Characterization of the phenylalanine ammonia lyase gene from the rubber tree (Hevea brasiliensis Müll. Arg.) and differential response during Rigidoporus microporus infection

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Introduction

The white root rot disease, caused by Rigidoporus microporus is the most destructive root disease in Asian and African rubber tree (Hevea brasiliensis Müll. Arg.) plantations (Mohammed et al. 2014). An International Rubber Research and Development Board (IRRDB) survey indicated that white root disease is severe in Cote D’Ivoire, Nigeria and Sri-Lanka, and is a significant endemic problem in Gabon, Indonesia, Malaysia and Thailand (Ogbebor et al. 2013). It also affects other tropical woody crops such as cocoa, coffee, tea, coconut, oil palm, Ceylon breadfruit and Obeche (Nandris et al. 1987). In practice, the most effective solution to control white root rot disease is through the utilization of tolerant rubber clones as the rootstock. Therefore, gene expression analysis of defense related genes could assist plant breeders to select tolerant rubber clones. Phenylpropanoid compounds are precursors to a wide range of phenolic compounds that play a vital role in plant defense. The phenylalanine ammonia lyase (PAL) gene controls a key branch point in the pathway of flavonoid biosynthesis, which generates antimicrobial phytoalexins and plays important roles in the response to biotics (Bowles 1990). Many studies have shown the importance of salicylic acid (SA) for both local and systemic induced resistance in plant responses to pathogens (Mur et al. 1996; Shirasu et al. 1997; Clarke et al. 2000). Suzuki et al. (2004) reported that SA was synthesized by a branch of the phenylpropanoid pathway, through which cinnamic acid, formed from phenylalanine by the action of L-phenylalanine ammonia lyase, underwent chain shortening to form benzoic acid, which was then hydroxylated by a specific benzoate 2-hydroxylase to yield SA. This pathway is thought to be genetic because it has been observed that plants down-regulated the induction of the phenylpropanoid pathway (Araujo et al. 2009). In mango, the induction of four enzymes in the phenylpropanoid pathway is important for resistance to ascochyta blight disease in the chickpea (Kavousi et al. 2009). In mango, the induction of the phenylpropanoid pathway increases resistance to Ceratocystis fimbriata infection (Araujo et al. 2015). In addition, in the pepper plant, PAL1 enzymatic activity in the phenylpropanoid pathway acts as a positive regulator of SA-dependent defense signaling to combat microbial pathogens (Kim and Hwang 2014) and in a member of Euphorbiaceae family, PAL1 and PAL2 were...
cloned from *Manihot esculenta* Crantz. Finally, Pereira et al. (1999) examined the role of the *PAL* gene in the disease resistance of cassava. Phenylalanine ammonia lyase enzyme activity in the resistance interaction group was significantly higher than in the susceptible reaction group or the control group. Therefore, early or high PAL activity in response to infection is considered to be an indicator of pathogen resistance.

Understanding *H. brasiliensis* plant defense mechanisms is important for breeding resistant rubber clones. However, little is known about the molecular mechanisms regulating the defense response in the rubber tree. In recent years, many genes expressed in rubber tree tissues have been detected at translational and transcriptional levels by different methods. To date, no published data are available about the nucleotide sequence and tissue expression of the *PAL* gene in the rubber tree. The main goal of the current study was to clone and analyze the phylogeny of the *PAL* cDNA from the rubber tree. The study is also the first to report the tissue specific expression pattern of the *PAL* gene and its gene expression during *R. microporus* infection.

### Materials and Methods

#### Plant material

In this study, the PBS/51 clone was used as a tolerant clone as well as the RRIM 600 and BPM24 clones which are moderate and highly susceptible to infection with *R. microporus*. The seeds of these clones were collected from Songkhla and Trang provinces, Thailand. The plants were grown in a growth chamber under controlled conditions. The clones were arranged in a completely randomized design (CRD). Rubber seedlings with approximately the same height and diameter were chosen for inoculation. The clones were grown in a growth chamber under controlled conditions. The seeds of the clones used in the study were germinated for 3 months growth. In order to determine the same height and diameter were chosen for inoculations. The clones were arranged in a completely randomized design (CRD). Rubber seedings with approximately the seeds of these clones were collected from a diseased *H. brasiliensis* clone. The mycelium of the fungus was freshly grown on Potato Dextrose Agar (PDA) for 7 days and used for the inoculation.

#### Preparation of fungal inoculation

*Rigidoporus microporus* was obtained from the Department of Pest Management, Prince of Songkla University, Thailand. It was isolated from basidiocarps collected from a diseased *H. brasiliensis* clone. The mycelium of the fungus was freshly grown on Potato Dextrose Agar (PDA) for 7 days and used for the inoculation.

#### Treatment of rubber seeding with *Rigidoporus microporus*

The seeds of the clones used in the study were germinated in a growth chamber. During the 3-month seedling phase, the plants were inoculated with *R. microporus* with three replications being inoculated. Inoculation was carried out by creating wounds on stems which were close to the root. The area surface to be inoculated was sterilized with 70% ethanol. A sterilized surgical blade was used to create a wound around the upper part of the taproot. Using a cork borer agar of actively growing mycelia of the fungus was placed close to the wounds. Mock inoculation was carried out with sterile agar, which served as the control. Samples from the inoculated seedlings were collected 0, 12, 24, 48, 72 and 96 hours post inoculation (hpi) and immediately frozen in liquid nitrogen then stored at −80°C until RNA isolation was conducted.

#### RNA isolation and cDNA synthesis

RNA was isolated using a pH 9.0 extraction buffer [100 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 100 mM lithium chloride (LiCl), 2% sodium dodecyl sulfate (SDS)]. The samples of extracted RNA were kept at −80°C. The blended samples were extracted with phenol : chloroform-isooamyl. The RNA was precipitated in 8 M LiCl at 4°C overnight, followed by a 13,000 g centrifugation at 4°C for 20 min. The RNA pellets were dissolved in diethyl pyrocarbonate (DEPC)-treated water (Applichem, Germany). To eliminate genomic DNA, the extracted RNA was treated with RNase-free RQ1 DNase (Promega, USA). RNA quantification was carried out using a BioDrop DUO UV/VIS spectrophotometer and gel electrophoresis was performed to test the RNA quality and purity as described by Sambrook et al. (1989). Complementary DNA (cDNA) was synthesized using a Maxima H Minus First Strand cDNA synthesis kit following the manufacturer’s procedures (Thermo Scientific, USA). To confirm the absence of genomic DNA, all the cDNA samples were tested with specific primers of the gene encoding 18S rRNA. The primers were designed to anneal to sequences in exons on both sides of an 18S intron to discriminate the size of amplicons generated from cDNA and genomic DNA templates.

#### Cloning of cDNA encoding *PAL* gene using the rapid amplification of cDNA ends (RACE) method

*PAL* genes were cloned using degenerate polymerase chain reaction (PCR) and RACE approaches (Frohman et al. 1988). The internal, conserved fragments of the target genes were amplified with degenerate primers as shown in Table 1. Their design was based on the conserved regions among known nucleotide sequences of the corresponding genes from other plant species available at the National Centre of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The design of the degenerate primers was based on conserved blocks using the GENETYX program (Software Development Co., Ltd., Tokyo, Japan). A polymerase chain reaction mixture was prepared with 0.05 g template, 2.5 μl 10X PCR buffer, 0.5 μl dNTP (10 mM), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), and 0.3 μl Taq polymerase mixed in a total volume of 25 μl. Polymerase chain reaction was performed as follows: 94°C for 5 min; 35 cycles at 94°C for 30 sec, gradient PCR at 50–60°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 10 min. The PCR products were separated on 1% agarose gel. Subsequently, the ampli-
fied fragments of interest were cut from the agarose gel and purified using a Biospin Gel Extraction Kit (BioFlux). The fragments were then cloned into an RBCTA-cloning vector kit [RBC Bioscience (Taiwan)], and used to transform the DH5α competent cells according to the manufacturer’s protocols. Eight to ten white colonies were selected to be sequenced in order to obtain enough replicated sequences. The recombinant clones were sequenced using the automated sequencing facility of a BigDye® Terminator v3.1 cycle sequencing kit at 1st Base DNA Sequencing Services, Malaysia. Candidate cDNA sequences were analyzed using the nucleotide BLAST (Basic Local Alignment Search Tool) option from the NCBI website. Partial sequences of a fragment obtained from degenerated PCR enabled the design of *H. brasiliensis* gene specific primers for RACE. To obtain full-length cDNA *HbPAL*, specific primers were designed using Primer Premier 5.0 software (Table 1). Both 3′ and 5′ RACE were performed using Terminal Deoxynucleotidyl Transferase (Thermo Scientific, USA).

### Sequence analysis and multiple alignments

The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) encoded target gene were analyzed and sequence comparisons were conducted with BLAST (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were generated using the ClustalW program (Thompson et al. 1994).

### Quantitative real-time PCR analysis

The transcript levels of the *PAL* gene were analyzed using qRT-PCR with the ABI System. The design of the primers was based on the analogous *H. brasiliensis* coding sequences obtained from gene cloning results. Polymerase chain reaction was performed for primer selection to test the specificity of each primer with Melting temperature (Tm) at 60°C. The products were run on 2% (w/v) agarose gels containing Gold-PCR View to visualize the product after electrophoresis, in a 0.5% TBE (Tris/Borate/EDTA) buffer. The primers used for qRT-PCR are listed in Table 1. All the reactions were performed using the Fast Start, Universal SYBR Green Master Mix (Roche), following the procedure recommended by the manufacturer. To transform the fluorescent intensity measurements into relative mRNA levels, a 5-fold dilution cDNA sample was used to generate a standard curve. Polymerase chain reaction efficiencies (E) were calculated for each gene using the slopes of the standard curves derived from Light Cycler software, using serial dilutions according to the equation: $E = 10^{-\text{slope}}$ as described by Rasmussen (2000). Non infected *H. brasiliensis* was chosen as a calibrator for each group of samples and assigned a nominal value of 1.0. Three replicate PCR reactions were performed using about 1 μl of cDNA (50 ng · μl−1 of total RNA), 0.3 μM of each specific primer, and 10 μl of 1× SYBR Green PCR master mix (Roche, Switzerland) in a 20 μl volume. A negative control was included using water as a template in each reaction. The reaction mixtures were initially denatured at 95°C for 10 min, followed by a quantification program of 40 cycles at 95°C for 15 sec and 60°C for 60 sec. At the end of each run, melting curve analyses were conducted following the instrument’s instructions by slowly increasing the temperature from 60 to 95°C to ensure the specificity of the primer and the purity of the amplified product. Relative expression levels were calculated using the $\Delta\Delta$Ct method (Pfaffl 2001) and normalized Ct data obtained from a target gene with Ct values from the 18S rRNA gene as an internal control. The Ct values presented are the means of three independent biological replicates, and each reaction had three technical replicates.

### Results and Discussion

#### Isolation and characterization of *Hevea brasiliensis* PAL (*HbPAL*)

Using the degenerate oligonucleotide primers, a 1,352 bp fragment was amplified from leaves of a RRIM600 clone. After cloning, sequencing and alignment, the fragment of PAL with the highest identity to the plant’s *PAL* genes...

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequences (5′→3′)</th>
<th>Primer efficiency [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAL-F1</td>
<td>F: GCTGCGATTTTGYGGCAA</td>
<td></td>
</tr>
<tr>
<td>PAL-R1</td>
<td>R: CACTGTATTCATAGTACATCA</td>
<td></td>
</tr>
<tr>
<td>PAL-3GP1</td>
<td>SF2: GGAAGAGATCTGAGGCA</td>
<td></td>
</tr>
<tr>
<td>PAL-3GP2</td>
<td>SF3: GGTCTAACTACGGGTGTCT</td>
<td></td>
</tr>
<tr>
<td>PAL-5GP1</td>
<td>SR2: AATGGCTACCAAATGGTTCTT</td>
<td></td>
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<tr>
<td>PAL-5GP2</td>
<td>SR1: TGATCCCTACAGATAGCTCT</td>
<td></td>
</tr>
<tr>
<td>B26</td>
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<td>95.4</td>
</tr>
<tr>
<td>B25</td>
<td>GACCTTAGAGCAGATCTGATT</td>
<td>97.5</td>
</tr>
<tr>
<td>AAP</td>
<td>GACCCACCGCTGACTAGTACGGGGGGGGGGGGGGG</td>
<td></td>
</tr>
<tr>
<td>AUAP</td>
<td>GACCCACCGCTGACTAGTACGGGGGGGGGGGGGGG</td>
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</tr>
<tr>
<td>QHbPAL-F</td>
<td>GGATCTGCAAAAGTTGTGG</td>
<td></td>
</tr>
<tr>
<td>QHbPAL-R</td>
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</tr>
<tr>
<td>Q18S-F</td>
<td>AAGCCTTACGCTTGATACATT</td>
<td></td>
</tr>
<tr>
<td>Q18S-R</td>
<td>CCCGACTTCTCCCTGTAACT</td>
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Characterization of the phenylalanine ammonia lyase gene from the rubber tree…

was used to conduct 5’ and 3’ RACE, and a full-length cDNA of HbPAL was obtained as shown in Figure 1. The full length cDNA of HbPAL contained a 2,145 bp ORF and the deduced protein consisted of 721 amino acid residues. Multiple alignment analysis indicated that the HbPAL had a high homology to PAL from other plants (Fig. 2). The HbPAL shared 94%, 92%, 91%, 89% and 87% identity with PAL from M. esculenta, Jatropha curcas L., Ricinus communis L., Populus spp. and Theobroma cacao L., respectively. Many sites essential to PAL activities conserved in different plant species were also found in the HbPAL (Fig. 2). For example, a highly conserved domain known as phenylalanine and histidine ammonia lyase signature (GTITASGDLVPLSYIA) was found in position 196-211. Within this motif, there was the key active site Ala-Ser-Gly tripeptide (200-202) which is thought to be the key active site forming the MIO group (3,5-dihydro-5-methyl-4H-imidazol-4-one) (Gao et al. 2012). These two sites are assumed to play an important role in the function of this protein, and the presence of these sites in the HbPAL suggests that it has a similar function to that of other PAL proteins. Moreover, the strictly conserved residues, Y106, L134, S201, N258, Q346, Y349, R352, F398 and Q486 were found in the HbPAL. In addition, two key types of sites, deamination sites such as L-204, V-205, L-254, and A-255, and catalytic active sites such as N-258, G-259, NDN (380–382 aa), H-394 and HNQDV (482–488 aa) were found in the HbPAL protein (Jin et al. 2013). Moreover, a phosphorylation site, which is involved in the Phaseolus vulgaris L. turnover PAL subunits (Allwood et al. 1999) was also found at VAKRVLTT (541-548) of the HbPAL. All the active sites mentioned above were similar and at the counterpart positions with J. curcas PAL (JcPAL) (Gao et al. 2012), indicating that HbPAL is a member of the PAL family. This suggests that the predicted amino acid sequence of HbPAL is accurate. Also, several studies have concluded that homogenous sequences of the PAL gene are very highly conserved in many plant species. The predicted amino acid sequence of the gene is highly consistent with the plant PAL protein obtained (greater than 80%).

Expression levels of HbPAL in different tissues

Total RNAs were extracted from the stem, young and mature leaves, immature and mature seeds from the RRIM600 clone. The 18S gene was used as an internal control for all the samples. The results of qRT-PCR showed that the HbPAL mRNA transcribes in a clearly tissue specific pattern. The lowest expression level occurred in the mature seed (normalized to 1). The highest expression level occurred in the mature leaf and the relative expression levels followed in this order: young leaf, 95.76 > stem 21.69 > immature seed 18.53 > mature leaf 1.76 > mature seed 1.0 (Fig. 4). It is suggested that the influence of PAL probably varies, depending on the biological process (Jin et al. 2013). Phenylalanine ammonia lyase genes expressed in different tissues have been investigated in various plants (Ma et al. 2013, Song and Wang 2009). The PAL gene in J. curcas was most highly expressed in its flowers followed by its stem and leaf, with the lowest expression in its root and seed (Gao et al. 2012). Hevea brasiliensis PAL showed a similar expression pattern.
Fig. 2. Multiple alignments of the deduced amino acid sequences of the HbPAL homologue gene with other PAL protein sequences. Those underlined show the conserved domain phenylalanine and histidine ammonia lyase signature (GTITASGDLVPLSYIA) and the possible phosphorylation site (VAKRVLTT) is shown in a box. The catalytic active sites are indicated by black arrows.

The sequences compared are from *Manihot esculenta* (MePAL2, AAK60275.1), *Jatropha curcas* (JcPAL, ABI33979.1), *Ricinus communis* (RcPAL, XP002519521), *Theobroma cacao* (TcPAL, EOY18683.1), *Ziziphus jujuba* (ZjPAL, XP015866644), *Glycine max* (GmPAL, NP001236956) and *Vitis vinifera* (VvPAL, XP002268732).
pattern to the PAL gene in *Salvia miltiorrhiza* Bunge (Song and Wang 2009), *Solanum tuberosum* L. (Joos and Hahlbrock 1992), *Petroselinum crispum* (Mill.) Fuss. (Appert et al. 1994) and *Rehmannia glutinosa* L. (Lee et al. 2003), which had a higher expression in its leaves. Moreover, the PAL genes in *Oryza sativa* Libosch. and *Rhus chinensis* Mill. were expressed predominately in stems (Zhu et al. 1995; Ma et al. 2013) which differed from PAL genes in *Nicotiana tabacum* L. (Pellegrini et al. 1994), *Astragalus membranaceus* (Fisch.) Bunge (Liu et al. 2006) and *Rubus idaeus* L. (Ku...
mar and Ellis 2001), which were expressed more highly in roots. The results of the present study suggest that the rate of HbPAL1 gene activation in young leaves is much more important than that in stems or seeds.

**PAL transcription level during Rigidoporus microporus infection**

Plants have evolved multiple defense signaling pathways in response to environmental conditions and pathogen attack. Phenylalanine ammonia lyase is also a key enzyme involved in the biosynthesis of signal molecules such as SA, which has been shown to accumulate in the cells undergoing a hypersensitive response and to be essential for local and systemic resistance responses (Yang et al. 2011). Among the pathways induced in infected plants, the biosynthesis of phenylpropanoids has long been the subject of great attention, as it is strongly activated by elicitors and provides antimicrobial phytoalexins, lignin monomers and precursors of SA. The PAL gene seems to be one of the most critical elements of this system, catalyzing deamination of L-phenylalanine to trans-cinnamic acid and thus linking primary metabolism to the conversion of plant phenolics (MacDonald and D’Cunha 2007). In this study, the white root rot disease-tolerant rubber clone PB5/51 was used together with the RRIM600 and BPM24 clones which are representative of susceptible clones. These three clones demonstrated different levels of tolerance to *R. microporus* infection. The increase in Hb-PAL expression of the RRIM600 and BPM24 clones due to *R. microporus* infection was less than that of PB5/51. The HbPAL gene showed an expression two or more times higher than that of the susceptible clones. The HbPAL gene expression in the RRIM600 clone rapidly increased during the early stages of infection at 12 hpi before dramatically declining and remained relatively unchanged during the latter stages of infection (Fig. 5). In the BPM24 clone, the expression of the HbPAL gene remained slightly unstable during the early stages of infection, except for an increase at 72 hpi followed by a decrease at 96 hpi. On the other hand, the expression of the PAL gene in infected PB5/51 showed a unique induction pattern, in which gene expression increased slightly for the first 24 hpi and then showed a strong induction peak at 72 hpi. This was similar to the findings of Oghenekaro et al. (2016) who demonstrated that HbPAL was significantly increased in the highly susceptible RRIM612 clone and in the least susceptible PR107 clone 5 months after inoculation with *R. microporus*, slightly higher for RRIM612 than for PR107. In addition, Sayari et al. (2014) evaluated the transcription level of the PAL gene between resistant (Tarom) and susceptible (Khazar) rice cultivars in response to inoculation with *Rhizoctonia solani*. The PAL gene showed induction at 12 hpi and continued to elevate until 48 hpi in both cultivars. The maximum level of PAL transcripts in the Tarom cultivar was 6 times higher than the level observed in the Khazar cultivar at that stage. Also, genes involved in phenylpropanoid biosynthesis were differentially ex-

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Fig. 5. Differential expression patterns of HbPAL gene in PB5/51 (A), RRIM600 (B) and BPM24 (C) after infection with *Rigidoporus microporus* at 0, 12, 24, 48, 72 and 96 hours post inoculation.
pressed between ZC108 (anthracnose-resistant tea cultivar) and LJ43 (anthracnose-susceptible tea cultivar), suggesting that genes related to phenylpropanoid metabolism may play important roles in the phenotype of ZC108 (Wang et al. 2016). In contrast, Sherif et al. (2016) found that the expression of the PAL gene did not show any significant difference between tolerant and susceptible American elm clones at most sampling times post-inoculation, but its transcription level was higher in inoculated compared to control tissue. However, PAL increased to a significantly higher level in the susceptible clone only at 24 hpi, which is in agreement with PAL gene expression in the RRIM600 clone in the present study. Moreover, Fondevilla et al. (2011) found that the PAL gene is among those most highly up-regulated in a pea variety developing partial resistance reactions to Mycosphaerella pinodes. Shadle et al. (2003) reported that over-expression of PAL effectively reduced disease severity in tobacco plants infected with a virulent fungal pathogen (Cercospora nicotiana) which was presumed to result from high levels of chlorogenic acid production. Finally, a PAL-knockdown anaeme which was presumed to result from high levels of tolerance can be detected in their seedling stages.

Considered together, the differences in PAL expression levels of disease defense induction depend on the species of plant. The current study indicates that the level of transcription of the PAL genes can distinguish between tolerant and susceptible clones (Fig. 5). It is suggested that the over-expression of this gene constitutes a good indicator of the onset of a defense response in H. brasiliensis, and that candidate clones for white root rot disease tolerance can be detected in their seedling stages.

Conclusion

PAL genes have been identified and cloned in many plant species. However, no previous studies have been reported describing the PAL gene in H. brasiliensis. In order to elucidate the disease defense mechanism of H. brasiliensis PAL, which is the first key enzyme of the phenylpropanoid pathway, was chosen for gene cloning. In this study, based on the sequence of the HbPAL gene, specific primers were designed and cloned from HbPAL gene fragments from leaves. This study is the first to obtain the full-length cDNA sequence of the PAL gene (HbPAL). Tissue expression analysis by qRT-PCR revealed that HbPAL was expressed in all the tissues tested, especially in young leaves. Hveca brasiliensis PAL gene expression profiles from different H. brasiliensis clones after infection with R. microsporus were observed. In particular, the HbPAL gene was continuously up-regulated in the PBS/51 clone by an amount that is double or more in contrast to the susceptible clones RRIM600 and BPM24. These results suggest that HbPAL might play an important role in higher tolerance to R. microsporus in PBS/51. Additional studies to measure phenylpropanoid compound content and transcriptomes after inoculation will expand the understanding of the mechanisms of disease tolerance in the rubber tree.

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References


