BMC Genomics

RESEARCH Open Access

GWAS and transcriptome analyses unravel *ZmGRAS15* regulates drought tolerance and root elongation in maize

Dongmei Wang[1†], Xuyang Liu[1†], Guanhua He[1], Kailiang Wang[1], Yongxiang Li[1], Honghui Guan[1], Tianyu Wang[1], Dengfeng Zhang[1*], Chunhui Li[1*] and Yu Li[1*]

Abstract

Background Drought is a major abiotic stress affecting maize development and growth. Unravelling the molecular mechanisms underlying maize drought tolerance and enhancing the drought tolerance of maize is of great importance. However, due to the complexity of the maize genome and the multiplicity of drought tolerance mechanisms, identifying the genetic effects of drought tolerance remains great challenging.

Results Using a mixed linear model (MLM) based on 362 maize inbred lines, we identified 40 associated loci and 150 candidate genes associated with survival rates. Concurrently, transcriptome analysis was conducted for five drought - tolerant and five drought - sensitive lines under Well-Watered (WW) and Water-Stressed (WS) conditions. Additionally, through co-expression network analysis (WGCNA), we identified five modules significantly associated with the leaf relative water content (RWC) under drought treatment. By integrating the results of GWAS, DEGs, and WGCNA, four candidate genes (*Zm00001d006947*, *Zm00001d038753*, *Zm00001d003429* and *Zm00001d003553*) significantly associated with survival rate were successfully identified. Among them, *ZmGRAS15* (*Zm00001d003553*), a GRAS transcription factor considered as a key hub gene, was selected for further functional validation. The overexpression of *ZmGRAS15* in maize could significantly enhance drought tolerance through regulating primary root length at the seedling stage.

Conclusion This study provides valuable information for understanding the genetic basis of drought tolerance and gene resources for maize drought tolerance breeding.

Keywords Maize (*Zea mays* L.), Drought tolerance, GWAS, WGCNA, *ZmGRAS15*, Root

†Dongmei Wang and Xuyang Liu contributed equally to this work.

*Correspondence:
Dengfeng Zhang
zhangdengfeng@caas.cn
Chunhui Li
lichunhui@caas.cn
Yu Li
liyu03@caas.cn
[1]State Key Laboratory of Crop Gene Resources and Breeding, Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China

Background

Drought is a major abiotic stress affecting crop growth and reducing yields. Maize (*Zea mays* L.), being one of the most widely cultivated crops worldwide, is especially sensitive to drought stress. According to the Food and Agriculture Organization (FAO, 2022), annual maize yield losses across the world due to drought stress ranged from 15 to 20%. Therefore, improving the drought tolerance of maize and increasing its yield under drought stress are greatly important.

Drought tolerance is a complex quantitative trait involving coordinated morphological, physiological, and molecular mechanisms. In order to identify the genetic effects of drought tolerance in maize, many traits including root traits, grain yield, and survival rate were used to evaluate the drought tolerance at different development stage. Specifically, survival rate has become common physiological indexe used for assessing drought tolerance at the seedling stage [1]. During early development stage (V3-V6), maize exhibit heightened sensitivity to drought stress [2]. Survival rate was used as evaluation indicator, which reflects the ability of maize to maintain life activities and resume growth under extremely arid conditions. Root traits reflect soil moisture acquisition efficiency but require labour-intensive phenotyping methods like root excavation or imaging systems [3]. Grain yield remains the ultimate agronomic indicator, yet its evaluation is developmentally restricted to reproductive stages and confounded by genotype-environment interactions [4]. Collectively, survival rate assessment at the seedling stage offers two distinctive advantages: (1) Under controlled stress conditions, high experimental repeatability can be attained (2) The survival ability of plants is directly demonstrated. These characteristics make survival rate become an essential and integral part of the integrated drought evaluation system.

Genome-wide association studies (GWAS) have been a powerful method for excavating the candidate genes associated with target traits [5]. Currently, several genes related to drought tolerance have been identified by GWAS in maize, such as *ZmVPP1*, *ZmRtn16*, *ZmTIP1*, *ZmcPGM2 ZmSRO1d*, and *ZmCIPK3* [6–10]. Wang et al. (2016) used an association panel comprising of 368 maize inbred lines to identify 42 candidate genes associated with drought tolerance at the seedling stage [6]. Wu et al. (2021) evaluated the phenotypes of 368 maize inbred lines at multiple growth stages under normal watering and drought stress conditions and identified 2,318 candidate genes related to drought tolerance via GWAS, among which *ZmcPGM2*, which was involved in sugar metabolism, regulates the corresponding phenotypes and negatively regulates drought tolerance in maize [8]. Li et al. (2023) discovered 75 candidate genes related to maize root architecture through GWAS, among them, *ZmCIPK3* encodes calcineurin B-like-interacting protein kinase 3, was overexpressed, resulting in the promotion of root elongation and drought tolerance in maize [10].

RNA sequencing (RNA-seq) has been widely used for detecting genome-wide gene expression patterns in studies of the drought stress response in maize [11, 12]. Weighted gene co-expression network analysis (WGCNA), which is based on RNA-seq, is an effective method to narrow down the range of candidate genes [13], and WGCNA has become a popular technique for facilitating the discovery of core gene networks related to target traits [14]. GWAS combined with WGCNA has been applied to identify the genes responsible for stress tolerance in maize. For example, Ma et al. (2021) identified two hub genes involved in salt tolerance [15]. Li et al. (2021) identified 168 candidate genes associated with root architecture in response to salt, among which two candidate genes, *ZmIAA1* and *ZmGRAS43*, improve salt tolerance by regulating plant hormone signal transduction, phenylpropanoid biosynthesis, and fatty acid biosynthesis [16]. However, only a few genes related to drought tolerance in maize have been successfully identified by integrating GWAS and WGCNA.

Transcription factors play crucial roles in mediating the hubs of transcription networks and have been shown to function to improve stress tolerance in plants. Well-known stress-related TFs include members of the dehydration-responsive element-binding (DREB) basic helix–loop–helix (bHLH), NAM-ATAF-CUC2 (NAC) and basic leucine zipper (bZIP), WRKY, and MYB protein families [17–21]. Although transcription factor genes have been extensively studied, further research is still needed to identify additional novel transcription factors that are involved in stress responses. GRAS proteins are an important plant-specific transcription factor family that play crucial roles in gibberellin signal transduction, root development, light signaling, biotic stress, and abiotic stress responses [22]. GRAS transcription factors are induced by various stress signals, such as drought, high salinity, or extreme temperatures [23]. In the past few years, studies have demonstrated that the GRAS transcription factor *PAT1* (*Vitis amurensis*), which was overexpressed in plants, confered more drought, cold, and salinity tolerance in *Arabidopsis* [24]. Moreover, the GRAS protein AtSCL14 interacts with the TGACG motif-binding factor (TGA) TF and modulates the stress response in *Arabidopsis* [25]. Additionally, overexpression of *GmGRAS37* improved drought and salt tolerance in soybean hairy roots [26].

In this study, to explore the genetic architecture underlying drought tolerance in maize, we performed a GWAS with a panel of 362 maize inbred lines and conducted a transcriptome analysis based on 10 maize lines. By integrating the results of GWAS and WGCNA, we aimed

to uncover candidate genes underlying drought stress at the seedling stage in maize. Among the identified candidate genes, we specifically selected *ZmGRAS15*, a GRAS transcription factor, for further functional validation. This study aims to provide a theoretical basis for genetic improvement of drought tolerance and valuable gene resources for more efficient and targeted maize drought tolerance breeding.

Materials and methods

Plant materials and growth conditions

In total, 362 maize inbred lines were selected as members of the association panel used in this study [27]. The experiment was performed in a greenhouse (28 °C, 14 h light/10 h dark cycle) at the Chinese Academy of Agricultural Sciences (Beijing). This pot experiment was repeated three times from 2022 to 2023. The materials were planted in boxes (60 cm × 40 cm × 10 cm) containing 12 kg of enriched soil (nutrient soil: vermiculite = 1:1). Moreover, the enriched soil is mixed with water in advance to ensure that the soil moisture in the boxes is consistent. In each box, ten inbred lines were grown in ten rows with fifteen plants per row. For the drought treatment, irrigation was stopped at the three-leaf stage, which lasted for 25 days. The plants were subsequently rewatered, and the survival rate of each maize inbred line was determined after seven days. The test was repeated three times, and the results were averaged. Ten inbred lines, including Huangzaosi (HZS), K12, G71, G90, Chang7-2 (C7-2), Lv28, Qi319, Zheng58 (Z58), B73, and Ye478, were selected for transcriptomic analysis. The seeds were germinated in paper rolls (Anchor, USA). After two weeks, the seedlings were moved to a $60 \times 40 \times 10$ cm cultivation box containing 12 kg of growth medium (growth substrate: soil = 1:1). For each cultivation box (replicates), ten inbred lines were grown in ten rows, and 15 plants were included in each row. Two water treatments were used: (i) drought treatments, in which the plants were watered with 12 kg of water when the seedlings were moved to cultivation boxes, were no longer irrigated and drought was continued for 25 days, and (ii) the Well-Watered treatments, in which the plants were watered with 12 kg of water when the seedlings were moved and watered every five days to maintain the water content. Three replicates were set for both the WS and WW treatments.

GWAS

The association panel comprising 362 inbred lines was genotyped via the resequencing method. After quality control (missing rate ≤ 20%, heterozygosity ≤ 20% and minor allele frequency (MAF) ≥ 0.05), 8,219,596 high-quality SNPs were retained for GWAS in this study. A GWAS was performed for survival rates via a linear mixed model (MLM) in EMMAX software [27]. The number of effective SNPs was calculated in the simpleM program in R [28, 29]. A total of 760,662 independent SNPs were ultimately obtained, and the threshold for significant trait marker associations was set to 6.5×10^{-8} (Bonferroni-corrected threshold of $\alpha = 0.05$). Given the rigor of the mixed linear model, we conservatively chose 1.0×10^{-5} as the suggestive threshold. The significant SNPs within 60 kb were grouped into quantitative trait loci (QTLs), and the most significant SNP was selected as the leading SNP. On the basis of the significant SNPs under the threshold of $P < 1 \times 10^{-5}$, the alleles with higher survival rates were defined as favourable alleles. The number of favourable alleles in each inbred line was counted, and the correlation between the cumulative number of favourable alleles and the trait value was analysed.

RNA sequencing and data analysis

When the water content of the drought treatment reached 30% (25 days after drought treatment in the greenhouse), the leaves of the plant materials were harvested for measurement of the leaf relative water content (RWC) and for RNA sequencing. Three replicates were set for both the WS and WW treatments. Briefly, the leaves of five randomly selected plants in each row were sampled, and the leaf RWC was measured [30]. The remaining sample was frozen in liquid nitrogen immediately and stored at -80 °C for subsequent RNA sequencing. The soil water content was obtained on the basis of the weights of the water and soil in each cultivation box. A total of 40 samples (ten maize genotypes from each of the two water treatments) were used for RNA isolation. Total RNA from the leaf samples was isolated via TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. The degradation of RNA was assessed by agarose gel electrophoresis. The quality and integrity of the RNA were examined via a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, DE) and a Bioanalyzer 2100 (Agilent Technologies, USA). The concentration of RNA was measured with a Qubit 2.0 fluorometer (Life Technologies, USA). A total of 3 μg of RNA per sample was used for library preparation, which was generated via the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's protocol. Library quality was assessed on an Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). The prepared libraries were subsequently sequenced on an Illumina HiSeq platform (Illumina, USA). The raw data in fastq format were first processed with quality control. The raw reads that contained adapters, reads containing poly-N sequences and low-quality reads (with greater than 50% bases whose Q_{phred} values were lower than 30) were removed to obtain clean data. The sequence and gene

annotation of the maize reference genome B73_RefGen_v4 were downloaded from ftp://ftp.ensemblgenomes.org/pub/plants/release-47/fasta/zea_mays/ and ftp://ftp.ensemblgenomes.org/pub/plants/release-47/gtf/zea_mays/. The alignment index of the reference genome was built via Bowtie v2.2.3 [31]. The pair-end clean sequences were aligned to the reference genome via TopHat v2.0.12 [32].

Gene expression analysis

The fragments per kilobase of transcript per million fragments mapped (FPKM) values of genes were calculated via Cufflinks v2.2.1 software [33]. The relationships of the samples were analysed via correlation, clustering, and principal component analysis (PCA) on the basis of the FPKM values of the genes. To identify the drought-responsive genes, the DEGs were detected via pairwise comparisons between the different water treatments of each genotype via DESeq v1.18.0 [34] on the basis of the read counts of genes obtained via HTSeq v0.6.1 [35]. DESeq provides statistical routines to determine differential expression via a model that is based on the negative binomial distribution. The resulting *P* values were adjusted via Benjamini and Hochberg's approach to control the false discovery rate (FDR). The genes with|log2 (fold change)| > 1 and FDR < 0.05 were considered differentially expressed.

WGCNA and hub gene identification

WGCNA was used to construct co-expression networks. Gene co-expression analysis was performed in 10 samples under WS and WW conditions via the step-by-step method in the "WGCNA" package of Rv3.5.1 [13]. The soft threshold of the co-expression network was selected by $R^2 > 0.9$ based on the FPKM of all genes in the samples, the soft threshold of the samples under drought stress was set to 16. All the FPKMs were transformed into a topological overlap matrix (TOM), hierarchical clustering analysis was performed, and the genes were classified into different co-expression modules via the dynamic tree cut method. The minimum number of genes for each co-expression module was subsequently set to 30. With 25 as the boundary, the clustered similar co-expression modules were merged, and the degree of association (MM) of the genes within the modules was calculated. For the results of the gene co-expression analysis under drought stress, the leaf relative water content data were combined, the correlations between different co-expression modules and the relative leaf water content were calculated via correlation analysis, and the correlations between gene expression and the relative RWC were calculated.

Gene expression analysis by qRT-PCR

To validate DEGs identified via RNA-seq, a real-time quantitative reverse transcription PCR (qRT-PCR) approach was implemented. Leaf tissues from four maize genotypes (comprising two drought-tolerant and two drought-sensitive lines) under WW and WS treatments were processed for RNA isolation. The relative quantitative outcomes were computed by normalizing to the endogenous reference gene, GAPDH. The relative gene expression levels were calculated in accordance with the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were executed for all the reactions. The primers used in this study were listed in Additional file: Table S6.

Drought - tolerance phenotypic identification of Transgenic maize

To elucidate the function of *ZmGRAS15* under drought stress, two overexpression lines were obtained from the Center for Crop Functional Genomics and Molecular Breeding of China Agricultural University. The drought tolerance experiments were conducted in a greenhouse (28 °C, 16 h light/8 h dark light cycle) at the Chinese Academy of Agricultural Sciences (Beijing). The seeds were rolled with germination paper. After growing for 4–5 days, samples were taken, and bar test strips were used to detect positive and negative samples. The positive plants were subsequently transplanted into cultivation boxes. After normal growth for 14 d in the cultivation box, wild-type (WT) ND101 and transgenic plants with consistent growth were transplanted into soil pots (containing a peat soil: nutrient: vermiculite mixture at a volume ratio of 1:1:1). At the three-leaf seedling stage, the plants were subjected to water deprivation for 25 d to simulate drought conditions, followed by rehydration. After 5 d of rehydration, the surviving plants were counted and photographed. To measure the primary root length of the transgenic and WT plants, we used the hydroponic method for phenotypic analysis at the seedling stage. When the seedlings had grown for 7 d under normal conditions, 20% PEG treatment was performed to simulate drought stress, and those without treatment were used as controls. After 7 d of drought stress, the plants were sampled to measure the primary root length.

Results

GWAS for maize drought tolerance at seedling stage

To systematically assess the impact of genetic variants on drought tolerance at the maize seedling stage, we conducted phenotypic analysis on the drought tolerance of a natural population consisting of 362 elite inbred lines. We used the survival rate to represent drought tolerance at the maize seedling stage. There is a great deal of phenotypic variation in drought tolerance within the population. Approximately 22% of the maize lines had survival rates greater than 0.5, while 13% of the maize lines were highly sensitive to drought, with a survival rates approaching zero. Using a MLM based on

approximately 8,219,596 M high-quality SNPs, we performed a GWAS for survival rates. Under the suggestive threshold of $P < 1 \times 10^{-5}$, we obtained 178 associated SNPs and 40 associated loci (Fig. 1; Additional file: Table S1). In particular, a cluster of 89 associated SNPs was detected on Chromosome 2. A total of 150 genes were considered maize drought tolerance candidate genes (Additional file: Table S2).

These candidate genes were significantly enriched in several Gene Ontology (GO) terms (https://plants.ensembl.org/biomart/martview/) (Additional file: Table S3), such as metabolic process, biological regulation, reproductive process, response to stimulus and signaling. Combining the GWAS results and the gene function annotations obtained through GO analysis, numerous crucial candidate genes were identified. Among these genes (Additional file: Table S2), three transcription factors were detected, including two MYB transcription factors, *MYB140* (*Zm00001d020569*) and *MYBR73* (*Zm00001d004681*), and one bHLH transcription factor, *ICE2* (*Zm00001d049294*). Additionally, *ZEP1* (*Zm00001d003512*) and *ZmBGal1* (*Zm00001d048783*) are involved in plant hormone signaling pathways. Moreover, nine genes were found to be involved in the stress pathway based on the functions of their homologues in *Arabidopsis* and rice, including *ZmPRX99* (*Zm00001d047514*), *C0P8J4* (*Zm00001d003554*), *Dmas2* (*Zm00001d003525*), *DSM1* (*Zm00001d018025*), *ZmNHL21* (*Zm00001d034942*), *ZmDnaJ21* (*Zm00001d003459*), *ZmRGLG1* (*Zm00001d048784*), *CYP709C14* (*Zm00001d006947*), and *CYP709C21* (*Zm00001d006948*). Furthermore, we identified dozens of promising candidate genes that have been demonstrated to function in regulating plant stress tolerance in *Arabidopsis* and rice. *MYB140* (*Zm00001d020569*), which is homologous to *Arabidopsis ATMYB36*, encodes a MYB family protein involved in root development and the regulation of plant stress tolerance [36]; *MYBR73* (*Zm00001d004681*), encoding a MYB transcription family member homologous to *Arabidopsis AtMYB52* and involved in the ABA response; *AtMYB52* overexpression lines are drought tolerant [37]; *C0P8J4* (*Zm00001d003554*), which is homologous to *Arabidopsis AtHSP22.0*, is a Columbia endomembrane-localized small heat shock protein involved in the response to salt and drought stress [38]; and *ZmRGLG1* (*Zm00001d048784*), encoding an E3 ubiquitin–protein ligase and homologous to *Arabidopsis RGLG1*, negatively regulates drought stress by mediating *ERF53* transcriptional activity [39]. Owing to their gene function annotation and homologues in rice and *Arabidopsis*, these genes could be considered important candidates for further functional studies.

Transcriptome sequencing analysis

Transcriptome analysis was performed to assess the whole-genome gene expression levels in the five drought-tolerant lines and five drought-sensitive lines under WW and WS conditions. The degree of drought was evaluated by the soil water content and leaf RWC of ten maize inbred lines. The results revealed that the leaf RWCs of Huangzaosi (96.53%), K12 (93.18%), G71 (91.99%), G90 (90.96%), and Cang7-2 (87.12%) were greater than 85% under the drought treatment (Additional file: Fig S1). In contrast, the leaf RWCs of Lv28 (78.35%), Qi319 (72.97%), Zheng58 (61.57%), B73 (61.06%), and Ye478 (53.53%) decreased to lower than 80% under drought control. More than 2.27 billion clean reads were obtained, with an average of 56.42 million reads per library from 40 libraries for subsequent analysis. The mean of Q20 and

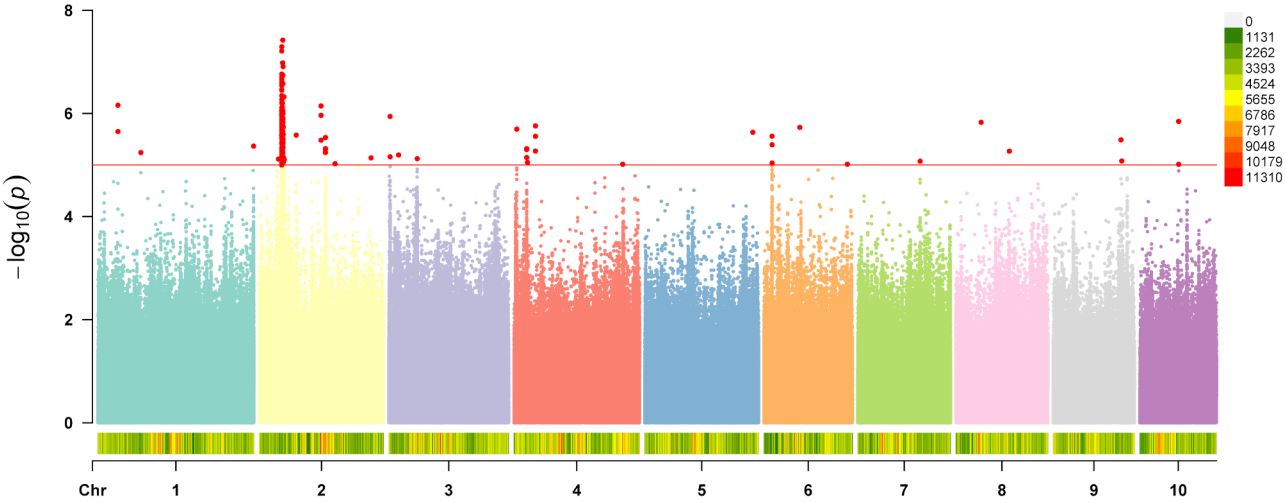

Fig. 1 Manhattan of GWAS for survival rates of maize seedlings under drought stress. The dashed horizontal line indicates the Bonferroni-adjusted significance threshold ($P = 1.0 \times 10^{-5}$). The SNPs within the candidate gene, identified by the GWAS of the entire population, is labeled as red dots

Q30 of raw data were 97.03% and 92.34, respectively, suggesting the high quality of sequencing. After data filtering, a total of 2.27 billion clean reads, with an average of 56.42 million reads per library, were obtained for subsequent analysis. The uniquely mapped clean reads, which accounted for more than 95% of the mapped reads in all the libraries, were used for gene expression analysis. The expression of gene was calculated by normalizing the reads to FPKM value. The results showed that 56.12% (54.01–57.58% in each sequencing library, based on the total number of 33,997 genes in B73_RefGen_v4.0) of genes were expressed (FPKM > 1) on average. Approximately 95% (94.81–95.80% in each sequencing library) of the expressed genes were enriched in the FPKM of 1-100, while only 5% of the expressed genes were in the FPKM range of 100-10000 (Additional file: Fig S2). The number of expressed genes in different ranges showed no obvious changes between maize lines and water treatments. In addition, PCA of the transcriptomes clearly revealed that the samples under WS and WW treatment could be divided into two groups, indicating that drought stress affects gene expression in maize.

Identification of drought-responsive genes

To identify the genes involved in the drought response, a total of 7,278 drought-responsive genes were detected through differential expression analysis between the WW and WS treatments in ten maize inbred lines. GO enrichment analysis of the drought-responsive genes was subsequently performed. Although the functions of the drought-responsive genes in each line were diverse, their functions were enriched mainly in drought response-related annotations, such as gene ontology classes related to response to stimulus, response to abiotic stimulus, and response to water deprivation (Additional file: Table S4). To identify common DEGs between different genotypes, comparison assays were conducted for the drought-tolerant lines and drought-sensitive lines. Among the five drought-tolerant lines, 28 genes were commonly responsive to drought (Additional file: Table S5). In total, the expression patterns of 28 genes, including 16 up-regulated genes and 12 down-regulated genes, were observed in all five drought-tolerant lines. There were 147 common drought-responsive genes in the drought-sensitive lines (Additional file: Table S5), including 56 up-regulated genes and 91 down-regulated genes. Among these common DEGs, only 10 genes (4 up-regulated and 6 down-regulated) responded to drought in all ten lines (Fig. 2). With respect to their functional annotations and the functions of homologous genes, five candidate genes were identified. These genes include two zinc finger protein genes, *col10* (*Zm00001d037327)* and *OZ1* (*Zm00001d043095*); a heat stress transcription factor gene, *ZmHSFA2-4* (*Zm00001d005888*); a heat shock protein, *Hsp28* (*Zm00001d018298*); and a CBL-interacting protein kinase gene, *ZmSnRK3.3* (*Zm00001d029075*). The expression of *Zm00001d005888* and *Zm00001d018298* was induced in all ten lines, whereas the expression of *Zm00001d037327* and *Zm00001d043095* was significantly down-regulated by drought stress in the ten lines. We randomly selected four genes from the transcriptome results and analyzed their expression levels in drought-tolerant and drought-sensitive lines under WW and WS conditions as shown in Additional file: Fig S3A. The expression results were consistent with those of the transcriptome (Additional file: Fig S3B). The four genes were differentially expressed in both drought-tolerant and drought-sensitive materials under normal and drought stress conditions.

Gene co-expression networks related to drought tolerance

To understand drought - related gene regulation networks in ten maize lines and identify key drought - tolerance genes, we used WGCNA to construct gene co - expression networks of drought - responsive genes. (Fig. 3A). Under drought treatments, the drought responsive genes were clustered into 28 co-expression modules. Among these, module-trait correlation analysis revealed that five modules were significantly associated with leaf RWC (Fig. 3B), including the darkolivegreen (Module I, r = -0.82, $P = 8 \times 10^{-6}$), steelblue (Module II, r = -0.79, $P = 4 \times 10^{-5}$), tan (Module III, $r = 0.67$, $P = 0.001$), darkmagenta (Module IV, $r = 0.62$, $P = 0.003$), and skyblue (Module V, r = -0.58, $P = 0.007$) modules. The MEtan module was significantly positively correlated with the leaf RWC. The GO enrichment analysis of the 210 genes within the MEtan module revealed that these genes were enriched in protein modification processes, cell communication, kinase activity, responses to external stimuli, and metabolic processes. Cystoscope software was subsequently used to visualize the network and within-module connections of the target module computed via WGCNA. In the MEtan module, eight transcription factors were highly associated with drought tolerance (Fig. 3C). The eight important transcription factors include two NAC transcription factors (*Zm00001d019207* and *Zm00001d006106*), one MADS transcription factor (*Zm00001d031625*), one RAV transcription factor (*Zm00001d009468*), one TALE transcription factor (*Zm00001d033898*), one WRKY transcription factor (*Zm00001d002794*) and two GRAS transcription factors (*Zm00001d048681* and *Zm00001d003553*). Among these genes, *Zm00001d048681* and *Zm00001d031625*, which are associated with the drought-tolerant phenotype, had the most significant differences at 0.69 and 0.66, respectively. These hub genes might be important candidate drought tolerance genes for further study.

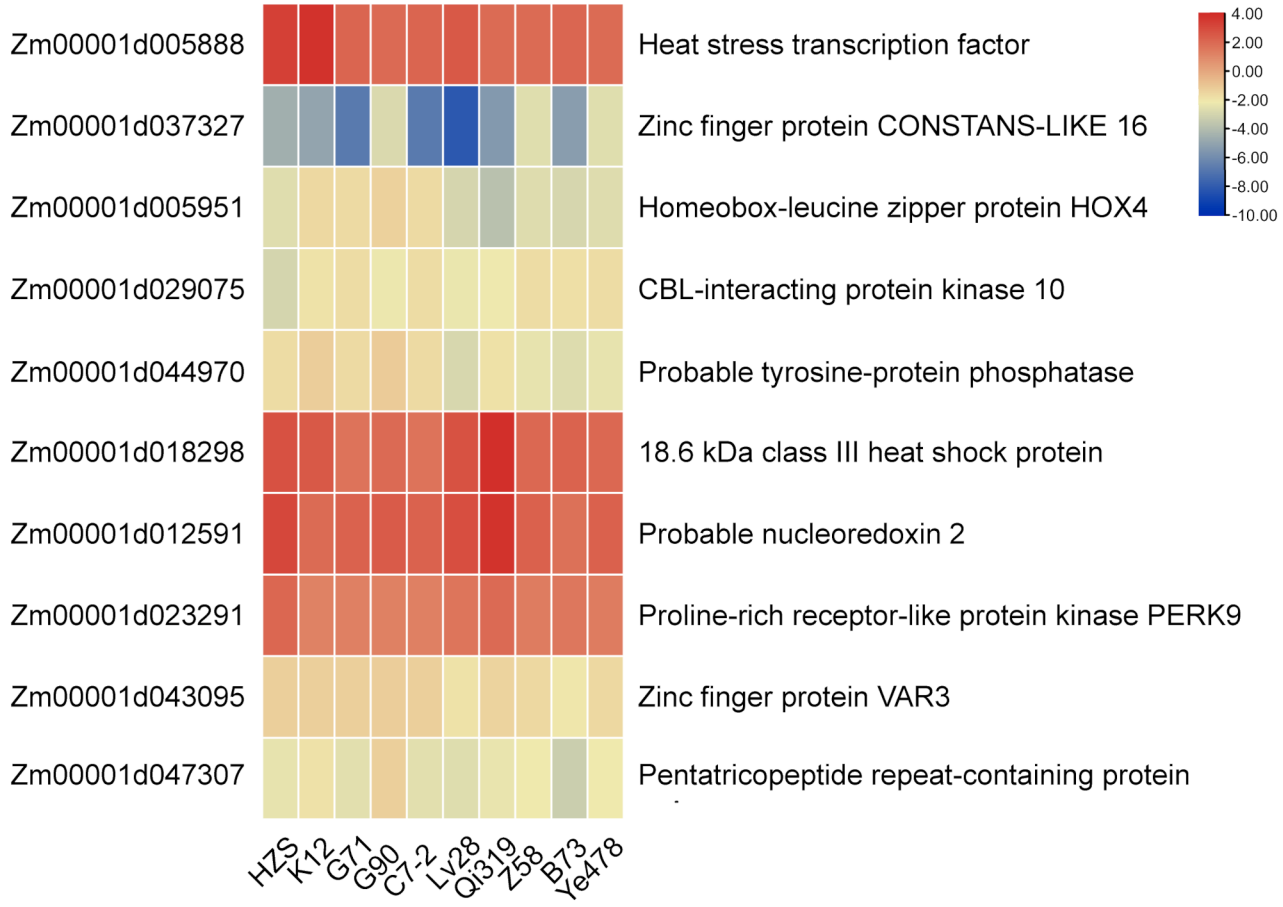

Fig. 2 The common drought responsive genes in all 10 maize lines

Identification of candidate genes by integrating GWAS and transcriptome data

The potential candidate genes were prioritized by integrating DEGs identified from transcriptome data of all drought-tolerant and drought-sensitive lines with the results of the GWAS. Based on the transcriptome data, 28 genes were found to be commonly responsive to drought in the five drought-tolerant lines, while 147 genes commonly responsed to drought in the drought-sensitive lines. Among the 150 genes detected via GWAS, we successfully identified three candidate genes associated with drought tolerance in maize seedlings *Zm00001d006947*, *Zm00001d038753*, and *Zm00001d003429*. *Zm00001d006947* was detected in all drought-tolerant lines, whereas *Zm00001d038753* and *Zm00001d003429* were detected in all drought-sensitive lines. *Zm00001d006947* encodes a cytochrome P450 protein involved in hormone and stress responses [40] and was simultaneously detected in all drought-tolerant lines. *Zm00001d038753* and *Zm00001d003429* were commonly detected in all the drought-sensitive lines. The gene *Zm00001d038753* encodes a ubiquitin domain-containing protein involved in the response to abiotic stresses. *Zm00001d003429* encodes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is homologous to rice *GAPA* and improves drought tolerance by regulating NADPH homeostasis [41].

Additionally, by integrating GWAS with WGCNA, we also identified a significant hub gene, *Zm00001d003553*, also known as GRAS transcription factor 51 (*ZmGRAS15*), which plays a role in the drought tolerance in maize seedlings. *Zm00001d003553* is homologous to rice *OsGRAS23* and plays a known role in improving the stress tolerance of rice [42]. To reveal the genetic variations affecting drought tolerance and identify favourable haplotypes, we performed association analysis for the priority candidate genes. The significant SNPs located in the promoter of *Zm00001d006947* formed two alleles in the population, with the G allele conferring better survival rates. The RNA-seq data revealed that the genes presented significantly different expression levels between the WW and WS conditions. Thus, the G allele was confirmed as the favourable allele (Fig. 4A-C). For *Zm00001d038753* and *Zm00001d003553*, the significant SNP located in the promoter separately formed two alleles, with the A allele conferring higher survival

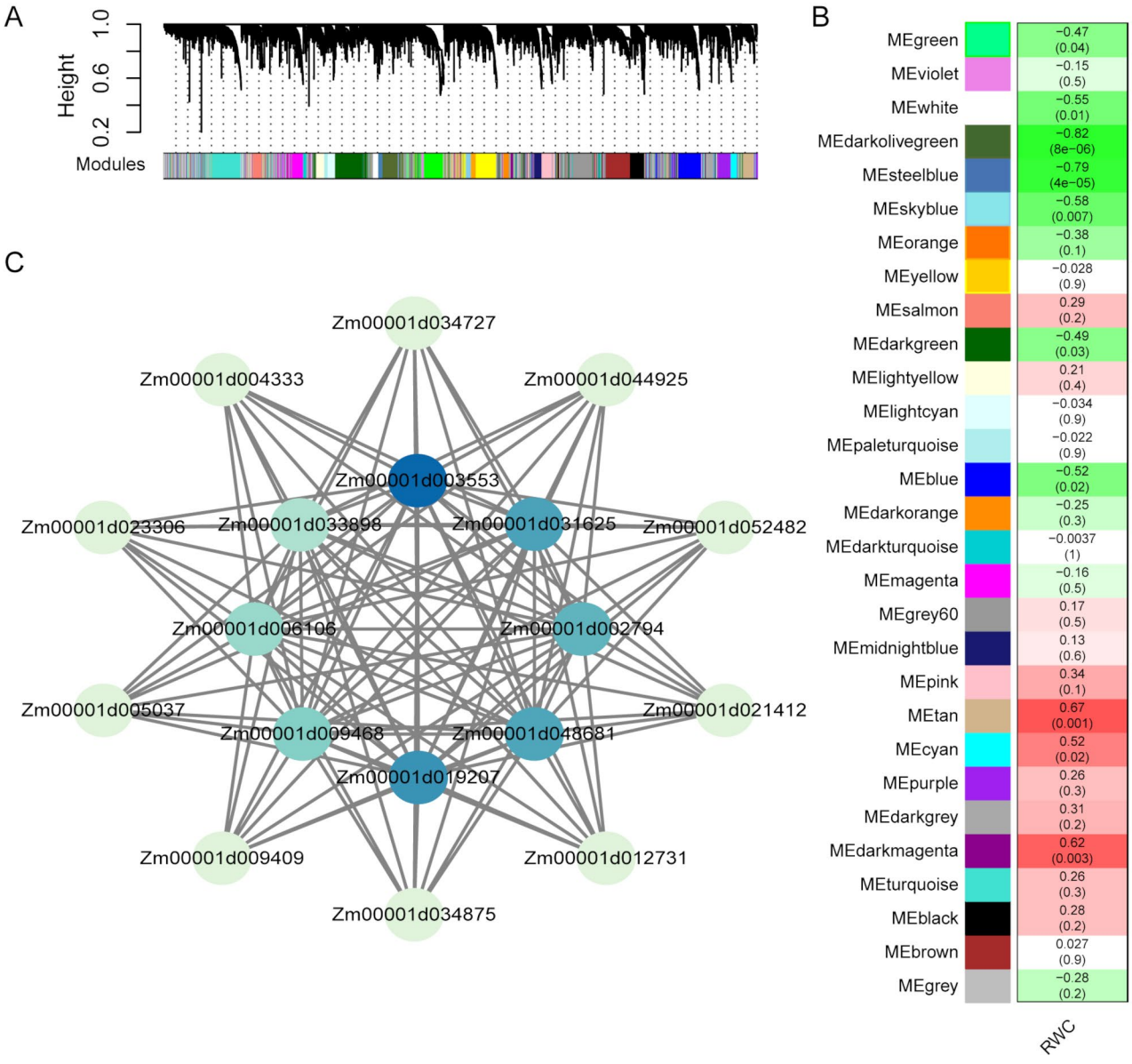

Fig. 3 Construction of gene co-expression networks. (**A**) The co-expression networks of drought-responsive genes were constructed using the samples under drought treatment by WGCNA. (**B**) The correlations of module-traits were detected for samples under drought treatment. For each module, the correlation coefficients (the upper number in heat map) and Student asymptotic *P* values (the lower number in brackets) were calculated. (**C**) The regulatory network of transcription factor genes in MEtan co-expression module

rates. Thus, the A allele was confirmed as the favourable allele (Fig. 4D-F and G-I). The RNA-seq data revealed that the expression levels of *Zm00001d038753* were significantly greater under WS conditions than under WW conditions and that *Zm00001d003553* was significantly greater in drought-tolerant lines than in drought-sensitive lines. The significant SNPs located in the intron of *Zm00001d003429* formed four haplotypes in the population, with the CAT and TAT haplotypes conferring better survival rates. The RNA-seq data revealed that the genes presented significantly different expression levels between the drought-tolerant lines and drought-sensitive lines under WS conditions. The drought-tolerant lines presented significantly greater expression than did the drought-sensitive lines under WS, but there was no significant difference under WW conditions (Fig. 4J-L). These genes were viewed as priority candidate genes for drought tolerance in this study.

Overexpression of *ZmGRAS15* improves drought tolerance

As shown in Additional file: Fig S4, four candidate genes presented different expression patterns in response

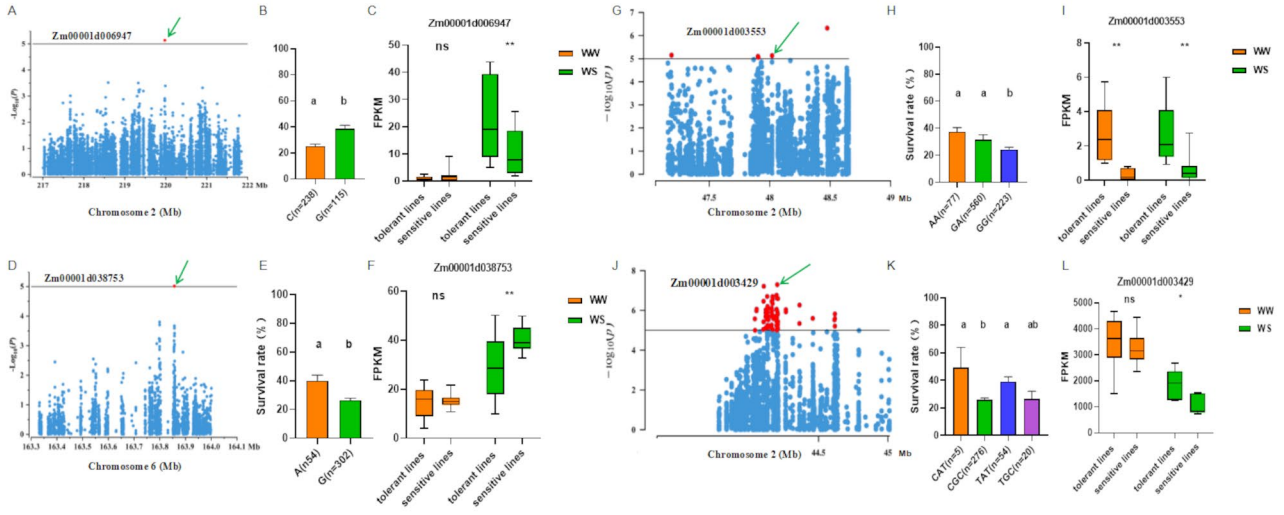

Fig. 4 Candidate genes identified by GWAS and RNA-seq under WW and WS conditions. (**A**, **D**, **G**, **J**) Identification of candidate genes by GWAS. The lines indicate the suggestive threshold of $P = 1.0 \times 10^{-5}$. (**B**, **E**, **H**, **K**) Phenotypic comparison of the different haplotypes based on significant SNPs. (**C**, **F**, **I**, **L**) Comparison of the expression of the candidate genes identified in drought-tolerant lines and drought-sensitive lines by RNA-seq data

to ABA and osmotic stress. *Zm00001d003429* and *Zm00001d003553* were up-regulated at the initial stage of ABA and PEG treatment but then gradually decreased. At the early stage of ABA and PEG treatment, *Zm00001d038753* and *Zm00001d006947* initially decreased but then increased. The expression of four candidate genes was induced by ABA and osmotic stress. To further verify the function of the high-confidence candidate genes discovered via GWAS and WGCNA, we chose *Zm00001d003553* as an example. *Zm00001d003553* encodes a GRAS transcription factor and is designated *ZmGRAS15*, which is homologous to rice *OsGRAS23*. Compared with the WT, the overexpression of *OsGRAS23* increased drought tolerance and oxidative stress tolerance and resulted in less H_2O_2 accumulation under drought stress [42]. Based on the transcriptome analyses of the ten inbred lines under WW and WS treatment, the expression of *ZmGRAS15* in the drought-tolerant lines was significantly greater than that in the drought-sensitive lines (Fig. 4I). To detect the tissue-specific expression of *ZmGRAS15*, the abundance of *ZmGRAS15* transcripts in different tissues was measured. The results of quantitative real-time polymerase chain reaction (qRT−PCR) revealed that the expression levels of *ZmGRAS15* were relatively high in maize roots and leaves (Fig. 5A).

To confirm the role of *ZmGRAS15* in drought tolerance, we further overexpressed the gene in maize and selected two overexpressing lines (OE1 and OE2) to investigate its function in drought tolerance. QRT−PCR analyses revealed that the transcript levels of *ZmGRAS15* in the transgenic lines were significantly greater than those in the control (WT) plants (Fig. 5B). We evaluated the drought tolerance of the transgenic maize and WT plants in the soil and found that the overexpressing lines (OE1 and OE2) presented significantly greater survival rates than the WT plants did (Fig. 5C and D). The tissue-specific expression of *ZmGRAS15* was significantly greater in root tissue. The primary root length of the transgenic lines and WT plants was measured under both WW and WS conditions. The *ZmGRAS15*-OE transgenic lines (OE1 and OE2) showed significantly longer for the primary root length than did the WT, respectively, for both WW and WS conditions (Fig. 5E and F). These results demonstrated that *ZmGRAS15* plays an important role in improving drought tolerance at the seedling stage.

Discussion

GWAS reveals candidate genes involved in drought tolerance

Linkage mapping and genome-wide association analysis are the two primary strategies for identifying drought tolerance in maize [6, 17, 43–46]. A considerable number of genes have been identified through GWAS strategies, including those involved in drought tolerance [6] and salt tolerance [47]. In this study, we used 362 inbred lines as an association population to conduct a GWAS analysis for survival rates. A total of 40 significantly associated loci and 150 candidate genes within 120 kb of these loci were detected. Among these genes, 147 significantly associated SNPs and 89 candidate genes were located on Chromosome 2, whereas the other genes were located on chromosomes 1, 6, 8 and 10. Through gene annotation, homologous gene function, and GO enrichment, 14 genes were identified as possible candidate genes, including two MYB transcription factors, one bHLH-transcription factor *ICE2*, two ABA pathway signaling-related

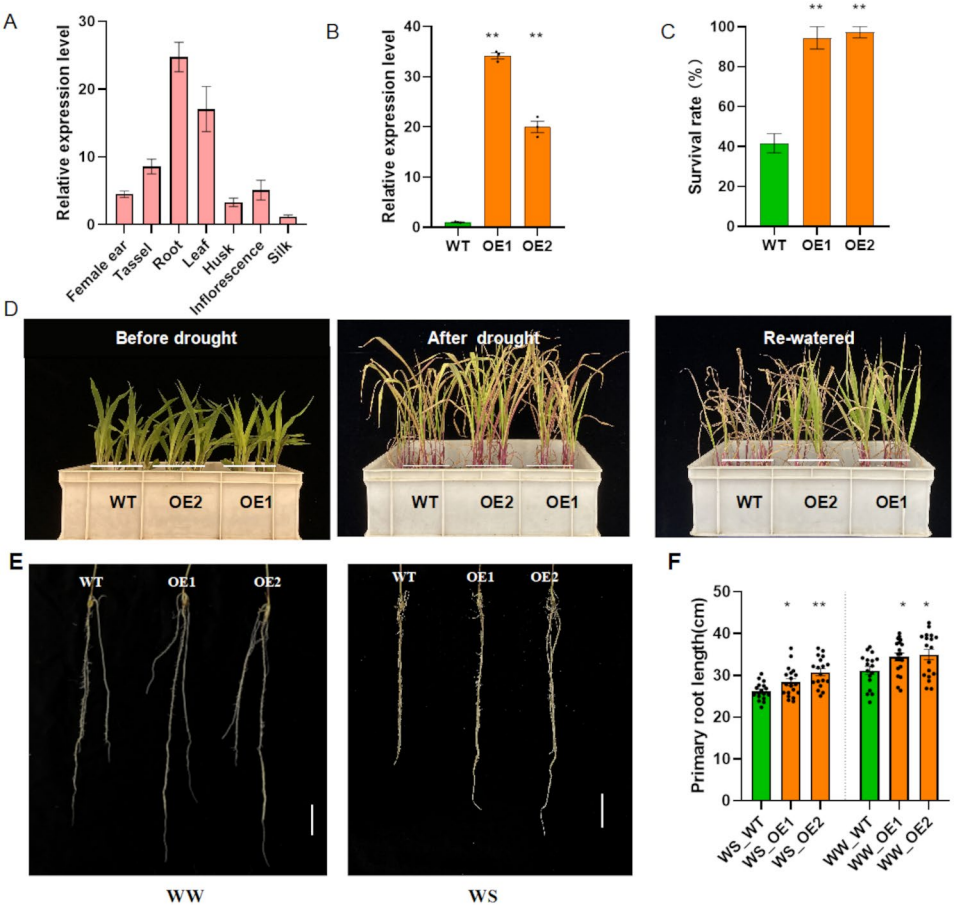

Fig. 5 Drought tolerance identification of *ZmGRAS15* overexpressing transgenic lines. (**A**) Reverse transcription quantitative PCR (qRT-PCR) expression analysis of *ZmGRAS15* in different tissues and organs of B73 under WW conditions. (**B**) Expression levels of *ZmGRAS15* in the leaves of *pUbi: ZmGRAS15* transgenic lines and WT plants. (**C**) Survival rate of *pUbi: ZmGRAS15* transgenic lines and WT plants after drought treatment. (**D**) Plant performance of *pUbi: ZmGRAS15* transgenic lines and WT plants before and after the drought treatment followed by a 5 d period of re-watering. (**E**) Phenotype of root traits in transgenic maize and wild-type (WT) under Well-Watered and Water-Stressed conditions. (**F**) Statistical analysis of primary root length. The experiment was conducted three times, and statistical significance was determined by a two-sided t-test (*$p < 0.05$; **$p < 0.01$; ns = not significant)

genes, one root development gene, and eight other genes associated with stress on the basis of the functions of their homologues in *Arabidopsis* and rice.

MYB transcription factors are among the largest families of transcription factors in plants and play important roles in regulating plant tolerance to drought stress by affecting biological processes such as ABA signalling, which is crucial in plant drought responsiveness [21, 48, 49]. Abscisic acid plays a crucial role in stress [50]. The orthologues of *Zm00001d003515* and *Zm00001d047514* have been reported to be involved in the regulation of ABA signalling. ICE genes, bHLH transcription factors, are key factors in the molecular mechanisms of drought and cold tolerance in plants [51–55]. *ICE2* (*Zm00001d049294*), which is homologous to rice *OsbHLH002*, may be involved in cold and osmotic stress because the OsMAPK3-OsbHLH002-OsTPP1 signaling pathway enhances chilling tolerance in rice [30]. *ZEP1* (*Zm00001d003512*) encodes zeaxanthin epoxidase 1, which is the key enzyme involved in ABA biosynthesis [56]. *ZmASR1* (*Zm00001d003515*) encodes an atypical aspartic protease that is homologous to *Arabidopsis ASPR1* and modulates lateral root development. *ASPR1* overexpression suppresses primary root growth and lateral root development [57]. *ZmPRX99* (*Zm00001d047514*) encodes a peroxidase protein that is homologous to *Arabidopsis PRX52* and is involved in the abiotic stress response [36]. *Dmas2* (*Zm00001d003525*), encoding a deoxymugineic acid synthase protein, is involved in the reactive oxygen species pathway. *Zm00001d034942*, which is homologous to *Arabidopsis NHL21*, belongs to the late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family and is involved in the defense response [58]. *ZmDnaJ21* (*Zm00001d003459*) encodes a DnaJ or heat shock protein 40 and participates in plant signal transduction and response to heat stress in *Arabidopsis* [59]. *CYP709C14* (*Zm00001d006947*) and *CYP709C21* (*Zm00001d006948*)

encode cytochrome P450 proteins involved in hormone and stress responses [40]. *ZmBGal1* (*Zm00001d048783*) encodes β-D-galactosidase, which is related to cell wall polysaccharide metabolism [60].

Transcriptome analysis reveals candidate genes involved in drought tolerance

In the present study, numerous genes that respond to drought stress were detected through transcriptome analysis of ten maize inbred lines with different degrees of drought tolerance in terms of RWC at a given key point of drought treatment. Gene ontology analysis revealed that these drought-responsive genes were significantly enriched in photosynthetic metabolism pathways, especially photosynthesis-related genes, which were differentially expressed in the drought-sensitive lines under different water treatments. In the drought-tolerant lines, the drought-responsive genes were significantly enriched in response to abscisic acid stimulation, whereas those in the drought-sensitive lines, with the exception of Lv28, were hardly enriched in this pathway, implying that these genes enriched in the drought-tolerant lines may regulate maize drought tolerance through ABA signalling.

In the present study, we found that 27 candidate genes were significantly expressed in the drought-tolerant lines, including five transcription factor genes, including *Zm00001d005888* (heat stress transcription factor B-3), *Zm00001d005951* (homeobox-leucine zipper protein), *Zm00001d008399* (NAC domain transcription factor superfamily protein), *Zm00001d045661* (zinc finger protein constans-like 16) and *Zm00001d037327* (zinc finger protein constans-like 7), the heat shock protein *Zm00001d018298*, three cytochrome P450 proteins *Zm00001d006947*, *Zm00001d045063* and *Zm00001d050323*, two CBL-interacting serine/threonine-protein kinases *Zm00001d029075* and *Zm00001d018429*, and the receptor-like protein kinase *Zm00001d010945*.

In addition, among these DEGs, *Zm00001d006947* and *Zm00001d006948* encode cytochrome P450 proteins involved in hormone and stress responses pointed out by Kurotani et al. (2015) [40]. Gene ontology function-based instructions to illustrate the response of *Zm00001d018298* to salt, hydrogen peroxide and reactive oxygen species. *Zmhdz10* (*Zm00001d005951*) belongs to the HD-Zip family, and the overexpression of *Zmhdz10* in plants increased tolerance to drought and salt stress and improved sensitivity to ABA [61]. *Zm00001d044970*, which is homologous to rice *LOC_Os06g10650* and encodes a tyrosine phosphatase family protein, might be a causal candidate gene for improving rice seed germination under salt stress [62]. *Zm00001d008399* is homologous to rice *OsNAP*. The overexpression of *OsNAP* significantly reduces the rate of water loss during the vegetative period, thus increasing tolerance to high salt, drought and low temperature [63]. *Zm00001d037327* encodes a B-box zinc finger transcription factor that is homologous to rice *OsBBX17*. The knockdown of *OsBBX17* significantly enhances saline-alkaline tolerance [64].

Co-localization of drought tolerance candidate genes with QTLs from linkage mapping

Recently, numerous candidate genes regulating maize drought tolerance have been reported [6, 65]. On the basis of the B73 reference genome, we analysed the co-localization of the hub genes identified in our study and drought tolerance genes identified in other studies. The candidate gene for GWAS screening, *Zm00001d048783*, was identified as a high-confidence candidate gene by Sha et al. (2023) [66]. The candidate gene *Zm00001d003438* was also consistent with the candidate genes significantly associated with drought tolerance reported in another study [6]. A total of 224 maize inbred lines under three water regimes were analysed for transcriptome data, and through Mendelian randomization analysis, 97 genes for maize drought tolerance, including *Zm00001d020568* and *Zm00001d047517*, were identified [67, 68]. *Zm00001d038753* co-localizes with *qKRE2-5-1* and *qKRE1-6-2* on Chromosome 6 [69]. In this study, transcriptome data revealed that the expression levels of *Zm00001d048783*, *Zm00001d003428*, and *Zm00001d038753* were significantly greater under WS conditions than under WW conditions. *Zm00001d047517* was down-regulated by drought stress, whereas *Zm00001d020568* was barely expressed. *Zm00001d048783*, *Zm00001d003428*, and *Zm00001d038753* are related to drought tolerance in maize.

Integrating GWAS and transcriptome analysis to reveal causal genes involved in drought tolerance

Integrating GWAS and WGCNA serves as an effective approach for identifying maize co-expression networks and hub genes. For example, Yang et al. (2021) reported that four hub genes (*Zm00001d018664*, *Zm00001d043797*, *Zm00001d034036*, and *Zm00001d048474*) affect maize flowering time by combining GWAS and WGCNA [70]. Similarly, Li et al. (2021b) revealed the genetic control of the maize response to salt stress through the combination of GWAS and WGCNA, revealing the role of *ZmIAA1* and *ZmGRAS43* in maize root plasticity in response to salt tolerance [16]. Two hub genes (*Zm00001d047306* and *Zm00001d024600*) were associated with tolerance to high salinity in maize seedlings according to GWAS, WGCNA, and gene-based association studies [15].

In this study, we identified four candidate genes and their favourable alleles related to drought tolerance by integrating GWAS and transcriptome analysis. Four candidate genes were induced by ABA and osmotic stress and presented a convergent expression pattern during treatment with ABA and PEG. For example, *CYP709C14* (*Zm00001d006947*) encodes a cytochrome P450 monooxygenase that catalyzes various steps of plant metabolic synthesis, including pigments, fatty acids, defense-related compounds, and phytohormones [71, 72]. Previous studies have also suggested that the cytochrome pathway was related to abiotic stress tolerance and ABA, as evidenced by *cyp709b3* deletion mutant of *Arabidopsis thaliana* showed sensitivity to salt stress during germination [73], and that the cytochrome P450 genes *Gh_D07G1197* and *Gh_A13G2057* enhanced drought and salt stress tolerance in *Gossypium hirsutum* [74]. *Zm00001d038753* encodes a ubiquitin domain-containing protein. The ubiquitin-binding protein OsDSK2a binds to polyubiquitin chains and interacts with the gibberellin (GA)-inactive enzyme elongated uppermost internode (EUI), mediating seedling growth and the salt response in rice [75]. *Zm00001d003429* (*GPA1*) encodes a glyceraldehyde-3-phosphate dehydrogenase and is homologous to rice *GAPA*, which plays a role in improving drought tolerance by regulating photosynthetic adaptation [41].

ZmGRAS15 positively regulates maize drought tolerance

Transcription factors play important roles in the response of plants to stress [76–79]. Several transcription factors, especially novel ones, remain to be studied in detail. GRAS proteins play crucial roles in controlling several features of development, growth and response to abiotic stress. GRAS family proteins are divided into several subfamilies, such as SCL3, HAM, LS, SCR, DELLA, SHR, PAT1 and LISCL [80], among which DELLA proteins are among the most comprehensively studied and serve as crucial regulatory targets within the gibberellin (GA) signalling pathway [24, 81, 82]. Compared with the wild type, the overexpression of *OsGRAS23* increased drought tolerance and oxidative stress tolerance and decreased H_2O_2 accumulation under drought stress [42]. The heterologous overexpression of *ZmGRAS72* significantly improved *Arabidopsis thaliana* tolerance to drought and salt stresses [83]). Additionally, *SHORTROOT* (*SHR*) and *SCARECROW* (*SCR*), two related members of the GRAS gene family, play important roles in cell elongation for root growth in *Arabidopsis* [84, 85]. In this study, *ZmGRAS15* was induced by ABA and osmotic stress. The expression level of *Zm00001d003553* was significantly up-regulated at 2 h under ABA and PEG treatment. *ZmGRAS15* was shown to belong to the LISCL subfamily and is an orthologue of *OsGRAS23*, *SCL9*, and *SCL14*. We discovered that *ZmGRAS15* was significantly associated with drought tolerance at the maize seedling stage. Additionally, a lead SNP was found to be significantly associated with *ZmGRAS15* and was located in the promoter of *ZmGRAS15*. All the primers used are listed in Additional file: Table S6.

Conclusion

In summary, in this study, we identified a total of four candidate genes associated with drought tolerance in maize seedlings by combining GWAS and transcriptome data. including *Zm00001d006947*, *Zm00001d038753*, *Zm00001d003429* and *ZmGRAS15*. The overexpression of *ZmGRAS15* can increase maize drought tolerance at the seedling stage by increasing primary root length. The genetic material developed, the candidate genes and the haplotypes identified in this study may be used for the breeding of drought-tolerant maize varieties.

Abbreviations

GWAS	Genome-wide association studies
WGCNA	co-expression network analysis
MLM	mixed linear model
RWC	relative water content
RNA-seq	RNA sequencing
DREB	dehydration-responsive element-binding
bHLH	basic helix–loop–helix
NAC	NAM-ATAF-CUC2
bZIP	basic leucine zipper
TGA	TGACG motif-binding factor
SNPs	single nucleotide polymorphisms
WW	Well-Watered
WS	Water-Stressed
DEGs	differentially expressed genes
HZS	Huangzaosi
C7-2	Chang7-2
Z58	Zheng58
QTLs	quantitative trait loci
FDR	false discovery rate
TOM	topological overlap matrix
GO	Gene Ontology
PCA	Principal component analysis
GA	gibberellin
EUI	elongated uppermost internode
SHR	SHORTROOT
SCR	SCARECROW

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11435-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Acknowledgements
Not applicable.

Author contributions
D.M. W. and X.Y. L. analyzed data and wrote the main manuscript text. C.H. L., Y. L. and D.F. Z. oversaw the research direction, provided key guidance and revised the manuscript. G.H.H. revised the manuscript and conducted an investigation process. K.L.W. conducted the experiments. Y.X. L, H.H.G. and T.Y. W. revised the manuscript. All authors reviewed the manuscript.

Funding
This research was supported by the Guangxi Key Research and Development Projects, China (GuikeAB21238004), National Natural Science Foundation of China Grant (32201751), the China Agriculture Research System of MOF and MARA (CARS-02-04) and Innovation Program of Chinese Academy of Agricultural Sciences.

Data availability
All the sequencing data are deposited in SRA under the Bioproject accession number PRJNA1185924. Accession "PRJNA1185924" has been released on 2025-03-04.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 8 November 2024 / Accepted: 4 March 2025
Published online: 13 March 2025

References

1. Liu SX, Wang HW, Qin F. Genetic dissection of drought resistance for trait improvement in crops. Crop J. 2023;11(4):975–85. https://doi.org/10.1016/j.cj.2023.05.002
2. Xie XW, Ren ZZ, Su HH, Abou-Elwafa SF, Shao J, Ku LX, Jia L, Tian ZQ, Wei L. Functional study of *ZmHDZ4* in maize (*Zea mays*) seedlings under drought stress. BMC Plant Biol. 2024;24:1471–2229. https://doi.org/10.1186/s12870-024-05951-3
3. Song P, Wang J, Guo X, Yang W, Zhao C. High-throughput phenotyping: breaking through the bottleneck in future crop breeding. Crop J. 2021;9(3):633–45. https://doi.org/10.1016/j.cj.2021.03.015
4. Tollenaar M, Lee EA. Yield potential, yield stability and stress tolerance in maize. Field Crops Res. 2002;75(2–3):161–9. https://doi.org/10.1016/s0378-4290(02)00024-2
5. Korte A, Farlow A. The advantages and limitations of trait analysis with GWAS: a review. Plant Methods. 2013;9:29. https://doi.org/10.1186/1746-4811-9-29
6. Wang XL, Wang HW, Liu SX, Ferjani A, Li JS, Yan JB, Yang XH, Qin F. Genetic variation in *ZmVPP1* contributes to drought tolerance in maize seedlings. Nat Genet. 2016;48(10):1233–41. https://doi.org/10.1038/ng.3636
7. Zhang XM, Mi Y, Mao HD, Liu SX, Chen LM, Qin F. Genetic variation in *ZmTIP1* contributes to root hair elongation and drought tolerance in maize. Plant Biotechnol J. 2020;18(5):1271–83. https://doi.org/10.1111/pbi.13290
8. Wu X, Feng H, Wu D, Yan SJ, Zhang P, Wang WB, Zhang J, Ye JL, Dai GX, Fan Y, et al. Using high-throughput multiple optical phenotyping to Decipher the genetic architecture of maize drought tolerance. Genome Biol. 2021;22(1):185. https://doi.org/10.1186/s13059-021-02377-0
9. Gao HJ, Cui JJ, Liu SX, Wang SH, Lian YY, Bai YT, Zhu TF, Wu HH, Wang YJ, Yang SP, et al. Natural variations of modulate the trade-off between drought resistance and yield by affecting ZmRBOHC-mediated stomatal ROS production in maize. Mol Plant. 2022;15(10):1558–74. https://doi.org/10.1016/j.molp.2022.08.009
10. Li CH, Guo J, Wang DM, Chen XJ, Guan HH, Li YX, Zhang DF, Liu XY, He GH, Wang TY, et al. Genomic insight into changes of root architecture under drought stress in maize. Plant Cell Environ. 2023;46(6):1860–72. https://doi.org/10.1111/pce.14567
11. Opitz N, Paschold A, Marcon C, Malik WA, Lanz C, Piepho HP, Hochholdinger F. Transcriptomic complexity in young maize primary roots in response to low water potentials. BMC Genomics. 2014;15:741. https://doi.org/10.1186/1471-2164-15-741
12. Danilevskaya ON, Yu GX, Meng X, Xu J, Stephenson E, Estrada S, Chilakamarri S, Zastrow-Hayes G, Thatcher S. Developmental and transcriptional responses of maize to drought stress under field conditions. Plant Direct. 2019;3(5). https://doi.org/10.1002/pld3.129
13. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 2008;9:559. https://doi.org/10.1186/1471-2105-9-559
14. Childs KL, Davidson RM, Buell CR. Gene coexpression network analysis as a source of functional annotation for rice genes. PLoS ONE. 2011;6(7):e22196. https://doi.org/10.1371/journal.pone.0022196
15. Ma LL, Zhang MY, Chen J, Qing CY, He SJ, Zou CY, Yuan GS, Yang C, Peng H, Pan GT, et al. GWAS and WGCNA uncover hub genes controlling salt tolerance in maize (*Zea mays* L.) seedlings. Theor Appl Genet. 2021;134(10):3305–18. https://doi.org/10.1007/s00122-021-03897-w
16. Li PC, Yang XY, Wang HM, Pan T, Wang YY, Xu Y, Xu CW, Yang ZF. Genetic control of root plasticity in response to salt stress in maize. Theor Appl Genet. 2021;134(5):1475–92. https://doi.org/10.1007/s00122-021-03784-4
17. Liu SX, Wang XL, Wang HW, Xin HB, Yang XH, Yan JB, Li JS, Tran LSP, Shinozaki K, Yamaguchi-Shinozaki K, et al. Genome-Wide analysis of *ZmDREB* genes and their association with natural variation in drought tolerance at seedling stage of *Zea mays* L. Plos Genet. 2013;9(9):e1003790. https://doi.org/10.1371/journal.pgen.1003790
18. Wei SW, Xia R, Chen CX, Shang XL, Ge FY, Wei HM, Chen HB, Wu YR, Xie Q. *ZmbHLH124* identified in maize Recombinant inbred lines contributes to drought tolerance in crops. Plant Biotechnol J. 2021;19(10):2069–81. https://doi.org/10.1111/pbi.13637
19. Xiang Y, Sun XJ, Bian XL, Wei TH, Han T, Yan JW, Zhang AY. The transcription factor *ZmNAC49* reduces stomatal density and improves drought tolerance in maize. J Exp Bot. 2021;72(4):1399–410. https://doi.org/10.1093/jxb/eraa507
20. Li ZFD, Wang X, Zeng R, Zhang X, Tian J, Zhang S, Yang X, Tian F, Lai J, Shi Y, Yang S. The transcription factor *bZIP68* negatively regulates cold tolerance in maize. Plant Cell. 2022;34(8):2833–51. https://doi.org/10.1093/plcell/koac137
21. Ren ZZ, Zhang PY, Su HH, Xie XW, Shao J, Ku LX, Tian ZQ, Deng DZ, Wei L. Regulatory mechanisms used by *ZmMYB39* to enhance drought tolerance in maize (*Zea mays*) seedlings. Plant Physiol Bioch. 2024;211:108696. https://doi.org/10.1016/j.plaphy.2024.108696
22. Bolle C. The role of GRAS proteins in plant signal transduction and development. Planta. 2004;218(5):683–92. https://doi.org/10.1007/s00425-004-1203-z
23. Li P, Zhang B, Su TB, Li PR, Xin XY, Wang WH, Zhao XY, Yu YJ, Zhang DH, Yu SC et al. *BrLAS*, a GRAS transcription factor from Brassica rapa, is involved in drought stress tolerance in transgenic *Arabidopsis*. Frontiers in Plant Science. 2018;9:1792. https://doi.org/10.3389/fpls.2018.01792
24. Peng JR, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP, Harberd NP. The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates Gibberellin responses. Gene Dev. 1997;11(23):3194–205. https://doi.org/10.1101/gad.11.23.3194
25. Fode B, Siemsen T, Thurow C, Weigel R, Gatz C. The GRAS protein SCL14 interacts with class II TGA transcription factors and is essential for the activation of stress-inducible promoters. Plant Cell. 2008;20(11):3122–35. https://doi.org/10.1105/tpc.108.058974
26. Wang TT, Yu TF, Fu JD, Su HG, Chen J, Zhou YB, Chen M, Guo J, Ma YZ, Wei WL, et al. Genome-Wide analysis of the GRAS gene family and functional identification of *GmGRAS37* in drought and salt tolerance. Front Plant Sci. 2020;11:604690. https://doi.org/10.3389/fpls.2020.604690
27. Li CH, Guan HH, Jing X, Li YY, Wang BB, Li YX, Liu XY, Zhang DF, Liu C, Xie XQ, et al. Genomic insights into historical improvement of heterotic groups during modern hybrid maize breeding. Nat Plants. 2022;8(7):750–63. https://doi.org/10.1038/s41477-022-01190-2
28. Gao XY, Becker LC, Becker DM, Starmer JD, Province MA. Avoiding the high bonferroni penalty in genome-wide association studies. Genet Epidemiol. 2010;34(1):100–5. https://doi.org/10.1002/gepi.20430

29. Johnson RC, Nelson GW, Troyer JL, Lautenberger JA, Kessing BD, Winkler CA, O'Brien SJ. Accounting for multiple comparisons in a genome-wide association study (GWAS). BMC Genomics. 2010;11:724. https://doi.org/10.1186/1471-2164-11-724
30. Zhang XJ, Liu XY, Zhang DF, Tang HJ, Sun BC, Li CH, Hao LY, Liu C, Li YX, Shi YS, et al. Genome-wide identification of gene expression in contrasting maize inbred lines under field drought conditions reveals the significance of transcription factors in drought tolerance. PLoS ONE. 2017;12(7):e179477. https://doi.org/10.1371/journal.pone.0179477
31. Langmead B. Aligning short sequencing reads with bowtie. Curr Protoc Bioinf. 2010;32(1):11. https://doi.org/10.1002/0471250953.bi1107s32
32. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 2013;14(4):R36. https://doi.org/10.1186/gb-2013-14-4-r36
33. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L. Differential gene and transcript expression analysis of RNA-seq experiments with tophat and cufflinks. Nat Protoc. 2012;7(3):562–78. https://doi.org/10.1038/nprot.2012.016
34. Anders S, McCarthy DJ, Chen YS, Okoniewski M, Smyth GK, Huber W, Robinson MD. Count-based differential expression analysis of RNA sequencing data using R and bioconductor. Nat Protoc. 2013;8(9):1765–86. https://doi.org/10.1038/nprot.2013.099
35. Anders S, Pyl PT, Huber W. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31(2):166–9. https://doi.org/10.1093/bioinformatics/btu638
36. Deng MD, Wang Y, Kuzma M, Chalifoux M, Tremblay L, Yang SJ, Ying JF, Sample A, Wang HM, Griffiths R, et al. Activation tagging identifies *Arabidopsis* transcription factor *AtMYB68* for heat and drought tolerance at yield determining reproductive stages. Plant J. 2020;104(6):1535–50. https://doi.org/10.1111/tpj.15019
37. Park MY, Kang JY, Kim SY. Overexpression of *AtMYB52* confers ABA hypersensitivity and drought tolerance. Mol Cells. 2011;31(5):447–54. https://doi.org/10.1007/s10059-011-0300-7
38. Yamaguchi N, Matsubara S, Yoshimizu K, Seki M, Hamada K, Kamitani M, Kurita Y, Nomura Y, Nagashima K, Inagaki S et al. H3K27me3 demethylases alter *HSP22* and *HSP17.6C* expression in response to recurring heat in *Arabidopsis*. Nat Commun. 2021;12(1):3480. https://doi.org/10.1038/s41467-021-23766-w
39. Cheng MC, Hsieh EJ, Chen JH, Chen HY, Lin TP. *Arabidopsis* RGLG2, functioning as a RING E3 ligase, interacts with AtERF53 and negatively regulates the plant drought stress response. Plant Physiol. 2012;158(1):363–75. https://doi.org/10.1104/pp.111.189738
40. Kurotani K-I, Hattori T, Takeda S. Overexpression of a *CYP94* family gene *CYP94C2b* increases intern ode length and plant height in rice. Plant Signal Behav. 2015;10(7):e1046667. https://doi.org/10.1080/15592324.2015.1046667
41. Chintakovid N, Maipoka M, Phaonakrop N, Mickelbart MV, Roytrakul S, Chadchawan S. Proteomic analysis of drought-responsive proteins in rice reveals photosynthesis-related adaptations to drought stress. Acta Physiol Plant. 2017;39(10):240. https://doi.org/10.1007/s11738-017-2532-4
42. Xu K, Chen SJ, Li TF, Ma XS, Liang XH, Ding XF, Liu HY, Luo LJ. *OsGRAS23*, a rice *GRAS* transcription factor gene, is involved in drought stress response through regulating expression of stress-responsive genes. BMC Plant Biol. 2015;15:141. https://doi.org/10.1186/s12870-015-0532-3
43. Hao Z, Liu X, Li X, Xie C, Li M, Zhang D, Zhang S, Xu Y. Identification of quantitative trait loci for drought tolerance at seedling stage by screening a large number of introgression lines in maize. Plant Breeding. 2009;128(4):337–41. https://doi.org/10.1111/j.1439-0523.2009.01642.x
44. Ruta N, Liedgens M, Fracheboud Y, Stamp P, Hund A. QTLs for the elongation of axile and lateral roots of maize in response to low water potential. Theor Appl Genet. 2010;120(3):621–31. https://doi.org/10.1007/s00122-009-1180-5
45. Mao HD, Wang HW, Liu SX, Li Z, Yang XH, Yan JB, Li JS, Tran LSP, Qin F. A transposable element in a *NAC* gene is associated with drought tolerance in maize seedlings. Nat Commun. 2015;6:8326. https://doi.org/10.1038/ncomms9326
46. Li PC, Zhang YY, Yin SY, Zhu PF, Pan T, Xu Y, Wang JY, Hao DR, Fang HM, Xu CW, et al. QTL-by-environment interaction in the response of maize root and shoot traits to different water regimes. Front Plant Sci. 2018;9:229. https://doi.org/10.3389/fpls.2018.00229
47. Luo X, Wang BC, Gao S, Zhang F, Terzaghi W, Dai MQ. Genome-wide association study dissects the genetic bases of salt tolerance in maize seedlings. J Integr Plant Biol. 2019;61(6):658–74. https://doi.org/10.1111/jipb.12797
48. Sun M, Xu Q-Y, Zhu Z-P, Liu P-Z, Yu J-X, Guo Y-X, Tang S, Yu Z-F, Xiong A-S. AgMYB5, an MYB transcription factor from celery, enhanced β-carotene synthesis and promoted drought tolerance in Transgenic Arabidopsis. BMC Plant Biol. 2023;23(1):151. https://doi.org/10.1186/s12870-023-04157-3
49. Zhu N, Duan BL, Zheng HL, Mu RR, Zhao YY, Ke LP, Sun YQ, An, editors. gene *GhMYB3* functions in drought stress by negatively regulating stomata movement and ROS accumulation. Plant Physiol Bioch. 2023;197:107648. https://doi.org/10.1016/j.plaphy.2023.107648
50. He ZH, Zhong JW, Sun XP, Wang BC, Terzaghi W, Dai MQ. The maize ABA receptors *ZmPYL8*, *9*, and *12* facilitate plant drought resistance. Front Plant Sci. 2018;9:422. https://doi.org/10.3389/fpls.2018.00422
51. Peng PH, Lin CH, Tsai HW, Lin TY. Cold response in Phalaenopsis aphrodite and characterization of *PaCBF1* and *PaICE1*. Plant Cell Physiol. 2014;55(9):1623–35. https://doi.org/10.1093/pcp/pcu093
52. Lu X, Yang L, Yu MY, Lai JB, Wang C, McNeil D, Zhou MX, Yang CW. A novel *Zea mays* Ssp. mexicana L. MYC-type *ICE*-like transcription factor gene *ZmmICE1*, enhances freezing tolerance in Transgenic Arabidopsis thaliana. Plant Physiol Bioch. 2017;113:78–88. https://doi.org/10.1016/j.plaphy.2017.02.002
53. Verma RK, Kumar VVS, Yadav SK, Kumar TS, Rao MV, Chinnusamy V. Overexpression of *Arabidopsis ICE1* enhances yield and multiple abiotic stress tolerance in indica rice. Plant Signal Behav. 2020;15(11):1814547. https://doi.org/10.1080/15592324.2020.1814547
54. Duan YD, Han JX, Guo BT, Zhao WB, Zhou S, Zhou CW, Zhang L, Li XG, Han DG. *MbICE1* confers drought and cold tolerance through up-regulating antioxidant capacity and stress-resistant genes in. Int J Mol Sci. 2022;23(24):16072. https://doi.org/10.3390/ijms232416072
55. Han J, Jawad Umer M, Yang M, Hou Y, Gereziher Mehari T, Zheng J, Wang H, Liu J, Dong W, Xu Y, et al. Genome-wide identification and functional analysis of *ICE* genes reveal that *Gossypium thurberi GthICE2* is responsible for cold and drought stress tolerance. Plant Physiol Bioch. 2023;199. https://doi.org/10.1016/j.plaphy.2023.107708
56. Audran C, Liotenberg S, Gonneau M, North H, Frey A, Tap-Waksman K, Vartanian N, Marion-Poll A. Localisation and expression of Zeaxanthin epoxidase mRNA in *Arabidopsis* in response to drought stress and during seed development. Aust J Plant Physiol. 2001;28(12):1161–73. https://doi.org/10.1071/PP00134
57. Soares A, Niedermaier S, Faro R, Loos A, Manadas B, Faro C, Huesgen PF, Cheung AY, Simoes I. An atypical aspartic protease modulates lateral root development in *Arabidopsis thaliana*. J Exp Bot. 2019;70(7):2157–71. https://doi.org/10.1093/jxb/erz059
58. Bao Y, Song WM, Zhang HX. Role of *Arabidopsis* NHL family in ABA and stress response. Plant Signal Behav. 2016;11(5):e1180493. https://doi.org/10.1080/15592324.2016.1180493
59. Cao LR, Wang GR, Fahim AM, Pang YY, Zhang QJ, Zhang X, Wang ZH, Lu XM. Comprehensive analysis of the *DnaJ/HSP40* gene Gamily in maize (*Zea mays* L.) reveals that enhances abiotic stress tolerance. J Plant Growth Regul. 2024;43(5):1548–69. https://doi.org/10.1007/s00344-023-11206-6
60. Li T, Zhang YM, Liu Y, Li XD, Hao GL, Han QH, Dirk LMA, Downie AB, Ruan YL, Wang JM, et al. Raffinose synthase enhances drought tolerance through raffinose synthesis or galactinol hydrolysis in maize and *Arabidopsis* plants. J Biol Chem. 2020;295(23):8064–77. https://doi.org/10.1074/jbc.RA120.013948
61. Zhao Y, Ma Q, Jin XL, Peng XJ, Liu JY, Deng L, Yan HW, Sheng L, Jiang HY, Cheng BJ. A novel maize homeodomain–leucine zipper (HD-Zip) I gene, *Zmhdz10*, positively regulates drought and salt tolerance in both rice and *Arabidopsis*. Plant Cell Physiol. 2014;55(6):1142–56. https://doi.org/10.1093/pcp/pcu054
62. Zeng P, Zhu PW, Qian LF, Qian XM, Mi YX, Lin ZF, Dong SN, Aronsson H, Zhang HS, Cheng JP. Identification and fine mapping of *qGR6.2*, a novel locus controlling rice seed germination under salt stress. BMC Plant Biol. 2021;21(1):36. https://doi.org/10.1186/s12870-020-02820-7
63. Chen X, Wang YF, Lv B, Li J, Luo LQ, Lu SC, Zhang X, Ma H, Ming F. The *NAC* family transcription factor *OsNAP* confers abiotic stress response through the ABA pathway. Plant Cell Physiol. 2014;55(3):604–19. https://doi.org/10.1093/pcp/pct204
64. Shen T, Xu FJ, Chen D, Yan RJ, Wang QW, Li KY, Zhang G, Ni L, Jiang MY. A B-box transcription factor *OsBBX17* regulates saline-alkaline tolerance through the MAPK cascade pathway in rice. New Phytol. 2024;241(5):2158–75. https://doi.org/10.1111/nph.19480
65. Guo J, Li CH, Zhang XQ, Li YX, Zhang DF, Shi YS, Song YC, Li Y, Yang DG, Wang TY. Transcriptome and GWAS analyses reveal candidate gene for seminal root length of maize seedlings under drought stress. Plant Sci. 2020;292:110380. https://doi.org/10.1016/j.plantsci.2019.110380

66. Sha XQ, Guan HH, Zhou YQ, Su EH, Guo J, Li YX, Zhang DF, Liu XY, He GH, Li Y, et al. Genetic dissection of crown root traits and their relationships with aboveground agronomic traits in maize. J Integr Agr. 2023;22(11):3394–407. https://doi.org/10.1016/j.jia.2023.04.022
67. Liu SX, Li CP, Wang HW, Wang SH, Yang SP, Liu XH, Yan JB, Li BL, Beatty M, Zastrow-Hayes G, et al. Mapping regulatory variants controlling gene expression in drought response and tolerance in maize. Genome Biol. 2020;21(1):163. https://doi.org/10.1186/s13059-020-02069-1
68. Khan SU, Zheng YX, Chachar Z, Zhang XH, Zhou GY, Zong N, Leng PF, Zhao J. Dissection of maize drought tolerance at the flowering stage using genome-wide association studies. Genes-Basel. 2022;13(4):564. https://doi.org/10.3390/genes13040564
69. TanWW, Li YX, Wang Y, Liu C, Liu ZZ, Peng B, Wang D, Zhang Y, Sun BC, Shi YS. QTL mapping of ear traits of maize under different water regimes. Acta Agron Sin. 2011;37:235–48. https://doi.org/10.3724/sp.j.1006.2011.00235
70. Yang YX, Sang ZQ, Du QG, Guo ZF, Li ZW, Kong XY, Xu YB, Zou C. Flowering time regulation model revisited by pooled sequencing of mass selection populations. Plant Sci. 2021;304:110797. https://doi.org/10.1016/j.plantsci.2020.110797
71. Werck-Reichhart D, Bak S, Paquette S. Cytochromes p450. Arabidopsis Book. 2002;1:e0028. https://doi.org/10.1199/tab.0028
72. Schuler MA, Werck-Reichhart D. Functional genomics of P450s. Annual Eeview of plant biology. 2003; 54:629–67. https://doi.org/10.1146/annurev.arplant.54.031902.134840
73. Mao GH, Seebeck T, Schrenker D, Yu O. *CYP709B3*, a cytochrome P450 monooxygenase gene involved in salt tolerance in *Arabidopsis thaliana*. BMC Plant Biol. 2013;13:169. https://doi.org/10.1186/1471-2229-13-169
74. Magwanga RO, Lu P, Kirungu JN, Dong Q, Cai XY, Zhou ZL, Wang XX, Hou YQ, Xu YC, Peng RH, et al. Knockdown of cytochrome P450 genes *Gh_D07G1197* and *Gh_A13G2057* on chromosomes D07 and A13 reveals their putative role in enhancing drought and salt stress tolerance in *Gossypium hirsutum*. Genes-Basel. 2019;10(3). https://doi.org/10.3390/genes10030226
75. Wang J, Qin H, Zhou SR, Wei PC, Zhang HW, Zhou Y, Miao YC, Huang RF. The ubiquitin-binding protein OsDSK2a mediates seedling growth and salt responses by regulating Gibberellin metabolism in rice. Plant Cell. 2020;32(2):414–28. https://doi.org/10.1105/tpc.19.00593
76. Choi HI, Hong JH, Ha JO, Kang JY, Kim SY. *ABFs*, a family of ABA-responsive element binding factors. J Biol Inorg Chem. 2000;275(3):1723–1730. https://doi.org/10.1074/jbc.275.3.1723
77. Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. Basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. P Natl Acad Sci USA. 2000;97(21):11632–7. https://doi.org/10.1073/pnas.190309197
78. Shinozaki K, Yamaguchi-Shinozaki K, Seki M. Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol. 2003;6(5):410–7. https://doi.org/10.1016/S1369-5266(03)00092-X
79. Tran LSP, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. Isolation and functional analysis of *Arabidopsis* stress-inducible *NAC* transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promote. Plant Cell. 2004;16(9):2481–98. https://doi.org/10.1105/tpc.104.022699
80. Guo T, Wang XW, Shan K, Sun WX, Guo LY. The loricrin-like protein (LLP) of phytophthora infestans is required for oospore formation and plant infection. Front Plant Sci. 2017;8. https://doi.org/10.3389/fpls.2017.00142
81. Murase K, Hirano Y, Sun TP, Hakoshima T. Gibberellin-induced DELLA recognition by the Gibberellin receptor GID1. Nature. 2008;456(7221):459–63. https://doi.org/10.1038/nature07519
82. Schwechheimer C. Understanding gibberellic acid signaling - are we there yet? Curr Opin Plant Biol. 2008;11(1):9–15. https://doi.org/10.1016/j.pbi.2007.10.011
83. She M, Zheng D, Zhang S, Ke Z, Wu Z, Zou H, Zhang Z. Functional analysis of maize *GRAS* transcription factor gene *ZmGRAS72* in response to drought and salt stresses. Agric Commun. 2024;2(3):100054. https://doi.org/10.1016/j.agrcom.2024.100054
84. DiLaurenzio L, WysockaDiller J, Malamy JE, Pysh L, Helariutta Y, Freshour G, Hahn MG, Feldmann KA, Benfey PN. The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* root. Cell. 1996;86(3):423–33. https://doi.org/10.1016/S0092-8674(00)80115-4
85. Levesque MP, Vernoux T, Busch W, Cui HC, Wang JY, Blilou I, Hassan H, Nakajima K, Matsumoto N, Lohmann JU, et al. Whole-genome analysis of the SHORT-ROOT developmental pathway in *Arabidopsis*. Plos Biol. 2006;4(5):e143. https://doi.org/10.1371/journal.pbio.0040249

Publisher's note