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Genome-wide Association Functional Studies (GWAFS) of Candidate Genes for Germination-related Traits of Low Temperature Tolerance in Maize (*Zea mays* L.)

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Abstract 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.) originated in tropical and subtropical areas and is naturally sensitive to low-temperature stress, especially during seed germination. As spring maize, seed germination and seedling growth at an early stage are usually subjected to low-temperature stress. In this study, 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 decrease of root length ranges from 0.105 to 0.936, which conforms to the normal distribution and can distinguish the tolerance of low-temperature germination, the 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%. The results of $F_{2:3}$ population linkage analysis were consistent with the gene candidate region predicted by GWAS. *BSA-seq* analysis showed that there were 13 all indexes on chromosome 3 between 220.9 Mb and 221.3 Mb. The average value of all index in the extreme mixed pool of the two offspring was -0.089. Therefore, the candidate interval of this study is located on chromosome 3 220.9 Mb-221.3 Mb. There are 21 candidate genes in the localization interval.

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

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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]. 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 [6,7]. 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. 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 [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 [7,8]. 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 [7]. 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. The optimum temperature of seed germination is about 25 C in maize, and temperatures below 15°C may cause low-temperature stress [9,10]. There is almost a complete lack of development in maize exposed to temperatures below 10 C [11]. Under low-temperature stress, plants undergo a series of physiological and morphological changes. The physiological changes include cell membrane hardening, reactive oxygen species (ROS) accumulation, protein instability, and metabolic disorders [12]. The morphological changes include delayed germination, decreased germination rate, and inhibited seedling growth [13]. ROS are also considered a signal molecule in different organisms, which can affect various physiological processes in plants. To eliminate the toxicity of ROS, plants have established an excellent antioxidant defense system, including a large number of enzymatic antioxidants and nonenzymatic antioxidants. Some ROS scavengers can resist environmental stress by regulating the expression of antioxidant enzyme genes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) [14,15,16]. As a cofactor, Vitamin B6 (VB6) plays an essential role in the enzymatic reaction and is related to cellular oxidative stress defense [17]. Ribosomes convert the genetic information of messenger RNA into functional proteins [18]. The essential, complex, and energy-consuming process, ribosome biosynthesis is accomplished by endogenous signals [19] and environment stimuli [20], such as ambient temperature [21,22]. Previous studies identified the processing pathway of precursor ribosomal RNA in rice and showed that ribosomal biogenesis was rapidly inhibited by low temperature [23]. The mechanism underlying maize seed germination under low-temperature stress is still ambiguous. The objective of this study was to investigate the dynamic changes in seed germination under low-temperature stress in maize, especially at the transcriptome level. We first detected the morphological and physiological changes in seed germination under low-temperature stress in two maize inbred lines with different low-temperature resistance. Subsequently, we analyzed the transcriptome changes of seeds germinated at low temperatures for 0, 2, 4, and 6 days. The results provide new insights into maize seed germination in response to low-temperature stress.

2. Materials and Methods

The low-temperature sensitive maize (SM) B283-1 and low-temperature resistance maize (RM) 04Qun0522-1-1 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 Key Laboratory of Organic Dry Farming, China. Seeds were sown on 12 June 2020. The plant density was 67,500 plants/ha. Seeds used in this study were harvested 50 days after pollination.

2.1. 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.

2.2. *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). Three biological replicates were prepared per sample for the Illumina transcriptome sequencing analysis. The RNA-seq libraries were constructed and sequenced as previously described. 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 [24]. 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. The p values were adjusted according to the Benjamini and Hochberg method. The DEGs (i.e., adjusted $p < 0.05$ and log2 (fold-change) were considered for further analyses. The DEGs were functionally characterized using the GO database. 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 wasused 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. Similarly, the KEGG pathways with corrected padj < 0.05 were assigned as significantly enriched pathways.

2.3. Verification of *DEGs* by *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 Bio systems, Foster City, CA, USA) in a 20L reaction volume, containing 10L 2 SYBR Premix *ExTaq* (*TaKaRa*), 0.4L ROX, 0.4 L 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 Real-time PCR Master Mix (Toyobo, Japan). The maize Actin gene (GenBank accession number: *Zm00001d010159*) was used as an internal reference control. The primers for the six selected genes were designed using the Primer Premier software (version 6.0). Three biological replicate were included in the *qRT-PCR* analysis. The generated data were analyzed according to the 2DDCT method.

2.4. 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. Results

3.1. Evolutionary Analysis of *ZmPP2C* Family Proteins in Maize

In order to identify the genes encoding PP2CA subfamily in maize, the amino acid sequences annotated as PP2CA subfamily genes of Arabidopsis were downloaded from Esemble Plants in the database. A total of 13 phosphatase-encoding *ZmPP2CA* genes were identified by the sequence alignment of maize reference genome B73 (Table 1). A total of 10 phosphatase coding *ZmPP2CA* genes were identified by the same method in rice. Phylogenetic analysis software MEGA8.0 was used to construct evolutionary tree, and it was found that PP2CA family proteins were divided into two distinct groups, indicating that PP2CA genes were much conserved in the three species (Figure 1). According to the evolutionary tree, PP2CA proteins of maize and rice are obviously separated from PP2CA proteins of Arabidopsis thaliana, suggesting that maize and rice are more closely related to each other than Arabidopsis.

Many genes in plants that respond to low temperature are regulated by ABA signaling pathways. In Arabidopsis thaliana, ABI1 gene was found to be regulated by ABA signaling pathway. Therefore, PLANT CARE was used to predict the cis-acting elements of ZmPP2CA promoter to analyze whether the promoter contains ABRE elements responding to ABA signaling. The promoter of ZmPP2CA was found to contain one or more ABRE elements (Figure 2). The results indicated that ZmPP2CA may be regulated by ABA signaling pathway.

3.2. Subcellular Localization of *ZmPP2C* Gene

According to Method 2.10, the vector was constructed, the protoplasm was prepared, and the recombinant transient expression vector was transformed with 40% PEG4000 to enter the protoplast. After 12h induction culture in the dark, the expression of *ZmPP2C*, YFP fusion protein in cells was observed by YFP excitation light. Results As shown in (Figure 3), YFP protein fluorescence was uniformly distributed in the cell, and *ZmPP2C*, YFP fusion protein fluorescence was mainly located in the cytoplasm. Results showed that the protein *ZmPP2C* mainly set in the cytoplasm.

3.3. Obtaining Arabidopsis Plants Overexpressing the *ZmPP2C* Gene

Target gene *ZmPP2C* clone, see method for the specific operation of constructing the vector containing the target gene, carry out colony PCR verification on the vector obtained for the target gene, cut the gel and send it to Liuhe Huada Gene Co., Ltd. for sequencing to ensure the correctness of the target gene. It can be seen from Figure 33 that the PCR band sizes of E (Figure 4). Coli and Agrobacterium colonies were the same as the target band, indicating that the expression vector of WMV12-ZmPP2C was constructed correctly, and the transgenic Arabidopsis was obtained later by the tidbit infection method.

3.4. Identification of Overexpressed Arabidopsis Plants at the DNA Level

Three overexpressed Arabidopsis plants were randomly selected for DNA extraction. The wild-type Arabidopsis

was used as a negative control, and water was used as a blank control. PCR amplification was performed using the 3' end of 35S-F and ZmPP2C as primers. The mplified PCR product was subjected to gel electrophoresis test, and the electrophoresis results are shown in (Figure 5). Except that the target fragment was amplified in the transgenic Arabidopsis, the blank control and negative control did not amplify the target fragment. This indicated that the *ZmPP2C* gene was successfully transferred into wild-type Arabidopsis.

Table 1. Maize PP2C family genes

Gen ID	Chr	Genome location	cDNA Size	Exons	Protein length (aa)
Zm00001d042886	3	185,093,948-185,095,922	1705	4	408
Zm00001d012401	8	175,000,529-175,002,418	1600	4	413
Zm00001d028574	1	39,067,264-39,069,360	1847	3	396
Zm00001d020100	7	93,421,327-93,422,962	1636	1	358
Zm00001d005609	2	180,274,627-180,276,009	1383	1	350
Zm00001d044015	3	218,456,535-218,462,539	1604	3	410
Zm00001d009626	8	73,966,748-73,968,804	1128	3	375
Zm00001d004357	2	106,146,770-106,150,162	1635	6	544
Zm00001d025055	10	103,573,333-103,577,996	1395	4	464
Zm00001d038846	6	173,043,461-173,046,048	1113	4	370
Zm00001d044301	3	226,455,732-226,460,323	1455	4	484
Zm00001d039163	6	178,866,492-178,868,781	1290	4	429
Zm00001d009747	8	79,569,443-79,571,945	1272	4	423

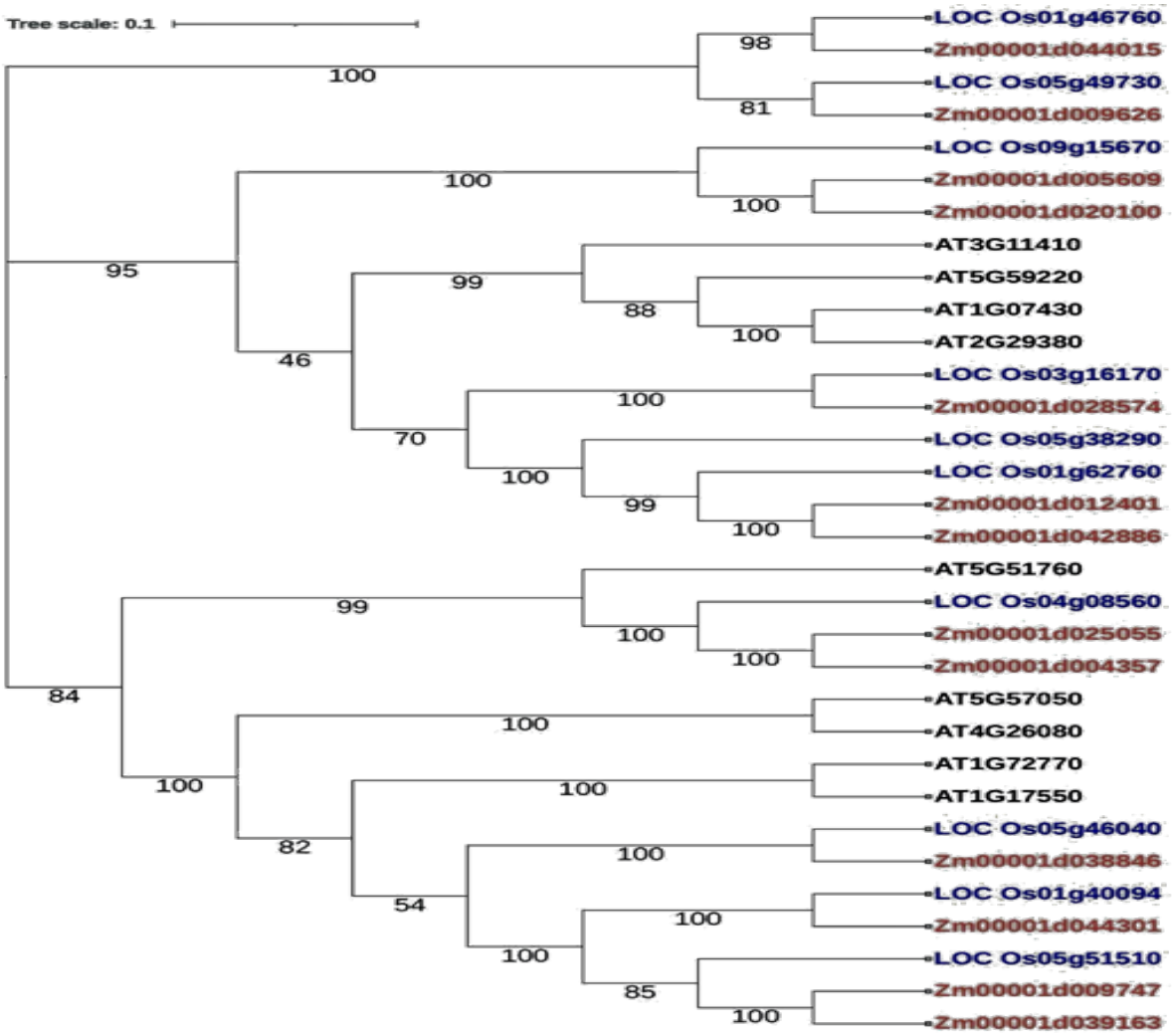

Figure 1. Evolutionary analysis of PP2C family proteins in maize, rice and Arabidopsi

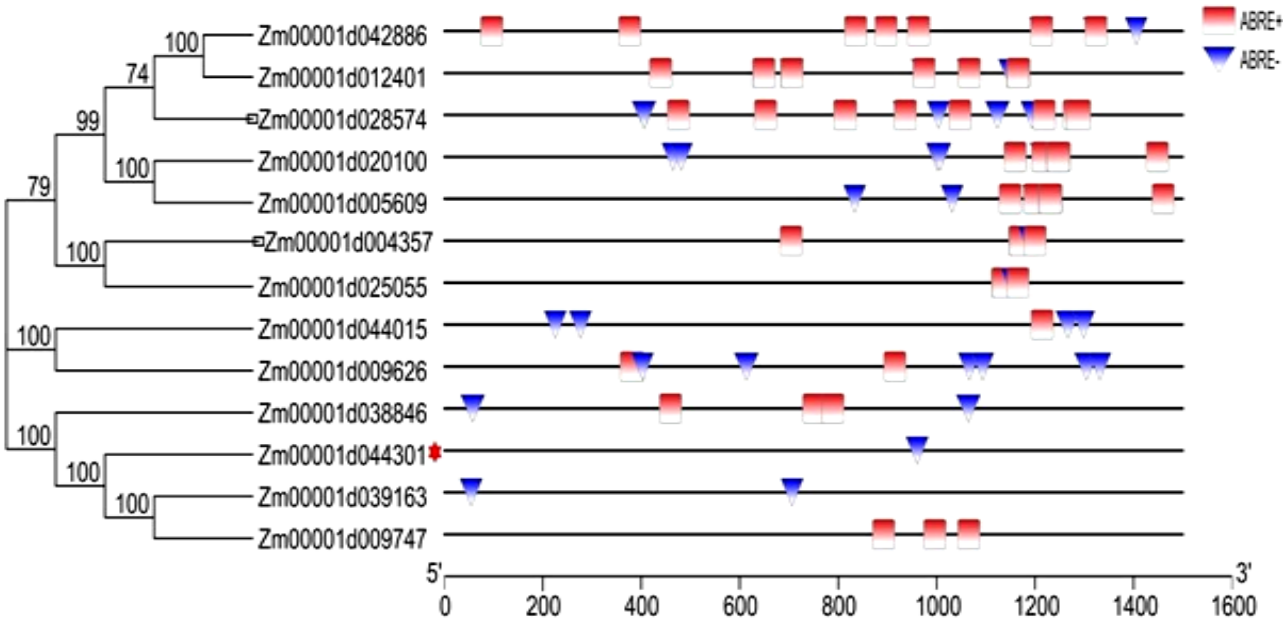

Figure 2. Phylogenetic analysis of *zm00001d044301* and other pp2ca proteins in Maize

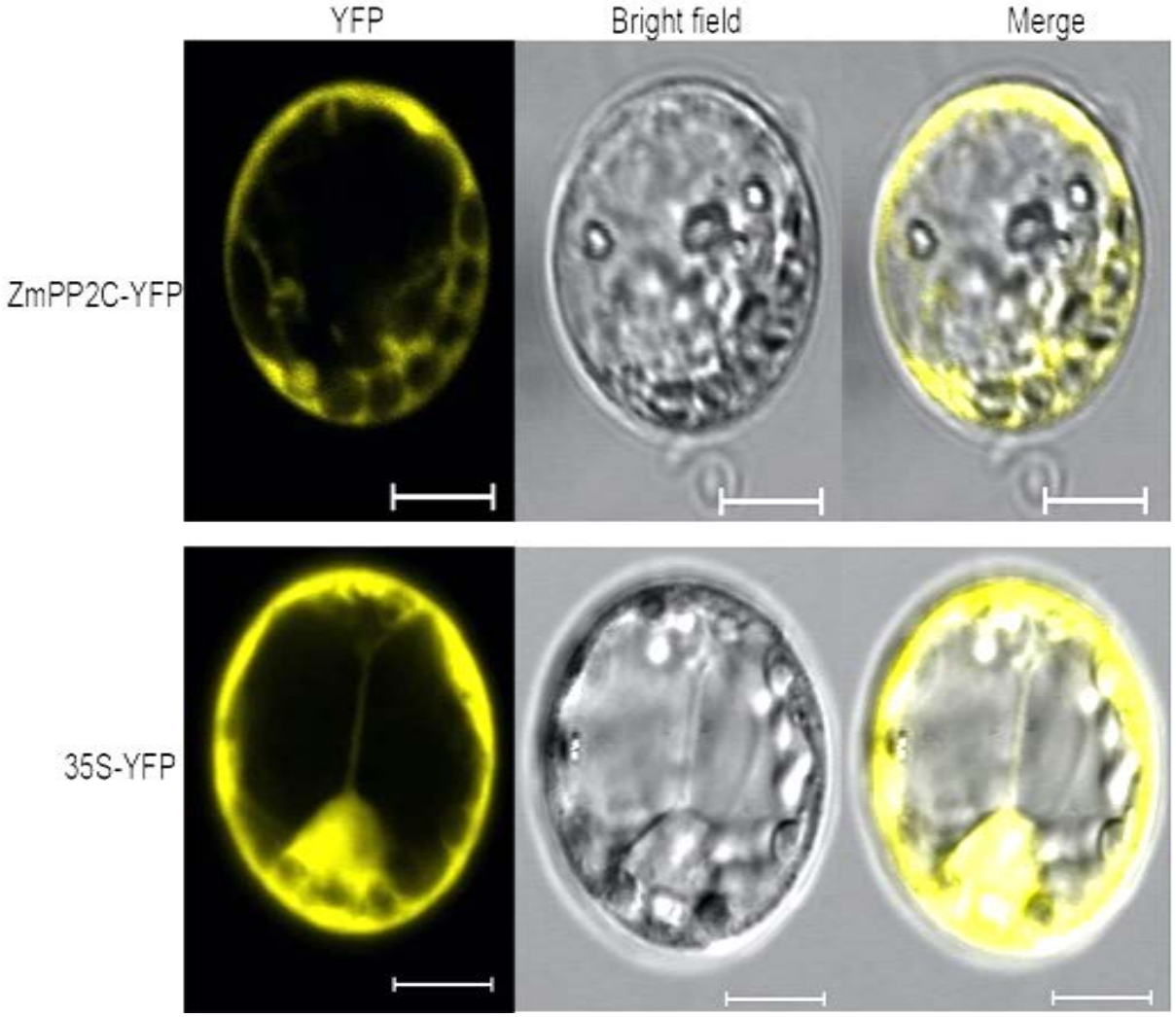

Figure 3. Subcellular localization of YFP labeled *ZmPP2C:* YFP fusion protein

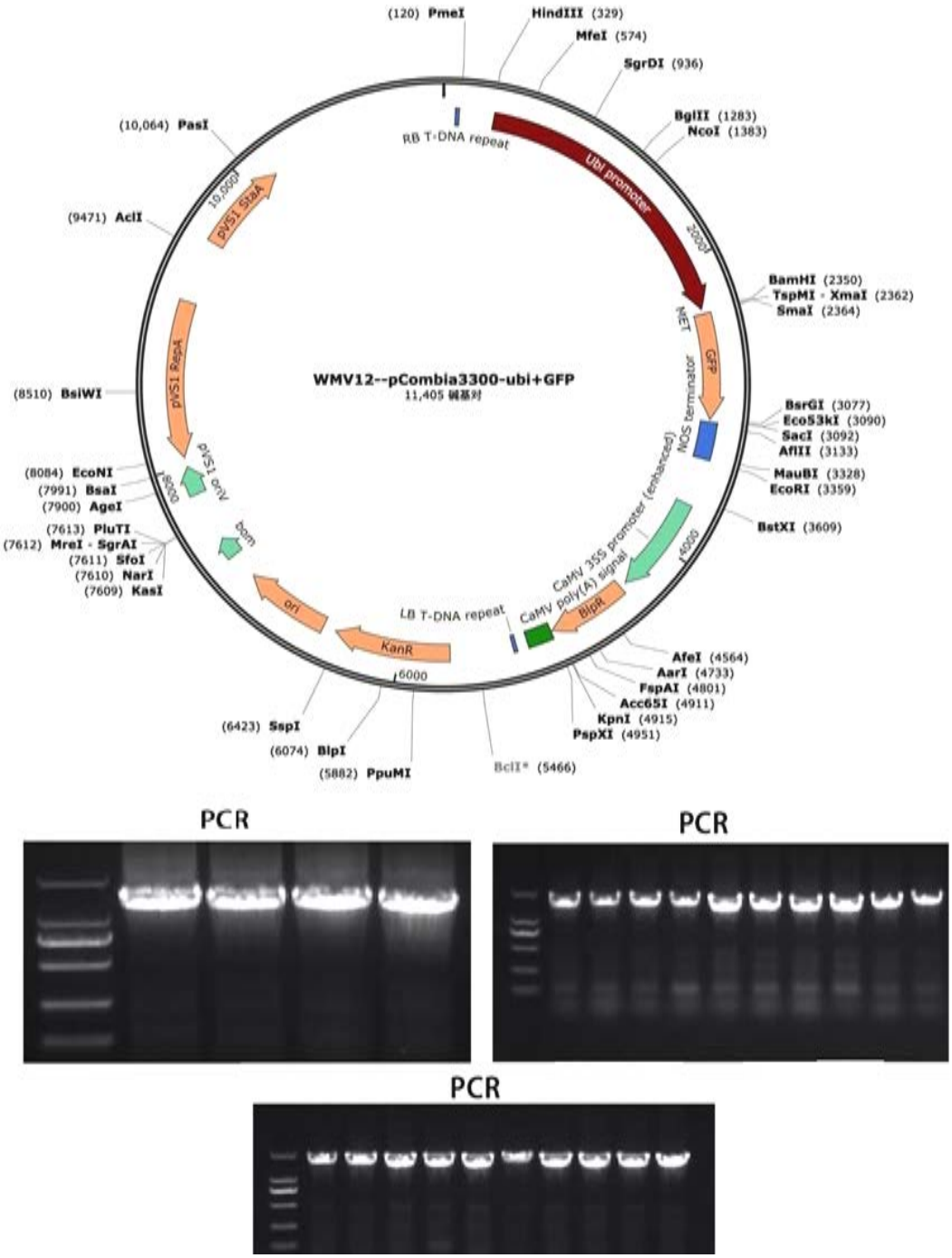

Figure 4. Colony PCR detection of *ZmPP2C* linked wmv12 expression vector

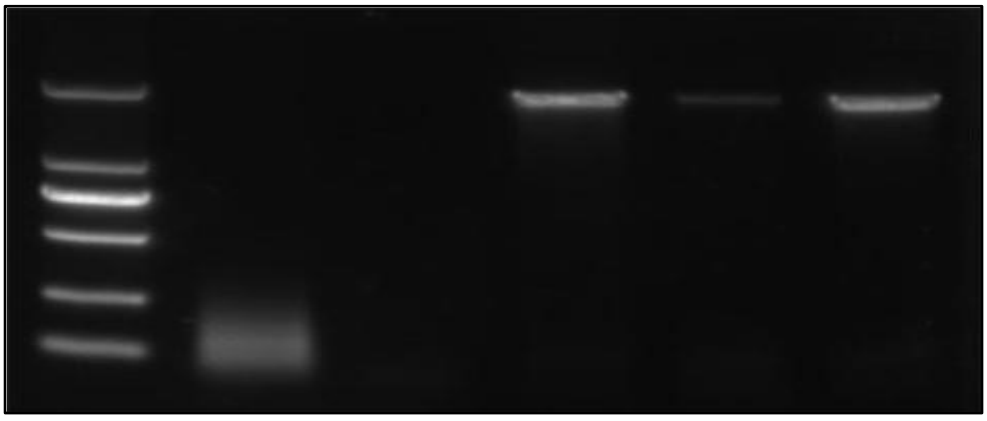

Figure 5. Detection of overexpression Arabidopsis strains at DNA level

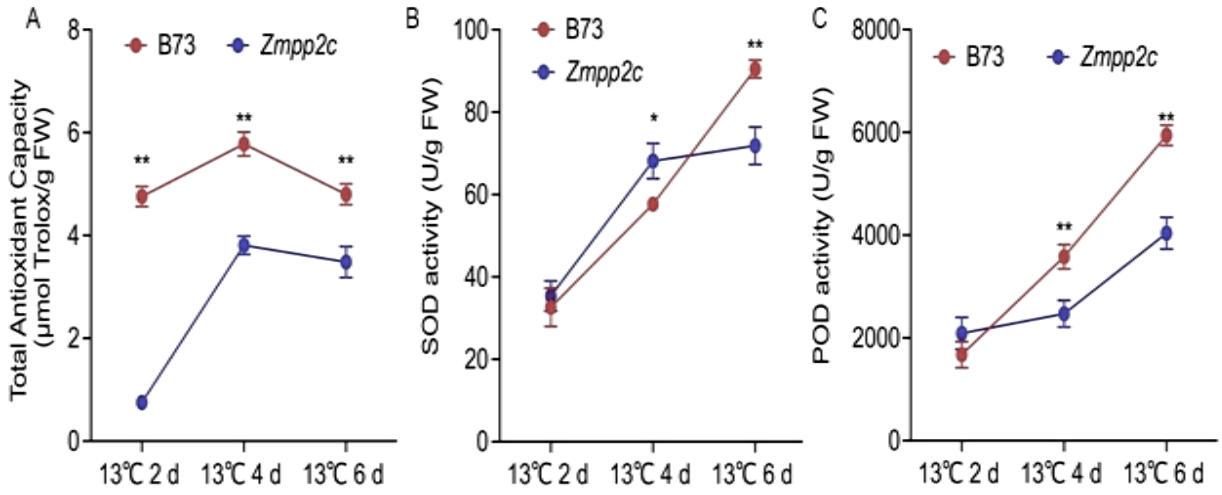

Figure 6. Antioxidant enzyme activity. (A) Total antioxidant capacity; (B) SOD activity; (C) POD activity

3.5. Antioxidant Enzyme Activity Identification of *ZmPP2C* MU Mutants Germinated at Low Temperature

In order to study the changes of antioxidant enzyme activities of ZmPP2C MU mutants germinated at low temperature, the total antioxidant capacity, antioxidant enzymes (SOD and POD) activity was measured. The results showed that the total antioxidant capacity of the two materials showed a trend of first increasing and then decreasing. The total antioxidant capacity of B73 was always significantly greater than that of the MU mutant of ZmPP2C. At the three time points of low temperature germination, 2 days, 4 days and 6 days, the total antioxidant capacity of the two materials was higher at 4 days of low temperature germination, which may be related to the radicle breaks through the seed coat. Interestingly, this is consistent with the largest difference in the expression of ZmPP2C between B73 and MU mutants after 4 days of low temperature germination (Figure 6A). In terms of SOD activity, the SOD activity of B73 germinated at low temperature for 6 days was extremely significantly higher than that of the MU mutant of Zmpp2c (Figure 6B). There was no significant difference in POD activity between the two materials after germination at low temperature for 2 days, and the POD activity of B73 was significantly higher than that of the MU mutant of Zmpp2c after 4 days (Figure 6C). This result indicated that ZmPP2C may affect the antioxidant activity of maize, and then affect the low temperature tolerance of maize.

4. Discussion

4.1. Dynamic Expression Changes of Heat Shock Protein-related Genes during Maize Germination at Low Temperature

Heat shock proteins (HSP), as a molecular chaperone, play an important role in resisting adversity. In Arabidopsis thaliana, overexpression of RcHSP17.8 increased SOD activity. Overexpression of ZmHSP16.9 in tobacco increased the activities of POD, CAT and SOD. Overexpression of GmHSP90A in Arabidopsis thaliana reduced chlorophyll loss and lipid peroxidation [25]. In this study, the changes of SOD and CAT activities and lipid peroxidation were consistent with the expression pattern of HSP gene. Protein is an essential molecular chaperone for protein homeostasis and protein complex stability under stress. Chloroplast-targeted DnaJ protein plays an important role in maintaining PSII under low temperature stress in tomato. In this study, DnaJ2 (*Zm00001d00666*) and chaperone protein DnaJ3 (*Zm00001d03669*) were only significantly down-regulated in SM-2dvs0d. Other genes encoding DnaJ protein were significantly down-regulated in both inbred lines, but RM was less down-regulated than SM. Interestingly, the gene expression pattern of PSII was similar to that of HSP. Therefore, the susceptibility of SM to early PSII injury may be caused by downregulation of HSP-related genes.

4.2. Dynamic Changes of Unsaturated Fatty Acid Related Gene Expression during Maize Germination at Low Temperature

Plant membrane is the first barrier for plants to resist the stimulation of external environment, which has typical fluidity and certain protective characteristics. Low temperature stress increased the unsaturated properties of fatty acids and the fluidity of plant membranes [26]. For example, rice mutants lacking the omega-3 fatty acid desaturase reduced membrane fluidity and increased sensitivity to low temperatur and cotton's low temperature tolerance was associated with increased desaturated fatty acids [27]. KEGG enrichment analysis showed that SM was significantly enriched in 4dvs2d fatty acid metabolism, but in general, the up-regulation range of fatty acid metabolisation-related genes in RM was greater than that in SM. Interestingly, most studies have shown that linolenic acid plays an important role in maintaining a low temperature phenotype because linoleic acid maintains membrane fluidity and chloroplast function at low temperatures [28]. In this study, the linoleic acid metabolic pathway was significantly enriched in RM for 2 to 4 days, while SM was significantly enriched at low temperature for 4 to 6 days. The metabolism of linoleic acid requires energy, while the metabolism of starch and

sucrose is significantly enriched in RM-4DvS2D, indicating that RM responds faster to low temperature to adapt to low temperature stress.

4.3. Dynamic Changes of Maize Low-temperature Eruption Ribosome-related Gene Expression

Ribosomes convert genetic information from mRNA into functional proteins. Ribosomes are essential for plant growth and environmental adaptation [23]. In this study, ribosome GO term of two maize inbred lines was significantly enriched in down-regulated genes, but the number of ribosom-related down-regulated genes in RM inbred lines was more than that in SM. After 2 to 4 days, the up-regulated genes were significantly enriched in the ribosome GO term, and the number of ribosome-related up-regulated genes in RM was higher than that in SM. We conclude that maize can be buffered by preexisting untranslated ribosomes before de novo synthesis meets the temperature-induced requirements, and the seeds begin to break through the seed coat at low temperatures for 2 to 4 days, requiring large amounts of ribosomes for protein synthesis in the absence of sufficient demand. Therefore, under low temperature stress, both SM and RM showed down-regulation of ribosome-related genes in early germination, while RM was more up-regulated than SM before radical breakthrough of seed coating. Both SM inbred lines and RM inbred lines germinated at 13°C for 4 days or at 25°C for 1.5 days, and the radicum broke through the seed coat. To verify this hypothesis, we analyzed the transcriptomes of seeds germinated at 13°C for 4 days and those germinated at 25°C for 1.5 days. Interestingly, 47 ribosome-related genes were down-regulated in SM inbred lines, while one ribosome-related gene was down-regulated and one ribosome-related gene was up-regulated in RM inbred lines. Therefore, the slow germination of SM line under low temperature stress may also be related to the downregulation of ribosome related genes.

5. Conclusion

From present study it was concluded that, cluster analysis relative decrease of root length was similar to those of comprehensive evaluation cluster analysis of 12 low temperature tolerance indexes. The relative decrease of root length ranges from 0.105 to 0.936, which conforms to the normal distribution and can distinguish the tolerance of low-temperature germination. Therefore, the relative decrease of root length was used as the evaluation index of low-temperature tolerance.

Acknowledgements

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