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Full Length Research Paper

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

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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 *HSP70*s 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 *Xushu*18 was obtained from Sichuan Academy of Agricultural Sciences, and planted in the field with exposure to natural sunlight. Various tissues/organs 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$^+$ Δ(lac-proAB)e14-[F'traD36 proAB$^+$ lacI^q lacZΔM15] hsdR17($r_K^-m_K^+$)} and BL21(DE3){F$^-$ ompT gal dcm lon hsdS_B($r_B^-m_B^-$)λ(DE3[lacI lacUV5-T7 gene 1 ind1 sam7 nin5])} 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 *Xushu*18 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'-GAAAACCTGTACTTCCAGGGTATGGCCGGAAAAGGT-3') and the reverse primer HSP70-R (5'-GTTTAGAGGCCCCAAGGGGTTAGTCCACCTCCTCAATC-3'). The nucleotide sequences of the aforementioned 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., 2010b).

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 pET32a(+) sequence. The primers contain the following sequences: pET-F (5'-TTTTCTACTTCAGCAACAAGTATAACCCCTTGGGGCCTCTAAAC-3'), pET-R (5'-ACCCTGGAAGTACAGGTTTTCACCAGAAGAATGATGATGATGATGG-3'). The underlined sequences are complemented to the *HSP70* gene. The PCR reaction was performed under the following conditions: 94°C for 2 min, 30 cycles with 98°C for 10 s, 54°C for 30 s and 68°C for 3 min. The amplified vector sequence was recovered from the agarose gel.

The purified *HSP70* and vector DNA fragments were treated by T4 DNA pol (TaKaRa) for 30 s at 37°C. Afterward, these two PCR products were mixed and annealed under the condition of an initial denaturation at 75°C for 10 min and cooled down to the room-temperature naturally. Finally, the annealed DNA mixture was directly transformed into *E. coli* competent cells. The resulting recombinants were verified by PCR and *Xba* I digestion, and then nucleotide sequencing. The confirmed recombinant plasmid was named as pE32-IPHSP70.

Isolation of genomic sequence

Total DNA of fresh tissues was extracted as template for PCR to obtain genomic sequence of *HSP70*. One pairs 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 DNAMAN. 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 Muticolor 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 ddH_2O. 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-IPHSP70 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 A_{600} 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 A_{600} up to about 0.5, 1, 0.5, 1 and 2 µl IPTG

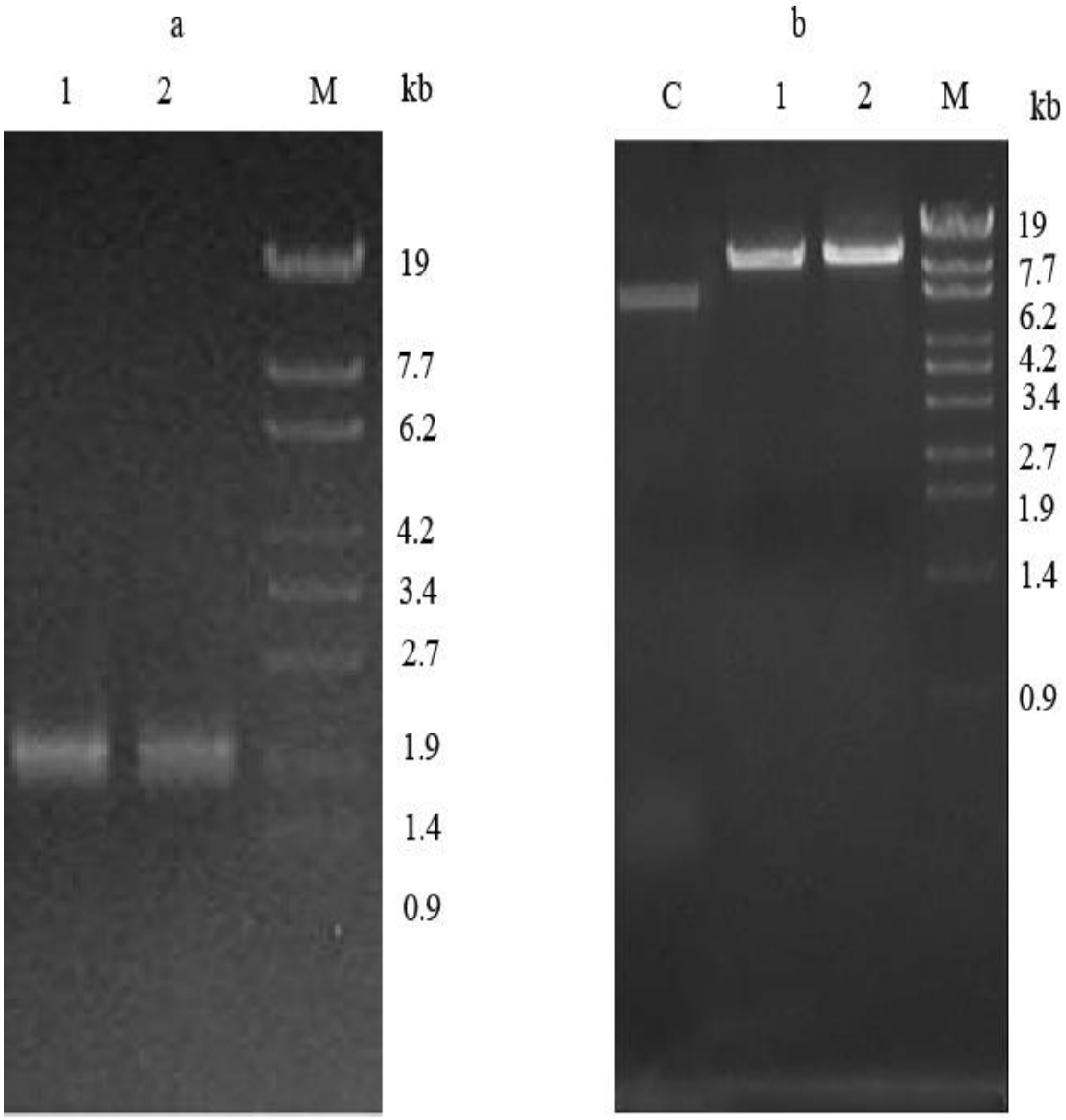

Figure 1. Agarose electrophoresis of PCR products (a) and of the enzyme digestion of cDNA clones (b). a: lane M, λEcoT14 I DNA marker; lanes 1 and 2, *HSP70*-1 and *HSP70*-2. b: lane M, λEcoT14 I DNA marker; lane C, *Xba* I-digested pET-32a (+); lanes 1 and 2, *Xba* I-digested pE32- *HSP70*-1 and pE32- *HSP70*-3.

(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,

Table 1. Different bases at three sites in four recombinants.

Site	51	1233	1674
Hsp70-1	A	C	G
Hsp70-2	T	T	G
Hsp70-3	A	T	C
Hsp70-4	T	C	G
Amino acid	Threonine	Threonine	Threonine

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 pI 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 *HSP70*s 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 *HSP70*s 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 extron 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 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 *trx*A. 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.

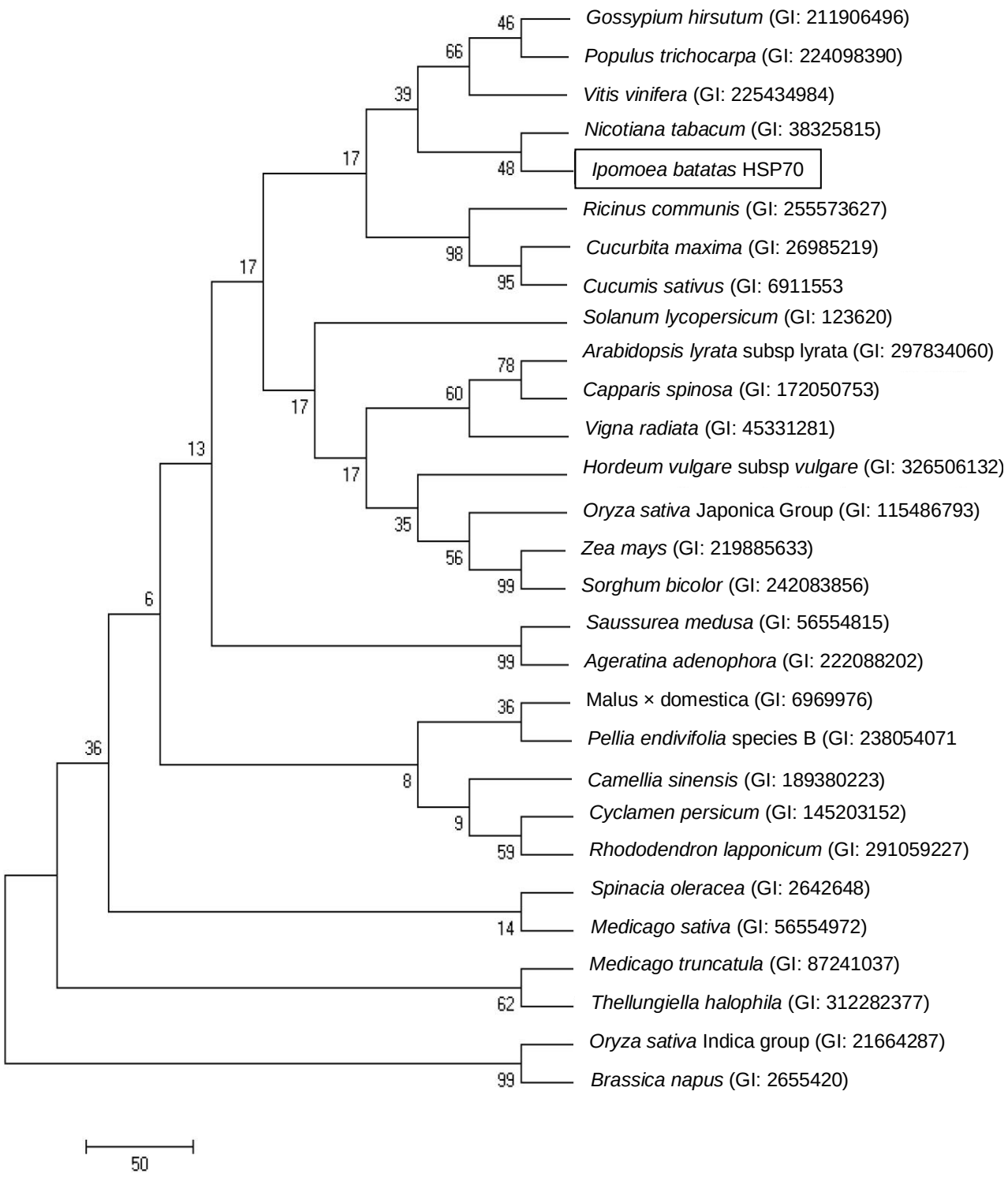

Figure 2. Amino acid sequence phylogenetic tree (neighbor-joining) of *HSP70* polypeptide from 29 plant species.

Protein sequence analysis showed that the protein of *HSP70* may possess 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

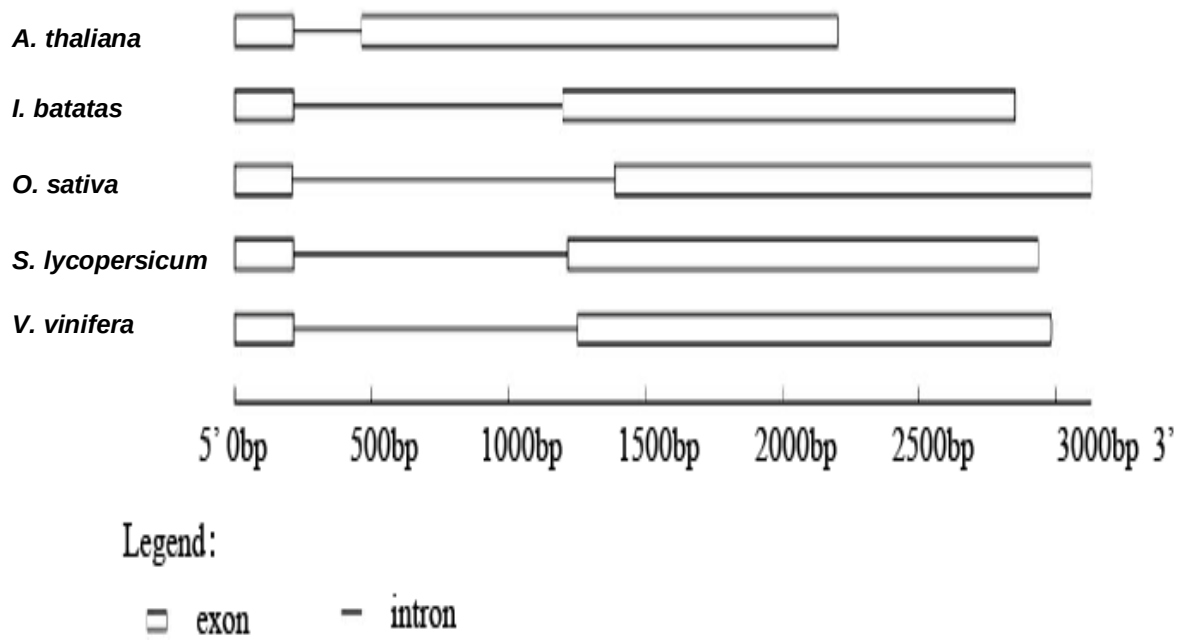

Figure 3. The gene structure displaying diagram of *HSP70* from 5 plant species.

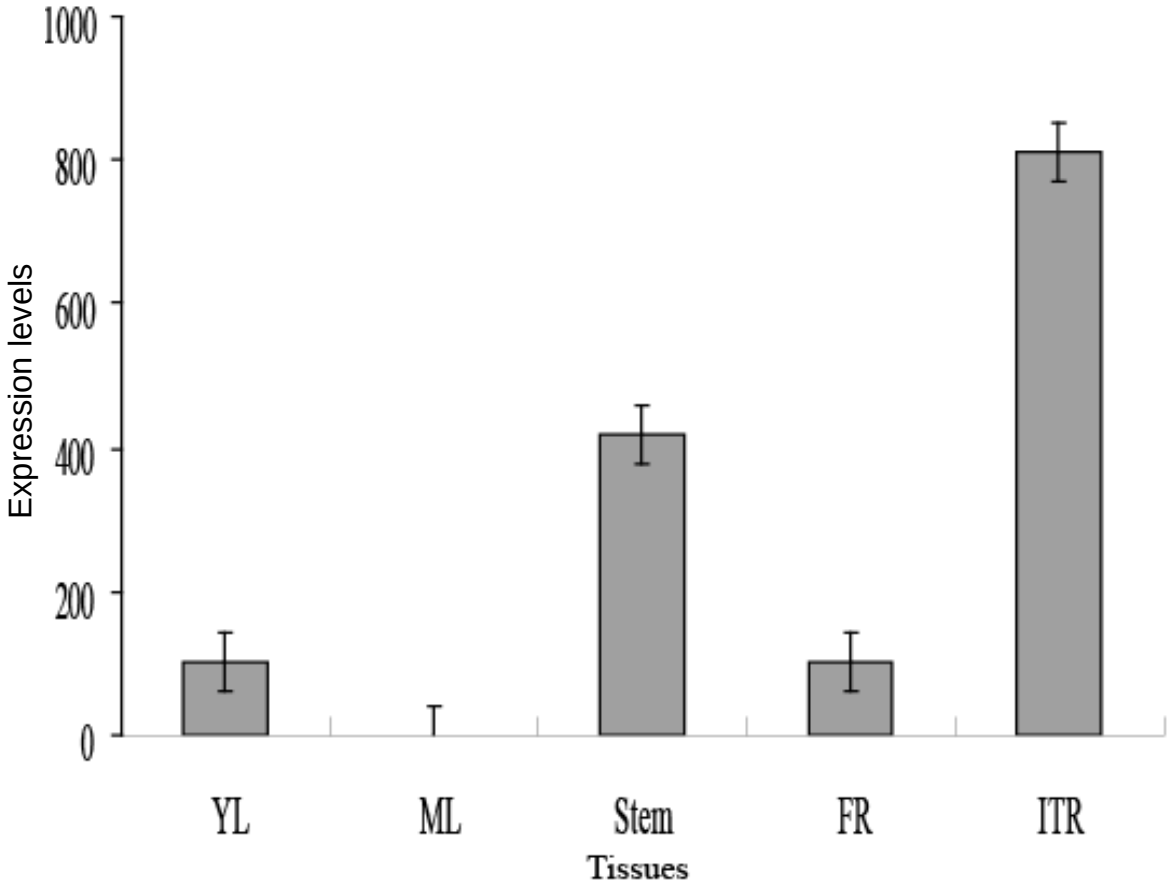

Figure 4. Quantitative real-time RT-PCR analysis of *HSP70* gene in various tissues of sweet potato. YL, young leaf; ML, mature leaf; Stem; FR, fibrous root; ITR, initial tuberous root.

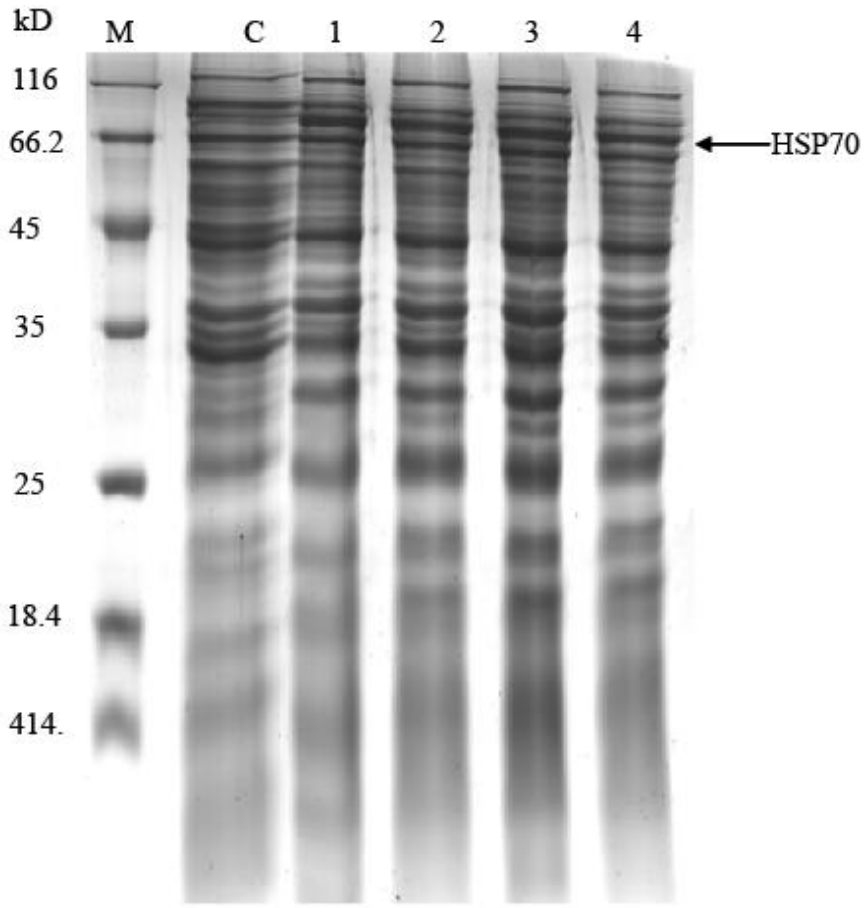

Figure 5. SDS-PAGE analysis of the expression of sweet potato *HSP70* in *E. coli*. M. Protein marker; lane C, pET-32a(+) induced by IPTG; Lines 1 to 4, pE32-IP *HSP70* induced by 0.1, 0.5, 1 and 2 mM IPTG, respectively.

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

tolerance to stresses or execute deep function research of *HSP70* from sweet potato in the future.

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