response to abiotic stimulus	26	2.80E−09	4.10E−07
GO:0009719	Response to endogenous stimulus	17	7.90E−07	4.90E−05

FDR, false discovery rate

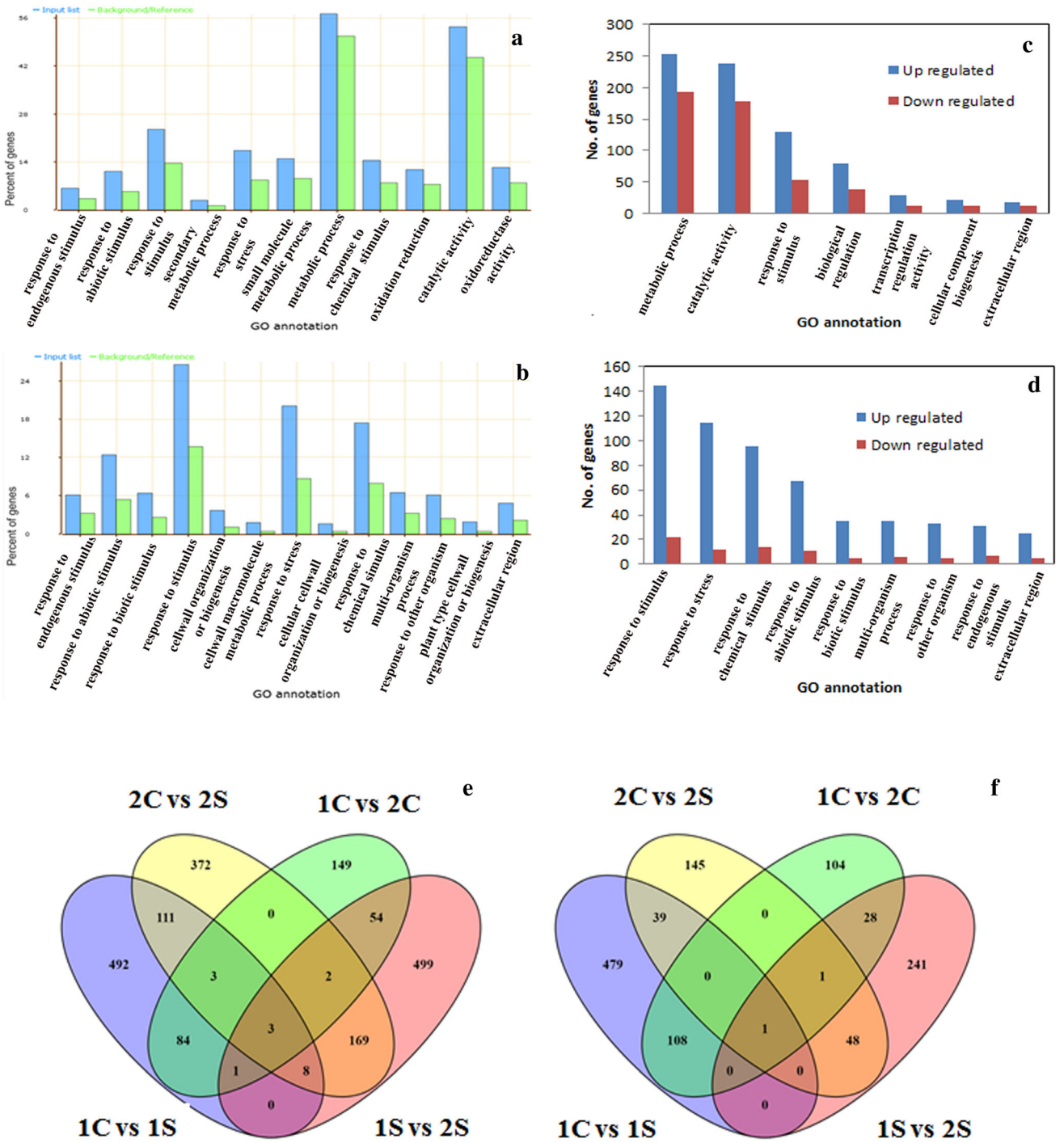

Fig. 2 Gene ontology enrichment analysis of genotype-specific responsive genes in **a** 1C versus 1S and **b** 2C versus 2S. The numbers of genes upregulated and down regulated of each category in **c** 1C versus 1S and **d** 2C versus 2S. The number and overlap of differentially expressed genes **e** upregulated, **f** down regulated in four comparing groups: 1C versus 1S, 2C versus 2S, 1C versus 2C and 1S versus 2S where 1: HKI161, 2: SNJ201126, C: well-watered, S: water deficit stress

common water deficit stress responsive genes expressed by both genotypes. For the genotype-specific responsive genes, there were 492 upregulated genes that specifically responded to water deficit in HKI161 while there were 372 genes uniquely responded to water deficit in SNJ201126. There were 125 common water deficit responsive genes in HKI161 and SNJ201126 (Fig. 2e). Among the down regulated genes, there were 145 genes uniquely responded to water deficit in SNJ201126, while there were 479 genes that specifically responded to water deficit in HKI161.

There were 40 common water deficit responsive genes in HKI161 and SNJ201126 (Fig. 2f).

While the comparison of DEGs under WW (1C vs 2C) and WD conditions (1S vs 2S) revealed uniquely expressed 149 and 104 up and down regulated genes respectively under 1C vs 2C and 499 and 241 uniquely expressed up and down regulated genes respectively under 1S vs 2S.

Pathway analysis for drought tolerance

A total of 266 and 239 up regulated genes, 211 and 75 down regulated genes could be annotated by KEGG pathway in HKI161 and SNJ201126 genotypes respectively. The major up and down regulated pathways are given in Supplementary Table 2a, b. The number of pathways affected by water deficit stress was more in HKI161 as compared to SNJ201126. In the genotype HKI161, upregulated genes were involved in pathways of phenyl propanoid biosynthesis (KO00940), MAPK signaling pathway—plant (KO04016) and plant hormone signal transduction (KO04075). The pathways that were significantly down regulated were glycolysis / gluconeogenesis (KO00010), photosynthesis (KO00195), carbon fixation in photosynthetic organisms (KO00710), starch and sucrose metabolism (KO00500) and purine metabolism (KO00230).

While, in the genotype SNJ201126, major pathways which were upregulated were protein processing in endoplasmic reticulum (KO04141), MAPK signaling pathway – plant (KO04016) and amino sugar and nucleotide sugar metabolism (KO00520). Downregulated genes were significantly over represented in pathways of starch and sucrose metabolism (KO00500), glycolysis / gluconeogenesis (KO00010), glycine, serine and threonine metabolism (KO00260), methane metabolism (KO00680) and purine metabolism (KO00230).

Efficient regulation of stress signal perception and transduction is involved in tolerance to water deficit

The phytohormone abscisic acid (ABA) accumulated in response to water deficit stress was evident by the genes encoding enzymes of ABA biosynthesis pathway in both the genotypes. Genotype HKI161 showed upregulation of nine cis-epoxy carotenoid dioxygenase (NCED) genes, *NCED 6* and *NCED 8* with a log2 fold change of 2.2 and 3.73 respectively while in case of SNJ201126, *NCED 5* was upregulated with a log2 fold change of 3.4 indicating an active ABA induced pathway in both the genotypes (Supplementary Table 3a).

Several genes of Ca^{2+} signaling related genes were upregulated in both the genotypes indicating changes in Ca^{2+} signals. The commonly upregulated genes in both the genotypes were Calmodulin binding protein, Calcium-binding protein CML42, Calcium-transporting ATPase and Calcium-binding EF-hand family protein. Apart from these in the genotype HKI161 Calmodulin-binding transcription activator 2 was upregulated while in SNJ201126 Calcium-binding proteins CML38, 27, 45, Calmodulin-like protein 2, Calmodulin-binding protein 60 G, Calcium-transporting ATPase 9 Plasma membrane-type and Plant calmodulin-binding related-protein were upregulated (Supplementary Table 3b). With the changes in Ca^{+} signals, the various signaling component genes activated in the MAPK pathway in HKI161 were putative WRKY DNA-binding domain superfamily protein (WRKY33), Pathogenesis related protein 1 (PR1), Chitinase B (chiB), 2C-type protein phosphatase (PP2C), Serine/threonine-protein kinase SRK2E (SnRK2), Mitogen activated protein kinase kinase 1/2 (MAPK 1/2), Calmodulin-binding protein 60 G (CAM4), Respiratory burst oxidase protein D (RboHD) and Mitogen-activated protein kinase kinase kinase YODA (YODA) while in case of SNJ201126, LRR receptor-like serine/threonine-protein kinase FLS2 (FSL2), Mitogen activated protein kinase 1/2 (MKK1/2), PR1, ETHYLENE INSENSITIVE 3-like 2 protein (EIN3/EIL), ChiB, Mitogen activated protein kinase kinase 2 (MKK2), Abscisic acid receptor PYL9 (PYR/PYL), SnRK2 and CAM4 genes were upregulated. The expression levels of these genes were low under well-watered conditions but under water deficit the levels increased indicating their stress inducibility. In case of HKI161, the end products of the signaling were upregulated, while in case of SNJ201126, the signaling components were upregulated (Supplementary Table 3c).

Membrane transporters and drought tolerance

The genes encoding for ATP-binding cassette (ABC) transporters involved in the primary transfer of ABA into the guard cells transporters were upregulated in both the genotypes. In the genotype SNJ201126, ABC transporter A family member 7, ABC transporter G family member 40 and ABC transporter B family member 19 were upregulated while in HKI161, ABC transporter G family member 40 and multidrug resistance protein ABC transporter family protein were upregulated. Secondary ABA transporter protein NRT1/ PTR FAMILY 2.9 and 6.2 were upregulated in SNJ201126 while in HKI161 high affinity nitrate transporter 2.7 and nitrate transport 4 were upregulated (Supplementary Table 3d).

Aquaporins of the category tonoplast intrinsic protein (TIP) 4 which mediate the outflow of water resulting in reduction of guard cell turgor and stomatal closure were upregulated in genotype SNJ201126, while in the genotype HKI161 Plasma membrane intrinsic protein (PIP2) and

TIP1 were upregulated. The expression levels of these transporters were higher in SNJ201126 compared to HKI161.

Also in the genotype SNJ201126 transporters involved in the transport of various osmolytes such as sugars, sugar alcohols (polyols and cyclitols), amino acids and quaternary ammonium compounds have been upregulated i.e., arabinose-proton symporter/putative polyol transporter 4, putative polyol transporter 1, amino acid transport protein (ANTL2), nucleotide/sugar transporter family protein and ammonium transporter were upregulated (Supplementary Table 3d).

The regulation of protein processing in endoplasmic reticulum is closely related to the tolerance to water deficit

The number of genes in protein processing in endoplasmic reticulum pathway that have been up regulated in the genotypes SNJ201126 and HKI161 was 11 and 6 respectively. The genes involved in the protein translocation complex, Protein transport protein Sec 61 subunit beta (Sec16A), Heat shock 70 kDa protein 17 (HSP70), Heat shock protein 26 (NEF) and Heat shock factor protein HSF30 (ER-sHSF) and endoplasmin like protein (GRP94) were commonly expressed in both the genotypes. However, the genes of calnexin / calreticulin protein folding machinery (CNX-CRT) calnexin homolog 2 (CNX), calreticulin-2 (CRT), protein disulfide isomerase 1 (PDIs) and DNAJ heat shock N-terminal domain-containing protein (HSP40) have been upregulated exclusively in SNJ201126 only. The levels of the commonly expressed genes were inherently high in the genotype SNJ201126 whereas in HKI161 genotype they were upregulated only in response to stress (Supplementary Table 3e). One of the heat shock transcription factor (ER-sHSF) was induced under stress in both the genotypes but the level of expression was almost three times in SNJ201126 compared to HKI161.

ROS scavenging

The enzymes involved in ROS scavenging upregulated in HKI161 were Peroxidase, Glutathione S-transferase (GST) U6, GST U10 glutathione transferase (GT) 41, and Putative L-Ascorbate Peroxidase 6 while in case of SNJ201126 the genes upregulated were Peroxidase, GST T1, GT19, GT25 and Flavone synthase type 12. These genes were induced upon exposure to stress only and not expressed under well-watered conditions in HKI161 however in case of SNJ201126 they were inherently expressed under unstressed conditions and upregulated in response to stress (Supplementary Table 3f).

Differential expression profiles of transcription factors associated with drought tolerance in the tolerant and susceptible lines

In the genotype HKI161, the number of transcription factors upregulated was 27 while in case of SNJ201126 it was 15. In the genotype HKI161, the major TF family upregulated was ethylene responsive factor (ERF) gene family in which 10 genes were upregulated i.e., ERF domain protein 9, ERF domain protein 11, Ethylene response factor 7, Ethylene response factor 73, Ethylene responsive element binding factor 1, Ethylene-responsive element binding factor 13, Ethylene responsive element binding factor 4, DREB subfamily A-4 of ERF/AP2 transcription, AP24 related, DREB and EAR motif protein 3. Three transcription factors belonging to MYB group, myb domain protein 19 myb domain protein 86 and myb domain protein 96 were also upregulated (Supplementary Table 4a). The down regulated transcription factors belonged to GRAS family protein, NAC domain containing protein 36, MIKC_MADS family protein, AGAMOUS-like 12 and myb domain protein 5 respectively (Supplementary Table 4b).

In the genotype SNJ201126 about 15 transcription factors were upregulated. Of these the major family belonged to NAC and MYB families in which 4 genes each were upregulated i.e., NAC domain containing protein 47, NAC family protein, NAC domain containing protein 83 and NAC domain containing protein 100, myb domain protein 63, myb domain protein 86, myb domain protein 7 and MYB related family protein (Supplementary Table 4c). The down regulated TFs in SNJ201126 were bHLH family protein, ERF family protein, redox responsive transcription factor 1, RAD-like 6, MYB_related family protein, B-box type zinc finger protein with CCT domain, MIKC_MADS family protein and Nin-like family protein (Supplementary Table 4d). The common transcription factors upregulated in both the genotypes were G2-like family protein and related to AP24, while MIKC_MADS family protein was down regulated in both. The expression levels of commonly expressed TFs were higher in the genotype SNJ201126 compared to HKI161 (Supplementary Table 4e).

Water deficit stress response may be related to the regulation of photosynthesis pathway and glycolysis

The different responses of the two genotypes to water deficit stress were also observed in photosynthesis. Six genes belonging to the biological process of photosynthesis were down regulated in the genotype HKI161, while in case of SNJ201126 the process remained unaffected. In the

genotype HKI161 the genes *PsbM*, *PsbQ* encoding different protein subunits of photosystem II reaction centre (PSII-RC), *PsaN* encoding protein subunit of photosystem I reaction centre pigment protein complexes (PSI-RC), *pet F*, *pet H* encoding protein subunits of photosynthesis electron transfer, a hydrolase (EC 3.6.3.14) and a ferredoxin NADP $^{+}$ reductase (EC1.18.1.12) were down regulated (Supplementary Table 5a). The decreased transcript abundance of these genes indicates that the photosynthetic machinery had been affected in HKI161.

Also the genes involved in the category of carbohydrate metabolism fructose 1,6, diphosphatase (EC3.1.3.11), fructose bi phosphate aldolase (EC 4.1.2.13), Ribulose-5-phosphate kinase (EC 2.7.1.19), malate dehydrogenase (EC 1.1.1.37) and alanine transaminase (EC 2.6.1.2) were down regulated in HKI161 while their expression levels were unaltered in SNJ201126. Thus, the adverse effect on light reactions was also reflected in carbon fixation process (Supplementary Table 5b).

However, DEGs involved in starch metabolism were down regulated in both HKI161 (5 genes) and SNJ201126 (4 genes). The genes downregulated in HKI161 were, trehalose 6-phosphate synthase [EC:2.4.1.15 2.4.1.347], trehalose 6-phosphate phosphatase [EC:3.1.3.12], beta-amylase [EC:3.2.1.2], phosphoglucomutase [EC:5.4.2.2] and beta-glucosidase [EC:3.2.1.21] while in SNJ201126 alpha, alpha trehalose synthase (EC 2.4.1.15), trehalose-6-phosphate phosphatase (EC 3.1.3.12), starch synthase (EC 2.4.1.21) and NDP-glucose-starch glucosyltransferase (EC 2.4.1.242) (Supplementary Table 5c,d).

Phenyl propanoid pathway differential expression profiles in the two genotypes

The number of genes of the phenyl propanoid pathway upregulated in HKI161 and SNJ201126 genotypes was 9 and 5 respectively. The enzymes that were commonly upregulated in both the genotypes were phenylalanine/tyrosine ammonia-lyase [EC:4.3.1.25], shikimate O-hydroxycinnamoyltransferase [EC:2.3.1.133], 5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase [EC:1.14.14.96], and caffeoyl-CoA O-methyltransferase [EC:2.1.1.104] and peroxidase (EC 1.11.1.7) while genes expressed only in the susceptible genotype were trans-cinnamate 4-monooxygenase [EC:1.14.14.91], ferulate-5-hydroxylase [EC:1.14.-.-], cinnamyl-alcohol dehydrogenase [EC:1.1.1.195] and beta-glucosidase [EC:3.2.1.21] (Supplementary Table 5e).

Validation of DEG expression profiles using qRT-PCR

In order to validate the RNA-seq results, 8 genes involved in the major pathways were analyzed using qPCR. The results of the qPCR analysis clearly corroborated those of RNA-seq (Fig. 3). The tolerant genotype had higher levels of expression of genes involved in protein processing in endoplasmic reticulum, membrane transporters and MAPK signaling pathways induced in response to water deficit stress treatment.

Discussion

Differential response of stress tolerance pathways is evident in the tested genotypes

Growth and yield of *Zea mays* is relatively sensitive to water deficit stress. In this study, genotypes HKI161 and SNJ201126 presented distinct patterns of stress response representing several changes in gene expression and metabolic pathways.

Differentially expressed genes of ABA biosynthesis, intracellular Ca^{+2} signaling and transmembrane transporter categories involved in turgor homeostasis determine the water deficit stress tolerance

The accumulation of ABA was evident by the genes coding enzymes of ABA biosynthesis in both the genotypes. Similarly, several Ca^{2+} signaling related genes were upregulated in both genotypes indicating changes in Ca^{2+} signals. The upregulation of the three components of ABA signaling *PYR*, *PP2C*, and *SNRK* in the genotype SNJ201126 and two components *PP2C*, *SNRK* in HKI161 along with downstream *MAPK18* indicates that the genotype HKI161 was affected earlier leading to the activation of downstream components. In the genotype SNJ201126 the primary ABA transporters ABC transporter A family member 7, ABC transporter G family member 40 were upregulated the expression of few genes was also validated with qPCR analysis. This family is one of the most important transporters involved in import of ABA into guard cells. Loss of function *abcg40* mutants have guard cells with reduced sensitivity to ABA and more susceptible to drought stress (Kang et al. 2010). While in the genotype HKI161 Multidrug resistance protein ABC transporter family protein and secondary ABA transporters, high affinity nitrate transporter 2.7, nitrate transport 4 were upregulated. Higher expression of nitrate transporters reportedly involved in stomatal opening probably causes excess water loss in sensitive genotype (Parmar et al. 2019).

Response of the genotype SNJ201126 was more rapid and efficient when challenged with water deficit. Up regulation of TIP4 in SNJ201126 might have possibly resulted

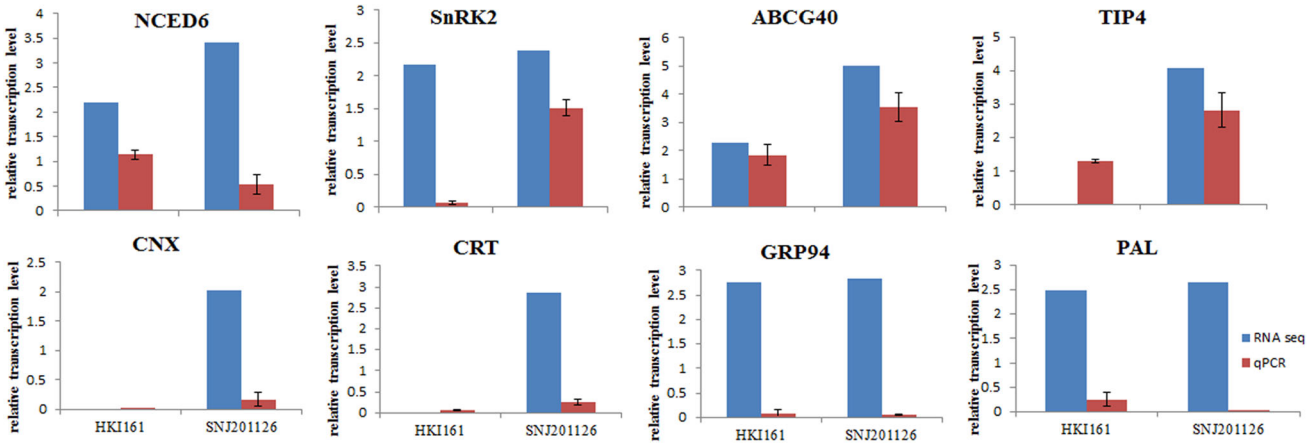

Fig. 3 qRT-PCR validation of RNA-seq data of 8 differentially expressed genes. The y-axis represents the relative gene expression levels (fold changes) in the real-time PCR analysis and fold changes in RNA-seq data. Maize gene GAPDH (accession number: X07156) was used as the endogenous control. Error bars represent the SE (n = 3)

in stomatal closure thus protecting the plant from further dehydration. The upregulation of TIP4 was confirmed by both RNAseq and qPCR. PIP2 and TIP1 were also upregulated in HKI161 although their expression levels were much lower. The expression of *PIP1* from *V. faba* and *B. juncea* improved drought resistance in *A. thaliana* and *N. tobaccum* plants respectively through the promotion of stomatal closure (Cui et al. 2008; Zhang et al. 2008). Our results of real time evaluation on stomatal conductance and transpiration in these genotypes (Table 2) corroborate the observations on gene expression. The genotype SNJ201126 maintained higher stomatal conductance (0.32 mmol H_2O $m^{-2}S^{-1}$) and transpiration (8.12 mmol H_2O $m^{-2}S^{-1}$) when compared to HKI161 (0.24, 5.52 mmol H_2O $m^{-2}S^{-1}$) respectively under water stress conditions in the field. Also the genotype SNJ201126 maintained lower leaf temperature under well-watered and water stress conditions, while there was a two degree rise in HKI161. Further, the genotype SNJ201126 maintained better relative water content under stress when compared to HKI161 under field as well as controlled conditions (Table 2, Fig. 1a).

The performance of the genotype SNJ201126 in maintaining higher water relations was consistent as indicated by the higher relative water content under field (78.15%) and controlled conditions at ASI (76.79%) and seedling stage (77.42%).

The regulation of protein processing in endoplasmic reticulum is closely related to the water deficit stress tolerance

Protein aggregation induces an unfolded protein response pathway (UPR) which activates either the calnexin/calreticulin protein folding cycle or the ER associated degradation system (ERAD) depending on the stress severity (Liu and Howell 2010). In the present study the genes of CNX/CRT protein folding machinery viz., calnexin, calreticulin, protein disulfide isomerase (PDIs) and DNJ proteins (HSP40) were upregulated only in the genotype SNJ201126 and not in HKI161. Various genes involved in protein processing in ER viz., *sec16A*, *HSP70*, *NEF* and *sHSF* and chaperone *GRP94* were relatively higher in genotype SNJ201126 (Supplementary Table 3e). The expression of calnexin, calreticulin and *GRP94* genes was also further confirmed using qPCR analysis. Thus, protein folding cycle was differentially expressed only in SNJ201126 although the ERAD pathway was operative in both the genotypes. Thus, the proteins which were probably folded might have been transported to Golgi complex while the misfolded proteins were eliminated through ERAD system (Liu and Howell 2010). In response to water deficit stress the ER protein folding machinery reaches a limit as the demands for protein folding exceeds the capacity of the system. Hence under stress conditions misfolded/unfolded protein accumulation in ER triggers an unfolded protein response which may eventually lead to apopotosis or programmed cell death (Park and Park 2019). Thus, maintenance of protein folding machinery is crucial in imparting stress tolerance (Fig. 4). A wheat CRT (*Ta-CRT*) is induced by drought and overexpression of *Ta-CRT* in tobacco plants enhanced drought resistance (Jia et al. 2008). Further, the three fold increase in expression level of ER-sHSF in SNJ201126 might have contributed to enhanced stress tolerance. Salt tolerance was enhanced in tomato plants constitutively expressing ER-sHSF (Fu et al. 2016).

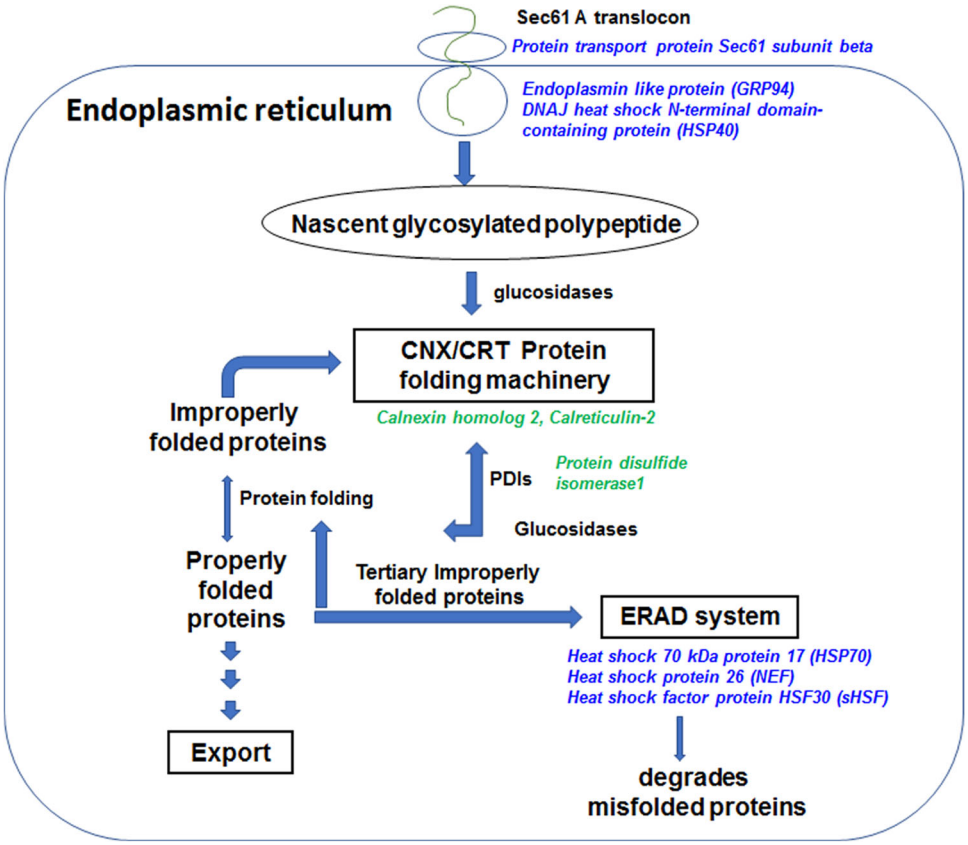

Fig. 4 Upregulated protein processing in endoplasmic reticulum pathway genes in the genotype SNJ201126. Genes indicated in green: Upregulated in response to water deficit stress only in SNJ201126. Genes indicated in blue: Upregulated in response to water deficit stress in both the genotypes. However, the upregulation was several folds higher in the genotype SNJ201126 (color figure online)

Antioxidative enzyme genes display distinct expression profiles in the two genotypes

The higher expression of anti-oxidative enzyme genes viz. peroxidases, glutathione—S-transferase, ascorbate peroxidase and flavone synthase observed in SNJ201126 as compared to HKI161 might be associated with conferring tolerance through a delay in the programmed cell death. The results on gene expression of anti-oxidative enzymes in our study are corroborated by the results on in vitro activities of anti-oxidative enzymes (Table 3). The activities of antioxidative enzymes SOD and catalase were higher in SNJ201126 indicating that the genotype was efficient in removing the bulk of ROS generated. The maintenance of higher activities of SOD, catalase, peroxidase and water relations resulted in the higher tolerance of drought tolerant rice cultivar (Wang et al. 2019).

Transcription factors associated with drought tolerance have differential expression profiles in the two genotypes

In the genotype HKI161, the AP2 / ERF TF family were majorly upregulated which are known to be involved in the synthesis of secondary metabolites (Seo and Choi 2015). Several genes of the phenyl propanoid pathway involved in the synthesis of secondary metabolite lignin as well as MYB96 and WRKY33 were upregulated in this genotype. Enhanced expression of TFs leading to the genes involved in secondary metabolite lignin biosynthesis, and defense response genes probably indicates higher susceptibility of this genotype to water deficit. In the genotype SNJ201126, the NAC genes were upregulated which are plant specific multifunctional transcription factors known to impart higher multiple abiotic stress tolerance in crops (Zhang et al. 2017).

Four transcription factors belonging to MYB family myb domain protein 63, myb domain protein 86, myb domain protein 7 and MYB related family protein were upregulated in the genotype SNJ201126. MYB TFs have been identified to be involved in drought response in some other crops by regulating stomatal movement and cuticular wax synthesis (Zhang et al. 2017; Baldoni et al. 2015; Li et al. 2016). The higher levels of stomatal conductance and transpiration observed under water deficit in this genotype might be indicative of the stress tolerance role of the MYB transcription factor overexpression.

Water deficit stress response may be related to the regulation of photosynthesis and glycolysis

Amongst the impacts of water deficit stress on several aspects of plant metabolism photosynthesis is most crucial (Pinheiro and Chaves 2011; Maheswari et al. 2012). In the genotype HKI161, genes involved in light reactions of photosynthesis pathway and carbon fixation were down regulated, while in case of SNJ201126 such down regulation was not evident. The down regulation of photosystem II related genes viz., *Psb*M & *Psb*Q as well as protein complexes of PSI (*Psa* N) was seriously affected and electron transport mechanism was inhibited. The down regulated gene *Psb* M in the genotype HKI161 is one of the components of the core complex of PSII and is involved in dimerization of PSII. *Psb*Q, another enzyme down regulated in this genotype is an electron transporter transferring electrons within the cyclic electron transport pathway. *Psa* N, gene of PSI protein complex was also down regulated, which plays an important role in docking plastocyanin to the PSI complex.

Ribulose-5-phosphate kinase, fructose 1, 6, bi phosphatase and fructose bi phosphate aldolase and malate dehydrogenase and alanine transaminase are important enzymes of CO_2 fixation, the transcripts of which were down regulated in HKI161. Overall, the transcriptome data indicated that the photosynthesis process was adversely affected due to the decreased transcript abundance of a wide range of genes required, which eventually led to a lower level of net CO_2 assimilation rate in HKI161 compared to SNJ201126 (Table 2, Fig. 1c) under water—deficit stress. The glycolytic pathway was decreased in HKI161 probably due to a blockage of hexoses entering this pathway. Further the results on chlorophyll content, net CO_2 assimilation rate and seed yield (Table 2, Fig. 1b, c) were consistent with the results on relative leaf turgidity (Table 2) which suggests that enhanced tolerance to water deficit stress in SNJ201126 may be due to enhanced leaf turgidity leading to better net CO_2 assimilation rate and higher grain yield compared to HKI161.

Phenyl propanoid pathway display differential expression profiles in the two genotypes

Phenylalanine ammonia lyase (PAL), catalyzing the deamination of phenylalanine to produce cinnamic acid, is the key enzyme of phenyl propanoid pathway. The upregulation of PAL gene was evident by both RNAseq & qPCR analysis in both the genotypes. The enzymes that were commonly upregulated in both the genotypes were phenylalanine ammonia-lyase, shikimate O-hydroxycinnamoyl transferase, 5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase, caffeoyl-CoA O-methyltransferase and peroxidase. On the other hand, trans-cinnamate 4-monooxygenase, ferulate-5-hydroxylase, cinnamyl-alcohol dehydrogenase and beta-glucosidase were upregulated only in HKI161. These enzymes catalyze reactions leading from 4-coumarate via a series of intermediates to coniferyl or synapyl alcohols (Hamberger et al. 2007). The genes upregulated mostly belonged to the pathways which led to biosynthesis of lignin. But a greater number of genes involved in catalyzing the reactions leading to the biosynthesis of various lignin monomers have been up regulated in HKI161 when compared to the SNJ201126 indicating that the stage of stress effect was more advanced in HKI161 compared to SNJ201126.

Thus a few key mechanisms of water deficit stress tolerance from signal transduction to eventual phenotypic expression were identified in maize. ABA and calcium signaling involved in turgor homeostasis and protein processing in endoplasmic reticulum for maintenance of growth and metabolism under stress are predominantly associated with stress tolerance. Genes encoding enzymes of antioxidative metabolism, ABA biosynthesis, as well as various transcription factors belonging to MYB, NAC and ERF families were found to be associated with water relations and stomatal movements and detoxification mechanisms have also contributed to stress tolerance. The genes of these major pathways were upregulated in the genotype SNJ201126 thus contributing to its better performance under water deficit from metabolic to grain yield levels. On the other hand, down regulation of light reactions as well as C_4 and Calvin cycle enzymes and upregulation of phenyl propanoid pathway leading to lignin biosynthesis have been observed as associated with injury and sensitivity to water deficit stress (Fig. 5). Down regulation of the genes involved the above pathways seems to have eventually contributed to the relative susceptibility of HKI161 in terms of reduced photosynthesis, biomass and grain yield. These results would also be useful in elucidating the possible molecular mechanisms of tolerance in maize to water deficit integrating water status, protein processing in ER and antioxidative response for enhanced growth and productivity under climate change so as to ensure food security. The role of negative regulators and their characterization in the entire pathway needs to be focused in future.

Conclusion

Water deficit stress transcriptome analysis of two maize genotypes under well-watered and water deficit stress conditions revealed several changes in gene expression manifesting in stress tolerance or sensitivity in the two genotypes tested. Based on the phenotypic and metabolic

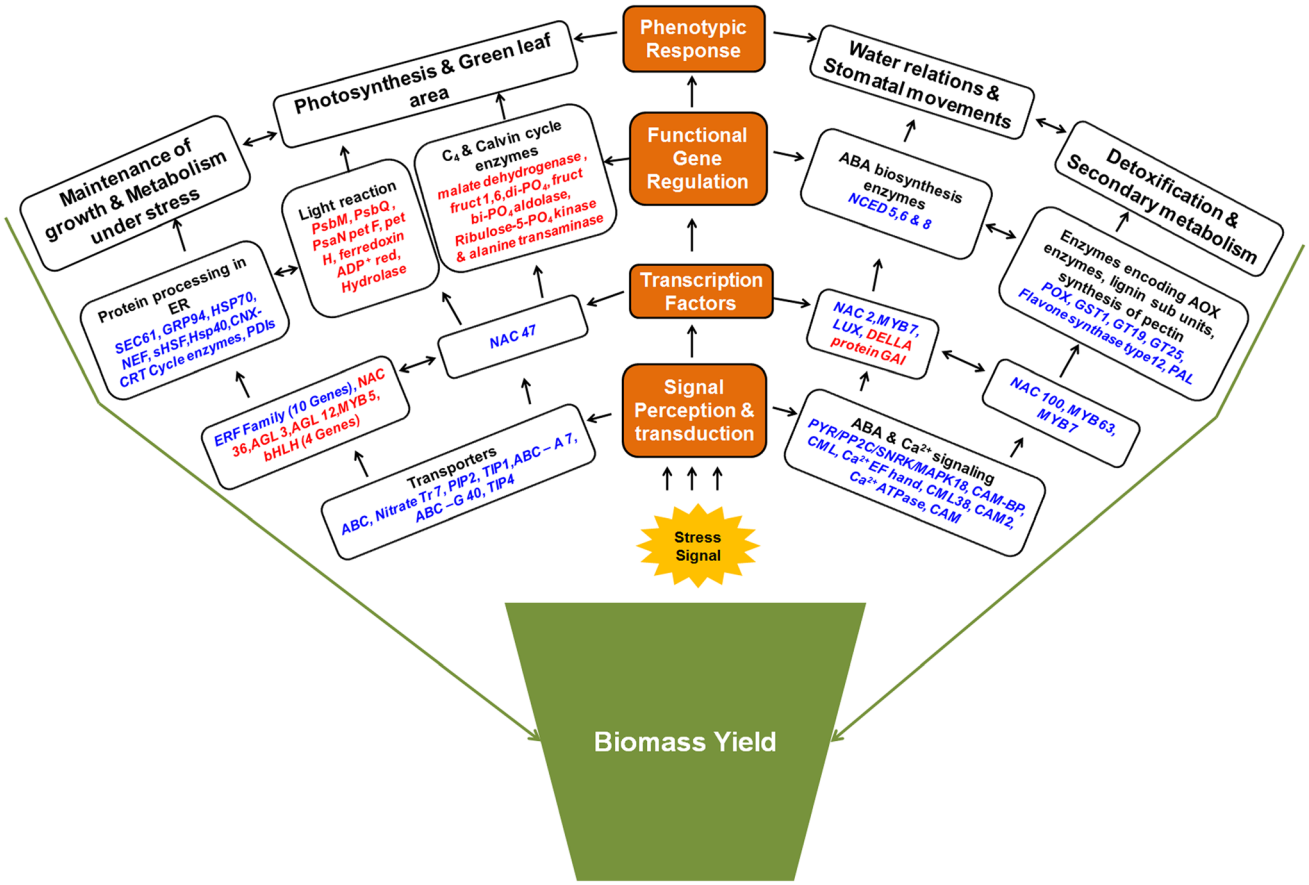

Fig. 5 Functional mechanisms of water deficit stress tolerance from stress signal transduction to expression of phenotype in maize. Illustrative representation of upregulated (blue) and down regulated genes (red) (color figure online)

traits of the two genotypes it is inferred that the enhanced stress tolerance in SNJ201126 is possibly due to high water retention ability, efficient protein processing in endoplasmic reticulum and robust photosynthetic capacity and AOX metabolism.

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Data availability The transcriptome data of two maize genotypes HKI161 and SNJ201126 under well-watered and water deficit stress conditions has been deposited at NCBI under the BioProject number PRJNA436078. The sequencing data was deposited in the Sequence Read Archive database under the accession number SRP133547.

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