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Identification of candidate genes for low temperature tolerance during germination of maize using a genome-wide association study and RNA-sequencing

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Abstract

Background: Maize (*Zea mays* L.) is the largest agricultural crop in the world based on acreage and yield, however, it is inherently sensitive to low temperatures. The growth and yield of maize can be affected by low temperature during its whole growth period, particularly during germination. Therefore, it is urgent to identify the new gene(s) related to the low temperature tolerance during maize germination.

Results: In this study, 14 phenotypic traits related to seed germination were used to explore the genetic architecture of maize through genome-wide association analysis (GWAS). A total of 30 single nucleotide polymorphisms (SNPs) associated with low temperature tolerance were detected ($-\log10(P) > 4$); 14 candidate genes were detected as being directly associated with these SNPs and 81 candidate genes were identified when the screen was extended to a distance of 30 kb from these SNPs. The candidate genes were predicted by conjoint analysis with RNA-sequencing (RNA-seq) to evaluate

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whole-genome gene expression levels. A total of nine differentially expressed genes (DEGs) (|log2foldchange|≥0.585， P<0.05) were found within distance of 30 kb, including two DEGs (*GRMZM2G101383* and *GRMZM2G402584*), which were associated with SNPs directly. The differential expression of these candidate genes was verified using qRT-PCR. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms of DEGs *GRMZM2G052129* and *GRMZM2G038964* were, 'fatty acid metabolic process', 'Mitogen-activated protein kinase (MAPK) signal transduction', and so on, which are related to the fluidity of the cell membrane and low temperature signal transduction.

Conclusion: Therefore, further functional analysis of *GRMZM2G052129* and *GRMZM2G038964* will provide valuable information for understanding the genetic mechanism of low temperature tolerance during germination in maize.

Keywords: maize, low temperature, germination, genome-wide association analysis, RNA-seq, candidate genes

Background

Maize (*Zea mays* L.) originated from tropical and subtropical regions and has a relatively high temperature threshold for germination [1]. As a consequence, it is inherently sensitive to low temperatures, particularly during germination, and so is seldom cultivated in higher latitudes or on mountains. When maize is cultivated at cold zones, the plants grow more slowly and have a shorter growing season which leads to lower seedling vitality and reduced yields. It is known that the minimum temperature for maize seed germination is about 10℃ [2]. When the temperature drops to 6 to 8℃,

irreversible damage will occur to cells and tissues. Under these conditions, seeds normally won't germinate and the growth of seedlings will stop [1]. Thus 10℃ has normally been chosen as the growth conditions for the identification and screen of proper maize germplasm [3, 4, 5]. In recent years, the occurrence of cold weather becomes more uncertain and low temperature weather occurs more frequently, especially during the germination period and early developmental stage of the seedlings. Low temperature stress not only reduces the emergence rate of maize seeds and seedling vigor, but also increases the chance of pathogenic infection by soil bacteria, which can seriously affect maize yields. Therefore, it is urgent to identify the new gene(s) related to the low temperature tolerance during maize germination.

At present, significant progress has been made in the identification of genetic loci associated with low temperature tolerance in maize. Their corresponding genes are normally identified by gene mapping and GWAS. Some indices, such as emergence rate, seedling emergence index, seedling dry weight, relative average germination time, and percentage of relative viability, have been used for genetic loci identification in maize inbred lines and populations [3, 6, 7]. For instance, a major QTL for low temperature tolerance of photosynthesis was detected on chromosome 6 using Ac7643×Ac7729/TZ population [8]. Two other QTL on chromosomes 3.01 and 6.03 were reported that affect leaf color at low temperatures, and a candidate gene, *luteus11*, was identified [9]. Using a panel of European flint maize inbred lines, 47 lines were found to harbor favorable alleles for six significant QTL [10]. In another study, 12 QTL controlling low-temperature germination rate and primary radicle length were detected

on chromosomes 4, 5, 6, 7 and 9 using 243 lines of the intermated B73×Mo17 (IBM) Syn4 recombinant inbred line (RIL) population [11]. Later work identified 43 QTL that explained 0.62%~39.44% of phenotypic variance for low temperature seed germination in maize using three connected $F_{2:3}$ populations with inbred lines of two low temperature tolerant inbred lines 220 and P9-10 and two susceptible lines Y1518 and PH4CV [5]. Several SNPs associated with low temperature tolerance traits at the seedling and seed germination stages were detected using GWAS. For example, 43 SNPs associated with 10 low temperature tolerance traits in maize seedlings or seed germination were found although no overlapping SNPs were identified at both development stages [12]. A total of 19 markers related to low temperature tolerance were identified through GWAS of 375 inbred lines in outdoor and artificial climate chambers, which explained 5.7%~52.5% of the phenotypic genetic variation for seedling growth stage and chlorophyll fluorescence parameters [13]. A further GWAS study identified 18 candidate genes from 17 genetic loci associated with low temperature tolerant germination, where 10 candidate genes were supported by previous QTL studies [14].

Many low temperature tolerance genes have been identified in many crops and the molecular mechanisms have been studied. In rice, *COLD1* interacts with the G-protein alpha subunit which accelerates its GTPase activity resulting in activation of Ca^{2+} channels for low temperature responses [15]. During the reproductive growth period of rice, the protein kinase gene *CTB4a* is involved in maintaining high pollen fertility under low temperature conditions which increases seed setting rate and yield. In

addition, *CTB4a* interacts with *AtpB* to regulate ATP content under low temperatures to enhance low temperatures tolerance [16]. The MADS-box family transcription factor OsMADS57 interacting with OsTB1 also regulates low-temperature tolerance in rice and helps balance growth and defense responses, which is dependent on *OsWRKY94*, their common target gene [17]. In addition to the transcriptional control, phosphorylation of Basic Transcription Factor 3 Like (BTF3L) protein by protein kinase OST1 promotes the interaction between BTF3L and CBFs, which increases the stability of CBF proteins and enhances plant low temperature tolerance [18]. Similarly, numerous studies investigating the mechanisms of low temperature tolerance have been reported in maize, but they have had limited impact on maize breeding. Several maize genes, including *CAT3*, *ZmCDPK1*, *ZmSEC14p*, with roles in low temperature tolerance have been identified. Their expression alleviates photodamage and increases expression of stress response proteins [19, 20, 21, 22]. Some genes that control kernel weight or kernel number at low temperatures have also been cloned, including genes that were shown to induce the expression of bZIP type and ERF/AP2 type transcription factors [23, 24].

There have been some studies on low temperature tolerance of maize during germination, but the detailed evaluation indices are missing. Thus, we established the standard evaluation system in the present study for genetic mapping study with particular consideration of the actual conditions of sowing in early spring in Northeast China. Low temperature evaluation methods for maize often include both field and indoor identification methods. The field methods are more objective. They can be used

105 to evaluate low temperature tolerance of maize during each growth period and to
106 analyze genotype by environment interactions. However, such field studies are easily
107 affected by changes in climatic conditions at different locations and different years, and
108 environmental conditions are difficult to control. There are many interfering factors and
109 repeatability is generally poor. Indoor evaluation methods have several advantages.
110 They are not limited by seasons, they have fewer interfering factors, the environmental
111 conditions are easily controlled, and different temperature gradients can be set during
112 the experiment to accurately assess the tolerance of different maize varieties to low
113 temperature, but one disadvantage to this method is that it is generally unable to assess
114 genotypes. Because of environmental interaction effects, two methods of evaluation,
115 both indoor and outdoor, were used and an improved technical system was established
116 for present study. Many performance indicators have been applied to evaluate low
117 temperature tolerance of maize germination, such as germination potential, germination
118 rate, germination index, radicle length and germ length [5, 14]. In order to eliminate
119 genetic background differences, the ratio of relative values of low temperature to
120 normal temperature for various traits is usually used as an indicator to measure the
121 strength of low temperature resistance [3].

122 With the above established evaluation system, a maize population panel of 222
123 diverse inbred lines were used for the analysis of the low temperature tolerance
124 correlated traits via GWAS. The candidate genes were predicted based on RNA-seq
125 data to achieve the following objectives: (1) identify potential SNPs responsible for low
126 temperature tolerance during development of seed germination; (2) find extremely low-

temperature tolerant maize inbred lines; (3) predict and identify the involved candidate genes. (4) select candidate genes for future studies and agriculture application.

Results

Low-temperature germination ability of the maize lines

The germination rate (Fig. 1a) and seedling (Fig. 1b, c, d, e) performance of the 222 maize lines were evaluated. The following 14 traits were measured: relative germination rate (RGR), relative germ length (RGL), relative radicle length (RRL), relative radicle surface area (RRSA), relative radicle volume (RRV), relative germination index (RGI), relative vitality index (RVI), relative simple vitality index (RSVI), XiangYang relative germination rate (XYRGR), XiangYang relative germ length (XYRGL), XiangYang relative simple vitality index (XYRSVI), KeShan relative germination rate (KSRGR), KeShan relative germ length (KSRGL), and KeShan relative simple vitality index (KSRSVI). Descriptive statistics on the relative values of germination traits under low temperature conditions and normal conditions, including the number of observations (n), mean, median, standard deviation (SD), and range, were calculated. For most of the traits, considerable phenotypic variation was detected among the lines, with means that ranged from 0.141 for RVI to 0.787 for RGR. RGR varied from 0.027 to 0.999 with an average of 0.737. RVI varied from 0.001 to 0.812 with an average of 0.162 (Table 1).

In total, 14 traits, which had mono-modal distributions, were measured (Fig. 2). Correlation analysis was carried out on the relative values of each index under low temperature stress. The traits in the germination period were closely related to the

149 regulation of maize growth (P<0.1), except for relative germination rate and relative
150 germ length. For the indoor traits, RSVI and RGL (0.83), RVI and RGI (0.82), and
151 RRSA and RRL (0.78), showed high correlation (P<0.01). There was significant
152 positive correlation among the other traits, indicating that the 8 traits selected could be
153 used as important indicators for low temperature tolerance in maize germination (Fig.
154 2a). Except for the XYRGL and KSRGL, most field traits reached a significant level of
155 correlation (P<0.1). More significant positive correlation was found between the other
156 traits of field such as XYRGR and XYRSVI (0.94), KSRGR and KSRSVI (0.89)
157 (P<0.01). Thus, the 6 selected traits could be used for evaluation of low temperature
158 tolerance in maize germination in the field (Fig. 2b). However, correlations were weak
159 between each trait under indoor and field conditions, such as RSVI and XYRGL (0.11)
160 (P>0.1) (Fig. 2c).

161 **Associated SNPs for GWAS**

162 GWAS was conducted on the BLUPs of the 14 relative traits (RGR, RGL, RRL,
163 RRSA, RRV, RGI, RVI, RSVI, XYRGR, XYRGL, XYRSVI, KSRGR, KSRGL,
164 KSRSVI) for 222 maize inbred lines. These lines were genotyped by 40,757 SNPs,
165 using the Tassel method and software package (http://www.maizegenetics.net/tassel).
166 GWAS of SNP markers and traits was performed using the Mixed-linear model
167 (MLM) combined with population structure and kinship using TASSEL5.0 software.
168 A total of 30 SNPs showed highly significant association with 14 traits (P values were
169 from 1.70E-07 to 9.84E-05; Fig. 3).

170 Among the 30 associated SNPs, three SNPs (SYN30173, PZE-105034900, PZE-

171 109053558) were associated with two traits (RSL and RSVI, RRV and RVI, XYRGR
172 and XYRSVI). These SNPs were distributed on all 10 maize chromosomes with the
173 highest number on chromosomes 2, which contained five SNPs. For each trait as RGR,
174 RRL, XYRGR, XYRSVI, XYRGL, KSRGR and KSRSVI, one SNP was found. Two
175 SNPs were detected for RGL, and the most significant association was for SYN30173,
176 with a *P* value of 3.75E-05. Ten SNPs were associated with RRV, they are located on
177 chromosomes 1 (SYN25470), 2 (SYN21841), 4 (PZE-104024779), 5 (PZE-
178 105034900), and 7 (PZE-107036384). Three SNPs were located on chromosome 6
179 (PZE-106003222, PZE-106129965, PZE-106130106), and two SNPs (PZE-108064544
180 and SYN26538) were found on chromosome 8. Four SNPs were found for RRSA
181 (SYN29778, PZE-108068725, PZE-103072583, PZE-110029252), RGI (PZE-
182 101120376, PZE-102099570, PZE-106097864, SYN5516) and RVI (SYN25470, PZE-
183 102100684, PZE-105034900, PZE-110057591). Two SNPs were associated with RSVI,
184 one on chromosomes 5 (SYN30173) and the other on chromosome 7 (SYN4961) (Table
185 2).

186 **Identification of candidate genes in maize**

187 Total of 14 candidate genes were found directly associated with SNPs using the B73
188 RefGen_v1 Maize Gene Database (http:// www.maizegdb.org/) (Table 3). Two of them,
189 which are directly associated with SNPs (*GRMZM2G107309* and *GRMZM2G158359*),
190 were correlated with RSVI, while *GRMZM2G158359* was also correlated with RGL.
191 Three candidate genes (*GRMZM2G101383*, *GRMZM2G173195* and
192 *GRMZM2G390374*) were associated with RRSA. Three genes (*GRMZM2G007734*,

193 *GRMZM2G092000* and *GRMZM2G001772*) were associated with RRV. And two genes
194 (*GRMZM2G402584* and *GRMZM2G001772*) were associated with RVI, while
195 *GRMZM2G001772* was both correlated with RRV and RVI. For RGR
196 (*GRMZM2G012088*), RGI (*GRMZM2G118286*), XYRGL (*GRMZM2G059110*),
197 KSRGR (*GRMZM2G152921*), and KSRSVI (*GRMZM2G005980*), only one candidate
198 gene was associated. A 30 kb window was selected to fall within the estimated window
199 of LD decay in our association panel [25]. In addition, extra 81 candidate genes were
200 identified within 30 kb of 30 associated SNPs (Additional file 1: Table S1).

201 **Differential gene expression and determination of candidate genes**

202 To help identify candidate genes for the identified SNPs, the low temperature resistant
203 maize line (Zao8-3, named 55) and low temperature sensitive line (Ji853, named 102)
204 were selected from among the 222 inbred lines and RNA-seq analysis was performed
205 to evaluate genome-wide gene expression levels. A total of 4982 DEGs were identified
206 at CT_102vsCT_55 down, 5550 DEGs were identified at CT_102vsCT_55 up, 5477
207 DEGs were identified at LT_102vsLT_55 down, 5661 DEGs were identified at
208 LT_102vsLT_55 up. The result showed that 1022 DEGs were down-regulated only in
209 LT_102vsLT_55 comparison groups, and that 985 DEGs were up-regulated only in
210 LT_102vsLT_55 comparison groups. Four DEGs ($|\log_2 \text{foldchange}| \geq 0.585$, $P<0.05$)
211 were down-regulated in LT_102vsLT_55 comparison groups and were up-regulated in
212 CT_102vsCT_55 comparison groups. Three DEGs ($|\log_2 \text{foldchange}| \geq 0.585$, $P<0.05$)
213 were up-regulated in LT_102vsLT_55 comparison groups while they were down-
214 regulated in CT_102vsCT_55 comparison groups (Fig. 4).

A total of 95 candidate genes were found with associated SNPs, and nine important DEGs were identified, including six candidate genes (*GRMZM2G038964*, *GRMZM2G059042, GRMZM2G009223*, *GRMZM2G052129*, *GRMZM2G073861*, and *GRMZM2G101408*) that were down-regulated only in LT_102vsLT_55 comparison groups and three candidate genes (*GRMZM2G101383*, *AC213621.5_FG004*, and *GRMZM2G402584*) that were up-regulated only in LT_102vsLT_55 comparison groups. The putative identity of the proteins encoded by these genes is shown in (Table 4): *GRMZM2G038964* encodes a inositol phosphoryl ceramide-B C-26 hydroxylase, *GRMZM2G059042* encodes a photoperiod responsive protein, *GRMZM2G009223* a glucose-6-phosphate/phosphate translocator 2, *GRMZM2G052129* a Pleckstrin homology (PH) domain-containing protein, *GRMZM2G073861* an AT hook motif family protein, *GRMZM2G101408* a coiled-coil domain-containing protein 124, *GRMZM2G101383* a pseudouridine synthase family protein, *AC213621.5_FG004* a Fasciclin-like arabinogalactan family protein (SOS5), and *GRMZM2G402584* a monocopper oxidase.

Functional prediction of candidate genes

Total of 29 GO and three KEGG terms were found associated with the nine candidate genes (Tables 5, 6; Additional file 2: Table S2). These GO terms belong to multiple functions. The first type of function is related to biological processes, including protein phosphorylation, signal transduction, carbohydrate transmembrane transport (*GRMZM2G009223*), fatty acid metabolic process, protein ubiquitination (*GRMZM2G038964*), and protein glycosylation (*AC213621.5_FG004*). The second

type is related to molecular functions, which involve copper ion binding (*GRMZM2G402584*), fatty acid alpha-hydroxylase activity (*GRMZM2G038964*), ubiquitin-protein transferase activity (*GRMZM2G059042*), transferase activity, transferring glycosyl groups (AC213621.5_FG004), oxidoreductase activity and transferase activity (*GRMZM2G402584*), and nucleoside diphosphate kinase activity (*GRMZM2G052129*). The third type of function is related to cellular components involving the endoplasmic reticulum membrane and plasma membrane (*GRMZM2G152921*), chloroplast (*GRMZM2G052129*), plant-type cell wall, plasmodesma and mitochondrion (*GRMZM2G402584*), endoplasmic reticulum (*GRMZM2G038964*), Golgi apparatus and membrane (*AC213621.5_FG004*), cytoplasm (*GRMZM2G059042* and *GRMZM2G101408*), nucleus (*GRMZM2G052129* and *GRMZM2G101383*), and integral component of membrane (*AC213621.5_FG004* and *GRMZM2G402584*) (Table 5; Fig. 5a). The KEGGs contains two pathways. The first type of function is related to MAPK signal transduction and the second type of function is related to nucleotide metabolism, including Purine metabolism, and Pyrimidine metabolism (Table 6; Fig. 5b).

Validation of qRT-PCR for differentially expressed genes

Six candidate genes that showed significant differential expression levels in RNA seq were chosen for further qRT-PCR analysis. They are *GRMZM2G009223*, *GRMZM2G038964*, *GRMZM2G059042*, *GRMZM2G101383*, *GRMZM2G402584* and *GRMZM2G052129*. Comparison of qRT-PCR results and RNA-Seq data showed consistent expression trends for all six genes. Four genes (*GRMZM2G052129*,

GRMZM2G009223, *GRMZM2G038964*, and *GRMZM2G059042*) showed significantly higher expression levels in 55 than in 102 under 2h and 4h treatment of low temperatures. and two genes (*GRMZM2G402584* and GRMZM2G101383) showed significantly lower expression levels in 55 than in 102 (Fig. 6).

Two candidate genes are of particular interest, *GRMZM2G052129* and *GRMZM2G038964*. They showed significantly up-regulated expression in 55 and showed significant down-regulated expression in 102 under all conditions (both low-temperatures 2h and 4h), and are involved in functions related to low temperature resistance, such as the MAPK signaling pathway, and fatty acid metabolic processes.

Discussion

Relationship between phenotypic characteristics and low tolerance of maize

The entire growth process of maize is affected by low temperature stress beginning at the seed germination stage. The most important indicator of low temperature tolerance during germination is root emergence [1], which is a critical factor for plant development and yield. Low temperature stress decreases root activity, shortens root length, and results in fewer lateral roots in affected plants [26]. In accordance with previous studies, root and shoot growth were evaluated in controlled environmental chambers in a range of temperature regimes [27]. After germination, low temperature stress also affects maize seedlings. It was found that relative water content, leaf area and leaf dry weight, plant height, root length, stem length and dry weight, and whole plant fresh weight can all be affected [14]. In our study, low temperature condition was defined as 10℃, and 25℃ for normal conditions. The germination traits assessed were

281 radicle length, radicle surface area, and radicle volume. We observed a large phenotypic
282 variation in radicle length among the 222 maize inbred line under low temperature
283 conditions and a strong correlation between radicle length and germination rate.
284 However, their genetic loci were different indicating different mechanisms are involved.
285 Our study focused on germination, which was defined as radicle emergence from seed,
286 under low temperature conditions. The 30 associated SNPs and two candidate genes
287 identified in this study provide valuable resources for future studies to enhance the
288 understanding of the genetic underpinnings of low temperature tolerance in maize and
289 to improve maize varieties through breeding.

290 **Consistent SNPs in previous reports**

291 To evaluate the reliability of SNPs detected in this work, we compared the 30 SNPs
292 identified to those identified in several related publications. Three SNPs overlapped
293 with published QTL at the physical position B73 RefGen_v3 (Additional file 3: Table
294 S3). A major QTL for chlorophyll content on chromosome 2 (bnlg1909) [28] and QTL-
295 8 for ΦPSII at the seedling stage [29] are consistent with PUT-163a-149007696-748.
296 One SNP (PZE-106003222) was located at a chromosome 6 region that was reported to
297 harbor cold related QTL (bnlg249) for traits related to plant height at the seedling stage
298 [30]. One SNP (PZE-107036384) was within a QTL on chromosome 7 (qOTGR7-1)
299 for traits related to optimum-temperature germination rate at the germination stage [11].
300 In addition, a candidate gene (*GRMZM2G380561*) that was highly correlated with a
301 nearby SNP (PZE-108068725) was supported by previously identified candidate gene
302 [5]. Thus, our analysis successfully detected the known SNPs associated with low

303 temperature tolerance, indicating the identified SNPs from the present study are highly
304 reliable for use in gene cloning and maize breeding.

305 **MAPK signaling pathway in low temperature stress**

306 MAPKs are serine–threonine kinases that mediate intracellular signaling and play vital
307 roles in regulating plant growth, development, and stress responses [31]. At present,
308 many protein kinase genes, including MAPKs, have been shown to mediate
309 transduction of abiotic stress response signals [32]. In plants, the accumulation of
310 permeants and antioxidants can be induced by low temperature, drought and salt stress,
311 which is mediated by MAPK pathways in yeast and animals [33]. These MAPK
312 pathways are activated by different stimuli via receptors such as protein tyrosine kinases,
313 G protein coupled receptors and two-component histidine kinases. Arabidopsis has
314 approximately 60 MAPKKK, 10 MAPKK, and 20 MAPK. They can be activated by
315 low temperature and other abiotic stresses and are thought to be an important
316 component of abiotic stress signaling [34]. In *Medicago sativa*, low temperature
317 treatment resulted in the activation of a MAPK within ten min [35]. Similarly, in
318 tobacco cells, a MAPK and another protein kinase were activated by osmotic stress in
319 Ca^{2+} or ABA-independent manner [36]. The maize gene *ZmMAPK5* showed increased
320 expression in response to specific low temperature treatments [23]. So far, some
321 different mechanisms underlying low temperature response have been proposed and the
322 coordinated regulatory networks have been analyzed in rice and Arabidopsis. In
323 particular, the signal transduction pathways between MPK activation and ICE1 stability
324 at low temperatures were established. This marks an important breakthrough in the field

of plant low temperature response regulation [37, 38, 39] and reveals the important role of MAPK cascade signals. A candidate gene (*GRMZM2G052129*) reported in this study is related to the MAPK signaling pathway, which may be related to low temperature tolerance in maize.

Functional analysis of candidate genes

Two candidate genes (*GRMZM2G052129* and *GRMZM2G038964*) have putative functions related to low temperature resistance, such as MAPK signaling [23] and fatty acid hydroxylase activity [40], in maize or other species. *GRMZM2G052129* encodes a Pleckstrin homology (PH) domain-containing protein, which is homologous to Arabidopsis *AT4G23895* and rice *LOC_Os05g51710.1*. Pleckstrin homology (PH) domains are typically involved in targeting proteins to the appropriate cellular location or in protein-protein interactions. Despite minimal sequence conservation, they share a common electrostatically polarized fold. Some (<10%) PH domains bind phosphoinositide phosphates (PIPs) with high specificity and affinity. They are found in wide range of cellular signaling proteins including serine/threonine kinase, adaptors, cytoskeletal associated molecules, lipid associated enzymes, tyrosine kinases, regulators of G-proteins, and endocytotic GTPases [41]. The putative protein encoded by *GRMZM2G038964* is an inositolphosphorylceramide-B C-26 hydroxylase which belongs to fatty acid hydroxylase superfamily, sharing homology with the fatty acid hydroxylase in Arabidopsis (*AT2G34770*) and in rice (*LOC_Os03g56820.1*). The fatty acid hydroxylase superfamily includes fatty acid and carotene hydroxylases and sterol desaturases. Beta-carotene hydroxylase hydroxylates beta-carotene in zeaxanthin

synthesis, and may be involved in other pathways. Other family members include C-5 sterol desaturases and C-4 sterol methyl oxidases. The family members containing two copies of a HXHH motif are involved in cholesterol biosynthesis and biosynthesis of plant cuticular wax. They are typically integral membrane proteins [41]. Maize cell membrane fluidity significantly decreases under low temperature stress, and the normal physiological function of membrane-bound proteins is lost. Alteration of the composition of membrane lipid fatty acids through genetic manipulation was shown to improve low temperature tolerance of plants [40].

In conclusion, the two candidate genes (*GRMZM2G052129* and *GRMZM2G038964*) were found to have significantly different gene expression levels under low temperature treatment in resistant and sensitive maize lines. Homologs of these genes in Arabidopsis and rice have functions related to low temperature resistance to stress, so these genes are attractive candidate genes for involvement in low temperature tolerance in maize.

Conclusion

In the present study, contributes to the understanding of genetic control of low temperature tolerance in maize at germination stage using a panel of 222 maize inbred lines. Through genome-wide association analysis (GWAS), RNA-seq analysis, and validation of qRT-PCR, the two candidate genes (*GRMZM2G052129* and *GRMZM2G038964*) were found to have significantly different gene expression levels under low temperature treatment in resistant and sensitive maize lines.

Methods

Plant materials

A panel consisting of 222 maize inbred lines that belong to the five heterotic groups Lan (Lancaster Sure Crop), LRC (Lv da Red Cob), PB (Partner B), Reid, and SPT (Sipingtou) was used for the association study (This seeds were originally acquired from researcher Xinhai Li and associate researcher Jianfeng Weng, Institute of Crop Science, Chinese Academy of Agricultural Sciences) [42, 43]. These inbred lines were shown to have a wide range of variation in yield components and biotic stress tolerance. They are generally grown in the Yellow and Huai River valley regions which are in the northeastern and south-western China [42]. Seeds were planted in temperate Harbin City, Heilongjiang province in spring 2016. Plants were well managed without any disease, insect and weed issues during the whole growing seasons. Harvested seeds were fully dried and then stored at 4℃. Only seeds with a germination rate greater than 90% at 25℃ were used for the next test.

Two maize lines, which were resistant (Zao8-3, named 55) and sensitive (Ji853, named 102) to low temperature were selected from among the 222 inbred lines and RNA-seq was performed to evaluate whole-genome gene expression levels.

Seed germination and field experiment

The seed suface was disinfected with 1% NaOCl (sodium hypochlorite) for 5 minutes and the seeds were rinsed three times with sterile distilled water for germination experiments. After 6h of seed swelling at normal temperature(25℃), following the ISTA protocol (International Seed Testing Association), germination experiments were performed in germinating paper in a dark chamber at 10℃ for low temperature conditions (treatment) and at 25℃ for normal conditions (control). The germinating

391 paper were wetted and 50 sterilized seeds were sown on top it and then covered by
392 another piece of wet paper towel [5]. Seed germination was defined as an observed
393 radicle emergence of 0.5cm. After incubation at 10℃ for 31 days and at 25℃ for 6
394 days, radicle traits were measured by the Epson PerfectionV800, and Regent WinRhizo
395 Canada software was used for data analysis. For each condition (treatment and control),
396 three independent experiments were conducted for each line.
397 These field experimental sites were located in Xiangyang, Harbin and Keshan of
398 Qiqihar in 2018. The treatment group with low temperature were sown when the ground
399 temperature was stable around 6℃. The normal sowing time was used as a control. The
400 experiment used a random block design with three repetitions and seed germination
401 number and seedling length were determined.
402 **Collection of phenotypic data**
403 Germination rate (GR) was expressed as percentage of germinating plants at the last
404 day (10℃ for 31 days and 25℃ for 6 days) to the total number of seed used.
405 The germ traits and radicle traits were measured using the Epson PerfectionV800.
406 The germ trait was germ length (GL), and radicle traits were radicle length (RL), radicle
407 surface area (RAS) and radicle volume (RV). Germination of plants was calculated from
408 19 to 31 days after sowing (DAS) at 2-day intervals at 10℃ and was calculated daily,
409 from 3 to 6 days at 25℃. All traits were measured as the average of 10 seedlings.
410 Traits calculated were as follows: germination index (GI), vitality index (VI) and
411 simple vitality index (SVI).
412 $$\mathrm{GI} = \sum \frac{\mathrm{Gt}}{\mathrm{Dt}}$$

where Gt is the number of germinating plants at a given day (Dt, the days after sowing).

$VI = TL \times GI$, where TL is the total length of seedling (including germ length and radicle length) on last day and GI was the germination index. $SVI = GL \times GR$, where GR was the germination rate, GL was germ length.

Field experiment statistics of germination rate on the last day of Xiangyang (XYGR) and Keshan (KSGR) tests, germ length in XiangYang (XYGL) and KeShan (KSGL) was measured by a ruler, simple vitality index was calculated for two locations (XYRSVI and KSRSVI). Each experimental replicate contained 30 seedlings and measured the average of 10 seedlings as XYGL and KSGL.

The 14 phenotypic traits were described as the relative value to the ones of the control group, such as RGR, RGL, RRL, RRSA, RRV, RGI, RVI, RSVI, XYRGR, XYRGL, XYRSVI, KSRGR, KSRGL, and KSRSVI.

All the above traits were recorded and analyzed. The corresponding standard deviation, range, mean, and median were calculated for each trait. The IBM SPSS Statistics version 20.0 software and the R statistical package (R Core Team, 2016) were used for statistical analysis.

Genotypic data and genome-wide association analysis

Genotyping was carried out on the association panel using the Illumina Maize SNP50 BeadChip [25, 42, 44]. In total, 40,757 SNPs were used for the association analysis with a minor allele frequency (MAF) of >0.05 in the population. The software STRUCTURE version 2.3 [45] and SPAGeDi [46] with 5000 SNPs (MAF$0.2) were

used to estimate the population structure and analyze the kinship of the 222 inbred lines.

GWAS was performed with a mixed linear model (MLM) in Tassel and the following 14 traits were used for association analysis: RGR, RGL, RRL, RRSA, RRV, RGI, RVI, RSVI, XYRGR, XYRGL, XYRSVI, KSRGR, KSRGL and KSRSVI. Because a Bonferroni correction (1/40757=2.44−05) was too conservative, a less stringent threshold of –log10(*P)* > 4 was used to detect significant association signals [43]. A 30 kb window was selected to fall within the estimated window of LD decay in our association panel [25] where the average LD decay was about 27.7 kb. The genes were identified through MaizeGDB (https://www.maizegdb.org/) based on the locations of the closest flanking SNPs (P<0.0001). All potential candidate genes within 60 kb, ±30 kb centered on the lead SNP of the detected loci, were identified.

Transcriptome sequencing and analysis of differentially expressed genes

The low temperature resistant lines Zao8-3 (named 55) and one low temperature sensitive line Ji853 (named 102) were used in this study. Before imbibition, the seeds were sterilized and rinsed as described above. Seeds swelling at normal temperature (25℃) for 6h, then seeds were sampled at 2h imbibition within the germinating paper under the 'normal' condition (25℃) for CK_102 and CK_55 and low temperature condition (10℃) for LT_102 and LT_55. Embryos were isolated from the seeds of each sample, frozen in liquid nitrogen, and stored at −80℃ for RNA extraction. There were three biological replicates for each treatment. The maize RNA samples were sent for library construction and sequencing in LC-BIO (Hangzhou, China). Briefly, for each total RNA sample, the genomic DNA was removed by DNase I treatment and the

457 mRNA was enriched using oligo (dT) magnetic beads and was subsequently fragmented.
458 Random hexamer-primer was used for the synthesis of double strand cDNA which was
459 further modified by end repair and 3′ -end single nucleotide A (adenine) addition. The
460 fragments were amplified for sequencing via Illumina Novaseq™ 6000. The maize
461 genome database ZmB73RefGenv4
462 (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/005/005/GCF_000005005.2_B73_R
463 efGen_v4) was used as the reference genome. Read count for each gene was obtained
464 from the mapping results and normalized to FPKM (Fragments Per Kilobase of
465 transcript per Million mapped reads) The default threshold for significant difference in
466 gene expression was set at $|\log_2 \text{foldchange}| \geqslant 0.585$, $p<0.05$. Raw sequence data are
467 available in the NCBI's GEO database with accession number
468 GSE146666(GSM4403922- GSM4403933).

469 **Identification and annotation of candidate genes**

470 GO enrichment analysis was used to provide all GO terms of the genes whose
471 expression was significantly enriched compared to background in the lists of DEGs,
472 and to filter the DEGs corresponding to specific biological functions. All DEGs were
473 categorized and grouped based on their GO terms using the public database at
474 http://www.geneontology.org/. The gene numbers were calculated for every term. The
475 hypergeometric test and GO Term Finder
476 (http://www.yeastgenome.org/help/analyze/go-term-finder) were used to find
477 significantly enriched GO terms. The three categories, ‘biological process’, ‘cellular
478 component’, and ‘molecular function’, were over represented and were further filtered

using Bonferroni multi-test adjustment method and Fisher's exact test [47, 48]. To further understand the biological functions of DEGs, the significantly enriched metabolic or signal transduction pathways were identified using KEGGs [49] (http://www.genome.ad.jp/kegg/kegg2.html). Genes were annotated using the maize GDB (http://www.maizegdb.org) and NCBI databases (http://www.ncbi.nlm.nih.gov/).

Validation of qRT-PCR for candidate genes

The total RNA samples used for transcriptome sequencing of low temperature resistant lines Zao8-3 (named 55) and low temperature sensitive lines Ji853 (named 102) were also used for qRT-PCR. For each sample, 500 ng of RNA was used for reverse-transcription with the kit, *TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). qRT-PCR reactions were carried out using the Analytik Jena Real Time PCR Detection System with maize actin gene as the control. Gene specific primers were designed using the Primer5 software (Additional file 4: Table S4). The 20 μl reaction mixtures contained 2 μl of diluted cDNA, 0.4 μl of reverse and forward primers, 7.2 μl of ddH_2O and 10 μl of the 2×*TransStart*® Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China). The gene expression level was calculated using the relative 2^ -ΔΔCT analytical method [50]. For each sample, three biological replicates were included while each biological replicate was technically repeated three times. All data were presented as the mean ±SD after normalization.

Additional files

Additional file 1: Table S1. Functions of extra 81 candidate genes were identified within 30 kb of 30 associated SNPs. (DOCX 24 kb)

Additional file 2: Table S2. Total of 29 GO terms of associated with the nine candidate genes (DOCX 14 kb)

Additional file 3: Table S3. SNPs or candidate genes overlapped with published QTL. (DOCX 14 kb)

Additional file 4: Table S4. Primer sequences of Real-Time quantitative PCR. (DOCX 14 kb)

Abbreviations

GWAS: Genome-wide sssociation analysis; SNP(s): Single nucleotide polymorphism(s); RNA-seq: RNA-sequencing; DEGs: Differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; qRT-PCR: Real time quantitative PCR; RGR: Relative germination rate; RGL: Relative germ length; RRL: Relative radicle length; RRSA: Relative radicle surface area; RRV: Relative radicle volume; RGI: Relative germination index; RVI: Relative vitality index; RSVI: Relative simple vitality index; XYRGR: XiangYang relative germination rate; XYRGL: XiangYang relative germ length; XYRSVI: XiangYang relative simple vitality index; KSRGR: KeShan relative germination rate; KSRGL: KeShan relative germ length; KSRSVI: KeShan relative simple vitality index; MLM: Mixed-linear model; FPKM: Fragments Per Kilobase of transcript per Million mapped reads; SD: Standard deviation; MAPK: Mitogen-activated protein kinase; PH: Pleckstrin homology; PIPs: phosphoinositide phosphates.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data charts supporting the results and conclusions are included in the article and additional files. All the transcriptome data have been deposited in the NCBI' GEO under accession number GSE146666 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146666).

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

HZ and JZ performed the experiments and wrote the paper. QX, DW, HD, JH, XY, ZW, LZ, and LD performed the experiments. ZW and YZ designed the experiments and revised the paper. All Authors read and approved the manuscript in the "Authors' Contributions" section

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Table 1 Descriptive statistics for germination traits under low temperature and normal conditions

Trait	n	Mean	Median	Range	SD
RGR	222	0.737	0.787	0.027-0.999	0.220
RGL	222	0.486	0.434	0.087-1.229	0.220
RRL	222	0.704	0.718	0.100-1.838	0.285
RRSA	222	0.722	0.769	0.133-2.352	0.282
RRV	222	0.739	0.737	0.173-2.650	0.325
RGI	222	0.270	0.263	0.009-0.977	0.124
RVI	222	0.162	0.141	0.001-0.812	0.111
RSVI	222	0.360	0.340	0.002-0.917	0.186
XYRGR	222	0.403	0.420	0.014-0.753	0.172
XYRGL	222	0.542	0.538	0.001-0.897	0.869
XYRSVI	222	0.222	0.225	0.001-0.557	0.105
KSRGR	222	0.510	0.537	0.084-0.857	0.175
KSRGL	222	0.500	0.496	0.212-0.857	0.111
KSRSVI	222	0.261	0.266	0.018-0.535	0.115

The traits were evaluated for 222 inbred lines during germination tests under normal (25℃), and low temperature conditions (10℃). Statistics include means, median, standard deviation (SD), and range. 14 relative (R) traits were derived by dividing the directly measured trait values (GR, GL, RL, RSA, RV, GI, VI SVI, XYGR, XYGL, XYSVI, KSGR, KSGL and KSVI), under low temperature conditions by their corresponding values under normal conditions

Table 2 SNPs for low temperature germination related traits detected in 222 maize inbred lines

SNP	Chromosome	Position	MAF	P value	Trait
SYN25470	1	60897507	0.34	5.87E-05	RVI
PZE-101120376	1	148125053	0.138	1.85E-06	RGI
PZE-101192647	1	238680213	0.111	3.53E-07	RRV
PUT-163a-88747038-4526	1	282891355	0.279	4.23E-05	KSRGR
PUT-163a-149007696-748	2	8500779	0.077	3.74E-05	RGR
SYN29778	2	17713628	0.318	6.89E-05	RRSA
PZE-102099570	2	114621446	0.361	1.54E-06	RGI
PZE-102100684	2	117468629	0.267	4.92E-05	RVI
SYN21841	2	219117623	0.253	3.73E-07	RRV
PZE-103072583	3	115856771	0.346	8.88E-05	RRSA
PZE-103094415	3	151499271	0.182	6.34E-05	RGL
PZE-104024779	4	28770811	0.095	8.24E-05	RRV
SYN30173	5	10372835	0.401	3.75E-05/7.8E-05	RGL/RSVI
PZE-105034900	5	19776726	0.089	2.34E-06/3.47E-05	RRV/RVI
PZE-106003222	6	4152307	0.141	2.15E-07	RRV
PZE-106097864	6	151745619	0.137	2.34E-05	RGI
PZE-106129965	6	168030463	0.382	1.96E-07	RRV
PZE-106130106	6	168266461	0.359	2.88E-07	RRV
SYN4961	7	7156626	0.144	0.000035996	RSVI
PZE-107036384	7	56177181	0.257	1.70E-07	RRV
PZE-107098845	7	148186320	0.161	7.77E-05	RRL
PUT-163a-78076151-4108	8	1508965	0.282	9.59E-05	KSRSVI
PZE-108064544	8	113952611	0.141	2.80E-07	RRV
PZE-108068725	8	119362385	0.286	9.81E-05	RRSA
SYN26538	8	169361414	0.224	2.24E-07	RRV
PZE-109053558	9	89605923	0.4	5.75E-05/5.02E-05	XYRGR/XYRSVI
SYN5516	10	8952194	0.301	6.64E-07	RGI
PZE-110029252	10	50842298	0.128	9.84E-05	RRSA
PZE-110057591	10	110369493	0.113	4.22E-05	RVI
PZE-110060997	10	115266833	0.122	4.08E-05	XYRGL

The location is indicated by chromosome and base pair position. The P values less than 10^{-4} corresponding to 5% type I error are displayed as scientific notations.The frequency is indicated by the Minor Allele Frequency (MAF). MAF: Minor Allele Frequency.

Table 3 Functions of candidate genes directly associated with derived germination traits.

Trait	SNP	Gene ID	Gene function
RGR	PUT-163a-149007696-748	*GRMZM2G012088*	uncharacterized
RGL/RSVI	SYN30173	*GRMZM2G158359*	probable receptor protein kinase TMK1
RSVI	SYN4961	*GRMZM2G107309*	histone deacetylase
RRSA	PZE-103072583	*GRMZM2G390374*	protein ROOT HAIR DEFECTIVE 3 homolog 1
RRSA	SYN29778	*GRMZM2G101383*	Pseudouridine synthase family protein
RRSA	PZE-108068725	*GRMZM2G173195*	glycerol-3-phosphate dehydrogenase
RRV	PZE-104024779	*GRMZM2G007734*	polyadenylation and cleavage factor homolog 4
RRV	PZE-108064544	*GRMZM2G092000*	uncharacterized
RRV/RVI	PZE-105034900	*GRMZM2G001772*	NA
RGI	SYN5516	*GRMZM2G118286*	long chain acyl-CoA synthetase 9, chloroplastic
RVI	SYN25470	*GRMZM2G402584*	uncharacterized
XYRGL	PZE-110060997	*GRMZM2G059110*	uncharacterized
KSRGR	PUT-163a-88747038-4526	*GRMZM2G152921*	seed maturation protein
KSRSVI	PUT-163a-78076151-4108	*GRMZM2G005980*	DNA-binding protein

All candidate genes contain either an associated SNP.

Table 4 Candidate genes contained in DEGs inconsistent between the CT_102vsCT_55 and LT_102vsLT_55 comparison groups

Gene ID	CT_102vsCT_55	LT_102vsLT_55	Gene function	Arabidopsis best hit	Rice best hit
GRMZM2G038964	no	down	inositolphosphorylceramide-B C-26 hydroxylase	(ATFAH1, FAH1) fatty acid hydroxylase 1	fatty acid hydroxylase putative expressed
GRMZM2G059042	no	down	photoperiod responsive protein	(ATCMPG2, CMPG2) CYS MET PRO and GLY protein 2	U-box domain containing protein expressed
GRMZM2G009223	no	down	glucose-6-phosphate/phosphate translocator 2	(ATGPT2, GPT2) glucose-6-phosphate/phosphate translocator 2	transporter family protein putative expressed
GRMZM2G052129	no	down	uncharacterized	Pleckstrin homology (PH) domain-containing protein	pleckstrin homology domain-containing protein-related taxo putative expressed
GRMZM2G073861	no	down	uncharacterized	no	AT hook motif family protein expressed
GRMZM2G101408	no	down	uncharacterized	no	coiled-coil domain-containing protein 124 putative expressed
GRMZM2G101383	no	up	Pseudouridine synthase family protein	Pseudouridine synthase family protein	coiled-coil domain-containing protein 139 putative expressed
AC213621.5_FG004	no	up	uncharacterized	(SOS5) Fasciclin-like arabinogalactan family protein	fasciclin-like arabinogalactan protein putative expressed
GRMZM2G402584	no	up	uncharacterized	(sks3) SKU5 similar 3	monocopper oxidase putative expressed

The list consists of six parts: gene ID, gene function description, up or down regulation in CT_102vsCT_55 and LT_102vsLT_55 comparison groups, *Arabidopsis* and rice best hit.

Table 5 GO analysis of candidate genes related to RNA-Seq result

Gene ID	GO
GRMZM2G038964	GO:0005783; GO:0006631; GO:0080132
GRMZM2G059042	GO:0004842; GO:0005737; GO:0010200; GO:0016567
GRMZM2G009223	GO:0034219; GO:0034219
GRMZM2G052129	GO:0004550; GO:0005634; GO:0009507
GRMZM2G073861	no
GRMZM2G101408	GO:0003674; GO:0005737; GO:0008150
GRMZM2G101383	GO:0005634
AC213621.5_FG004	GO:0000139; GO:0005794; GO:0006004; GO:0006486; GO:0007155; GO:0016021; GO:0016757; GO:0071555
GRMZM2G402584	GO:0005507; GO:0009505; GO:0009506; GO:0016722; GO:0003674; GO:0005739; GO:0008150; GO:0016021

Table 6 Distribution of genes and pathway elated to RNA-Seq results

pathway_id	pathway_name	Gene ID	KO_Entry	EC
ko00230	Purine metabolism	si618049h06(54)	K00940	EC:2.7.4.6
ko00240	Pyrimidine metabolism	si618049h06(54)	K00940	EC:2.7.4.6
ko04016	MAPK signaling pathway - plant	si618049h06(54)	K00940	EC:2.7.4.6

Fig. 1 Seed germination of the diverse maize panel under low temperature conditions. **a** Germination rate comparison among four maize inbred lines under low temperature treatment (10℃) and control (25℃); **b** Comparison of germ length and radicle length in maize inbred line Mo17; **c** in inbred line Ji853; **d** in bred line K10; **e** in maize inbred line Shen5003

Fig. 2 Distributions and correlations among 14 direct and derived germination traits. **a** 222 maize inbred lines under low temperature stress in each indoor index correlation analysis; **b** 222 maize inbred lines under low temperature stress in each field index correlation analysis; **c** 222 maize inbred lines under low temperature stress each indoor and field index correlation analysis. * means 10% significance level, ** means 5% significance level, ***means 1% significance level

Fig. 3 Manhattan plots of GWAS on 14 derived germination traits. relative germination rate (RGR), relative Germ length (RGL), relative radicle length (RRL), relative radicle surface area (RRSA), relative radicle volume (RRV), relative germination index (RGI), relative vitality index (RVI), relative simple vitality index (RSVI), XiangYang relative germination rate (XYRGR), XiangYang relative germ length (XYRGL), XiangYang relative simple vitality index (XYRSVI), KeShan relative germination rate (KSRGR), KeShan relative germ length (KSRGL), KeShan relative simple vitality index (KSRSVI)

Fig. 4 Venn diagram of DEGs distributed in maize inbred line 102 and 55. Low temperature treatments are Normal Control (CT) and Low temperature (LT). The low temperature tolerant line (Zao8-3) and low temperature sensitive line (Ji853) are labeled “55” and “102,” respectively. The five treatment-line biological samples are low temperature treatments line 102 VS low temperature treatments 55 up-

regulated expression (LT_102vsLT_55.up), low temperature treatments line 102 VS low temperature treatments 55 down-regulated expression (LT_102vsLT_55.down), normal control treatments line 102 VS normal control treatments 55 up-regulated expression (CT_102vsCT_55.up), normal control treatments line 102 VS normal control treatments 55 down-regulated expression (CT_102vsCT_55.down) and candidate genes.

Fig. 5 GO annotations and KEGG pathway of DEGs. **a** Bubble chart of GO classifications of the DEGs. The green round represents the GO annotations common to the genes of *GRMZM2G052129* and *GRMZM2G101383*. The purple round represents the GO annotations common to the genes of *GRMZM2G402584* and *AC213621.5_FG004*. The blue square represents the GO annotations to the genes of *GRMZM2G038964*. A total of 8 DEGs were annotated; **b** histogram of KEGG pathway of the annotated DEGs

Fig. 6 The real-time quantitative PCR (qRT-PCR) validation of GWAS and RNA-Seq results. Expression of six candidate genes in low temperature resistant lines Zao8-3 (named 55) and low temperature sensitive lines Ji853 (named 102). Expression analysis was conducted on embryos that were collected at 2h and 4h under low (10℃) and normal (25℃) temperature, respectively. * means 0.05 significance level, ** means 0.01 significance level

Figures

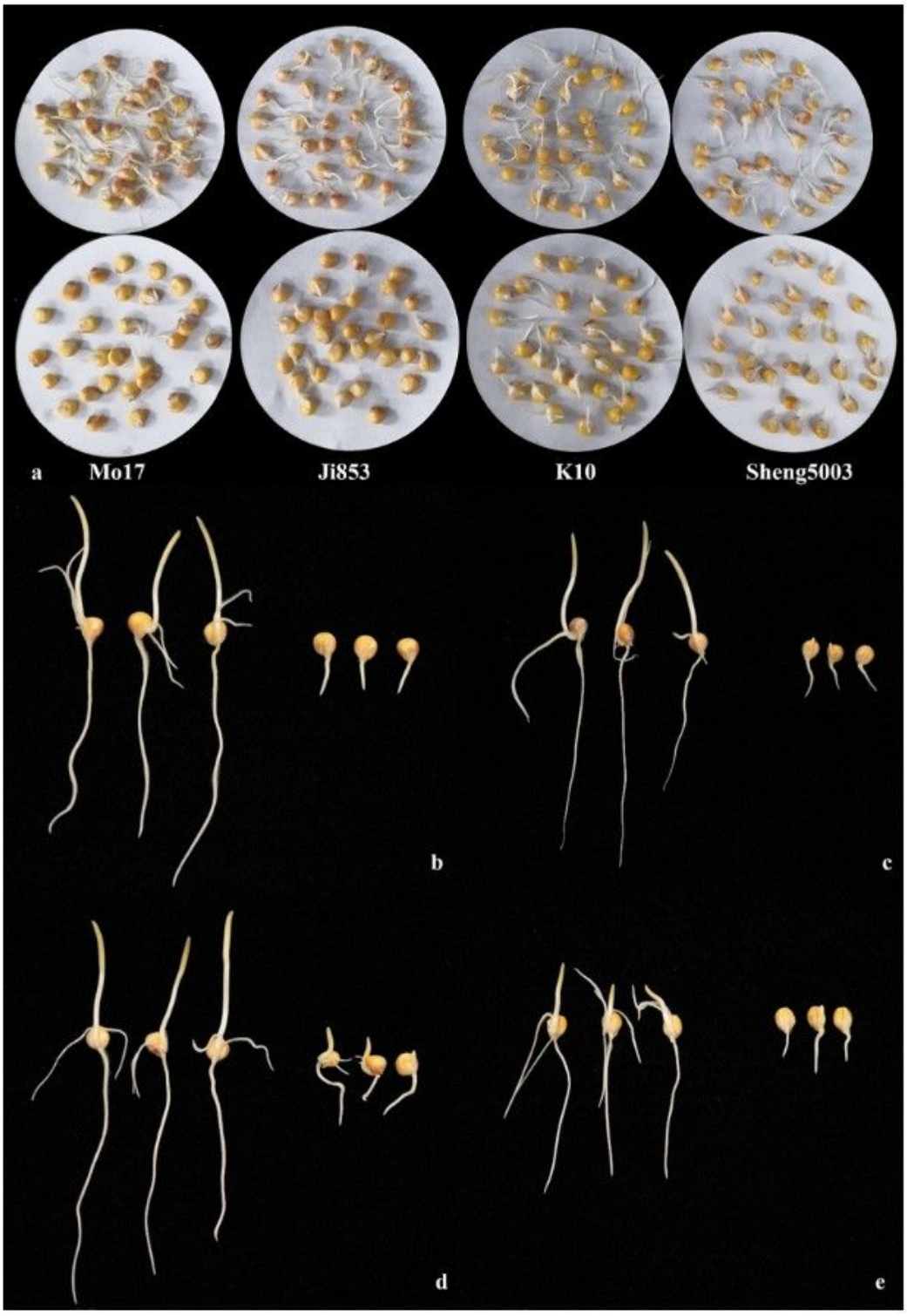


Figure 1

Seed germination of the diverse maize panel under low temperature conditions. a Germination rate comparison among four maize inbred lines under low temperature treatment (10℃) and control (25℃); b

Comparison of germ length and radicle length in maize inbred line Mo17; c in inbred line Ji853; d in bred line K10; e in maize inbred line Shen5003

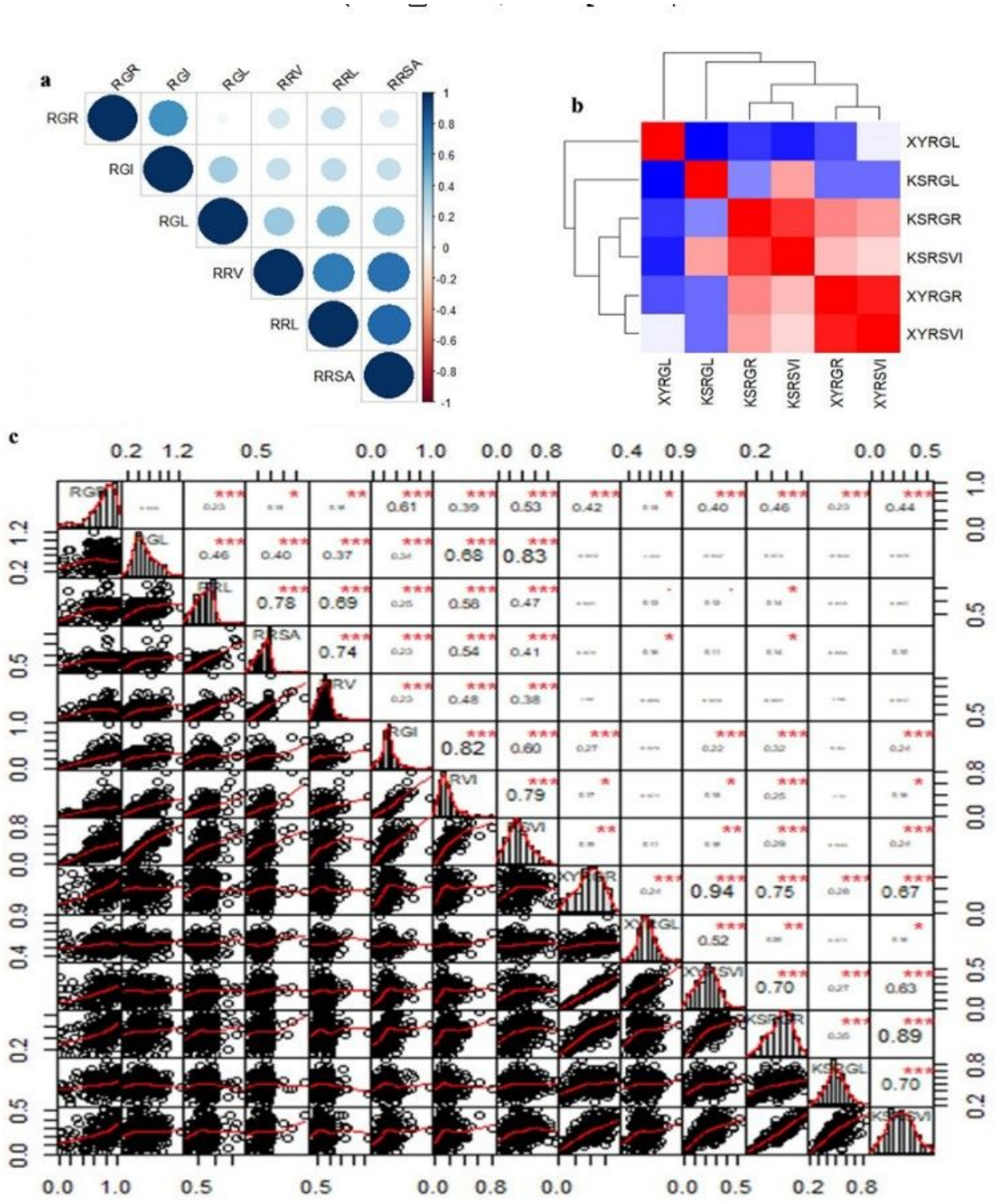


Figure 2

Distributions and correlations among 14 direct and derived germination traits. a 222 maize inbred lines under low temperature stress in each indoor index correlation analysis; b 222 maize inbred lines under low temperature stress in each field index correlation analysis; c 222 maize inbred lines under low

temperature stress each indoor and field index correlation analysis. * means 10% significance level, ** means 5% significance level, ***means 1% significance level

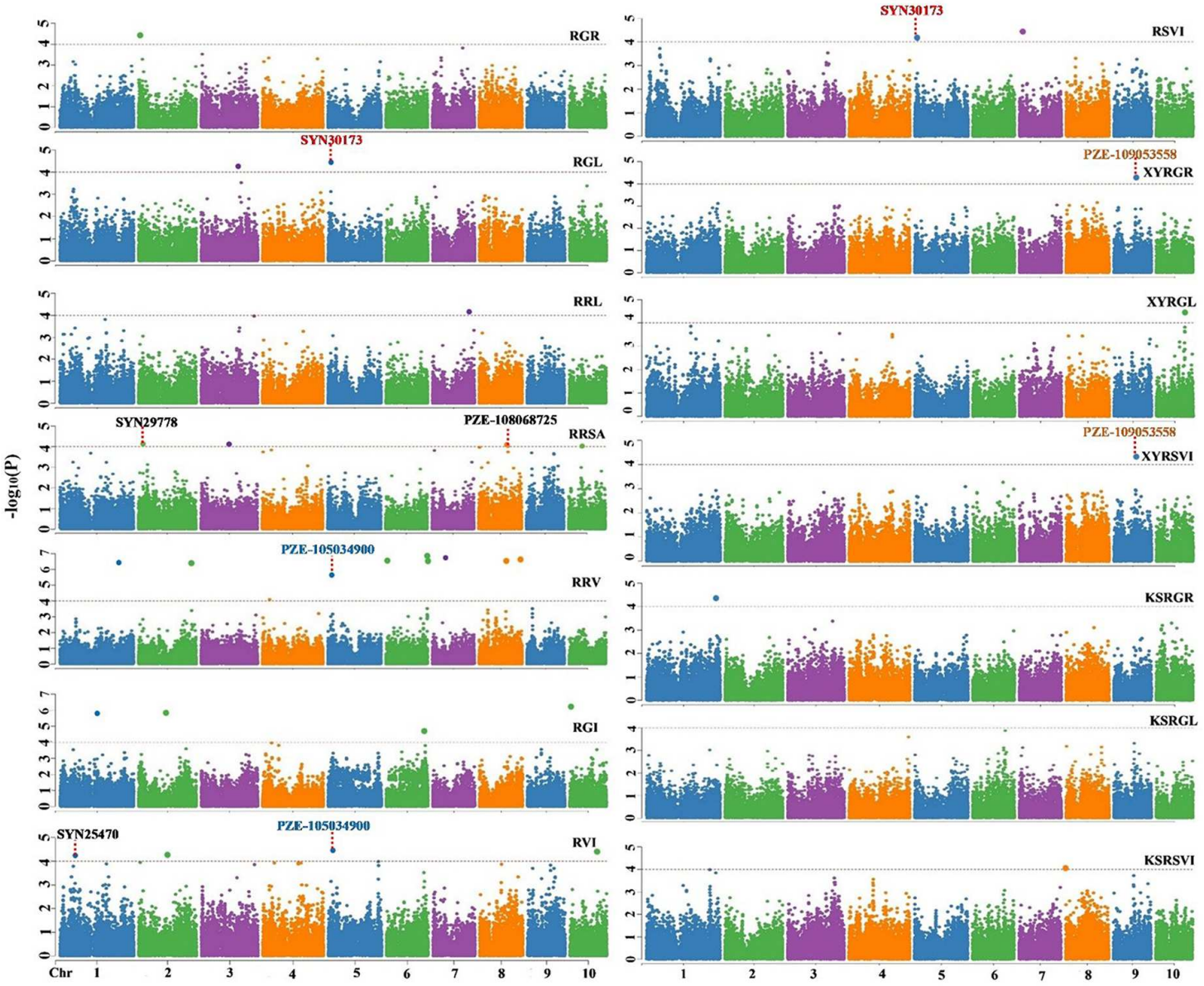


Figure 3

Manhattan plots of GWAS on 14 derived germination traits. relative germination rate (RGR), relative Germ length (RGL), relative radicle length (RRL), relative radicle surface area (RRSA), relative radicle volume (RRV), relative germination index (RGI), relative vitality index (RVI), relative simple vitality index (RSVI), XiangYang relative germination rate (XYRGR), XiangYang relative germ length (XYRGL), XiangYang relative simple vitality index (XYRSVI), KeShan relative germination rate (KSRGR), KeShan relative germ length (KSRGL), KeShan relative simple vitality index (KSRSVI)

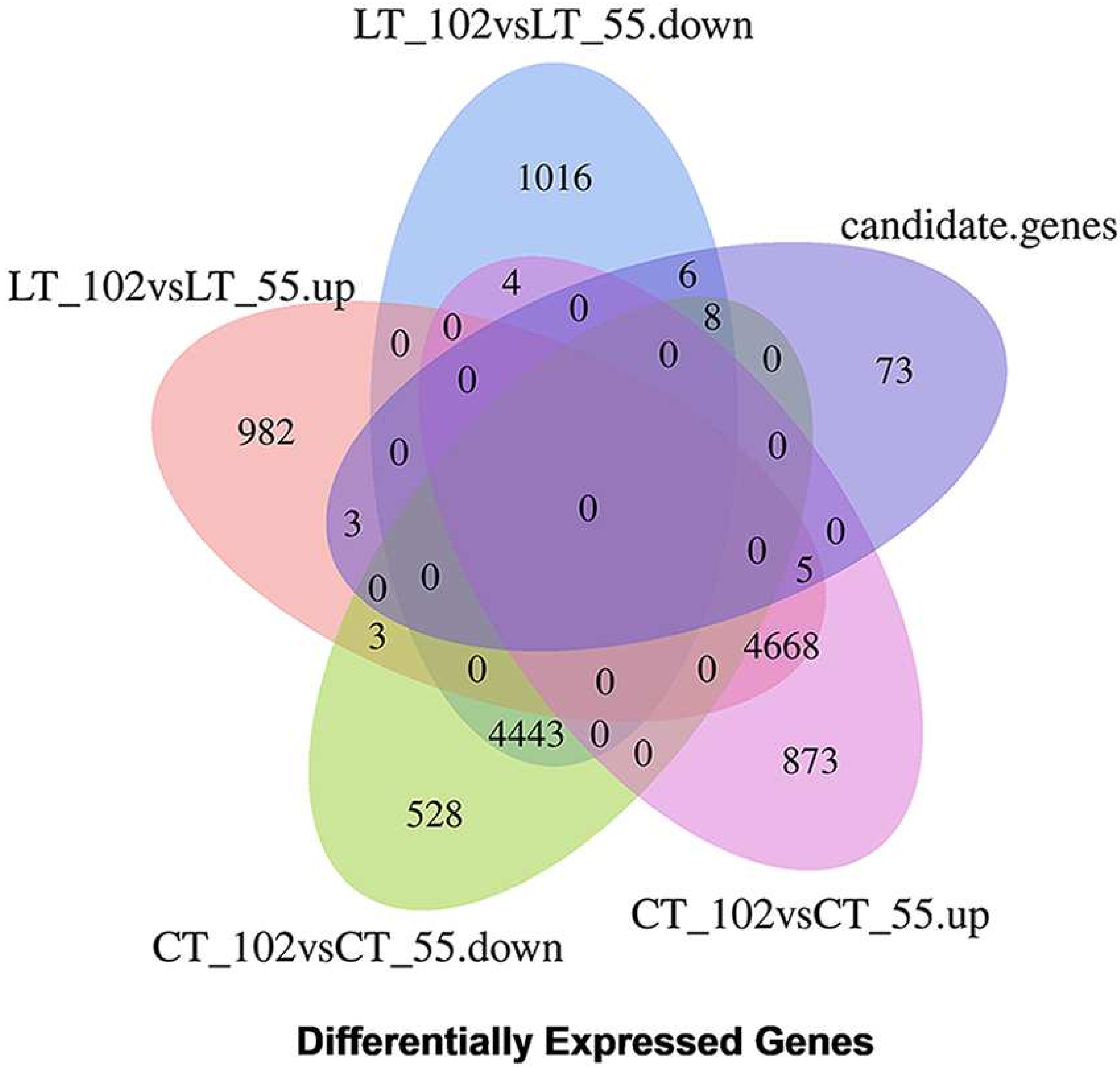
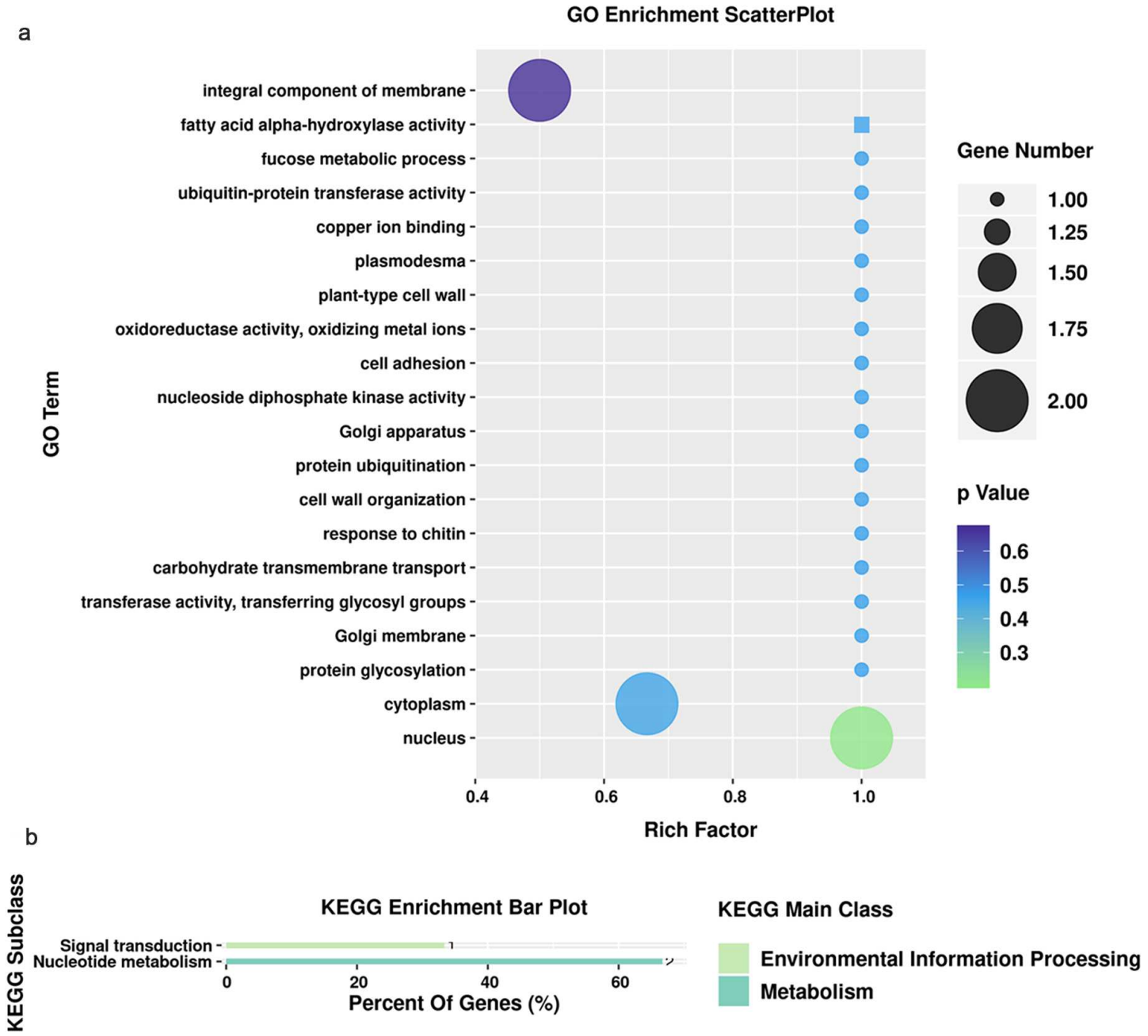


Figure 4

Venn diagram of DEGs distributed in maize inbred line 102 and 55. Low temperature treatments are Normal Control (CT) and Low temperature (LT). The low temperature tolerant line (Zao8-3) and low temperature sensitive line (Ji853) are labeled “55” and “102,” respectively. The five treatment-line biological samples are low temperature treatments line 102 VS low temperature treatments 55 up-regulated expression (LT_102vsLT_55.up), low temperature treatments line 102 VS low temperature treatments 55 down-regulated expression (LT_102vsLT_55.down), normal control treatments line 102 VS normal control treatments 55 up-regulated expression (CT_102vsCT_55.up), normal control treatments

line 102 VS normal control treatments 55 down-regulated expression (CT_102vsCT_55.down) and candidate genes.



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GO annotations and KEGG pathway of DEGs. a Bubble chart of GO classifications of the DEGs. The green round represents the GO annotations common to the genes of GRMZM2G052129 and GRMZM2G101383. The purple round represents the GO annotations common to the genes of GRMZM2G402584 and AC213621.5_FG004. The blue square represents the GO annotations to the genes of GRMZM2G038964. A total of 8 DEGs were annotated; b histogram of KEGG pathway of the annotated DEGs

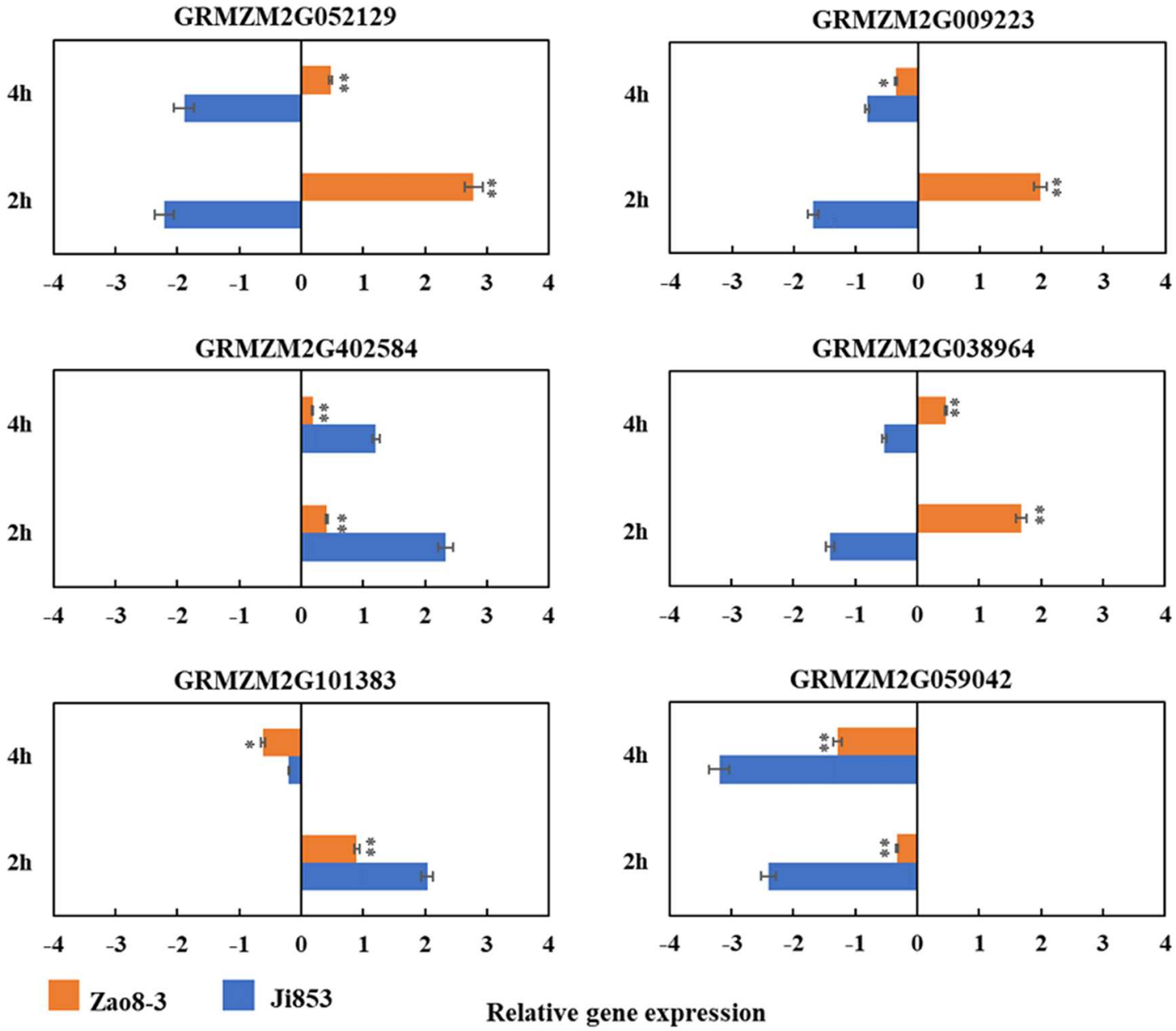

Figure 6

The real-time quantitative PCR (qRT-PCR) validation of GWAS and RNA-Seq results. Expression of six candidate genes in low temperature resistant lines Zao8-3 (named 55) and low temperature sensitive lines Ji853 (named 102). Expression analysis was conducted on embryos that were collected at 2h and 4h under low (10℃) and normal (25℃) temperature, respectively. * means 0.05 significance level, ** means 0.01 significance level

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