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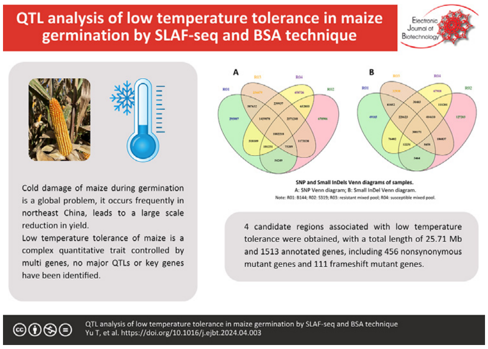
Research article

QTL analysis of low-temperature tolerance in maize germination by SLAF-seq and BSA technique [☆,☆☆]

Tao Yu [a,b,c,1,*], Jianguo Zhang [a,b,c,1,*], Jingsheng Cao [a,b,c], Xuena Ma [a], Shiliang Cao [a,b,c], Wenyue Li [a], Gengbin Yang [a,b], Sinan Li [a,b,c]

[a] *Maize Research Institute, Heilongjiang Academy of Agricultural Sciences, Harbin, Heilongjiang 150086, China*
[b] *Key Laboratory of Biology and Genetics Improvement of Maize in Northern Northeast Region, Ministry of Agriculture and Rural Affairs, Harbin, Heilongjiang 150086, China*
[c] *Key Laboratory of Germplasm Resources Creation and Utilization of Maize, Harbin, Heilongjiang 150086, China*

GRAPHICAL ABSTRACT



QTL analysis of low temperature tolerance in maize germination by SLAF-seq and BSA technique

Cold damage of maize during germination is a global problem, it occurs frequently in northeast China, leads to a large scale reduction in yield.
Low temperature tolerance of maize is a complex quantitative trait controlled by multi genes, no major QTLs or key genes have been identified.

SNP and Small InDels Venn diagrams of samples.
A: SNP Venn diagram; B: Small InDel Venn diagram.
Note: R01: B144; R02: S319; R03: resistant mixed pool; R04: susceptible mixed pool.

4 candidate regions associated with low temperature tolerance were obtained, with a total length of 25.71 Mb and 1513 annotated genes, including 456 nonsynonymous mutant genes and 111 frameshift mutant genes.

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ABSTRACT

Background: Cold damage of maize during germination is a global problem; it occurs frequently in northeast China, and leads to a large-scale reduction in yield. Low temperature tolerance of maize in germination is a complex quantitative trait controlled by multigenes, and no major QTLs or key genes have been identified.
Results: An F_2 isolation population with S319 and R144 as parents was constructed. The bulked segregant analysis (BSA) and specific-locus amplified fragment-sequencing (SLAF-seq) methods were applied to locate the chromosomal association regions related to low-temperature tolerance of maize during germination. Sequencing obtained 221.72 Gbp clean data, with an average sequencing depth of 25.96X. Four candidate regions associated with low-temperature tolerance trait of maize in germination were obtained, with a total length of 25.71 Mb and 1513 annotated genes, including 456 nonsynonymous mutant genes and 111 frameshift mutant genes.

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[*] Corresponding authors.
E-mail addresses: yutaoweiwei@163.com (T. Yu), zhangjianguo72@163.com (J. Zhang).
[1] These authors contributed equally to this work.

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sequencing (SLAF-seq)
Stress

Conclusions: This study aimed to lay the foundation for the mining of candidate genes of low-temperature tolerance in maize during germination, and accelerate the process of targeted improvement of maize low-temperature tolerance molecular marker-assisted breeding.

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1. Introduction

Maize (*Zea mays* L.) originated in Mexico, with an optimal growth temperature of 28 °C ~ 32 °C [1]. The process of seed germination and plant growth is very sensitive to temperature [2], especially in the early stages of growth, requiring higher temperatures for germination. When the temperature is below 5 °C, plant growth stops, and prolonging the low-temperature time can cause irreversible damage to plant cells and tissues [3]. Low-temperature injury of maize during germination refers to the low temperature of 0 ~ 10 °C suffered by maize from sowing to emergence, resulting in slow germination and emergence of maize, resulting in powdery and rotten seeds. Low temperature and cold damage of maize during germination is a global problem; it occurs frequently in northeast China, and seeds are susceptible to cold damage during germination, leading to delayed emergence and even death. This seriously reduces the emergence rate and plant growth rate and even leads to a large-scale reduction in yield [4]. The frequency of cold damage in spring in Heilongjiang province is the highest in northeast China. Therefore, it is an important direction of maize breeding in Heilongjiang province to select maize varieties tolerant to low temperatures during germination.

Bulked segregant analysis (BSA) is a method of selecting a certain number of individuals to mix their DNA and analyze it. The principle is to determine the linkage between markers and target traits based on the frequencies of the two extreme pool alleles [5,6]. BSA requires neither the creation of near-isogenic lines nor single-plant genotyping and is widely applicable for both qualitative and quantitative traits. Studies have shown that when the population size is 3000, the proportion of extreme individuals selected is 10%, and the distance between markers is 5 cM, a QTL with a phenotypic contribution rate of only 1% can be detected [7]. Therefore, BSA is widely used for QTL localization of any population with significant phenotypic differences in target traits among individuals due to its advantages of easy operation, low cost, fast, and efficient linkage marker detection. BSA was first applied by Michmore to screen the tightly linked marker of the downy mildew resistance gene *Dm5/8* in the F_2 isolate population of lettuce. Hayes and Saghai Maroof [8] used BSA-AFLP technology to locate resistance genes for soybean mosaic disease. Chen et al. [9] used BSA-SSR to detect the linkage molecular marker of resistance gene in maize coarse shrinkage disease. Liu et al. [10] applied SRAP-BSA to obtain cotton disease resistance gene linkage molecular markers. Boopathi et al. [11] utilized BSA-SSR to locate a closely linked marker with QTL (qtll2.l), the main potency of rice yield, under drought stress. Ghazvini et al. [12] used 423 SSR markers to analyze 267 DH populations constructed by 10 F_1 individuals, and mapped the wheat stalk rust gene *Sr54* on chromosome 2D to obtain its tightly linked marker barc228.

With the rapid development of sequencing technology, the combination of BSA and DNA sequencing can quickly carry out gene localization and molecular marker screening. The SLAF-seq (Specific-locus Amplified Fragment-sequencing) technology is a large-scale genome sequencing technology based on restriction enzymes and high-throughput sequencing. It has the advantages of not being limited by reference genomes, flexible enzyme digestion schemes, avoiding repeated sequences, simplifying complex genomes, and developing SNP molecular markers with high cost-effectiveness, good stability, and uniform distribution in the genome. It has been widely used in research such as QTL mapping. Yang et al. [13] extracted the DNA of 430 low-temperature sensitive and 385 low-temperature insensitive seedlings screened out of 10,800 rice F_3 seedlings, then used BSA combined sequencing technology for genotype identification, and a total of 6 low-temperature QTL sites of rice at the seedling stage were detected on chromosomes 1, 2, 5, 8, and 10. Takagi et al. [14] used BSA and resequencing methods to analyze the mixing pool of extreme traits in inbred recombinant line populations constructed by hybridization of extreme phenotypic difference lines and used the QTL-seq method to locate the QTL associated with rice blast and seed seedling viability. Meng et al. [15] used SLAF-Super BSA technology to locate the anti-phytophthora traits of *Arabidopsis thaliana* in the 7.1 ~ 11.2 Mb region of chromosome 4.

In an associated study, we screened a large set of maize inbred lines and identified two lines S319 and R144 that are sensitive and insensitive to cold stress, respectively. In this study, S319 and R144 were used as parents to construct the F_2 isolation population. BSA and SLAF-seq methods were applied to locate the chromosomal association regions related to low-temperature tolerance during germination and conduct enrichment analysis of genes within the association regions. This study aimed to lay the foundation for the mining of candidate genes of low-temperature tolerance in maize during germination, and accelerate the process of targeted improvement of maize low-temperature tolerance molecular marker-assisted breeding.

2. Materials and methods

2.1. Plant materials

R144 belongs to the SS group. The low-temperature tolerance evaluation in the three-year field has shown a relative seedling index of 92 ~ 96%. The germination rate of the low-temperature stress test was 98%, and the stress level at the germination stage showed high tolerance to low temperature. S319 belongs to the PB group. The low-temperature tolerance evaluation in the three-year field has shown a relative seedling index of 36 ~ 49%. The germination rate of the low-temperature stress test was 35%, and the stress level at the germination stage showed low tolerance to low temperature.

R144 was selected as a low-temperature tolerant donor for maize, and S319 was selected as a receptor. F_1 seeds were obtained by hybridization in Sanya, Hainan in the winter of 2018, and F_1 seeds were planted in Harbin, Heilongjiang in the summer of 2019, with individual plant numbers. Leaves were collected at the three-leaf stage and stored at −80°C for DNA extraction. All individual plants were self-bred to obtain F_2, and F_2 ears were harvested for indoor identification of low-temperature tolerance in maize.

2.2. Indoor identification of low-temperature tolerance during germination and screening of individuals with extreme phenotypes

Seeds of consistent size were selected and soaked in 0.5% NaClO solution for 5 min; then, the seeds were rinsed with tap water 3 times, and the seeds were placed in a Petri dish covered with filter paper on top and bottom, keeping the filter paper wet but no water flowing out. Seeds were cultivated in the artificial climate box at 5 °C/4d; the germination rate was calculated after 25 °C/3 d, and the relative germination rate and relative germination index were calculated. Three replicates were set with 100 capsules per replicate. The control was cultured in a 25 °C/7 d. The germination rate of F_2 under low-temperature stress conditions was statistically calculated, and extreme phenotypic individuals in the F_2 population were determined according to the germination rate.

$$Germinationrate(\%) = \frac{\text{number of germinated seeds}}{\text{number of seeds tested}} \times 100$$

$$Relati\,vegerminationrate(\%) = \frac{\text{germination rate of low temperature treatment}}{\text{germination rate of suitable temperature treatment}} \times 100$$

$$Germinationindex = \sum \frac{\mathrm{Gt}}{\mathrm{Dt}}$$

Gt: number of germination in t days, Dt: corresponding germination days.

$$Relati\,vegerminationindex = \frac{\text{germination index of low temperature treatment}}{\text{germination index of suitable temperature treatment}} \times 100$$

2.3. SLAF-BSA resequencing

The modified CTAB method was used to extract the genomic DNA of R144, S319 and the extreme phenotypic individuals of F_2 population, respectively. The concentration and purity of DNA were detected, and the DNA of qualified extreme phenotype individuals was mixed in equal amounts to construct offspring-resistant and susceptible DNA pools, respectively. Randomly interrupted the DNA to be sequenced to a fragment size of about 350 bp, modified the end of the fragment, constructed a sequencing library, and resequencing was performed with Illumina HiSeq (Bimax Biotechnology Co., LTD.).

2.4. Resequencing data analysis

2.4.1. Data quality control

Filter the raw data, evaluate the quality from the aspects of base sequencing quality distribution and base type distribution, filter the raw data after the evaluation, and remove the jointed and low-quality sequences, to obtain a sequence without redundancy.

2.4.2. Compare the reference genome

The redundant sequences were located on the maize reference genome (B73 RefGen-v4), and the bwa software was used to locate the position of the non-redundant sequences on B73 RefGen-v4.

2.4.3. Detect and annotate SNPs and Small InDels

SNP and Small InDels were detected using GATK software, and according to the localization results of the non-redundant sequence in B73 RefGen-v4, Picard was used to remove duplicates and GATK was used to realignment, detect, and filter SNP and Small InDels. SnpEff software annotated and predicted the obtained SNP and Small InDels, located the region where the SNP and Small InDels were according to the position information of SNP and Small InDels on B73 RefGen-v4 and analyzed whether the SNP and Small InDels variants produced synonymous mutations.

2.4.4. Association analysis of SNP and Small InDels

The low-quality SNP and Small InDels were filtered, the Euclidean distance (ED) method was used to calculate the ED value, the DISTANCE method was used to fit the ED value, and the median+3SD of the fitted value was selected as the correlation threshold for analysis to determine the correlation region. SNP-index and InDels-index methods were used to screen markers of significant differences in genotype frequency between mixing pools, ΔSNP-index and ΔInDels-index were fitted by DISTANCE method, and the regions above the association threshold were selected as the associated regions related to the low-temperature tolerance traits of maize during germination. The associated region results obtained by the combined ED algorithm, SNP-index and InDels-index methods were identified as candidate regions related to low-temperature tolerance traits in the germination stage of maize.

2.4.5. SNP and InDels function annotations

Non-synonymous mutations and frameshift mutant genes in the candidate regions related to low-temperature tolerance traits in germination were screened, and BLAST software was used to annotate databases such as NR, GO, KEGG, and COG, etc.

3. Results

3.1. Individual screening of extreme phenotypes for the identification of low-temperature tolerance at the germination stage of maize

The F_2 population constructed by R144 and S319 included 2600 individuals, and the seeds of individuals were used for the indoor identification of low-temperature tolerance. The relative germination rate of individuals was as follows: there were 131 lines with a relative germination rate of more than 86%, 1127 lines with a relative germination rate of 70 ~ 85%, 794 lines with a relative germination rate of 55 ~ 69%, 406 lines with a relative germination rate of 36 ~ 54%, 142 lines with a relative germination rate of 0 ~ 35%. Sixty individuals with extreme phenotypes were selected, including 30 individuals with a relative germination rate of 100% and 30 individuals with a relative germination rate of 0%. The results of the relative germination rate under low-temperature stress showed continuous variation, which conformed to the normal distribution and showed the characteristics of quantitative gene inheritance (Fig. 1). The phenotypic data of the relative germination rate under low-temperature stress were concentrated and wide-ranging, indicating that the constructed F_2 sequencing population was suitable for QTL mapping of quantitative traits.

3.2. Resequencing data analysis

3.2.1. Quality control of sequencing data

When constructing a library, the error rate of the first few bases and terminal bases at the 5′ end of the sequence is usually high. In this study, the error rate of sequencing data is low, with an average Q30 of both ends above 94. There was no separation of bases, indicating that the whole sequencing quality can be used for subsequent data analysis. Filtered the original sequence and ultimately obtained a non-redundant sequence of 221.72 Gbp for subsequent

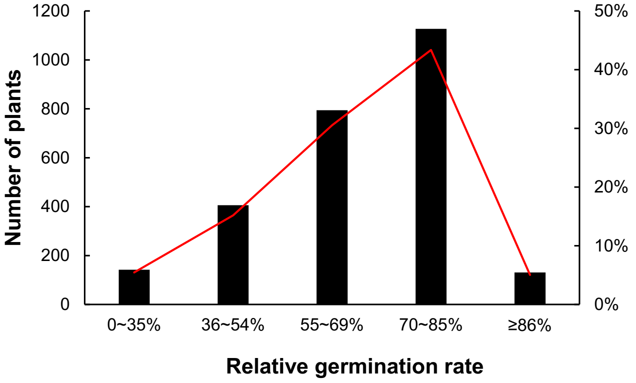

Fig. 1. Distribution of relative germination rate of the population.

information analysis, and the specific evaluation results were shown in Table 1.

3.2.2. Comparison of reference genomes

The bwa software was used to locate the non-redundant sequence to B73 RefGen-v4. The results showed that the average alignment rate of the four samples and B73 RefGen-v4 was 99.23%, with a successful alignment rate ranging from 91.78 to 93.81% (Table 1), indicating that the sequencing data were consistent with the maize reference genomes.

The starting and ending positions of the double-ended sequence on B73 RefGen-v4 were detected with the CollectInsertSizeMetric.jar software. The size of the inserted fragment obtained conformed to the normal distribution, the average sequencing depth was 25.96X, the coverage depth was 22.50X, and the chromosome coverage was 92.03%. The sequencing results had good randomness, which was suitable for the next step of data analysis.

3.2.3. Detection and annotation of SNPs and Small InDels

Using GATK and Picard to detect SNPs and Small InDels between samples, 7,026,691 SNPs and 1,126,691 small InDels were obtained between B144 and S319, including 95,846 non-synonymous mutant SNPs; 4,786,278 SNPs and 767,831 Small InDels were obtained between the resistant and susceptible mixed pools, of which 5420 were non-synonymous mutant SNPs (Fig. 2). The Small InDels variation was significantly less than the SNP variation. The specific annotation information of the SNPs and Small InDels in this study is shown in Table 2, and the rich differential loci are conducive to QTL mining.

3.2.4. Association analysis of SNPs and Small InDels

The low-quality SNPs and small InDels were filtered, and finally, a total of 3,429,319 high-quality SNPs and 589,152 InDels were obtained.

The ED method was used for SNP association analysis. A total of 12 candidate regions related to low-temperature tolerance trait were obtained, with a total length of 34.23 Mb, containing 1860 genes, including 509 non-synonymous mutant genes. In order to eliminate the false positive sites, 0.667 was used as the correlation threshold, and 4 regions on the threshold line were screened, with a total length of 27.69 Mb, containing 1923 genes, including 511 non-synonymous mutant genes. Based on SNP, the intersection of the associated regions of ED and SNP-index was taken, and four candidate regions associated with low-temperature tolerance trait of maize in germination were obtained on chromosomes 1 and 7, with a total length of 25.71 Mb and containing 1605 genes (Table 3).

InDels association analysis was performed by ED method, 1.91 was taken as the association threshold, and 8 candidate regions related to low-temperature resistance traits were obtained, with a total length of 28.68 Mb and 1899 genes, including 119 frameshift mutant genes. InDels-index association analysis was performed, 0.667 was taken as the association threshold, and 4 regions on the threshold line were screened out, with a total length of 76.90 Mb, containing 6087 genes, including 327 frameshift mutant genes. Based on InDels, the two methods of ED and SNP-index were taken to intersect the associated regions, and four candidate regions located on chromosomes 1, 6, and 7 were obtained

Table 1
Quality evaluation results of the sequencing.

Sample	Total-reads	Clean-Reads	Clean-Base	Q30 (%)	GC (%)	Mapped (%)	Properly-mapped (%)
R144	351,713,070	175,856,535	52,692,582,612	94.37	47.12	99.38	93.64
S319	323,686,736	161,843,368	48,481,828,704	94.24	46.22	99.12	91.78
R mix pool	327,165,116	163,582,558	48,959,280,218	94.37	46.43	99.21	93.35
S mix pool	478,893,390	239,446,695	71,590,115,640	94.31	45.61	99.2	93.81

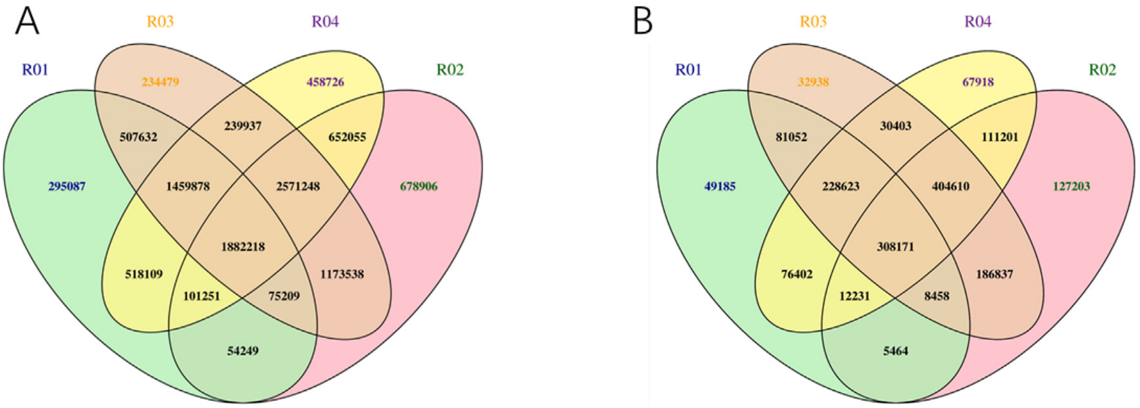

Fig. 2. SNP and Small InDels Venn diagrams of samples. (A) SNP Venn diagram; (B) Small InDel Venn diagram. Note: R01: B144; R02: S319; R03: resistant mixed pool; R04: susceptible mixed pool.

Table 2
Annotation results of SNP and Small InDels.

Small InDels Type	R01vsR02	R03vsR04	SNP Type	R01vsR02	R03vsR04
INTERGENIC	558,789	394,826	INTERGENIC	4,973,624	3,451,754
INTRAGENIC	298	194	INTRAGENIC	32	27
INTRON	142,599	91,998	INTRON	457,193	293,507
UPSTREAM	194,422	129,373	UPSTREAM	693,925	456,282
DOWNSTREAM	164,476	109,533	DOWNSTREAM	616,108	407,411
UTR-5-PRIME	14,103	9226	UTR-5-PRIME	20,793	13,376
UTR-3-PRIME	22,571	14,488	UTR-3-PRIME	46,398	29,840
UTR-3-DELETED	1	1	SPLICE-SITE-ACCEPTOR	890	550
SPLICE-SITE-ACCEPTOR	530	329	SPLICE-SITE-DONOR	793	486
SPLICE-SITE-DONOR	661	432	SPLICE-SITE-REGION	9379	5850
SPLICE-SITE-REGION	3536	2268	START-GAINED	13,195	8245
START-LOST	136	85	START-LOST	295	172
FRAME-SHIFT	10,936	6825	NON-SYNONYMOUS-START	39	27
CODON-DELETION	4002	2349	SYNONYMOUS-CODING	94,930	58,386
EXON-DELETED	1	1	NON-SYNONYMOUS-CODING	95,846	58,420
CODON-INSERTION	5202	3197	SYNONYMOUS-STOP	200	127
CODON-CHANGE-PLUS-CODON-DELETION	2473	1482			
CODON-CHANGE-PLUS-CODON-INSERTION	1472	910			
STOP-GAINED	309	212	STOP-GAINED	2462	1450
STOP-LOST	174	102	STOP-LOST	589	368
Other	0	0	Other	0	0
Total	1,126,691	767,831	Total	7,026,691	4,786,278

Table 3
SNP-associated regions detected by ED and SNP-index methods.

Chromosome-ID	Start	End	Size(Mb)	Gene-Number
1	236,240,000	260,090,000	23.85	1363
7	160,610,000	162,380,000	1.77	242
Total	–	–	–	1605

Table 4
InDels associated regions detected by ED and SNP-index methods.

Chromosome-ID	Start	End	Size (Mb)	Gene-Number
1	236,200,000	260,400,000	24.20	1484
6	27,610,000	28,170,000	0.560	14
7	160,160,000	162,880,000	2.72	387
Total	–	–	–	1885

that were associated with low-temperature resistance trait, with a total length of 27.49 Mb and 1885 genes (Table 4). The results of the intersection association regions corresponding to SNP and InDels were intersected again, and four candidate regions related to the low-temperature tolerance trait in the germination stage of maize were obtained on chromosomes 1 and 7, with a total length of 25.71 Mb.

3.2.5. Functional annotations for SNPs and InDels

Annotate the non-synonymous mutant SNPs and the InDels of the frameshift mutation within the four candidate regions, and the results showed that there were 1452 non-synonymous mutant SNPs between R144 and S319, and 1085 non-synonymous mutant SNPs between mixing pools. There were 165 frameshift mutant InDels between R144 and S319, and 123 frameshift mutations between mixing pools. These non-synonymous mutants of SNPs and InDels of frameshift mutations are likely to be directly related to the low-temperature tolerance trait of maize in germination.

BLAST software was used to annotate the non-synonymous mutant genes and frameshift mutant genes in the candidate regions by GO, KEGG, etc. A total of 1513 genes were annotated, including 456 non-synonymous mutant genes and 111 frameshift mutant genes between R144 and S319.

Using the GO database, a total of 1294 genes were annotated in the four candidate regions of this study, and the GO classification statistics of the annotated genes are shown in Fig. 3, including 378 non-synonymous mutant genes and 90 frameshift mutant genes. Annotated genes were classified according to biological process, cellular component and molecular function, with significant enrichment in GO annotation classification for cellular processes (GO: 0009987) and metabolic processes (GO: 0008152) in biological process function, cells (GO: 0005623) and cell parts (GO: 0044464) in cellular component function, and binding (GO: 0005488) and catalytic activity (GO: 0003824) in molecular function.

Using the KEGG database, a total of 716 genes were annotated in the 4 candidate regions related to the low-temperature tolerance trait in this study. The KEGG classification statistical results of the annotated genes in the candidate region are shown in Fig. 4, including 177 non-synonymous mutant genes and 41 frameshift mutant genes, involving 50 pathways such as cell processing and environmental signal processing, and significantly enriched in pathways such as plant hormone signal transduction, phenylpropane biosynthesis, sulfur metabolism and other pathways. It indicated that low-temperature stress can affect the utilization of energy and the synthesis of secondary metabolites in plants, and plants induce the expression of related genes and protein synthesis through signal transduction, causing a series of positive responses in plants, and ultimately prompting plants to adapt to the adversity environment or enhance plant stress resistance.

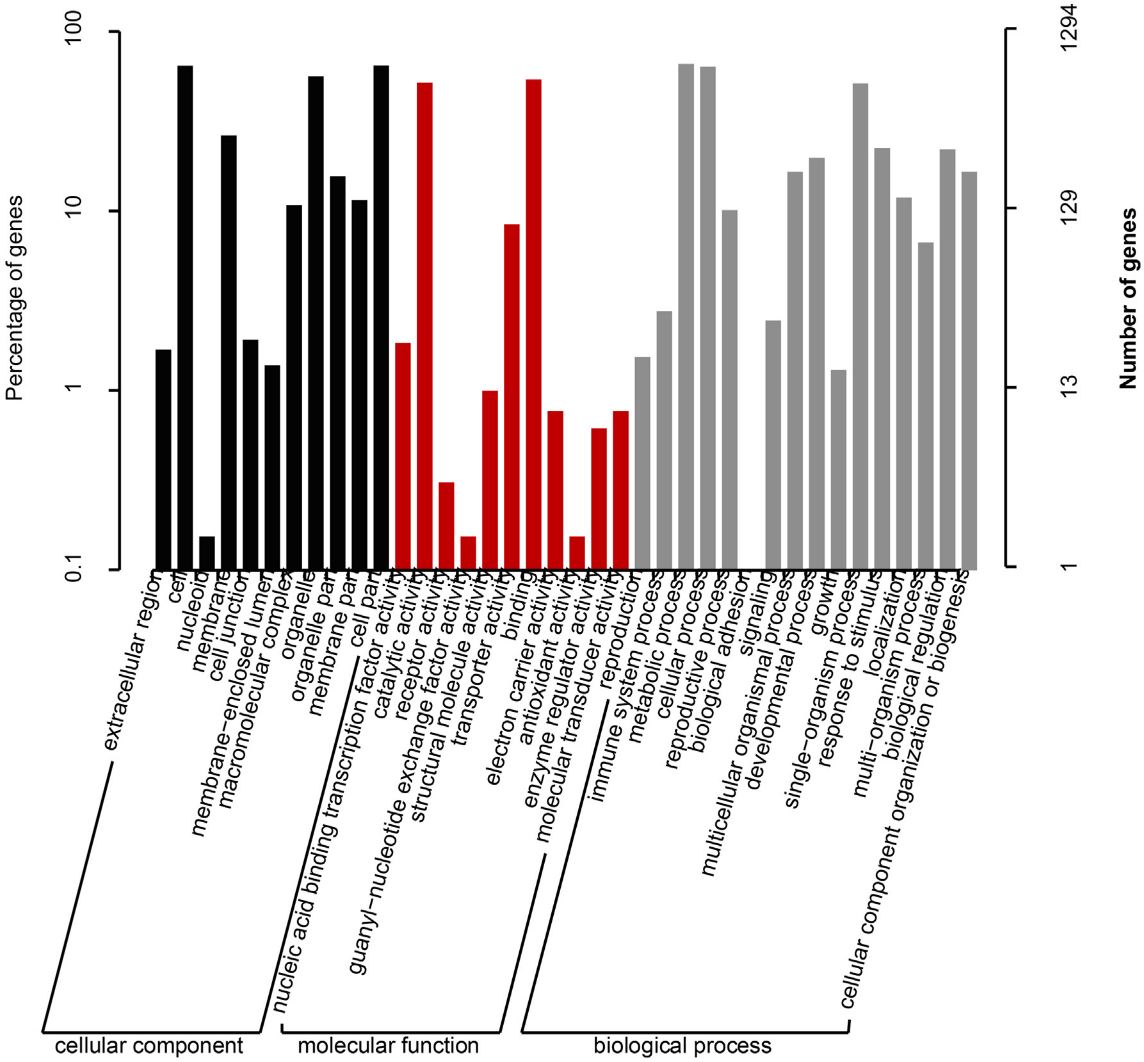

Fig. 3. GO annotation cluster diagram of genes in candidate regions.

4. Discussion

The maize genome is huge, 80% of which are repetitive sequences, and the low-temperature tolerance trait in the germination stage of maize are complex quantitative traits, so it is difficult to locate the genes related to the low-temperature tolerance trait in the germination stage of maize. With the rapid development of sequencing technology, resequencing combined with BSA technology provides a fast and efficient method for the localization of quantitative traits in complex genomes. Zhao et al. [16] combined SLAF-Seq and BSA technology to locate genes related to hemp fiber content; they found four candidate regions with a length of 8.68 Mb and 389 annotated genes on Chromosome 1. Zhou et al. [17] used three typical inbred lines (Huangzao4, Jing7 and Chang7-2) to construct two F_2 populations, mixing gene pools based on typical tassel symptoms by BSA technology, they located the QTL controlling tassel symptoms on the bin 1.06 region in Huangzao4 and Jing7 lines, between the markers of umc1590 and bnlg1598, in the Jing7 and Chang7-2 lines, the QTL was located on the bin 2.07 region, between the markers of umc1042 and bnlg1335. Yang et al. [13] extracted the DNA of 430 low-temperature sensitive and 385 low-temperature tolerant seedlings screened out of 10,800 rice F_3 seedlings, mixed them separately, and used BSA combined sequencing technology for genotype identification, and a total of 6 low-temperature tolerance QTL sites at the seedling stage of rice were detected on the 1st, 2nd, 5th, 8th, and 10th chromosomes. Takagi et al. [14] used BSA and resequencing methods to analyze the mixing pool of extreme traits in inbred recombinant line populations (RILs) constructed by hybridization of extreme phenotypic difference lines, and used the QTL-seq method to locate a QTL associated with rice blast and seed seedling viability. Zhu et al. [18] constructed the F_2 population derived from the cross between AS5 and NX420 to identify new genes for modulating salt resistance in maize, through the BSA-seq technology, they mapped one hundred and six candidate regions for salt tolerance on all of the chromosomes, finally, the *Zm00001d053925* was identified as a new functional gene responding to salt stress in the seedling stage of maize. Wang et al. [19] used an $F_{2:3}$ population derived from a JING724 and JING724A1 cross, through the bulk segregant analysis, they detected one QTL located on chromosome3: 14.00–19.28 Mb for stalk fracture. *Zm00001d039769* and *Zm00001d039913a* were identified as candidate genes that are associated with high stalk fracture angle by transcriptome analysis [19]. Luo et al. [20] used BSA to identify cold tolerance in rice and located 58 candidate genes, of which 5 candidate genes were significantly differentially expressed. Meng et al. [15] applied BSA-SLAF technology to locate the phytophthora-resistant QTL of *Arabidopsis thaliana* in the 7.1 ~ 11.2 Mb region of chromosome

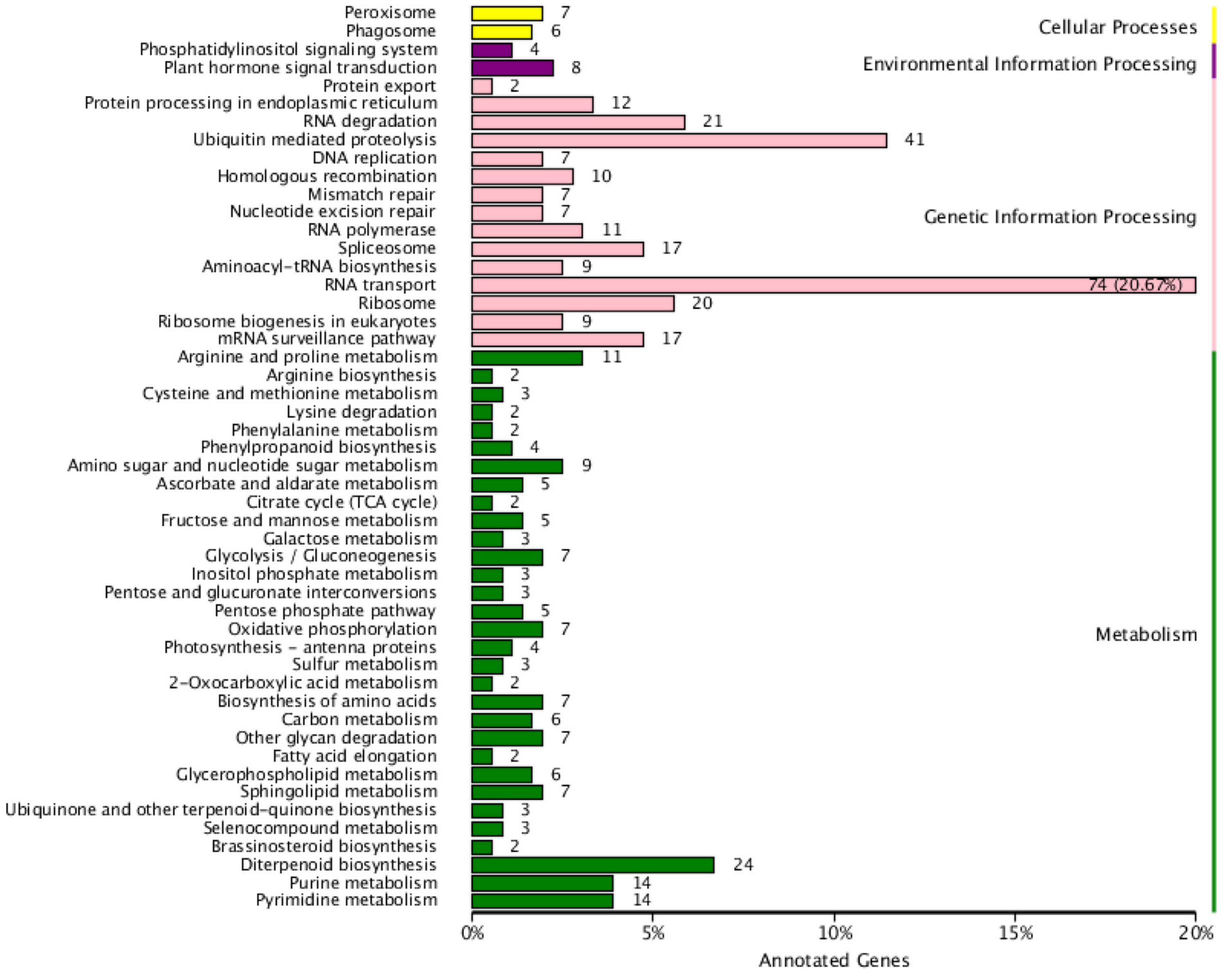

Fig. 4. KEGG analysis of genes in candidate regions.

4. Han et al. [21] mapped six novel QTLs related to pod dehiscence resistance in soybean by using specific–locus amplified fragment sequencing, it can explain 7.22–24.44% of the phenotypic variance and three stable QTLs (qPD01, qPD05-1 and qPD08-1) would be developed into CAPS/dCAPS markers in the future. In this study, R144, the inbred line of strong low-temperature tolerant maize, as the low-temperature resistance donor of maize, and the inbred line S319 of extremely weak low-temperature tolerant maize as the low-temperature resistance receptor of maize, were used to construct an F_2 population containing 2600 individual plants, and 30 individuals with extreme phenotypes of resistance and sensitivity were selected from F_2 population to form a mixed pool of resistance and sensitivity, together with the parents for resequencing. Sequencing obtained 221.72 Gbp data, with a sequencing depth of 25.96X, and a coverage depth of 22.50X. There were 7,026,691 SNPs between parents and 95,846 SNPs with nonsynonymous mutations. There were 4,786,278 SNPs and 58,420 nonsynonymous mutation SNPs between mixed pools. 1,126,691 small InDels were obtained between parents; 767,831 Small InDels were awarded between the mixed pools. Association analysis obtained 4 candidate regions related to low-temperature tolerance trait in the maize germination stage on chromosomes 1 and 7, with a total length of 25.71 Mb and 1513 annotated genes, including 456 nonsynonymous mutant genes and 111 frameshift mutant genes, which were significantly enriched in RNA transport, ubiquitin-mediated protein hydrolysis, and diterpene biosynthesis.

The accuracy of BSA depends on many factors such as population size, proportion of extreme individuals, and marker density. Among them, the population size mainly considers the distance between the marker and target gene, the recombination rate of the target region and the genetic characteristics of target traits [22]. However, selecting the suitable population size is not easy, Huang et al. [23] has shown that population size is not as large as it will be better, meanwhile, the population size is also restricted by the cost of phenotyping, because population size depends on the heritability of each QTL, so they suggested that the population size is based on the typical minor QTL. In the case of quantitative traits, other factors such as gene number, effects, interactions, and relative position need to be considered [24]. The proportion of extreme individuals is generally determined according to the population size and QTL effect. For small- and medium-sized groups containing 200–500 individuals, the optimal proportion of extreme individuals is 20–30% [25,26]. However, when the QTL effect is fixed, the proportion of extreme individual selection tends to decrease with the increase in group size. For QTLs with a phenotypic contribution rate greater than 10%, at least 20 individuals should be selected from a population of size 200, with a selection ratio greater than 10%. For QTLs with phenotypic contribution rates of 3–10%, extreme individuals need to select at least 50 from groups of 500–1000 in size, that is, the selection ratio is 5–10%. For QTLs with a phenotypic contribution rate of <3%, extreme individuals need to select at least 100 from groups of 3000–5000 in size, that is, the selection ratio is <5% [7]. The marker density determines the average distance and maximum distance between two adjacent markers on the genetic map, that is, the average density of the genetic linkage map and the genome coverage of the entire map [27]. High-density molecular markers are easier to detect the possibility of chromosome crossover in large populations and individuals with high-selection proportion. When the population size is 200, the extreme individual is 10, and the distance between adjacent markers is 15 cM, the target gene can only be initially located. When the population size is 500, the extreme individuals are 20, and the distance between adjacent markers is 1 cM, the target gene may be limited to a small region for precise localization [28]. With the construction of more and more high-density genetic linkage maps, the localization of complex quantitative traits of multigene control will be greatly accelerated [14,29]. In this study, R144 and S319 were used as parents to construct an F_2 mapping population consisting of 2600 individual plants. The mapping population was relatively large and suitable for locating complex quantities controlled by micro effective multigenes. Under indoor low-temperature stress, the relative germination rate of individual plants in the population showed continuous variation, which conformed to the normal distribution, showed the characteristics of quantitative gene inheritance, and was suitable for the localization of quantitative traits. In this study, 30 plants with resistant and susceptible extreme phenotypes in the F_2 population were selected to form a mixed pool of resistance and susceptibility, and the proportion of extreme individuals selected was 2%, which was helpful to locate the micro-effective QTLs with a contribution rate of less than 3% to the low-temperature resistant phenotype in the germination stage of maize.

As the next-generation sequencing technologies improve, BSA becomes a popular method to improve plant breeding by quickly developing markers, assisting marker selection, analyzing agronomic genomics, and identifying mutant loci and selective phenotypes [30]. BSA-Seq is widely used in a variety of crop species like maize, rice, cotton, soybean, barley and cucumber [31,32,33,34,35,36]. However, the most important advantage of using BSA-Seq is its simplicity, both sample collection and data analysis. Moreover, BSA-seq localization does not require screening of markers, which saves costs and improves detection efficiency, thus BSA-Seq offers many advantages that are useful for plant breeding. In this study, the BSA-seq method was used to narrow the candidate region of low-temperature tolerance in the germination stage of maize to 25.71 Mb on chromosomes 1 and 7, and 1513 genes were annotated, including 456 nonsynonymous mutant genes and 111 frameshift mutant genes. These genes may have undergone corresponding mutations in the coding sequences due to external low-temperature stress treatment, resulting in changes in the structure and expression of gene products in response to external stress. At the same time, the variation information of candidate sequences of resistant and susceptible extreme materials was obtained through resequencing, which was convenient for the development of functional markers related to low-temperature resistance in maize germination, which provided a rich marker basis for subsequent fine localization, and has great significance for accelerating molecular breeding for low temperature resistant in maize germination.

5. Conclusions

Low temperature tolerance of maize in germination is a complex quantitative trait controlled by multigenes, no major QTLs or key genes have been identified. With the rapid development of sequencing technology, the combination of BSA and SLAF-seq can quickly carry out gene localization and molecular marker screening of complex quantitative trait. In this study, an F_2 isolation population with S319 and R144 as parents was constructed. Sequencing obtained 221.72 Gbp clean data, with an average sequencing depth of 25.96X. Four candidate regions associated with low-temperature tolerance trait of maize in germination were obtained, with a total length of 25.71 Mb and 1513 annotated genes, including 456 nonsynonymous mutant genes and 111 frameshift mutant genes. The results presented here lay the foundation for the mining of candidate genes of low-temperature tolerance in maize during germination, and accelerate the process of targeted improvement of maize low-temperature tolerance molecular marker-assisted breeding.

Author contributions

- Study conception and design: T Yu; J Zhang; J Cao
- Data collection: T Yu; X Ma
- Analysis and interpretation of results: T Yu; S Cao; W Li; G Yang
- Draft manuscript preparation: T Yu; X Ma
- Revision of the results and approval of the final version of the manuscript: T Yu; J Zhang; J Cao; S Li

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Conflicts of interest

The authors declare no conflict of interest.

Supplementary material

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