

Genome-wide Association Studies and Genes for Seed Germination Chromosome under Low-Temperature Stress in Maize (*Zea mays* L) Cropping System

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Received February 02, 2023; Revised March 05, 2023; Accepted March 13, 2023

Abstract Low-temperature stress delays seed germination in maize. Different maize inbred lines display various low-temperature resistance, but the dynamic changes in seed germination under low-temperature stress in maize remain unknown, especially at the transcriptome level. Maize (*Zea mays* L.). 18 evaluation indexes of low temperature tolerance germination of 238 maize inbred lines were statistically analyzed, of which 12 evaluation indexes of low temperature tolerance had large coefficient of variation. The results of cluster analysis relative decrease of root length is similar to those of comprehensive evaluation cluster analysis of 12 low temperature tolerance indexes. The relative K + Q model was used to set the threshold - log₁₀P = 4.61 to score the GWAS of evaluation indexes of low temperature tolerance. A total of 47 SNP loci significantly associated with low temperature tolerance were detected, of which the SNP loci significantly associated with chromosome 8 were the most, and the interpretation range of phenotypic contribution rate was 10.88% - 16.31%. It can be used as a candidate interval for subsequent screening and analysis. GWAS analysis showed that there were at least 2 evaluation indexes of low temperature tolerance, and a total of 6 SNPs were detected, of which 1 was on chromosome 5 and the other 5 were on chromosome 3. The consistent loci on chromosome 3 were located between PZE-103174386 (220,933,020) and PZE-103174950 (221,386,301).

Keywords: seed germination, seedling growth, low-temperature stress, maize, peroxidase activity

Cite This Article: Xiaofen Li, Rongzhen Wang, Kaiyuan Cui, Hafeeze Noor, Pengcheng Ding, and Fida Noor, "Genome-wide Association Studies and Genes for Seed Germination Chromosome under Low-Temperature Stress in Maize (*Zea mays* L) Cropping System." *Journal of Food and Nutrition Research*, vol. 11, no. 3 (2023): 193-204. doi: 10.12691/jfmr-11-3-4.

1. Introduction

Maize (*Zea mays* L.) has many exciting characteristics relevant to basic and applied research [1,2]. For example, its highly diverse genome may contribute to its dispersal from the origin area to regions with substantially different environmental conditions [2,3]. As a thermophilic crop, maize is vulnerable to cold damage in early spring, which inhibits seed germination and seedling growth. At the germination stage, resistance to low temperatures is essential for plants growing in a temperate region. Rapid germination and seedling growth under low-temperature stress could enable early sowing in spring. However, the molecular mechanism underlying low-temperature resistance or sensitivity during seed germination in maize remains unknown, in contrast to the available information about the effects of cold stress on seedlings or at the whole-plant level [4,5,6]. Maize is one of the world's

major food, economic and energy crops, mainly distributed in tropical and temperate regions. Seed germination rate and emergence rate are closely related to crop yield. With the change of environment, low temperature stress caused by low temperature (0-15°C) has become one of the main abiotic stresses affecting maize seed germination, propagation and biomass accumulation. In high latitudes or mountainous areas, extending the growth period in early spring can accumulate more biomass and thus increase maize yield [4]. However, the cold wave in late spring seriously affected seed germination, resulting in a decrease in yield. Improving seed germination resistance to low temperature is the key to ensure the smooth emergence and normal development of early sowing maize. Demonstrating tolerance to low temperatures above 0°C during the first stage of a plant's life cycle is an important characteristic of warm-season crops, even if they are grown in temperate regions. In fact, continuous germination and rapid growth in cold soils improves tolerance to environmental

properties and is able to allow early sowing, resulting in important agronomic advantages such as flowering before the onset of the hottest and driest periods of the year [5]. Rapid and normal seedling morphogenesis is essential for maize production. However, as a thermophilic crop, maize is susceptible to cold injury when sown in early spring, which seriously inhibits seed germination and seedling growth [6]. Germination is a complex process that reactivates important cellular events from a resting state, including various metabolic responses and signal transduction pathways. The germination process can be divided into three stages based on the time course of water absorption, the rapid water absorption stage, the imbibition stage, (II) the delayed or plateau stage of water absorption, and (III) the post-germination stage, when embryo growth is significantly accelerated. In the early stage of seed imbibition, the cell membrane has not been completely repaired, and a large amount of cell solute leakage often occurs, especially at low temperatures [7]. Cell membrane repair is required for seed germination and subsequent seedling morphogenesis, but the underlying mechanism is unknown and is one of the most interesting and important topics in seed science [8]. Compared to the available information on the effects of cold stress on seedlings or on the whole plant level, little is known about the molecular mechanisms of cold tolerance or sensitivity during germination [9]. Low temperature stress can not only reduce the emergence rate and seedling vitality of corn seeds, but also increase the chance of soil pathogen infection, and seriously reduce the yield of corn [10]. Therefore, in the context of crop spread and climate change, a better understanding of the effects of low temperature on maize seed germination is urgently needed.

2. Materials and Methods

2.1. Materials

The low-temperature sensitive maize (SM) B283-1 and low-temperature resistance maize (RM) 04Qun0522-1-maize inbred lines used in this study were bred in our laboratory. They were grown at the experimental station (36°90'N, 117°90'E) of Shanxi Agricultural University, China. Seeds were sown on 14 June 2021. The plant density was 67,500 plants/ha. Seeds used in this study were harvested 50 days after pollination.

2.2. Evaluation of Seed Germination

The bottom of a sprouting bed consisted of 4 cm height silica sand (diameter of 0.05–0.8 mm) with 60% saturation moisture content in a germination box. Randomly selected 30 maize seeds were sown on the surface of the sprouting bed, and then they were covered with 2 cm height silica sand with 60% saturation moisture content. Subsequently, the germination boxes were placed in different germination conditions for various treatments. The germination boxes were placed in a growth chamber at 13°C for 4 days to detect the percentage of seeds showing radicle protrusion. The germination boxes were placed in a growth chamber at 25°C for 7 days or at 13°C for 7 days to measure germination percentage. A seed was

considered as germinating seed when the radicle was similar to seed length and the germ was similar with half of the seed length. Moreover, some germination boxes were placed in a growth chamber at 25°C for 4 days (NT) as control, and some germination boxes were placed in a growth chamber at 13°C for 4 days followed by 25°C for 2 days (LNT) as low-temperature treatment. All tissues of the two inbred lines under NT and LNT were used for later experiments. Each treatment included three replicates.

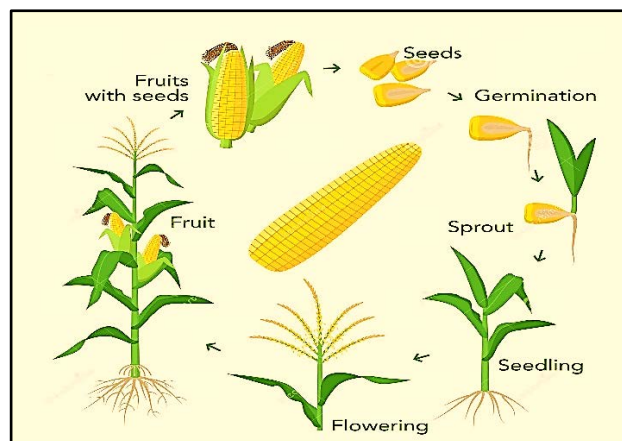


Figure 1. Maize plant growth infographic elements from seeds to fruits, mature corn ears. Seedling, germination, planting, flowering. Vector encyclopedic illustration. Corn life cycle in flat design

2.3. RNA-seq and Data Analysis

Seeds germinated at 13°C for 0, 2, 4, and 6 days were used for RNA extraction. Thirty germinated seeds were pooled together for each sampling and then stored at 80°C before the RNA extraction. The frozen tissue samples were ground into a powder using a ball mill. Subsequently, the sample (0.1 g) was used for total RNA extraction using a Plant Total RNA Purification Kit (Bioflux, Beijing, China). The quality of RNA-seq data was tested. The retained clean reads were mapped to the *RefGen_V4* maize reference genome and gene sequences (<http://www.gramene.org/>) using HISAT2 [11]. To quantify gene expression levels, feature Counts (v1.5.0-p3) were used to determine the number of reads mapped to each gene. The fragments per kilobase of transcript per million mapped reads method was used to estimate gene expression levels in the 24 analyzed samples. The analysis of differential expression between two conditions/groups (three biological replicates per condition) was performed using the *DESeq2* R package (1.16.1) [12]. The *p* values were adjusted according to the Benjamini and Hochberg method. The *DEGs* (i.e., adjusted *p* < 0.05 and log₂ (fold-change-1)) were considered for further analyses. The *DEGs* were functionally characterized using the GO database [13]. All *DEGs* were grouped into the three main GO categories (biological process, cellular component, and molecular function) according to their GO terms using a publicly available database (<http://www.geneontology.org/>). In this study, the GO term with corrected *padj* < 0.05 was used as the GO term with significant enrichment of *DEGs*. To further clarify the biological functions of the *DEGs*, the significantly enriched metabolic or signal transduction pathways were identified based on a KEGG pathway

enrichment analysis [14]. Similarly, the KEGG pathways with corrected $\text{padj} < 0.05$ were assigned as significantly enriched pathways.

2.4. Verification of DEGs by a qRT-PCR Analysis

To validate the DEGs identified from the RNA-seq analysis, we re-extracted the RNA of seeds germinated at 13°C for 0, 2, 4, and 6 d in two lines. The following six genes were randomly selected for a qRT-PCR assay: *Zm00001d017241*, *Zm00001d044301*, *Zm00001d044303*, *Zm00001d021291*, *Zm00001d023994*, and *Zm00001d045512*. About 800 ng total RNA was used to synthesize cDNA by using the *PrimeScript RT* reagent Kit (Takara, Dalian, China). Subsequently, cDNA was diluted to 100L with ddH₂O. All the QrtPCR reactions were performed on an ABI Stepone plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a 20L reaction volume, containing 10L SYBR Premix *ExTaq* (TaKaRa), 0.4L ROX, 0.4L of 10m primers, 2L cDNA, and 6.8L ddH₂O. The qRT-PCR analysis was performed using the ABI StepOne Plus Real-Time PCR System (Applied Biosystems, CA, USA) and the SYBR® Green Realtime PCR Master Mix (Toyobo, Japan). The maize Actin gene (GenBank accession number: *Zm00001d010159*) was used as an internal reference control [15]. The primers for the six selected genes were designed using the Primer Premier software (version 6.0). Three biological replicates were included in the qRT-PCR analysis. The generated data were analyzed according to the 2DDCT method [16].

2.5. Statistical Analysis

Multiple comparisons were performed using Duncan's test at the 0.05 significance level. All the tests were conducted using SPSS Version 21.0 for Windows (SPSS, Chicago, IL, United States).

3. Result

3.1. Eighteen Phenotypic Traits of Low Temperature Tolerance in 238 Maize Inbred Lines were Statistically Analyzed

Germination rate (GR), germination index (GI), root length (RL), length (SL), the fresh weight of root (RFW), MiaoXian heavy (SFW), root dry weight (RDW) and shoot dry weight (SDW), plant fresh weight (PFW), plant dry weight (PDW), root length, vigor index (RLVI), long seedling vigor index (SLVI), fresh weight of root vigor index (RFWVI), seedling fresh weight vigor index (SFWVI), root dry weight vigor index (RDWVI), seedling dry weight vigor index (SDWVI), plant fresh weight vigor index (PFWVI), plant dry weight vigor index (PDWVI). However, the traits measured under low temperature stress are not only related to the response to low temperature, but also to their own growth. In order to exclude the effect of seeds themselves, the relative reduction of germination traits at low temperature and under normal conditions is more indicative of low temperature tolerance. In this

experiment, the relative reduction value of each low temperature resistance index is taken as the evaluation index of low temperature resistance. Descriptive statistical analysis was carried out on the low temperature tolerance indexes, including observation number (N), minimum, maximum, average, standard deviation, coefficient of variation, kurtosis and skewness (Table 1). For most traits, considerable phenotypic variation was detected in strains, with a minimum of 0.002 relative reduction in root fresh weight and a minimum of 0.887 relative growth vigor index *RDGP*, *RDGI*, *RDRLVI*, *RDSLVI*, *RDRFWVI*, *RDSFWVI*, *RDRDWVI*, *RDSDWVI*, *RDPFWVI*, *RDPDWVI*. The maximum value is 1. This suggests that maize inbred lines are rich in phenotypic variation. In terms of the mean value of relative reduction of low-temperature tolerance phenotypes, the minimum value of relative reduction of root fresh weight was 0.404 (*RDRFW*), and the maximum value of relative reduction of seedling growth vigor index was 0.977 (*RDSLVI*). The results indicated that the seedling growth vigor index was affected by low temperature. From the point of view of the coefficient of variation, the coefficient of variation from large to small is *RDGP* > *RDRFW* > *RDRDW* > *RDRL* > *RDPFW* > *RDPDW* > *RDGI* > *RDSDW* > *RDSFW* > *RDSDWVI* > *RDSL* > *RDRFWVI* > *RDRLVI* > *RDRDWVI* > *RDPDWVI* > *RDPFWVI* > *RDSFWVI* > *RDSLVI*. You can see from the skewness and kurtosis values. *RDRL*, *RDSL*, *RDRFW*, *RDSFW*, *RDRDW*, *RDSDW*, *RDPFW*, *RDPDW* It's basically a normal distribution, but *RDGP*, *RDGI*, *RDRLVI*, *RDSLVI*, *RDRFWVI*, *RDSFWVI*, *RDRDWVI*, *RDSDWVI*, *RDPFWVI*, *RDPDWVI*. The distribution is skewed to the right. The results showed that the radicle and germ of maize inbred lines were inhibited by low temperature treatment.

3.2. Correlation Analysis of Evaluation Indexes of Maize Germination Tolerance to Low temperature

Correlation analysis was carried out on 18 evaluation indexes of low temperature tolerance, and it could be seen from (Figure 2). That all showed unimodal distribution. There was a significant positive correlation between the two indexes ($P < 0.001$). There were 238 maize inbred lines germinated at low temperature, and the correlation coefficients of 18 low temperature germination evaluation indexes were pairings $r = 0.40-1$, *RDGP* *RDGI*, *RDPDW*, *RDPFW*, *RDRFWVI*, *RDRLVI*, *RDRLVI*, *RDRDWVI*, *RDRDWVI*, *RDPDWVI*, *RDPDWVI*, *RDSFWVI*, *RDPFWVI*, *RDSFWVI*, *RDSFWVI*, and *RDSLVI*. Pearson correlation coefficients are achieved 0.9 Above, *RDGP*: Relative decrease of germination percentage; *RDRFW*: Relative decrease of root fresh weight; *RDRDW*: Relative decrease of root dry weight; *RDRL*: Relative decrease of root length; *RDPFW*: Relative decrease of plant fresh weight; *RDPDW*: Relative decrease of plant dry weight; *RDGI*: Relative decrease of germination index; *RDSDW*: Relative decrease of seedling dry weight; *RDSFW*: Relative decrease of seedling fresh weight; *RDSDWVI*: Relative decrease of seedling dry weight vitality index; *RDSL*: Relative decrease of seedling length; *RDRFWVI*: Relative decrease of root fresh weight vitality index;

RDRLVI: Relative decrease of root length vitality index; *RDRDWVI*: Relative decrease of root dry weight vitality index; *RDPDWVI*: Relative decrease of plant dry weight vitality index; *RDPFWVI*: Relative decrease of plant fresh weight vitality index; *RDSFWVI*: Relative decrease of seedling fresh weight vitality index; *RDSLVI*: Relative decrease of seedling length vitality index. The frequency distribution of each trait is shown on a central diagonal in the form of a histogram. Scatter plots of between every pair of traits are shown in the areas below the diagonal, and numerical correlation coefficients between every pair of traits are shown in the areas above in the diagonal. *, ** and *** indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

3.3. Cluster Analysis of Evaluation Indexes of Corn Germination Tolerance to Low Temperature

According to the statistical analysis of 18 evaluation indexes of low temperature tolerance, there were 12 indexes with large coefficient of variation. We calculated the Euclidean distance of these 12 evaluation indexes, and used Neighbor joining method to cluster analysis 238 maize inbred lines. Among them, *RDGP*, *RDRFW*, *RDRDW*, *RDRL* were low temperature resistance evaluation indexes with large variation coefficients. *RDGP* cluster analysis can be divided into four types: very low temperature tolerant inbred lines (0-0.298), low temperature tolerant inbred lines (0.315-0.582), low temperature sensitive inbred lines (0.598-0.784) and very low temperature sensitive inbred lines (0.794-1). *RDRFW* was divided into two types: extremely low temperature tolerant inbred lines (0.002-0.586) and low temperature tolerant inbred lines (0.593-0.673), low temperature sensitive inbred lines (0.676-0.735) and low temperature extremely sensitive inbred lines (0.740~0.951). There are four types. *RDRDW* was divided into three types: extremely low temperature tolerant inbred lines (0.082 ~ 0.541), low temperature tolerant inbred lines (0.548~0.755) and extremely low temperature sensitive inbred lines

(0.762~0.938). *RDRL* can be divided into four types: extremely low temperature tolerant inbred lines (0.105 ~ 0.527), low temperature tolerant inbred lines (0.541~0.602), low temperature sensitive inbred lines (0.608~0.709) and extremely low temperature sensitive inbred lines (0.713~0.936) (Table 2). In order to screen low-temperature resistant materials, 12 maize inbred lines with high coefficient of variation were analyzed among 238 maize inbred lines. Evaluation indexes of low temperature resistance (*RDGP*, *RDRFW*, *RDRDW*, *RDRL*, *RDPFW*, *RDPDW*, *RDGI*, *RDSDW*, *RDSFW*, *RDSDWVI*, *RDSL*, *RDRFWVI*) adopted comprehensive evaluation method, that is, comprehensive evaluation of low temperature resistance evaluation indexes based on membership function values in fuzzy mathematics. The comprehensive evaluation index was also calculated by Euclidean distance, and the clustering analysis results of 238 maize inbred lines were performed by using Neighbor joining method, as shown in (Figure 3, and Table 3). The results were divided into extremely low-temperature tolerant inbred lines (2.170~5.882) and low-temperature tolerant self-bred lines. There were four types of inbred lines (6.120-8.290), low temperature sensitive inbred lines (8.357-10.009) and low temperature extremely sensitive inbred lines (10.102-11.848). 10 low temperature tolerant inbred lines and 10 low temperature sensitive inbred lines were selected by comprehensive evaluation cluster analysis based on membership function values (Table 4). Low temperature tolerance inbred lines, Anhui-3, Y36-1, B50-1-1, 8112, Longkang 11 (Longkang11), 04 Group 0603-6-2-2-1 (*04qun0603-6*), 04 Group 0522-1-1 (*04Qun022-1-1*), Brigade 28 (Lv28), KWS mother (KWSM), HB08F28-1-1. Low temperature sensitive inbred lines, B283-1, HB0858-1, B209, HB089E, Pr07112-1-2, HN0715-2-1-1, 04 Group 0613-2 (*04qun0613-2*), Pr07278-2, B302, CQ0775-2. Among them, low temperature tolerant low temperature inbred line 04 Group 0522-1-1 and low temperature sensitive inbred line B283-1 were used for linkage analysis of F2:3 population construction and late extreme material transcriptome analysis.

Table 1. Phenotypic analysis of low temperature germination related traits

Trait	No.	Min	Max	Mean	SD	CV(%)	Skewness	Kurtosis
<i>RDGP</i>	216	0.010	1.000	0.404	0.281	69.554	0.393	-0.988
<i>RDRFW</i>	216	0.002	0.951	0.641	0.149	23.310	-1.082	2.616
<i>RDRDW</i>	216	0.082	0.938	0.657	0.126	19.228	-0.970	2.556
<i>RDRL</i>	216	0.105	0.936	0.645	0.117	18.091	-0.908	3.482
<i>RDPFW</i>	216	0.320	0.998	0.724	0.102	14.078	-0.751	1.894
<i>RDPDW</i>	216	0.410	0.915	0.709	0.085	11.963	-0.608	0.717
<i>RDGI</i>	216	0.637	1.000	0.894	0.100	11.151	-0.691	-0.733
<i>RDSDW</i>	216	0.424	0.900	0.746	0.079	10.649	-0.796	1.241
<i>RDSFW</i>	216	0.483	0.943	0.780	0.080	10.291	-0.624	0.647
<i>RDSDWVI</i>	216	0.507	1.000	0.949	0.067	7.064	-2.583	10.310
<i>RDSL</i>	216	0.592	0.946	0.818	0.054	6.608	-0.644	1.361
<i>RDRFWVI</i>	216	0.630	1.000	0.953	0.057	5.949	-2.332	8.719
<i>RDRLVI</i>	216	0.796	1.000	0.957	0.046	4.760	-1.176	0.832
<i>RDRDWVI</i>	216	0.763	1.000	0.962	0.042	4.344	-1.391	2.265
<i>RDPDWVI</i>	216	0.815	1.000	0.964	0.040	4.130	-1.315	1.447
<i>RDPFWVI</i>	216	0.808	1.000	0.965	0.039	4.069	-1.411	1.917
<i>RDSFWVI</i>	216	0.838	1.000	0.971	0.033	3.400	-1.502	2.078
<i>RDSLVI</i>	216	0.887	1.000	0.977	0.026	2.612	-1.322	1.357

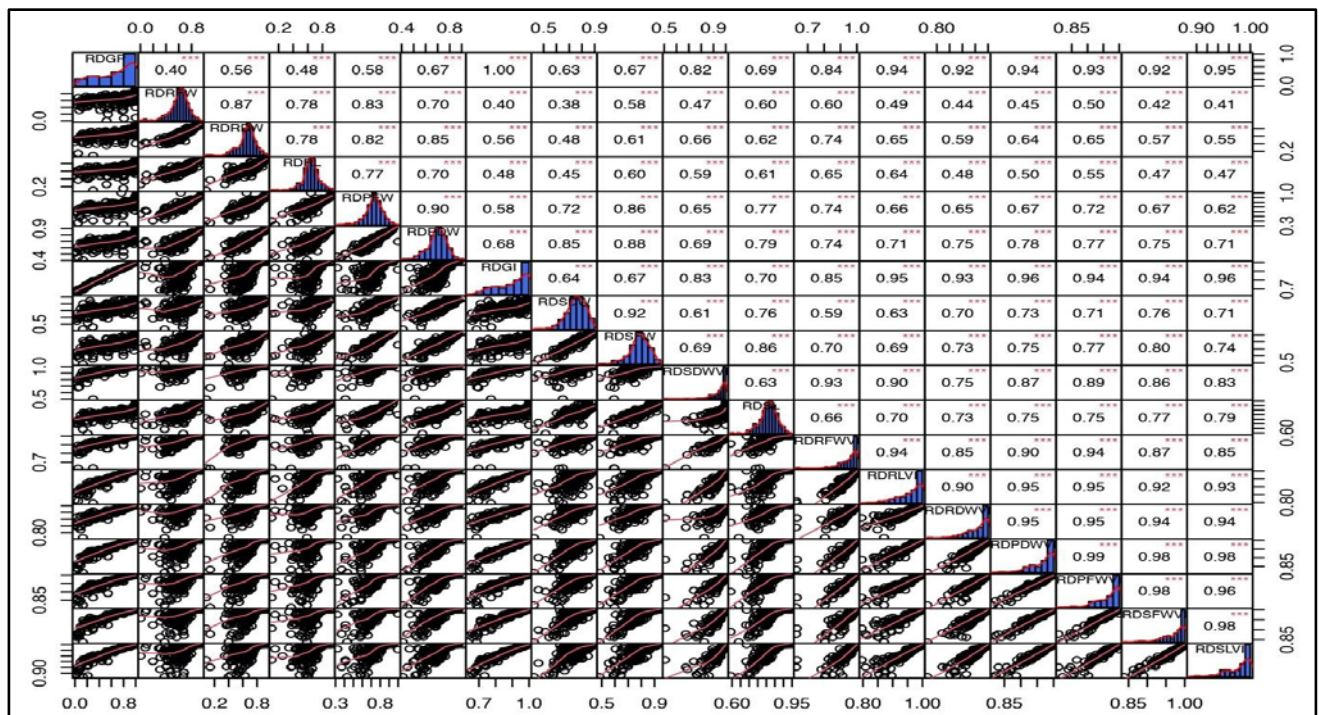


Figure 2. Distribution and correlation of 18 low temperature evaluation indexes

Table 2. Description of cluster analysis of low temperature resistance index

Trait	Classification	N	Min	Max	Mean	SD
RDGP	Low temperature tolerance inbred lines	39	0.010	0.298	0.182	0.082
	Better than low temperature tolerance inbred lines	38	0.315	0.582	0.451	0.081
	Low temperature sensitive inbred line	39	0.598	0.784	0.697	0.059
	Low temperature extremely sensitive inbred line	100	0.794	1.000	0.936	0.071
RDRFW	Extremely low temperature tolerant inbred line	60	0.002	0.586	0.461	0.129
	Low temperature tolerant inbred line	60	0.593	0.673	0.637	0.024
	Low temperature extremely sensitive inbred line	45	0.676	0.735	0.707	0.019
	Low temperature extremely sensitive inbred line	48	0.740	0.951	0.811	0.059
RDRDW	Extremely low temperature tolerant inbred line	35	0.082	0.541	0.442	0.098
	Extremely low temperature tolerant inbred line	143	0.548	0.755	0.667	0.052
	Low temperature extremely sensitive inbred line	37	0.762	0.938	0.820	0.049
	Low temperature tolerant inbred line	25	0.105	0.527	0.424	0.109
RDRL	Low temperature tolerant inbred line	101	0.608	0.709	0.657	0.028
	Low temperature sensitive inbred lines	51	0.713	0.936	0.782	0.061
	Low temperature sensitive inbred lines	52	0.543	0.680	0.631	0.040
	Low temperature tolerant inbred line	41	0.807	0.998	0.856	0.043
RDPFW	Low temperature tolerant inbred line	114	0.683	0.800	0.741	0.032
	Low temperature extremely sensitive inbred line	26	0.410	0.595	0.547	0.048
	Low temperature sensitive inbred line	112	0.609	0.745	0.690	0.035
	Low temperature sensitive inbred line	59	0.637	0.820	0.754	0.047
RDGI	Low temperature tolerant inbred line	157	0.827	1.000	0.947	0.051
	Low temperature sensitive inbred line	12	0.424	0.603	0.555	0.059
	Low temperature sensitive inbred line	73	0.612	0.733	0.688	0.032
	Low temperature tolerant inbred line	10	0.483	0.609	0.578	0.037
RDSW	Extremely resistant to low temperature self-pollination	34	0.631	0.724	0.686	0.028
	Low temperature tolerant inbred line	202	0.852	1.000	0.961	0.041
	Low temperature sensitive inbred line	14	0.507	0.842	0.762	0.090
	Low temperature tolerant inbred line	17	0.890	0.946	0.911	0.015
RDSFW	Low temperature tolerant inbred line	75	0.592	0.802	0.762	0.041
	Low temperature sensitive inbred line	159	0.931	1.000	0.980	0.021
	Low temperature tolerant inbred line	57	0.630	0.927	0.879	0.059
	Low temperature tolerant inbred line					



Table 3. Comprehensive evaluation of membership function cluster analysis description

Trait	Classification	N	Min	Max	Mean	SD
Comprehensive assessment	Extremely low temperature tolerant inbred line	23	2.170	5.882	4.756	1.083
	Low temperature tolerant inbred line	73	6.120	8.290	7.234	0.611
	Low temperature sensitive inbred line	85	8.357	10.009	9.156	0.454
	Low temperature extremely sensitive inbred line	35	10.102	11.848	10.687	0.458

Table 4. Selection of extreme materials for low temperature germination

Inbred line name	Membership function value	Classification
Anhui-3	3.20	Low temperature tolerant inbred line
Y36-1	3.45	Low temperature tolerant inbred line
B50-1-1	4.47	Low temperature tolerant inbred line
8112	4.97	Low temperature tolerant inbred line
Longkang11	5.54	Low temperature tolerant inbred line
04qun0603-6	5.81	Low temperature tolerant inbred line
04qun0522-1	6.13	Low temperature tolerant inbred line
Lv28	6.76	Low temperature tolerant inbred line
KWSM	6.90	Low temperature tolerant inbred line
HB08F28-1-1	7.30	Low temperature tolerant inbred line
CQ07335-2-2	16.95	Low temperature tolerant inbred line
B302	16.95	Low temperature tolerant inbred line
Pr07278-2-2	16.96	Low temperature tolerant inbred line
04qun0613-2	16.99	Low temperature tolerant inbred line
HN0715-2-1-	17.09	Low temperature tolerant inbred line
Pr07112-1-2	17.15	Low temperature tolerant inbred line
HB089E	17.48	Low temperature tolerant inbred line
B209	17.51	Low temperature tolerant inbred line
HB0858-1	17.80	Low temperature tolerant inbred line
B283-1	17.85	Low temperature tolerant inbred line

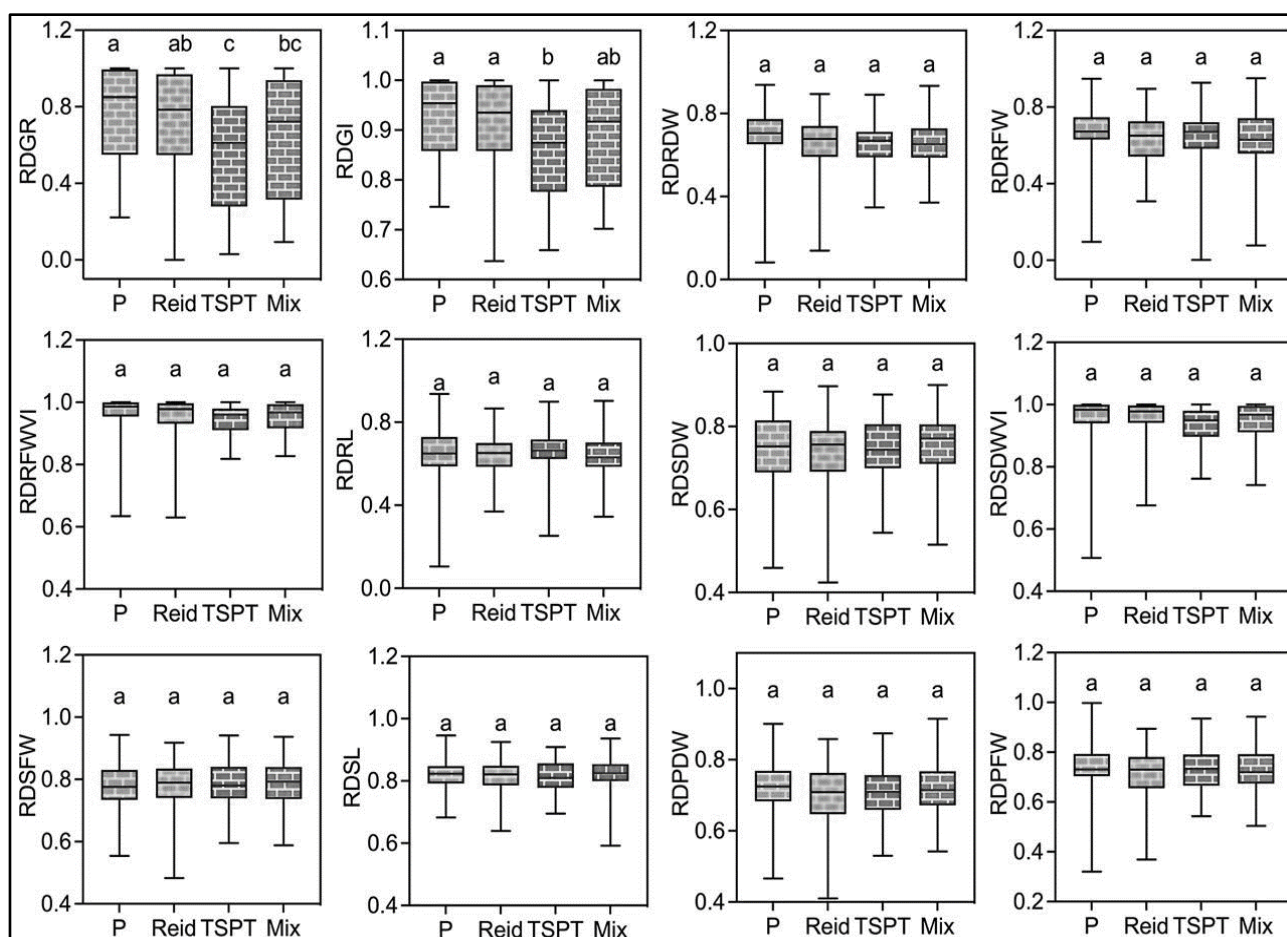


Figure 4. Distribution of phenotypic traits of low temperature tolerance in different subgroups (Note: P group (45 inbred lines); Reid group (71 inbred lines); TSPT Tangsipingtong group (45 inbred lines); mix mixed subgroup (55 inbred lines))

3.4. Different Germplasm Group of Genome-wide Association Analysis in the Low Temperature Germination Index Distribution

In this laboratory, SNP sequencing of 238 maize inbred lines was completed by Illumina. The population structure was divided into four subgroups named P group, Reid Group, Tangsipingtong Group (TSPT) and mixed subgroup (Mix). According to the results of population division, phenotypic distribution of different germplasm groups under each evaluation index of low temperature tolerance was observed (Figure 4). The results showed that there were significant differences between the P group and the Reid group with the relative reduction value of germination rate, and the P group and Reid group with the relative reduction value of vitality index were significantly different from the Tangsi flathead group, and there were also significant differences between the P group and the Tangsi flathead group with the relative reduction value of dry weight and the relative reduction value of seedling length. There was no significant difference in other evaluation indexes of low temperature tolerance among groups. These results not only indicate that different subpopulations have effects on the two evaluation indexes

of low temperature tolerance, i.e., relative decrease value of germination rate and relative decrease value of vitality index, but also indicate that there are some good variation of low temperature tolerance traits in Tangsi Pingtong group.

3.5. Maize Resistant to Low Temperature Germination Traits Relating the Correlation Analysis

Genome-wide association analysis of possible existence of false positive and false negative evaluation, this study used the two matrix model Q matrix (Q) model and matrix Q + K + K model (Q). Two model analyses were conducted on 12 evaluation indexes of low temperature tolerance with large phenotypic variation. It can be seen from (Figure 5) that among the two models, most of the Q model deviated from the theoretical value and there were many false positive sites, while the K+Q model was closer to the theoretical value, and more false positives could be filtered out by correcting this model. Therefore, Q+K model was used to conduct whole-gene association analysis for evaluation indexes of low temperature tolerance in this experiment.

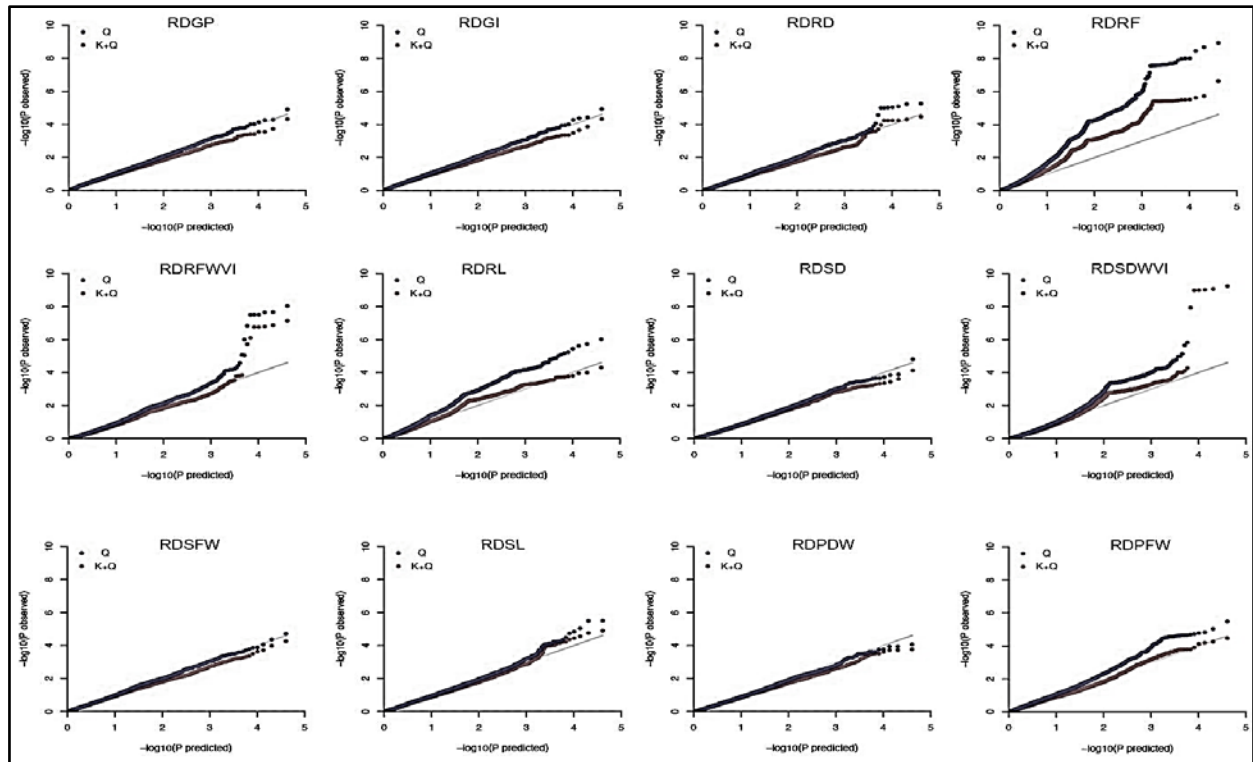


Figure 5. QQ chart of genome-wide association analysis of various phenotypic traits of low temperature tolerance

Based on the mixed linear model of K+Q, genome-wide association analysis was conducted for each evaluation index of low temperature tolerance, and the threshold value $P < 2.43 \times 10^{-5}$ ($\text{Log}_{10}P = 4.61$) was set. As shown in (Figure 6 and Table 5), eight significant SNPs were detected on the phenotype of RDRFWVI. It was distributed on chromosomes 3, 5 and 7, and the highest significant loci, PUT-163a-60355202-2755, accounted for 16.75% of phenotypic contribution. Five significantly correlated SNPs were detected on the phenotypic traits of RDRL, which were distributed on chromosomes 3 and 7, respectively, with the highest point being ZIP-103174386, and the explainable phenotypic variation rate was 11.42%. Six significantly correlated SNPs were detected on chromosome 3 and chromosome 5, respectively, with the highest point being PUT-163a-60355202-2755, and the explainable phenotypic contribution rate was 22.1%. A total of 36 significant SNPs were detected on the phenotype of RDRFW, which were distributed on

chromosomes 1, 2, 3, 5 and 8, respectively. Most significant SNPs were found on chromosome 8, which explained the contribution rate of phenotype from 10.88%-16.31%. Two significant SNPs on the phenotypic traits of RDSL were located on chromosomes 2 and 5, respectively, with the highest point SYN6137, representing an explainable phenotypic variation rate of 11.21%. A total of 6 SNPs were detected with at least 2 phenotypic traits, among which 1 SNPs was on chromosome 5 and the rest 5 SNPs were detected both are on chromosome 3, and the consistent sites on chromosome 3 all fall in ZIP-103174386 (220,933,020) and PZE-103174950 (221,386,301). When $-\text{Log}_{10}P = 4.61$, only 5 related SNPs were detected for phenotypic traits. In order to find more common SNPs, this study used $-\text{Log}_{10}P = 3.61$ for screening, and a total of 229 significantly related SNPs were screened. In addition, 29 SNPs appeared between at least two phenotypic traits, unequally distributed on chromosome 2, 3, 4, 5, 7 and 8.

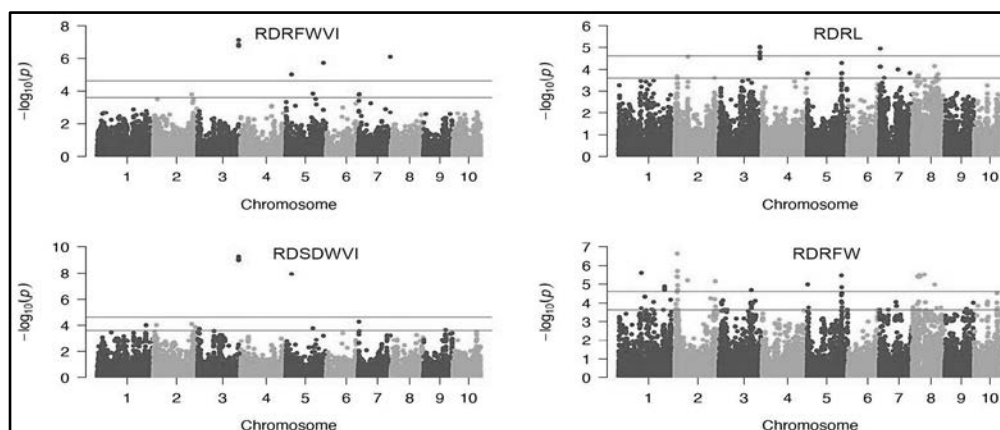


Figure 6. MLM Manhattan diagram of genome-wide association analysis

Table 5. MLM significantly associated SNPs

SNP	Chromosome	Position(bp)	-Log ₁₀ (P)	R ² (%)	Trait
PZE-101198749	1	247232050	4.7	11.56	RDRFW
PZE-101198766	1	247307676	4.81	11.48	RDRFW
PZE-101198751	1	247232249	4.88	11.27	RDRFW
PZE-101111994	1	122412228	5.63	13.58	RDRFW
SYN24701	2	12692172	4.64	11.06	RDRFW
SYN7480	2	15988377	4.69	10.88	RDRFW
SYN6137	2	188472331	4.9	11.21	RDRL
PZE-102032700	2	15354344	4.94	11.44	RDRFW
PZE-102175032	2	218127320	5.15	12.36	RDRFW
PZE-102082381	2	68919521	5.2	13	RDRFW
SYN16703	2	14233473	5.38	13.07	RDRFW
SYN7619	2	12136902	5.4	12.56	RDRFW
PZE-102026116	2	12111842	5.42	13.6	RDRFW
PZE-102030524	2	14279052	5.72	13.68	RDRFW
PZE-102027520	2	12806878	6.64	16.36	RDRFW
PUT-163a-60355202-2755	3	220967615	4.61	10.39	RDRL
PZE-103114887	3	174817636	4.69	11.75	RDRFW
PZE-103174950	3	221386301	4.78	10.79	RDRL
PZE-103174823	3	221359551	5	11.31	RDRL
PZE-103174386	3	220933020	5.05	11.42	RDRL
PZE-103174823	3	221359551	6.76	15.78	RDRFWVI
PZE-103174950	3	221386301	6.76	15.78	RDRFWVI
PZE-103174386	3	220933020	6.79	15.86	RDRFWVI
PZA03360.1	3	221242330	6.88	16.62	RDRFWVI
PUT-163a-60355202-2755	3	220967615	7.14	16.75	RDRFWVI
PZE-103174386	3	220933020	8.99	21.44	RDSDWVI
PZA03360.1	3	221242330	9	21.7	RDSDWVI
PZE-103174950	3	221386301	9.03	21.54	RDSDWVI
PZE-103174823	3	221359551	9.08	21.67	RDSDWVI
PUT-163a-60355202-2755	3	220967615	9.25	22.13	RDSDWVI
PZA02040.2	5	172138658	4.76	10.87	RDRL
PZE-105128030	5	185549432	4.84	11.18	RDRFW
SYN35365	5	2182381	4.98	11.53	RDRFW
PZE-105043202	5	30619422	5.04	11.55	RDRFWVI
PZE-105126664	5	184114608	5.46	12.71	RDRFW
PZE-105127434	5	184481750	5.49	12.78	RDRFW
PZE-105150892	5	202342547	5.73	17.15	RDRFWVI
PZE-105043202	5	30619422	7.94	18.7	RDSDWVI
SYN18499	7	4550449	4.95	9.6	RDRL
SYN15613	7	173788699	6.1	15.78	RDRFWVI
PZE-108068736	8	120293164	4.97	11.51	RDRFW
PZE-108029721	8	33079206	5.4	12.62	RDRFW
PZE-108033115	8	39463361	5.4	12.56	RDRFW
PZE-108033126	8	39474701	5.4	12.56	RDRFW
PZE-108033191	8	39598339	5.4	12.56	RDRFW
PZE-108033244	8	39752826	5.4	12.56	RDRFW
PZE-108033339	8	39932676	5.4	12.56	RDRFW
PZE-108027686	8	29014669	5.4	12.56	RDRFW
PZE-108033264	8	39761658	5.41	12.59	RDRFW
PZE-108027783	8	29235663	5.42	12.62	RDRFW
SYN18660	8	29151950	5.42	12.68	RDRFW
PZE-108030869	8	34707945	5.42	12.62	RDRFW
PZE-108027738	8	29152851	5.42	12.61	RDRFW
PZE-108033030	8	39088700	5.43	12.62	RDRFW
PZE-108027746	8	29168805	5.44	12.89	RDRFW
PZE-108033118	8	39465426	5.5	12.81	RDRFW
PZE-108040318	8	65300210	5.52	12.85	RDRFW

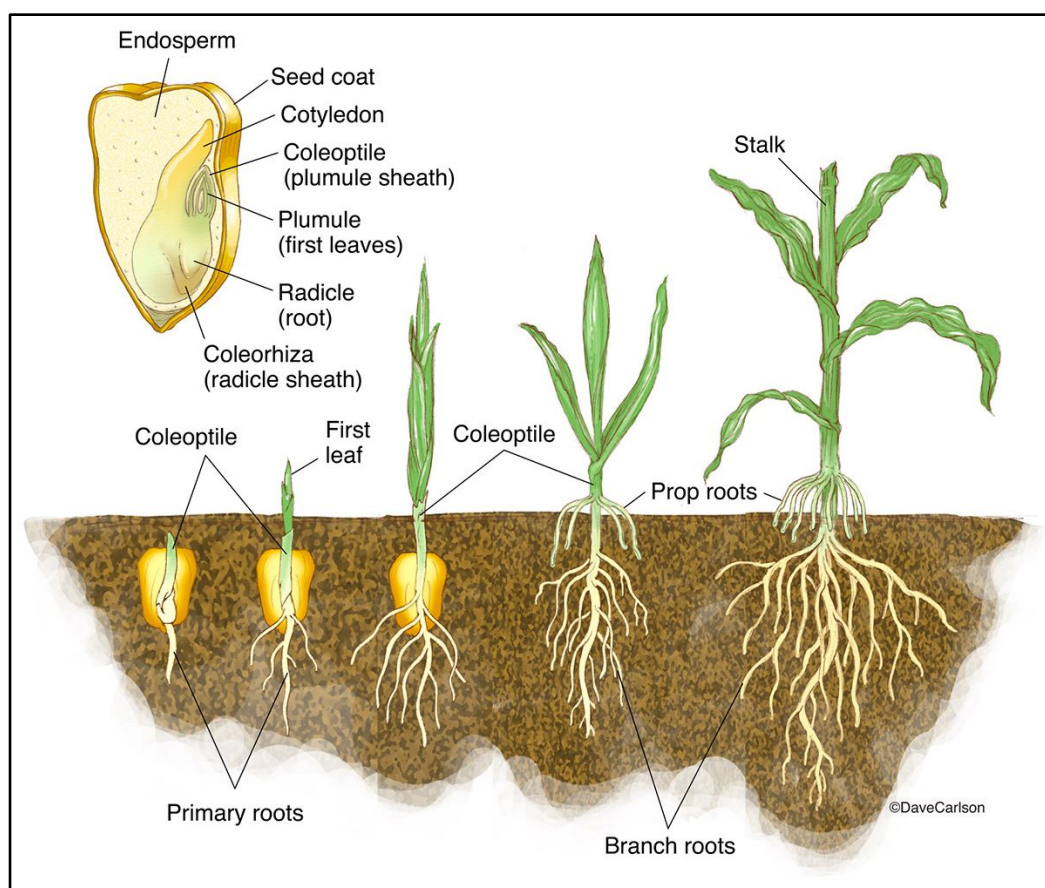


Figure 7. Effect mechanism of maize seed germination at low temperature on Seedling Growth

4. Discussion

4.1. Germplasm Evaluation of Low Temperature Germination Tolerance of Maize

The most important indicator of low temperature tolerance during germination is root growth, which is a key factor in plant development and yield. Low temperature stress reduced root vitality, shortened root length, and led to a decrease in lateral roots [17]. In a controlled growth chamber, root and bud growth was evaluated under normal and low temperature conditions, and the results showed that low temperature had the greatest influence on the number of root strips in the germination stage of maize [18]. Low temperature stress can also affect the growth of maize seedlings after germination, including leaf area, dry leaf weight, plant height, root length, stem length and whole plant fresh weight [19,20,21]. In this study, the low temperature condition was 13°C and the normal condition was 25°C. Eighteen evaluation indexes of low temperature germination tolerance were used to evaluate 238 maize inbred lines, and phenotypic variation analysis was conducted on maize low temperature germination tolerance traits. As can be seen from Table 1, coefficient of variation $RDGP > RDRFW > RDRDW > RDRL$, dry weight should be dried in oven to constant weight. Considering the time and benefit cost, RDRDW and RDRL had little difference in coefficient of variation. RDRL and the membership function of 12 indexes with

large coefficient of variation comprehensively evaluated the results of cluster analysis. RDRL had 25 extremely low temperature resistant materials, 51 extremely low temperature sensitive materials, and 23 extremely low temperature resistant materials comprehensively evaluated. Low temperature sensitive material 35 material partition results are similar. Study on Low Temperature of maize: American C and Ye478 are low temperature resistant materials [22]. Among the comprehensive evaluation indexes, the membership functions of the two materials are 6.128 ~ 8.290, and the RDRL of MeC and Ye478 are within 0.541 ~ 0.602, which belong to the low temperature resistant materials. In conclusion, our low temperature tolerance evaluation system is real and reliable, and comprehensive evaluation < 8.290 and RDRL < 0.602 can be used as screening materials for low temperature germination tolerance.

4.2. Genetic Basis of low Temperature Germination Tolerance of Maize

Genome-wide association analysis combined with linkage analysis was used to analyze the genetic basis of maize low temperature germination tolerance through associated common QTLs, which has important guiding significance for the mining of maize low temperature germination tolerance genes and the screening of excellent mutant germplasm. A total of 19 markers related to low temperature tolerance were identified by the identification of GWAS in 375 inbred lines in the field and growing room. These marker sites accounted for 5.7% to 52.5% of genetic variation in phenotype and chlorophyll

fluorescence parameter variation at seedling stage [17]. In this study, K+Q model was used to conduct genome-wide correlation analysis for 18 traits related to hypothermia tolerance, and 47 SNPs significantly associated with hypothermia tolerance were detected. Among them, chromosome 8 had the largest number of significantly associated SNP sites, and the explanation range of phenotypic contribution rate was 10.88%-16.31%. It can be used as a candidate interval for subsequent screening and analysis. A total of 6 SNPs were detected for at least 2 phenotypic traits according to GWAS analysis, among which 1 SNP was on chromosome 5, and the other 5 SNPs were on chromosome 3. The consistent sites on chromosome 3 all fall between ZIP-103174386 (220,933,020) and ZIP-103174950 (221,386,301). Linkage analysis of QTL sites can not only detect the accuracy of GWAS analysis results, but also reduce the QTL interval by using the SNP sites detected by GWAS. Genetic linkage analysis was performed on 574 F2:3 populations constructed from B283-1 X 04 group 0522-1-1 using *BSA-seq* method and KASP genotyping technique. The linkage interval was consistent with the interval obtained by GWAS analysis, and there were 13 All-indexes on chromosome 3 from 220.9Mb-221.6Mb analyzed by *BSA-seq*, and the mean value of the extreme mixed pool Δ All-index of the two progeny was -0.089. There is a linkage imbalance in this interval. In summary, the localization interval is reliable, there are 21 candidate genes in the localization interval, and only 12 candidate genes have transcriptional expression in this transcription analysis combined with transcriptome analysis, which can be used as candidate genes for subsequent screening and analysis.

5. Conclusion

In summary, maize seed germination under low-temperature stress displayed an increase of lipid peroxidation and inhibited subsequent seedling growth under normal temperature. According to the analysis of phenotypic variation of low temperature tolerance in 238 maize inbred lines and F2:3 population, the relative reduction values of root fresh weight and root length could be used as evaluation indexes for low temperature germination tolerance of maize. Therefore, this study provides new insights into maize seed germination in response to low-temperature stress.

Acknowledgements

This paper was financed by The Global Crop Diversity. This research was supported by the Research Program Sponsored by Ministerial and Provincial Co-Innovation Centre for Endemic Crops Production with High-quality and Efficiency in Loess Plateau, Taigu 030801, China (No. SBGJXTZX-15), the Key Laboratory of Crop Ecology and Dryland Cultivation Physiology of Shanxi Province (201705D111007), the Key Innovation Team of the 1331 Project of Shanxi Province, the Scientific and Technological Innovation Project of colleges in Shanxi

Province (2021L178), Science and technology innovation fund of Shanxi Agricultural University (2018YJ18).

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