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Cloning and molecular characterization of a cDNA encoding a small GTPase from *Hevea brasiliensis*

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ABSTRACT. Small GTPases play a critical role in the regulation of a range of cellular processes including growth, differentiation, and intracellular transportation. The cDNA encoding a small GTPase, designated as *HbGTPase1*, was isolated from *Hevea brasiliensis*. *HbGTPase1* was 882 bp long containing a 612-bp open reading frame encoding a putative protein of 203 amino acids, flanked by an 83-bp 5'-untranslated region (UTR) and a 187-bp 3'-UTR. The predicted molecular mass of HbGTPase1 is 22.62 kDa, with an isoelectric point of 5.06. The HbGTPase1 protein was predicted to possess the conserved functional regions of the small GTPase superfamily of proteins. Quantitative polymerase chain reaction analysis revealed that *HbGTPase1* was constitutively expressed in all tissues tested. *HbGTPase1* transcripts accumulated at relatively low levels in the flower, latex, and leaves, while *HbGTPase1* transcripts accumulated at

relatively high levels in bark. Transcription of *HbGTPase1* in the latex was induced by jasmonate.

Key words: GTPase; *Hevea brasiliensis*; Latex; Jasmonate

INTRODUCTION

GTPases are grouped into 2 major classes, the small GTPases and the heterotrimeric G proteins (Assmann, 2002). Small GTPases, proteins with molecular masses between 21 and 30 kDa, are monomeric guanine nucleotide-binding proteins related to a subunit of heterotrimeric G proteins (Yang, 2002). They are universal molecular switches, regulating several cellular processes such as vesicle trafficking, cytoskeletal dynamics, cell polarity, and gene expression, by cycling between an "activated" state when bound to GTP and a GDP-bound inactive state (Takai et al., 2001; Vernoud et al., 2003). Upon stimulation by an upstream signal, the GTP-bound active form interacts with specific downstream effector proteins, which leads to the regulation of cellular responses and developmental processes that are highly conserved throughout eukaryotes (Molendijk et al., 2004; Wu et al., 2011). The small GTPase superfamily is divided into at least 5 families, including Ras, Rho, Arf, Ran, and Rab, although there are no Ras GTPases in plants (Vernoud et al., 2003). Members of this class of proteins are among the largest families of signaling proteins in eukaryotic cells (Yang, 2002; Vernoud et al., 2003).

Hevea brasiliensis Muell-Arg is the only cultivated species, and the main source, of natural rubber (Gouvêa et al., 2010). The economic importance of natural rubber has prompted investigations regarding the biochemistry and cell biology of latex biogenesis (Kush, 1994), but the physiological processes of latex remain mysterious (Ko et al., 2003). Latex constitutes the cytoplasmic component of the laticifers and latex vessels in *H. brasiliensis*. Although laticifers are the major location for rubber biosynthesis, the molecular mechanisms of this pathway and the regulation of natural rubber production in *H. brasiliensis* are not well known (Chow et al., 2007). In this study, we report results of the cloning and characterization of the small GTPase gene from *H. brasiliensis* (GenBank No. JQ943654). To our knowledge, this is the first small GTPase gene cloned and characterized from *H. brasiliensis*. The expression profiles of HbGTPase1 in *H. brasiliensis* were also investigated. The present study contributes to an understanding of the molecular characterization of the small GTPase and its possible function in the rubber tree.

MATERIAL AND METHODS

Plant material

Virgin rubber trees (*H. brasiliensis*) of clone RY-7-33-97 were planted in 2007 in the Experimental Farm of the Chinese Academy of Tropical Agriculture Science. The selected trees with a girth of 50-55 cm were treated with 0.2% ethephon and 0.1% jasmonic acid (JA), following the Hao method (Hao and Wu, 2000). For latex RNA extraction, rubber trees were tapped using a tapping knife. The first few drops of latex, which contained mostly debris from the plant, were discarded. The latex was allowed to drop directly into liquid nitrogen in an ice kettle. The frozen latex powder was either stored at -70°C or used immediately. Rubber tree

leaves, flowers, and buds were washed with double-distilled H_2O to remove latex and immediately frozen in liquid nitrogen. Frozen leaves, flowers, and bark were stored at -70°C or immediately used.

Isolation of RNA

Total RNA was extracted according to methods described in Tang et al., 2007. The quality and concentration of the extracted RNA were checked by agarose gel electrophoresis and measured by spectrophotometry (DU-70, Beckman, USA).

3'-Rapid amplification of cDNA ends (RACE) of *HbGTPase1*

3'-Ready cDNA was synthesized by reversely transcribing 1 µg total RNA with 3'-CDS primer A (provided in the kit). 3'-RACE primer 3P1 (5'-AGCTTCTGGTTGGGAACAA GTGTGATC-3') was designed and synthesized based on the expressed sequence tag (EST) from the latex cDNA library. 3'-RACE was carried out in a total volume of 25 mL, containing 1 µL 3'-ready cDNA, and run for 35 cycles of amplification (94°C for 15 s, 68°C for 30 s, and 72°C for 1 min). The product was purified and cloned into a pGEM-T easy vector (Promega), followed by sequencing.

5'-RACE of *HbGTPase1*

An aliquot of 1 µg total RNA was reverse transcribed with 5'-CDS primer A and SMART II A oligonucleotides (provided in the kit) to obtain the 5'-ready cDNA, using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). 5'-RACE primer 5P1 (5'-AATGCCATGTGCTCCACGGTAATAGCT-3') and nested primer 5P2 (5'-CGTTCTTGTCCTGCAGTATCCCAAATT-3') were designed and synthesized based on the EST from the latex cDNA library. Primary 5'-RACE polymerase chain reaction (PCR) was performed with the 5P1 and UPM primers (provided in the kit) in a total volume of 25 µL containing cDNA, and was denatured at 94°C for 3 min, followed by 32 cycles of amplification (5 s denaturation at 94°C, 10 s annealing at 68°C, and a 1-min extension at 72°C). The product of the primary PCR was diluted 50-fold, and was used as a template for nested PCR performed with the primers 5P2 and NUP (provided in the kit). The product was purified and cloned into the pGEM-T easy vector (Promega) and sequenced.

Cloning of the full-length cDNA of *HbGTPase1*

After alignment and assembly of the internal conserved sequence and the 3'- and 5'-RACE products, the full-length cDNA sequence of the *HbGTPase1* gene was deduced and subsequently obtained by PCR. The open reading frame (ORF) of *HbGTPase1* was amplified by reverse transcription PCR (RT-PCR), using the primers HbGTPase1F (5'-AAGAGGAGA ATCAGAGC-3') and HbGTPase1R (5'-TTTACAGCTCTCTATGCGGCAAC-3') with the TAKARA RNA PCR kit. An aliquot of 1 µg total RNA was reversely transcribed according to manufacturer protocols, and 2 µL cDNA was used in PCR in a total volume of 50 µL under the following program: 94°C for 2 min, followed by 32 cycles of amplification (94°C for 15 s,

58°C for 30 s, and 72°C for 2 min). The PCR product was purified and cloned into the pGEM-T easy vector (Promega) followed by sequencing.

Multiple alignments and bioinformatic analyses

The nucleotide sequence, deduced amino acid sequence, and ORF encoded by *HbGTPase1* were analyzed. The sequence comparison was conducted by a database search using the BLAST program (http://www.ncbi.nlm.nih.gov). Multiple alignments were carried out using the ClustalX software, version 1.81 (Thompson et al., 1997).

Analysis of *HbGTPase1* expression

The quantitative PCR (qPCR) primer P1 (5'-CAGAAGTCTGGATGCTGCTCTTCT-3') and primer P2 (5'-TGGTTTAAGGTGGGAATAGTCTCT-3') were designed and synthesized based on the *HbGTPase1* sequence. The qPCR was performed using the fluorescent dye SYBR-Green (Takara, Dalian, China) and the BIO-RAD CFX96 Real-Time PCR system (Bio-Rad, USA). The reactions were carried out as follows: 30 s at 95°C for denaturation, 5 s at 94°C, 20 s at 60°C, and 20 s at 72°C for amplification. Three biological replicates were carried out and triplicate quantitative assays for each replicate were performed. A rubber tree *Actin* gene was amplified as an internal control. The relative abundance of transcripts was calculated according to the Bio-Rad CFX Manager (Version 1.5.534) of BIO-RAD CFX96.

RESULTS

Cloning and characterization of *HbGTPase1*

The EST from the latex cDNA library, which showed similarity to other plant small GTPase genes based on a BLASTX search, was obtained in a previous study (Liang et al., 2009). The BLASTX search revealed that the EST contained part of the ORF of the small GTPase gene. Based on the sequence of the fragment, 3'- and 5'-RACE primers were designed to amplify unknown 3'- and 5'-end sequences of *HbGTPase1* cDNAs, using 3'- and 5'-RACE strategies. The amplified products of the 3'- and 5'-end cDNA fragments were 289 and 453 bp in length, respectively. By alignment and assembly of these 2 sequences with the EST, the full-length cDNA sequence of *HbGTPase1* was deduced, amplified by PCR, and ultimately confirmed by sequencing. The full-length cDNA was 882 bp in length, and it contained a 612-bp ORF, with a 3'-untranslated region (UTR) of 187 bp downstream of the stop codon, and a 5'-UTR of 83 bp upstream of the start codon (Figure 1).

The deduced HbGTPase1 protein consisted of 203-amino acid residues with a calculated molecular weight of 22.62 kDa and an isoelectric point of 5.06. The HbGTPase1 protein contains the conserved functional regions and domains of the small GTPase superfamily of proteins (Figure 1), which are shared by the majority of GTPases (Bourne et al., 1991; Dombrowski et al., 2008). Domains G1-G3 are involved in binding magnesium and GTP phosphate residues and domains G4 and G5 are involved in binding the guanine moiety (Bourne et al., 1991).

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ctctcccaccctcgtcgcttctacctgcaatttccaattcacttcccgcctggaccggtccaatccctgtcgacctcaccgtc  83
ATGAATCCTGAATATGACTATCTGTTTAAGCTTTTGCTAATTGGGGATTCTGGAGTTGGCAAATCATGTCTTCTTCTGAGGTTT  167
 M  N  P  E  Y  D  Y  L  F  K  L  L  L  I  G  D  S  G  V  G  K  S  C  L  L  L  R  F   28
                                 G1
GCGGATGATTCATATCTGGACAGTTACATCAGTACAATTGGAGTTGACTTTAAAATTCGCACTGTAGAGCAAGATGGGAAGACC  251
 A  D  D  S  Y  L  D  S  Y  I  S  T  I  G  V  D  F  K  I  R  T  V  E  Q  D  G  K  T   56
                      G2
ATTAAACTTCAAATTTGGGATACTGCAGGACAAGAACGATTCAGGACAATCACAAGTAGCTATTACCGTGGAGCACATGGCATT  335
 I  K  L  Q  I  W  D  T  A  G  Q  E  R  F  R  T  I  T  S  S  Y  Y  R  G  A  H  G  I   84
          G3
ATAATAGTTTATGATGTGACAGACCAAGAGAGTTTCAACAATGTTAAGCAATGGTTGAACGAAATTGATCGTTATGCTAGTGAA  419
 I  I  V  Y  D  V  T  D  Q  E  S  F  N  N  V  K  Q  W  L  N  E  I  D  R  Y  A  S  E  112
       F5
AATGTAAACAAGCTTCTGGTTGGGAACAAGTGTGATCTCACTGCTAACAAAGTTGTTTCTTATGAAACAGCCAAAGCATTTGCT  503
 N  V  N  K  L  L  V  G  N  K  C  D  L  T  A  N  K  V  V  S  Y  E  T  A  K  A  F  A  140
                   G4
GATGAAATTGGCATTCCATTCATGGAGACAAGTGCAAAAAATGCTACCAATGTAGAGGAGGCTTTTATGGCAATGGCTGCTGAC  587
 D  E  I  G  I  P  F  M  E  T  S  A  K  N  A  T  N  V  E  E  A  F  M  A  M  A  A  D  168
                      G5
ATCAAGAACAGGATGGCAAGTCAACCAGCAGCAAACAATGCAAGGCCACCGACTGTGCAGATACGTGGGCAACCGGTTAACCAG  671
 I  K  N  R  M  A  S  Q  P  A  A  N  N  A  R  P  P  T  V  Q  I  R  G  Q  P  V  N  Q  196
AAGTCTGGATGCTGCTCTTCTTAAgtggctctggactgacctcacgtggcatcatcggtgcatccaaatgtggcttctcggttg  755
 K  S  G  C  C  S  S  *                                                              203
tcctggagttagagactattcccaccttaaaccataaaatgattgcgtcatcaattgttctgcatgcaattcaaaagagtttgt  839
tttcttttttatttattgttaatgtcttgtaaaaaaaaaaaaa                                          882
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Figure 1. Nucleotide and deduced amino acid sequences of *HbGTPase1*. Numbers corresponding to the nucleotide and amino acid sequence are indicated on the left and right, respectively. Conserved domains of the GTPase superfamily of proteins are highlighted in gray, labeled G1-G5. Domains G1-G3 are involved in binding magnesium and GTP phosphate residues and G4 and G5 are involved in binding the guanine moiety.

The phylogenetic analysis of HbGTPase1 was constructed using closely related small GTPase members from *Arabidopsis thaliana*. The tree revealed that the HbGTPase1 protein is located on a branch with the *A. thaliana* Rab GTPase (Figure 2).

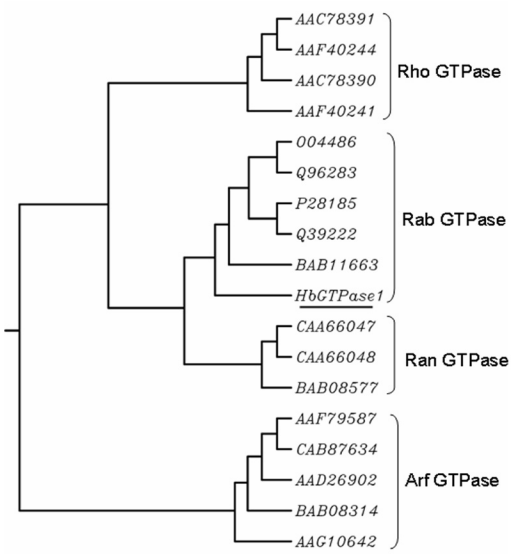

Figure 2. Phylogenetic tree of HbGTPase1 and small GTPase members from *Arabidopsis thaliana* with indicated GenBank accession numbers.

Differential expression of *HbGTPase1* in different tissues

Accumulation of *HbGTPase1* transcripts in different tissues was examined using qPCR analysis. The results showed that *HbGTPase1* was constitutively expressed in all tissues tested. *HbGTPase1* transcripts accumulated at relatively low levels in flowers, latex, and leaves, while *HbGTPase1* transcripts accumulated at relatively high levels in bark (Figure 3).

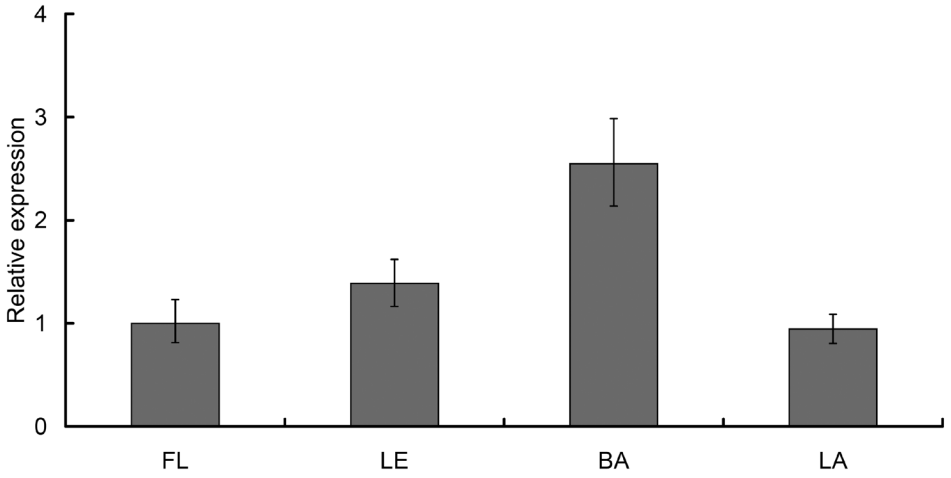

Figure 3. Transcription pattern analysis of *HbGTPase1*. The qPCR was performed on RNA derived from various tissues from the rubber tree. *Actin* was amplified for normalization. FL = flowers; LE = leaves; BA = bark; LA = latex.

Effects of ethephon and JA on *HbGTPase1* expression in latex

Ethephon, a stimulant of latex production in the rubber tree, has been widely used in commercial latex production practices (Zhu and Zhang, 2009). Furthermore, biosynthesis of natural rubber is enhanced in rubber trees by the endogenous accumulation and the exogenous application of JA (Yu et al., 2007). Therefore, we tested whether ethephon and JA application had any effect on *HbGTPase1* expression in laticifer cells. The qRT-PCR analysis demonstrated that the *HbGTPase1* transcript was induced by JA, while ethephon was not effective in inducing *HbGTPase1* expression (Figure 4).

DISCUSSION

Small GTPases regulate diverse processes in eukaryotic cells such as cell proliferation, cytoskeletal organization, signal transduction, and intracellular membrane trafficking (Yang, 2002; Vernoud et al., 2003; Gu et al., 2004; Nielsen et al., 2008). A phylogenetic analysis revealed that small GTPases are classified into 4 groups in plants: Rab, Rho, Arf, and Ran (Vernoud et al., 2003). In this study, we present results on the cloning and characterization of the gene encoding a small GTPase from the rubber tree. The HbGTPase1 protein contained highly conserved motifs typical of the GTPase superfamily. When the HbGTPase1 protein was compared to representative members of *Arabidopsis* small GTP binding proteins,

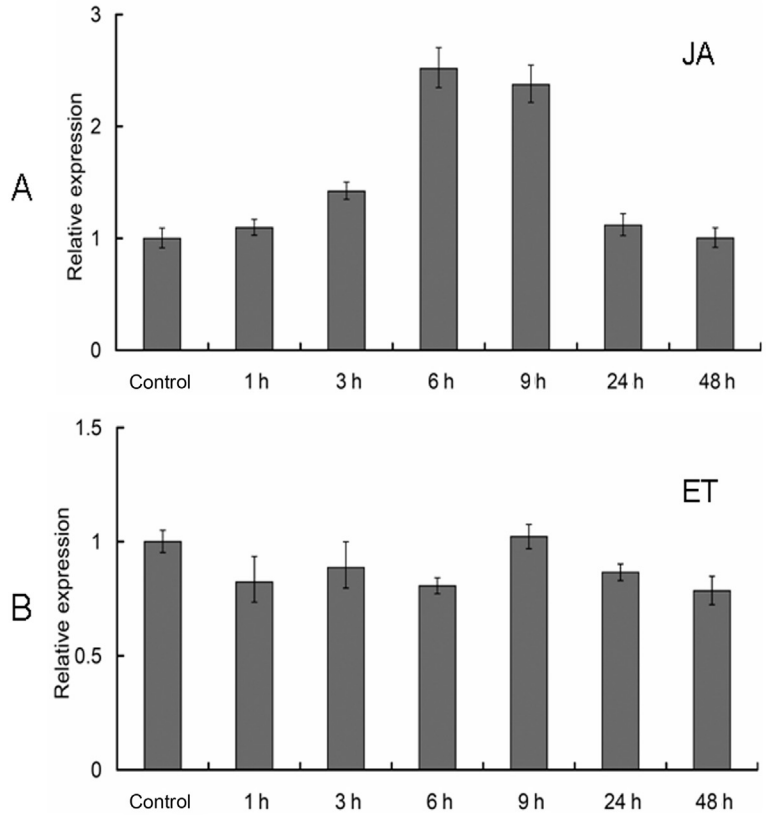

Figure 4. Effects of hormones on the expression of *HbGTPase1* in the latex. RNA extracted from the extruded latex of rubber trees treated with ethephon or JA for 0 (control), 1, 3, 6, 9, 24, or 48 h were subjected to qRCR assay; *Actin* was used as a control. **A.** The *HbGTPase1* transcript in latex is induced by jasmonic acid (JA) treatment. **B.** The *HbGTPase1* transcript is not induced following ethephon treatment (ET).

it was grouped with the Rab subfamily. The Rab subfamily of GTPases has been shown to be involved with stress tolerance. In jojoba and ice plants, a Rab5 GTPase family member was upregulated in response to salt stress (Bolte et al., 2000; Mizrahi-Aviv et al., 2002). The *OsRab7* gene was differentially regulated in response to cold, salt, dehydration, and abscisic acid in rice (Nahm et al., 2003). The *smGTP* gene, which is related to the *Rab2* gene family of GTPases, was strongly induced by salt stress in root, crown, and leaf tissues in *Lolium temulentum* (Dombrowski et al., 2008). In the present study, qRT-PCR analysis showed that the *HbGTPase1* transcript was upregulated in response to JA.

JA is a key signal in the elicitation of secondary metabolism, an important defense response. Elicitors of the processes of secondary metabolism induce JA biosynthesis as an early response, and JA subsequently induces expression of genes that code for the synthesis of enzymes involved in the biosynthetic processes involved in secondary metabolism (Menke et al., 1999; van der Fits and Memelink, 2000). JA can induce laticifer differentiation and may regulate rubber biosynthesis (Hao and Wu, 2000; Wu et al., 2002; Yu et al., 2007; Peng et al.,

2009). It will be of great interest to elucidate whether or not rubber biosynthesis is regulated by small GTPases. Cloning more small GTPase genes should provide further understanding about the relationship between JA signal transduction, small GTPases, and the biosynthesis and regulation of natural rubber in the rubber tree.

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