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Identification of loci contributing to maize drought tolerance in a genome-wide association study

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Abstract Genome-wide association studies (GWAS) have been used widely to analyze the genetic control of complex traits in crops. In the present study, seven related phenotypic traits were analyzed in combination to study their association with 41,101 SNPs in 201 maize inbred lines that had been evaluated in seven environments (year/location combinations) under water-stressed (WS) or well-watered (WW) regimes. By comparing the association signals with a fixed P value, GWAS showed that the number of association signals identified varied among traits and in different environments. Data that were missing under the severe water stress treatment had a great impact on the results of this GWAS. A total of 206 significant SNPs were associated with 115 candidate genes for drought tolerance and related traits including final grain yield, total number of ears per plot, kernel number per row, plant height, anthesis-silking interval, days to anthesis (DtA), and days to silking (DtS). Among these, four genes were associated with at least two different related traits, and six genes associated with traits were detected in at least two environments under water stress. Nine candidate QTL identified by GWAS were also discovered, three of which co-located to a consensus QTL region meta-analyzed by linkage mapping for drought tolerance. Some regulatory genes related to abiotic stress responses might also make a strong contribution to drought tolerance. The comprehensive information presented here regarding consensus QTL combined with candidate genes derived from GWAS provides an important reference tool for improving maize drought tolerance.

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Introduction

Maize (*Zea mays* L.) is the world's largest cereal crop for food, forage, oil, and raw material for the production of energy (http://faostat.fao.org/, 2009). Statistics have shown that overall growth in cereal yields slowed in the 1990s. However, maize yields in developing countries maintained their upward momentum, while gains in wheat and rice yields slowed markedly (http://www.fao.org/docrep/004/y3557e/y3557e08.htm). This trend indicates that maize will likely become the main staple crop for ensuring future food security. However, drought and other abiotic stresses often affect maize yield, especially in the rain-fed cultivation areas that account for about 74 % of total crop-growing areas and in marginal areas into which farming continues to expand (Biradar et al. 2009). Although drought stress might happen whenever maize is grown without irrigation, the plants area cutely susceptible to the negative effects of drought stress immediately before and during flowering (Agrama and Moussa 1996). When drought stress occurs during this critical period, maize plants stop growing and initiate a series of drought-stress defense mechanisms to ensure survival. At the same time, the length of the anthesis-silking interval (ASI) increases significantly, and ear number, grain number, and final grain yield decrease. Morphological changes also occur, including reduced plant height, leaf curling, and barren ears (Hao et al. 2011).

Drought tolerance is a complex trait whose expression includes changes in morphology, physiology, and biochemistry. Identifying the functional polymorphic variations lied on candidate genes is an efficient way to enhance drought tolerance by marker selection in maize and to improve crop yield in affected areas (Hadiarto and Tran 2011; Zhang et al. 2011). Until now, many quantitative trait loci (QTL) related to drought tolerance have been identified on all maize chromosomes by linkage mapping in populations derived from crosses between two parents, and some linked markers have already been used successfully for selection in backcross breeding programs (Hao et al. 2010; Liu et al. 2015b; Tuberosa and Salvi 2006). Undoubtedly, QTL research has promoted our understanding of the mechanisms of drought tolerance in general; Many genes that influence stress-tolerance have been identified and their function analyzed in transgenic plants (Yang et al. 2010) (http://www.plantstress.com/). However, traits such as drought stress tolerance that are under highly complex genetic control are difficult to manipulate and have thus far been deployed less often in improved cultivars.

Since the sequencing of the maize inbred lines B73 and Mo17, many new genes have been discovered. Furthermore, numerous single nucleotide polymorphisms (SNPs) and insertion–deletion polymorphisms (Indels) have been discovered by comparing the sequences of 27 diverse inbred lines (Gore et al. 2009). Several SNP variants and Indels have been linked to phenotypic traits such as adaptation to abiotic stresses in plants (Rafalski 2002). In human genetics, the development of SNP assays and the appropriate statistical methods enabled genome-wide association studies (GWAS) based on linkage disequilibrium (LD) to scan for novel loci associated with human disease phenotypes (Klein et al. 2005). GWAS is also a powerful tool for analyzing quantitative traits in plants that supplements QTL mapping in populations derived from biparental crosses and has been used in a wide range of crops (Yan et al. 2011). In maize and rice, a metabolome-based GWAS was used to identify candidate genes involved in important metabolic traits (Matsuda et al. 2015; Wen et al. 2014a, b). Another GWAS corrected for population structure identified 42 SNPs in 33 genes associated with 126 trait × environment × treatment combinations (Xue et al. 2013). Further, GWAS of quantitative resistance to maize rough dwarf disease (MRDD) in maize was performed and identified SNPs and fragments within bin 8.03 associated with MRDD resistance (Liu et al. 2014). With the MaizeSNP50 Genotyping BeadChip containing 55,000 SNPs developed by Illumina Inc., complex heterotic traits can now be analyzed and candidate genes affecting plant height can be studied (Riedelsheimer et al. 2012; Weng et al. 2011). The 55,000 SNPs on the BeadChip are evenly spaced markers that represent two-thirds of the 19,350 genes predicted to exist in the maize genome, with from one to 17 SNPs per gene (Weng et al. 2011; Xue et al. 2013; Yang et al. 2014). In the present study, phenotypic data fora 201-line sub-group from a panel of 284 diverse inbred lines evaluated for drought responses in multiple environments were used with results from the MaizeSNP50 BeadChip for GWAS. With these high-density SNP markers and drought-tolerance phenotypes for multiple environments,

associated SNPs and some underlying genetic loci that contribute to maize drought tolerance have been revealed.

Materials and methods

Plant materials, growth conditions, and tissue sampling for GWAS

A total of 201 diverse inbred lines (Hao et al. 2011; Weng et al. 2011) from five representative maize subpopulations in breeding programme, most of which were derived from commercial Chinese hybrid parents with clear pedigrees, were chosen for genotyping and SNP calling based on our previous studies (Liu et al. 2015a; Xie et al. 2007; Zhang et al. 2011). Four lines were added as replicated quality controls in six independent BeadChip panels. All plant materials were grown in the greenhouse under normal conditions to the three-leaf stage only for sampling frozen seedling leaves for genotyping. DNA was extracted from each sample using the CTAB method (Murray and Thompson 1980). Details regarding sample preparation have been described previously in Weng et al. (2011). The genomic DNAs of four common inbred lines (Qi319, Huangzao4, Ye478, and Dan340) were used as control sand were genotyped twice to evaluate genotyping quality. SNP genotyping was performed by EmeiTongde (Beijing) using the Illumina MaizeSNP50 BeadChip (Illumina, Inc.) (Liu et al. 2015a).

Field evaluation and selection criteria for drought tolerance

In the present study, a sub-group of 201 lines was used for GWAS of drought tolerance, which was evaluated by phenotyping plants in the field and designed as an alpha lattice design in seven environments (designated as 2006HN, 2007HN, 2008HN, 2007XJ, 2008XJ1, 2008XJ2, and 2009XJ) under either a WW or WS regime, as described in detail in Zhang et al. (2011). The investigation method for agronomical and morphological traits followed the standard of new variety selection used in regional trials of China and after detailed phenotypic analysis, seven traits including final grain yield (GY) of each plot, total number of ears per plot (EarN), kernel number per row (KerN) of ear,

plant height (PlaH), and ASI [calculated from days to anthesis (DtA) and days to silking (DtS)] were chosen as the representative phenotypic traits related to drought tolerance, as described in detail in Zhang et al. (2011). Repeatability (w^2) was calculated for all of the traits, in which environment was defined as a field $\times$ year combination, and measured as: $w^2 = \sigma_G^2/[\sigma_G^2 + (\sigma_{GE}^2/n) + \sigma_e^2/(nr)]$, where σ_G^2 is genotypic variance, σ_{GE}^2 represents genotype-by-environment interaction, σ_e^2 stands for error variance, n is the number of environments, and r is the number of replications (Xue et al. 2013). Correlations between GY and other traits were calculated using SPSS Statistics 19 software (IBM SPSS Inc.). For statistical purposes, the data for each of the phenotypes were divided into 10 classes (*n*) with spacing of 0.5 standard deviations. The Shannon–Weaver index (H′) was used to calculate the diversity of each trait: $H' = -\sum_{i=1}^{n} p_i \ln p_i$, where p_i is the relative proportion of entries belonging to the ith level of a particular trait (Waldman and Shannon 1948).

GWAS and statistical analysis of drought tolerance-related traits

The software PLINK (Purcell et al. 2007) was used to estimate LD as the parameter r^2 ($r^2 \geq 0.1$) between all SNPs on each chromosome with less than 20 % missing data. PowerMarker 3.25 and STRUCTURE version 2.3 were used to calculate SNP alleles (Liu and Muse 2005) and population structure, respectively. Kinship was measured with SPAGeDi (Hardy and Vekemans 2002) using 5000 SNPs with minor allele frequencies (MAF) $\geq$ 0.2. STRUCTURE was set to K = 5 according to results of our previous study and run three times with a burn-in period of 500,000 iterations and 500,000 replications (Liu et al. 2015a). SNPs with low MAF (<5 %) were excluded, which left 41,101 high-quality SNPs for use in the association analysis. Average marker density was one marker every 50 kb and 53.48 % of SNPs were within a 10-kb interval of the neighboring markers. A mixed linear model (MLM) implemented in the program TASSEL version 5.0 (Bradbury et al. 2007) was used for GWAS with 41,101 high-quality SNPs (MAF $\geq$ 0.05) and SNPs with $-\log_{10}(P) \geq 4.0$ were selected for candidate gene analysis. Two significant associated SNPs on the same chromosome within a distance of less than

6 kb were combined as a single candidate QTL. The physical distances between SNPs were based on the Maize B73 RefGen_v2. GO analysis to predict the functions of genes underlying each SNP was performed using the online analysis tool on the AgriGO website (http://bioinfo.cau.edu.cn/agriGO/).

Results

Phenotypic analysis of related traits for assessing drought tolerance responses under well-watered and water-stressed conditions

Drought stress had relatively negative impacts on each trait. The average performance across environments of the seven abovementioned traits is shown in Table 1. GY under drought in each plot varied greatly and exhibited a large standard deviation, with an average value of 594.32 g under both watering regimes and much lower GY under drought, with an average value of 345.41 g. These data indicated that this collection of natural populations was sufficiently diverse for the subsequent GWAS using GY as a major trait for the analysis of drought tolerance. PlaH under the WS treatment was also lower than normal, with a reduction in average PlaH of 48.89 cm under water stress. Additionally, under WS conditions, ASI increased in duration and other yield component traits all decreased, which showed that drought had an adverse influence on yield in many different ways. In contrast, ASI was longer and w^2 decreased observably under WS conditions, which was expected because drought stress tends to reveal genetic variation in ASI (Almeida et al. 2013). More severe stress results in longer ASI, which might reflect the sensitivity and drought-adaptive nature of this trait. Yield component values decreased, as did repeatability, which implied that the plants under the WS regime had experienced varying degrees of drought and that the performance of these traits could indicate drought response as selection criterion for drought tolerance. The seven traits were significant different between inbred lines or environments under WW and WS conditions (Table S1). Because some GY data for susceptible lines was missing due to evaluation under severe natural drought stress conditions, not all of the phenotypes investigated were subsequently used in GWAS.

There were relatively strong correlations between GY and other yield component traits, except for ASI under the WS condition, as expected. Correlation coefficients between traits are shown in Table 2. Correlations between GY and DtS, EarN, KerN, and PlaH under WS were relatively higher and significant, while the correlation between GY and DtA under WS was lower than under the WW condition. The frequency distributions of these six traits are shown in Fig. S1. Overall, the frequency distributions of traits in different treatments are close to normal ($|\chi| \leq 1$), except for GY under the WS condition and ASI, The lack of normality for these two traits resulted from missing data in several environments. However, data for the other five traits were entirely concentrated in standard deviation classes 4–7 (68.27–72.80 % of entries). Data for 77.96 % of the entries for PlaH under the WS condition were concentrated in standard deviation classes 3–7. Thus, the remaining data met the requirements for the genetic analysis of quantitative traits (Table 1). The diversity of each trait under these two distinct watering treatments was calculated using the Shannon–Weaver index, which indicated that there was abundant diversity in these seven traits in our inbred lines (Fig. 1).

Genome-wide association of markers with seven major phenotypic traits related to maize drought tolerance

To determine the exact contribution of each trait to drought tolerance, seven representative phenotypic traits measured under both WW and WS conditions in seven environments were chosen to evaluate the feasibility of GWAS in maize, as in Zhang et al. (2011). Using a MLM that combined population structure and kinship data, a total of 206 significant marker-trait associations were identified for seven maize traits under both watering regimes ($P \leq 1 \times 10^{-4}$) and significant association signals varied among these seven traits under both watering regimes in these seven environments (Fig. 2; Table S2). Most SNPs detected were associated with flowering time. A total of 38 SNPs were associated with DtA, 63 SNPs were associated with DtS, and 38 SNPs were associated with ASI. A total of 92 SNPs were detected under the WS treatment, and 113 were detected under the WW treatment, while 15 SNPs were detected under both watering conditions.

Table 1 Performance of seven maize traits across seven environments

| Trait	Treatment	Max	Min	Average	SD	WS/WW	Skew	Kurt	w²
PlaH	WS	261.40	62.67	148.39	42.57	0.75	0.25	−0.73	0.52
	WW	299.70	70.00	197.28	38.68		−0.06	−0.48	0.78
ASI	WS	21.00	−6.00	4.75	3.10	1.36	1.28	2.46	0.64
	WW	17.00	−3.50	3.50	2.54		1.76	5.31	0.89
DtA	WS	107.00	51.00	70.73	7.97	1.00	0.45	0.69	0.81
	WW	104.00	49.50	70.50	7.26		0.09	0.61	0.85
DtS	WS	107.00	54.00	74.59	7.78	1.02	0.36	0.67	0.79
	WW	105.00	52.00	73.38	8.14		0.21	0.43	0.84
KerN	WS	34.15	2.00	16.48	5.27	0.79	0.52	0.00	0.61
	WW	41.14	3.48	20.93	5.26		−0.06	0.12	0.89
EarN	WS	21.00	1.00	8.95	4.02	0.72	0.39	−0.36	0.61
	WW	24.50	1.50	12.37	3.70		−0.31	0.29	0.96
GY	WS	2015.00	6.67	345.41	352.02	0.41	1.96	2.96	0.41
	WW	2505.00	30.00	843.23	490.51		0.73	−0.04	0.78

GY grain yield, *EarN* total ears number per plot, *KerN* kernel number per row, *PlaH* plant height, *ASI* anthesis-silking interval, *DtA* days to anthesis, *DtS* days to silking, *WW* well-watered regime, *WS* water-stressed regime, *Skew* skewness, *kurt* kurtosis

Table 2 Phenotypic correlations between seven maize traits under WW (above diagonal) and WS (under diagonal) conditions

	DtA	DtS	ASI	PlaH	KerN	EarN	GY
DtA	1	0.95**	0.20**	0.46**	−0.16*	−0.08*	−0.15*
DtS	0.93**	1	0.44**	0.46**	−0.22**	−0.12*	−0.21*
ASI	−0.15*	0.12	1	0.11	−0.27**	−0.17*	−0.29**
PlaH	0.51**	0.51**	−0.01	1	0.22**	0.21**	0.31**
KerN	−0.06	−0.10	0.08	0.26**	1	0.44**	0.77**
EarN	−0.18*	−0.22*	−0.04	0.01	0.40**	1	0.71**
GY	−0.20**	−0.25**	−0.07	0.18*	0.57**	0.63**	1

*, ** Significance at $P < 0.05$ or 0.01, respectively

Fig. 1 Comparison of Shannon–Weaver indices for seven maize traits under WS or WW conditions
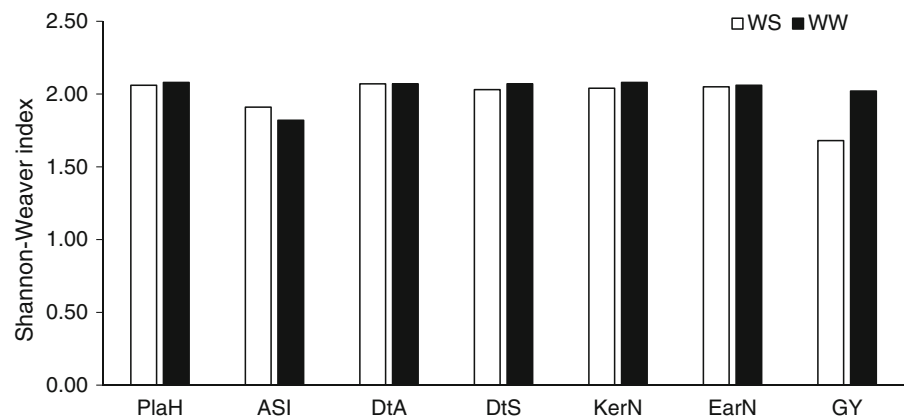
Moreover, five SNPs were related to two different phenotypic traits and six SNPs were detected in at least two environments under WS (Table S3). By searching for functional annotations regarding the genes in which each SNP is located, a total of 115 candidate genes were identified, including 75 genes of known function. The genes detected under the WS condition were involved in fewer processes than were those detected under the WW condition. SNPs in genes involved in multicellular organismal
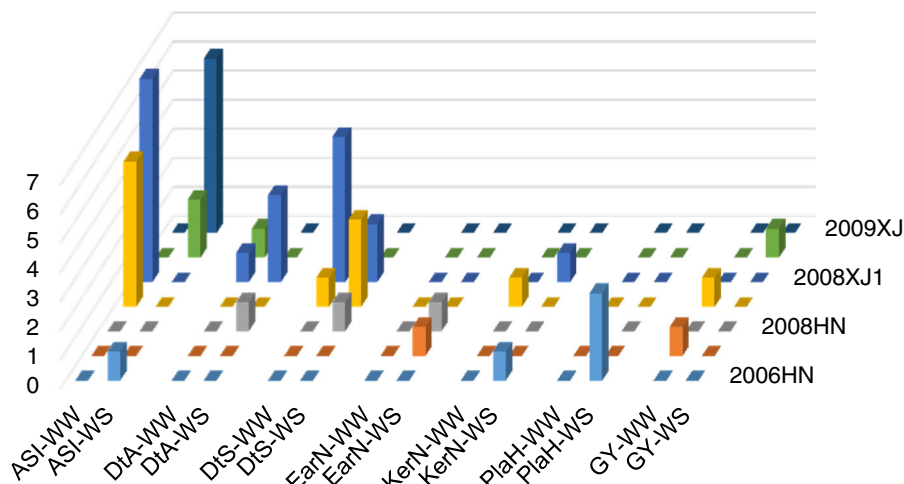
Fig. 2 The number of significant associations identified using different $-\log_{10}$ (P value) thresholds for seven traits related to drought tolerance under both regimes and seven environments. For each phenotype, the numbers of distinct peaks of association that were significant at nominal P value thresholds (*color scale*) are shown. In this figure, the seven environments are designated as 2006HN, 2007HN, 2008HN, 2007XJ, 2008XJ1, 2008XJ2, and 2009XJ, respectively. The phenotypic traits are abbreviated as grain yield (GY), total ears number per plot (EarN), kernel number per row (KerN), plant height (PlaH), anthesis-silking interval (ASI), days to anthesis (DtA), and days to silking (DtS), respectively. (Color figure online)

processes, macromolecular complexes, responses to stimuli, and biological regulation were not identified under WS conditions. This could be because some biological processes related to energy consumption might have slowed in the plants during adaptation to drought stress. SNPs in genes involved catalysis were relatively more abundant under WS conditions (Fig. 3).

Identification of candidate genes derived from QTL mapping that are related to drought tolerance in maize

Among the 206 significant associations we identified, a total of 92 SNPs were associated with 61 genes under the WS condition. Among these, five SNPs including PZE-108108866, PZE-108117143, PZE-110050326, PZE-110051604, and SYN1030 were related to both DtA and DtS, and were located within the four genes *GRMZM2G107498*, *GRMZM2G171716*, *GRMZM2G 397788*, and *GRMZM2G107491*, respectively (both SNPs PZE-108117143 and PZE-110050326 were located within the gene *GRMZM2G171716*) (Table S3). However, only one gene, *GRMZM2G107491*, has an annotated function; it is a zinc finger CCCH-type domain-containing protein ZFN-like 2, which is related to abiotic stress in *Oryza sativa* and *Arabidopsis thaliana*

(Wang et al. 2008).The functions of the other three identified genes need to be explored further to test their roles in abiotic stress tolerance.

Another six SNPs showed significant associations with drought response phenotypes in more than one environment (Table S3). PUT163a169259221125 was associated with PlaH; PZE-103126852 and PZE-103126857 were associated with DtA; and PZE-110050326, PZE-110051604, and PZE-110051621 were associated with DtS to significant degrees in at least two different environments (Table S3). The gene *GRMZM2G315769* is associated with DtA, contains two SNPs (PZE-103126852 and PZE-103126857), and encodesa CBL-interacting serine/threonine-protein kinase 15 that has been related to salt tolerance and ABA metabolism in rice, sorghum, and *Arabidopsis* (Chen et al. 2013; Gu et al. 2010; Guo et al. 2015; Pandey et al. 2015; Xiang et al. 2007).

Fifteen significant SNPs were revealed under both WW and WS conditions (Table S4). The SNPs SYN26509, SYN26514, and PZE-110017902 are associated with *GRMZM2G040968*, which encodes pyruvate, phosphate dikinase, a key enzyme in gluconeogenesis and photosynthesis. The SNP PZE-110017983 is associated with an aldehyde dehydrogenase gene, *GRMZM2G331368*, which plays an important role in stress responses (Huang et al. 2008).

Fig. 3 GO analysis of significant associated SNPs detected by GWAS. **a** The genes detected under the WS condition; **b** the genes detected under the WW condition
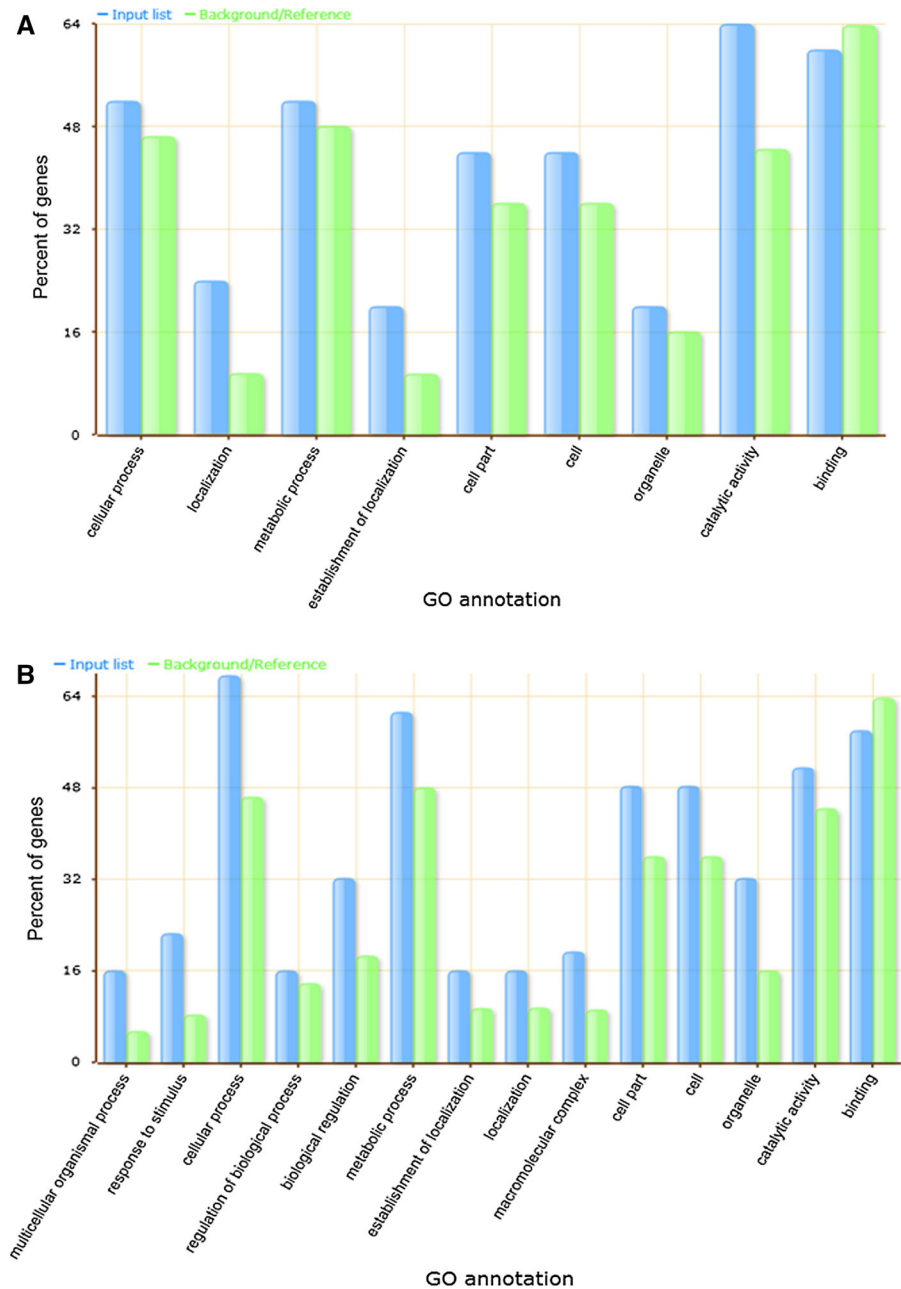
Finally, nine candidate QTL distributed on nearly every maize chromosome were identified (Table 3) in the present study. Three of these were located in the consensus QTL for drought tolerance that had previously been detected by meta-analysis of linkage mapping data (Hao et al. 2010; Li et al. 2010). These hot spots are now even more credibly related to

drought tolerance and should be studied further. Combined with the physical coordinates of the consensus genomic regions based on the B73 maize reference sequence version 2, 19 of the 115 associated genes identified in our study overlapped mQTL (meta-QTL; which are derived by combining results from previous QTL reports into consensus genomic regions

Table 3 Candidate QTL associated with drought tolerance identified by GWAS

No.	Chr.	SNP		Position	Candidate gene	Function
		Left	Right			
1	1	SYN35039	SYN35041	192278356–192281694	*GRMZM2G019806*	Helix-loop-helix DNA-binding
2	2	SYN18368	SYN18369	219554914–219554997	*GRMZM2G155849*	Cyclin-like F-box
3	3	PZE-103087821	PZE-103087822	141919832–141919862	*GRMZM2G174249*	Concanavalin A-like lectin/glucanase
4	3	PZE-103126852	PZE-103126857	182444667–182446866	*GRMZM2G315769*	Sodium:dicarboxylate symporter/CBL-interacting serine/threonine-protein kinase 15
5	5	SYN3700	PUT-163a-71328889-3180	215235057–215236168	*GRMZM2G101571*	Target SNARE coiled-coil region
6	6	SYN11615	SYN11613	77659356–77661610	*GRMZM2G342243*	Harpin-induced 1
7	8	SYN1030	PZE-108108866	160930651–160936029	*GRMZM2G107491*	U2 auxiliary factor small subunit/zinc finger CCCH type domain-containing protein ZFN-like 2
8	10	PZE-110036801	PZE-110036805	70074866–70075041	NA	NA
9	10	PZE-110051617	PZE-110051621	97242285–97242839	*GRMZM2G099172*	unknown

Two significant associated SNPs on the same chromosome separated by a distance of less than 6 kb were combined as a single candidate QTL

associated with trait variation)(Fig. 4). Among these 19 genes, eight have annotated functions and most are involved in abiotic stress regulation, protein synthesis, and transportation. These genes include proteinase inhibitor 12, a eukaryotic proteinase inhibitor that can inhibit serine peptidases belonging to the S1 family (Rawlings et al. 2004), a kinesin motor that might play a role in organelle transport (Brady 1995), a VHS subgroup gene involved in intracellular membrane trafficking (Misra et al. 2000), and a ribosomal protein L5. The transcript GRMZM2G107114_P01, which contains SNPPUT-163a-148967626-627, encodes a cytoplasmic anti-proliferative protein known as prohibitin, which might be related to maize development (Wen et al. 2014a, b). The functions of other genes identified here that are related to stress responses need to be further explored. Certain regulatory genes related to abiotic stress-response traits might contribute strongly to drought tolerance, because several of the SNPs with high $-\log_{10}$ (P) values identified here were all associated with regulatory genes.

Discussion

The importance of secondary traits for analyzing maize drought tolerance

Drought tolerance is difficult to measure and define. Plant breeders know that plant secondary traits are important indicators of stress tolerance and that their evaluation can help breeders to make selection decisions to improve stress tolerance. According to previous studies (Fischer and International Rice Research Institute 2003; Zhang et al. 2011), selection for secondary traits should proceed with the following points in mind: traits should be (1) heritable and variable, (2) genetically correlated with grain yield, and (3) easy and inexpensive to measure. Secondary traits such as flowering time, plant architecture, and yield components have been widely used for evaluating drought tolerance among different species (Cabello et al. 2014; Gomez et al. 2010; Lu et al. 2011; Trijatmiko et al. 2014). The present study evaluated a highly diverse maize germplasm collection in which flowering time, yield components, and final GY varied

Fig. 4 Meta-QTL for drought tolerance and significant SNPs distributed on the maize chromosomes. From outside to inside, the *circles* represent the 10 maize chromosomes, SNPs, candidate QTL identified in our research, and the consensus QTL detected by Li et al. (2010) and Hao et al. (2010). All of the positions refer to the B73 RefGen_v2. The positions of consensus QTL DCQ1 to DCQ39 were transformed using the online tool Genome Browser at the MaizeGDB (http://www. maizegdb.org/). MQTL1 to mQTL79 are the same loci as those published in Li et al. (2010)
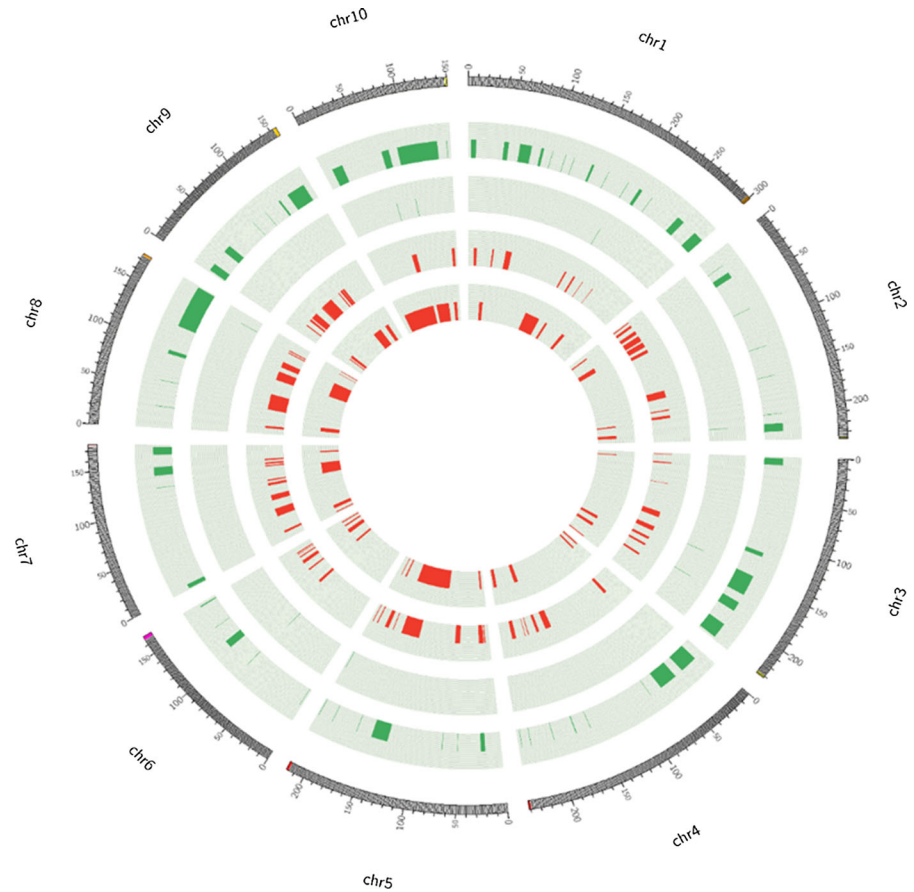
significantly, especially under WS conditions. Moreover, there were also correlations between GY and other traits, except for ASI under WS conditions, a result similar to that of Xue et al. (2013), which indicates that these secondary traits are good predictors of drought tolerance (Table 2). EarN instead of KerN in WS treatments, was the most closely correlated agronomic trait with GY, indicating EarN was greatly impacted by WS conditions. The correlations between GY and DtA or DtS became closer under WS and the GWAS result also indicated that most of the significant SNPs detected were associated with DtA and DtS. The detection of these SNPs might indicate that control of flowering time could help improve maize drought tolerance and that these SNPs will be useful for the further identification of genetic loci controlling drought tolerance.

The number of distinct peaks of significant association signals identified for each phenotype using gradual *P* value thresholds is shown in Table 4. The number of signals identified in each of seven environments varied greatly and the signal intensities for all seven traits varied. Missing data under severe water stress had a great impact on the GWAS. The level and duration of water stress should be better controlled to avoid missing data that can interfere with genetic analysis. Quantile–quantile plots (Fig. 5) showed that variation was reduced and more accurate results were obtained compared with the GLM method, which indicates that kinship could effectively reduce false positives that occur due to population stratification and relatedness between individuals, as also shown by other research (Akhatar and Banga 2015; Gajardo et al. 2015; Gouy et al. 2015). Due to missing data in some environments or the absence of variation among populations, association analysis was not performed for all traits across all seven environments in our study.

Table 4 Significant SNP-trait associations for seven maize traits

Trait	2006HN	2007HN	2008HN	2007XJ	2008XJ1	2008XJ2	2009XJ	Total	SNPs	Genes
PlaH										
WW	–	3	0	0	4	0	2	24	22	19
WS	12	0	–	1	2	–	0			
ASI										
WW	–	1	–	7	13	2	1	38	36	25
WS	2	–	0	1	0	3	8			
DtA										
WW	–	0	0	7	9	6	3	38	30	23
WS	0	–	5	5	3	–	0			
DtS										
WW	–	–	2	7	25	1	0	63	44	28
WS	0	–	5	6	16	–	1			
EarN										
WW	–	5	0	3	–	0	0	14	14	8
WS	1	0	2	3	0	0	0			
KerN										
WW	–	2	–	4	0	1	1	17	17	16
WS	4	0	–	1	1	2	1			
GY										
WW	–	2	–	3	–	0	0	12	12	8
WS	0	2	–	0	2	2	1			
Total								206	175	127

The phenotypic traits are abbreviated as *GY* grain yield, *EarN* total ears number, *KerN* kernel number per row, *PlaH* plant height, *ASI* anthesis-silking interval, *DtA* days to anthesis, *DtS* days to silking. Treatments are abbreviated as *WW* well-watered, *WS* water stressed, "–" indicates that the trait was not analyzed due to missing data. All of the associations were measured at $P \leq 1 \times 10^{-4}$

GWAS for identifying associations between genes and responses to abiotic stress in maize

Following the development of high-throughput automated sequencing and the completion of the genome sequences of many plant species, GWAS technology has been widely used for the genetic interpretation of many important plant phenotypes (Atwell et al. 2010; Liu et al. 2014; Xue et al. 2013). This application depends on the identification of large numbers of molecular markers such as SNPs across entire plant genomes. It also depends on the precise phenotyping of traits in multiple environments. Thus, the maizeSNP50 BeadChip used in our study has been useful for GWAS of traits related to abiotic stress tolerance (Weng et al. 2011). If the size of the maize genome is estimated as 2500 Mb, the 56,000 SNPs on the SNP50 BeadChip result in an average of one SNP per 44,642

base pairs. And although the density of one SNP per 500 bases on the *Arabidopsis* chip is considerably higher than the density on the maize SNP50 BeadChip, it is lower than the SNP density commonly used in human studies (Atwell et al. 2010). Further, the 30-kb window (with an average LD decay of ~ 27.7 kb) was selected to fall within the estimated window for LD decay in our association panel, which was estimated as 44 kb. The genes within windows of this size were identified in the MaizeGDB (http://gbrowse. maizegdb.org/cgi-bin/gbrowse/maize_v1/) according to the positions of the closest flanking SNPs ($P \leq 1 \times 10^{-4}$) or supporting intervals. Many studies have shown that most SNPs associated with phenotypes are located very close to a genetic variant that influences the trait (Lu et al. 2012). Although there is a marked reduction in the number of associations of moderate significance (for example at $P = 10^{-5}$)
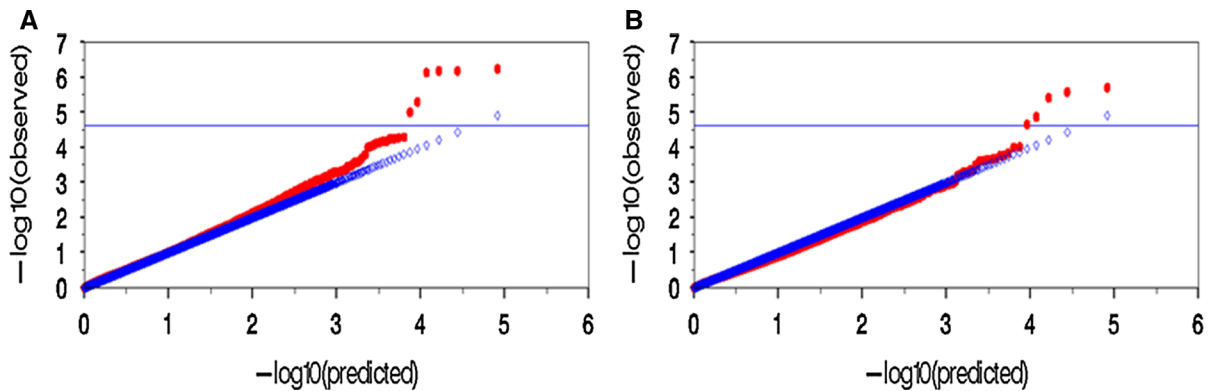
Fig. 5 Quantile–quantile plot for associations with ASI in the well-watered condition in Xinjiang in 2007 (2007XJ). **a, b** The differing results when associations were calculated with a GLM or a MLM

across phenotypes, an excess of highly significant associations persists (or has even become greater). Prediction accuracies ranging from 0.71 to 0.81 for SNPs on the SNP50 BeadChip have been verified in 285 inbred lines and their hybrid testcrosses with two testers for complex, highly polygenic traits (Riedel-sheimer et al. 2012). Using the same maize SNP50 BeadChip, other SNPs associated with drought tolerance have previously been detected (Xue et al. 2013); however, the SNPs revealed in that experiment and the present study differ. As with linkage mapping, the variation that results from the use of diverse of plant materials and environments indicates the complexity of environmental effects on drought tolerance phenotypes that are controlled by multiple genes. However, GWAS and QTL mapping are complementary techniques that can compensate for each other's limitations during the analysis of complex traits (Korte and Farlow 2013), such as when these methods were combined to study water-logging tolerance in maize seedlings (Zhang et al. 2013).

Significant marker-trait association signals related to drought tolerance further identified by mapping

The goal of GWAS is to resolve quantitative traits to their causal genetic loci and is typically followed by further functional analysis of genes that might be key metabolic regulators affecting traits of interest. For instance, GWAS has identified SNPs in genes affecting ABA levels in maize floral tissues during drought stress in a study that identified 1229 SNPs in 540 candidate genes. SNPs significantly associated with

maize drought tolerance were detected in the maize homologues of a MADS-box gene, a phaseic acid biosynthetic gene, a pyruvate dehydrogenase kinase gene, and an aldehyde oxidase gene (Setter et al. 2011). Using the same SNP assay and detailed phenotyping in 80 maize inbred lines under water stress, Hao et al. (2011), associated 29 SNPs in or near *dhn1*, *ivr1*, *myb*, and other genes related to drought tolerance response that were associated with at least two phenotypic traits in at least one environment. Similarly, 49 other significant SNPs associated with drought tolerance have been discovered on all maize chromosomes except chromosome 6 by GWAS using the Illumina chip (Farfan et al. 2015; Xue et al. 2013). Xu et al. (2014) reported that most of the non-synonymous SNPs related to phenotypic variation in drought tolerance in that study were located in bin 1.07. Combined with our results, these studies identified SNPs distributed on all chromosomes, with the most (39 SNPs) on chromosome 10 and the fewest (8 SNPs) on chromosome 6 (Fig. 4). Meta-analyses detected consensus QTL (Hao et al. 2010; Li et al. 2010), including nine candidate QTL for drought tolerance in maize, that were also identified in this study by GWAS.A total of 118 QTL related to drought tolerance have been found dispersed on all maize chromosomes, which will help to reveal candidate genes. Some hotspots have also identified in bins 1.06, 3.06, 6.05, and 10.04 using 203 QTL for secondary traits under WW and WS conditions (Fig. 3). Among these, an ∼8-Mb hotspot in bin 3.06 has been associated with drought tolerance in tropical maize (Almeida et al. 2014). These results indicate that the

Fig. 6 Manhattan plot of DtS-WS (days to silking under water-stressed condition) in 2009, Xinjiang
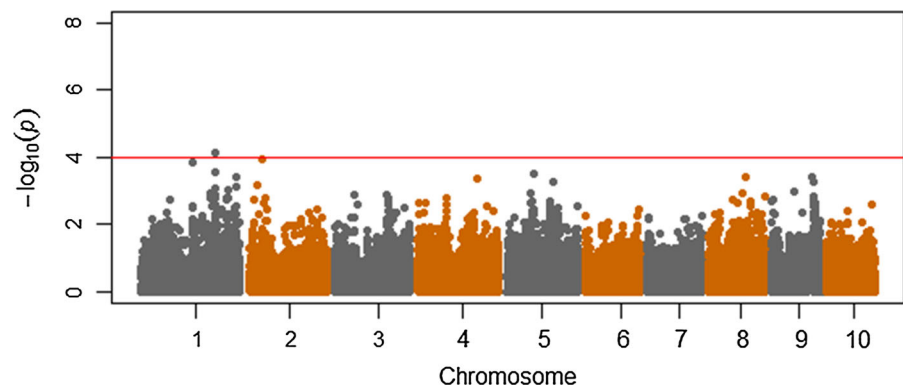
hotspot regions related to drought-tolerance are not evenly distributed. In addition, some studies have indicated that bin 1.03 (Hao et al. 2010) and bin 1.07 (Xu et al. 2014) might contain some genes related to drought tolerance. Our study also detected five SNPs in bin 1.07, but did not detect any significant signals in bin 1.03, which could be a false-negative result according to our Manhattan plot (Fig. 6). Further research could focus in more detail on these two regions.

Molecular markers associated with Vitamin A deficiency had been validated to efficiently increase proA carotenoid concentration in maize, and two functional markers associated with drought resistance were also been identified useful in MAS, which the markers were derived from linkage and association mapping (Babu et al. 2012; Liu et al. 2015b). For most complex quantitative traits such as drought tolerance and grain yield, transgenic strategies have not yet been very successful for genetic improvement. However, genomic selection and targeted genome editing should improve the prospects for engineering such traits after GWAS (Morrell et al. 2011). Traditional marker-assisted selection has also been relatively ineffective for complex traits. Because genomic selection predicts performance based on information from all markers, it can predict trait performance more accurately than can single associated loci (Jannink et al. 2010). The analysis in finer detail of regions that we had previously scanned by linkage mapping and identification by GWAS of associations between important loci and drought tolerance, will enhance breeding efforts for improved drought tolerance. For example, the drought related SNPs can be used as function makers in marker assisted selection (Li et al. 2012; Liu

et al. 2015b; Pan et al. 2012; Xu et al. 2012). The control of drought tolerance is complex, and many genes with minor effects are involved. So our next steps following GWAS will include more detailed sequencing and evaluation of genomic regions that influence drought tolerance to different degrees, and joint analyses to find support for candidate genes associated with drought tolerance.

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