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Molecular cloning and characterization of an intronless farnesyl diphosphate synthase (FDP) gene from Indian rubber clone (*Hevea brasiliensis* Muell. Arg. RRII105): A gene involved in isoprenoid biosynthesis

P. Venkatachalam [a,d,*], N. Geetha [b], A. Thulaseedharan [c], Shivendra V. Sahi [d]

[a] Department of Biotechnology, Periyar University, Salem 636 011, TN, India
[b] Department of Biotechnology, Mother Teresa Women's University, Kodaikanal 624 101, TN, India
[c] Advanced Centre for Biotechnology and Molecular Biology, Rubber Research Institute of India, Rubber Board, Kottayam 686 009, Kerala, India
[d] Department of Biology, Western Kentucky University, Bowling Green, KY 42101, USA

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ABSTRACT

Farnesyl diphosphate synthase (FDP) is a key intermediate in the biosynthesis of isoprenoid compounds and it is also the allylic diphosphate initiator of natural rubber (*cis*-1,4-polyisoprene) biosynthesis in plants. This report describes the isolation and characterization of full-length cDNA as well as genomic DNA encoding *HbFDP* from an elite Indian rubber clone (RRII 105) and its expression analysis in *Arabidopsis* plant. A 1.2 kb cDNA encoding farnesyl diphosphate synthase was isolated and the nucleotide sequence analysis revealed an open reading frame of 1029 nucleotides which encoded a protein of 342 amino acids with a predicated molecular mass of 39.4 kDa. It is interesting to note that the genomic FDP gene fragment did not contain any introns (intronless) and has a continuous open reading frame encoding a peptide of 342 amino acids. The deduced amino acid sequence of the *HbFDP* named HbFDP exhibited a high homology with other plant FDPs. Phylogenetic tree analysis showed that HbFDP belonged to the plant FDP group. The putative protein sequence analysis of predicated *HbFDP* cDNA showed the presence of 2 transmembrane motifs (DDIMD; DDYLD) in the catalytic site. It also contains the DDXXD motifs that are characteristic of prenyltransferases. Genomic southern blot analysis indicated that *HbFDP* is a member of small gene family in *Hevea*. Semi-quantitative RT-PCR results clearly indicated that *HbFDP* gene was differentially expressed in various tissues examined. However, the abundance of mRNA transcript level was found to be high in laticifer cells which indicate the possible involvement of *HbFDP* gene in natural rubber biosynthesis in *Hevea*. Further to validate the role of *HbFDP* gene in plants, *HbFDP* cDNA was over-expressed in transgenic *Arabidopsis* plants. Semi-quantitative RT-PCR analysis showed that accumulation of *HbFDP* mRNA transcripts was 3–5 folds higher in transgenic *Arabidopsis* plants than control. This data suggest that *HbFDP* gene might play an important role in the regulation of plant growth and isoprenoid biosynthesis.

1. Introduction

Hevea brasiliensis (Muell.) Arg., also known as Para rubber tree, is a perennial tree crop which is the principal commercial source for natural rubber (NR) production. Rubber trees start producing latex after attaining 5–6 years immaturity period and have a latex productive lifespan of 25–30 years. It is reported that the global production of NR reached nearly 11 million tons in 2011 with Asia accounting for about 93% of the supply (Rahman et al., 2013). However, the demand for rubber (natural and synthetic) has steadily increased now and is expected to continue to increase in the years to come. Evans (2011) pointed out that in 2010 the consumption of natural rubber was 10.7 million tons for all rubber industry (tire and non-tire) which is predicted to rise to 15.4 million tons by 2020. Also the natural rubber will continue to play key roles in rubber product based industries across the world. *H. brasiliensis*, a tropical tree originating from South America, is widely cultivated in South America, Africa and South East Asia for the production of natural rubber (*cis*-1,4-polyisoprene) which is a mixture of high molecular weight polymers present in the latex. Among more than 2000 plant species known to produce natural rubber, the Brazilian rubber tree (*H. brasiliensis*) is the only commercial source at

Abbreviations: HbFDP, *Hevea brasiliensis* farnesyl diphosphate synthase; PCR, Polymerase chain reaction; RT-PCR, Reverse transcription polymerase chain reaction; RRII, Rubber Research Institute of India; RRIM, Rubber Research Institute of Malaysia.

* Corresponding author at: Department of Biotechnology, Periyar University, Salem 636 011, TN, India.
E-mail address: pvenkat67@yahoo.com (P. Venkatachalam).

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present (Venkatachalam et al., 2009). Due to the depletion in reserve fossil fuel for synthetic rubber, nowadays a great preference is provided by consumer for natural rubber production. Furthermore, to meet the anticipated demand for enhanced production of latex yield could depend on the available new rubber clones that are addressing various threats to rubber cultivation including long immaturity and low yield (Nugawela and Jom, 2011).

Improvement of various tree species including rubber tree by conventional breeding is hindered by their long gestation periods, loss of desired genetic recombination, and occurrence of high degree of heterozygosity due to cross pollination. Biotechnological tools such as genetic transformation offer an attractive supplement to the conventional crop breeding program, because it provides the potential to rapid transfer of desirable genes for specific traits into selected clones without affecting their desirable genetic background (Jayashree et al., 2003). Therefore, isolation and cloning of key rubber biosynthetic genes are one of the prerequisites to develop genetically engineered rubber tree clones with enhanced latex production. A series of efforts has been made to isolate and characterize key genes or enzymes involved in rubber biosynthesis in *H. brasiliensis* (Attanyaka et al., 1991; Goyvaerts et al., 1991; Adiwilaga and Kush, 1996; Oh et al., 1999; Priya et al., 2006, 2007). However, the function and role of the proteins suggested to be involved in rubber biosynthesis in these reports remain to be verified. Rubber is biosynthesized by the sequential condensation of isopentenyl diphosphate (IDP) to the initiating allylic diphosphates such as geranyl diphosphate, farnesyl diphosphate (FDP), and geranylgeranyl diphosphate. farnesyl diphosphate synthase enzyme catalyzes 1'4 condensation of the 5-carbon isoprenoid compounds isopentenyl diphosphate (IPP) and 10-carbon geranyl diphosphate (GPP) to form the 15-carbon product farnesyl diphosphate (FPP).

FDP is considered as key intermediate in the isoprenoid biosynthetic pathway, since it is the common precursor of polyisoprene units and farnesylated proteins (Cao et al., 2012). In order to understand the role of FDP gene in rubber-producing plants it is of critical importance to clone and characterize the genes and/or enzymes involved in rubber biosynthesis in different rubber clones. Due to the important role of FDP in isoprenoid biosynthesis, it has already been isolated and characterized from many plant species including *H. brasiliensis* (clone RRIM 600) (Adiwilaga and Kush, 1996), *Artemisia annua* (Hemmerlin et al., 2003), *Arabidopsis* (Closa et al., 2010; Keim et al., 2012), *Chimonanthus praecox* (Xiang et al., 2010), *Withania somnifera* (Gupta et al., 2011), *Euphorbia pekinensis* (Cao et al., 2012), *Hedychium coronarium* (Lan et al., 2013), *Medicago sativa* (Sun et al., 2013), *Rhizosolenia setigera* (Ferriols et al., 2015), *Tripterygium wilfordii* (Zhao et al., 2015), and *Matricaria recutita* (Su et al., 2015). It has been reported that FDP synthase catalyzes the synthesis of the last common substrate in the isoprenoid biosynthesis. Hence, it is a branch point and a likely regulatory enzyme in the pathway, the availability of the cloned FDP gene from Indian *Hevea* clone should be useful for further investigation of the regulation of rubber biosynthesis and generation transgenic rubber plants for enhanced latex yield. However, as far as we know, the cDNA encoding for *FDP* gene has been reported from RRIM 600 a Malaysian rubber clone, there were no reports from Indian high yielding rubber clone RRII105 till now. The major reason is that RRIM 600 rubber clone is a low latex yielder in India and the RRII105 is the most popular high yielding rubber clone which is being cultivated and occupied in rubber growing regions of India. Therefore, isolation and characterization of FDP gene from Indian popular clone RRII 105 will be highly useful for understanding the molecular mechanism of this gene and also for overexpression of this gene under a constitutive promoter for enhanced latex yield production in transgenic rubber plant in the future.

In this communication, we report the cloning and characterization of an *HbFDP* gene from genomic DNA as well as cDNA from RRII 105 rubber clone. We also demonstrated the expression pattern of *FDP* gene in different types of tissues by semi-quantitative RT-PCR analysis. In addition, the *HbFDP* gene was over-expressed by cloning into a binary vector and expression pattern was studied in transgenic *Arabidopsis* plants.

2. Materials and methods

2.1. Plant material

Rubber trees (clone RRII 105; Rubber Research Institute of India, RRII) grown in the experimental field were used for all experiments. Leaf samples (free from any infection) were collected and transported to the lab for genomic DNA extraction. Fresh latex sample from tapped trees was collected into a falcon centrifuge tube with extraction buffer for RNA isolation.

2.2. Genomic DNA extraction and PCR amplification of HbFDP gene

Total genomic DNA was extracted from the leaf tissues of *H. brasiliensis*, clone RRII 105 as described by Venkatachalam et al. (2002). FDP gene specific oligonucleotide primers were designed based on the published sequence from RRIM 600 clone (Malaysian clone) by Adiwilaga and Kush (1996) viz., forward primer: 5′-CTCTCC GTTTGAATCCATGGCGGAT-3′ and reverse primer: 5′-TAGTTTGGAGTT GTATCTTAGAA-3′ and were used for PCR amplification of FDP gene from genomic DNA, PCR reaction was performed in a 20 μl reaction volume, which consisted of 10× buffers, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTPs, 0.5 U of Taq DNA polymerase, 25 ng template DNA and 250 nM of forward primer and reverse primer. The DNA amplification was performed in a thermal cycler (Perkin Elmer, USA) and PCR cycling parameters were 94 °C for 4 min followed by 30 cycles of 60 s at 94 °C, 90 s at 55 °C, 2 min at 72 °C and then followed by a 10 min final extension at 72 °C.

2.3. Total RNA extraction and FDP cDNA synthesis by RT-PCR

RNA was isolated as described previously by Venkatachalam et al. (1999), treated with DNaseI and subsequently reverse transcription (RT) of total RNA was carried out. The 50 μl RT reaction contained 5 μg total RNA, 50 pmol oligo dT primer, 50 mM dNTPs mix, 10 mM DTT, 1 μl RNase inhibitor and 1 μl (40 U) Superscript II reverse transcriptase (Invitrogen, CA) in 10× buffer supplied by the manufacturer. The RNA and primers were preheated to 70 °C for 10 min and snap-cooled in ice before adding the remaining components, the RT reactions (1 h, 42 °C), were terminated by heating at 70 °C for 15 min. The cDNA (2 μl) was then used for PCR amplification of FDP gene using specific primers (5′-TCTCCGTTTGAATCCATGGC-3′ and 5′-ATTCTGCTTGTAGC ACATGG-3′) as described above and the amplified cDNA insert was used for cloning and further characterization analysis.

2.4. Cloning and nucleotide sequence analysis

The PCR amplified FDP gene from genomic DNA as well as cDNA fragments were purified and cloned into pGEM-T plasmid vector as per the manufacturer's instructions. Presence of the cloned inserts in the recombinant plasmid was further confirmed through the release of insert by restriction digestion with *Not*I enzyme as well as by PCR analysis. The nucleotide sequence of the cloned DNA fragments was determined using the automated sequencing facility (Indian Institute of Science, Bangalore). The nucleotide sequence of FDP was edited to discard the vector contamination sequences at either ends and compared with published sequences in the NCBI database using BLASTN program (Altschul et al., 1990). Sequence alignment and comparison were made using the ClustalW program (Thompson et al., 1994).