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EXPRESSION RESPONSES OF PATHOGENESIS-RELATED PROTEINS IN TOLERANT AND SUSCEPTIBLE *Hevea brasiliensis* CLONES TO THE WHITE ROOT DISEASE

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ABSTRACT

Pathogenesis-related proteins play crucial functions in the plant defense with antimicrobial activity response to pathogen infections. In this study, cDNA parts of selected antifungal genes (*HbPR-2*, *HbPR-4* and *HbPR-5*) were isolated from *Hevea brasiliensis*, the parts spanning 1,120, 233, and 517 bp, respectively. In BLASTP analysis *HbPR-2* showed a Glyco_hydro_17 domain; *HbPR-4* showed a Barwin domain; and *HbPR-5* contained a GHG-TLP-SF domain. The sequence alignment of amino acids indicated high similarities to the *PR-2, PR-4* and *PR-5* genes found in other plants. Transcriptional profiles from quantitative real time-PCR were compared in three *Hevea* clones, namely PB5/51 (a tolerant clone), BPM24 and RRIM600 (susceptible clones), after inoculation with *Rigidoporus microporus* at 0, 12, 24, 48, 72 and 96 h. The results revealed that these gene transcripts were more active in the tolerant clone than in the susceptible clones. These clearly indicate varied defense responses across clonal varieties, in the important context of host-pathogen interactions between the white root disease and rubber trees. This gives the priming strategy for tolerant rubber selection.

Keywords: *Hevea brasiliensis*, *Rigidoporus microporus*, white root disease, gene expression, pathogenesis-related proteins

INTRODUCTION

Hevea brasiliensis Muell. Arg. is an economically important crop in South-East Asia, particularly in Thailand. However, there is a risk of attack by the soil borne fungal pathogens with long-term cultivation under tropical weather conditions. *Rigidoporus microporus* is one of fungal pathogens, causing the white root disease in various tropical crops, especially in *H. brasiliensis.* It produces rhizomorphs at roots in the deep soil and infects nearby tree roots. The attacked plants commonly die, and this directly reduces the latex productivity of a rubber plantation (Nicole *et al.*, 1986; Nandris *et al.*, 1987; Nandris *et al.*, 1988).

Plants do not have a similar immune system to animals that have mobile white blood cells. Instead, plants have developed various other mechanisms to defend themselves against the pathogen invasion. When the fungal pathogen attacks host plant tissues, the pathogenesis-related proteins (PR-proteins) are produced with the large quantities both locally and systemically areas, and these proteins tend to accumulate and localize to the interactions, particularly in case of resistant interactions (Benhamou *et al.*, 1991; Rasmussen *et al.*, 1992; Stintzi *et al.*, 1993; Van Loon , 1997; Ebrahim *et al.*, 2011; Rai *et al.*, 2014; Fatima and Anjum, 2016).

The PR-proteins are grouped into 17 families according to their amino acid sequences, serological relationship, properties, and functions. Prior studies have been reported on functions of PR-proteins, including PR-2 (β-1,3-glucanases), PR-4, and PR-5 that have shown antifungal activity (Van Loon and Van Strien, 1999; Ebrahim *et al.*, 2011). These three PR-families, PR-2, PR-4, and PR-5, are the antifungal proteins that participated in induced responses to the fungal invasion (Selitrennikoff, 2001). The PR-2 is a class of hydrolytic enzymes that catalyze the cleavage of 1,3-β-D-glucosidic linkages in 1,3-β-glucans. It degrades the cell walls of the fungal pathogen, or release cell wall-derived materials that elicit active defense reactions in the plant (Doxey *et al.*, 2007; Sudisha *et al.*, 2012). Furthermore, PR-4 is considered an endochitinase as CBP20 in tobacco. It could adhere to chitin and exhibited weak chitinase activity (Ponstein *et al.*, 1994; Brunner *et al.*, 1998; Lu *et al.*, 2012). Several studies have reported antifungal activity associated with PR-4, such as that of wheatwin1 and wheatwin2 toward *Fusarium culmorum*, and of *LrPR4* toward *Magnaporthe grisea* (Caruso *et al.*, 1996; Li *et al.*, 2010). In addition, the PR-5 proteins are called thaumatin-like proteins (TLPs) or osmotins. Members in this family possess antifungal activity against fungal pathogens and enhance the resistance to a pathogen attack (Hejgaard *et al.*, 1991; Liu *et al.*, 1994; Abad *et al.*, 1996; Hu and Reddy, 1997).

The classical molecular approaches to tag resistance genes are complicated to employ with rubber trees, while developing such biotechnological methods is essential for rubber crop improvement (Jamali and Arain, 2005). The molecular aspects of plant defense response could provide

insights for more sophisticated molecular tool developments as mediated by the PR-proteins, and thereby use for rubber tree selection that carry more resistant capacity to white root disease. In order to obtain potentially tolerant rubber trees and to better understand the mechanisms associated with resistance genes related to *Hevea* defense responses against *R. microporus* infections, the antifungal genes (*HbPR-2*, *HbPR*-4 and *HbPR-5* genes) were isolated. The characteristics of their cDNA and amino acid sequences were examined with bioinformatics tools. In addition, the expression profiles of *HbPR-2*, *HbPR-4* and *HbPR-5* genes in *Hevea* after inoculation of seedlings were determined using quantitative Real Time-PCR (qP CR). The data from this study could facilitate developing tolerant rubber clones to white root disease.

MATERIALS AND METHODS

Plant material: Three clonal varieties of rubber trees, namely PB5/51, RRIM600 and BPM24 were used in this study. Their seeds were collected from Songkhla and Trang provinces, in Southern Thailand. PB5/51 was used to represent the tolerant clone. RRIM600 and BPM24 were used as moderate and highly susceptible clones, respectively. The rubber seedlings were grown in growth chambers under controlled conditions, subject to a Completely Randomized Design (CRD). For the inoculation studies, rubber seedling with approximately similar heights and diameters were chosen, to rule out effects from a wide range of plant maturation.

Preparation of fungal inoculums: The virulent *R. microporus* was derived from the Department of Pest Management, Prince of Songkla University. It has been isolated from basidiocarps that were collected from the white root disease in rubber plantation. The mycelium of *R. microporus* grown on potato dextrose agar (PDA) medium for 7 days was used for the inoculation.

Treatment of rubber trees with *R. microporus* inoculation and RNA extraction: The seedlings representing 3 clones were grown in the chambers for germination under controlled conditions. Three biological replicates were inoculated with *R. microporus*. The stem close to the root was disinfected with 70% ethanol and made an artificial wound around the bark on the trunk using a sterile scalpel prior to the fungal infection. An agar plug (0.7 cm in diameter) with actively growing fungal mycelia was placed close to the wound. Mock inoculation was performed using an agar plug, serving as control. Leaves from all inoculated rubber trees were kept at 0, 12, 24, 48, 72 and 96 h post inoculation (hpi). The RNA was isolated according to Sangsil *et al*. (2016). The RNA pellet was suspended in RNase-free water and treated with DNase (Promega) to eliminate DNA. Quantity of RNA was carried out with nanodrop spectrophotometer.

Molecular amplification of *PR*-genes: The single strand cDNA was synthesized in accordance with the manufacturer's instructions of Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific, USA). cDNA templates coding for *PR-2*, *PR-4* and *PR-5* were produced using RT-PCR amplification, with degenerate specific primers (Table 1). The thermal conditions consisted of predenaturation at 95 °C, 4 min and followed by 35 cycles of denature (95 °C, 1 min), annealing (55 °C, 1 min) and extension step (72 °C, 1 min) with a final extension at 72 °C, 10 min. The PCR products were verified using electrophoresis technique (1.5 % agarose gel) and stained by ethidium bromide (Sigma, Japan). GeneJET Gel Extraction Kit (Thermo Scientific, USA) was used to purify the excision of DNA target bands out of the agarose gel. The obtained DNA was cloned with RBC TA-cloning vector kit from RBC Bioscience (Taiwan). The recombinant clones were analyzed the sequence by BigDye® Terminator v3.1 cycle sequencing kit, First Base DNA Sequencing Services, Malaysia.

Bioinformatics Analyses: Reference sequences of the PR-genes and proteins in this study were obtained from GenBank databases (http://www.ncbi.mlm.nih.gov). Multiple sequence alignment was carried out by ClustalX 1.81 (Thompson *et al.*, 1997) and GENEDOC programs. DNA Comparison and the predicted amino acid sequences were performed by BLAST analysis.

Gene expression analysis of PR-genes: The qPCR was used for the expression quantification, the specific primers were designed by Primer3 program as shown in Table 2. The transcript levels were investigated by S so Advanced™ Universal SYBR® Green Supermix (BIO-RAD). The thermal cycle program of PCR machine composed of an pre-denaturation step (95 °C, 10 min) followed by 35 cycles of two-step, denaturation (95 °C, 15 sec) and annealing step (59 °C, 1 min). Afterwards, the melting curves of the PCR products were analyzed to verify the amplified product purity. The 18S rRNA was used for gene expression normalization, internal reference gene, and the relative expression levels were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The experimental conditions were also confirmed for 18S rRNA stability across samples from the cycle threshold values. There were three replicates in each treat-

ment for gene expression assays. Statistical analysis was carried out with R program version 3.2.3. The raw data were transformed by taking logarithms. The relative transcript abundance of gene expression at each time point was compared between treated and control group by Tukey's HSD test, and statistically significant differences are called based on P-value lower than 0.05.

Table 1 The degenerate nucleotide sequences of primers used in molecular cloning

Gene	Direction	Primer Sequence (5'→3')
PR2	Forward	GTGGAGYWGGAACCATCAAG
	Reverse	ATTTGCWAKAAGRTARGCCTCAA
PR4	Forward	GAGCAACWTACMATWWCTACAA
	Reverse	GCAYTGATCMACKATTCTCAC
PR5	Forward	AACAACTGCCCCWACMCKRTCT
	Reverse	TTAGGGTARCTRTAAGCATC

RESULTS

Molecular cloning and sequence analysis: The bands of *HbPR-2, HbPR-4,* and *HbPR-5* were obtained from amplification of cDNA with degenerate primers (Table 1), from the total RNA of a healthy rubber tree. The part of *HbPR-2* that was isolated had 1,120 bp. It matched the β-1,3-glucanase available in the NCBI database, and showed 98-99% homology with *H. brasiliensis.* BLASTP analysis of *HbPR-2* showed a Glyco _hydro_17 domain (Glycoside hydrolase family 17; PF00332) with the sequence LEVVV-SESG-WPSAG (Fig. 1A). Glyco_hydro_17 is a hydrolytic enzyme, and these catalyze the cleavage of 1,3-β-D-glucosidic linkages in β-1,3-glucans and are found in bacteria, fungi, and metazoans. The partial *HbPR-4* that was isolated had 233 bp, and this part from *H. brasiliensis* was 79-81% similar to those found in *Vitis vinifera*, *Vaccinium virgatum*, and *Populus euphratica*. It contained a Barwin domain (Consensus pattern: CGKCLSVTN) which is a highly conserved structural feature of the PR-4 proteins analyzed by BLASTP search (Fig. 1B). The *HbPR-5* section isolated had 517 bp, and shares significant 79-81% sequence similarity with TPLs from other plants. The data are shown in Table 3. The section of HbPR-5 protein contained a GH64 - TLP-SF domain which fit into the superfamily glycoside hydrolase family 64 (GH64) and TPLs (Fig. 1C). The superfamily are mostly β-1,3-glucanases which hydrolyze the β-1,3-glucans, bacterial and fungal cell walls, and their expression is induced by biotic and abiotic stresses such as pathogen attack, drought or cold stresses.

Table 2: The nucleotide sequences of primers in real-time PCR

Gene	Direction	Sequence (5'→3')	Amplicon size (bp)
HbPR-2	Forward	AGCCCTTAGAGGCTCAAACA	119
	Reverse	ACCAGAAGCCACGAACATTT	
HbPR-4	Forward	GAGCAACTTACCATTTCTACAA	233
	Reverse	GCATTGATCACCGATTCTCAC	
HbPR-5	Forward	CTTGGCCGAATATGCACTAA	174
	Reverse	TCCAGGGACCTTCAATTCAT	

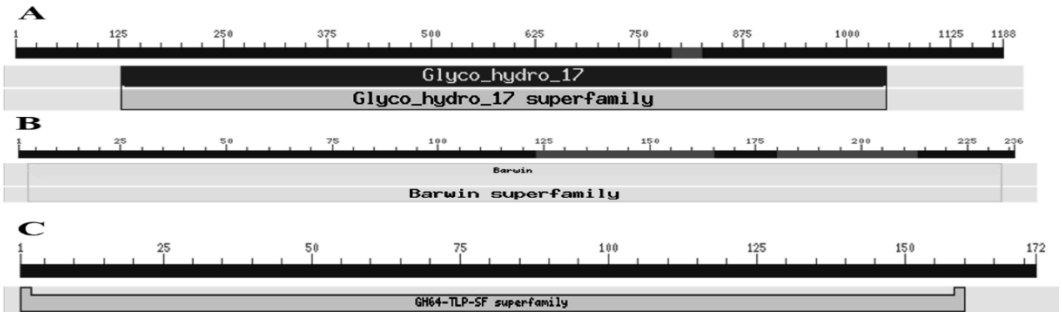

Figure 1: Conserved domain analysis of the deduced HbPR-2, HbPR-4 and HbPR-5 proteins; A: Glyco_hydro_17 domain of HbPR-2, B: Barwin domain of HbPR-4 and C: GHG4-TLP-SF domain of HbPR-5

Gene expression profiles of *HbPR-2, HbPR-4* and *HbPR-5* in response to fungal pathogen infection: In this study, the tolerant clone (PB5/51) and susceptible clones (RRIM600 and BPM24) to the white root disease were used to investigate the expression profiles of *HbPR-2*, *HbPR-4* and *HbPR -5* after *R. microporus* inoculation at 0, 12, 24, 48, 72, and 96 hpi. The 18S was used for qPCR data normalization in gene expression. There were variations in the gene expression levels of the PR-gene in response to the pathogen. The express-ion of *HbPR-2* was up-regulated in RRIM600 and PB5/51 at 12 h ($P \leq 0.05$) while BPM24 had significant down-regulation (Fig. 2A). Transcript levels of *HbPR-4* were significantly up-regulated in PB5 /51 and BPM24 at 12 h, and thereafter stable. However, this gene was significantly down-regulated in the RRIM600 clone (Fig. 2B). The expression

responses of *HbPR-5*, a thaumatin-like pathogen-nesis-related protein, varied across the three clones. This gene was strongly up-regulated in PB5/51, RRIM 600 and BPM24, respectively (Fig. 2C). However, the expressions of *HbPR-2*, *HbPR-4*, and *HbPR-5* Gradually decreased less from 72 h to 96 h, which reflected the inhibition of the three genes in the susceptible species of *H. brasiliensis*.

The tolerant clone, PB5/51, and the susceptible clones, RRIM600 and BPM24, were examined using qPCR with the specific primers shown in Table 2. All our focal genes had significantly up-regulated expression levels in the PB5/51 clone. *HbPR-4* and *HbPR-5* were expressed at higher level than *HbPR-2* ($P \leq 0.05$) as shown in Fig. 3A. The expression of *HbPR-5* in RRIM600 was strongly up-regulated, while *HbPR-2* and *HbPR-4* were eventually significantly down-regulated, despite their early up-regulation (Fig. 3B). In BPM24, the expression profiles of *HbPR-4* and *HbPR-5* showed significant up-regulation, but *HbPR-2* was down-regulated (Fig. 3C).

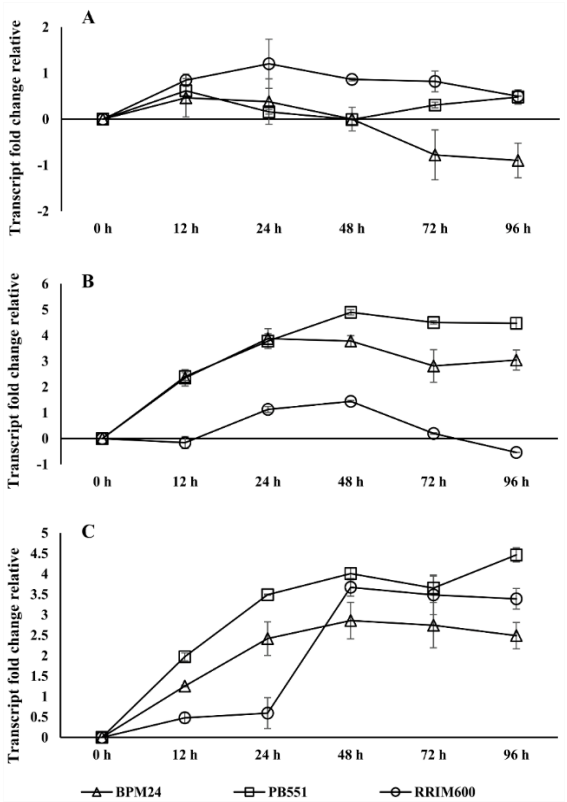

Figure 2: qPCR determined time profiles of the expressions of *HbPR-2* (A), *HbPR-4* (B), and *HbPR-5* (C) in the leaves of tolerant (PB5/51) and susceptible clonal varieties (RRIM 600 and BPM24) after inoculation with *R.microporus* compare with mock inoculation. The data are normalized to 18S rRNA expression. The transcripts levels are represented as fold changes relative to control. Each value shown is the mean of three biological replicates.

Table 3: BLAST results (sequences with significant alignments) for *HbPR-2*, *HbPR-4* and *HbPR-5*

Gene	Description	Accession	Identity (%)
HbPR-2	*Hevea brasiliensis* clone RRII 414, β-1,3-glucanase gene	EF222319.1	99
	Hevea brasiliensis clone PB86, β-1,3-glucanase (Glu I) gene	DQ649474.1	99
	Hevea brasiliensis, β-1,3-glucanase (Gln5) gene	JQ650524.1	99
	Hevea brasiliensis clone RRIM 600, β-1,3-glucanase (Glu1) gene	EF208894.1	98
HbPR-4	*Populus trichocarpa*, Pathogenesis-related protein-4	XP_002319077.1	81
	Vitis vinifera, Pathogenesis-related protein-4	AAC33732.1	81
	Vitis pseudoreticulata, Pathogenesis-related protein-4	AEW12795.1	81
	Ziziphus jujube, Pathogenesis-related protein-4 like	XP_015875395.1	79
HbPR-5	*Citrus sinensis*, thaumatin-like protein	XP_006477433.1	80
	Litchi chinensis, thaumatin-like protein	AEB77709.1	78
	Theobroma cacao, Osmotin	XP_007040162.1	77
	Medicago truncatula, pathogenesis-related thaumatin family protein	XP_013447000.1	75

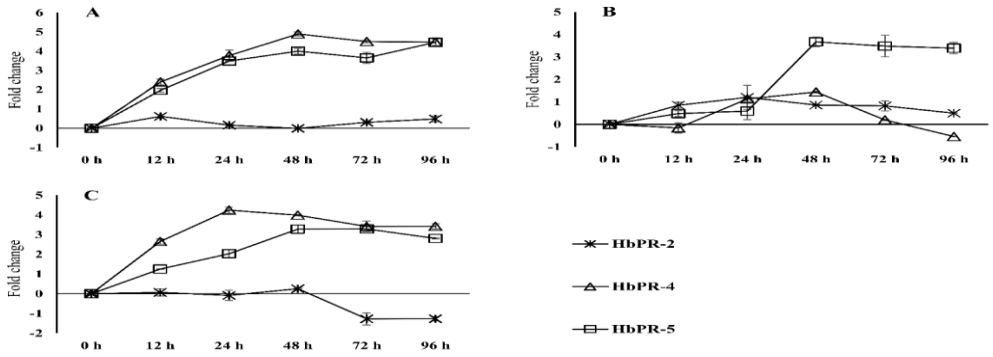

Figure 3: The expression profiles of *HbPR-2, HbPR-4* and *HbPR-5* in the leaves of three rubber plant clonal varieties; A: PB5/51, B: RRIM600, and C: BPM24 after inoculation with *R. microporus* compare with mock inoculation. The data are normalized to 18S rRNA expression level. Each value shown is the mean of three independent biological replicates.

DISCUSSION

In this study, the isolation and characterization of the genes, *HbPR-2*, *HbPR-4* and *HbPR-5*, from *H. brasiliensis* were described. The β-1,3-glucanase gene belongs to *PR-2* and is induced by pathogens. A BLASTP search showed that the partial protein sequence of HbPR-2 had high similarity to a β-1,3-glucanase in other plant species. It contained the glycosyl hydrolases family 17 domain LEVVVSESGWPSAG (Su *et al.*, 2013). The glycoside hydrolase family 17 inhibited the mycelial fungal growth by cleaving long-chain polysaccharide of cell wall β-1,3-glucans and would be more efficacy when synergistic action with chitinases (Mauch *et al.*, 1988; Higa-Nishiyama *et al.*, 2006). Therefore, *PR-2* genes might potently contribute to plant resistance to pathogens (Sels *et al.*, 2008).

HbPR-4 was isolated from leaves of rubber trees. It contained a highly conserved structural feature of the PR-4 protein, namely a Barwin domain. The aligned *HbPR-4* proteins are typically rich in cysteine (Lu *et al.*, 2012). ChtBD from *H. brasiliensis* has sequence similarity to hevein with chitin-binding properties and antifungal activity (Van Parijs *et al.*, 1991). Wheatwin1 and wheatwin2 isolated from wheat belonged to members of PR-4 proteins, and their recombinant proteins showed antifungal properties, suppressing fungal mycelial growth (Caruso *et al.*, 2001). Moreover, apart from antifungal activity, wheatwin1 was also able to interact with RNA from wheat and fungal pathogen, *Fusarium culmorum*. The antifungal activity of PR-4 proteins was exerted by their ribonuclease activity (Caporale *et al.*, 2004; Bertini *et al.*, 2009). Furthermore, the PR-4 proteins exhibited both deoxyribonuclease and ribonuclease activities (Guevara-Morato *et al.*, 2010; Bertini *et al.*, 2012). Therefore, these could be implied that the PR-4 protein accumulation displayed a role in the first-line defense responses.

PR-5 is one of the key members of the PR-protein families that play an important function in the plant defenses against pathogen attacks. Conserved regions in several plants showed that all variants of PR-5 have the same thaumatin and osmotin structures. The PR-5 protein contains 16 cysteine residues that may potentially form eight disulfide bonds, a signal position common to PR-5 proteins (Liu *et al.*, 2011; Rather *et al.*, 2015). In this study, we cloned partly the *HbPR-5* that encodes an antifungal protein in *H. brasiliensis*. The *HbPR-5* gene section showed a high degree of homology with thaumatin-like proteins. Moreover, the PR-5 proteins of other plants have been extensive study that possessed the certain activity to inhibit fungal growth and spore germination of various fungi (Midoro-Horiuti *et al.*, 2001; Thompson *et al.*, 2006; Liu *et al.*, 2011; Rather *et al.*, 2015).

In the study, three clonal rubber varieties including a tolerant clone (PB5/51) and susceptible clones (RRIM600 and BPM24) were used to detect expression of these defense genes after inoculation with *R. microporus*. The leaf samples were investigated at 0, 12, 24, 48, 72 and 96 hpi and used to analyze the gene expression profiles. The expression profiles of the pathogenesis related protein transcripts *HbPR-2*, *HbPR-4*, and *HbPR-5* were studied, with a view to molecular screening of white root disease tolerant rubber clones. The expression patterns of these genes were significantly up-regulated in the tolerant PB5/51 clone, while the transcript levels in the susceptible clones of BPM24 and RRIM600 eventually decreased despite early up-regulation. These patterns were similar to those in expression of *PR-2* that was studied in tolerant and susceptible rubber clones, in their responses to *Phytophthora meadii* infection. The results showed a tolerant RRII105 clone had higher *PR-2* transcript levels and prolonged induction for 4 days after inoculation. Meanwhile, the *PR-2* transcript levels in RRIM600 significantly decreased after 48 hpi (Thanseem *et al.*, 2005). Furthermore, the *PR-4* encoding *LcPR4a* gene in the lentil (*Lens culinaris*) was induced by *Ascochyta lentis* infection. The gene expression analysis demonstrated differential expression of the *LcPR4a* after infection with *A. lentis* in both resistant and susceptible rubber clones. The antifungal activities of LcPR4a protein expressed in *E. coli* showed a potentially significant function of *LcPR4a* in the defense response of lentil to ascochyta blight, caused by *A. lentis* (Vaghefi *et al.*, 2013). In 2010, Mukherjee studied the proteome of *Arabidopsis thaliana* infected with *Alternaria brassicicola*. The results showed that the *PR-4* and *PR-5* (osmotins) were greatly up-regulated. In addition, the study of *VpPR4-1* in a wild Chinese grape (*Vitis pseudo-reticulata*) showed significantly increased transcription after infection with powdery mildew fungus. The overexpression of *VpPR4-1* in 30 regenerated *V. vinifera* cv. Red Globe induced by *Agrobacterium tumefaciens*-mediated transformation showed that 26 transgenic grapevines had higher transcript levels of PR-4 proteins than the wild-type vines. Six of them suppressed the growth of powdery mildew**.** That study suggests that PR-4 protein acts an important function in the defense with resistance to powdery

mildew disease (Dai *et al.,* 2016). Similar to PR-5 protein, it was associated with plant disease resistance and its antifungal activities were exhibited in some plant species. A study of *PR-5* in *Zea mays* against *Rhizoctonia solani* showed that the transcript of *ZmPR5* was more stable in the resistant inbred maize line R15 than in the susceptible inbred line 478, and the transcription level of *ZmPR5* was high expressed after challenge with *R. solani* (Liu *et al.*, 2011). Moreover, the recombinant ZmPR5 protein suppressed hyphal growth of *R. solani*. The study indicated that the accumulation of ZmPR5 protein was closely related to sheath blight resistance in maize. Further, the antifungal assay of *ZmPR5* showed that it could act an important function in the disease resistance response. In order to understand the defense response pathways and to enhance resistance to fungal infection, *PR-5* was studied and *PdPR5-1* was isolated from *Prunus domestica*. The expression of *PdPR5-1* was studied in relation to infection with *Monilinia fructicola*. The results showed that the resistant cultivars showed much higher transcript levels than the susceptible cultivars, after infection with *M. fructicola* (El-kereamy *et al.*, 2011). However, the expression levels of five PR-proteins, including *PR-1, PR-3, PR-5, PR-8* and *PR-9* were studied in two rubber clones, RRIM612 (highly susceptible clone) and PR107 (least susceptible clone). Although the transcription of *PR-5* with antifungal properties was up-regulated in both clones' responses, the transcript levels of the other studied genes were variable in the clones. This suggests that the genetic characteristics of rubber clones may play an important function in defense related gene expression (Oghenekaro *et al.*, 2016).

In short, we report the expressional variability of three genes that possibly contribute to the establishment of white root disease tolerance in the rubber tree. Three PR-Proteins, *HbPR-2, HbPR-4* and *HbPR-5*, were characterized and identified the expression profiles after *R. microporus* inoculation. *HbPR-2*, *HbPR-4* and *HbPR-5* were isolated with the parts spanning 1,120, 233, and 517 bp, respectively. Although the antifungal genes were induced and appeared in both tolerant and susceptible rubber clones, the predominant difference between these types of rubber clones was in the intensity and duration of the response. The tolerant clone, PB5/51, may be associated with its prolonged expression of key genes following infection.

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