Isolation and expression studies of the \textit{ERD15} gene involved in drought-stressed responses

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\textbf{ABSTRACT}. The early response to the dehydration 15 (\textit{ERD15}) gene is widely involved in the processes of signal transduction, programmed cell death, gene transcription, and stress tolerance in plants. In a previous study, the \textit{ERD15} gene was shown to be an important regulator of the abscisic acid response and salicylic acid-dependent defense pathway, acting as an important negative regulator of abscisic acid. The complete \textit{IbERD15} gene (accession No. KF723428) was isolated by reverse transcription-polymerase chain reaction. The \textit{IbERD15} gene contains an open reading frame of 504 bp, encodes a peptide of 167 amino acids, and has a molecular mass of 18.725 kDa. The transcript levels of the \textit{IbERD15} gene in a variety of tissues were examined by digital gene expression profiling. The roots of the sweet potato were treated by 3 degrees of polyethylene glycol, and the results indicate that the \textit{IbERD15} gene might play an important role in the defense response to drought.
stress. Moreover, the *IbERD15* gene was successfully transformed into yeast cells for analysis of drought tolerance in transgenic yeast.

**Key words:** *ERD15* gene; Sweet potato; Quantitative analysis; Digital gene expression profiling; Yeast transformation

**INTRODUCTION**

The sweet potato [*Ipomoea batatas* (L.) Lam.] is the fourth most important crop in China after wheat, rice, and maize. In China, the planting area of the sweet potato is steadily growing, and its total production and acreage make up 70-85% of the world’s totals (Zhang et al., 2009). Sweet potato is not only used as food or feed, but also serves as a very important feedstock in industrial applications. At present, the world is facing an increasingly serious energy crisis, and there is an urgent demand for new energy. Due to its rich starch content, the sweet potato shows great promise for use in industrial ethanol production.

Plant rootage, growth, and productivity are influenced heavily by biotic and abiotic stresses such as drought, salinity, low temperature, pests, and germs. To adapt to their surroundings, plants have developed a series of complex mechanisms to minimize the stress-induced damages. Abscisic acid (ABA), a sesquiterpenoid, plays an important role in plant germination, development, maturation, and more (Finkelstein and Gibson, 2002; Xiong et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). It accumulates in response to different stresses and helps plants to acclimate to changes in their environment. There are many genes that are characterized as positive or negative regulators of ABA; one of them has been termed the early response to dehydration (ERD) protein (Ziaf et al., 2011). Expression levels of *ERD* genes increase in response to drought, low temperature, high salt content, and ABA, which leads to a rapid and effective response mechanism (Taji et al., 1999). *ERD* genes have a variety of functions. For example, *ERD1* prevents injury to the chloroplast membrane in *Arabidopsis*, but the *ERD15* gene in the soybean acts as a transcription factor, which regulates gene transcription related to programmed cell death (Steponkus et al., 1998; Alves et al., 2011).

*ERD15* was originally characterized as a rapid drought-responsive gene (Kiyosue et al., 1994c) in *Arabidopsis*; recently, it has been reported as a negative regulator of ABA that can also be induced by salicylic acid (SA), wounding, and pathogenic infection to mediate cross-talk between abiotic and biotic stress responses (Kariola et al., 2006; Ziaf et al., 2011). As a transcription factor, *ERD15* binds and activates expression of the endoplasmic reticulum stress- and osmotic stress-induced NRP-B promoter to transduce a stress-induced cell death signal (Alves et al., 2011). As has been shown, the *ERD15* gene has many important functions in plants; however, the *ERD15* gene in the sweet potato has not been cloned or studied, to date. For this purpose, we report the cloning and characterization of the sweet potato *ERD15* gene.

**MATERIAL AND METHODS**

**Strains and materials**

The sweet potato cultivar Xushu 18 was used in this research, which was obtained from the Sichuan Academy of Agricultural Sciences. Various tissues such as young leaves, mature leaves, and stems were sampled, washed, and frozen immediately in liquid nitrogen for
temporary storage. For the drought treatment, 2-month-old Xushu 18 was irrigated with different concentrations (i.e., 5, 10, and 20 mM) of polyethylene glycol (PEG) solution once every 2 days for 20 days. Samples from expanding tuberous roots were collected on days 3, 10, and 20 and immediately frozen in liquid nitrogen. pET32a(+) was used for constructing the prokaryotic duplication and expression vector. *Escherichia coli* strains of JM109 and BL21(DE3) were used for routine cloning and recombinant expression, respectively. *Saccharomyces cerevisiae* strain INVSC1 (genotype, MATα-a his3±1 leu2 trp1-289, ura3-52; Invitrogen, USA) was used for yeast transformation. The yeast plasmid, pYES2.0 (Invitrogen), with the inducible GAL1 promoter, was used for the protein expression vector.

**RNA extraction and first-strand cDNA synthesis**

The methods of RNA extraction and complementary DNA (cDNA) synthesis were similar to previously reported methods (Shao et al., 2011; Cao et al., 2012). Total RNA of the samples was isolated from the sweet potato using the TRIzol Reagent (Invitrogen). The RNA sample was subjected to DNaseI digestion (Fermentas, USA). All RNA samples were stored at -80°C until use. First-strand cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Invitrogen). Reverse transcription (RT) was performed using the primer oligo(dT)15.

**Gene isolation**

To verify the contig 3490, which contains annotation information for *ERD15*, a pair of primers was designed based on the upstream and downstream sequences of *ERD15* (i.e., 3490F: 5’-TGTTTCTCTTGGCGATTACAG-3’ and 3490R: 5’-GCAGACATAAAGCGACTCC-3’). Then, the complete *ERD15* gene sequence was amplified by polymerase chain reaction (PCR) with the forward primer ERD15-F (5’-GGATCCATGGCCTAGTTTCTGGAA-3’; the underlined sequence is the restriction endonuclease site BamHI) and reverse primer ERD15-R (5’-GAATTCATGGCCTAGTTTCTGGAA-3’; the underlined sequence is the restriction endonuclease site EcoRI) after obtaining the sequencing results from the first PCR. All above primers were designed based on the related *ERD15* gene from other plants and the sweet potato transcriptome database (http://cfgbi.scu.edu.cn/). KOD-FX-Neo (TOYOBO, Japan) was used for PCR, and the PCRs were subjected to one cycle of 94°C for 2 min; 30 cycles of 98°C for 10 s, 53°C for 2 min, and 72°C for 30 s; and one cycle of 68°C for 10 min. PCR products of a single objective band were purified and cloned into the pET32a(+) vector for sequencing. Finally, the ligation product was directly transformed into *E. coli* JM109 competent cells. The resulting recombinants were verified by PCR, single digestion, and double digestion.

**Bioinformatic analysis of sequence data**

Protein motif and protein domain predictions of the deduced amino acid sequence were performed using Pfam (http://pfam.sanger.ac.uk/search). The National Center for Biotechnology Information (NCBI) server and DNAMAN were used for protein similarity analyses. A protein property analysis was performed by using a variety of tools, such as ProtParam, Superfamily, SignalP 4.1, TMpred, NetPhosK 1.0 Server, and SWISS-MODEL. Phylogenetic analysis and neighbor-joining tree construction were completed using MEGA4 (Tamura et al., 2007).
Phylogenetic tree and alignment

A BLAST of the NCBI server was used to search similar nucleotide and amino acid sequences to the IbERD15 protein. The other ERD genes from 14 plant species were chosen for protein sequence alignment and phylogenetic tree construction. The 14 ERD15 homologous sequences registered in GenBank were ERD15 (XP_004236564.1) from Solanum lycopersicum, ERD1 protein (ADE74634.1) from Nicotiana tabacum, ERD15 (ABB89735.1) from Capsicum annum, ERD15 (AFX66978.1) from Solanum tuberosum, ERD15 (XP_004303625.1) from Fragaria vesca subsp. vesca, ERD15 (ADP37978.1) from Brassica napus, ERD15 (XP_004513370.1) from Cicer arietinum, ERD15 (ABB89735.1) from Capsicum annum, ERD15 (AFX66978.1) from Solanum tuberosum, ERD15 (XP_004303625.1) from Fragaria vesca subsp. vesca, ERD15 (ADP37978.1) from Brassica napus, ERD15 (XP_004513370.1) from Cicer arietinum, ERD15 (ABB89735.1) from Capsicum annum, ERD15 (AFX66978.1) from Solanum tuberosum, ERD15 (XP_004303625.1) from Fragaria vesca subsp. vesca, ERD15 (ADP37978.1) from Brassica napus, ERD15 (XP_004513370.1) from Cicer arietinum, ERD15 (ABB89735.1) from Capsicum annum, ERD15 (AFX66978.1) from Solanum tuberosum, ERD15 (XP_004303625.1) from Fragaria vesca subsp. vesca, ERD15 (ADP37978.1) from Brassica napus, ERD15 (XP_004513370.1) from Cicer arietinum, ERD15 (ABB89735.1) from Capsicum annum, ERD15 (AFX66978.1) from Solanum tuberosum, ERD15 (XP_004303625.1) from Fragaria vesca subsp. vesca, ERD15 (ADP37978.1) from Brassica napus, ERD15 (XP_004513370.1) from Cicer arietinum, ERD15 (ABB89735.1) from Capsicum annum, ERD15 (AFX66978.1) from Solanum tuberosum, ERD15 (XP_004303625.1) from Fragaria vesca subsp. vesca, ERD15 (ADP37978.1) from Brassica napus, ERD15 (XP_004513370.1) from Cicer arietinum, ERD15 (ABB89735.1) from Capsicum annum, ERD15 (AFX66978.1) from Solanum tuberosum.

The phylogenetic tree was generated from the deduced amino acid sequences for ERD15 homologues from other species using the MEGA 5.0 software with 1000 bootstrapping iterations.

Digital gene expression (DGE) profiling

The raw 21-bp DGE tags that were generated according to the Illumina pipeline were obtained from 7 different tissues, including mature leaves, young leaves, stems, fibrous roots, expanding tuberous roots, initial tuberous roots, and harvested tuberous roots. Bowtie was used for mapping clean tags to the ERD15 gene at Galaxy’s web platform allowing only one base mismatch. The edgeR package was used to normalize the different sequencing library sizes and expression levels by estimating the bias introduced by RNA composition. Via normalizing analysis, the expression of ERD15 in different tissues can be described accurately (Shao et al., 2011; Tao et al., 2012).

Expression patterns of IbERD15 in response to drought treatment

Total RNAs from control (untreated) and treated samples under drought stress obtained from the tuberous roots of the sweet potato were reverse transcribed. Semi-quantitative RT-PCR was performed to evaluate the expression profiles of ERD15 in the sweet potato by drought stress based on 2X EasyTaq PCR SuperMix (TransGen Biotech, China). The housekeeping reference gene (actin) was employed to normalize the PCR efficiency of the samples. The primers contained the following sequences: ERD15F (5’-AGGCTGGATTTGGTGATGATG-3’), ERD15R (5’-CAAGGGATTTTGGACCTCTCTC-3’); the primers for the reference gene were actin-F (5’-GGTGTTATGTTGGATGATG-3’) and actin-R (5’-CGGTAAGAGGACAGGGTG-3’).

Construction of the yeast expression vector and transformation

The IbERD15 gene was amplified by the above PCR method, and the amplified product and pYES2 were digested with BamHI plus EcoRI, then they were ligated to construct the expression vector, pYES2-ERD15. The recombinant was verified by BamHI digestion and
PCR. pYES2-ERD15 and pYES2 (empty vector) were transferred to *E. coli* JM109 for amplification. The pYES2-ERD15 and pYES2 (empty vector) were transformed into *S. cerevisiae* INVSc1 by means of the lithium acetate method following manufacturer instructions (Li et al., 2012). The selected yeast clones were grown at 30°C in SD-URA medium without uracil. The yeast genome was extracted as a template, and PCR was used to detect whether the *ERD15* gene was integrated into the genome.

### Analysis of the tolerance to drought stress in transgenic yeast

A single colony of the control transformant (empty vector) and identified transgenic transformant were inoculated into 3 mL SD-URA liquid medium and grown at 30°C for 24 h. The above culture was inoculated into 20 mL medium, for a second time, and then grown until the A600 reached 0.4. Then, cultures were induced for 30 h. The concentrations of the 2 types of cultures were determined, and they were diluted to the same concentration (i.e., OD<sub>600</sub> up to 0.2). The cells from 1 mL of each culture were precipitated by centrifugation and suspended with 1 mL 8 M Sorbitol solution at 4°C for 24 h. The treated solutions were diluted 1000-fold, and 50 μL liquid was distributed to the SD-URA solid medium at 30°C for 48 h (Deng et al., 2010; Li et al., 2012). To compare survival rates, photographs of bacteria in the solid medium were taken and survival rate of bacteria was counted.

### RESULTS

#### Isolation of the *ERD15* gene

The recombinant plasmid that contained the *ERD15* gene was confirmed by BamHI digestion and PCR. Verified by sequencing, the *ERD15* gene of the sweet potato had a coding sequence of 504 bp and encoded a protein of 167 amino acids. The ERD15 protein is a small and acidic protein that plays a key role as a negative regulator in the ABA response. The predicted molecular weight of the IbERD15 protein was 18.725 kDa, and this gene was named *IbERD15*. The sequence of *IbERD15* has been deposited in GenBank (accession No. KF723428). Sequencing of 10 clones revealed the presence of one cDNA, indicating that there might be no other isoforms present in the sweet potato.

#### Bioinformatic analysis of ERD15

The data showed that the IbERD15 protein had an aliphatic index of 62.72, and a grand average hydrophaticity (GRAVY) of -0.699, proving that it is a hydrophilic protein. In addition, the NetPhosK analysis indicated that the IbERD15 protein may possess 13 phosphorylation sites (i.e., 10 serine, 1 threonine, and 2 tyrosine predictions). Therefore, it implies that ERD15 may serve as a substrate for some kinases involved in signal transduction or protein regulation. No signal peptide sequence or transmembrane regions were found in IbERD15, suggesting that IbERD15 is an intracellular protein that could not be transported to cytosol or the cytomembrane. In the second structure assay, the IbERD15 peptide largely included random coils (114 amino acids, 68.26%) and some α helices (47 amino acids, 28.14%), indicating a relatively incompact structure. Protein domain prediction revealed that IbERD15 contained a predicted domain structure with PAM2 (6-23) in the N-terminus, which is consistent with the
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ERD15 genes from the soybean, Arabidopsis, and more (Kiyosue et al., 1994c; Albrecht and Lengauer 2004; Alves and Fietto, 2013).

Phylogenetic tree and alignment analysis

Using the online sequence alignment tool BLASTN and the BLASTP program to search the NCBI database, the IbERD15 gene shared a high similarity in nucleotide and amino acid sequences with those of other ERD15 genes from other plants. Multiple-amino acid sequence alignments of the ERD15 peptides were collected from the sweet potato (accession No. KF723428) and other species by DNAMAN and MEGA. The amino acid sequences of the ERD15 genes from the sweet potato, S. lycopersicum, and C. annuum showed 59-63% similarity. To research the possible role of the ERD15 gene, a phylogenetic tree based on the amino acid sequences from other plant species was constructed.

![Phylogenetic tree](image)

**Figure 1.** Phylogenetic tree (neighbor-joining) of the early response to the dehydration 15 (ERD15) protein from a total of 15 plant species.

According to the results of the phylogenetic tree, the IbERD15 protein shared higher similarities to the ERD15 proteins from C. annuum, B. napus, and A. thaliana. The phylogenetic results may indicate the origin of the IbERD15 gene.

DGE profiling

With the results of the DGE profiling, the transcript levels of the sweet potato IbERD15 gene in 7 organs were counted as follows: young leaf [342.41 transcript per million (TPM)], stem (219.48 TPM), mature leaf (44.03 TPM), initial tuberous roots (51.23 TPM), harvested...
tuberous roots (194.85 TPM), fibrous roots (41.58 TPM), and expanding tuberous roots (22.71 TPM). It is clear that the *ERD15* gene of the sweet potato was predominantly expressed in the young leaf; fairly highly expressed in the stem and harvested tuberous roots; minimally expressed in the mature leaf, initial tuberous roots, and fibrous roots; and lowest in the expanding tuberous roots.

Expression of *ERD15* under drought tolerance

Previous studies have shown that the *ERD* genes are involved in a wide variety of processes related to signal transduction in plants, and the expression of *ERD* genes increases abundantly under many types of stress. The *ERD15* gene always acts as a negative regulator of ABA, and its overexpression is related to programmed cell death. In order to obtain a wide array of knowledge on the expression levels and molecular action mechanisms of the *ERD15* gene under drought stress conditions, we treated the sweet potato to 3 degrees of PEG, as stated above. The results of the digital expression profiling proved that *ERD15* had the lowest expression level in expanding tuberous roots, which may be related to the steady conditions in the soil. The expanding tuberous roots of the sweet potato are the initial sites to suffer PEG treatment, which may be of great important. Therefore, the total RNA of the expanding tuberous roots was extracted for expression analysis. The expression patterns of *IbERD15* in expanding tuberous root by 3 degrees of PEG collected on days 3, 10, and 20 are presented in Figure 3.

**Figure 2.** Results of digital gene expression profiling of the *ERD15* gene in various tissues of the sweet potato. *TPM* indicates the transcript per million. YL = young leaf; ML = mature leaf; ST = stem; FR = fibrous roots; ITR = initial tuberous roots; ETR = expanding tuberous roots; HTR = harvested tuberous root.

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It is clear that in comparison to the control the expression levels of the \textit{ERD15} gene began to increase rapidly in the first sample, reaching a maximum at the second or third samples, and then dropped down to <10 and 20 mM PEG. To sum up, the above results further indicate that the \textit{IbERD15} gene may play an important role in the response to drought stress.

**Tolerance to drought stress in transgenic yeast**

In Figure 4, we can see that the transgenic and control yeast survived under the stress of 8 M sorbitol, even though they grew slower than the yeast cultured under normal conditions. Apart from this finding, there were no obvious differences in growth between the transgenic and control yeasts. This phenomenon is probably due to a lack of corresponding effect factors for the \textit{IbERD15} gene, or the \textit{IbERD15} gene cannot perform its functions in yeast cells.

**Figure 3.** Semi-quantitative polymerase chain reaction analysis of the \textit{IbERD15} gene in the expanding tuberous roots of the sweet potato.

**Figure 4.** Growth of control transformant and transgenic yeast with \textit{IbERD15} under drought stress conditions.\textbf{A.} Control transformant (empty vector). \textbf{B.} Transgenic yeast with \textit{IbERD15}.
DISCUSSION

The ERD gene family was originally discovered in *A. thaliana* and was considered a dehydration-inducible gene (Kiyosue et al., 1993b, 1994b). Subsequently, a number of studies indicated that different ERD genes had various functions; for example, the *Arabidopsis* homolog ERD1 prevents injury to plant cells (Steponkus et al., 1998; Dunáeva and Adamska, 2001). ERD16 is a ubiquitination extension protein (Kiyosue et al., 1994a). ERD13 (or AtGSTF10) is a plant phi specific class GST (glutathione S-transferase) that interacts with the protein of BAK1 (Kiyosue et al., 1993a; Ryu et al., 2009). ERD15 from *Arabidopsis* has been shown to be an important regulator of the ABA response and SA-dependent defense pathway (Kariola et al., 2006), acting as a negative regulator of ABA. Its overexpression reduces ABA sensitivity, which leads to a decline in drought and freezing tolerances. Moreover, the *GmERD15* gene, belonging to the ERD15 gene family in the soybean, is regarded as a transcription factor that regulates gene transcription related to programmed cell death (Alves et al., 2011). The expression of ERD15 in plants is altered in the presence of different stress signals (e.g., ABA and SA). Simultaneously, the downstream genes of ERD15 are induced to enact corresponding changes. Finally, plants will enact defense mechanisms against external stresses (Liu et al., 2009). Presently, a total of 16 ERD genes have been cloned from *A. thaliana*, and many have also been obtained from the soybean, *Solanum pennellii*, *B. napus*, and *C. annuum*, among others (Chen et al., 2010; Ziaf et al., 2011; Alves and Fietto, 2013).

Until recently, there were no reports on ERD genes in the sweet potato; moreover, drought stress and related regulation pathways research on ERD15 in the sweet potato cannot be found. In this study, we demonstrated that the *IbERD15* gene fulfills an important function in response to drought stress. It was apparent that the expression level of *IbERD15* was consistently higher than that of the control sample. At the initial stage of experimentation, the ERD15 gene was induced in response to drought stress so that expression increased, reaching a maximum at the second sample, which then declined. The results we obtained from the sweet potato were in accordance with previous studies in *Arabidopsis* (Kariola et al., 2006). Although transgenic yeast with the *IbERD15* gene did not exhibit obvious differences when compared to the control, this finding may be attributed to the lack of corresponding effect factors or the *IbERD15* gene may be unable to function in yeast cells. To sum up, the above results further indicate that the *IbERD15* gene might play an important role in the defense response to drought stress. In addition, a signal peptide or transmembrane sequences were not found, implying that *IbERD15* could not be transported out of the cell membrane. By the above digital gene expression profiling analysis, the transcript level of the *IbERD15* gene in 7 different tissues was expatiated. The *ERD15* gene of the sweet potato was highly expressed in the young leaf, stem, and harvested tuberous roots; lowly expressed in the mature leaf, initial tuberous roots, fibrous roots, and expanding tuberous roots. In general, the expression level of *IbERD15* was higher under relatively unstable states in comparison to that under stable states. The members of the ERD15 family are characterized by similar sizes (120-170 amino acids), and they all share a highly conserved domain structure with the PAM2 motif (an acidic domain) in the N-terminus, which is also found in IbERD15. Similar to other ERD15 genes, *IbERD15* also contains a 12-amino acid core sequence (9-
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20) in the conserved PAM2 motif, suggesting that it might have a similar function to that of other members of the ERD15 family (Aalto et al., 2012).

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