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Real Time RT-PCR as a Tool for Quantitative Hevea *Gene Expression Studies*

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We describe a real time quantitative RT-PCR (qRT-PCR) approach to measure gene expression of selected latex genes with SYBR Green I as the reporter dye. In this study, the target genes were the small rubber particle protein (SRPP) and farnesyl diphosphate synthase (FPPS) while the reference was the 18S ribosomal RNA (rRNA) gene. As a first requirement, amplification efficiencies of primer pairs specific to target and reference genes were determined by two-step RT-PCR. In the RT (reverse transcription) step, single-stranded cDNA was synthesised from latex total RNA. Then, ten-fold serial dilutions were amplified with gene-specific primers to generate cycle threshold (C_t) values for constructing a cDNA standard curve for each gene. Subsequently, one-step qRT-PCR reactions were set up to generate C_t values for the target and reference genes from the same RNA sample. A relative quantification method which employs an efficiency-compensated comparative C_t approach was used to calculate target gene transcript level relative to the 18S rRNA gene.

Keywords: *Hevea brasiliensis*; latex; real time PCR; qRT-PCR; cycle threshold; relative quantification; gene expression; transcript

Quantitative RT-PCR (qRT-PCR) is currently a widely adopted method for quantifying temporal and spatial changes in gene transcript (mRNA) levels. Due to its high sensitivity of detection, qRT-PCR supersedes traditional gene expression analysis approaches such as Northern blots, *in situ* hybridisations, RNAse protection assays, semi-quantitative RT-PCR and cDNA array profiling[1,2]. Additionally, it requires a small amount of starting material and can be executed on a high-throughput scale, thus making multiple target gene quantification possible[3,4]. Monitoring of amplification in real time is enabled by the optical detector system of a thermocycler which detects fluorescence emitted by amplification products in real time[5]. The simplest means of amplicon detection uses a fluorescent intercalating dye such as SYBR Green I which binds to double-stranded DNA while other methods are based on amplicon-specific hybridisation by fluorogenic probes[6]. Examples of real time PCR instruments are the Rotor Gene (Corbett Life Science, Australia), the iCycler (BioRad, USA) and the Light Cycler (Roche, Switzerland).

Central to qRT-PCR is the calculation of mRNA abundance using the cycle threshold (C_t) value generated by a real time PCR instrument. The C_t is defined as the PCR

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cycle at which target amplicon fluorescence exceeds a threshold that is selected to be above background fluorescence[5]. Two approaches of amplicon quantification are available: absolute and relative quantification[6–8]. In absolute quantification, amplification of a target gene is compared to a standard curve based on known copy numbers of the target[1]. Relative quantification however, relates target gene amplification level to that of an internal control such as a housekeeping gene[9–12].

With steady improvements in methodological and statistical aspects of the technique, qRT-PCR has been applied in expression studies of numerous types of genes in many plant species[13–18]. This paper describes a step-by-step procedure for quantifying levels of gene transcripts in *Hevea* latex using a Rotor Gene real time thermocycler (Corbett Life Science, Australia) by qRT-PCR. Two genes encoding enzymes of the mevalonate pathway of *Hevea* rubber biosynthesis were chosen as examples of target genes: the small rubber particle protein (SRPP) and farnesyl diphosphate synthase (FPPS). The *Hevea* 18S ribosomal RNA (rRNA) gene was used as a reference gene. Relative quantification based on the mathematical model of Pfaffl[10] was used to calculate target gene transcript level in comparison with the reference gene transcript.

MATERIALS AND METHODS

Plant Material

Latex was collected by tapping the *Hevea* trees (clone RRIM 600) grown on the fields of the Sungai Buloh Experiment Station, Malaysian Rubber Board for RNA isolation. Latex was collected directly into an equal volume of 2X RNA extraction buffer (0.1 M Tris-HCl, 0.3 M LiCl, 10 mM EDTA, 10% SDS, pH 9.5). Total RNA from latex was extracted essentially according to the method of Kush *et al.*[19]. To visualise its integrity, latex total RNA was electrophoresed using 1% agarose gel according to Sambrook *et al*.[20].

RNA quality was assessed by using the Agilent 2100 bioanalyzer (Agilent Technologies, USA) and the RNA 6000 Nano LabChip® Kit (Agilent Technologies, USA). The RNA Integrity Number (RIN), fragment size distribution, concentration and rRNA ratios were generated by the Agilent 2100 Bioanalyzer system software version B.01.03.

Primer Design

Primers were designed using the Beacon Designer version 4.0 software (Premier Biosoft International, USA). The sequences of three primer pairs were specific to *Hevea* cDNA sequences: SRPP, FPPS and 18S rRNA (*Table 1*).

Determination of Primer Efficiency

Determination of primer efficiency was performed by employing two-step RT-PCR using the Rotor Gene 3000 Real Time Thermal Cycler (Corbett Life Science, Australia). Prior to generating first-strand cDNA, latex total RNA was subjected to the elimination of genomic DNA using the QuantiTect Reverse Transcription Kit (Qiagen, USA). Next, 1 µg latex total RNA was reverse-transcribed into first-strand cDNA [primed by oligo(dT) and random primers] according to the vendor's manual under the following conditions: 15 min at 42ºC and inactivation of the reverse transcriptase at 95ºC for 3 min. The single-stranded cDNA product was diluted into a series of five arbitrary ten-fold dilutions with sterile distilled water.

TABLE 1. PRIMER SEQUENCES AND FEATURES FOR TARGET AND REFERENCE GENES

Gene	Primer sequence (FW: forward; RV: reverse)	Amplicon length (bp)	Melting temperature (T_m)	GenBank Accession no. of cDNA
SRPP	FW: GCTGGAGTTTATGCTGTAGATTC	104	55.0	AF051317
	RV: TTCACCACATTCTCAATAGTATCG		54.5	
FPPS	FW: ACACCAGATTCCCGTCAATG	169	54.9	AY349419
	RV: ATTCAATACACCAACCAAGAGC		54.4	
18S rRNA	FW: CCATAAACGATGCCGACCAG	110	56.1	AY496880
	RV: CAGCCTTGCGACCATACTC		55.1	

PCR reactions were performed with each primer pair using serial dilutions of single stranded latex cDNA (QuantiTect® SYBR Green PCR Kit; Qiagen, USA). To overcome inter-sample variation, all reactions were performed in triplicate and master mixes of reaction components were made whenever possible. A 'No Template Control' (NTC) was included for each primer pair. The PCR profile was: activation of the hot start *Taq* polymerase at 95ºC for 15 min, followed by 45 cycles of nucleic acids denaturation at 94ºC for 15 sec, primer annealing at 55ºC for 30 sec and polymerisation at 72ºC for 30 sec.

The Rotor Gene analysis software version 6.0 (Build 38) was used to visualise amplification and to generate cDNA standard curves. For each gene, PCR sigmoid curves were generated by the Rotor Gene software from amplification of five ten-fold serially diluted cDNAs. Next, these PCR sigmoid curves were log transformed by the software for C_t determination. Log transformed curves from replicated reactions should overlap closely with one another to indicate minimal systematic error occurring during reaction set-ups. Subsequently, a threshold line was set manually at the exponential phase at a point which showed significant fluorescence signal above background noise. The point where the threshold line intersects with the serial dilution curve determines the C_t value for a particular gene transcript in the log template concentration graph. For each gene, the NTC should not show any amplification earlier than the 25th PCR cycle to indicate absence of contamination leading to non-specific amplification. The C_t values reported by the software were used to generate a standard curve of C_t values against log of template concentration for each gene. Finally, the software calculated the slope value (M) and reaction efficiency [equals to $10^{(1/-M)} - 1$] from the standard curves.

Quantification of Relative Gene Transcripts

To determine the expression level of selected *Hevea* genes, individual one-step RT-PCR reactions were set up using the QuantiTect® SYBR Green RT-PCR Kit (Qiagen, USA). The concentration of latex total RNA template used for RT-PCR reaction set up was 400 ng/µl. Each reaction contained a gene-specific primer pair. One-step RT-PCR conditions were: reverse transcription at 50ºC for 50 min, then activation of *Taq* polymerase at 95ºC for 15 min, followed by 45 cycles of denaturation phase at 94ºC for 15 s, primer annealing at 55ºC for 30 s and polymerisation at 72ºC for 30 s.

In order to minimise errors from sample-to-sample variation, reactions were performed

in triplicate. Master mixes of reaction components were made whenever possible. Negative controls were set up for each primer pair: the "No Template Control" (NTC) which included all PCR and RT components except template RNA and the "No Reverse Transcription Control" (NRTC) which included the template RNA and all PCR components except RT enzyme.

The Rotor Gene analysis software version 6.0 (Build 38) was used to monitor amplification cycles and to analyse results. For each gene, PCR sigmoid curves were generated by the Rotor Gene software. Next, PCR sigmoid curves were log transformed by the software for C_t determination. As indication of minimal systematic error occurring during reaction set-ups, log transformed curves from replicated reactions should overlap closely with one another. The threshold was set in the log transformed plot of each gene by importing the threshold setting from the cDNA standard curve of the same gene (generated during primer optimisation). The negative controls should not show any amplification earlier than the 25th PCR cycle to indicate absence of non-specific amplification products. Next, the C_t values calculated by the software were used to determine transcript level of each target gene according to the efficiency-compensated relative quantification method by Pfaffl[10]. The Pfaffl equation used was based on the mathematical model of relative expression where the reference gene (18S rRNA gene in this study) was also the control or housekeeping gene, the sample refers to the target and $E = 10^{(1/-M)}$.

$$\text{Pfaffl ratio} = (E_{target})^{\Delta C_t \text{ target(control-sample)}}$$

Melt Curve Analysis

Reaction products from one-step and two-step qRT-PCR were subjected to melt curve analysis to ensure that the PCR products are amplified from specific primers and not primer dimers. Using the Rotor Gene analysis software version 6.0 (Build 38), melt curve analysis was carried out using a ramp temperature of 72ºC – 95ºC. The melt curve phase started immediately after the PCR cycles. The detection of a single peak and a narrow range of T_m variation (up to 1ºC) between replicates indicate single species amplicons. Controls should not show any peak if no amplification was detected.

DNA Sequencing and Analysis

One-step qRT-PCR amplicons were purified using the Montage PCR Purification Kit (Millipore, USA). The purified DNA was sequenced with the ABI 3730XL DNA Analyzer using the ABI Big Dye Terminator Cycle Sequencing Ready Kit version.3.1 (Applied Biosystems, USA). Gene-specific primers that had been used for amplification were used for bidirectional sequencing of purified qRT-PCR products. Sequences were edited using the ChromasPro version 1.42 software (Technelysium Pty. Ltd., Australia). Sequence alignment was performed using ClustalW program from the European Bioinformatic Institute (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

RESULTS AND DISCUSSION

Integrity of Plant Material

Total RNA was isolated from latex of field-grown rubber trees for expression analysis of two genes, SRPP and FPPS. High quality RNA is critical for accurate and reproducible qRT-PCR results. Agarose gel electrophoresis (*Figure 1*), and microcapillary electrophoresis using the Agilent 2100 bioanalyzer (*Figure 2*) confirmed that latex total RNA was intact

based on observation of distinct 18S and 28S rRNA components. The bioanalyzer also generated a RIN value (*Table 2*) which is an indispensible parameter for RNA quality evaluation due to the high sensitivity of qRT-PCR. RIN determination is based on a combination of several features which contribute to RNA integrity that are extracted from microcapillary analysis data[21]. A RIN value of greater than 5 and a PCR amplicon length of up to 200 bp was recommended to ensure successful RT-PCR quantification[12,22.] Therefore, based on an average RIN value of 7.3 (*Table 2*) and the amplicon sizes of genes of interest in this study (*Table 1*), the latex RNA sample was confirmed to be suitable for qRT-PCR analysis.

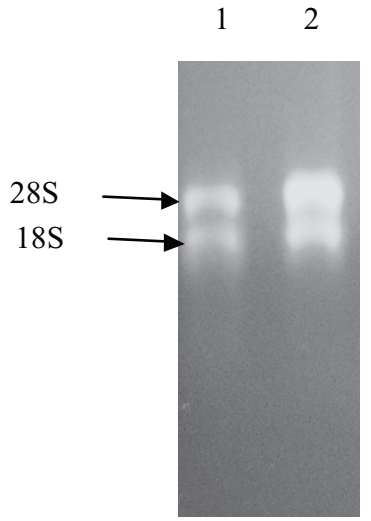

Figure 1. Electrophoresis of latex total RNA in 1% agarose gel. The 18S and 28S rRNA bands in RNA diluted 100X (lane 1) and 20X (lane 2) are indicated by arrows.

Primer Design and Efficiency Optimisation

In qRT-PCR analysis of *Hevea* gene expression, primer pairs specific to the SRPP, FPPS and 18S rRNA cDNAs were designed to amplify 104, 169 and 110 bp amplicons respectively (*Table 1*). Only *Hevea*-specific cDNAs were used for primer design because specificity of transcript detection is increased by primers designed according to precise target sequences[5,23].

The purpose of primer optimisation is to verify that gene-specific primer pairs show high amplification efficiency so that relative quantification can be determined accurately. cDNA (from RT of latex total RNA) is preferable as template for two-step qRT-PCR because DNA is not as easily degradable as RNA. In addition, the same cDNA pool

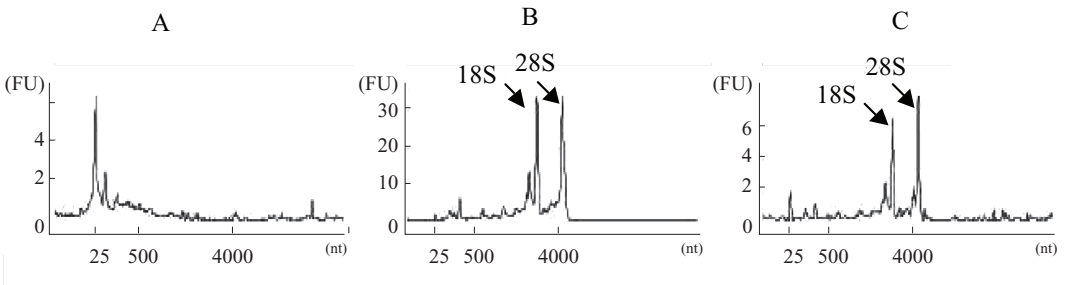

Figure 2. Latex total RNA fractions generated by the Agilent 2100 bioanalyzer software. Undiluted latex total RNA showed poor resolution (A) while RNAs diluted 20X (B) and 100X (C) displayed distinct 18S and 28S rRNA peaks indicating intact RNA.

TABLE 2. BIOANALYZER ANALYSIS OF LATEX TOTAL RNA

Bioanalyzer parameters	Latex total RNA	
	20 × dilution	100 × dilution
RNA concentration	904 ng/μL	134 ng/μL
RNA integrity number (RIN)	7.1	7.5

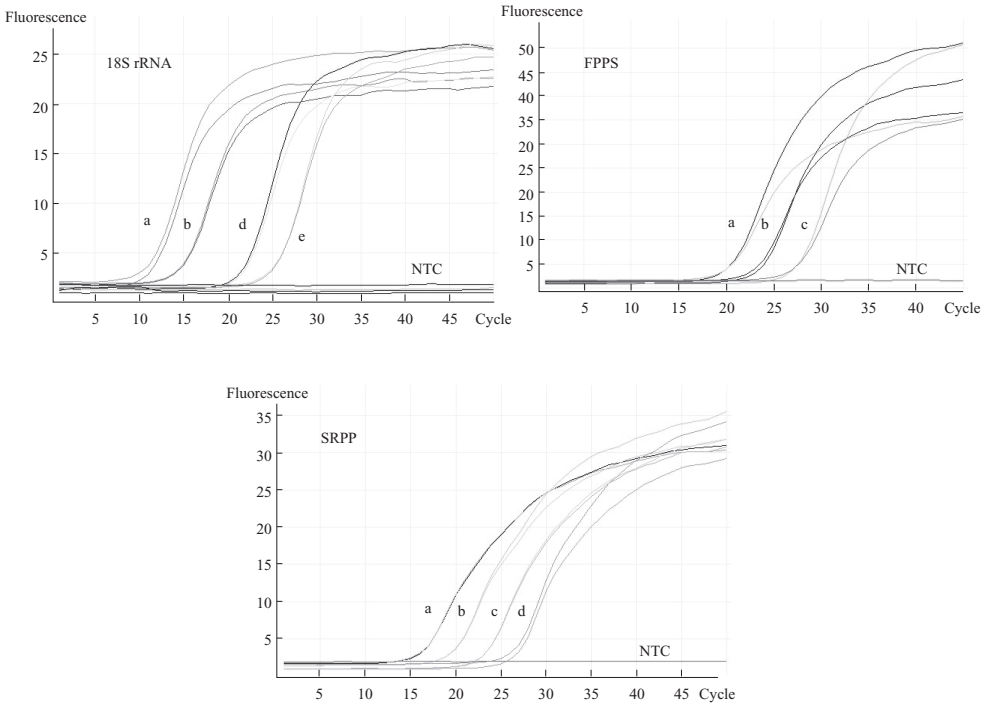

Figure 3. PCR sigmoid curves generated from amplification of ten-fold serially diluted cDNA templates. For each gene, five cDNA dilutions were set up but the higher dilutions of SRPP and FPPS did not show any amplification due to low transcript abundance. The c dilution of 18S rRNA was removed as it was an outlier value. The negative control (NTC) appeared as a flat line as there was no amplification product. a: 1:10 dilution, b: 1:100 dilution, c: 1:1000 dilution, d: 1:10 000 dilution, e: 1:100 000 dilution.

can be used to validate many primer pairs. *Figure 3* shows the PCR sigmoid curves of ten-fold serial dilutions for the target and reference genes generated by the Rotor Gene software. The calculation of efficiency parameters for each primer pair was performed by first determining C_t values from log transformed curves. For each gene transcript, the Rotor Gene software constructed a standard curve by plotting C_t values corresponding to each serial dilution against the log of template concentrations expressed as number of copies (*Figure 4*). Corresponding standard curves were also generated (*Figure 5*).

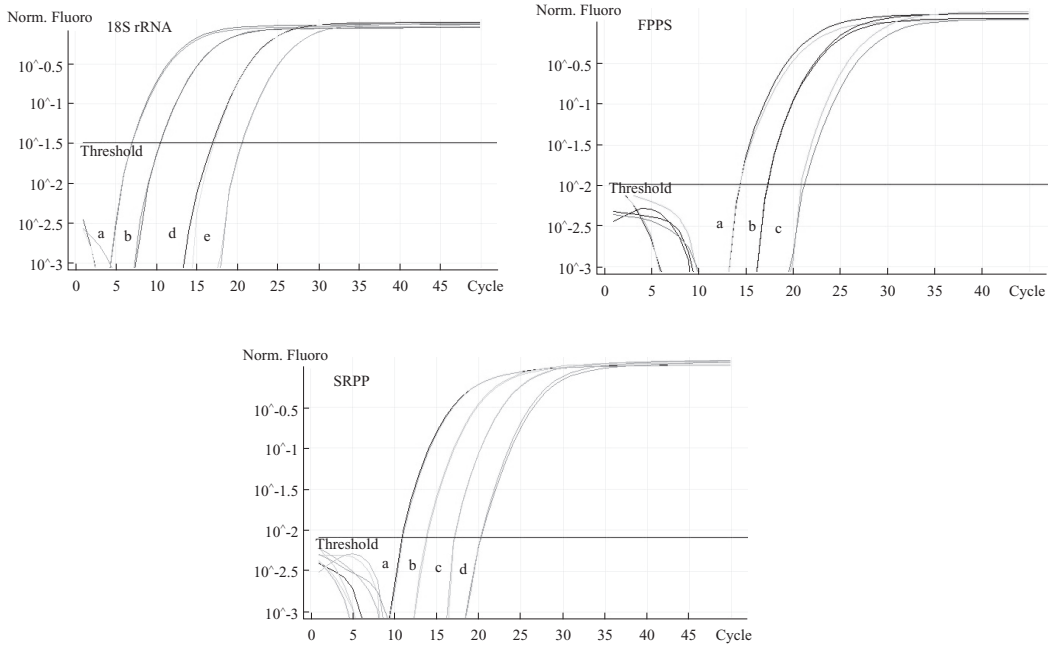

Figure 4. Log transformation of PCR sigmoid curves of ten-fold serial dilutions. Higher dilutions of SRPP and FPPS did not show any amplification due to low transcript abundance. The c dilution of 18S was removed as it was an outlier value. For each gene, the Ct is the point where the threshold intersects each curve. The log template concentrations were equivalent to arbitrary concentrations of 100 000(a), 10 000(b), 1 000 (c), 100 (d) and 10 (e) copies.

Amplification efficiency is reflected by the "reaction efficiency" parameter in the Rotor Gene software. A 100% efficient amplification indicates doubling of PCR product at every cycle from its initial amount of template resulting in a slope value (M) of –3.32 (based on the equation, reaction efficiency = $10^{(1/-M)}$–1). Practically, a reaction efficiency value of close to 1 is usually accepted[24,25]. As seen in *Table 3*, reaction efficiency and M calculations for all gene transcripts approximated the desired values and hence, primer optimisation was successful due to high amplification efficiency of the primer pairs. Also, the standard curves exhibited three to five orders of magnitude of amplification (*Figure 5*), a requirement which is necessary for producing a reliable slope[26]. We also found manual threshold setting to be preferable as it allowed outlier C_t values to be eliminated and as a result, a better fit for the standard curves. However, if primer pairs cannot be optimised even after following all precautions and recommendations, gene-specific primers would have to be redesigned and tested again.

Relative Quantification of Gene Expression

The expression of target genes in latex was determined by performing one-step qRT-PCR containing their respective optimised primer pairs. One-step qRT-PCR is more convenient and the RT step may be assumed

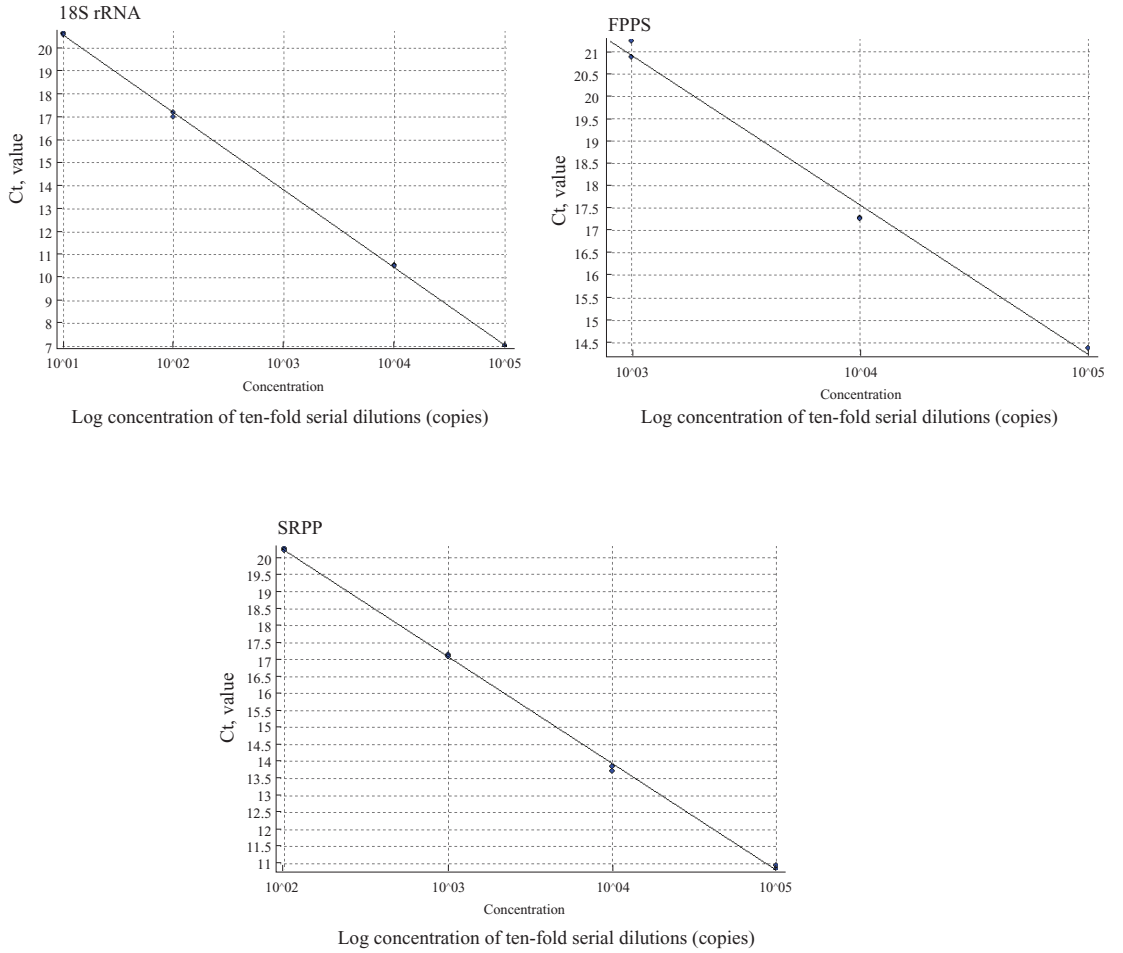

Figure 5. cDNA standard curves of target and reference genes.

to be performing equally efficient as long as the same reagents and reaction conditions are maintained[23,27]. For each gene, PCR sigmoid curves were generated by the Rotor Gene software after the RT incubation step (*Figure 6*). As before, these plots were log transformed for determination of the C_t value for each gene (*Table 4*).

A comparative C_t method is available for relative quantification of transcript abundance[7,9]. However, this method requires efficiencies of target and reference genes to be equal without which transcript level determination may be misleading[6,10]. To overcome this, we used the efficiency-calibrated mathematical model published by Pfaffl[10] to calculate *Hevea* transcript expression level. Expression of the target genes, SRPP and FPPS, was calculated in relation to the reference gene, 18S rRNA (*Table 4*). The 18S rRNA gene was chosen

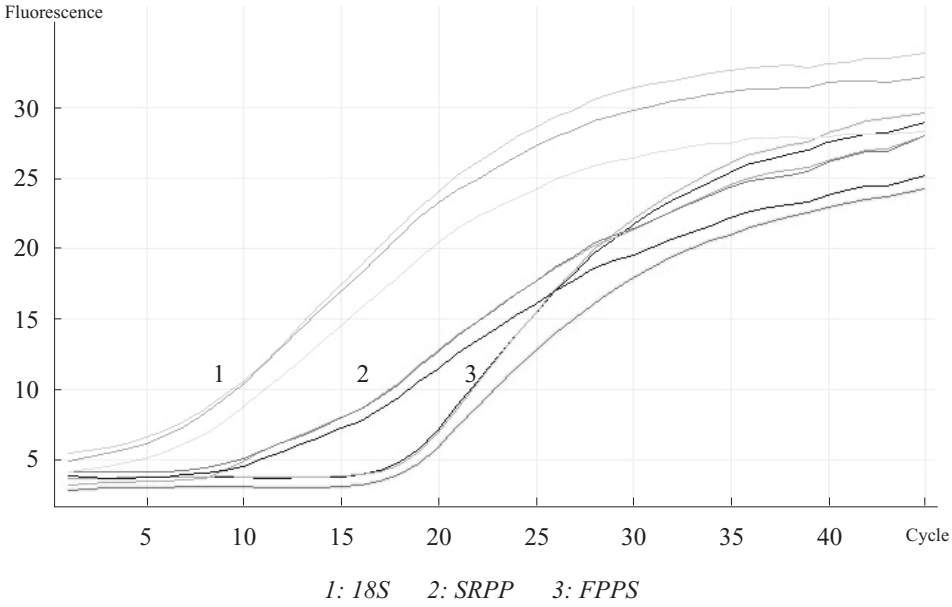

Figure 6. PCR sigmoid curves generated from one-step qRT-PCR of target and reference genes.

TABLE 3. PRIMER OPTIMISATION PARAMETERS FOR TARGET AND REFERENCE GENES

	Reaction efficiency	Slope (M)
18S	0.98	–3.37
SRPP	1.08	–3.14
FPPS	0.99	–3.35

TABLE 4. CYCLE THRESHOLDS OF TARGET AND REFERENCE GENES AND RELATIVE GENE EXPRESSION BY PFAFFL RATIO

Gene	C_t	Average C_t (Target gene)	18S rRNA	Average C_t (18S rRNA)	Pfaffl ratio (18S rRNA =10 000)
SRPP	4.77	5.22	3.84	3.88	3748
	5.35		3.99		
	5.54		3.82		
FPPS	14.39	14.18	3.84	3.88	8
	13.90		3.99		
	14.26		3.82		

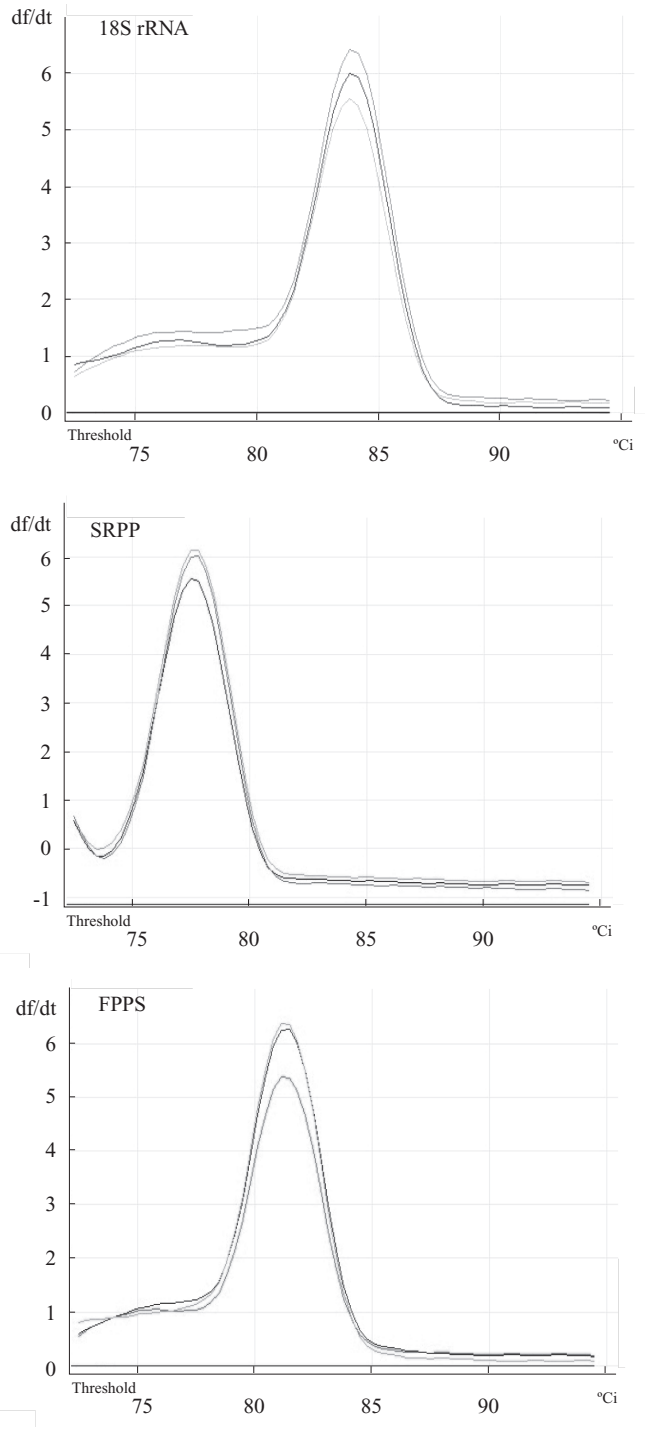

Figure 7. Melt curve analysis of products from one-step qRT-PCR of target and reference genes.

based on evidence for it being a housekeeping gene in experiments involving different tissues and physiological conditions of the rubber tree[28,29,30]. Based on Pfaffl ratio calculation, our results showed that the SRPP transcript level was nearly 40% than that of 18S rRNA while FPPS transcript was less than 1% (*Table 4*).

Quantification of gene expression can be extended to other latex genes of interest. In another study using the Pfaffl model of relative gene expression analysis, one-step qRT-PCR measurement of a panel of thirteen rubber biosynthesis pathway genes was used to verify the EST expression profile of the same genes[31]. For the quantification of a large number of genes, it is convenient to design primer pairs with a T_m range of not more than 2ºC difference between genes. This is to accommodate as many genes as possible into a single rotor run in the Rotor Gene instrument. However, each run should include the 18S rRNA reference gene to normalise variation between runs.

Validating qRT-PCR Results

Post-PCR validation of amplification specificity is an important step and is routinely performed using melt curve analysis. This applies to amplification products from primer optimisation and relative quantification reactions. Melt curve analysis provides greater certainty that the amplicon is a *bona fide* product and not a result of template independent amplification[23,32]. Non-specific products such as primer dimers would produce a melting temperature different from that of the desired amplicon[33]. *Figure 7* shows the melt curve profiles of target and reference genes following one-step qRT-PCR. Amplification of single species PCR product was indicated by the presence of only one prominent peak for each gene.

Control reactions were also routinely verified by melt curve analysis. In our experiments, the average C_t values from control reactions during primer optimisation or relative gene expression were usually zero (no product) or more than 25. For the former, no peak would appear but for controls with C_t values, the melting temperatures of amplification products of control and target reactions should not be the same.

In our experiments, an additional step of sequence determination of products from one-step qRT-PCR was taken even though this is not a requirement. Since the alignment of each amplicon with its respective *Hevea*-specific cDNA was 100% (data not shown), amplicons were confirmed to be gene-specific, this further supporting the melt curve analysis results.

CONCLUSION

A qRT-PCR protocol for quantifying *Hevea* latex transcripts based on the Rotor Gene real time PCR instrument is presented in this paper. Real time qRT-PCR is an efficient way of quantifying gene expression in *Hevea* as it consumes less time and labour compared to traditional methods of mRNA abundance determination. However, due to the sensitivity of this method, it is important to adhere closely to the experimental and mathematical requirements to ensure reliability of gene expression results.

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