Dissection of Maize Drought Tolerance at the Flowering Stage Using Ge-Nome-Wide Association Study

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Abstract

Drought is one of the most critical environmental factors constraining corn production especially when it occurs during flowering, resulting in serious yield losses. In this study, anthesis to silk interval (ASI), plant height (PH), and ear biomass at the silking date (EBM) of 279 inbred lines were evaluated under water-stress (WS) and well-water (WW) field conditions for three consecutive years. Averagely, ASI was extended by 25.96%, ear biomass was decreased by 17.54%, and the PH was reduced by 12.47% under drought stress conditions. Genome wide association studies (GWAS) were carried out using phenotypic values under WS, WW and drought-tolerance index (WS-WW or WS/WW) applying mixed linear model controlling both population structure and relative kinship. Totally, 71, 159, and 21 SNPs were significantly ($P < 10^{-5}$) associated with ASI, ear biomass, and PH, respectively. Candidate genes encoding ARABIDILLO 1 protein, glycoprotein, Tic22-like and Zinc finger family protein for ASI, and 26S proteasome non-ATPase regulatory subunit-9 for EBM, were identified under both WW and WS conditions. Pyridoxal phosphate transferase was associated with EBM under drought stress treatment in consecutive two years. Furthermore, most candidate genes were evidenced to be drought responsive in the association panel. Meanwhile, the favourable/drought tolerance haplotypes were identified based on haplotype analysis. These findings provide insights into the genetic basis of drought tolerance at the flowering stage especially for the female inflorescence development and will facilitate high drought tolerant maize breeding.

Key Message

Six candidate genes contributing to maize drought tolerance at the flowering stage were identified by combining GWAS, expression profiling, and favorable/drought tolerance haplotype analysis.

Introduction

Maize is the most widely grown crop worldwide, and has incredible importance for food, feed and other industrial products (Shiferaw et al. 2011). It was estimated that production of maize needs to boost by 67% to cope with the increased population growth and food demand in 2050 (Ray et al. 2013). Thus, it is of great significance to increase maize yield productivity and reduce yield loss caused by biotic and abiotic stresses.

Drought is considered as one of the most detrimental curbs of agriculture which endangers maize (*Zea mays* L.) production globally because of its erratic nature (Daryanto et al. 2016; Gupta et al. 2020). Maize is an open-pollinating crop which is extremely sensitive to drought stress throughout its live span, especially from the one week before and three weeks after flowering period, causing severe yield loss by 15-25% (Bänziger et al. 2000). Drought occurs at this stage inhibits ear development, causing abnormal differentiation of spikelet, and changes of hormone signalling involved in cell division, growth and primordium development in maize (Wu et al. 2021), leading to the asynchronous development in tassel and ear, tremendous extension of anthesis and silking interval (ASI), and reduction of silk receptivity (NeSmith and Ritchie 1992; Saini and Westgate 1999). Consequently, this time slack between pollen release and silk emergence adversely affects pollination and kernel set and results in the reduction of grain yield (Bolaños and Edmeades 1996; Bruce et al. 2002). It has also been reported that drought stress reduces plant height as well as ear height, decreases the availability of photosynthate for the grain production, ultimately leading to yield reduction (Lopes et al. 2011; Sari-Gorla et al. 1999). Due to global warming and the increasing shortage of water resources, water deficit during maize flowering period has become more and

more prominent. Breeding maize for drought tolerance at flowering stage is thus of significant importance for global food productivity.

Association analysis based on linkage disequilibrium (LD) had acquired increasing popularity and accuracy in the genetic architecture of polygenic traits in crops due to broader genetic variation, larger numbers of alleles and the maximum number of recombinants obtained (Mackay 2001; Yu and Buckler 2006). Genome-wide association study (GWAS), taking advantages of natural variation and historical recombination, has emerged as an alternative tool to linkage mapping for identifying superior alleles for complex traits with reduced time consumption and increased mapping resolution (Rosenberg et al. 2010; Yan et al. 2011). GWAS has been employed to identify numerous SNPs directly associated with drought tolerance especially at the seedling stage. *ZmVPP1*, was associated with dehydration tolerance in maize seedlings, encoding a vacuolar-type H⁺ pyrophosphatase, which improved seedling drought tolerance in maize due to enhanced photosynthetic activity and root development (Wang et al. 2013). Significant associations were detected between maize seedling drought tolerance and functional genes promoter variation, such as SNPs located in promoter of *ZmDREB2.7* and miniature inverted repeat transposable element in *ZmNAC111* promoter, which determined gene expression for dehydration tolerance in maize seedlings (Liu et al. 2013; Mao et al. 2015). A 368 maize association panel was used to conduct an association analysis on *ZmPP2C-A* family genes and found that *ZmPP2C-A-10* was closely related to drought stress, through regulating the ABA signalling pathway (Xiang et al. 2017). This study reveals the correlation between endoplasmic reticulum stress response and drought resistance.

Drought tolerance in maize is a complex and quantitative trait and its molecular mechanism is extremely complicated. Research on maize drought-tolerant genes, especially the drought-tolerant genes during flowering period, is relatively lagging. However, rare genes, contributing to drought tolerance, were identified through GWAS or linkage mapping using larger scale population due to the uncontrollable field condition. At present, few genes were found to improve the drought-tolerance during the flowering period of maize. NAC transcription factor NUT1 was found specifically expressed in protoxylem at the flowering stage, and functions to manipulate water transport by maintaining protoxylem vessel integrity through activating genes necessary for secondary cell wall reinforcement, thus affecting drought tolerance in NUT1 mutants (Dong et al. 2020). A recent study found that *ZmEXPA4*, identified through transcriptomics analysis, functions during ear growth and silk elongation, alleviating the drought-induced elongation of the ASI without affecting PH and other traits (Liu et al. 2021). More recently, using a high-throughput phenotyping platform, 368 maize inbred lines were continuous non-destructive tested under multiple growth periods, normal watering and drought stress, resulting 2318 candidate genes associated with i-traits and drought tolerance in maize (Wu et al. 2021). Mutant-based functional validation has shown that *ZmcPGM2* (involved in sugar metabolism) and *ZmFAB1A* (involved in phosphoinositide metabolism) can negatively regulates drought resistance in maize during flowering.

In this study, an association population of 279 inbred lines, genotyped by 776,254 high-density SNP markers, was evaluated under field WW and WS conditions. We aimed to dissect the candidate genes associated with drought-related traits, such as plant height, ASI and ear biomass at the silking date, and to provide potential gene resources for breeding drought tolerant maize varieties.

Materials And Methods

Plant materials and experimental design

The association panel used for GWAS, comprised of a total of 279 inbred lines with diverse genetic variation, were selected from various geographical regions including tropical, sub-tropical and temperate of the world. Among these lines, 268 were selected from previously published 368 inbred lines for eQTL mapping (Fu et al. 2013), and the rest 11 lines were newly added from temperate regions.

The field trials were conducted in Urumqi (Xinjiang province: 43°54'N, 87°28' E), from May to September. The association panel was planted in three independent repeats in an incomplete randomized block design (RCBD), during the years 2017, 2018 and 2020. These lines were divided in three patches based on the flowering time in order to get the synchronized flowering for all the lines. Each plot was 3.6 m in length with planting space of 0.24 m. The spacing between the rows was 1.1 m. Each row had 15 plants, and each line had 3 replications. Three seeds were planted per hole and thinned to one plant three weeks after planting. Every line was planted side by side, one row for water stress treatment (WS) and the other for well-watered regime (WW), with an independent valve to control the irrigation. The fertilizers, herbicides and insecticides were applied as per requirement according to the local recommendation practices.

Daily moisture level was recoded using the sensors that were installed in each patch at different position in the field in both water stress and well-watered regimes. Drought stress was applied in WS regime following the CIMMYT's protocol. Plant rows were irrigated in WS regime through drip irrigation method at intervals of 14 days, till 3 weeks before estimated date of anthesis (AD) for the association panel. The expected date of flowering time for each line was calculated on the basis of Growing degree days (GDD). Daily temperature was recorded, and GDD was calculated for each line using the following formula.

$$GDD = \frac{(L + H)}{5} - 50$$

Where L means Daily lowest temperature (°F) and H means daily highest temperature (°F).

Managed drought stress trial was conducted in WS regime by withdrawing irrigation initiated at the 21st day (-21 D) before anthesis estimated according to GDD. The soil moisture was maintained at < 40 centibars before -15 D and at 80-120 centibars from -15 D to -7 D of anthesis initiation. At -7 D of anthesis, the soil moisture reached 120-150 centibars and was kept at 150-200 centibars until 14 days after anthesis when irrigation was resumed. In WW regime, supplemental irrigation was provided as needed to avoid moisture stress.

Phenotyping for drought stress related traits

PH and flowering traits such as anthesis date, silking date, and ear biomass were examined in inbred lines under WS and WW conditions. Days to anthesis (AD) and days to silking (SD) were determined by the number of days from planting to pollen shedding of 50% of plants, and 50% of plants having clearly visible silks respectively in each repeat. The plants were considered having reached anthesis and silking, when one anther extruded (termed as pollen shading) or one silk was visible respectively. The ASI was calculated as the interval between anthesis date and silking date (ASI = SD - AD) was counted in days (Bolaños and Edmeades 1996). PH was measured from soil level to the lowest tassel branch of each plant using a meter scale, and data was recorded in centimetres (cm). For ear biomass, ear at its silking date of each inbred lines was harvested and dried to a consistent weight at 72°C. The ear biomasses were weighed with electronic weighing balance and recorded in grams (g). Drought tolerant index was calculated by dividing the mean values of studied traits in WS regime by

WW regime. To estimate the random errors, each measurement per inbred line comprised of 6-8 individual plants with 3 independent repeats.

Genotyping, SNP calling and imputation

For association analysis, 279 genotypes (5 DAP) were genotyped containing high quality 776, 254 SNP markers with MAF > 0.05 published earlier was used for association analysis (Fu et al. 2013; Yang et al. 2011). GATK v3.6 (McKenna et al. 2010) was used for SNP calling. Briefly, each sample SNPs were called independently by adjusting the minimum threshold of phred scaled confidence to call variants 20, Fisher strand bias < 30.0 was used for filtering SNPs and quality by depth > 2.0. The generated different samples SNPs were merged together and the loci having missing information were recalled through Haplotype Caller. Reported Indels for maize were downloaded from the Ensemble Plants database. Before imputation, SNPs with missing rate > 60% and heterozygote rate > 10% were filtered out. The heterozygous (0/1) genotypes were replaced with missing (./.). Then SNP with MAF < 2% were also filtered out. For the imputation of missing genotypes, BEAGLE v4.1 (Browning and Browning 2016) was used. First all the sites were imputed without reference panel and then with reference (Bukowski et al. 2018). The two sets of imputation were merged together. After imputation, both heterozygous genotypes and imputed genotypes were marked as missing (./.). The called SNPs of the study were compared with 15 DAP maize SNPs (Fu et al. 2013). The rate of concordance calculated was done on the basis of SNPs of the same maize lines and two datasets of SNPs were merged together for further study. The called genotypes (5 DAP) from current study were retained for overlapped SNPs loci. Variant Annotation package (Obenchain et al. 2014) was used for SNPs annotation and grouping them into the UTR, CDS, Intron and Intergenic regions.

GWAS for ASI, EBM, and PH

The associations between SNPs markers (776, 254) and traits phenotypes were detected using mixed linear model (MLM) imbedded in TASSEL V5.0 (Bradbury et al. 2007). The SNPs with MAF $\geq$ 0.05 were used in the analysis. In the MLM, population structure (Q) and kinship (K) was estimated (Pang et al. 2019). Briefly, kinship matrices and principal components were estimated based on 236, 205 SNPs with MAF > 0.05 and for which there was no missing using TASSEL. Top 3 principal components were selected as population structure, and for kinship estimation, the 'Normalized_IBS' method was used. The population structure and kinship were added as covariates in the MLM. The regression-based coefficient of determination values of all significantly associated SNPs was recorded to determine the variations explained by each SNP locus. Non-independence of SNPs in maize genome leading to strong LD, therefore, a less strict significant association threshold of $P < 10^{-5}$ was applied in this study. Manhattan and QQ plots were drawn through R software.

Based on reference genome of maize B73 (V4), the genes co-localized with or nearby significantly associated SNPs were considered as candidates. The gene annotation, function of candidate genes and reported genes for relevant traits in maize and other crops and the orthologues in Arabidopsis with function of regulating the ASI, ear biomass, and PH were retrieved from Maize genome database (<http://www.maizegdb.org/>), Gramene database (<http://gramene.org/>), NCBI (<https://www.ncbi.nlm.nih.gov/>) and other available literature.

Drought responsive, linkage disequilibrium, and haplotype analysis of candidate genes

The expression levels of candidate genes *Zm00001d013992*, *Zm00001d020506*, *Zm00001d029937*, *Zm00001d029938*, and *Zm00001d039319* were analyzed using their FPKM value from 197 diverse inbred lines

under WS and WW conditions (unpublished data from Dr. Mingqiu Dai, Huazhong Agricultural University). Allele effects of the most significant SNPs for the six overlapping candidate genes across multiple years or conditions, and their corresponding phenotypic data were used for haplotype analysis, following a two-pair t-test, to analyze their allelic effect in corresponding phenotypic performance. LD analysis of these candidate genes were conducted by SNPs within a gene using “LD heatmap” in the R software.

Statistical analysis

The data was cleaned by removing suspicious value of each replicate by *Q-test*. The mean values of multiple environments were added as the final phenotype value of each family for subsequent correlation analysis, association analysis and linkage mapping, etc. Best linear unbiased estimators (BLUEs) were calculated by using the genotype and covariate as fixed factor, and the rest as random factor. Phenotypic data distribution, correlation among the traits and differences in traits among the inbred lines were determined using analysis of variance (ANOVA) through statistical software package (SPSS 25.0, Chicago, USA). On ANOVA basis, genetic parameters, including the genotypic and phenotypic coefficient of variance (CV, σ_p^2 , σ_g^2) for mean values, were calculated. The broad sense heritability (H^2) was estimated on the basis of mean of genotypes for every trait and each environment as the ratio of genotypic to phenotypic variance, using the variance components according to the following equation:

$$H^2 = \frac{\sigma_g^2}{(\sigma_g^2 + \sigma_{GE}^2 + \sigma_e^2)/lr}$$

Where σ_g^2 represents genetic variance, σ_{GE}^2 shows the Genetic and environmental interaction variation, σ_e^2 shows residual error variance, *l* shows the number of environments and *r* shows number of replicates (Holland et al. 2010).

Results

Evaluation of drought-tolerance phenotypes in the association panel

For the association panel, three traits ASI, ear biomass and PH were tested in field condition under both drought stress at the flowering stage and WW conditions for three years. The test of normality revealed that the frequency distributions for all the studied traits were near to normal for most of the traits in the association panel (Fig. 1; Table 1). While variations still exist which might be caused by fluctuating environment in the field or genotypic differences. The descriptive statistics, heritability analysis and coefficient of variance for the phenotypic traits of all the three years are listed in Table 1. Generally, drought stress significantly decreased the ear biomass and PH, while increased the ASI, suggesting that water stress at the flowering stage had diverse effects on drought-related traits.

Table 1
Descriptive statistics and heritability estimate for the traits of association panel.

Traits	Range	Mean $\pm$ SD	C.V. (%)	(H^2) %
ASI-WS	1.67-21.42	5.58 $\pm$ 2.39	42.97	89.31
ASI-WW	0.56-18.15	4.43 $\pm$ 2.17	48.90	87.45
EBM-WS	0.48-3.08	1.42 $\pm$ 0.41	29.16	86.78
EBM-WW	0.48-3.81	1.71 $\pm$ 0.47	27.21	86.64
PH-WS	43.21-169.72	111.36 $\pm$ 21.54	19.34	92.52
PH-WW	51.23-183.74	127.22 $\pm$ 21.53	16.93	94.60
ASI, anthesis-silking interval, days; EBM, ear biomass, g; PH, plant height, cm; SD, standard deviation; C.V., coefficient of variance; H^2 , Broad-sense heritability.				

The mean values of ASI under drought stress was 5.58 days with the coefficient of variance (CV) 42.97%, while under WW condition, the ASI was 4.43 days with the CV of 48.89% (Table 1). The averaged ASI across the three years under WS regime was 1.15 days larger than that of the WW regime. Under drought stress, ASI was increased by 25.96% across the three years, indicating that drought stress significantly enlarges the ASI. Drought stress caused a 17.54% reduction in EBM with the minimum of 0.48 g and maximum 3.08 g under WS condition, while they were 0.48 g and 3.81 g under well water regime. The mean value of ear biomass was 1.71 g under WW condition with the CV 27.20%, while it was 1.41 g under WS condition with the CV 29.15% for the three-year environments, respectively (Table 1). Likewise, drought stress caused a 12.47% reduction in the PH, and the minimum of PH was 43.21 cm and maximum 169.72 cm under WS condition, while they were 51.23 cm and 183.74 cm under WW condition. The mean value of PH under WS regime were 11.36 cm with the CV 19.34%, while under WW regime PH was 127.22 cm with the CV 16.92% for the three years, respectively (Table 1). These results indicated that drought stress at the flowering stage severely affects the ear biomass as well as PH, causing suppressed plant growth and development. The estimated heritability (H^2) of ASI, EBM and PH was higher than 80% under the two water treatments across the three years respectively (Table 1), indicating that these traits were significantly affected by the genotype.

Correlations among drought related traits

The correlations among the ASI, EBM and PH under WS and WW conditions are listed in Table 2. Significant positive correlation ($P < 0.05$) was found between ASI and PH under WS (0.13) and WW (0.15) conditions. Also, significant correlations were found between ear biomass and PH under both WS (0.22) and WW (0.18) conditions. No correlation was found between ASI and ear biomass.

Table 2
Correlation analysis based on BLUP values across three-year environments under drought (WS, above diagonal) and well-watered (WW, under diagonal) regimes

Trait	ASI	EBM	PH
ASI		-0.09	0.13*
EBM	-0.04		0.22**
PH	0.15*	0.18**	
ASI, anthesis-silking interval; EBM, ear biomass; PH, plant height;			
*, ** Significant at $P < 0.05$, 0.01 , respectively.			

GWAS for maize drought tolerance genes

GWAS was performed for the association panel between 776,254 SNP markers and phenotypic information of 279 inbred lines using Tassel software where the threshold was kept at $P < 10^{-5}$. A total of 71, 159 and 21 SNPs significantly associated with ASI, EBM and PH located in 36, 81 and 16 genes, scattered over all 10 chromosomes, with R^2 ranged from 7.19–15.52%. The number of identified SNPs in each chromosome ranged from 9 to 31 on chromosomes 9 and 1, respectively. The information about all the SNPs and candidate genes of all the three traits and three-year environments are shown in Tables S1-S9. The association analysis identified a total of 17, 48 and 6 SNPs located in 13, 18 and 5 genes for ASI under WS, WW and drought tolerance index (WS-WW) with R^2 ranging from 7.73%-14.32% (Fig. 2; Table 3 and Table S1-S3). Totally, 49, 93 and 17 SNPs located in 23, 43 and 15 genes were associated with EBM under WS, WW and drought tolerance index (WS/WW) with R^2 ranging from 7.99%-12.52% (Fig. 3; Table 3 and Table S4-S6). GWAS analysis showed that total 9, 9 and 3 SNPs were associated with PH and located in 7, 6 and 3 genes under WS, WW and WS/WW with R^2 ranging from 7.19%-15.52% across the three-year environments (Fig. 4; Table 3 and Table S7-S9).

Table 3
Annotation of SNPs associated with ASI and EBM under multiple conditions

Traits	Marker	Chr.	Position	P value	R ²	Gene ID	Annotation
ASI-WS-18	S1_93513564	1	93513564	5.38E-06	0.08207	<i>Zm00001d029938</i>	Protein ARABIDILLO 1
	S1_93277641	1	93277641	6.06E-06	0.08113	<i>Zm00001d029937</i>	Glycoprotein
	PZE-103003226	3	2449913	1.03E-07	0.14322	<i>Zm00001d039319</i>	Tic22-like family protein
	chr3.S_183263192	3	183319292	1.01E-05	0.07963	<i>Zm00001d042997</i>	HIT-type Zinc finger family protein
ASI-WW-18	S1_93277641	1	93277641	2.20E-07	0.1079	<i>Zm00001d029937</i>	Glycoprotein
	S1_93277775	1	93277775	3.28E-07	0.10511		
	S1_93278150	1	93278150	7.29E-07	0.09852		
	S1_93513564	1	93513564	1.01E-06	0.09549	<i>Zm00001d029938</i>	Protein ARABIDILLO 1
	S1_93507046	1	93507046	2.48E-06	0.08831		
	S1_93505855	1	93505855	3.76E-06	0.08489		
	S1_93509892	1	93509892	3.76E-06	0.08489		
	S1_93510646	1	93510646	3.76E-06	0.08489		
	S1_93511155	1	93511155	3.76E-06	0.08489		
	S1_93510058	1	93510058	8.64E-06	0.07831		
	S1_93511521	1	93511521	8.64E-06	0.07831		
	S1_93513096	1	93513096	8.64E-06	0.07831		
	PZE-103003226	3	2449913	1.64E-06	0.10835	<i>Zm00001d039319</i>	Tic22-like family protein

Traits	Marker	Chr.	Position	P value	R ²	Gene ID	Annotation
	chr3.S_183263192	3	183319292	1.66E-06	0.09449	<i>Zm00001d042997</i>	HIT-type Zinc finger family protein
	S3_183315457	3	183315457	1.91E-06	0.09027		
	S3_183315658	3	183315658	1.91E-06	0.09027		
	S3_183316916	3	183316916	1.91E-06	0.09027		
	S3_183318642	3	183318642	1.91E-06	0.09027		
	S3_183315400	3	183315400	5.78E-06	0.08148		
	S3_183311733	3	183311733	7.14E-06	0.07982		
	S3_183311777	3	183311777	7.14E-06	0.07982		
EBM-WS-17	chr7.S_116288756	7	116316709	5.92E-06	0.1034	<i>Zm00001d020506</i>	26S proteasome non-ATPase regulatory subunit 9
	chr7.S_116288791	7	116316744	5.92E-06	0.1034		
	chr7.S_116288792	7	116316745	5.92E-06	0.1034		
	chr7.S_116285652	7	116313605	1.01E-05	0.09798		
	chr7.S_116285655	7	116313608	1.01E-05	0.09798		
EBM-WW-17	S7_116315576	7	116315576	1.17E-06	0.11793	<i>Zm00001d020506</i>	26S proteasome non-ATPase regulatory subunit9
	S7_116316425	7	116316425	1.17E-06	0.11793		
	S7_116316559	7	116316559	1.17E-06	0.11793		
	chr7.S_116288756	7	116316709	1.29E-06	0.12102		
	chr7.S_116288791	7	116316744	1.29E-06	0.12102		

Traits	Marker	Chr.	Position	<i>P</i> value	R ²	Gene ID	Annotation
	chr7.S_116288792	7	116316745	1.29E-06	0.12102		
	chr7.S_116285652	7	116313605	3.42E-06	0.11084		
	chr7.S_116285655	7	116313608	3.42E-06	0.11084		
	S7_116314423	7	116314423	1.81E-06	0.11403		
	S7_116316667	7	116316667	2.11E-06	0.11193		
EBM-WS-18	S5_27121944	5	27121944	3.25E-06	0.08944	<i>Zm00001d013992</i>	Pyridoxal phosphate dependent transferase family protein
EBM-WS-20	S5_27121944	5	27121944	9.15E-06	0.09491	<i>Zm00001d013992</i>	Pyridoxal phosphate dependent transferase family protein

Common genes identified across multiple years or conditions

Several overlapping genes were identified among different years and water treatments (Table 3). Four candidate genes encoding ARABIDILLO 1 protein (*Zm00001d029938*) and Glycoprotein (*Zm00001d029937*) on Chr. 1, Tic22-like family protein (*Zm00001d039319*) and Zinc finger family protein (*Zm00001d042997*) on Chr. 3, were identified for ASI under both WS and WW conditions in 2018 (Fig. 2), suggesting that these genes might be promising candidates that function in inflorescence development no matter there is drought stress or not. A SNP (S5_27121944), located in Pyridoxal phosphate transferase encoding gene (*Zm00001d013992*), was significantly associated ($P < 10^{-5}$) with EBM under drought stress condition on Chr. 5 was consistently detected for two years environments of 2018 and 2020 (Fig. 3), suggesting the drought tolerance role of this gene in developing ear. A candidate gene (*Zm00001d020506*) encoding 26S proteasome non-ATPase regulatory subunit-9 was detected for EBM on Chr. 7 under both WS and WW regimes during 2017 field trial (Fig. 3), suggests its possible role in maize female ear development.

Candidate genes drought responsive pattern

Most quantitative traits functional genes are responsive at the transcriptional level. Drought tolerance is complex and regulated by many quantitative trait loci (QTLs) with minor effects. In order to determine whether these candidate genes were drought regulated, we analysed their expression level using the expression data from 197 diverse inbred lines under WS and WW conditions, of which 135 inbred lines were involved in our association panel. Unsurprisingly, significant differential expression exists in most candidate genes between WW and WS treatment. *Zm00001d013992*, *Zm00001d029938*, and *Zm00001d039319* were increased by 45.44%, 17.46%, and

6.01%, while *Zm00001d029937* was reduced by 30.26% compared to their expression under WW condition (Fig. 5). The responsive patterns are consistent with our findings of drought stress at the seedling stage in Zheng58, *Zm00001d013992*, *Zm00001d029938*, and *Zm00001d039319* were significantly up-regulated, and *Zm00001d029937* was down-regulated by drought stress (unpublished data), suggesting their potential roles in drought tolerance. However, no obvious difference was observed for *Zm00001d039319*.

Allele effect of common candidate genes

SNPs within a gene might determine its function, therefore, it is of great interest to reveal the true association between SNP variation and target traits in a population. Haplotype analysis is a useful strategy to extract more and helpful to identify associations with the rare causal variants. According to the most significant SNP (S5_27121944) variation in *Zm00001d013992* (Table 3, Fig. 6A), 226 (2018) and 213 (2020) inbred lines were grouped into two haplotypes *HapA* (A) and *HapB* (G). Compare to *HapA*, rare *HapB* group had significant higher EBM (2.10 g) under WS condition in both 2018 and 2020 (Fig. 6B, Table S10). Five polymorphisms chr7.S_116288756, chr7.S_116288791, chr7.S_116288792, chr7.S_116285652, and chr7.S_116285655 were identified in *Zm00001d020506* (Fig. 6C, Table S11). 101 lines belong to the *HapA* (AACCT) with averaged EBM of 1.58 g and 1.79 g under WS and WW in 2017, while the other 107 lines carry the GTTTC haplotype with significant lower EBM of 1.35 g and 1.55 g (Fig. 6D). Three SNPs in *Zm00001d029937* were significantly associated with ASI under WW condition in 2018 (Table 3, Fig. 6E), and accordingly two haplotype AAC (*HapA*) and GGG (*HapB*) were obtained. Rare haplotype *HapA* showed longer ASI (> 10 days) than *HapB* whose ASI are 5.6 days and 6.6 days under WW and WS conditions (Fig. 6F, Table S12). The similar case was found for adjacent gene *Zm00001d029938*, that is the same 15 lines belongs to the *HapA* (TCGATAATC) group presenting longer ASI under both WW and WS condition (Fig. 6G, H, Table S13). PZE-103003226 was the only SNP identified in *Zm00001d039319* (Fig. 6I). 165 lines were grouped into *HapA* (G) shows weaker sensitivity to drought stress with shorter ASI and both WW and WS conditions (Fig. 6J, Table S14). For *Zm00001d042997*, 9 SNPs classify the 237 inbred lines into three groups (Fig. 6K, Table S15), 17 lines belong to lower frequency of *HapC* (TGACTTAA) presenting a larger ASI under both WS and WW conditions compared to *HapA* (AACTCCCG) and *HapB* (AACTCTAA), while no statistical difference was found between *HapA* and *HapB* (Fig. 6L).

Discussion

The current study showed that drought stress significantly increased the ASI (from 4.4 to 5.6 days) and reduced the PH (from 127.2 cm to 111.4 cm), indicating that silk extrusion is significantly delayed and plant architecture is greatly affected under drought stress. These results are in pipeline with the previous reports, which also reported significant extension in ASI and reduction in plant and ear height under WS condition (Messmer et al. 2009; Xue et al. 2013). Water deficit at the flowering stage delays or inhibits the growth and female ear development leading to reduced ear biomass, from 1.71 g to 1.41 g (Table 1). It was reported that osmotic stress limits the dry matter accumulation by approximately 50% during serious water shortage (Hejník et al. 2015; Zafar et al. 2020). Thus, considering the higher estimated heritability and vulnerable to drought stress, ear biomass could be an option for improving maize selection under water scarce condition (Edmeades et al. 2020; Setter 2012). In this study, PH was significantly correlated with yield related traits under both WW and WS conditions, plant height decreases less when exposed to drought can ensure sufficient "source" and exhibit better drought resistance. PH was positively correlated with EBM, suggesting a potential role of EBM in drought tolerance. No significant correlation was found between the ASI and ear biomass indicating the delayed silk extrusion has no

relationship with the ear development. Therefore, the three traits may not be tightly correlated with each other or the correlation may be disturbed due to the variation in the fluctuating environment in the field. Based on our funding, we proposed that the selection of taller plants, shorter ASI and bigger EBM might boost yield under water deficit.

GWAS is a powerful strategy for genetic dissection of complex traits in plants. In this study, a total of 71, 159 and 21 SNPs were significantly ($P < 10^{-5}$) associated with ASI, EBM, and PH by GWAS, which were located in 36, 81 and 16 genes, respectively. To date, only few GWAS studies have been conducted in maize drought tolerance under complex field condition, and few stable genomic regions were detected across various mapping populations and environments (Li et al. 2016; Xue et al. 2013). Multiple maize nested association mapping populations were tested under two contrasting water regimes for seven drought-related traits including ASI, PH, and yield related traits, and resulted in hundreds of promising QTLs and candidate genes through GWAS and linkage mapping (Li et al. 2016). In addition, many other candidate genes were detected to be associated with drought tolerance correlated yield and agronomic traits (Farfan et al. 2015; Hao et al. 2011; Lu et al. 2010; Xue et al. 2013), however, none of them were found in this study, which may be due to the different growth and climate conditions, and drought treatment. Fortunately, few candidate genes co-localized with reported QTLs in our study. Uncharacterized gene *GRMZM2G173084*, associated with ASI-WW in 2017, overlapped in the QTL for both ASI-WW and ASI-WS which was detected by joint linkage analysis in a CN-NAM population (Li et al. 2016). *Zm00001d003939*, encoding a 11- β -hydroxysteroid dehydrogenase 2, is the candidate gene for PH-WS-2017 located in a consistent QTL for WW-PH which was identified in both CN-NAM and US-NAM population. In addition, several overlapping genes were identified under water treatments (Table 3) among different years, while only *Zm00001d013992* was commonly identified in two-year environment, which implied maize drought tolerance is a complex trait, highly affected by environment and treatment.

Members of zinc finger family protein play critical roles in plants growth and developmental processes, including flowering, senescence, and also abiotic stress responses (de Lorenzo et al. 2007; Yan et al. 2017). A C₂H₂ zinc finger transcription factor determines stomatal closure by regulating genes related to H₂O₂ homeostasis, such as peroxidases, glutathione S-transferase and cytochrome P450s, thereby modulate drought response in rice (Huang et al. 2009). Here, we identified *Zm00001d042997*, encoding a HIT-type Zinc finger family protein was associated with ASI under both WW and WS conditions, indicating a potential conserved abiotic stress tolerant role of Zinc finger family protein. F-box domain containing protein ARABIDILLO-1 is conserved in plants, involved in root architecture development and functions during rice abiotic stress mainly through regulating root branching and lateral root development (Mu et al. 2010; Sharma et al. 2014). It was reported that Arabidillo-1 mediated protein degradation most probably through modulating the GA3 signalling pathway (Mu et al. 2010). However, another research revealed that ARABIDILLO 1 knock out and overexpression plants responded normally to auxin and abscisic acid (Nibau et al. 2011). *Zm00001d029938*, encoding ARABIDILLO 1 in maize, was associated with ASI in this study. However, whether it contributes to drought tolerance through a hormone dependent or independent way is still unknown. For EBM, a SNP (S5_27121944) which annotated as Pyridoxal phosphate dependent transferase (*Zm00001d013992*) was consistently associated with EBM for consecutive two years under drought regime (Table 3). Pyridoxal phosphate (PLP) is an active form of pyridoxine (vitamin B6) which functions as coenzyme in several reactions such as decarboxylation, deamination as well as transamination. The PLP dependent enzymes mainly perform in amino acid biosynthesis and the metabolism of its derived metabolites.

Therefore, it is interesting to speculate that *Zm00001d013992* might involve in amino acid metabolism and then promotes ear development in maize under drought stress condition.

The drought responsive patterns of these genes suggest that *Zm00001d013992*, *Zm00001d029938*, and *Zm00001d039319* might positively while *Zm00001d029937* might negatively correlate with drought tolerance, suggesting their potential roles in the drought tolerance. Based on the haplotype analysis, we identified favourable and rare allele for candidate genes. The lead SNP S5_27121944 (A/G) in *Zm00001d013992* separates the association panel into two groups, and only around 6% lines (BY855 and BY4960, etc) carries the favourable haplotype exhibits higher ear biomass, 3 g under WS (Fig. 6A). These lines and causal SNPs could be selected to cultivate drought tolerant lines with big ear biomass. The abovementioned results might be meaningful for genetic improvement of drought tolerant maize aimed at shorten ASI under both WW and WS. Haplotype analysis displayed potential causal SNPs of candidate drought tolerant genes and thereby will benefit maize breeding in both genome selection and genome editing.

The findings of this study provide insights into the genetic basis of drought tolerance at the flowering stage especially for the female inflorescence's development. Those overlapping genes are proposed as candidate genes for drought tolerance in maize. Moreover, it also provides genetic resources which could be used for drought-tolerance marker development and benefit for future marker assisted or genome-wide selection for drought tolerant maize breeding. Future research might explore the association signals in depth and the role of nonsynonymous SNPs, candidate genes function through mutation and gene editing method as well as the underlying molecular mechanism of maize ear and silk development under water deficit condition.

Declarations

Supplementary Information The online version contains supplementary tables available at XX.

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Author contribution statement JZ and PL designed this project. SUK, YZ, ZC, XZ, GZ, NZ, and PL performed phenotype investigation and data analysis. SUK and PL wrote the manuscript. PL and JZ edited this draft. All authors have read and agreed to the published version of the manuscript

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Compliance with ethical standards

Conflicts of Interest The authors declare that they have no conflict of interest.

Data Availability GWAS results are provided in supplementary data and the genotypic data of the association panel is available on request from the corresponding author.

References

1. Bänziger M, Edmeades GO, Beck D, Bellon M (2000) Breeding for Drought and Nitrogen Stress Tolerance in Maize: From Theory to Practice. Mexico, CIMMYT
2. Bolaños J, Edmeades GO (1996) The Importance of the Anthesis-Silking Interval in Breeding for Drought Tolerance in Tropical Maize. *Field Crop Res* 48:65–80
3. Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES (2007) TASSEL: Software for Association Mapping of Complex Traits in Diverse Samples. *Bioinformatics* 23:2633–2635
4. Browning BL, Browning SR (2016) Genotype Imputation with Millions of Reference Samples. *Am J Hum Genet* 98:116–126
5. Bruce WB, Edmeades GO, Barker TC (2002) Molecular and Physiological Approaches to Maize Improvement for Drought Tolerance. *J Exp Bot* 53:13–25
6. Bukowski R, Guo X, Lu Y, Zou C, He B, Rong Z, Wang B, Xu D, Yang B, Xie C (2018) Construction of the Third-Generation *Zea Mays* Haplotype Map. *Gigascience* 7:1–12
7. Daryanto S, Wang L, Jacinthe PA (2016) Global Synthesis of Drought Effects on Maize and Wheat Production. *PLoS ONE* 11:e0156362
8. de Lorenzo L, Merchan F, Blanchet S, Megías M, Frugier F, Crespi M, Sousa C (2007) Differential expression of the TFIIIA regulatory pathway in response to salt stress between *Medicago truncatula* genotypes. *Plant Physiol* 145:1521–1532
9. Dong Z, Xu Z, Xu L, Galli M, Gallavotti A, Dooner H K, Chuck G (2020) Necrotic upper tips1 mimics heat and drought stress and encodes a protoxylem-specific transcription factor in maize. *Proc Natl Acad Sci U S A* 117:20908–20919
10. Edmeades GO, Bolanos J, Elings A, Ribaut JM, Bänziger M, Westgate ME (2000) The Role and Regulation of the Anthesis-silking Interval in Maize. In: Westgate M, Boote K, Knievel D, Kiniry J. *Physiology and modeling kernel set in maize*. CSSA Special Publications, pp 43–73
11. Farfan ID, De La Fuente GN, Murray SC, Isakeit T, Huang PC, Warburton M, Williams P, Windham GL, Kolomiets M (2015) Genome Wide Association Study for Drought Aflatoxin Resistance and Important Agronomic Traits of Maize Hybrids in the Sub-Tropics. *PLoS ONE* 10:e0117737
12. Fu J, Cheng Y, Linghu J, Yang X, Kang L, Zhang Z, Zhang J, He C, Du X, Peng Z (013) RNA Sequencing Reveals the Complex Regulatory Network in the Maize Kernel. *Nat Commun* 4:1–12
13. Gupta A, Rico-Medina A, Caño-Delgado AI (2020) The Physiology of Plant Responses to Drought. *Science* 368:266–269
14. Hao Z, Li X, Xie C, Weng J, Li M, Zhang D (2011) Identification of Functional Genetic Variations Underlying Drought Tolerance in Maize Using SNP Markers. *J Integr Plant Biol* 53:641–652
15. Hejrnák V, Tatar Atasoy GD, Martinková J, Skalicky M (2015) Growth and photosynthesis of upland and pima cotton: Response to drought and heat stress. *Plant Soil Environ* 62:507–514
16. Holland JB, Nyquist WE, Cervantes-Martínez CT (2010) Estimating and Interpreting Heritability for Plant Breeding: An Update. John Wiley & Sons Ltd
17. Huang X, Chao D, Gao J, Zhu M, Shi M, Lin H (2009) A previously unknown zinc finger protein DST regulates drought and salt tolerance in rice via stomatal aperture control. *Genes & development* 23:1805–1817

18. Li C, Sun B, Li Y, Liu C, Wu X, Zhang D, Shi Y, Song Y, Buckler E, Zhang Z, Wang T, Li Y (2016) Numerous genetic loci identified for drought tolerance in the maize nested association mapping populations. *BMC Genomics* 17:1–11
19. Liu B, Zhang B, Yang Z, Liu Y, Yang S, Shi Y, Jiang C, Qin F (2021) Manipulating *ZmEXPA4* Expression Ameliorates the Drought-Induced Prolonged Anthesis and Silking Interval in Maize. *Plant Cell* 33: 2058–2071
20. Liu S, Wang X, Wang H, Xin H, Yang X, Yan J, Li J, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K, Qin F (2013) Genome-wide analysis of *ZmDREB* genes and their association with natural variation in drought tolerance at seedling stage of *Zea mays* L. *PLoS Genet* 9:e1003790
21. Lopes MS, Araus JL, Van Heerden PDR, Foyer CH (2011) Enhancing Drought Tolerance in C4 Crops. *J Exp Bot* 62:3135–3153
22. Lu Y, Zhang S, Shah T, Xie C, Hao Z, Li X, Farkhari M, Ribaut JM, Cao M, Rong T (2010) Joint Linkage-Linkage Disequilibrium Mapping Is a Powerful Approach to Detecting Quantitative Trait Loci Underlying Drought Tolerance in Maize. *Proc Natl Acad Sci U S A* 107:19585–19590
23. Mackay TFC (2001) Quantitative Trait Loci in *Drosophila*. *Nat Rev Genet* 2:11–20
24. Mao H, Wang H, Liu S, Li Z, Yang X, Yan J, Li J, Tran LS, Qin F (2015) A transposable element in a NAC gene is associated with drought tolerance in maize seedlings. *Nat Commun* 6:1–13
25. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M (2010) The Genome Analysis Toolkit: A MapReduce Framework for Analyzing Next-Generation DNA Sequencing Data. *Genome Res* 20:1297–1303
26. Messmer R, Fracheboud Y, Bänziger M, Vargas M, Stamp P, Ribaut JM (2009) Drought Stress and Tropical Maize: QTL-by-Environment Interactions and Stability of QTLs across Environments for Yield Components and Secondary Traits. *Theor Appl Genet* 119:913–930
27. Mu C, Chen N, Li X, Jia P, Wang Z, Liu H (2010) F-box protein Arabidillo-1 promotes lateral root development by depressing the functioning of GA3 in Arabidopsis. *J Plant Biol* 53:374–380
28. NeSmith DS, Ritchie JT (1992) Effects of Soil Water-Deficits during Tassel Emergence on Development and Yield Component of Maize (*Zea Mays*). *Field Crop Res* 28:251–256
29. Nibau C, Gibbs D, J Bunting KA, Moody LA, Smiles EJ, Tubby JA, Bradshaw SJ, Coates JC (2011) ARABIDILLO proteins have a novel and conserved domain structure important for the regulation of their stability. *Plant Mol Biol* 75:77–92
30. Obenchain V, Lawrence M, Carey V, Gogarten S, Shannon P, Morgan M (2014) Variant Annotation: A Bioconductor Package for Exploration and Annotation of Genetic Variants. *Bioinformatics* 30:2076–2078
31. Pang J, Fu J, Zong N, Wang J, Song D, Zhang X, He C, Fang T, Zhang H, Fan Y, Wang G, Zhao J (2019) Kernel Size-related Genes Revealed by an Integrated eQTL Analysis during Early Maize Kernel Development. *Plant J* 98:19–32
32. Ray DK, Mueller ND, West PC, Foley JA (2013) Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PLoS ONE* 8: e66428
33. Rosenberg NA, Huang L, Jewett EM, Szpiech ZA, Jankovic I, Boehnke M (2010) Genome-Wide Association Studies in Diverse Populations. *Nat Rev Genet* 11:356–366
34. Saini HS, Westgate ME (1999) Reproductive Development in Grain Crops during Drought. *Adv Agron* 68:59–96

35. Sari-Gorla M, Krajewski P, Di Fonzo N, Villa M, Frova C (1999) Genetic Analysis of Drought Tolerance in Maize by Molecular Markers II Plant Height and Flowering. *Theor Appl Genet* 99:289–295
36. Setter TL (2012) Analysis of Constituents for Phenotyping Drought Tolerance in Crop Improvement. *Front Physiol* 3:180
37. Sharma M, Singh A, Shankar A, Pandey A, Baranwal V, Kapoor S, Tyagi AK, Pandey GK (2014) Comprehensive expression analysis of rice Armadillo gene family during abiotic stress and development. *DNA Res* 21:267–283
38. Shiferaw B, Prasanna BM, Hellin J, Bänziger M (2011) Crops That Feed the World 6 Past Successes and Future Challenges to the Role Played by Maize in Global Food Security. *Food Secur* 3:307–327
39. Wang X, Wang H, Liu S, Ferjani A, Li J, Yan J, Yang X, Qin F (2016) Genetic Variation in *ZmVPP1* Contributes to Drought Tolerance in Maize Seedlings. *Nat Genet* 48:1233–1241
40. Wu X, Feng H, Wu D, Yan S, Zhang P, Wang W, Zhang J, Ye J, Dai G, Fan Y, Li W, Song B, Geng Z, Yang W, Chen G, Qin F, Terzaghi W, Stitzer M, Li L, Xiong L, Yan J, Buckler E, Yang W, Dai M (2021) Using High-Throughput Multiple Optical Phenotyping to Decipher the Genetic Architecture of Maize Drought Tolerance. *Genome Biol* 22:1–26
41. Xiang Y, Sun X, Gao S, Qin F, Dai M (2017) Deletion of an Endoplasmic Reticulum Stress Response Element in a *ZmPP2C-A* Gene Facilitates Drought Tolerance of Maize Seedlings. *Mol Plant* 10:456–469
42. Xue Y, Warburton ML, Sawkins M, Zhang X, Setter T, Xu Y, Grudloyma P, Gethi J, Ribaut JM, Li W (2013) Genome-Wide Association Analysis for Nine Agronomic Traits in Maize under Well-Watered and Water-Stressed Conditions. *Theor Appl Genet* 126:2587–2596
43. Yan J, Warburton M, Crouch J (2011) Association Mapping for Enhancing Maize (*Zea Mays* L) Genetic Improvement. *Crop Sci* 51:433–449
44. Yan Z, Jia J, Yan X, Shi H, Han Y (2017) Arabidopsis KHZ1 and KHZ2 Two Novel Non-Tandem CCCH Zinc-Finger and K-Homolog Domain Proteins Have Redundant Roles in the Regulation of Flowering and Senescence. *Plant Mol Biol* 95:549–565
45. Yang X, Gao S, Xu S, Zhang Z, Prasanna BM, Li L, Li J, Yan J (2011) Characterization of a Global Germplasm Collection and Its Potential Utilization for Analysis of Complex Quantitative Traits in Maize. *Mol Breeding* 28:511–526
46. Yu J, Buckler ES (2006) Genetic Association Mapping and Genome Organization of Maize. *Curr Opin Biotechnol* 17:155–160
47. Zafar SA, Patil SB, Uzair M, Fang J, Zhao J, Guo T, Yuan S, Uzair M, Luo Q, Shi J, Schreiber L, Li X (2020) *DEGENERATED PANICLE AND PARTIAL STERILITY 1 (DPS 1)* encodes a cystathionine β -synthase domain containing protein required for anther cuticle and panicle development in rice. *New Phytol* 225:356–375

Figures

Figure 1

Frequency distributions of ASI, EBM and PH under two water treatments.

Figure 2

Manhattan plots of ASI using MLM model in three years

Figure 3

Manhattan plots of ear biomass using MLM model in three years

Figure 4

Manhattan plots of plant height using MLM model in three years

Figure 5

Drought responsive pattern of candidate genes under WS and WW conditions in 197 inbred lines.

Figure 6

LD patterns and the allele effects of the most significant SNPs for candidate genes *Zm00001d013992* (A), *Zm00001d020506* (C), *Zm00001d029937* (E), *Zm00001d029938* (G), *Zm00001d039319* (I), and *Zm00001d042997* (K). Results of haplotype analysis for *Zm00001d013992* (B), *Zm00001d020506* (D), *Zm00001d029937* (F), *Zm00001d029938* (H), *Zm00001d039319* (J), and *Zm00001d042997* (L). Blue: HapA; Pink: HapB; Green: HapC

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