Cloning, characterization and expression analysis of HSP70 gene from sweet potato

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Accepted 23 April, 2012

The 70-kDa heat shock protein is a key factor for stress resistance and cellular network of plant. It plays an important role in protein assembly, folding, localization, degradation, tumor immunity etc. In this study, the complete HSP70-coding sequences were cloned from sweet potato by RT-PCR. They contain an open reading frame (ORF) of 1959 bp coding for a peptide of 652 amino acids with a molecular mass of 71.39 kD. Sequence analysis showed that sweet potato HSP70 gene has high homology with other plant HSP70 genes. The results from quantitative real-time RT-PCR demonstrated that the transcription level of the HSP70 gene in initial tuberous roots was much higher than that in young leaves, stems, mature leaves and fibrous roots. HSP70 gene was inserted into the vector pET-32a(+) by SLIC (sequence and ligation independent cloning) and successfully expressed in Escherichia coli BL21(DE3).

Key words: Sweet potato, HSP70 gene, gene cloning, gene expression, quantitative RT-PCR analysis.

INTRODUCTION

Sweet potato has the highest energy yields per unit area per unit time among many plants. It is grown widely in the world due to its high and stable yields, strong adaptability, rich nutrient content, low land and input requirement, easy to manage and multiple uses.

70-kDa heat shock proteins (HSP70) exist widely in the cytosol of plants, animals and microorganisms as well as within eukaryotic organelles, such as mitochondria and endoplasmic reticulum (Hartl and Hayer-Hartl, 2002). Under various environmental and physiological stresses the HSP70s are involved in protein assembly, folding (Bukau et al., 2006), localization (Ryan and Pfanner, 2002), degradation (Wickner et al., 1999), tumor immunity (Nicchitta, 2003) etc. High temperature stress causes cellular proteins extensive denaturation and aggregation, which if kept up leads to cell death. Through HSP70's chaperoning activity, HSP70 helps plant cells to deal with heat-induced damage to cellular proteins (Krishna, 2004). Interestingly, recent studies revealed that the ABA signaling pathway(s) involved in HSP70 expression in response to heat stress. Under stress, the expression of HSP70 can be regulated by PP2A (protein phosphatase 2A), AMPK (AMP-activated protein kinase), CHIP (E3 ubiquitin ligase) etc, which takes part in ABA signal transduction pathways widely (Luo et al., 2006; Wang et al., 2010a; Hu et al., 2010a). However, the mechanism involved in HSP70 expression in response to heat stress in sweet potato is unclear. Thus, the first step to understanding the mechanism the characters of HSP70 gene of sweet potato should be known.

Here, we report the cloning and characterization of the sweet potato HSP70 gene, which contains an open reading frame (ORF) of 1959 bp coding for a peptide of 652 amino acids. Sequence analysis provided insights about its possible identity. Quantitative real-time RT-PCR was used for detecting the HSP70 expression level so as to understand the function of HSP70 gene of sweet potato and gene expression regulation mechanism in the future.

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MATERIALS AND METHODS

Strains and materials

The sweet potato cultivar Xushu18 was obtained from Sichuan Academy of Agricultural Sciences, and planted in the field with exposure to natural sunlight. Various tissues/organisms including young leaf, initial tuberous roots, fibrous roots, mature leaf and stems were sampled at 90 days after planting. Tissues of sweet potato were harvested, washed, and frozen immediately in liquid nitrogen till use.

pET32a(−) was used for constructing prokaryotic expression plasmid. Escherichia coli strains of JM109 (endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB− Δlac-proAB)Δ1−[F′traD36 proAB− lacIq lacZAM15] hsdR17(k2,mcr−) and BL21(DE3)(F− ompT gal dcm lon hsdS2(rK1m2)lacZΔM15[proAB]e14) were used for routine cloning and recombinant expression, respectively.

Isolation of total RNA and cloning of HSP70 gene

Total RNA was extracted using TRIzol (Invitrogen) following the instructions of the manufacturer. First-strand cDNA was synthesized from total RNA of sweet potato Xushu18 by M-MLV reverse transcriptase (Invitrogen). Reverse transcription (RT) was performed using the primer oligo(dt)15. The following conditions were used: incubation at 37°C for 2 min, then adding 1 μl (200 U) M-MLV reverse transcriptase, incubation at 37°C for 50 min, heating at 70°C to terminate the reaction. The resulting RT-products were used to amplify HSP70 gene by PCR with the forward primer HSP70-F (5'-GAAAACCTGTAATTCCAGGGTATGCGGGAACAGTT-3') and the reverse primer HSP70-R (5'-GTTTAGAGGCCTCCAGGTTTACGCTCCTCAATC-3'). The nucleotide sequences of the aforementioned primers were identical to the vector pET32a(−) and used to clone HSP70 gene into the vector via SLIC method (Cheo et al., 2004; Wang et al., 2010).

The PCR reaction was performed using the KOD-Plus-Neo (TOYOBO) DNA polymerase and under the condition consisting of a pre-denaturation at 94°C for 2 min, and 30 cycles of denaturation at 98°C for 10 s, annealing at 48°C for 30 s and extension at 68°C for 90 s, and final extension at 68°C for 10 min. The PCR product was analyzed and recovered from 1.5% (w/v) agarose gel.

For preparing the vector DNA fragment, a pair of primers were designed based on the corresponding nucleotide sequences of HSP70 from other plants and sweet potato transcriptome database (our laboratory database). The underlined nucleotide sequences are identical to the vector pET32a(−) and used to clone HSP70 gene into the vector via SLIC method (Cheo et al., 2004; Wang et al., 2010).

The PCR reaction was performed using the KOD-Plus-Neo (TOYOBO) DNA polymerase and under the condition consisting of a pre-denaturation at 94°C for 2 min, and 30 cycles of denaturation at 98°C for 10 s, annealing at 48°C for 30 s and extension at 68°C for 90 s, and final extension at 68°C for 10 min. The PCR product was analyzed and recovered from 1.5% (w/v) agarose gel.

Isolation of genomic sequence

Total DNA of fresh tissues was extracted as template for PCR to obtain genomic sequence of HSP70. One pair of PCR primers were used, which was the same as those of HSP70 cDNA. Genomic DNA fragment was amplified in a total volume of 25 μl containing 1 μl total DNA by using DNA polymerase KOD-Plus-Neo (TOYOBO). PCR conditions were pre-denaturation at 94°C for 2 min, and following 30 cycles of denaturation at 98°C for 10 s, annealing at 48°C for 30 s and extension at 68°C for 2 min 10 s, and final extension at 68°C for 10 min. The purified HSP70 fragment was also ligated to the amplified pET32a(+) vector DNA fragment as aforementioned by SLIC. Recombinants were verified by PCR and Xba I digestion, nucleotide sequencing was carried out soon after.

Bioinformatics analysis of sequence data

Database searches and the protein analysis (including molecular weight) were performed with the NCBI server (http://www.ncbi.nlm.nih.gov) and the ProtParam tool (http://www.expasy.ch/tools/protparam.html). Multiple protein sequence alignments were analyzed using DNAAMAN. Phylogenetic analysis and tree construction were completed using MEGA4.0 (Tamura et al., 2007). Functional protein annotations of the deduced amino acid sequence were performed using Superfamily database (http://supfam.cs.bris.ac.uk/SUPERFAMILY/). The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) and TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) were used to predict the HSP70 signal peptide and transmembrane regions. The NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) was used to predict kinase-specific phosphorylation sites. Protein subcellular localization prediction was carried out by WoLF PSORT (http://wolfpsort.org/).

Quantitative real-time RT-PCR

The quantitative real-time RT-PCR was used to evaluate the expression profiles of HSP70 in five different tissues of sweet potato and carried out on a Multicolor Real-time PCR detection system (Bio-Rad, USA), based on SYBR Green Realtime PCR Master Mix-plus (TOYOBO, Japan). Each 20 μl reaction consisted of 1 μl template, 10 μl 2×SYBR Green Realtime PCR Master Mix-plus, 2 μl Plus Solution, 1 μl 10 μM of each primer, and 5 μl ddH2O. Independent reference gene (actin) was included to improve the level of reliability for the samples. The PCR amplification conditions included an initial heating step at 95°C for 1 min, followed by 40 cycles of three-step reactions (95°C for 15 s, 58°C for 15 s, and 72°C for 45 s). The data were analyzed by iCycler IQ 3.1. Each experiment was repeated at least three times.

Recombinant expression of HSP70 gene

The recombinant plasmid pE32-1PHSP70 was transformed into E. coli BL21(DE3) competent cells and the recombinants were selected on LB plates amended with 50 μg/ml ampicillin according to the Molecular Cloning (Sambrook et al., 1989). 3 ml of LB medium (containing 50 μg/ml ampicillin) were inoculated with a single colony of the transformed E. coli and grown at 37°C overnight until the A600 reached up to about 0.5. Then, each 10 ml of LB medium plus 50 μg/ml ampicillin was inoculated with 0.5 ml of the aforementioned culture and grown at 37°C. When cell density of the cultures reached an A600 up to about 0.5, 1, 0.5, 1 and 2 μl IPTG
(1 M) were separately added in each 10 ml of cultures to induce gene expression. The cultures were subsequently incubated at 18°C for 16 h. The cells from 1 ml of each culture were precipitated by centrifugation and suspended with 0.1 ml SDS-PAGE loading buffer. Finally, 30 μl sample were separated on 10% SDS-PAGE.

RESULTS

Cloning and analysis of HSP70 gene

The HSP70 gene encoding heat shock protein70 from sweet potato was successfully amplified by RT-PCR from total RNA using the specific primers. The recombinant clones were confirmed by Xba I digestion and PCR (Figure 1). DNA sequencing revealed that there were four kinds of recombinants, designated as HSP70-1 (accession no. JQ063089), HSP70-2 (accession no. JQ063090), HSP70-3 (accession no. JQ063091) and HSP70-4 (accession no. JQ063092), respectively. Highly homology among them except for three sites: 51, 1233, 1674. However, amino acids encoded were the same: threonine (Table 1). The result was confirmed by sequencing the RT-PCR products and double peaks were detected using Chromas at these sites,
simultaneously, which was in complete accord with bases occurring in four recombinants. The coding sequences of four cDNAs are 1959 bp in length, coding for an open reading frame of 652 amino acids with the predicted molecular weight of 71,3918 kD and pl of 5.11, respectively.

Amino acid (aa) sequence and phylogenetic analysis

The results of the ProtParam tool showed that this polypeptide has an aliphatic index of 82.13 and the grand average of hydropathicity (GRAVY) of -0.614, suggesting that it is a hydrophilic protein. In the high-level structure assay, HSP70 displayed a rather compacted structure which contained alpha helixs (232 aa, 35.58%), extended strands (109 aa, 16.72%), and some random coils (309 aa, 47.39%). By using InterProScan Sequence Search, two actin-like ATPase domains (6 to 192, 195 to 387), peptide-binding domain (391 to 549) and C-terminal subdomain (543 to 626) were found in HSP70 protein. Transmembrane region analysis of the amino acid sequence also demonstrated that one strong outside-to-inside transmembrane helix was from 405 to 427 residues. It is most likely a cytoplasmic protein by protein subcellular localization prediction.

NetPhosK analysis indicated that the HSP70 protein may possess multiple phosphorylation sites, such as PKA, PKB, PKC, PKG, CKII, etc, implying that HSP70 may be used as a substrate for some kinases and is involved in energy metabolism, signal transduction, protein assembly and folding in the regulation process of cell.

The results from Blastn and Blastp revealed that the HSP70 gene of sweet potato shared a high identity in nucleotide and amino acid sequence with the HSP70s from other plants. A phylogenetic tree based on the amino acid sequences from other 28 plant species was constructed (Figure 2). The amino acid sequence of HSP70 from sweet potato has an overall identity from 91.5 to 96.1% with those of 28 plants. The HSP70s of Nicotiana tabacum, Gossypium hirsutum, Ricinus communis had the closest relationship (99.0%) with that of sweet potato, which is in agreement with species taxonomy (Figure 2).

Sequence analysis and comparison of genomic DNA

The primers of HSP70-F and HSP70-R were also applied to amplify the genomic fragment in corresponding to the cDNA of HSP70. DNA sequencing revealed that DNA fragments (2942 bp) were obtained from genomic DNA, which contained the same 983 bp intron (214-1196) and two exon regions (1 to 213, 1197 to 2942). Analysis of the intron position of several species suggested that the intron position of HSP70 in A. thaliana, O. sativa, S. lycopersicum, V. vinifera is similar to the sweet potato (Figure 3). The results demonstrate that genomic HSP70 gene of sweet potato contains one intron, indicating that HSP70 in sweet potato is most likely encoded by the nuclear genome.

Tissue/organ-specific expression pattern

On the basis of the results of the quantitative real-time RT-PCR, the level of HSP70 expression varied among tissues/organs (Figure 4) and was the highest in the initial tuberous roots, which was up to almost 200-fold in mature leaf but 2-fold in stem. There was no obvious difference between young leaf and fibrous root.

Recombinant expression

The HSP70 gene was expressed in E. coli BL (DE3) as a fusion protein with trxA. And, an expected protein band with a molecular weight of about 82 kD on the SDS-PAGE was obviously observed (Figure 5). However, there is no distinct difference in protein expression level when different concentrations of IPTG (0.1, 0.5, 1 and 2 mM) were used. The recombinant protein of HSP70 may be used to raise antibody in the further research.

DISCUSSION

In this study, HSP70 genes which contain 1959 bp nucleotides and encode a peptide of 652 amino acids from sweet potato were isolated. By analyzing the HSP70 gene sequences, different bases at the sites of 51, 1233 and 1674 were observed, indicating that there are different HSP70 genes in sweet potato genome. The sequences from PCR cDNA products and genomic recombinants confirmed the results, which is accorded with that sweet potato is believed to be an allohexaploid plant (Gao et al., 2011). However, the amino acid sequences were not changed, which suggested that HSP70 genes were highly conservative in sweet potato. The conserved HSP70 genes are different from other genes cloned from the same plant. It was observed that the nucleotide mutations of other genes usually cause the changes of the encoded amino acids in sweet potato.
Protein sequence analysis showed that the protein of HSP70 may posses multiple protein kinase receptor sites which occur widely in eukaryotic proteins. Therefore, this protein may be likely involved in various regulatory reactions, especially via phosphorylation and dephosphorylation. HSP70 is predicated as a compact hydrophilic protein, which contains larger part of random coils, but less alpha helixs and some extended strands. The strong signal peptide sequence was not figured out, suggesting that Hsp70 can not be transported out of...
**Figure 3.** The gene structure displaying diagram of HSP70 from 5 plant species.

**Figure 4.** Quantitative real-time RT-PCR analysis of HSP70 gene in various tissues of sweet potato. YL, young leaf; ML, mature leaf; Stem; FR, fibrous root; ITR, initial tuberous root.
membrane.

By quantitative real-time RT-PCR analysis, the transcript level of HSP70 gene in different tissues was elaborated. The transcription level of HSP70 was the highest in initial tuberous roots. High expression of HSP70 in roots and relatively low in other organs suggests a specific role of HSP70 in root growth or function. The expression of HSP70 gene in Arabidopsis also proved that a role for HSP70 could be ascribed to seed maturation and germination (Sung and Vierling, 2001).

Responses to temperature changes may require an array of evolutionarily conserved proteins known as called heat shock proteins (Vierling, 1991). The results of transcriptome sequencing displayed that there were HSP11, HSP20, HSP83, HSP90, HSP70, etc. and other genes encoding small heat shock proteins in sweet potato (unpublished data). HSP70 are 70-kDa heat shock proteins which can be divided into two categories: HSP70, HSC70; HSP70 with low expression under normal conditions, however, could be quickly induced when heat stress occurs. Correspondingly, HSC70s are constitutively expressed under non-stress conditions, but with little or no induced expression after heat shock (Denlinger et al., 2001). The expression of HSP70 contributes to defending the harm caused by high temperature for plant. In addition, more and more experiments demonstrated that the expression of HSP70 could be induced under cold stress in different plant species such as Arabidopsis (Swindell et al., 2007), Hevea brasiliensis (Zhang et al., 2009), Lycopersicon esculentum (Li et al., 1999), and Oryza sativa (Huang et al., 2004).

To our knowledge, there was no report about HSP70 genes in sweet potato. The research of HSP70 function in sweet potato is also very limited. Therefore, isolation and characterization of the HSP70 could be the initial step for further studies on the stress tolerance in sweet potato. In addition, previous research results proved that there exist cross-talks between endogenous ABA and HSP70 in regulating the defense systems under heat or/and drought stress in plants. HSP70 may play a crucial role in ABA-induced stress defense (Hu et al., 2010b). Our results can provide a possible way to enhance the plant

Figure 5. SDS-PAGE analysis of the expression of sweet potato HSP70 in E. coli. M. Protein marker; lane C, pET-32a(+) induced by IPTG; Lines 1 to 4, pE32-IP HSP70 induced by 0.1, 0.5, 1 and 2 mM IPTG, respectively.
tolerance to stresses or execute deep function research of HSP70 from sweet potato in the future.

ACKNOWLEDGEMENTS

The work was supported by “Eleven-Five” key project of Sichuan Province (No. 07SG111-003-1) and National Science and Technology Pillar Program of China (No. 2007BAD78B03).

REFERENCES


