1. Background

During their development, crop plants are the subject of exposure to a number of biotic and abiotic stresses, such as drought, salt, freezing, viruses, and others, which inevitably affect the crop yields (1, 2, 3). For instance, one survey study showed that heat stress from 1980 to 2008 resulted in global maize and wheat production to decline by 3.8 and 5.5%, respectively (3). In order to adapt rapidly to the environmental changes, plants have evolved a large quantity of intricate and diverse measures to cope with different stresses through synthesizing and assembling adaptive molecules (4).

As one of the major abiotic stresses, dehydration can induce plants to express a large number of genes which can be divided into two categories based on the induction time: RD (responsive to dehydration) and ERD (early responsive to dehydration) genes (5). ERD genes rapidly respond to the dehydration and other abiotic stresses (6, 7). So far, ERD genes (1-16) had been cloned from 1 hour dehydrated Arabidopsis thaliana and their functions have...
almost been known. For example, ERD1 was revealed to encode an ATP-dependent chloroplast protease (8), ERD2, and ERD8 were reported to produce heat shock proteins (HSPs) (9, 10), but the function of ERD3 gene has been unknown yet.

2. Objectives
The aims of this study were: (1) to clone ZmERD3 gene and its promoter from Zea mays, and to analyze their bioinformatics properties by online software, and (2) to analyze the tissue-specific expression pattern of ZmERD3 gene and its response to the abiotic stresses in maize. In short, by doing the above-mentioned work, we hope to get enough information about the potential function of ZmERD3 gene and to provide a theoretical basis for the breeding of the resistant maize varieties.

3. Materials and Methods

3.1. Plant Materials and Growth Conditions
A drought-tolerant maize inbred line “Huangzao 4” was used in our experiments. The seeds were germinated and were grown to the 3-leaf stage on composite soil (soil: vermiculite: organic fertilizer with the ratio of 3:2:1, respectively) under the condition of a 14-h light (200 μE·m\(^{-2}\)·s\(^{-1}\)) /10-h dark cycle at 25 °C in an incubator.

3.2. Total RNA Extraction and ZmERD3 Clone
Total RNA was extracted from the entire plants using total RNA Isolation Kit (Invitrogen, USA), and RNA integrity was checked on 1% Agarose gel. The extracted RNA served as the template to synthesize the first cDNA strand using FastQuant RT Kit (Tiangen Biotech, China).

Based on the homology alignment with AtERD3 (XP_002867953) in MaizeGDB (http://www.maizegdb.org/), ZmERD3 cDNA sequence was obtained and its specific primers were designed. F: 5′-TCGACGACGGGAGAATA-3′, R: 5′-CCCACTCCATTGTTGCCATACTGAGCC-3′. ZmERD3 PCR was performed in a 25 μL reaction system including 12.5 μL 2× Taq PCR Master Mix (Tiangen Biotech, China), 1 μL cDNA template, 1 μL 10 μM forward primer, 1 μL 10 μM reverse primer, and 9.5 μL ddH\(_2\)O. The amplification was performed according to the following program: pre-denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C degeneration for 40 s, 55 °C annealing for 30 s and 72 °C extension for 2 min; at last 72 °C extension for 10 min. The PCR fragments were cloned into the pMD-19T vectors (Takara, Japan) and then sequenced (BGI, Beijing, China).

3.3. Bioinformatics Analysis of ZmERD3
The homology alignment with AtERD3 and the prediction of functional domains were respectively conducted by DNAman software and Blast tool of NCBI. The basic properties of ZmERD3 protein were predicted using the ExPASy ProtParam tool (http://us.expasy.org/tools/protparam.html). The subcellular localization was predicted by ProtComp 9.0 (http://www.softberry.com/beryl.plhtml), WoLF PSORT (http://www.genscript.com/wolf-psort.html), YLoc (http://www.multiloc.org/YLoc), and TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP). The prediction of the signal peptide was carried out using signalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The 3D structural model was constructed using SWISS-MODEL (http://swiss model.expasy.org/interactive). The phylogenetic tree was generated in the MEGA4.0 software using the Neighbor-Joining method.

3.4. Cloning and Analysis of the ZmERD3 Promoter
The promoter sequence, located at about 1.9 kb upstream of the ZmERD3 gene, was obtained by sequence alignment in the MaizeGDB database. A pair of specific primers for the promoter amplification was designed including pro-F: 5′-TTATGGTTGCCATACTGAGCC-3′, and pro-R: 5′-AGCGGAAACGGAGAGTAGAAG-3′. The amplification reaction was carried out in the 25 μL reaction system as above. DNA was extracted from maize plant using DNA Extraction Kit (Tiangen Biotech, China) and was taken as the template for promoter cloning. PCR parameter was set as follows: one cycle at 94 °C for 5 min, 35 cycles of amplification at 94 °C for 40 s, 56 °C for 40 s and 72 °C for 2 min, and one cycle at 72 °C for 10 min lastly. The PCR products were purified using DNA Recovery Kit (Takara, Japan) and then were ligated into pMD-19T vectors for sequencing (BGI, Beijing, China). The online software, PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), was used to analyze its cis-regulatory elements.

3.5. Tissue-Specific Expression Analysis of the ZmERD3 Gene
In order to analyze the tissue-specific expression of ZmERD3 gene, maize plant organs (leaf, stem, root, filament, and grain) were sampled at 3 different developmental stages: trefoil, jointing, and heading stage. The total RNA was respectively extracted from these tissues mentioned as above and then was converted into cDNA which was used as a template for the fluorescent qRT-PCR analysis. The primers for qRT-PCR were as follows:
3.6. Expression Analysis of the ZmERD3 in Response to Abiotic Stresses

The expression patterns of ZmERD3 gene responsive to 4 kinds of abiotic stress treatments (PEG-6000, NaCl, ABA, and low temperature) were also analyzed by qRT-PCR technique. Before imposing the treatments, all plants were grown under the same conditions. Maize seedlings of the three-leaf stage were exposed to the different stress factors, respectively: drought (20% PEG-6000), salinity (250 mM NaCl), low temperature (4 ℃), and ABA (100 μmoL.L\(^{-1}\)) for 0, 1, 3, 6, 12, and 24 h. At every time point, seedlings were harvested and immediately were frozen in the liquid nitrogen. Total RNA was extracted from these samples, and then first cDNA strands was synthesized. The qRT-PCR primers, the reaction system, and procedure were the same as that of “Tissue-specific expression analysis”.

3.7. Data Analysis

The statistical data were analyzed using SPSS 17.0 software. The data were analyzed by one-way ANOVA and expressed as mean ± SD. Values among groups were considered different at \(P < 0.05\).

4. Results

4.1. Cloning and Sequence Analysis of ZmERD3 Gene

MaizeGDB database search together with bioinformatics analysis resulted in the identification of ZmERD3 gene in Zea mays (accession number: KU360141). The ZmERD3 cDNA sequence cloned by PCR contains 2116 bp (Fig. 1) and has an 1833-bp ORF encoding a peptide of 610 amino acids. The basic properties of the ZmERD3 protein was known by the prediction using ExPASy ProtParam tool, and its molecular weight, isoelectric point (pI), and molecular formula are respectively about 67.82 kDa, 9.2, and C3039H4705N843O857S33. Ala, Leu, Gly, and Pro are rich in ZmERD3 protein and the predicted peptide contains 57 negatively charged residues (Asp + Glu), and 77 positively charged residues (Arg + Lys).

The subcellular location prediction was conducted using several online tools, and the prediction results, however, were inconsistent with each other. ProtComp 9.0 has predicted that ZmERD3 protein may be located in the Golgi complex, YLoc has predicted it in the cytoplasm with a probability of 56.2%, WoLFPSORT has predicted its localization in the ER, and the TargetP has predicted it in the others except for mitochondria and chloroplast, correspondingly. It was predicted that ZmERD3 protein has no signal peptide by SignalP 4.0 Server. To sum up, ZmERD3 protein has a high probability of location in the cytoplasm. The 3D structure prediction of the ZmERD3 protein is shown in Figure 2; its SWISS-MODEL template is SMTL (Id: 3grz.1) whose name is ribosomal protein L11 methylase from Lactobacillus delbrueckii subsp. Bulgaricus.

The prediction of the conserved domain exhibited...
that ZmERD3 protein has one conserved functional domain of S-adenosyl methionine (SAM- or Adomet-) methyltransferase, which is located between amino acids of 216<sup>th</sup>-308<sup>th</sup> (Fig. 3). The sequence homology alignment using DNAmel software showed that there is 67% homology between ZmERD3 and AtERD3 protein (Fig. 4) and their highest homology region covers the SAM- methyltransferase domain.

The BLAST analysis showed that ZmERD3 peptide shared more than 80% homology with that of *Setaria italica* (XP_014661336.1) and *Oryza brachyantha* (XP_006662118). For further analysis, Clustal X and MEGA 4.0 software were applied to align multiple sequences and construct a neighbor-joining phylogenetic tree (Fig. 5). The phylogenetic tree contains two big branches with PMT (putative methyltransferase) from other plants. Among them, ZmERD3 protein was clustered together with *Setaria italica* (XP_014661336), *Brachypodium distachyon* (XP_003573760), and *Oryza brachyantha* (XP_006662118).

Figure 3. Conserved domain of ZmERD3 protein. The Blast result showed that ZmERD3 protein has one conserved functional domain of SAM-methyltransferase which is located between amino acids 216<sup>th</sup>-308<sup>th</sup>.

Figure 4. Homology alignment between ZmERD3 and AtERD3 proteins. Deep blue line indicates the homologous sequences, and the highest homology between these two sequences mainly exists between amino acids of 95<sup>th</sup>-322<sup>th</sup> which covers the domain of SAM-methyltransferase.

Figure 5. Phylogenetic analysis of ZmERD3 protein with other putative methyltransferases. Bootstrap values indicate the separation between adjacent branches and the scale bar represents 0.2 substitutions per site. ZmERD3 protein has closest relationship with PMT from *Setaria italica* as relationship coefficient is about 99%.
4.2. Cloning and Analysis of the ZmERD3 Promoter

The promoter sequence containing 1877 bp from maize genomic DNA was cloned (Fig. 6) and its cis-regulatory elements were predicted by PlantCARE online software. The prediction result showed besides some important core elements (such as TATA-box and CAAT-box), there are also many cis-regulatory elements responsive to the light, heat, cold, dehydration, gibberellin, ethylene, and others (Table 1). Thus, it was predicted that ZmEDR3 gene may be involved in the multiple responsive pathways to abiotic stresses.

4.3. Tissue-Specific Expression of ZmERD3

ZmERD3 gene expression in maize plant organs (root, stem, young leaf, old leaf, filament, and grain) was analyzed by qRT-PCR technique (Fig. 7). The results showed that the expression level of ZmERD3 gene in leaves was lowest at 3 development stages compared to that in other organs. The abundance of ZmERD3 mRNA in roots became higher and higher as the development process went ahead and the amount of mRNA in filaments was highest; about 38 folds higher than that in leaves at heading stage.

4.4. ZmERD3 Expression Responsive to Abiotic Stresses

The regulation of ZmERD3 gene at transcriptional level was studied in order to dissect its induction in response to abiotic stresses in maize plants (Fig. 8). ZmERD3 expression was notably induced upon drought simulation by the PEG-6000 treatment, and the relative expression level reached its peak after exposure to 20% PEG-6000 for 3 h. Similar to PEG-6000, NaCl could also up-regulate ZmERD3 expression ability rapidly. As shown in Figure 8, treatment with 250 mM NaCl for 6 h led the ZmERD3 mRNA amount to increase about 20 folds more than that of the control; however, its expression then quickly began to decrease along with the extension of NaCl treatment time. The response of ZmERD3 expression to low-temperature stress was not as significant as that of PEG-6000 and NaCl treatments, and the highest increased rate was less than 4 times. In addition, the ZmERD3 mRNA accumulation rapidly increased during the first 3 h of ABA treatment; thereafter, its transcript level gradually decreased but still remained higher compared to the control group until 24 h.

Table 1. Predicting cis-acting elements of the ZmERD3 promoter from maize.

| Regulatory elements | Core sequence | Function                           |
|---------------------|---------------|------------------------------------|
| TATA-box            | TAATA         | Core promoter                      |
| CAAT-box            | CAAT          | Enhancer element                   |
| A-box               | CCGTCC        | Cis-acting regulatory element      |
| CCGTCC-box          | CCGTCC        | Element related to meristem specific activation |
| CGTCA-motif         | CGTCA         | Element involved in the meja-responsiveness |
| G-Box               | CACGTT        | Light responsive element           |
| GARE-motif          | AAACAGA       | Gibberellin-responsive element     |
| MBS                 | CAACGTG       | MYB binding site involved in drought-inducibility |
| HSE                 | AAAAAATTTCC   | Heat stress responsive element     |
| LTR                 | CCGAAA        | Low-temperature responsive element |
| TC-rich repeats     | ATTCTCTAAC    | Involved in defense and stress responsive element |
| circadian           | CAANNNNATC    | Element involved in circadian control |
| ERE                 | ATTTCAAA      | Ethylene-responsive element        |
5. Discussion

A variety of abiotic stresses, including high temperature, drought, high salinity and so on are the major threats to the plant development and yield. In order to improve plant productivity, many researchers have attempted to develop varieties resistant to several specific stresses (11, 12, 13). For example, the GmIMT1 gene (11) encoding methyltransferase in soybean was demonstrated to be involved in the multiple plant response pathways and GmIMT1 transgenic plants exhibited higher salt-tolerance compared to the wild-type plants.

In this study, we cloned and characterized the ZmERD3 cDNA which contains 1833-bp ORF encoding a polypeptide of 610 amino acids. The Blast result using online software NCBI CDS showed that the ZmERD3 peptide has a typical SAM-dependent methyltransferase
domain, and the subcellular location prediction exhibited that ZmERD3 protein has a high possibility of location in the cytoplasm, so it should be one non-genetic material methyltransferase. Methyltransferases can regulate a dynamic network of cellular signaling events and are required to keep intracellular homeostasis in face of external perturbations by catalyzing methylation reaction for production of physiologically active substances (such as glycinebetaine) (14, 15, 16, 17, 18). Meanwhile, these methylation products benefit crops to maintain a higher cytoplasmic osmotic pressure or others under salt/drought stress (19, 20, 21). For instance, Caffeoyl-CoA O-methyltransferase plays an important role in lignin biosynthesis, which can enhance the mechanical strength of the vascular bundle and facilitate plants to transport water and to resist malignant stresses (22).

Expression analysis using qRT-PCR showed ZmERD3 gene was expressed in all checked organs (root, stem, leaf, filament, and grain), but its expression level was different. Moreover, the abundance of ZmERD3 mRNA in the same organs at different development stage was different. These results provide a good evidence for the tissue-specificity and development process of ZmERD3 expression in the maize plants, similar to other methyltransferases (23, 24). Meanwhile, ZmERD3 expression patterns in response to different abiotic stresses were analyzed at the transcript level, and the results showed that its expression was up-regulated upon all indicated stress treatments. In comparison to the control plants, 3-6 h of treatments with 20% PEG-6000 and 250 mM NaCl rapidly induced ZmERD3 expression in maize plants to increase about 20 times. As one of abiotic stress signal substance, ABA could also enhance the expressivity of ZmERD3 gene and 3 h of ABA treatment made the mRNA level reach its peak. In short, consistent with the methyltransferases from other species (25, 26, 27, 28), ZmERD3 expression can be induced by NaCl, PEG-6000, and ABA. However, the effect of low temperature on ZmERD3 expression was less compared to other treatments.

In conclusion, ZmERD3 gene and its promoter were cloned from Zea mays in this study. ZmERD3 gene is highly orthologous to the AtERD3 (about 67%) from A. thaliana, and its ORF contains 1833 bp. It was predicted that ZmERD3 protein contains SAM-dependent methyltransferase domain. Its promoter was predicted to own many cis-regulatory elements responsive to dehydration and other stress factors. The qRT-PCR results showed ZmEDR3 expression has the tissue-specificity and depends on the development process. Moreover, ZmERD3 gene might be involved in multiple response pathways to the different abiotic stresses. In a word, these research results are hoped to provide a good foundation for further research investigation on the functions and biochemical characteristics of ZmERD3 gene.

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