

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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Abstract: Phenylalanine ammonia lyase (PAL) is a specific branch point enzyme of primary and secondary metabolism. It plays a key role in plant development and defense mechanisms. Phenylalanine ammonia lyase from *Hevea brasiliensis* (*HbPAL*) presented a complete open reading frame (ORF) of 2,145 bp with 721 encoded amino acids. The sequence alignment indicated that the amino acid sequence of *HbPAL* shared a high identity with PAL genes found in other plants. Phylogenetic tree analysis indicated that *HbPAL* was more closely related to PALs in *Manihot esculenta* and *Jatropha curcas* than to those from other plants. Transcription pattern analysis indicated that *HbPAL* was constitutively expressed in all tissues examined, most highly in young leaves. The *HbPAL* gene was evaluated by quantitative real-time PCR (qRT-PCR) after infection with *Rigidoporus microporus* at 0, 12, 24, 48, 72 and 96 hours post inoculation. The expression patterns of the PAL gene differed among the three rubber clones used in the study. The transcription level of the white root rot disease tolerant clone, PB5/51 increased sharply during the latter stages of infection, while it was relatively subdued in the white root rot disease susceptible clones, RRIM600 and BPM24. These results suggest that the *HbPAL* gene may play a role in the molecular defense response of *H. brasiliensis* to pathogen attack and could be used as a selection criterion for disease tolerance.

Key words: gene expression, *Hevea brasiliensis*, phenylalanine ammonia lyase, *Rigidoporus microporus*

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 in PAL expression had reduced levels of SA and compromised disease resistance (Maher *et al.* 1994; Pallas *et al.* 1996). For example, a functional analysis of tobacco PAL showed that PAL-suppressed transgenic tobacco has undeveloped systemic acquired resistance and reduced SA levels in its leaves (Pallas *et al.* 1996). Furthermore, 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

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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 PB5/51 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 after 3 months growth. In order to determine the organ-specific expression of the *PAL* gene in healthy rubber trees, the total RNA of RRIM600 clone was extracted from young and mature leaves, bark, as well as from premature and mature seeds. Three samples from each organ were analyzed in triplicate by quantitative real-time polymerase chain reaction (qRT-PCR).

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-isoamyl. 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 GENE-TYX 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 $50-60^{\circ}\text{C}$ 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-

Table 1. Primer sequences used to isolate the *PAL* gene from *Hevea brasiliensis* and quantitative real-time polymerase chain reaction (qRT-PCR) primers used for expression analysis

Primers	Primer sequences (5'→3')	Primer efficiency [%]
PAL-F1	F: GCTGGDATYTTYGGCAA	
PAL-R1	R: GCCTYAGYTTYTGCATCA	
PAL-3GSP1	SF2: GGAGGAGAATCTGAAGCA	
PAL-3GSP2	SF3: GGTCTAACTACGGGTGCT	
PAL-5GSP1	SR2: AATGGCTACCAAAATGGTTCTT	
PAL-5GSP2	SR1: TGATCCTACTAGCATAGCTGCT	
B26	GACTCTAGACGACATCGATTTTTTTTTTTTTTTTTTTT	
B25	GACTCTAGACGACATCGA	
AAP	GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGGG	
AUAP	GGCCACGCGTCGACTAGTAC	
QHbPAL-F	GGACATGCTCAAAGTTGTGG	95.4
QHbPAL-R	TGCTGGCATTCTTCTCATTC	
Q18S-F	AAGCCTACGCTCTGGATACATT	97.5
Q18S-R	CCCGACTGTCCCTGTTAATC	

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 DH5 α 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 (T_m) 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: $E = 10^{-1/\text{slope}}$ 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 \cdot μl^{-1} of total RNA), 0.3 μM of each specific primer, and 10 μl of 1 \times 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 $\Delta\Delta\text{Ct}$ 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

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-methylidene-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%).

Based on multiple sequence alignments and phylogenetic analysis, *M. esculenta* *PAL* (*MePAL*) and *JcPAL* have strong connections in terms of structure and features. Using alignments of multiple amino acid sequences, a phylogenetic tree was constructed to further identify the relationships between the *HbPAL* protein sequence and those of other plants that have already been obtained. As shown in Figure 3, *HbPAL*, *MePAL* and *JcPAL* appear to have similar structures and features, because *HbPAL* clustered with *MePAL* and *JcPAL*.

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* levels 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

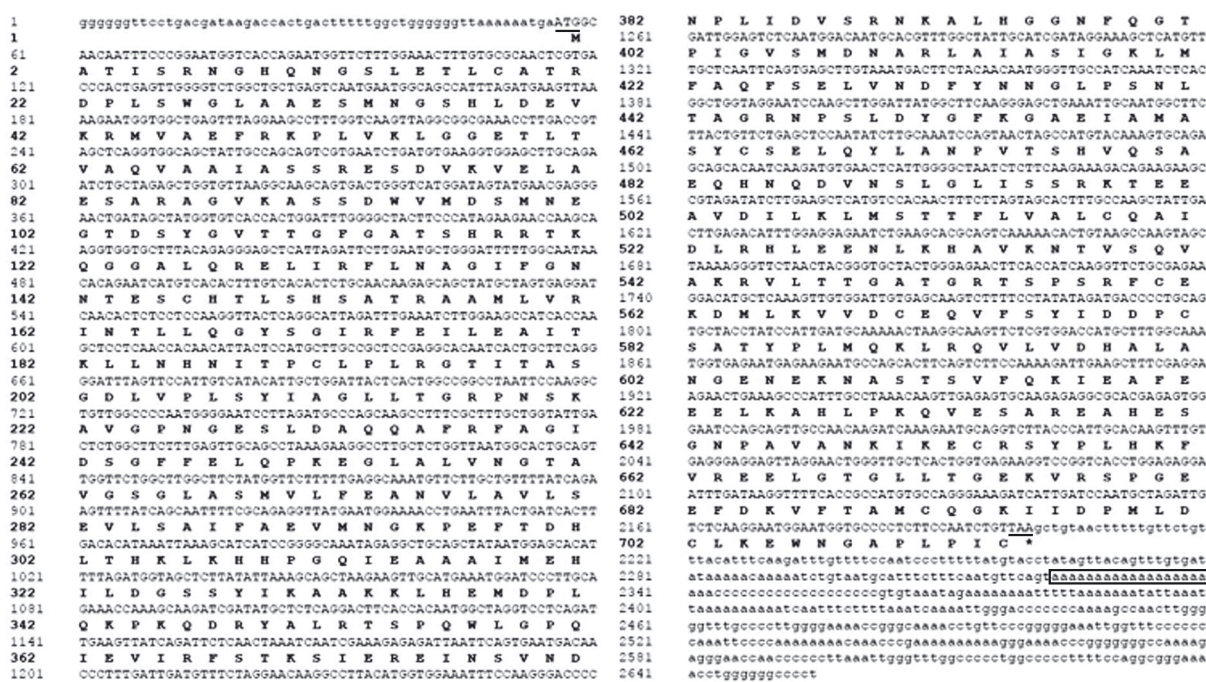


Fig. 1. The nucleotide sequence and the deduced amino acid sequence of *HbPAL*. The start codon (ATG) and stop codon (TAA) are underlined. The open black box represents the typical poly A signal sequence

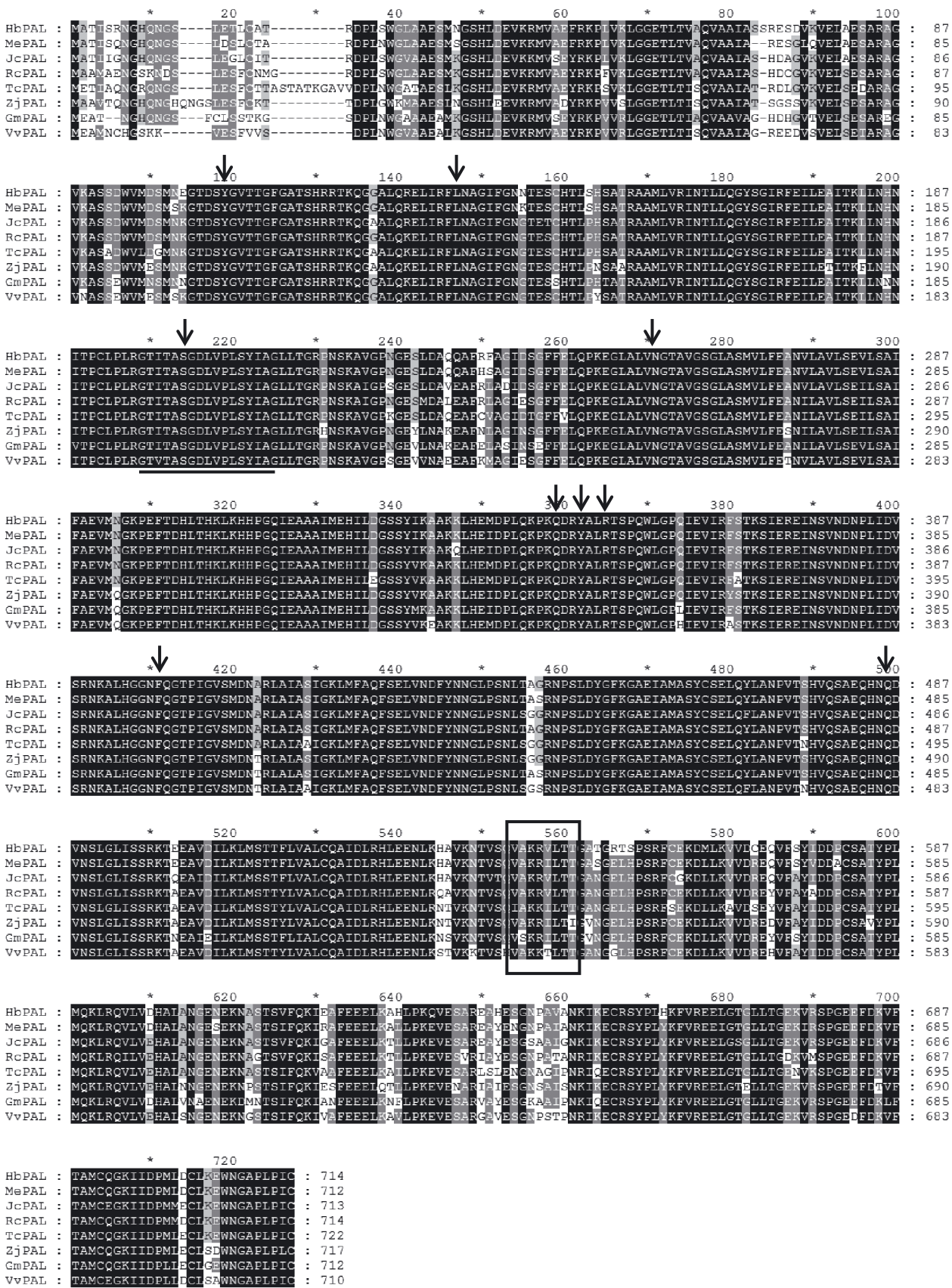


Fig. 2. Multiple alignments of the deduced amino acid sequences of the *HbPAL* homologue gene with other *PAL* protein sequences. Those underlined show the conserved domain phenylalanine and histidine ammonia lyase signature (GTITASGDLVPLSYIA) and the possible phosphorylation site (VAKRVLTT) is shown 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, XP 002268732)

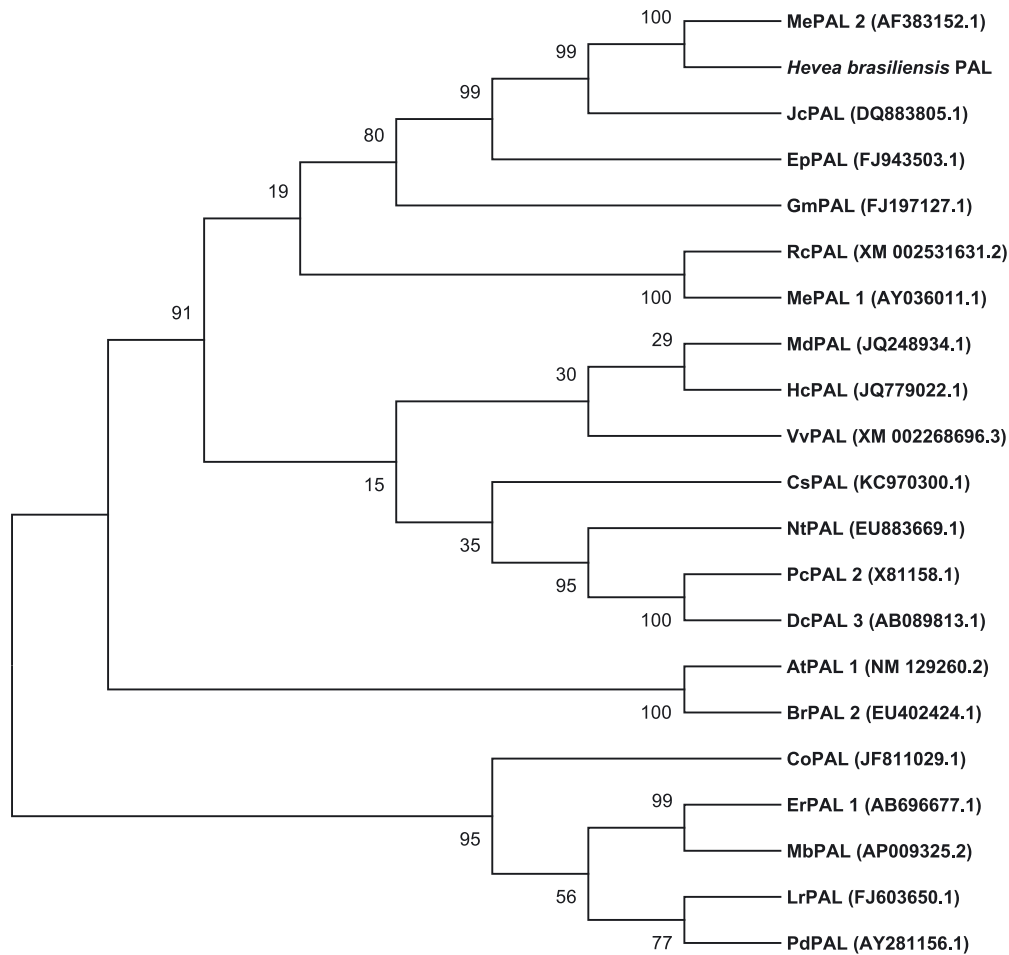


Fig. 3. Phylogenetic tree illustrating the genetic relationships between HbPAL and other plant PALs. Sequence name and GenBank accession No. in the figure are shown as follows: *Manihot esculenta* (MePAL2, AF383152.1), *Jatropha curcas* (JcPAL, DQ883805.1), *Euphorbia pulcherrima* (EpPAL, FJ943503.1), *Garcinia mangostana* (GmPAL, FJ197127.1) *Ricinus communis* (RcPAL, XM 002531631.2), *Manihot esculenta* (MePAL1, AY036011.1), *Malus hybrid* cultivar Royalty (MdPAL, JQ248934.1), *Hibiscus cannabinus* (HcPAL, JQ779022.1), *Vitis vinifera* (VvPAL, XM002268696.3), *Cannabis sativa* (CsPAL, KC970300.1), *Nicotiana tabacum* (NtPAL, EU883669.1), *Petroselinum crispum* (PcPAL2, X81158.1), *Daucus carota* (DcPAL3, AB089813.1), *Arabidopsis thaliana* (AtPAL1, NM 129260.2), *Brassica rapa* (BrPAL2, EU402424.1), *Cinnamomum osmophloeum* (CoPAL, JF811029.1), *Eucalyptus robusta* (ErPAL1, AB696677.1), *Musa balbisiana* (MbPAL, AP009325.2), *Lycoris radiata* (LrPAL, FJ603650.1) and *Phalaenopsis × Doritaenopsis* hybrid cultivar (PdPAL, AY281156.1)

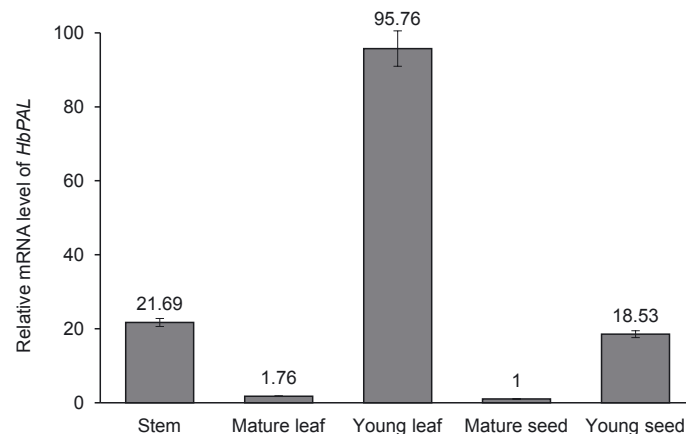


Fig. 4. Expression of HbPAL in different tissues by quantitative real-time PCR. Vertical bars represent the means±SE (n = 3)

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-

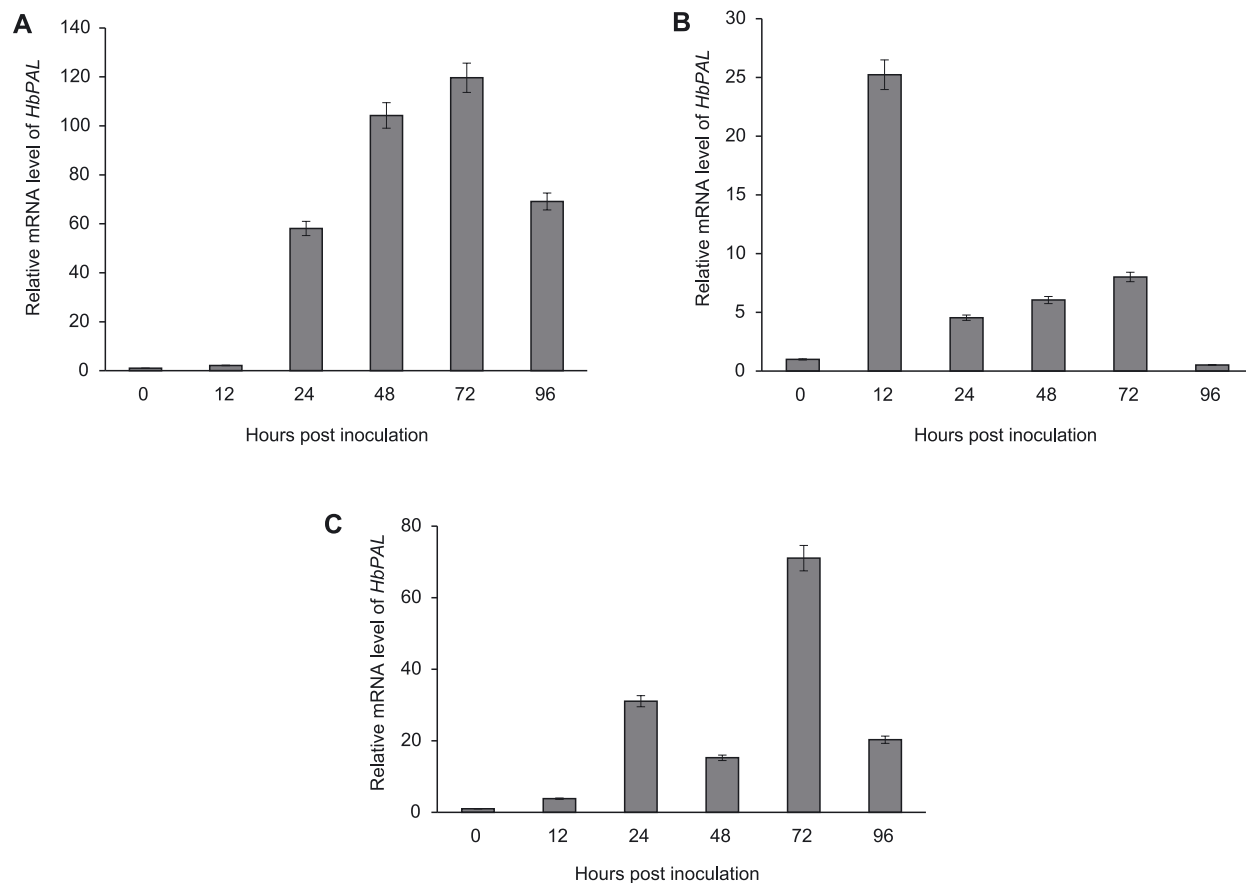


Fig. 5. Differential expression patterns of *HbPAL* gene in PB5/51 (A), RRIM600 (B) and BPM24 (C) after infection with *Rhizoglyphus microsporus* at 0, 12, 24, 48, 72 and 96 hours post inoculation

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 *Rhizoglyphus microsporus* 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. microsporus* infection. The increase in *Hb-*

PAL expression of the RRIM600 and BPM 24 clones due to *R. microsporus* 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. microsporus*, 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-

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 nicotianae*) which was presumed to result from high levels of chlorogenic acid production. Finally, a *PAL*-knockdown of *Brachypodium distachyon* exhibited delayed development and reduced root growth, along with increased susceptibility to the fungal pathogens, *Fusarium culmorum* and *Magnaporthe oryzae* (Cass *et al.* 2015).

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. *Hevea brasiliensis* *PAL* gene expression profiles from different *H. brasiliensis* clones after infection with *R. microporus* were observed. In particular, The *HbPAL* gene was continuously up-regulated in the PB5/51 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. microporus* in PB5/51. 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

- Allwood E.G., Davies D.R., Gerrish C., Ellis B.E., Bolwell G.P. 1999. Phosphorylation of phenylalanine ammonia-lyase: evidence for a novel protein kinase and identification of the phosphorylated residue. *FEBS Letters* 457 (1): 47–52.
- Appert C., Logemann E., Hahlbrock K., Schmid J., Amrhein N. 1994. Structural and catalytic properties of the four phenylalanine ammonia-lyase isoenzymes from parsley (*Petroselinum crispum* Nym.). *European Journal of Biochemistry* 225 (1): 491–499.
- Araujo L., Bispo W.M.S., Rios V.S., Fernandes S.A., Rodrigues F.A. 2015. Induction of the phenylpropanoid pathway by acibenzolar-s-methyl and potassium phosphite increases mango resistance to *Ceratocystis fimbriata* infection. *Plant Disease* 99 (4): 447–459.
- Bowles D.J. 1990. Defense-related proteins in higher plants. *Annual Review of Biochemistry* 59 (1): 873–907.
- Cass C.L., Peraldi A., Dowd P.F., Mottiar Y., Santoro N., Karlen S.D., Bukhman Y.V., Foster C.E., Thrower N., Bruno L.C., Moskvin O.V., Johnson E.T., Willhoit M.E., Phutane M., Ralph J., Mansfield S.D., Nicholson P., Sedbrook J.C. 2015. Effects of phenylalanine ammonia lyase (*PAL*) knockdown on cell wall composition, biomass digestibility, and biotic and abiotic stress responses in *Brachypodium*. *Journal of Experimental Botany* 66 (14): 4317–4335.
- Clarke J.D., Volko S.M., Ledford H., Ausubel F.M., Dong X. 2000. Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in *Arabidopsis*. *The Plant Cell* 12 (11): 2175–2190.
- Fondevilla S., Kuster H., Krajinski F., Cubero J.I., Rubiales D. 2011. Identification of genes differentially expressed in a resistant reaction to *Mycosphaerella pinodes* in pea using microarray technology. *BMC Genomics* 12 (1): 28.
- Frohman M.A., Dush M.K., Martin G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proceeding of the National Academy of Sciences of the United States of America* 85 (23): 8998–9002.
- Gao J., Zhang S., Cai F., Zheng X.J., Lin N., Qin X., Ou Y., Gu X., Zhu X., Xu Y., Chen F. 2012. Characterization, and expression profile of a phenylalanine ammonia lyase gene from *Jatropha curcas* L. *Molecular Biology Reports* 39 (4): 3443–3452.

- Jin Q., Yao Y., Cai Y.P., Lin Y. 2013. Molecular cloning and sequence analysis of a phenylalanine ammonia-lyase gene from *Dendrobium*. PLoS One 8 (4): e62352.
- Joos H.J., Hahlbrock K. 1992. Phenylalanine ammonia-lyase in potato (*Solanum tuberosum* L.). Genomic complexity, structural comparison of two selected genes and modes of expression. European Journal of Biochemistry 204 (2): 621–629.
- Kavousi H.R., Marashi H., Mozafari J., Bagheri A.R. 2009. Expression of phenylpropanoid pathway genes in chickpea defense against race 3 of *Ascochyta blight*. Plant Pathology Journal 8 (3): 127–132.
- Kim D.S., Hwang B.K. 2014. An important role of the pepper phenylalanine ammonia-lyase gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. Journal of Experimental Botany 65 (9): 2295–2306.
- Kumar A., Ellis B.E. 2001. The phenylalanine ammonia-lyase gene family in raspberry. Structure, expression, and evolution. Plant Physiology 127 (1): 230–239.
- Lee B.K., Park M.R., Srinivas B., Chun J.C., Kwon I.S., Chung I.M., Yoo N.H., Choi K.G., Yun S.J. 2003. Induction of phenylalanine ammonia-lyase gene expression by paraquat and stress-related hormones in *rehmannia glutinosa*. Molecules and Cells 16 (1): 34–39.
- Liu R., Xu S., Li J., Hu Y., Lin Z. 2006. Expression profile of a PAL gene from *Astragalus membranaceus* var. *Mongholicus* and its crucial role in flux into flavonoid biosynthesis. Plant Cell Reports 25 (7): 705–710.
- Ma W.L., Wu M., Wu Y., Ren Z.M., Zhong Y. 2013. Cloning and characterisation of a phenylalanine ammonia-lyase gene from *Rhus chinensis*. Plant Cell Reports 32 (8): 1179–1190.
- MacDonald M.J., D’Cunha G.B. 2007. A modern view of phenylalanine ammonia lyase. Biochemistry and Cell Biology 85 (3): 273–282.
- Maher E.A., Bate N.J., Ni W., Elbind Y., Dixon R.A., Lamb C.J. 1994. Increase disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proceedings of the National Academy of Sciences of the United States of America 91 (16): 7802–7806.
- Mohammed C.L., Rimbawanto A., Page D.E. 2014. Management of basidiomycete root- and stem-rot diseases in oil palm, rubber and tropical hardwood plantation crops. Forest Pathology 44 (6): 428–446.
- Mur L.A.J., Naylor G., Warner S.A.J., Sugars J.M., White R.F., Draper J. 1996. Salicylic acid potentiates defence gene expression in tissue exhibiting acquired resistance to pathogen attack. The Plant Journal 9 (4): 559–571.
- Nandris D., Nicole M., Geiger J.P. 1987. Root rot disease of rubber trees. Plant Disease 71 (4): 298–306.
- Ogbebor N.O., Adekunle A.T., Eghafona O.N., Ogboghodo A.I. 2013. Incidence of *Rigidoporus lignosus* (Klotzsch) imaz of para rubber in Nigeria. Researcher 5 (12): 181–184.
- Oghenekaro A.O., Omorusi V.I., Asiegbu F.O. 2016. Defence-related gene expression of *Hevea brasiliensis* clones in response to the white rot pathogen, *Rigidoporus microporus*. Forest Pathology 46 (4): 318–326. DOI:10.1111/efp.12260.
- Pallas J.A., Paiva N.L., Lamb C., Dixon R.A. 1996. Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. The Plant Journal 10 (2): 281–293.
- Pellegrini L., Rohfritsch O., Fritig B., Legrand M. 1994. Phenylalanine ammonia-lyase in tobacco. Molecular cloning and gene expression during the hypersensitive reaction to tobacco mosaic virus and the response to a fungal elicitor. Plant Physiology 106 (3): 877–886.
- Pereira L.F., Goodwin P.H., Erickson L. 1999. The role of a phenylalanine ammonia lyase gene during cassava bacterial bright and cassava bacterial necrosis. Journal of Plant Research 112 (1): 51–60.
- Pfaffl M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29 (9): e45.
- Rasmussen R. 2000. Quantification on the light cycler. p. 21–34. In: “Rapid Cycle Real-Time PCR, Methods and Applications” (S. Meuer, C. Wittwer, K.I. Nakagawara, eds.). Springer Press, Heidelberg, Germany, 408 pp.
- Sambrook J., Fritsch E.F., Maniatis T. 1989. Molecular Cloning: a Laboratory Manual. Vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 626 pp.
- Sayari M., Babaeizad V., Ghanbari M.A.T., Rahimian H. 2014. Expression of the pathogenesis related proteins, NH-1, PAL, and lipoxygenase in the Iranian Tarom and Khazar rice cultivars, in reaction to *Rhizoctonia solani* – the causal agent of rice sheath blight. Journal of Plant Protection Research 54 (1): 35–43.
- Shadle G.L., Wesley S.V., Korth K.L., Chen F., Lamb C., Dixon R.A. 2003. Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of l-phenylalanine ammonia-lyase. Phytochemistry 64 (1): 153–161.
- Sherif S.M., Shukla M.R., Murch S.J., Bernier L., Saxena P.K. 2016. Simultaneous induction of jasmonic acid and disease-responsive genes signifies tolerance of American elm to Dutch elm disease. Scientific Reports 6: 21934.
- Shirasu K., Nakajima H., Rajasekhar V.K., Dixon R.A., Lamb C. 1997. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. The Plant Cell 9 (2): 261–270.
- Song J., Wang Z. 2009. Molecular cloning, expression and characterization of a phenylalanine ammonia-lyase gene (*SmPAL1*) from *Salvia miltiorrhiza*. Molecular Biology Reports 36 (5): 939–952.
- Suzuki H., Xia Y., Cameron R., Shadle G., Blount J., Lamb C., Dixon R.A. 2004. Signals for local and systemic responses of plants to pathogen attack. Journal of Experimental Botany 55 (395): 169–179.
- Thompson J.D., Higgins D.G., Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22 (22): 4673–4680.
- Wang L., Wang Y., Cao H., Hao X., Zeng J., Yang Y., Wang X. 2016. Transcriptome analysis of an anthracnose-resistant tea plant cultivar reveals genes associated with resistance to *Colletotrichum camelliae*. PLoS One 11 (2): e0148535.
- Yang H.R., Tang K., Liu H.T., Huang W.D. 2011. Effect of salicylic acid on jasmonic acid-related defense response of pea seedlings to wounding. Scientia Horticulturae 128 (3): 166–173.
- Zhu Q., Dabi T., Beeche A., Yamamoto R., Lawton M.A., Lamb C. 1995. Cloning and properties of a rice gene encoding phenylalanine ammonia-lyase. Plant Molecular Biology 29 (3): 535–550.