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Full Length Research Paper

Molecular cloning and characterization of an actin-depolymerizing factor gene in *Hevea brasiliensis*

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Actin-depolymerizing factor (ADF) plays important roles in regulating actin dynamics by maintaining the optimum equilibrium between unpolymerized actin molecules and assembled actin filaments in different cellular processes. In this study, the first ADF gene in *Hevea brasiliensis* designated as *HbADF*, was isolated. The *HbADF* contained an open reading frame (ORF) encoding 139 amino acids. The deduced HbADF showed high identities to plants ADF proteins. Besides a conserved ADF domain, HbADF also contained putative actin and specific F-actin binding sites, phosphorylation site and possible CAM (calmodulin) combining region. The phylogenetic analysis indicated that HbADF was clustered in the subclass I. Being consistent with phylogenetic result, the expression of *HbADF* was constitutive. The *HbADF* transcripts were upregulated by ethephon and wounding treatments; whereas, *HbADF* was firstly induced, and then gradually downregulated by jasmonic acid. The expression profiles and characterizations of *HbADF* suggested that *HbADF* might be associated with latex regeneration and flow in *H. brasiliensis*.

Key words: Actin cytoskeleton, actin-depolymerizing factor, expression analysis, *Hevea brasiliensis*, semi-quantitative reverse-transcription polymerase chain reaction.

INTRODUCTION

The actin cytoskeleton is associated with several cellular processes in plants, and these processes include cyto-plasmic organization, establishment of cell polarity, cell elongation, polar tip growth, cold acclimation, defense response, intracellular traffickin and cytokinesis (Meagher and Williamson, 1994; Pollard et al., 2000; Staiger, 2000; Ouellet et al., 2001; Chen et al., 2002; Wasteneys and Galway, 2003; Augustine et al., 2008; Tian et al., 2009). Multiple actin binding proteins (ABPs), such as actin-depolymerizing factors (ADFs)/cofilins, profilins, etc, regulate actin dynamics by maintaining the optimum equilibrium between unpolymerized actin molecules (G-actin) and assembled actin filaments (F-actin) in different cellular processes (Hussey et al., 2006). ADF/cofilins are a large family of ubiquitous, low molecular mass (15 to 20 kDa) actin binding proteins in eukaryotic cells. They play critical roles in maintaining a cellular actin turnover rate significantly faster than the *in vitro* rates attained by purified actin filaments (Lappalainen and Drubin, 1997; Theriot, 1997; Carlier, 1998; Bamburg, 1999).

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Abbreviations: ADF, Actin-depolymerizing factor; **ABPs,** actin binding proteins; **bp,** base pair; **cDNA,** complementary DNA; **ET,** ethephon; **JA,** jasmonic acid; **EST,** expression sequence tag; **F,** forward primers; **R,** reverse primers; **NCBI,** National Center for Biotechnology Information; **ORF,** open reading frame; **RACE,** rapid amplification of complementary DNA ends; **CAM,** calmodulin; **RT-PCR,** reverse-transcription polymerase chain reaction.

In general, plant ADFs/cofilins has been referred to as ADFs (Staiger et al., 1997; Staiger, 2000). The DNA complementary to RNA (cDNAs) encoding plant ADFs have been cloned and characterized from *Lilium longiflorum* and *Brassica napus* (Kim et al., 1993), *Zea mays* (Kim et al., 1993; Rozycka et al., 1995; Lopez et al., 1996; Hussey et al., 1998), *Petunia hybrida* (Mun et al., 2000), *Arabidopsis* (Dong et al., 2001a, b), *Triticum aestivum* (Ouellet et al., 2001), *Vitis vinifera* (Thomas and Schiefelbein, 2002), *Nicotiana tabacum* (Chen et al., 2002), *Oryza sativa* (Feng et al., 2006), *Gossypium hirsutum* (Zhang et al., 2006 and 2007) and *Gossypium barbadense* (Chi et al., 2008). The expression patterns of multiple plant ADFs from *Z. mays*, *L. longiforum* and *N. tabacum* have been studied previously and classified as constitutive or pollen specific (Kim et al., 1993; Lopez et al., 1996; Jiang et al., 1997; Allwood et al., 2002; Chen et al., 2002). In *Arabidopsis*, the ancient subclasses of ADF genes exhibited novel and differential expression profiles (Ruzicka et al., 2007). These data point to the differential regulation of plant ADFs, and also provide a framework for a model where the differentially expressed actins and ABPs co-evolved in specific organs, tissues and cells. The phylogenetic analyses indicated that *Arabidopsis* and rice ADF proteins were divided into four ancient subclasses (Feng et al., 2006; Ruzicka et al., 2007).

In *Arabidopsis*, *AtADF1* regulates the root hair length (Dong et al., 2001a, b). Overexpression of *AtADF1*, *AtADF5* and *AtADF6* in different types of plant cells reorganized the actin cytoskeleton and reduced the length and number of filamentous actin structures (Dong et al., 2001b). The heterogenetic expression of *AtADF4* gene in tobacco resulted in waved hypocotyls of transgenic plants, especially in darkness (Peng and Huang, 2006). In addition, the mutant with less function of *AtADF4* specifically compromises AvrPphB (an avirulence Avr protein from the plant pathogen *Pseudomonas syringae*)-mediated resistance against *P. syringae* pv. tomato (Tian et al., 2009). The ectopic expression of barley *HvADF3* and several isovariants of *Arabidopsis* ADFs in barley epidermal cells were shown to compromise penetration resistance to powdery mildew fungi (Miklis et al., 2007).

Natural rubber can be synthesized in over 2000 plant species, representing about 300 genera from seven families (Backhaus, 1985). The rubber tree (*Hevea brasiliensis*) is the only species established as a key commercial rubber source due to the good yield and excellent physical properties of its rubber products (Auzac et al., 1989). Natural rubber synthesis takes place in the cytoplasm of highly specialized cells (laticifers or latex cells). Laticifers are periodically differentiated from the cambium and arranged in an isolated network in the inner bark of *H. brasiliensis* (He´bant and de Fay, 1980; de Fay and Jacob, 1989). The cytoplasm of laticifers, known as latex, is expelled when the bark is wounded or tapped to obtain the latex (Gomez, 1976; Gomez and Moir, 1979). Ultrastructural studies on mature laticifers showed that they had no plasmodesmata and were isolated from adjacent cells (He´bant, 1981; de Fay and Jacob, 1989). The actin cytoskeleton of laticifers might be associated with their transport and signaling networks. In addition, it was proposed that the actin cytoskeleton might play important roles in regulating latex flow and wound plugging of laticifers in *H. brasiliensis* (Gao et al., 2003). As a key regulator of actin cytoskeleton, the ADF genes might play important roles in rubber tree.

Although some plant ADFs have been cloned and characterized, no ADF genes in *H. brasiliensis* was reported. In this paper, we reported the cloning and characterization of an ADF gene from *H. brasiliensis* (*HbADF*, GenBank Accession Number: HM126477). In addition, the expression profiles of *HbADF* were also investigated by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis. The study not only contributes to understanding the molecular characterization of *HbADF*, but also provides new insights into *HbADF* in rubber tree.

MATERIALS AND METHODS

Plant materials

The Reyan 8-79 (RY8-79), a high-yielding clone, was planted at the experimental farm of Chinese Academy of Tropical Agricultural Sciences. In this experiment, the latex, flower, leaf and bark samples were collected from 17-year-old rubber tree, which was regularly tapped on the s/2 d/4 system with 1% ethylene stimulation for the past 11 years. As for the ethephon (ET) and jasmonic acid (JA) treatment, six-year-old virgin trees were treated with ET and JA prior to the first tapping according to the method of Hao and Wu (2000). The first few drops of latex containing the debris from the plant were discarded, and then the latex was allowed to drop directly into liquid nitrogen in an ice kettle for total RNA extraction. For the wounding treatment, the control plants were maintained under normal conditions; the wounding stress was induced by tapping the bark of seedlings with a knife. The leaves from wounding treatment and control samples were collected at different time, and immediately frozen in liquid nitrogen for total RNA extraction.

RNA exaction

For RT-PCR, total RNA was prepared from latex, leaves, flowers and barks according to Tang et al. (2007), and then treated with RNase-free RQ1 DNase (Promega). The RNA quantity and quality were determined using the spectrophotometer.

Molecular cloning of HbADF full length cDNA

Using total RNA as template, reverse transcriptions of first strand cDNA were performed with SuperScript II reverse transcriptase (Invitrogen). Rapid amplification of cDNA ends (RACE) technique was employed to obtain the full length cDNA of *HbADF*. The 3′ and 5′ends of *HbADF* were isolated with the SMART RACE cDNA amplification kit (Clontech) following the manufacturer's recommendations. The 3′-RACE and 5′-RACE gene-specific primers were 5′-TGATCCAACTGAGATGGGAC-3′ and 5′-CCAAGCTTCTCCACAATGAC-3′, respectively. All the fragments were cloned in pGEM-T

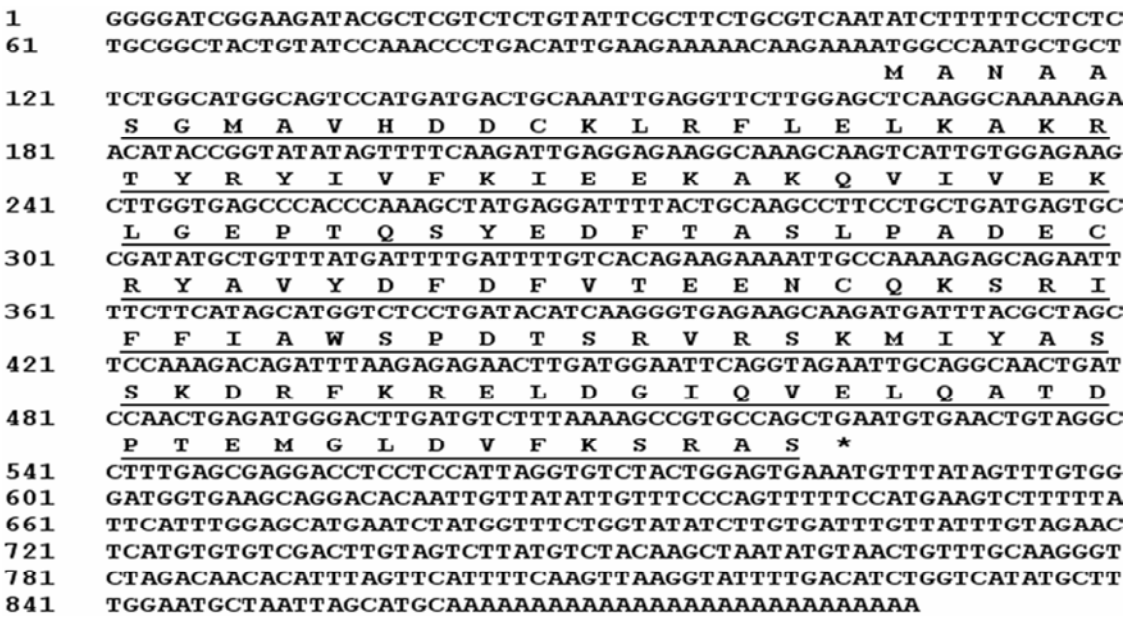

Figure 1. Nucleotide sequence of *HbADF* and its deduced amino acid sequence (GenBank Accession No. HM126477). The conversed ADF domain was underlined.

easy vector and sequenced. After aligning and assembling of the sequences of the internal expression sequence tag (EST), 3′-RACE and 5′-RACE products, the full-length cDNA sequence of the *HbADF* was gotten and testified by sequencing the PCR product. The forward and reverse primers were 5′-GGGGATCGGAAGATA CGCT-3′ and 5′-TTTGCATGCTAATTAGCATTCC-3′, respectively. The PCR reaction was performed with the following thermal cycling parameters: 94°C for 4 min followed by 32 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 2 min, and the final extension was performed at 72°C for 10 min. The PCR product was cloned into the pGEM-T easy vector and sequenced.

Semi-quantitative RT-PCR analysis

The cDNA synthesis was performed using 2 ug total RNA according to the manufacture's protocol (Invitrogen, USA). All semi-quantitative RT-PCR experiments described here were reproduced at least three times using independent cDNA preparations. In each PCR reaction, the gene-specific primers were used, and the internal reference (18s rRNA gene) was amplified with the target gene. Forward (F) and reverse (R) primers used for semi-quantitative RT-PCR analysis is as follows: 18s rRNA (F 5′-GGTCGCAAG GCTGAAACT-3′ and R 5′-ACGGGCGGTGTGTACAAA-3′); *HbADF* (F 5′-GAGGTTCTTGGAGCTCAAGG-3′ and R 5′-TGCTTCACCATC CCACAAAC-3′). Semi-quantitative RT-PCR was performed with the following thermal cycling parameters: 94°C for 4 min followed by 18 or 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, and the final extension was performed at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1.2% agarose gels.

Multiple alignments and bioinformatic analyses

The sequences of 20 ADF proteins were download from the National Center for Biotechnology Information (NCBI) database and aligned with Multiple Alignment Tool program in NCBI (http:// blast.ncbi.nlm.nih.gov/Blast.cgi). The secondary structures and CAM (calmodulin) binding sites of HbADF were analyzed with PSIPRED v2.6 (http://bioinf4.cs.ucl.ac.uk:3000/psipred) and CTDB program (http://calcium.uhnres.utoronto.ca), respectively. The conserved domain, actin binding sites, specific F-actin binding sites and phosphorylation sites of HbADF were predicted with Conserved Domain Database in NCBI (Marchler-Bauer et al., 2009). The phylogenetic tree was constructed by the neighbor joining method (1000 bootstrap replicates) using the MEGA program 4.1 (Kumar et al., 2008).

RESULTS

Isolation and characterization of HbADF

Analyzing the data from the latex cDNA library, we got an EST with high homology to plant actin-depolymerizing factor. In this research, the gene we studied was named as *HbADF*. Based on the known EST sequence, two primers were designed to amplify the 3′- and 5′-end sequences of *HbADF* cDNA by RACE strategy. The PCR products of 3′- and 5′-end were cloned into the pGEM-T easy vector and sequenced; the 212 and 365 bp sequences were obtained from the 3′- and 5′-end of *HbADF*, respectively. After aligning and assembling the 3′- and 5′-end sequences and the known EST sequences of *HbADF*, the full-length cDNA sequence of *HbADF* was gotten and confirmed by sequencing its PCR product. The full length cDNA was 888 bp in size with a predicted 420 bp open reading frame (ORF). The 5′- and 3′-UTR were 105 and 363 bp with 28 bp poly (A) tail, respectively (Figure 1).

The full-length cDNA of *HbADF* encodes a polypeptide

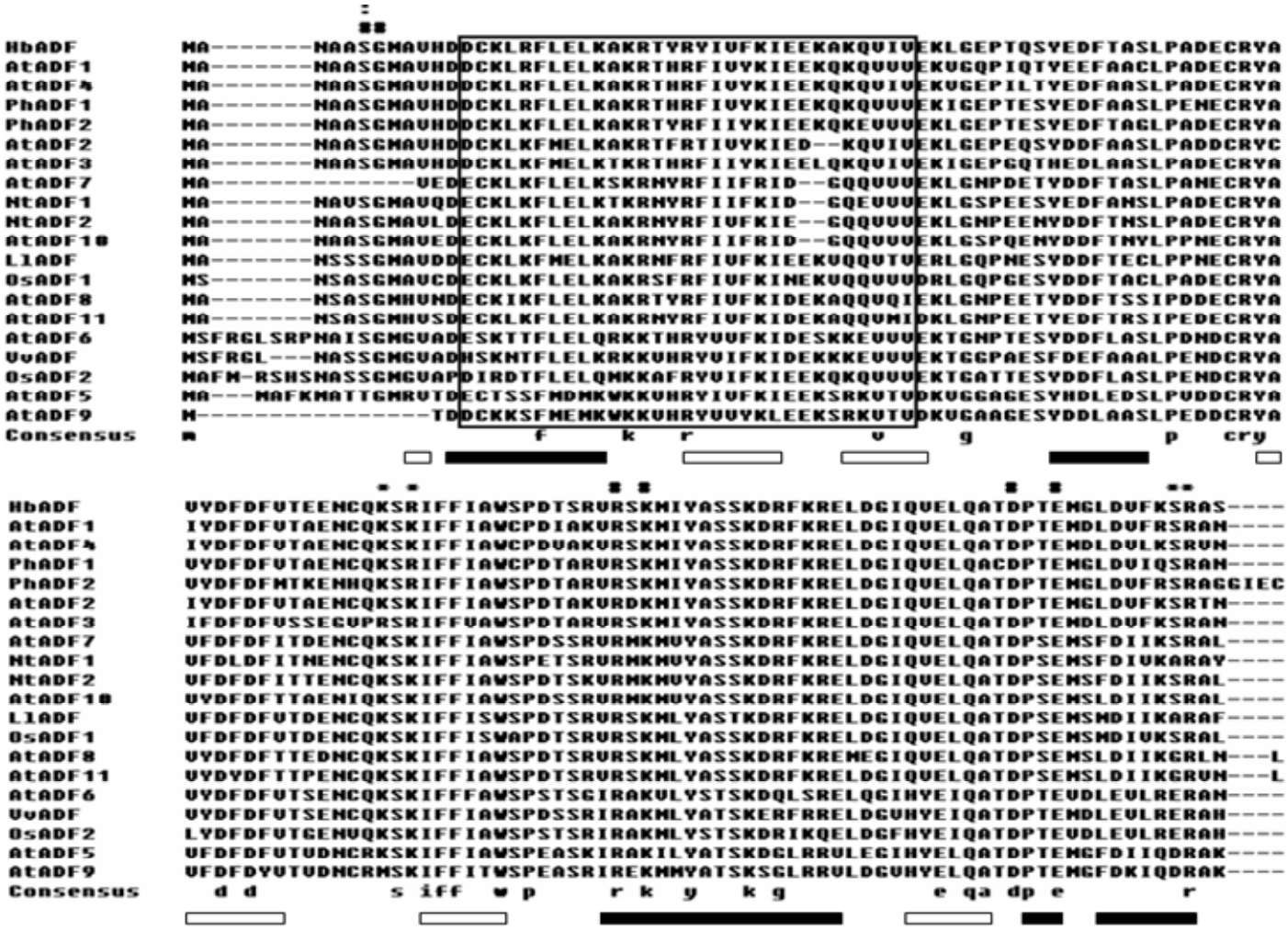

Figure 2. Sequence alignment of deduced *HbADF* and other plant ADFs. Above the alignment, the putative phosphorylation site, actin binding site and specific F-Actin binding site are marked with colon, number sign and asterisks, respectively. The boxed area indicates the putative CAM combining region. Below the alignment, rectangles represent β strands, whereas rectangles filled with black color represent α helices; the accession numbers of plant ADFs protein are as follows: AtADF1 (NP_190187), AtADF2 (NP_566882), AtADF3 (NP_851227), AtADF4 (NP_851228), AtADF5 (NP_565390), AtADF6 (NP_565719), AtADF7 (NP_194289), AtADF8 (NP_567182), AtADF9 (NP_195223), AtADF10 (NP_568769), AtADF11 (NP_171680), NtADF1 (AAL91666), NtADF2 (AAL91667), OsADF1 (NP_001047657), OsADF2 (NP_001051449), PhADF1 (AAK72617), PhADF2 (AAK72616), VvADF (Q8SAG3) and LIADF1 (S30935).

of 139 amino acids with a calculated molecular mass of 16.1 kDa and a pI of 6.01. The search in NCBI conserved domain database showed that *HbADF* contained a conserved actin depolymerisation factor domain (Figure 1). Moreover, the query result also indicated that the HbADF protein contained predicted actin binding sites, specific F-actin binding sites and phosphorylation site, and these potential sites are shown in Figure 2. Analyzing the potential CAM binding sites indicated that the possible CAM combining region was located at N terminus of HbADF. Like other plant ADF proteins, *HbADF* has six putative β strands and four α helices, suggesting that it contains a conversed secondary structure with other plant ADFs (Figure 2). These results suggested that HbADF might carry out the function of ADF proteins.

Phylogenetic relationship of ADF proteins

The deduced amino acid sequence of HbADF is highly conserved among plant ADF proteins, with 47 - 89% identities and 79 - 97% similarities to other plant ADFs at the amino acid level (data not shown). To evaluate the evolutionary relationships within the members of plants ADFs, the phylogenetic tree was constructed among 20 plant ADF proteins by the neighbor joining method using the MEGA 4.1 program (Kumar et al., 2008). As shown in Figure 3, the 20 ADF proteins were divided into four ancient subclasses. Among the four subclasses, the *HbADF*, Ph1,-2 and AtADF1,-2,-3,-4 were clustered in the subclass I. The expression of *Arabidopsis* ADFs in the subclass was constitutive in different tissues, which

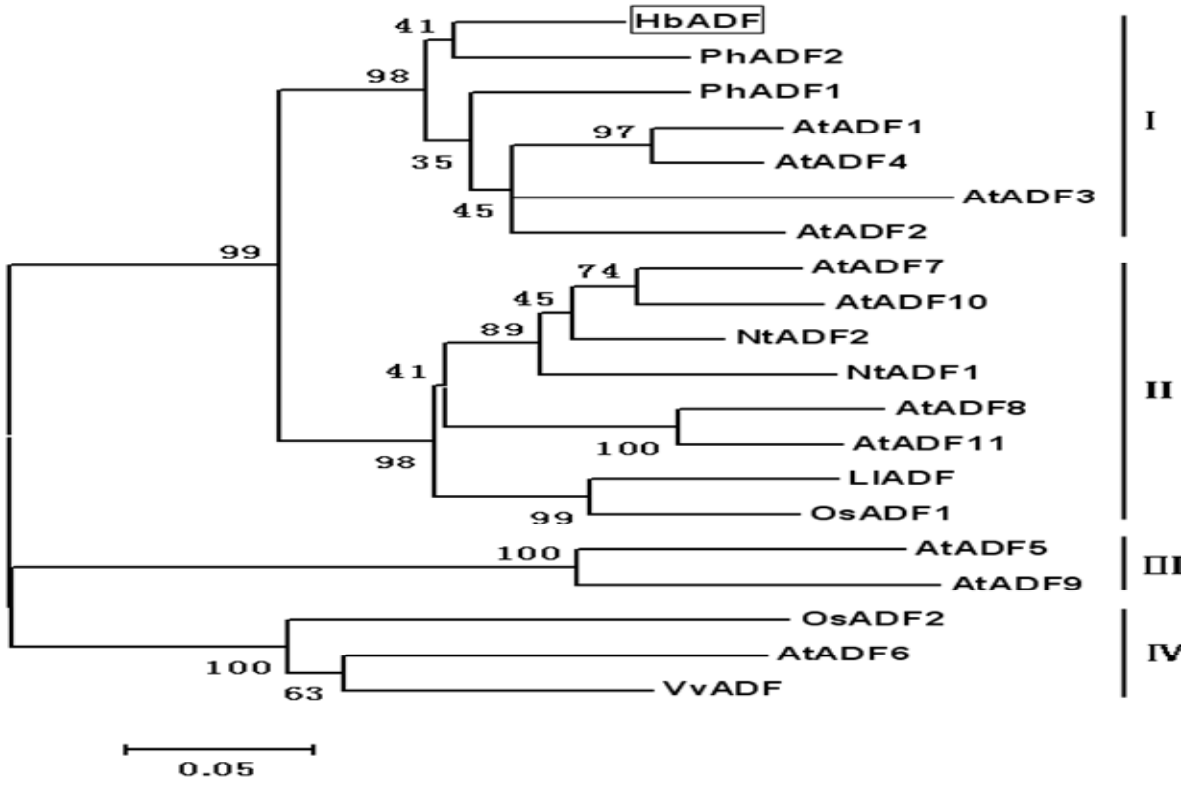

Figure 3. Phylogenetic analysis of *HbADF* and other plant ADF proteins. The tree was constructed by the MEGA program using the neighbor-joining method. Numbers on nodes indicate the bootstrap values after 1000 replicates. The scale bar indicates the estimated number of amino acid substitutions per site. Subclasses I to IV were indicated by vertical bars on the right. The accession numbers of plant ADFs protein in NCBI database are the same as in Figure 2.

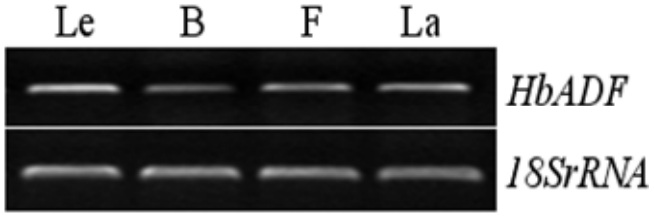

Figure 4. Semi-quantitative RT-PCR analysis of *HbADF* expression in different tissues. Le, B, F and La represent leaf, bark, flower and latex, respectively. The *18SrRNA* is used as the internal control for semi-quantitative RT-PCR.

suggested that *HbADF* might show similar expression profiles.

Expression analysis of *HbADF* in different tissues

The previous studies indicated that the expression patterns of plant ADFs were classified as constitutive or pollen specific (Kim et al., 1993; Lopez et al., 1996; Jiang et al., 1997; Allwood et al., 2002; Chen et al., 2002). Therefore, the expression patterns of *HbADF* were investigated in four tissues by semi-quantitative RT-PCR analysis. The results showed that the expression of the *HbADF* gene was constitutive, which was consistent with the phylogenetic result. As shown in Figure 4, the *HbADF* expression levels were different in four tissues, with the highest expression in leaves, followed by latex, flowers and bark.

Expression patterns of HbADF under different treatments

In plants, various studies have suggested that *ADF* plays an important role in signal transduction, defense response, pollen tube growth, root formation, tip growth, etc (Meagher and Williamson, 1994; Pollard et al., 2000; Staiger, 2000; Ouellet et al., 2001; Chen et al., 2002; Wasteneys and Galway, 2003; Augustine et al., 2008; Tian et al., 2009). To investigate the potential response of *HbADF* to different stimuli, the expression patterns of the *HbADF* gene were analyzed under ET, JA and wounding treatments. As shown in Figure 5, *HbADF* expression was regulated by three treatments, but it showed different expression profiles under different treatments. For wounding treatment, *HbADF* transcripts were slightly

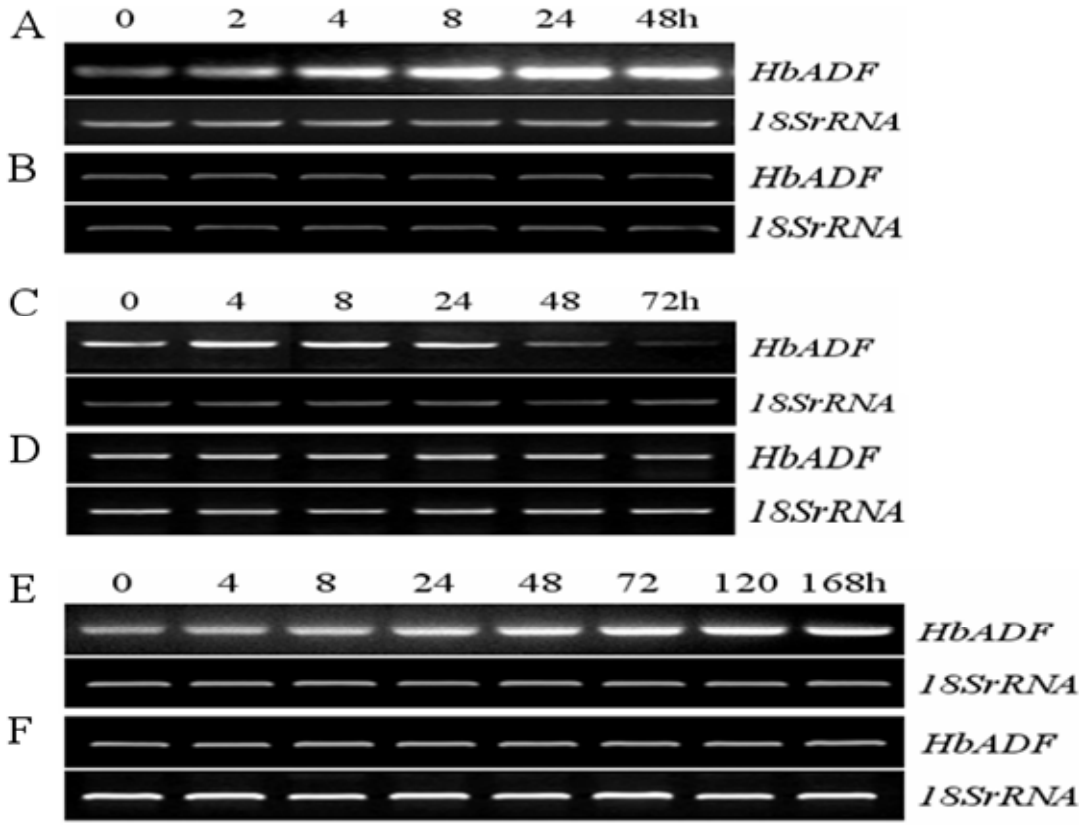

Figure 5. Semi-quantitative RT-PCR analysis of *HbADF* expression patterns under different treatments. A and B represent semi-quantitative RT-PCR analysis of *HbADF* expression profiles under wounding treatment and ck, respectively; C and D represent semi-quantitative RT-PCR analysis of *HbADF* expression profiles under JA treatment and ck, respectively; E and F represent semi-quantitative RT-PCR analysis of *HbADF* expression profiles under ET treatment and ck, respectively. *18SrRNA* is used as the internal control for semi-quantitative RT-PCR.

upregulated at 2 h, and then significantly induced from 4 h; the maximum accumulation occurred at 24 - 48h (Figure 5A). For JA treatment, *HbADF* firstly was induced and reached the highest level at 4 h, then constantly downregulated and reached the lowest level at 3 days (Figure 5C). After ET treatment, the expression of *HbADF* slightly increased from 4 h and reached a maximum at 3 days; the highest expression level continued until 7 days (Figure 5E). In contrast, the *HbADF* transcripts did not show obvious variation in all three control samples (Figures 5B, D and F). The results mentioned earlier suggested that *HbADF* might be related to ET, JA and wounding responses.

DISCUSSION

Based on the EST sequence from the latex SSH cDNA library in *H. brasiliensis*, *HbADF* was isolated and analyzed in this study. To our knowledge, *HbADF* was the first ADF gene cloned in *H. brasiliensis*. The *HbADF* encoded one 139 amino acids protein with a conversed ADF domain (Figure 1). The HbADF was highly conversed among plants ADF proteins; it contained predicted actin binding sites, specific F-actin binding sites, phosphorylation site and CAM binding sites. Moreover, HbADF had similar secondary structure to other plants ADF proteins (Figure 2). Although the biological activity of the HbADF protein need to be further determined, the sequence comparisons among plant ADF proteins suggested that the protein might have actin-binding properties and play important roles in actin dynamics by maintaining the optimum equilibrium between unpolymerized actin molecules and assembled actin filaments.

The plant ADFs could be divided into two major classes, a vegetative class and a reproductive class (Mun et al., 2000). Ruzicka et al (2007) reported the characterization of the tissue-specific and developmental expression of all *Arabidopsis* ADF genes and the subcellular localization of several protein isovariants. The phylogenic tree indicated that the two ancestral ADF genes, vegetative-expressed and pollen-expressed genes, had acquired their tissue specificity before monocot-dicot divergence, which was also supported by this research.

The flowering plants, *H. brasiliensis*, *Arabidopsis*, *Oryza sativa*, Tobacco, etc, evolve with a large pre-existing repertoire of ADFs, indicating that the ADFs might carry out important functions in flowering plants. In our study, the phylogenic tree indicated that the HbADF belonged to subclass I, which was classified into the predominant vegetative group of ADF isovariants in the four subclasses (Ruzicka et al., 2007). Being consistent with the phylogenic result, *HbADF* showed a constitutive expression in four tissues (Figure 4), suggesting that *HbADF* might play more important roles in rubber tree.

It is well-known that actin is a major dynamic component of the plant cytoskeleton. Several cellular processes, such as cell division and differentiation, stomatal movement, wound repair, response to pathogen attack, defense response, cell wall biosynthesis and transmembrane signaling, are reported to be associated with the actin cytoskeleton (Aon et al., 1999). As the key regulating proteins of actin dynamics, the ADF genes might be involved in these processes. The *HbADF* expression was regulated by ET, JA and wounding treatments. As we know, it was firstly reported that the member of ADF genes was associated with JA, ET and wounding treatments. The plant hormone regulates growth, development and stress responses. In rubber tree, the latex is collected by regularly tapping the bark, and ethylene is utilized to stimulate latex yield. On the other hand, JA and mechanical wounding can induce the secondary laticifers differentiation (Hao and Wu, 2000; Tian et al., 2003). In this research, *HbADF* was upregulated by ET and wounding treatments. Different from ET and wounding treatments, the *HbADF* transcripts was firstly upregulated and then gradually downregulated by JA treatment (Figure 5). These results implied that *HbADF* might be involved in wounding, JA and ethylene signaling pathways. By analyzing the relationship between the actin cytoskeleton in laticiferous cells and latex exploitation, Gao et al. (2003) proposed that actin cytoskeleton might play important roles in regulating latex flow and wound plugging of laticifers in *H. brasiliensis*. Five years later, Gao et al. (2008) further reported that the depolymerizing compounds of actin cytoskeleton could stimulate latex production in rubber tree. Based on the information mentioned earlier, *HbADF* might be related to latex exploitation by modifying the optimum equilibrium between unpolymerized actin molecules and assembled actin filaments in latex regeneration and flow. Therefore, it will be of importance to elucidate the relationships among *HbADF*, wounding and ethylene signaling pathway and latex yield in *H. brasiliensis*.

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