Cloning and Expression Analysis of RACE Mediated Farnesyl Diphosphate Synthase *(fpps)* Gene in Indian lac Insect *Kerria lacca* (kerr).

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Abstract

Lac insect is an economically important insect and the only animal species secreting resin. The main component of lac resin is sesquiterpenes, and farnesyl pyrophosphate (FPP) is the precursor for sesquiterpene formation in terpenoid biosynthetic pathway. The enzyme involved in the formation of FPP is the FPPS. The molecular cloning of coding region of FPPS gene provided the information helpful for further applied studies in the area of genetic manipulation in lac insects to increase lac resin productivity and its expression profile revealed the function of the enzyme and its role in biosynthetic process of resin secretion in different life stages of *K. lacca*. After the sequencing and assembly of the partial FPPS fragment and 3'RACE product, it resulted in 769 bp sequence of *K.lacca* FPPS encoding gene. The final product revealed a great homology with the mitochondrial isoprenyl diphosphate synthase gene reported in Bird cherry-oat aphid (*Rhopalosiphum padi*). The expression profile study revealed that this gene expression got upregulated in settled larvae and adult female lac insects in comparison to crawlers. The expression profile pattern reveals that the cloned *FPPS* gene may have some putative role in development of hormone biosynthesis or in resin biosynthesis.

***Index Terms* : *Kerria lacca*, Farnesyl Diphosphate Synthase (*fpps*) gene, RACE, cloning, RT-qPCR**

I.Introduction

Lac is a natural resin, that is cultivated and collected by tribes inhabiting the sub-hilly tracts of Jharkhand, Chhattisgarh, West Bengal, Madhya Pradesh, Maharashtra, Odisha and in some other states of India. Its importance is basically due to resin, dye and wax. Lac resin is used in painting varnishes, cosmetics (for example, nail polishes), ornaments (bangles), food industry (sweets), pharmaceutics (for example tablet coating) and polishing of fruits and vegetables, lac dyes are used in colouring silk and woollen fabrics and lac wax is used as a substitute for carnauba wax. Lac is the scarlet resinous secretion of a number of species of lac insects, of which the most commonly cultivated species in India is *Kerria lacca* (Kerr). Lac resin is a polymer of poly unsaturated fatty acids and sesquiterpenes connected by ester and lactide linkages. Sesquiterpenes are a class of terpenes that consist of three isoprene units and have the empirical formula $C_{15}H_{24}$. Biochemical modifications such as oxidation or rearrangement produce the related sesquiterpenoids. Sesquiterpenes are found naturally in plants and insects, as semiochemicals, e.g. defensive agents or pheromones. The reaction of geranyl pyrophosphate with isopentenyl pyrophosphate results in the 15-carbon farnesyl pyrophosphate which is an intermediate in the biosynthesis of sesquiterpenes. Oxidation of farnesene then provides sesquiterpenoids. The enzyme involved in the biosynthesis of farnesyl pyrophosphate is the farnesyl diphosphate synthase.

Gracia and Couillaud (1999) observed that the enzyme farnesyl-diphosphate synthase, which has been shown to play a key role in isoprenoid biosynthesis, catalyzes the synthesis of farnesyl diphosphate from isopentyl diphosphate and di-methylallyl diphosphate. FPP is considered to be a branching point in the synthesis of different types of natural isoprenoids. Furthermore, it catalyzes a key step in the biogenesis of isoprenoid phytoalexins as suggested by Stoessl et al. (1977). Wang et al. (2014) observed by using

Gas chromatography analysis the recombinant farnesyl pyrophosphate synthase (FPS) catalyzed the formation of farnesyl diphosphate (FPP) from geranyl diphosphate (GPP) and isopentenyl diphosphate (IPP), hence supposed to be an important enzyme involved in the resin biosynthesis of the Indian lac insect *K. lacca*.

The FPPS encoding gene sequence information and its expression profile are highly required to decipher the function of the enzyme and its role in biosynthetic process of resin secretion. This knowledge will also be helpful for further applied studies in the area of genetic manipulation in lac insects to increase lac resin productivity. Hence the cloning of the gene encoding for FPPS in lac insect is attempted for the first time in this study.

The molecular cloning of coding region of FPPS gene involves the amplification of cDNA using primers designed from the transcriptome data. At first, partial gene can be cloned by PCR with those primers. RACE is a PCR based technique which facilitates the cloning of full length cDNA sequences when only a partial cDNA sequence is available. FPPS gene expression in different life stages of *K. lacca* can also be studied by using the RT-qPCR technique. Gene-expression analysis is increasingly important in many fields of biological research. Understanding the patterns of expressed genes is expected to provide insight into complex regulatory networks and will most lead to the identification of genes related to new biological processes.

With this background, this work, the first in the field of cloning and studying the FPPS gene in *K. lacca* was initiated with the objectives, to isolate the partial gene coding for FPPS from the Indian lac insect, *K. lacca*, to carry out 3′ and 5′ RACE for FPPS coding gene, to analyze the sequence of FPPS gene obtained from *K. lacca* and to study the expression of FPPS gene in different life stages of *K. lacca*.

II. Materials and methods

2.1 Sample preparation

Lac insect samples LIK21 (Cross Breed) were collected from National lac Insect germplasm conservation (NATLIGEC) maintained at Research farm of ICAR-Indian Institute of Natural Resins and Gums, Namkum, Ranchi. They were kept in 70% alcohol and washed thrice to remove the resin. Then dried on a tissue paper to remove the alcohol and 30 mg was weighed in separate 1.5 ml eppendorf tubes. It was then kept at -60 °C.

2.2 Isolation of RNA and synthesis of cDNA

RNA was isolated using HiPurA total RNA miniprep purification kit (HiMedia Cat. no. MB602- 50PR) following the user's manual. The RNA thus obtained was quantified on the nanodrop spectrophotometer (Thermo Scientific Nanodrop 2000). Table 1 reveals the RNA concentrations. The elute (1e and 2e) containing pure RNA was stored at -20 °C. cDNA was synthesized in an RNase free microfuge tube on ice; using Ambion RLM-RACE Kit, (Invitrogen Cat. no.- AM1700) following the user's manual.

2.3 Designing of primers

Primers (FPPS_Y_F.1 and FPPS_Y_R.1) were used to clone the partial FPPS gene and were designed using the trancriptome of *Kerria lacca* (unpublished data). Table 2 deciphers the primer sequences.

2.4 Amplification of partial FPPS sequence

PCR was carried out for amplification of the partial gene sequence by preparing a cocktail of 25 µl volume containing 2.5 µl of 10X KCl Buffer, 2.0 µl of 25 mM $MgCl_2$, 0.5 µl 10 mM dNTPs Mix, 1.0 µl of each forward and reverse primer of 10 µM concentration, 0.25 µl of Taq Polymerase (5u/µl) and 1.0 µl of template cDNA. The reaction was carried out in SensoQuest, Lab cycler following the cycle, 94°C for 3 min, 35 cycles of 94°C for 30 sec, 43°C for 30 sec, 72°C for 30 sec and final extension step of 72°C for 7 min. The amplified band of the 3′ RACE was eluted by using; MinElute Gel Extraction Kit (50)

(Qiagen Cat.no. 28604) following the instructions and sent for sequencing to Xcelris Labs Pvt. Ltd., Ahmedabad.

2.5 PCR for RLM-RACE using gene specific primers

2.5.1 PCR for 3′ RLM-RACE

2.5.2 Outer 3′ RLM-RACE PCR

cDNA was diluted to 1:5 and 1:10 for PCR. All other components were same as that of partial sequence PCR except the primers. 3′ RACE gene specific outer primer1 was taken as forward primer whereas 3′ RACE outer primer acted as reverse primer. The primer sequences were included in Table 2. The PCR cycle followed was initial denaturation phase of 94°C for 3 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and final extension step of 72°C for 7 min.

2.5.3 Nested outer 3' RLM-RACE PCR

All the conditions were same as that of outer 3′ RLM-RACE PCR except the primers used. The 3′ RACE gene specific outer primer2 as forward primer and 3′ RACE inner primer as reverse primer for amplification.

2.5.4 Inner 3' RLM-RACE PCR

All the components and reaction conditions were same as that of nested outer 3′ RLM-RACE PCR except the primers. The 3′ RACE gene specific inner primer acted as forward primer and 3′ RACE inner primer was taken as reverse primer.

The amplified fragment size was checked at each step on 1% agarose gel.

2.6 5′ RLM-RACE

2.6.1 Processing of RNA

The processing of RNA was done following the user's manual of RLM-RACE Kit, (Invitrogen Cat. no.- AM1700) with a series of steps like, Calf Intestine Alkaline Phosphatase (CIP) treatment, then extraction with phenol:chloroform followed with chloroform. This step was followed by treatment with Tobacco Acid Pyrophosphate (TAP). Now, the CIP/TAP treated RNA was ligated to 5'-RACE adapter.

2.6.2 Reverse Transcription

The reverse transcription for the synthesis of cDNA with this ligated RNA was carried out by using Ambion RLM-RACE Kit, (Invitrogen Cat. no.- AM1700).

2.7 Nested PCR for 5′ RLM-RACE

2.7.1 First Outer 5′ RLM-RACE PCR

cDNA was diluted to 1:1 prior to PCR. The amplification was carried out with 5′ RACE gene specific outer primer2 as forward primer and 5′ RACE outer primer as reverse primer. The programme set for PCR was at 94°C for 3 min, 35 cycles of 94°C, 30 sec, 30 sec at 63°C and 30sec at 72°C followed by a final extension step of 7 min at 72°C.

2.7.2 Nested outer 5' RLM-RACE PCR

All the components and reaction conditions were same as that of outer 5′ RLM-RACE PCR except the primers. The primers used were, 5′ RACE gene specific outer primer1 and 5′ RACE inner primer.

2.7.3 Inner 5′ RLM-RACE PCR

PCR product was diluted as 1:5 and then used for inner 5′ RLM-RACE. The primers used were 5′ RACE gene specific inner primer and 5′ RACE inner primer and the PCR conditions were same.

The amplified products were checked on 1% agarose gel. Amplified products were eluted as done for partial FPPS fragment elution. The eluted DNA was quantified using the nanodrop spectrophotometer (Thermo Scientific Nanodrop 2000). The concentration were shown in table 3.

2.8 Cloning of FPPS gene

Cloning of FPPS was carried out using DH5α strain of E. coli following Chung et al. (1989) using 2X TSS solution (LB broth containing 20% (w/v) polyethylene glycol, 10% dimethyl sulfoxide and 1M $MgCl_2$ at pH 6.5). For preparation of competent cells, the overnight grown bacterial culture was diluted to 1:50 in LB Broth and incubated at 37°C until the cells reach the log phage (O.D. at 600nm is 0.4). 1ml aliquots of early log phage of bacterial culture were prepared and centrifuged at 4°C for 1-2 min. The supernatant was discarded and pellet was suspended in 1X TSS solution and stored at -70°C and used for transformation. The ligation procedure was done using TA Cloning Kit (Invitrogen Cat. no: 45-0030) following the user's manual. Frozen TSS-competent cells were thawed slowly on ice and the ligation mix (100pg -10 ng of DNA) was added to the tube of competent cells. The tubes were flicked to mix the cells and DNA and the cells were incubated on ice for 10 minutes. The tubes were then transferred to room temperature and incubated for 10 minutes. The tubes were again transferred to ice and incubated for an additional 10 minutes. 1 ml of LB broth was then added and the cells were incubated at 37°C for up to 1 hr with shaking (at 200 rpm). The cells were then plated onto the L.B Agar plates and incubated overnight at 37°C with Ampicillin (50mg/ml). For blue-white colony screening X-Gal and IPTG were used.

2.8.1 Colony PCR

Colony PCR was done with T7 and SP6 primers confirmed the transformed bacterial cells. The PCR product was then lyophilized and then sent for sequencing to Xcelris Labs Pvt. Ltd., Ahmedabad.

2.9 Expression of FPPS gene in different life stages

2.9.1 Validation of expression profiles using RT-qPCR

To study the fold difference for the expression of the FPPS gene RT-qPCR was performed for different life stages of lac insects. Three life stages of insects were taken, C (Crawlers), S (Settled Larva), and F (Female cells) (Sample ID: Simdega Stock). At First RNA was isolated from the insect samples by using HiPurA total RNA miniprep purification kit (HiMedia Cat. no. MB602-50PR). cDNA was then synthesised for the all three stages by using RevertAid First Strand cDNA Synthesis Kit (Fermentas Cat. no.-#K1621). Primers for the amplification of the FPPS gene were designed from the partial FPPS gene sequence of the Indian lac insect *K. lacca,* namely, Fs-q F1, Fs-q R1. Actin gene was used as an internal control. The actin primers used were U09207 F1 and U09207 R1. The qPCR was performed using specific primers on Agilent Technologies Stratagene MX-3005P qPCR equipment (Agilent Technologies India Pvt, Ltd).

First strand cDNA was used as template in 10 µl reactions including 5 µl 2X HiSyBr Green PCR Master Mix (HiMedia MBT074-50R) and 2.5 pmol of each primer. Two sets of reactions were set by using Actin primers (U09207 F1 and U09207 R1) in one reaction and FPPS primers (Fs-q F1 and Fs-q R1) in the other. The primer sequences were shown in table 2. All the reactions had two replications. The thermal cycle includes 5min at 95°C followed by 40 cycles of 30 sec at 95°C, 45 sec at 58°C and 30 sec at 72°C. The final segment bears 1 cycle of 95°C for 1 min, 58°C for 30 sec and 95°C for 30 sec. From the QPCR experiment, Ct values were obtained for all reactions. The average Ct was calculated for each reaction. In this study, Crawlers (C) were used as reference stage and actin was used as internal control.

Change in ct value (ΔCt) for different life stages was calculated by using the formula:

ΔCt.FPPS-ΔCt.Actin

For the calculation of ΔΔCt., the following formulae were used:

ΔΔCt.S= ΔCt..C-ΔCt.S; and ΔΔCt.F= ΔCt.C-ΔCt.F

The relative abundance of FPPS gene in different life stages was calculated by using the formula $2^{-\Delta\Delta Ct}$.

III. Results and Discussion

3.1 Cloning of the partial FPPS

To clone partial FPPS gene, PCR was carried out with the primers FPPS_Y_F.1 and FPPS_Y_R.1 using cDNA as template. This PCR yielded an amplified fragment of 807 bp (figure 1). The amplified fragment was then eluted followed by lyophilisation and sent for sequencing to Xcelris Labs Pvt. Ltd, Ahmedabad. The following sequence was obtained after sequencing the 807 bp amplicon with the primers FPPS_Y_F.1 and FPPS_Y_R.1.

3.1.1 Sequence of the partial FPPS

TCGAATAATAGAACTCTTCGAAAATCATATTTCGTTTTGGGACAACATTAGAGCTTTATCGA
TTGTTGCAACATACGAAGAATTAGCAAAAGATCGTGGTCATATTTTAACAGATGATGAATTA
TTTGATGTTTATATGCTGGGTTGGATTTTTGAAATATTCGAATCATATTCTGTGTTAGCGGAT
GACATTGTAGATGATGCTCCTTTACGATACGGAGAGCCCTCTTGGTTTTATAGGAATGATAG
AAGTCCAGTAGCATCTTATGATACATTCGCGATGATAAACTTTGGTCGAACTTTATTAAATA
AATATTTCAAGGATAAATTCTATTATTACGATATTCAAAGAAATATCACGCAGTGCACCTTG
ACATATATCTGGGGACAGCATACAGATGTATTAATACAAGATCCAAAACTTTTTGATATGAG
TAAAATGTTTAATTCTAAAGTGTATCATGATCTTGTACCATTCAAAGCTGGTTACAATTTTTC
TTGTCCCATTGATTTGGGTTTGCATATGAATGGTATATACGACGAAGAAGATGGTAGAAAA
ATTTATGAAGTTTGTCGTATAGCCGGATGCATATTACAATTTTGGGATGATTTCAAAGATTA
TCAAAAAGATTTCCTGAAGGAAAATGTTCGTGCATTATTG

3.2 Cloning of the 3′ FPPS

The cloning of 3′ FPPS was done by the 3′ RLM-RACE technique by using the Ambion RLM-RACE Kit. After initial reverse transcription, three nested PCRs were carried out using gene specific primers and 3'RACE primers given in the kit.

The first 3′ RLM-RACE outer PCR revealed a smeared band when run on 1% agarose gel (figure 2). Later on a nested outer PCR was carried out. The nested outer 3′ RLM-RACE PCR also revealed appearance of smear (figure 3).

The third PCR, that is; the 3′ RLM-RACE inner PCR resulted in the amplification of the cDNA at 600 bp, 500 bp, and 200 bp in the 1:5 diluted template, while a discrete 200 bp fragment was amplified in the 1:10 diluted template (figure 4). This amplified fragment was then eluted by using MinElute Gel Extraction Kit (Qiagen). The 3′ RACE fragment was cloned in a pCR 4-TOPO vector. A positive clone containing an insert of ≅200 bp was selected and sent for sequencing to Xcelris Labs Pvt. Ltd, Ahmedabad. The following sequence was obtained after sequencing the amplicon with 3′ RACE gene specific inner primer and 3′ RACE inner primer.

3.2.1 Sequence of the 3′ RACE FPPS

AATTTCCTGAAGAATGTTCGTGCATTATTGCCAGAGCTTTAGATGAAGCTAACGAAACACAA
AGGAAAATTCTTTTGGATAATTACGGCAAAAAAAAAAAACCCTTTGGGGGTCCTTTTTTTTT
CGACCCCGGGAAAA

3.3 Cloning of the 5′ RACE FPPS

5′ RLM-RACE failed to amplify the 5′ end of FPPS; it revealed smear after repeated attempts. Although it is a common problem with 5′ RACE, more trials or a different approach is required to amplify the 5′ end of FPPS gene.

3.4 Analysis of K. lacca FPPS sequence

Geneious software version 6.1.6 was used to perform the alignment of the resulting sequences. The sequences (partial FPPS and 3' RACE FPPS) were aligned by performing *De novo Assembly* using default parameters.

3.4.1 Performing De novo Assembly

The De novo assembled sequence was submitted to EMBL for accession number. The accession number for the gene is LT707635.

3.4.2 BLAST result of related sequences and Phylogenetic tree

Blastx was performed by using the default parameters except for excluding flowering plants and *Candida albicans*. The blast result showed that the *K. lacca* FPPS gene (LT707635) shared homology with the FPPS gene of various species including insects and vertebrates. By using the homologous sequences a phylogenetic tree by neighbour joining method was drawn using the Mega6 software (figure 5). The evolutionary tree was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA6. The evolutionary tree showed two clusters A and B. Cluster A bears 10 genotypes from both invertebrate (Green peach aphid, Pea aphid, Green aphid, Japanese blue crab, Ceratosolen, Brown spruce longhorn beetle, Green peach aphid, Red flour beetle, Panamanian leafcutter ant) and vertebrate group (Japanese rice fish). Cluster B bears 2 genotypes from invertebrate group which are lac insect from IINRG and Bird cherry-oat aphid.

K. lacca FPPS (LT707635) revealed closest resemblance with mitochondrial isoprenyl diphosphate synthase gene reported in Bird cherry-oat aphid (AEK32004), which was submitted (398 aa sequence) from China by Li and Sun (2011) (unpublished). The Bird cherry-oat aphid is an aphid in the superfamily Aphidoidea in the order Hemiptera. It is a true bug and sucks sap from plants. As evidenced from the phylogenetic tree, LIK0021 is most distantly related to an isoform, farnesyl diphosphate synthase 1 of the green peach aphid (*Myzus persicae*) bearing the accession number ABY19312 which shows close homology (30%) to that of putative mitochondrial isoprenyl diphosphate synthase precursor of pea aphid (*Acyrthosiphon pisum*) having the accession number AAY33490 (394 aa sequence). The 394 aa sequence of ABY19312 was submitted by Zhang and Li (2008) from China Agricultural University. Also, they reported the existence of two different farnesyl diphosphate synthase genes in the genome of the green peach aphid, *M. persicae*.

As observed in blast result, *K. lacca* FPPS (LT707635) revealed 30% homology to Japanese blue crab (AJT60334) also known as swimming crab which belongs to phylum Arthropoda. The 429 aa sequence of *Portunus trituberculatus* (swimming crab) was submitted by Zhu et al. in 2015 from China (unpublished). Cluster A includes two species from beetle, brown spruce longhorn beetle (AFR31785) and red flour beetle (NP_001164089). *Tetropium fuscum* (AFR31785) having 435 aa sequence is a species of beetle in the Cerambycidae family. *K. lacca* FPPS revealed 29% homology with putative farnesyl diphosphate synthase of (AFR31785). The Female lac insect from IINRG, showed 28% homology with farnesyl pyrophosphate synthase of *Tribolium castaneum* (NP_001164089). The red flour beetle having 425 amino acid sequence; it belongs to class insecta and is a species of beetle in the family Tenebrionidae, the darkling beetles. As evidenced from the evolutionary tree, LIK0021 showed close homology with *Oryzias latipes* (XP_004077958). The 409 amino acid sequence was submitted from China in 2015. Blast results revealed that 30% homology was observed with farnesyl pyrophosphate synthase isoform X1 of *O. latipes* with lac insect. *K. lacca* FPPS showed 29% homology with the PREDICTED: farnesyl pyrophosphate synthase-like isoform X2 of *Acromyrmex echinatior* (XP_011061595). This record was predicted by automated computational analysis. This record was derived from a genomic sequence (NW_011627342) annotated using gene prediction method: Gnomon. The 362 amino acid sequence was submitted from Panama in 2015. Commonly known as "leaf-cutter ants" they are the species of ant from one of the two genera of advanced attines within the tribe Attini. The evolutionary tree showed the 31% homology of putative mitochondrial isoprenyl diphosphate

synthase precursor of *Megoura viciae* having the accession number AAY33489 with *K. lacca* FPPS (LT707635).

3.5 Analysis of RT-qPCR

3.5.1 Amplification plot

Amplification plot generally depicts threshold cycle (Ct value) that is at which cycle the amplification is above baseline. The baseline is being selected to remove the background signal. Amplification plot also shows the pattern of amplification for each sample. In the present study, all the samples started to amplify beyond threshold between cycles 20-30 (figure 6a).

3.5.2 Dissociation curve

Two steps are required to interpret results from a SYBR Green I melt curve analysis. The first step is to review the PCR products produced by the samples in the reaction. The presence of a single homogeneous melt peak for all sample reactions confirms specific amplification. The data from this reaction are reliable and meaningful for analysis and interpretation. The second step is to evaluate the NTC (No Template Control) sample well for the presence of primer-dimer formation. Slight, high cycle amplification and a small wide peak at a lower temperature by melt is an indication of only primer amplification. It is acceptable to observe a small amount of primer-dimer formation in the NTC wells, but if there is a corresponding peak in the sample amplification plots the Ct values from these wells cannot be trusted as accurate.

During a dissociation curve (Melting Curve) analysis, all products generated during the PCR amplification reaction are melted at 95°C, then annealed at 55°C and subjected to gradual increase in temperature. During the incremental temperature increases, fluorescence data are collected until the reaction reaches 95°C. The result is a plot of raw fluorescence data units, R, versus temperature. This view of the data may appear difficult to interpret at first, but the rapid linear decrease in fluorescence to background is where the major PCR product melts to its single stranded form. In this study we found all products generated melting at around 76 °C for FPPS and 82 °C for Actin gene. At this stage, fluorescence data were collected (figure 6b).

3.5.3 Expression of FPPS gene in different life stages (Ct values)

Gracia and Couillaud (1999) studied the Expression of FPPS mRNA using RNAse protection assays. An RNAse protection assay constituted an extremely efficient method for specific detection of RNA, as it is reported to be 10-100 times more sensitive than Northern analysis. Real-time PCR is increasingly becoming the method of choice for high-throughput gene expression analysis. However, to get reliable results from real-time PCR analysis, accurate normalization of gene expression against a control gene is required. Mayo et al. (2013) reported the tissue-specific expression of *T. fuscum* farnesyl diphosphate synthase (TfFPPS), an enzyme expected to provide a key fuscumol precursor, was measured. TfFPPS transcripts were relatively abundant in male midguts, but were also present at significant levels in other tissues. Zhang and Li (2008) studied the juvenile hormone biosynthesis via the mevalonate pathway and they revealed that the pathway includes a precursor farnesyl diphosphate synthase I (*FPPS I*). The gene and its expression may regulate reproduction in insects. It may be predicted that *FPPS I* gene expression would be down-regulated during adult exposure corresponding to fecundity responses. Several studies reporting FPP synthase characterization show a complex pattern of expression of genes encoding these enzymes; in plants, it is possible for different genes to encode two FPS isoforms. In addition, the occurrence of different FPS mRNAs, which are transcribed from the same gene and hence share part of their sequence, has been reported in the plant as well as in the animal kingdom .

In this study, the expression of FPPS cDNA was studied using qPCR assay. The Ct or threshold cycle value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence (Table 3). Actin was used

as an internal control. Three life stages of *K. lacca* were used, Crawlers (C), Settled larva (S), and Female (F).

Level of expression was checked by using the formula $2^{-\Delta\Delta Ct}$. Livak et al. (2001) demonstrated the two most commonly used method to analyze the data from real-time, quantitative PCR experiments were absolute quantification and relative quantification.

Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as untreated control. The $2^{-\Delta\Delta Ct}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments.

Change in ct value (ΔCt) for different life stages was calculated by using the formula:

ΔCt.FPPS-ΔCt.Actin

ΔCt.C= 6.84; ΔCt.S= 10.88; ΔCt.F= 7.18

For the calculation of ΔΔCt., the following formulae were used:

ΔΔCt.S= ΔCt..C-ΔCt.S; and ΔΔCt.F= ΔCt.C-ΔCt.F

ΔΔCt.S= -4.04, ΔΔCt.F= -0.34

In the study, Crawlers (C) were used as reference stage. The qPCR analysis showed that the level of expression of FPPS gene in S. was observed, ~16.449 fold more than C. Similarly, level of expression in F was reported to be ~1.2657 fold more than C.

Gracia and cauilloud (1999) reported in insects, additional experiments are required to draw a complete picture of FPS activity in different tissues. They also speculated that the FPS of insects may have undergone specific evolutionary pressure related to their inability to synthesize cholesterol.

After sequencing and assembling the partial FPPS fragment and 3'RACE product resulted in 769 bp sequence of *K.lacca* FPPS encoding gene. The final product revealed a great homology with the mitochondrial isoprenyl diphosphate synthase gene reported in Bird cherry-oat aphid (*Rhopalosiphum padi*).

IV. Conclusion

The expression profile study revealed that this gene expression got up regulated in settled larvae and adult female lac insects in comparison to crawlers. The expression profile pattern reveals that the cloned FPPS gene may have some putative role in development of hormone biosynthesis or in resin biosynthesis.

V. Acknowledgement

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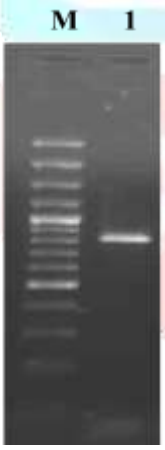


Figure 1: PCR of the partial FPPS gene showing a discrete band of 807 bp (lane 1). M: 100 bp ladder.

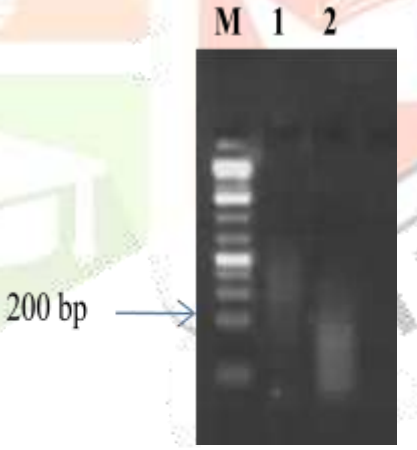


Figure 2: 3′ RACE outer PCR. Lane M- 100 bp ladder;Lane 1- diluted 1:5 cDNA template; and Lane 2- 1:10 cDNA template.

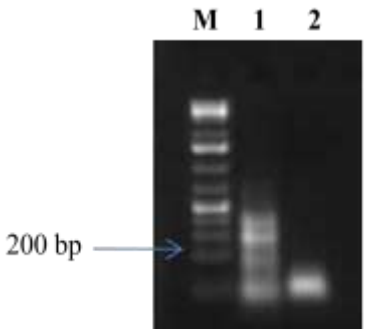


Figure 3: 3′ RACE nested outer PCR. Lane M- 100 bp ladder; Lane 1- diluted 1:5 cDNA outer PCR product as template; and Lane 2- 1:10 cDNA outer PCR product as template.

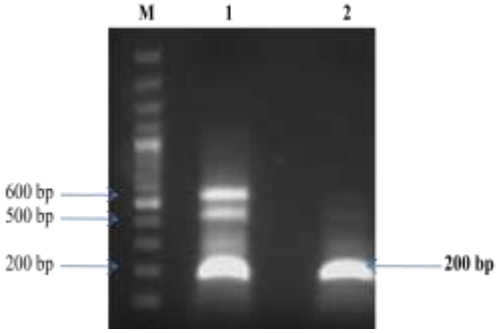


Figure 4: 3′ RACE inner PCR. Lane M- 100 bp ladder;
Lane 1- 1:5 diluted template; and
Lane 2- 1:10 diluted template.

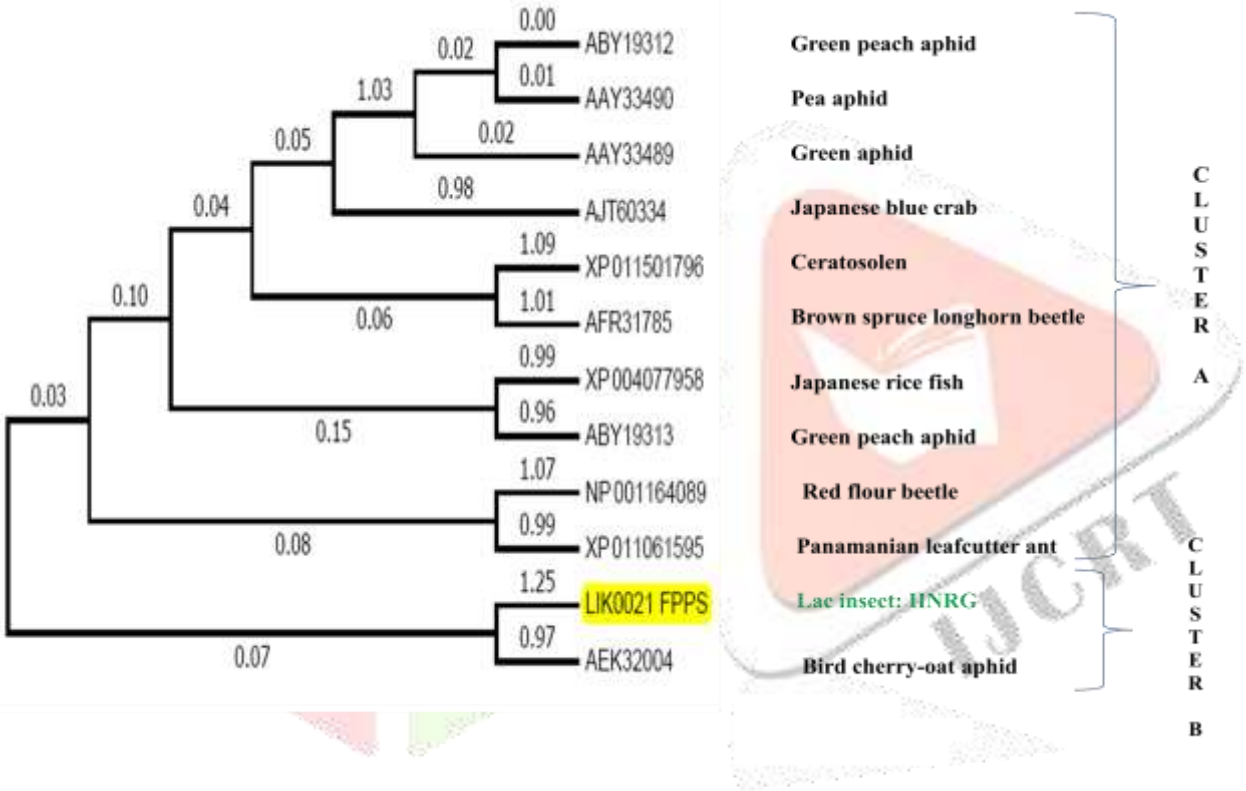
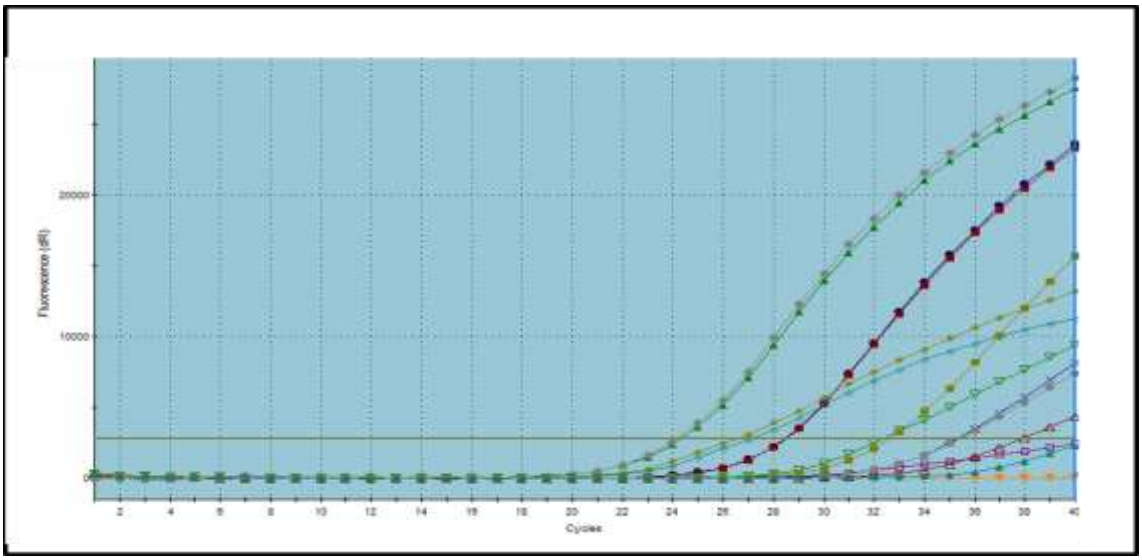
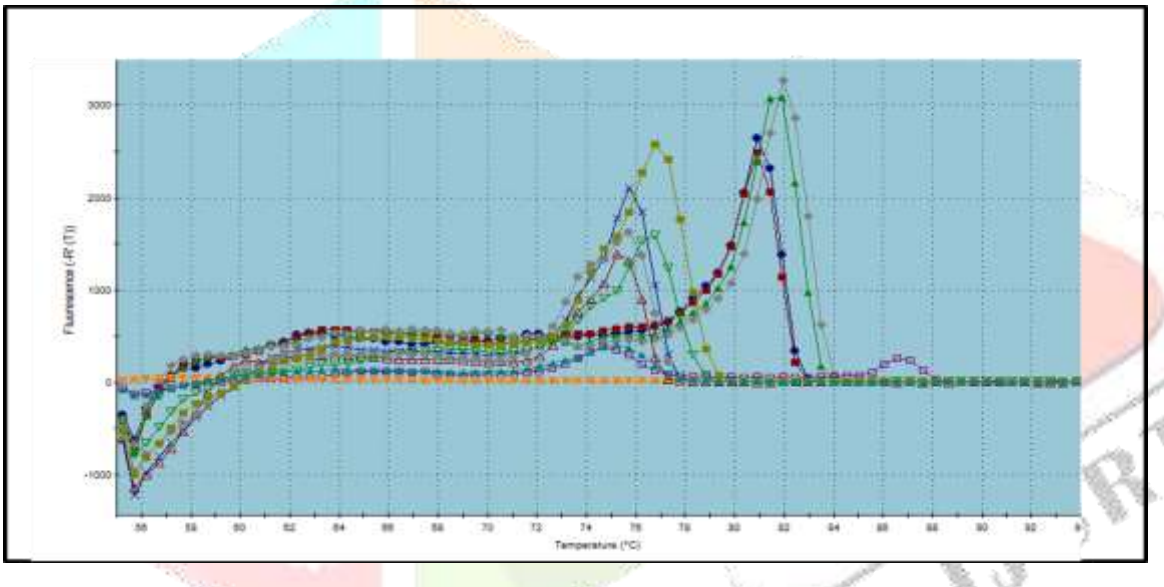


Figure 5: Evolutionary tree of the blast organisms using by Mega6 software. The evolutionary tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 10.98137707 is shown. Accession No. for LIK0021 is **LT707635**



(a)



(b)

Figure 6: RT-qPCR summarized results; (a) Amplification plots, (b) Dissociation curve

Table.1: Nanodrop readings for the eluted RNA.

Samples	Concentration (ng/µl)	O.D. (260/280)
1**(1E)**	334.1	2.20
2	784.3	2.15
3	356.1	2.18
1**(2E)**	399.4	2.18
2	297.0	2.17
3	140.0	2.15

Table 2: Sequence of the Primers used

Sl. No.	Primer name	Sequence (5'-3')	T_m
FPPS_Y_F.1		AAGCAATGAAACCGCTTGTT	47.68
FPPS_Y_R.1		CTCGGTTCTTTTTGCCGTAA	49.73
Pr. F 3′ RACE gene specific outer primer1		GTCCCATTGATTTGGGTTTGCA	64

Pr. R 3′ RACE outer primer	GCGAGCACAGAATTAATACGACT	64
Pr. F3′ RACE gene specific outer primer2	CATACAGATGTATTAATACAAGATC	64
Pr. R 3′ RACE inner primer	CGCGGATCCGAATTAATACGACTCACTATAGG	64
Pr. F 3′ RACE gene specific inner primer	TTGGGATGATTTCAAAGATTATCA	64
Pr. R 3′ RACE inner primer	CGCGGATCCGAATTAATACGACTCACTATAGG	64
Pr. F 5′ RACE gene specific outer primer2	AAGAGGGCTCTCCGTATCGT	64
Pr. R 5′ RACE outer primer	GCTGATGGCGATGAATGAACACTG	64
Pr. F<5′ RACE gene specific outer primer1	CATCTGTTAAAATATGACCACGAT	64
Pr. R<5′ RACE inner primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	64
Pr. F<5′ RACE gene specific inner primer	CAATCGATAAAGCTCTAATGTTGT	64
Pr. R<5′ RACE inner primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	64
Fs-q F1	TGATGCTCCTTTACGATACGG	52.4
Fs-q R1	GGTGCACTGCGTGATATTTCT	52.4
U09207 F1	GCGCTACACTGAAGGAATCA	51.8
U09207 R1	TTATGACTCGCGCTTACTGG	51.8

Table 3: Ct values of the amplified products

Well	Well Name	Well Type	Threshold (dR)	Ct (dR)
A1	A- c.1	unknown	2749.154	28.51
A2	A- c.2	unknown	2749.154	28.49
A3	A- s.1	unknown	2749.154	24.36
A4	A- s.2	unknown	2749.154	24.15
A5	A- f.1	unknown	2749.154	26.55
A6	A- f.2	unknown	2749.154	27.09
A7	----	NTC	2749.154	No Ct
B1	A- c.1	unknown	2749.154	34.33
B2	A- c.2	unknown	2749.154	36.34
B3	A- s.1	unknown	2749.154	37.91
B4	A- s.2	unknown	2749.154	32.35
B5	A- f.1	unknown	2749.154	35.39
B6	A- f.2	unknown	2749.154	32.62
B7	----	NTC	2749.154	No Ct