Plant Physiology and Biochemistry 47 (2009) 954–959

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Molecular characterization of an ethephon-induced Hsp70 involved in high and low-temperature responses in *Hevea brasiliensis*

Zhi-Li Zhang [a,b,*,1], Jia-Hong Zhu [a,1], Quan-QI Zhang [a], Yuan-Bao Cai [a]

[a] Key Laboratory of Tropical Crop Biotechnology, Ministry of Agriculture, Institute of Tropical Biosciences and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China
[b] Hainan Academy of Agricultural Sciences, Haikou 571101, China

ARTICLE INFO

Article history:
Received 31 December 2008
Accepted 5 June 2009
Available online 17 June 2009

Keywords:
Hevea brasiliens
Heat shock protein 70
Ethephon
Expression
Low temperature

ABSTRACT

Hsp70s have been shown to play important roles in helping cells to cope with adverse environments, especially in response to temperature. In this study a novel ethephon-induced *Hsp* gene, designated as *HbHsp70*, was isolated from *Hevea brasiliensis*. The *HbHsp70* cDNA contained a 1965 bp open reading frame encoding 655 amino acids. The deduced HbHsp70 protein showed high identities to Hsp70s from other plants. Expression studies revealed more significant accumulation of *HbHsp70* transcripts in leaves and stems than in roots, barks and latex. The transcription of *HbHsp70* was induced by ethephon, heat treatment and low temperature stress, whereas jasmonic acid had little effects. Recombinant *HbHsp70* was expressed in *Escherichia coli* and purified by Ni-NTA affinity chromatography. Measuring the light scattering of luciferase (Luc) revealed that HbHsp70 prevents the aggregation of luc during high-temperature stress. *In vitro* experiments showed that HbHsp70 had protective functions not only against heat stress but also against chilling stress. All these data suggest that *HbHsp70* may play roles in responses to heat shock and low temperature in *H. brasiliensis*.

1. Introduction

All organisms respond to high temperature by inducing the synthesis of a group of evolutionarily conserved polypeptides known as called heat shock proteins [1], which can also be induced by low temperature [2] and other environmental stresses such as drought [3], salinity or flooding [4]. Hsp70s are one of the most widely studied members and have been shown to play important roles in helping cells to cope with adverse environments, especially in response to temperature [5]. Hsp70 chaperones are involved in several processes such as translation, translocation into organelles, refolding of stress-denatured proteins, prevention of aggregation of denatured proteins and membrane protection [5]. Presently, accumulation of Hsp70s in response to low temperature has been demonstrated in different plant species such as *Spinacia oleracea L.* [6], *Lycopersicon esculentum* [7], *Arabidopsis* [8], *Secale cereale* [9], *Populus* [10] and *Oryza sativa* [11]. Similar results have also been obtained from cold stress studies using 2DE where Hsp70 has been found to be induced under low temperature [2,10,12].

Natural rubber is synthesized in over 2000 plant species, representing about 300 genera from seven families [13]. The rubber tree (*Hevea brasiliensis*) has been established as a key commercial rubber source due to the good yield and excellent physical properties of its rubber products. Rubber is the raw material of choice for heavy-duty tire and other industrial uses requiring elasticity, flexibility and resilience [14]. As a kind of tropical plant, rubber tree is mostly grown above 20 °C. Low temperature, especially under 10 °C would severely affect all metabolic activities and, in particular, result in permanent damages to the photosynthetic apparatus [15]. Latex yield during the winter season always decreases by approximately 8–40% depending on the clone of rubber tree every year [16]. Presently, the molecular mechanism by which the rubber tree perceives and tolerates low temperature is poorly understood.

Ethephon, a mimic of the plant hormone ethylene, has been applied regularly on the trunk of rubber tree to stimulate latex yield, but the underlying physiological and biochemical processes that result from this treatment are unclear [17]. In order to clarify the processes and mechanisms, an ethephon-induced latex SSH

Abbreviations: Luc, Luciferase; Hsp70, Heat shock protein 70; 2DE, 2-Dimensional electrophoresis; SSH, Suppression subtractive hybridization; RACE, Rapid amplification of cDNA ends; RT-PCR, Reverse transcription-polymerase chain reaction; IPTG, Isopropyl thiob-D-galactoside; LB, Luria Bertani; JA, Jasmonic acid.

* Corresponding author at: Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, Hainan Pronvince, China. Tel.: +86 898 65314866; fax: +86 898 66890978.
E-mail address: zzl_catas@hotmail.com (Z.-L. Zhang).

[1] Equally contributed.

0981-9428/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2009.06.003

cDNA library was constructed from latex in *H. brasiliensis* [18], in which an EST with high homology to *Hsp70* genes was identified. In this study, we report the cloning and characterization of the Hsp70 gene from *H. brasiliensis* (*HbHsp70*, GenBank Accession Number: EU627002). The expression profiles of *HbHsp70* and its function in *Escherichia coli* were also investigated. The present study contributes to an understanding of the molecular characterization of Hsp70 and its possible function in the rubber tree.

2. Results

2.1. Isolation and characterization of HbHsp70

Based on the EST sequence with high homology to *Hsp70* genes from the ethephon-induced latex SSH cDNA library, two primers were designed to amplify the unknown 3′- and 5′-end sequences of *Hsp70* cDNA by RACE strategy. PCR products of 1154 bp and 877 bp were obtained respectively. By aligning and assembling these three sequences, the full-length cDNA sequence of *HbHsp70* was deduced, amplified by PCR and confirmed by sequencing. The full-length cDNA was 2344 bp and contained a 1965 bp ORF flanked by a 172 bp 5′-UTR and a 204 bp 3′-UTR including a poly(A) tail of 37 bp.

The *HbHsp70* encodes 655 amino acid residues with predicted molecular masses of 70.17 kDa and calculated pI of 5.27. The deduced amino acid sequence showed high identities of 89%, 87% and 86% to those of the Hsp70 from *Pisum sativum* (GenBank accession No.CAA6786), *Petunia x hybrida* (GenBank accession No.CAA30018), *Glycine max* (GenBank accession No.CAA44620) (Fig. 1), in which the ATP binding domain and peptide-binding domain were strongly conserved, but diverged in the C-terminal motif. The C-terminal motif, depending on subcellular localization, tends to be conserved across evolutionarily distant organisms [19], but it is unique to each of the major subcellular localization sites in plants. For the cytosolic forms, the conserved sequence is GPKIEEVD; for the ER luminal member, it is HDEL; for the mitochondrial matrix member, it is PEAEYEEAKK; and for the stromal member, it is PEGDVIDADFTDSK [19]. In HbHsp70 there is an amino terminal leader sequence GPKIEEVD, showing that the HbHsp70 is a cytoplasmic Hsp70. Several special regulatory elements were identified in the promoter region of *HbHsp70*, including a cis-acting element LTR (^{20}CCGAAA) involved in low-temperature response [20], an AE-box (^{133}AGAAACAA) involved in light response [21] and a cis-acting element (^{163}ATTTTCTTCA) involved in defense and stress response [22].

2.2. Differential expression of HbHsp70 in H. brasiliensis organs

The expression patterns of the *HbHsp70* gene were investigated in various organs (L, leaves; S, stems; R, roots; BA, bark; LA, latex) by semi-quantitative RT-PCR analysis. The results demonstrating that the *HbHsp70* transcript was present in all plant organs, but its level was significantly changed in different organs. The *HbHsp70* gene was strongly expressed in leaves and stems, whereas it was weakly expressed in roots, latex and barks (Fig. 2).

2.3. Expression patterns of the HbHsp70 under various abiotic stresses

JA can induce laticifer differentiation in *H. brasiliensis* [23]. Ethephon has been regularly applied on the trunk of rubber tree to stimulate latex yield [17]. To test whether *HbHsp70* gene expression was regulated by JA or ethephon, total RNA was isolated from the latex of six-year-old virgin trees, which had been treated(except the control) with JA and ethephon at their first tapping and subjected to semi-quantitative RT-PCR analysis. It was shown that ethephon induced the transcription of *HbHSP70* in latex, whereas JA had little effects throughout the time course of JA-treatment (Fig. 3).

Most of the families of Hsp can response to temperature [24]. Here, the *HbHsp70* gene expression in leaves under heat shock and low temperature was investigated. The *HbHsp70* transcript was shown to increase 1 h after 42 °C heat shock, but to decline 2 h after 42 °C exposure. By 8 h of heat shock, the mRNA restored to prestress level (Fig. 4A). Low temperature also induced the expression of *HbHSP70* in *H. brasiliensis*. With the passage the time, *HbHsp70* transcript was gradually up-regulated and came to its peak at 48 h after 8 °C treatment. Upon return to 25 °C the *HbHsp70* transcript became less abundant, but higher than prestress level (Fig. 4B). It is true not only for leaves, but for roots and stems, although to less extend (Fig. 4C).

2.4. Expression and purification of recombinant HbHsp70 in E. coli

To analyze its possible function under heat and chilling stresses, the complete coding sequence for *HbHsp70* was introduced into *E. coli* using the pET-30a(+) expression vector. The vector lone was also introduced into *E. coli* as a control. Expression was confirmed by SDS-PAGE. Overexpression of HbHsp70 in *E. coli* at 37 °C for 2 h resulted in the production of a soluble protein of about 72 kDa, which corresponded to the predicted value. The protein was purified by Ni-NTA affinity chromatography (Fig. 5) and was used for further functional analysis.

2.5. Molecular chaperone activity of HbHsp70

The molecular chaperone activity of HbHsp70 was examined by monitoring the protection of aggregation of luc by the protein under thermal stress. A concentration-dependent effect was observed *in vitro* by measuring the light scattering of luc at 45 °C. At 45 °C and without any HbHsp70, 200 nM of luc aggregated rapidly in the first 20 min and the absorbance at 340 nm came to the peak in 40 min. Relative light scattering apparently decreased in the presence of 100 nM HbHsp70. Even if thermal stress for 60 min, luc remained soluble (Fig. 6). These data indicated that the recombinant HbHsp70 strongly suppressed aggregation of luc under thermal stress.

2.6. Cell viability under high temperature and chilling stresses

To evaluate the effect of recombinant HbHsp70 on cell survival under heat stress, the culture, after adding IPTG for 2 h, was diluted and then transferred to 50 °C for 5 h, cell viability decreased rapidly in both pET and pET-Hsp70 cultures, but the cell lysis rates of expressing HbHsp70 were lower than control (Fig. 7A). To test the hypothesis that HSPs may be involved in protection against chilling stress, the viability of recombinant cells at 4 °C was studied. The viability of recombinant cells at 4 °C was measured by OD600 values, showing that the OD600 value of pET-Hsp70 cells expressing HbHsp70 increased slowly from 0.628 to 0.824 in 24 h at 4 °C, whereas the OD600 value of control cells varied indistinctively (Fig. 7B).

3. Discussion

Based on the EST sequence information from an ethephon-induced latex SSH cDNA library from *H. brasiliensis*, a full-length cDNA encoding Hsp70, designated *HbHsp70* was isolated from *H. brasiliensis* in this study. Hsp70s are one of the most widely studied members, which have long been recognized as one of the most conserved protein families and perform universally basic and