Semi-quantitative RT-PCR analysis of transcripts encoding protease inhibitor in *Hevea brasiliensis* Muell. Arg latex

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Abstract. In this paper, the semi-quantitative RT-PCR was carried out on four *Hevea brasiliensis* protease inhibitor transcripts (*HbPI1-4*) of rubber tree clone PB 260 under abiotic and biotic stresses. The research aimed to understand whether the expressions of HbPIs are induced by either biotic or abiotic stresses. The RNA isolation was performed under strict procedure to synthesize the best quality of cDNA. The accumulation of gene transcript was determined by RT-PCR (Reverse Transcriptase – Polymerase Chain Reaction) at the 30th cycle. Pre-PCR experiments were carried out on 20th, 25th, 30th, and 35th cycles for each gene. At the 30th cycle, PCR products were clearly visible on an agarose gel and could be quantified in comparison to the reference gene (*HbActin*). In general, the value of gene expression of *HbPIs* ranged from 0.222 to 0.531 in various conditions. However, the data were not statistically different under abiotic (tapping panel dryness, TPD) and biotic (white root disease, WRD) stresses. The semi-quantitative RT-PCR was able to confirm the relatively stable expression of *HbPIs* under five tree conditions (healthy trees, TPD-affected trees at <50% dry cut length (DCL), TPD-affected trees at >50% DCL, WRD-affected trees and both of symptoms).

1. Introduction

The milky white latex is the main product of the para rubber tree, *Hevea brasiliensis* Muell. Arg [1]. It contains the natural rubber commercially used as the raw material of industrial products such as tire and rubber bearing due to its unique physical properties such as elasticity, impact resistance and abrasion [2, 3]. Latex is the cytoplasm of highly differentiated cells namely laticifers [4, 5]. Latex is harvested by tapping the soft bark truncating the laticifers. The latex flows out until the beginning of the coagulation processes plugs the severed vessels [6, 7].

Inside latex, various types of protein have been detected in which they have associated functions in plant defense against pathogens and wounding responses [8-10]. In a matter of fact, twenty-five per cent of the proteins in C- and B-serums are known as antimicrobial agents in *Hevea brasiliensis*. Most of these proteins have the activity of chitinase/lysozyme which can degrade fungal and bacterial cell wall components such as chitin and peptidoglycan, respectively [8, 11]. However, among these known proteins, small size proteins known as protease inhibitor were less studied in *Hevea* latex.

Limited numbers of research have reported the isolation and characterization of protease inhibitor from the latex of two *Hevea* tree clones RRIM 600 and PB 260 [11-15]. In molecular levels, high numbers of Expressed Sequence Tags (ESTs) encoding protease inhibitors were predicted in laticifers

[10]. Taken together, the studies showed that the protease inhibitors were encoded by numerous genes. A comparative genome analysis between *Arabidopsis thaliana* and *Hevea brasiliensis* has been carried out to putatively identify the gene family encoding *Hevea* protease inhibitors (HbPIs) [16]. Further transcript quantification analyses are necessary to confirm the presence of these genes in the latex. In addition, the expression analysis should be carried out during contrasted condition such as diseased and healthy rubber trees to determine the constitutive or inducible expression of HbPI encoding genes.

Nowadays, the accumulation of messenger RNA (mRNA) in the plant can be measured using a rapid, sensitive and reliable method [17-19]. Despite the greater accuracy of recently developed techniques such as real-time PCR, semi-quantitative PCR methods are still widely used and appropriate for many purposes in plant studies [20-23]. The main reason for its popular use is that the semi-quantitative method provides a combination of traditional PCR and transcript quantification at a relatively low cost [22]. The semi-quantitative PCR was equally confirmed to generate results as well as the more advanced methods [24].

In this paper, an alternative semi-quantitative RT-PCR method for comparing the expression of four transcripts encoding putative *Hevea brasiliensis* protease inhibitors under the abiotic and biotic stresses was presented. The research aimed to understand whether the expressions of HbPIs are induced by WRD, TPD or combination of both. Firstly, the RNA isolation was performed under strict procedure to synthesize the best quality of cDNA. Secondly, the accumulation of gene transcript was determined by RT-PCR. Thirdly, the quantification of transcript expression was normalized using the expression of a reference gene on a gel electrophoresis analyzer. Fourthly, data and statistical analysis were carried out.

2. Materials and Methods

2.1. Plant material

Latex samples were obtained from rubber tree clones PB 260 growing at the field trial of Sembawa Rubber Research Institute, Palembang, Indonesia (Latitude: S 2° 55' 38.687"; Longitude: E 104° 32' 18.918"). The sampling of latex was carried out at 7 to 8 am to obtain a sufficient amount of latex. The samples were collected in five conditions i.e. healthy trees, tapping panel dryness (TPD)-affected trees at <50% dry cut length (DCL), TPD-affected trees at >50% DCL, white root disease (WRD)-affected trees and both of symptoms (**Figure 1**). TPD as a physiological disease represents abiotic stress while WRD represents biotic stress. The determination of the tapping panel dryness syndrome followed the measurement of DCL [25]. The collected samples were brought to the laboratory on dry ice and stored in a deep freezer (-80°C) until RNA extraction.

2.2. RNA extraction and preparation

The RNA was extracted using the modified method of Chang [26]. The latex samples were briefly thawed in a water bath at 50°C and then transferred to centrifuge tubes. The thawed latex was centrifuged for 40 min at 15,000 g at 20°C. The white fraction was collected and transferred to new centrifuge tubes. Series of organic solution addition and centrifugation was performed. First, one volume of phenol/chloroform/isoamylalcohol (25/24/1; v/v/v) was added and mixed at room temperature followed by a centrifugation for 15 min at 10,000 g at 4°C. The resulted upper phase (aqueous) was collected into new centrifuge tubes. Secondly, one volume of phenol/chloroform/ isoamylalcohol (25/24/1; v/v/v) was added and mixed at room temperature followed by a centrifugation for 15 min at 10,000 g at 4°C. The upper phase (aqueous) was collected into new centrifuge tubes. Thirdly, one volume of chloroform/isoamylalcohol (24/1; v/v) was added and mixed at room temperature followed by a centrifugation for 15 min at 10,000 g at 4°C. An addition of 8M LiCl to the resulted aqueous phase was carried out to reach a final concentration of 2M. The solution was mixed by inverting tubes several times and incubated overnight at 4°C. The mixture was then centrifuged for 30 min at 10,000 g at 4°C. The supernatant was removed. A 400 μL of DEPC water was added and agitated periodically to help dissolve the RNA pellet. The RNA solution was transferred into 1.5 mL microtubes. A volume of 400 μL phenol/chloroform/isoamylalcohol (25/24/1;

v/v/v) was added and mixed followed by a centrifugation for 15 min at 13,000 g at 4°C. One volume of chloroform/isoamylalcohol (24/1; v/v) was added into the upper phase followed by a centrifugation for 10 min at 10,000 g at 4°C. The aqueous phase was collected. A 1/10 volume of sodium acetate 3M pH 5.2 and 3 volume of absolute ethanol were added to precipitate the RNA for 3 hours in a freezer. After centrifugation for 30 min at 13,000 g at 4°C, the supernatant was discarded. The pellet was rinsed with 70% ethanol and centrifuged for 5 min at 13,000 g at 4°C. The pellet was dried in a speed vacuum and resuspended in 30 μL of nuclease-free water.

To eliminate potential DNA contamination, total RNA was treated with DNase I (Sigma-Aldrich, USA). A volume of 1 μL DNase I was added to 8 μL RNA (1000 ng) for each sample. After incubation for 15 min, the RNA was dissolved in 1 μL STOP solution (50 mM EDTA) for 10 min at 70°C. Agarose gel electrophoresis was used to visualize the RNA integrity while the quantity of RNAs was measured using NanoDrop 2000 (ThermoScientific, USA). RNAs were conserved at -80°C.

Figure 1. Illustration of rubber tree clones PB 260 in the sampled field trial. (a) healthy bark (normal latex flow); (b) TPD-affected bark at ≤50% DCL (arrow: partial cessation of latex flow); (c) TPD-affected bark at >50% DCL (arrow: complete cessation of latex flow); (d) WRD-affected tree (arrow: fruit body of *Rigidosporus lignosus*); (e) WRD+TPD (arrow: complete bark dry and fruit body of the infectious fungi). WRD: white root disease; TPD: tapping panel dryness; DCL: dry cut length.

2.3. cDNA synthesis and semi-quantitative RT-PCR procedures

The semi-quantitative RT-PCR analysis was conducted as follows. cDNAs were synthesized from 1 μg of total RNA to the final 20 μL reaction mixture using Bioneer AccuPower Cycle Script RT PreMix kit (Bioneer, USA) according to the manufacturer's instructions. Full-length cDNA synthesis was checked on each cDNA sample by PCR amplification of the Actin cDNA using primers at the cDNA ends. Reverse Transcriptase (RT)-PCR was finally carried out using a thermocycler. The standard program comprised a cycle of 10 min at 95°C, 45 sec at 65°C and 60 sec at 72°C, followed by 45 sec at 95°C, 45 sec at 65°C and 60 sec at 72°C for 30 cycles. The 30th cycle was selected after pre-PCR on several cycles (20th, 25th, 30th, and 35th) for each gene. At the 30th cycle, the PCR product was clearly visible on an agarose gel and could be quantified in comparison to the reference gene

(*HbActin*). The primer pairs used in this study were as follow: *HbActin* (Genbank accession: JF270598.1) with 5'-AGTGTGATGTGGATATCAGG-3' (forward) and 5'-GGGATGCAAGG ATAGATC-3' (reverse), *HbPI1* (Genbank accession: KX101219.1 with 5'-GGGGAATCCACA GATTTCATT-3' (forward) and 5'-TTTAGCCAATGGCAGGAACT-3' (reverse), *HbPI2* (Genbank accession: KX101220.1) with 5'-GAATGGCAAGTCAGTGTCCA-3' (forward) and 5'-TTTAGCCAATGGCAGGAACT-3' (reverse), *HbPI3* (Genbank accession: KX101221.1) with 5'-TCGAGAGGAGAATGGCAAGT-3' (forward) and 5'-GAAAACCAGGACCCTGCATA-3' (reverse), *HbPI4* (Genbank accession: KX101222.1) with 5'-AGTGTGATGTGGATATCAGG-3' (forward) and 5'- GGGATGCAAGGATAGATC-3' (reverse).
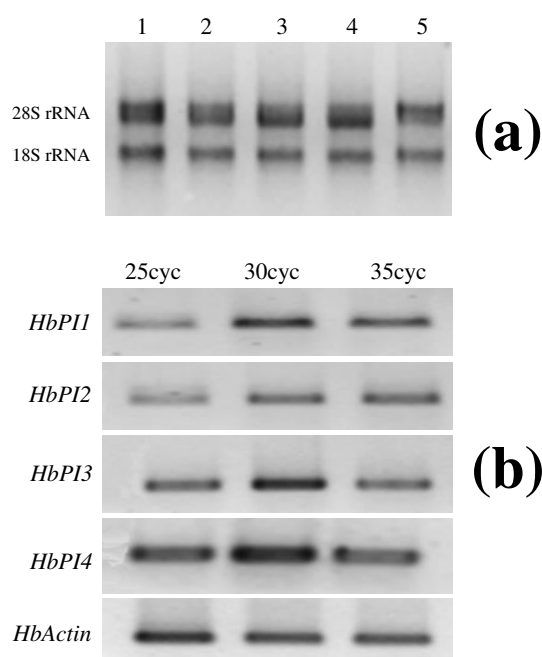
2.4. Acquisition of gel images and quantitative analysis
Fifteen µL of the PCR products were resolved by electrophoresis on ethidium bromide-stained gels, scanned using GelDock Imaging System (California, USA). The band quantification was carried out by scanning densitometry using Gel Analyzer 2010 (University of Leicester, UK). The measurement resulted in band intensity as the expression unit. The ratio between the sample RNA to be determined and *HbActin* was calculated to normalize for initial variations in sample concentration. Mean and standard deviation were calculated after normalization to *HbActin*. Relative expression (RE) of target genes against internal control was calculated by the equation: RE = expression unit of target genes/expression unit of internal control.

The mean values of normalized expression were obtained from three biological replicates. Statistical analysis was performed using the Analysis of Variance (ANOVA) at 5% level followed by Newman-Keuls Student test (XLStat, Addinsoft, USA).

3. Results and Discussion

3.1. Determination of RNA quality and PCR cycle for observation
The validation of RNA integrity is essential prior to the analytical pipeline for transcript accumulation analysis [27]. The quality and quantity of RNA determine largely the quality and quantity of synthesized cDNAs [28]. In this work, total RNA was isolated from every 5 samples of healthy and disease-affected rubber trees (**Figure 2a**).

Figure 2. RNA visualization (a) and determination of the exponential range of amplification at 25th, 30th and 35th cycles for four transcripts encoding *Hevea* protease inhibitors. (b) (1) healthy bark; (2) TPD-affected bark at ≤50% DCL; (3) TPD-affected bark at >50% DCL; (4) WRD-affected bark; (5) WRD+TPD-affected bark.
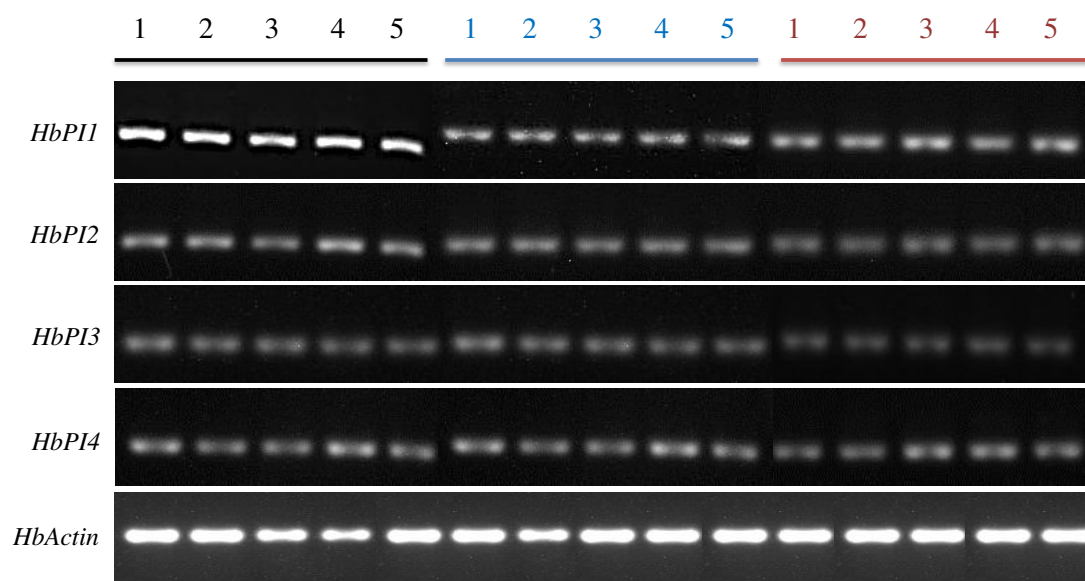
The agarose gel visualization showed a good RNA integration with the presence of 18S and 28S rRNAs. The purity of the total RNA samples (ratio of 260/280 nm) ranged from 1.79 to 1.95 while the RNA concentrations ranged from 600 to 990 ng/μL. According to the literature, the use of partially damaged or DNA contaminated RNA, approximately 7% of the transcript expression led to a less optimum result [29]. This could mean that the result of fold change expression analysis comparing two opposed conditions could result in a false positive [27]. In this work, the total RNA was ensured to be free from DNA contamination by carrying out a procedure of DNase.

One of the most important rules of thumb when performing a semi-quantitative RT-PCR is to select the appropriate number of cycles. This determination of the optimum range of amplification is indispensable (1) to have a clearly visible band on agarose gel and (2) to be able to be quantified using a visualization software. The optimal number of cycles on both internal control and the target RNA has to be in the same range so that both can be measured on the same gel [22]. In this work, the determination of optimum PCR cycle was carried out on 20th, 25th, 30th, and 35th cycles (**Figure 2b**). On the 20th cycle, the gel was unclear and cannot be visualized. However, the band was visually clear on the 30th cycle for all transcripts *HbPIs* and equally *HbActin* so that this cycle was selected for semi-quantitative RT-PCR visualization.

3.2. Semi-quantitative RT-PCR
In this paper, the semi-quantitative RT-PCR was successfully carried out on five cDNA samples representing healthy and disease-affected rubber tree latex. With each of the chosen RNA sample, 258-bp fragment of *HbPI1*, 216-bp of *HbPI2*, 179-bp of *HbPI3*, 312-bp of *HbPI3*, and 188-bp of *HbActin* were specifically amplified. Using the *HbActin* as an internal control, the mRNA levels of the above four transcripts were determined among various cDNA samples (**Figure 3**).
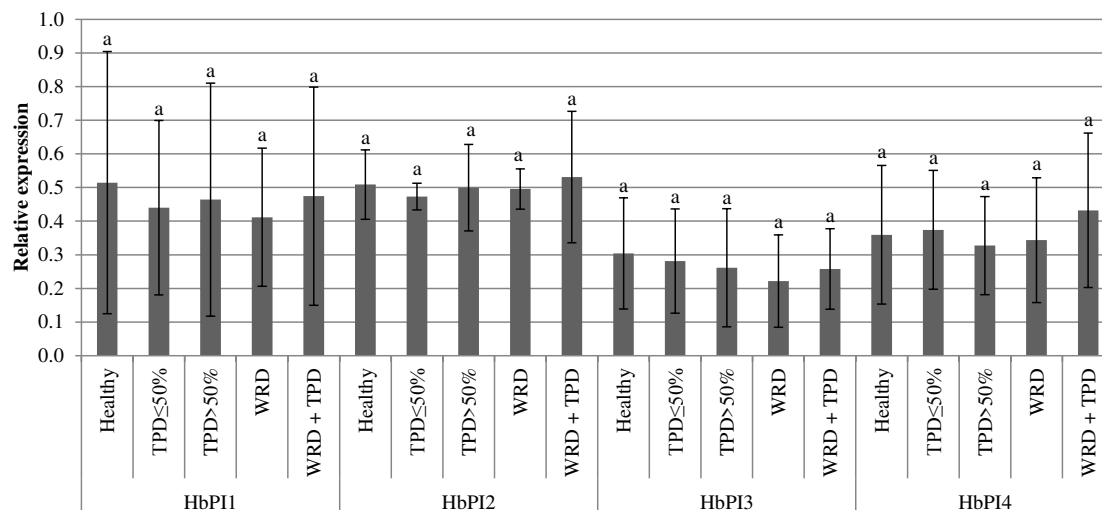
Figure 3. Semi-quantitative RT-PCR of four *Hevea brasiliensis* transcripts encoding protease inhibitors (*HbPIs*) under abiotic and biotic stresses. *HbActin* served as an internal control to normalize the expression levels of each *HbPI* transcripts. Each PCR was optimized for 30 cycles to achieve non-saturated gel image. Each colour bar represents one replicate. (1) healthy bark; (2) TPD-affected bark at ≤50% DCL; (3) TPD-affected bark at >50% DCL; (4) WRD-affected bark; (5) WRD+TPD-affected bark. WRD: white root disease; TPD: tapping panel dryness; DCL: dry cut length.

As shown in **Figure 3**, the band intensity of RNA transcripts of all test genes was visually lower than the internal control (*HbActin*). The expression of *HbActin* was stable throughout the different conditions of the cDNA sample which confirms the right choice of *HbActin* as the internal control. Meanwhile, the expression of all *HbPI* transcripts visually varied throughout the different sample conditions. The variation could lead to a potential assumption that *HbPI* transcripts were differentially regulated by abiotic and biotic stresses. After converting the visual image of agarose gel into semi-quantified data using Gel Analyzer, **Figure 4** confirmed the differential expression of *HbPI* transcripts among different cDNA samples. In general, the transcript accumulation of *HbPI1* and *HbPI2* were higher than *HbPI3* and *HbPI4* regardless of tree conditions (healthy or diseased). The combination of WRD and TPD had the highest relative expression of *HbPI2* transcript with the value of 0.531. On the other hand, *HbPI3* had the lowest relative expression on WRD-affected bark with the value of 0.222. However, the data was not statistically different under abiotic (TPD) and biotic (WRD) stresses. The mean values from three biological replicates showed a high variation. It means equally that the expression of *HbPI* transcripts was not regulated by either two diseases in a given time and tissue.

In the previous studies, the semi-quantitative RT-PCR analysis has been carried out in *Hevea brasiliensis*. The expression of *HbACO* genes in response to ethephon or ethylene was clearly observed using this technique [30]. The semi-quantitative RT-PCR has been successfully revealed the expression of the transcripts of two rubber biosynthesis genes (*HMGS2* and *HRT1*) and one sucrose transporter gene (*HbSUT2A*) [23]. In another experiment, the differential expression pattern of isolated cDNA fragments between healthy and TPD-affected trees was confirmed using semi-quantitative RT-PCR. This work revealed the down regulation of *HbTOM20* expression in TPD-affected trees meaning that the gene could play an important role on impaired latex biosynthesis during the occurrence of

TPD [31]. The recent semi-quantitative RT-PCR study showed that a protease inhibitor *PpEPI10* gene was significantly up regulated during colonization of rubber tree by *Phytophthora palmivora* which later interact with the subtilisin proteases in rubber tree. This research revealed the strength of semi-quantitative RT-PCR [32]. In this study, the semi-quantitative RT-PCR on *HbPI* transcripts was able to confirm the relatively stable expression among five conditions in rubber tree clone PB 260. This study has also shown its practical use as the alternative of transcript accumulation analysis for a group of genes in diverse conditions.

Figure 4. Transcript expression analysis using semi-quantitative RT-PCR for four *Hevea brasiliensis* transcripts encoding protease inhibitors (HbPIs). Each point represents tree biological replicates. Relative expression was calculated as the expression ratio of each treatment and *HbActin*. WRD: white root disease; TPD: tapping panel dryness. Statistical analysis was carried out using Newman-Keuls test of XL-STAT with p-value of 0.05.

3.3. Potential post-transcriptional and post-translational regulation of HbPIs

The stable expression of semi-quantitative RT-PCR analysis throughout different abiotic and biotic stresses highlighted a potential hypothesis of post-transcriptional or/and post-translational regulation of HbPIs. Transcriptional regulation of protease inhibitors has been demonstrated and reviewed [33]. However, the post-transcriptional and post-translational regulation of protease inhibitors in plants has not yet known. Even though so, the biological interaction of protease and its protease inhibitors is certain [34]. The expression of a serine protease inhibitor *KTI4* from tomato was regulated by a protease encoding gene *SlVPE3* in which this protease is post-transcriptionally regulated by miRNA [35]. Other demonstration showed that a cysteine protease inhibitor, cystatin, was transcriptionally regulated by a cysteine protease which was post-translationally regulated by protein modification [36]. The results revealed the complex regulation between protease inhibitors and its target proteases.

3.4. Future perspectives

Finally, the semi-quantitative RT-PCR method was shown to be consistently applicable for transcript expression studies. The work presented in this paper was an attempt to reveal the potential application of rubber tree latex not only as a raw material for tire industry but also for other purposes. The protease inhibitors are among other essential proteins contained inside laticifers. Future studies should be focused on deep functional characterization of *HbPI* gene family to reveal its wide and important role inside the latex.

4. Conclusions

In this study, the semi-quantitative RT-PCR on *HbPI* transcripts was able to confirm the relatively stable expression under abiotic and biotic stresses in rubber tree clone PB 260. The result highlighted the hypothesis of potential post-transcriptional and post-translational regulation of *HbPIs* which defines its abundance in the cell.

5. Acknowledgments

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