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Expression and promoter analysis of the *hevein* gene in rubber tree (*Hevea brasiliensis*) clones resistant and susceptible to *Phytophthora* spp.

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ABSTRACT: Hevein is a major latex protein that accumulates in the vacuolar compartments, known as lutoids, of the rubber tree (*Hevea brasiliensis*). It has been reported to play key roles in plant defense responses and to participate in the latex coagulation process. This study aims to investigate the molecular mechanism and relation of the *hevein* gene to the resistance of rubber trees against *Phytophthora* spp. Phylogenetic analysis was performed to evaluate the relationship of hevein from rubber trees with the other PR4 genes within the plant species. The phylogenetic analysis categorized the hevein homologs into two major clades based on the presence or absence of sap, demonstrating convergent evolution. The hevein precursor and prohevein from *H. brasiliensis* shared a close relationship with prohevein from cassava (Euphorbiaceae) and grouped together with *Ricinus communis* and *Populus alba* (order Malpighiales). The expression of the *hevein* gene was studied in rubber tree clones PB260, a *Phytophthora*-resistant clone, and RRIM600, a susceptible clone, under non-infected conditions through quantitative real-time polymerase chain reaction (qRT-PCR) technique. The results indicated that the expression level of the *hevein* gene was significantly higher in PB260 than in RRIM600. This result suggests that the expression level of the *hevein* gene may be related to pathogenic fungi resistance in rubber tree clone PB260. The potential *cis*-acting regulatory elements were identified on the *hevein* promoter of the RRIM600 rubber tree clone. Several hormone-, defense-, mineral-, organ specific-, light-, and water stress-responsive elements were present. The promoter of the *hevein* gene was found to harbor ethylene-, salicylic acid- and jasmonic acid-responsive elements known to be involved in plant defense against pathogens. The results from this study provide an understanding of the expression and regulation of the *hevein* gene in rubber trees and is beneficial for further improvement of pathogenic fungi-resistant rubber tree clones in rubber tree breeding programs.

Keywords: *Hevein*; pathogenesis-related genes; *Phytophthora*; rubber tree

INTRODUCTION

The rubber tree (*Hevea brasiliensis*) is one of the most important economic crops of Thailand and belongs to the Euphorbiaceae family. It is the major source of natural rubber, *cis*-1,4-polyisoprene, which is collected from laticifer cells located in the inner soft bark tissue (Ekchaweng et al., 2017). Thailand is the world leader in producing

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natural rubber, and most of the production occurs in the southern part of the country (Pethin et al., 2015). In the south, the long wet season and overall high humidity promote abnormal leaf fall (ALF), one of the most common diseases in rubber trees caused by *Phytophthora* species (Khompatara et al., 2019). In rubber trees, infection by *Phytophthora palmivora* causes extensive defoliation of mature leaves. It also attacks the tapping surfaces, causing dark vertical lines in the panels. These symptoms lead to reduced rubber tree growth and latex productivity (Deenamo et al., 2018). Rubber tree clones clearly exhibit variable levels of resistance to pathogenic fungal diseases (Narayanan and Mydin, 2012). Through an analysis of percentage disease index (Joseph et al., 1994) in Southern Thailand, rubber tree clones PB260 and RRIM600 were reported to be resistant and susceptible to *Phytophthora* sp., respectively (Rodesuchit et al., 2009). The widely cultivated clone RRIM600 also showed a high degree of susceptibility to ALF disease in India (Thanseem et al., 2005).

To protect against pathogen attack, plants have evolved numerous constitutive and induced basal defense mechanisms, i.e., the deposition of lignin, suberin and callose to reinforce cell walls at the pathogen penetration site; the accumulation of antimicrobial proteins and phytoalexins; oxidative bursts; defense signaling pathways and transcriptional expression of *pathogenesis-related* (*PR*) genes (Deenamo et al., 2018). PR-proteins are defined as proteins that are induced when the plant faces stress, pathogen attack or abiotic stimuli. The inhomogeneous group of PR-proteins has been classified into 17 families. Only PR-1–PR-5, PR-8, and PR-11–PR-16, have exhibited direct antifungal activity against phytopathogenic fungi (Moosa et al., 2017). The expression of *β-1,3-glucanase*, a *PR-2* gene, resulted in a marked increase in *Phytophthora* sp. tolerance by the RRII105 rubber tree clone, with a higher and more prolonged rate of tolerance than the susceptible RRIM600, after *P. meadii* inoculation.

Prohevein is a chitin-binding protein from rubber trees and is classified as a PR-4 protein. Post-translational modification of the hevein precursor (204 amino acids) generates prohevein (187 amino acids; 18.5 kDa) which is then processed into the N-terminal hevein peptide (43 amino acids; 4.7 kDa) and the C-terminal domain (144 amino acids; 13.3 kDa) (Berthelot et al., 2016). This process is homologous to the wound-inducible proteins WIN1 and WIN2 from potatoes (Stanford et al., 1989) and to PR-4 from tobacco (Friedrich et al., 1991). In the rubber tree, hevein is one of the major latex proteins that accumulates in the lysosomal microvacuoles called lutoids (Peumans et al., 2001). It represents about 70% of the soluble protein in the bottom fraction of latex (Archer et al., 1969). Hevein has been reported to play a role in plant defense. It has shown the capacity to inhibit the growth of several chitin-containing fungi (Van parijs et al., 1991) via penetration through the fungal cell wall, affecting the active-sites involved in cell wall morphogenesis. Hevein has also been proposed to participate in the latex coagulation processes, when released from lutoids into the cytosol, through its ability to bind to the *N*-acetyl-D-glucosamine moiety of the glycosylated protein located on the surface of rubber particles (Gidrol et al., 1994). Rubber particle aggregation leads to latex coagulation, which protects the rubber tree trunk by sealing the wound and providing a physical and biochemical barrier to pathogen invasion (Shi et al., 2019).

Since the difference in PR protein abundance between non-infected tolerant and susceptible clones allows tolerant rubber trees to respond more efficiently to pathogen attack (Havanapan et al., 2016), this study was performed to analyze the expression level of the *hevein* gene in a resistant rubber tree clone (PB260) and a susceptible clone (RRIM600), to verify its contribution to plant defense against *Phytophthora* spp.. Furthermore, to reveal its transcriptional regulation, the promoter of the *hevein* gene from rubber tree clone RRIM600 was also

analyzed *in silico*. The information from this study will improve our understanding of the expression and regulation of the *hevein* gene in rubber trees and will be beneficial for the improvement of pathogenic fungi resistant rubber tree clones in rubber tree breeding programs.

MATERIALS AND METHODS

Plant materials

Latex samples were collected from 10-year-old PB260 and RRIM600 rubber tree clones that had been harvested for rubber for 2 years. The rubber trees were located at Chiang Rai Horticultural Research Center, Thailand. All rubber trees used in the experiment were non-infected. Three trees of each clone were selected for their similar girth and latex yield. The tapping system used at the research center was a half spiral downward cut, on an alternating schedule of one day tapped and one day rest (S/2 d2). Six mL of latex from each tree was collected in an equal volume of 2X fixation/extraction buffer (50 mM Tris-HCl, 300 mM LiCl, 10 mM EDTA, 10% SDS, pH 9.0). The latex samples were immediately deep-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Phylogenetic tree construction

The phylogenetic tree was constructed based on the amino acid sequences of the hevein precursor from rubber tree and various plants retrieved from the NBCI database. For the multiple sequence alignment, amino acid sequences were aligned using the CLUSTALW algorithm and adjusted manually, which were visualized using BioEdit version 7.2.5 (Hall, 1999). The phylogenetic tree was constructed by MEGA-X (Kumar et al., 2018) using Maximum Likelihood with default settings and the Whelan and Goldman (WAG) model (Whelan and Goldman, 2001). Support for the tree obtained was assessed by using bootstrapping with 1000 replications (Felsenstein, 1985).

RNA extraction and cDNA synthesis

Total RNA was extracted using the LiCl precipitation method adapted from Pujade-Renaud et al. (1994) and developed by Kongsawadworakul et al. (2004). The amount and purity of RNA were determined by NanoDrop® ND-1000 UV-Vis Spectrophotometer at wavelengths of 260 and 280 nm. Total RNA was treated with rDNase I (DNA-free™, Ambion) to remove contaminating DNA from RNA preparation. First-strand cDNA synthesis was performed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). For each sample, first-strand cDNA synthesis and qRT-PCR amplifications were performed in triplicate.

Gene expression analysis by qRT-PCR

Specific primers [HevF: 5'-ACTGGACACAGATGGGAAAG-3'; HevR: 5'-GCCTTGTTGTTGCTTATTGC-3'] for the *hevein* gene were designed based on the nucleotide sequence (accession number M36986) from GenBank (http://www.ncbi.nlm.nih.gov) using the Primer3 Plus and OligoAnalyzer 3.1 programs. The qRT-PCR was performed with the ABI-7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Each 20 μL qRT-PCR reaction consisted of 2 μL of 10X PCR buffer, 0.8 μL of 50 mM $MgCl_2$, 0.4 μL of 10 mM dNTPs, 0.4 μL each of 20 mM forward and reverse primers, 0.24 μL of SYBR® Green I (Sigma), 0.16 μL of Platinum® *Taq* DNA polymerase (Invitrogen) and 2 μL of cDNA (dilution 1:25). Polymerase chain reaction (PCR) was performed for 40 cycles. Two-step PCR included denaturation at 95°C for 15 sec and annealing as well as polymerization at 57°C for 1 min. The relative quantity of target gene transcript, compared to a reference of housekeeping gene *UBC2a* (Li et al., 2011), was automatically calculated as $2^{-\Delta Ct}$ where ΔCt = Ct of target gene - Ct of reference gene.

Promoter analysis

The promoter region of the *hevein* gene was acquired from whole genome shotgun contigs of *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. (taxid:3981) in the NCBI database. The *cis*-acting responsive elements of the gene were analyzed through NewPLACE (https://sogo.dna.affrc.go.jp), a database of plant *cis*-acting regulatory DNA elements.

Statistical analysis

Significant differences in *hevein* gene expression between the rubber tree clones were analyzed with an Independent-Sample T-Test with 95% confidence intervals. Statistical analysis was conducted by PASW Statistics 18 and Microsoft Excel 2016.

RESULTS AND DISCUSSION

Phylogenetic analysis of hevein precursor

In this study, the phylogenetic tree was generated based on the 19 amino acid sequences of PR4 group proteins from *Hevea brasiliensis* and other plant species. Hevein precursor from *H. brasiliensis* exhibited a percent identity similar to the those from other plant species in the NCBI database with a range between 70-80%. The highest percent identity was shown with Mehevein from *Manihot esculenta* (80.81%), a plant belonging to the same family as the rubber tree (Euphorbiaceae). The lowest percent identity was found with a wound-induced protein from *Actinidia chinensis* (70.5%). The Hbhevein HEV1 precursor exhibited a sequence identity at 73.4 and 73.66% to PR-4-like protein from *Vigna unguiculata* and *Camellia sinensis*, respectively.

The homologs from phylogram were categorized into two major clades based on the presence or absence of sap (**Fig. 1**). Clade A indicates members with sap-present characteristics and was composed of *H. brasiliensis* (Van Welzen and Chayamarit, 2007), *M. esculenta* (Calatayud et al., 1994) and *Ricinus communis* (Holfelder et al., 1998) from the Euphorbiaceae family; *Populus alba* (Escher et al., 2004) from the Salicaceae family; as well as *Solanum tuberosum* (Duceppe et al., 2012) and *Capsicum annuum* (Sato et al., 2003) from the Solanaceae family. The several types of sap (e.g., latex, xylem sap, phloem sap) found in most plant species contain a diversity of biologically active compounds. Many of these compounds provide resistance to pathogens and herbivores via their toxicity or antinutritive effects (Lazreg-Aref et al., 2018). In addition, the hevein precursor and prohevein from *H. brasiliensis* clearly originated from the same ancestor. They were placed as sister to *Manihot* and grouped together with *Ricinus* and *Populus* in the Malpighiales order (Tangphatsornruang et al., 2011). Conversely, Clade B mainly contained members with sap-absent characteristics and was composed of *A. chinensis* from the Actinidiaceae family (Li et al., 2012a), *Quercus lobata* from the Fagaceae family (Huang et al., 1999), *C. sinensis* from the Theaceae family (Li et al., 2012b) as well as *Cicer arietinum*, *Trifolium pretense*, *Cajanus cajan*, *Glycine max* and *V. unguiculata* from Fabaceae, the legume family (Xu et al., 2010).

From an evolutionary aspect, the reconstructed phylogram in this study implies the diversification and differentiation of the PR4 group in several plant species (Fig. 1). Although all amino acid sequences from relevant taxa in the tree are in the same group of PR4 proteins, the presence of sap is not found in all taxa, evidenced by the existence of distinct clades. It has been reported that sap production is an adaptive function of plants stemming from convergent evolution (Agrawal and Konno, 2009). The defense mechanisms in plants via gene evolution plays

an important role in protection against microbes and pathogens (Zhang et al., 2019). Sap production is an advantageous mechanism and has a beneficial impact on plant defense (Deyett and Rolshausen, 2019). Both the structural and chemical composition of sap have apparently shown pathogenic toxicity and work together to weaken invaders (Konno, 2011). Accordingly, the latex of rubber trees is a type of sap that has a defensive role against plant enemies.

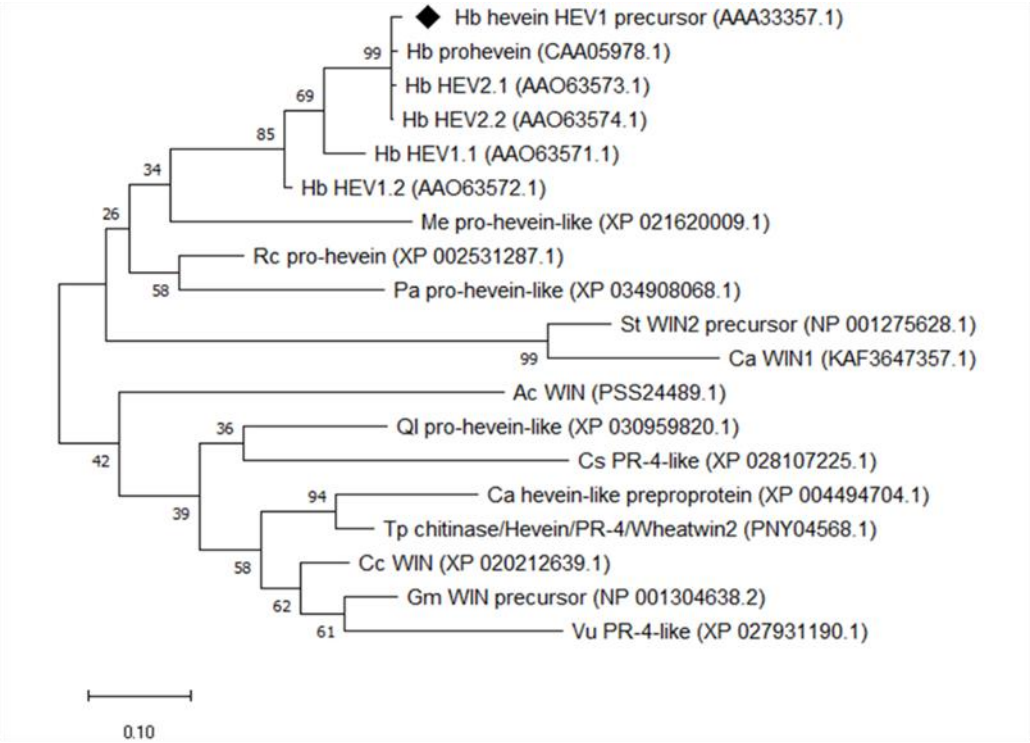

Figure 1 Phylogenetic tree of hevein precursor proteins

The amino acid sequences of *H. brasiliensis* hevein HEV1 precursor [AAA33357.1], HEV1.1 [AAO63571.1], HEV1.2 [AAO63572.1], HEV2.1 [AAO63573.1], HEV2.2 [AAO63574.1] and prohevein [CAA05978.1] were aligned with the sequences of *Actinidia chinensis* var. chinensis WIN (Ac [PSS24489.1]), *Capsicum annuum* WIN1 (Ca [KAF3647357.1]), *Cicer arietinum* hevein-like preproprotein (Ca [XP_004494704.1]), *Cajanus cajan* WIN (Cc [XP_020212639.1]), *Camellia sinensis* PR-4-like (Cs [XP_028107225.1]), *Glycine max* WIN precursor (Gm [NP_001304638.2]), *Manihot esculenta* pro-hevein-like (Me [XP_021620009.1]), *Populus alba* pro-hevein-like (Pa [XP_034908068.1]), *Quercus lobata* pro-hevein-like (Ql [XP_030959820.1]), *Ricinus communis* pro-hevein (Rc [XP_002531287.1]), *Solanum tuberosum* WIN2 precursor (St [NP_001275628.1]), *Trifolium pratense* chitinase/Hevein/PR-4/Wheatwin2 (Tp [PNY04568.1]), and *Vigna unguiculata* PR-4-like (Vu [XP_027931190.1]). Multiple sequence alignment was constructed by MEGA-X (Kumar et al., 2018) using Maximum Likelihood and the Whelan and Goldman (WAG) model (Whelan and Goldman, 2001). WIN: Wound-induced protein; PR: Pathogenesis-related protein.

Expression analysis of the *hevein* gene

In this study, the level of *hevein* gene expression was monitored in latex samples of PB260 and RRIM600, the rubber tree clones resistant and susceptible to *Phytophthora* sp., respectively. The results showed high expression of the *hevein* gene in latex, which is consistent with the fact that hevein is one of the major latex proteins of the rubber tree. The expression level of the *hevein* gene was significantly higher in the *Phytophthora* resistant

clone (PB260) than in the susceptible clone (RRIM600) (**Fig. 2**). This result suggests that the expression level of the *hevein* gene may be related to pathogenic fungi resistance in the rubber tree clone PB260.

Pn-AMPs, the hevein-like anti-microbial proteins from *Pharbitis nil*, exhibited considerable antifungal activity against both chitin and non-chitin containing fungi (Koo et al., 1998). Over-expression of hevein Pn-AMP in tobacco enhances resistance to the fungal pathogen *Phytophthora parasitica* (Koo et al., 2002). It has been reported that the expression of hevein-like antimicrobial peptides in transgenic tomato enhances resistance to *Phytophthora infestans* (Khaliluev et al., 2011). In addition, transgenic tomatoes with Pn-AMP2 showed enhanced resistance against both *Fusarium oxysporum* and *Phytophthora capsici* (Lee et al., 2003). Transgenic tomato plants with recombinant hevein also demonstrated effective antifungal properties (Lee and Raikhel, 1995).

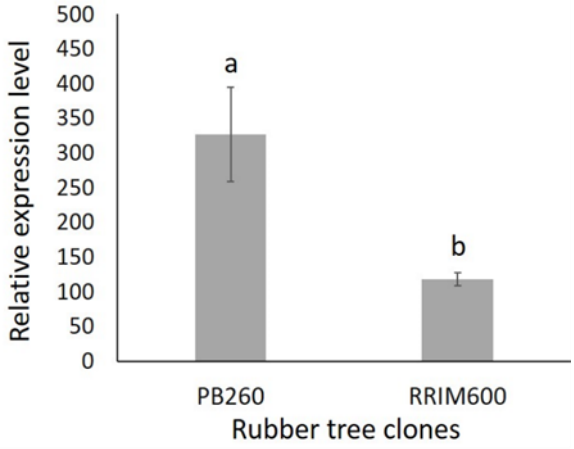

Figure 2 Relative expression levels of the *hevein* gene in PB260 and RRIM600 rubber tree clones

Data corresponds to the means and standard errors of nine independent replicates performed in triplicate (n=27). Different letters indicate the significant difference between the two rubber tree clones (Independent-Sample T-Test $t_{(52)}$= 5.813 , $P < 0.001$).

Promoter analysis of the *hevein* gene

To reveal *hevein* gene regulation at the transcriptional level, the 3000-bp upstream promoter regions of the RRIM600 rubber tree clone were retrieved from whole genome shotgun contigs of *H. brasiliensis* (Willd. ex A. Juss.) Müll. Arg. (taxid:3981) in the NCBI database to identify *cis*-acting regulatory elements. The *cis*-acting regulatory elements of the *hevein* gene were analyzed by NewPLACE and separated into 8 groups consisting of the promoter consensus element, hormone responsive element, defense responsive element, mineral responsive element, organ specific element, light responsive element, water stress responsive element, and miscellany. The percentage and composition of each *cis*-acting regulatory element group on the *hevein* promoter are shown in **Figure 3** and **Figure 4**, respectively. The promoter consensus elements, including CAATBOX and TATABOX, were commonly found on the *hevein* promoter of RRIM600, as these elements were reported to be essential for accurate initiation of basal transcription in most plant species. Auxin (CACGCAATGMGH3 and NTBBF1ARROLB)-, cytokinin (ARR1AT and CAREOSREP1)- and gibberellin (PYRIMIDINEBOXHVEPB1 and WRKY71OS)-responsive elements were present on the *hevein* promoter. This result suggests that auxin, cytokinin and gibberellin may regulate expression of the *hevein* gene.

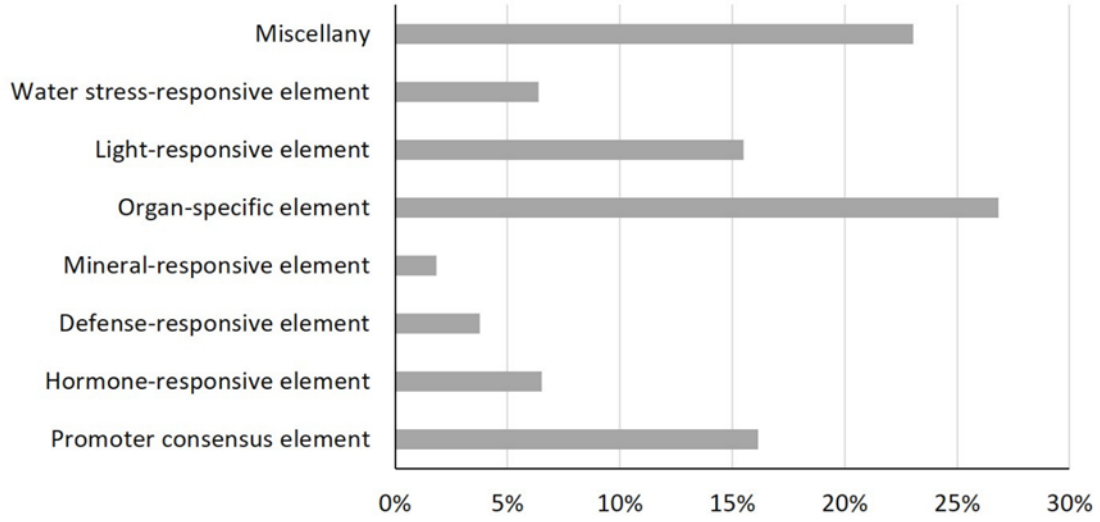

Figure 3 The bar chart represents the percentage of *cis*-acting regulatory elements on the *hevein* promoter of the RRIM600 rubber tree clone

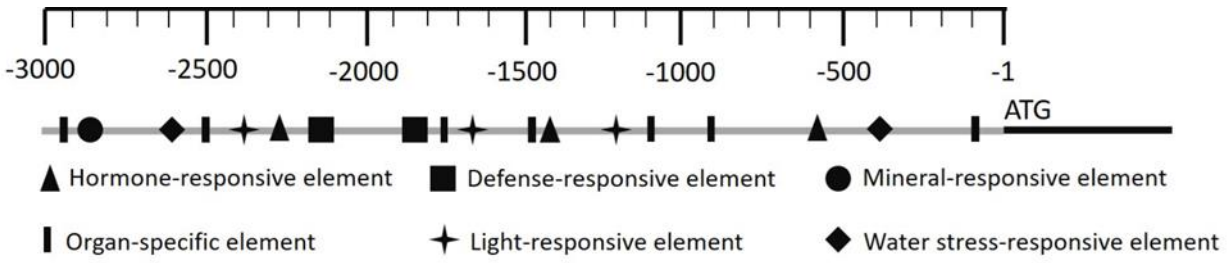

Figure 4 The composition of *cis*-acting regulatory elements on the *hevein* promoter of the RRIM600 rubber tree clone

The 3000-bp promoter region is displayed. Different shapes show different *cis*-acting regulatory elements.

Cis-acting regulatory elements related to ethylene response (ERELEE4) were found on the *hevein* promoter of the RRIM600 rubber tree clone. This finding is related to the up-regulation of the *hevein* gene in the leaves, stems and latex of rubber trees after treatment with ethephon (chloro-2-ethylphosphonic acid), an ethylene generator or releaser (Broekaert et al., 1990; Deng et al., 2002). Exogenous application of ethylene to plants resulted in the activation of antimicrobial pathogenesis-related (PR) genes (Thomma et al., 1999). In addition, over-expression of *ethylene-response factor ERF2* in *Arabidopsis* increased the transcript accumulation of hevein-like proteins and elevated its resistance to *F. oxysporum* (McGrath et al., 2005). Moreover, WBOXATNPR1 salicylic acid-responsive elements were also found on the *hevein* promoter of the RRIM600 rubber tree clone. It was reported that salicylic acid activated the expression of *wamp* genes, a new family of *hevein-like AMP* denoted as wheat antimicrobial peptide, in wheat seedlings (Istomina et al., 2016). The jasmonic acid-responsive element (QARBNEXTA) was found in the antisense orientation of the *hevein* promoter. Similar misallocation has been reported for the promoter region of the osmotin isoform in tobacco, suggesting the binding of transcription factors with less efficient responses to the

corresponding stimulus (Sato et al., 1996, Kitajima et al., 1998). In rubber trees, the expression of genes involved in plant defense, including *hevein*, *β-1,3-glucanase* and *chitinase*, was up-regulated by the application of methyl jasmonate (Yang et al., 2008). It has been reported that the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play important roles in crosstalk for plant defenses against pathogens and insects (Yang et al., 2015). The SA-signaling pathway triggered by biotrophic pathogens suppressed the JA-signaling pathway, which was effective against necrotrophic pathogens (Pettongkhao and Churngchow, 2019). Ethylene has been reported to be involved in the regulation of SA- and JA-signaling (Chen et al., 2011).

W-box, a wounding responsive element, and pathogen responsive elements, including AG-motif, BIHD1OS and GT-1, were found on the *hevein* promoter (**Table 1**). It was reported that expression of the *hevein* gene was induced after wounding (Broekaert et al., 1990). Functional analysis of PHev2.1, a promoter of the *hevein precursor*, in rice also showed a systemic response to wounding (Pujade-Renaud et al., 2005). The inoculation of *M. grisea*, the fungal pathogen of rice blast disease, significantly activated the PHev2. 1 promoter. Moreover, three mineral responsive elements, including CGCGBOX, CURECORECR and SURECOREATSULTR11, associated with calcium, copper and sulfur responses, were shown on the hevein promoter of the RRIM600 rubber tree clone.

The presence of organ specific elements, CACTFTPPCA1, EBOXBNNAPA, GTGANTG10, POLLEN1LELAT52, ROOTMOTIFTAPOX1 and TAAAGSTKST1, which were associated with mesophyll, seed, embryo, pollen, root and guard cell-specific responses, was verified. As shown in rice, GUS activity driven by PHev2.1 was observed in leaves, immature flowers, pollen bags, pollen grains, immature seeds and root vascular tissues (Pujade-Renaud et al., 2005). Five light responsive elements, including GT-1, GATA-box, I-box, INRNTPSADB and SEF4MOTIFGM7S, were identified in *hevein* promoter regions. Water stress-responsive elements, including ACGTATERD1 and MYCCONSENSUSAT, associated with dehydration response were also analyzed.

CONCLUSION

Based on the phylogenetic analysis from the amino acid sequences of PR4 proteins from *H. brasiliensis* and other plant species, the homologs were categorized into two major clades based on the presence or absence of sap. Under non-infected conditions, higher expression of the *hevein* gene was observed in *Phytophthora*-resistant rubber tree clone PB260 than in susceptible RRIM600. This study indicates that *hevein*, which is classified as a PR4 protein, may influence the resistance level to pathogenic diseases in rubber trees. In addition, the presence of *cis*-acting regulatory elements on the *hevein* promoter of rubber tree clone RRIM600 shows that *hevein* is regulated by hormones, defenses, minerals, light, water and is differentially expressed in different organs. Functional characterization and differential expression of the *hevein* gene are of interest for further investigation, as the *hevein* gene may be a candidate gene for molecular breeding of *Phytophthora*-resistant rubber tree clones.

Table 1 Defense responsive elements identified on the *hevein* promoter of the RRIM600 rubber tree clone

Identified motif	*Cis*-acting regulatory element	Transcription factor	NewPLACE ID	Expected function	Reference
AGATCCAA	AGMOTIFNTMYB2	C2C2-GATA family	S000444	Wound induced or elicitor treatment	Sugimoto et al., 2003
TGTCA	BIHD1OS	HB family	S000498	BELL recognition site involved in disease resistance responses	Luo et al., 2005
GAAAAA	GT1GMSCAM4	GT factor family	S000453	Pathogen- and salt-induced	Park et al., 2004
TGACY	WBOXNTERF3	WRKY family	S000457	Wound induced	Nishiuchi et al., 2004

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REFERENCES

Agrawal, A. A., and K. Konno. 2009. Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. Annual Review of Ecology, Evolution, and Systematics. 40: 311-331.

Archer, B. L., B. G. Audley, G. P. McSweeney, and T. C. Hong. 1969. Studies on composition of latex serum and bottom fraction particles. Journal of Rubber Research Institute of Malaysia. 21: 560-569.

Berthelot, K., F. Peruch, and S. Lecomte. 2016. Highlights on *Hevea brasiliensis* (pro) hevein proteins. Biochimie. 127: 258-270.

Broekaert, I., H. I. Lee, A. Kush, N. H. Chua, and N. Raikhel. 1990. Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). Proceedings of the National Academy of Sciences. 87(19): 7633-7637.

Calatayud, P. A., Y. Rahbé, B. Delobel, F. Khuong-Huu, M. Tertuliano, and B. Le Rü. 1994. Influence of secondary compounds in the phloem sap of cassava on expression of antibiosis towards the mealybug *Phenacoccus manihoti*. Entomologia Experimentalis et Applicata. 72(1): 47-57.

Chen, Y. Y., L. F. Wang, S. G. Yang, and W. M. Tian. 2011. Molecular characterization of *HbEREBP2*, a jasmonate-responsive transcription factor from *Hevea brasiliensis* Muell. Arg. African Journal of Biotechnology. 10(48): 9751-9759.

Deenamo, N., A. Kuyyogsuy, K. Khompatara, T. Chanwun, K. Ekchaweng, and N. Churngchow. 2018. Salicylic acid induces resistance in rubber tree against *Phytophthora palmivora*. International Journal of Molecular Sciences. 19(7): 1883.

Deng, X. D., X. W. Fei, J. S. Huang, and X. Q. Zheng. 2002. Isolation and analysis of rubber *hevein* gene and its promoter sequence. Acta Botanica Sinica. 44(8): 936-940.

Deyett, E., and P. E. Rolshausen. 2019. Temporal dynamics of the sap microbiome of grapevine under high Pierce's disease pressure. Frontiers in Plant Science. 10: 1-15.

Duceppe, M. O., C. Cloutier, and D. Michaud. 2012. Wounding, insect chewing and phloem sap feeding differentially alter the leaf proteome of potato, *Solanum tuberosum* L. Proteome Science. 10(1): 1-14.

Ekchaweng, K., E. Evangelisti, S. Schornack, M. Tian, and N. Churngchow. 2017. The plant defense and pathogen counterdefense mediated by *Hevea brasiliensis* serine protease *HbSPA* and *Phytophthora palmivora* extracellular protease inhibitor *PpEPI10*. PLOS ONE. 12(5): 1-16.

Escher, P., M. Eiblmeier, I. Hetzger, and H. Rennenberg. 2004. Spatial and seasonal variation in amino compounds in the xylem sap of a mistletoe (*Viscum album*) and its hosts (*Populus s*pp. and *Abies alba)*. Tree Physiology. 24(6): 639-650.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39: 783-791.

Friedrich, L., M. Moyer, E. Ward, and J. Ryals. 1991. Pathogenesis-related protein 4 is structurally homologous to the carboxy-terminal domains of hevein, Win-1 and Win-2. Molecular and General Genetics. 230(1): 113-119.

Gidrol, X., H. Chrestin, H. L. Tan, and A. Kush. 1994. Hevein, a lectin-like protein from *Hevea brasiliensis* (rubber tree) is involved in the coagulation of latex. Journal of Biological Chemistry. 269(12): 9278-9283.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41: 95-98.

Havanapan, P., A. Bourchookarn, A. J. Ketterman, and C. Krittanai. 2016. Comparative proteome analysis of rubber latex serum from pathogenic fungi tolerant and susceptible rubber tree (*Hevea brasiliensis*). Journal of Proteomics. 131: 82-92.

Holfelder, M. G., M. Steck, E. Komor, and K. Seifert. 1998. Ricinine in phloem sap of *Ricinus communis*. Phytochemistry. 47(8): 1461-1463.

Huang, C. C., Y. T. Zhang, and B. Bartholomew. 1999. Fagaceae. P. 380-400. In: Z. Y. Wu, and P. H. Raven. Flora of China 4. Missouri Botanical Garden Press, St. Louis.

Istomina, E. A., T. V. Korostyleva, N. A. Rozhnova, E. A. Rogozhin, V. A. Pukhalskiy, and T. I. Odintsova. 2016. Genes encoding hevein-like antimicrobial peptides WAMPs: Expression in response to phytohormones and environmental factors. Russian Journal of Genetics. 52(11): 1176-1185.

Joseph, A., S. P. Idicula, and V. K. Rajalakshmy. 1994. Control of *Gloeosporium* leaf disease in young rubber plantations. pp. 82-84. In Proceedings of International Rubber Research and Development Board (IRRDB) Symposium on Diseases of *Hevea* 21-22 November 1994. Cochin, India.

Khaliluev, M. R., A. G. Mamonov, A. N. Smirnov, P. N. Kharchenko, and S. V. Dolgov. 2011. Expression of genes encoding chitin-binding proteins (PR-4) and hevein-like antimicrobial peptides in transgenic tomato plants enhanced resistance to *Phytophthora infestance*. Russian Agricultural Sciences. 37(4): 297-302.

Khompatara, K., S. Pettongkhao, A. Kuyyogsuy, N. Deenamo, and N. Churngchow. 2019. Enhanced resistance to leaf fall disease caused by *Phytophthora palmivora* in rubber tree seedling by *Sargassum polycystum* extract. Plants. 8(6): 168-181.

Kitajima, S., T. Koyama, Y. Yamada, and F. Sato. 1998. Constitutive expression of the neutral PR-5 (OLP, PR-5d) gene in roots and cultured cells of tobacco is mediated by ethylene-responsive *cis*-element AGCCGCC sequences. Plant Cell Reports. 18(3): 173-179.

Kongsawadworakul, P., B. Peret, D. Morin, M. Lebrun, F. Pellegrin, D. Nandris, and H. Chrestin. 2004. Ethylene-cyanide metabolism/ACC-oxidase and cyanoalanine synthase cross-roads in *Hevea brasiliensis*: Effects induced by yield stimulation with Ethrel® and other stresses. pp. 331-345. In Proceedings of IRRDB Seminar on *Hevea* Physiology and Breeding 6-9 September 2004. Kunming, People's Republic of China.

Konno, K. 2011. Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein. Phytochemistry. 72(13): 1510-1530.

Koo, J. C., S. Y. Lee, H. J. Chun, Y. H. Cheong, J. S. Choi, S. I. Kawabata, M. Miyagi, S. Tsunasawa, K. S. Ha, D. W. Bae, C. D. Han, B. L. Lee, and M. J. Cho. 1998. Two hevein homologs isolated from the seed of *Pharbitis nil* L. exhibit potent antifungal activity. Biochimica et Biophysica Acta. 1382(1): 80-90.

Koo, J. C., H. J. Chun, H. C. Park, M. C. Kim, Y. D. Koo, S. C. Koo, H. M. Ok, S. J. Park, S. H. Lee, D. J. Yun, C. O. Lim, J. D. Bahk, S. Y. Lee, and M. J. Cho. 2002. Over-expression of a seed specific hevein-like antimicrobial peptide from *Pharbitis nil* enhances resistance to a fungal pathogen in transgenic tobacco plants. Plant Molecular Biology. 50: 441-452.

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution. 35: 1547-1549.

Lazreg-Aref, H., B. Gaaliche, A. Ladhari, M. Hammami, and S. O. Hammami. 2018. Co-evolution of enzyme activities and latex in fig (*Ficus carica* L.) during fruit maturity process. South African Journal of Botany. 115: 143-152.

Lee, H. I., and N. V. Raikhel. 1995. Prohevein is poorly processed but shows enhanced resistance to a chitin-binding fungus in transgenic tomato plants. Brazilian Journal of Medical and Biological Research. 28(7): 743-750.

Lee, O. S., B. Lee, N. Park, J. C. Koo, Y. H. Kim, T. Prasad, C. Karigar, H. J. Chun, B. R. Jeong, D. H. Kim, J. Nam, J. G. Yun, S. S. Kwak, M. J. Cho, and D. J. Yun. 2003. Pn-AMPs, the hevein-like proteins from *Pharbitis nil* confers disease resistance against phytopathogenic fungi in tomato, *Lycopersicum esculentum*. Phytochemistry. 62(7): 1073-1079.

Li, H., Y. Qin, X. Xiao, and C. Tang. 2011. Screening of valid reference genes for real-time RT-PCR data normalization in *Hevea brasiliensis* and expression validation of a sucrose transporter gene *HbSUT3*. Plant Science. 181(2): 132-139.

Li, J. Q., X. W. Li, and D. D. Soejarto. 2012a. Actinidiaceae. P. 334-360. In: Z. Y. Wu, P. H. Raven and D. Y. Hong. Flora of China 12. Missouri Botanical Garden Press, St. Louis.

Li, J. Q., X. W. Li, and D. D. Soejarto. 2012b. Theaceae. P. 366-478. In: Z. Y. Wu, P. H. Raven and D. Y. Hong. Flora of China 12. Missouri Botanical Garden Press, St. Louis.

Luo, H., F. Song, R. M. Goodman, and Z. Zheng. 2005. Up-regulation of *OsBIHD1*, a rice gene encoding BELL homeodomain transcriptional factor, in disease resistance responses. Plant Biology. 7(5): 459-468.

McGrath, K. C., B. Dombrecht, J. M. Manners, P. M. Schenk, C. I. Edgar, D. J. Maclean, W. R. Scheible, M. K. Udvardi, and K. Kazan. 2005. Repressor and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of *Arabidopsis* transcription factor gene expression. Plant Physiology. 139(2): 949-959.

Moosa, A., A. Farzand, S. T. Sahi, and S. A. Khan. 2017. Transgenic expression of antifungal pathogenesis-related proteins against phytopathogenic fungi–15 years of success. Israel Journal of Plant Sciences. 65(1-2): 38-54.

Narayanan, C., and K. K. Mydin. 2012. Breeding for disease resistance in *Hevea* spp.-status, potential threats, and possible strategies. pp. 240-251. In Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees 2012. California, United States of America.

Nishiuchi, T., H. Shinshi, and K. Suzuki. 2004. Rapid and transient activation of transcription of the *ERF3* gene by wounding in tobacco leaves: possible involvement of NtWRKYs and autorepression. Journal of Biological Chemistry. 279(53): 55355-55361.

Park, H. C., M. L. Kim, Y. H. Kang, J. M. Jeon, J. H. Yoo, M. C. Kim, C. Y. Park, J. C. Jeong, B. C. Moon, J. H. Lee, H. W. Yoon, S. H. Lee, W. S. Chung, C. O. Lim, S. Y. Lee, J. C. Hong, and M. J. Cho. 2004. Pathogen-and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. Plant Physiology. 135(4): 2150-2161.

Pethin, D., K. Nakkanong, and C. Nualsri. 2015. Performance and genetic assessment of rubber tree clones in Southern Thailand. Scientia Agricola. 72(4): 306-313.

Pettongkhao, S., and N. Churngchow. 2019. Novel cell death-inducing elicitors from *Phytophthora palmivora* promote infection on *Hevea brasiliensis*. Phytopathology. 109(10): 1769-1778.

Peumans, W. J., J. M. Van Damme, A. Barre, and P. Rougé. 2001. Classification of plant lectins in families of structurally and evolutionary related proteins. Molecular Immunology of Complex Carbohydrates. 2: 27-54.

Pujade-Renaud, V., A. Clement, C. Perrot-Rechenmann, J. C. Prevôt, H. Chrestin, J. L. Jacob, and J. Guern. 1994. Ethylene induced increase in glutamine synthetase activity and mRNA levels in *Hevea brasiliensis* latex cells. Plant Physiology. 105(1): 127-132.

Pujade-Renaud, V., C. Sanier, L. Cambillau, A. Pappusamy, H. Jones, N. Ruengsri, D. Tharreau, H. Chrestin, P. Montoro, and J. Narangajavana. 2005. Molecular characterization of new members of the *Hevea brasiliensis hevein* multigene family and analysis of their promoter region in rice. Biochimica et Biophysica Acta. 1727(3): 151-161.

Rodesuchit, A., S. Prukwarun, and S. Suchartgul. 2009. The epidemic of rubber diseases and the effect to the yields of some recommended clones (Research report). P.1-70. Department of Agriculture. Surat Thani.

Sato, F., S. Kitajima, T. Koyama, and Y. Yamada. 1996. Ethylene-induced gene expression of osmotin-like protein, a neutral isoform of tobacco PR-5, is mediated by the AGCCGCC *cis*-sequence. Plant and Cell Physiology. 37(3): 249-255.

Sato, S., S. Moreshet, M. Takagaki, Y. Shinohara, and T. Ito. 2003. Effects of drought stress on sap flow, stomatal conductance, and leaf water potential of pepper cultivars (*Capsicum annuum* L.). Japanese Journal of Tropical Agriculture. 47(2): 61-69.

Shi, M., Y. Li, S. Deng, D. Wang, Y. Chen, S. Yang, J. Wu, and W. M. Tian. 2019. The formation and accumulation of protein networks by physical interactions in the rapid occlusion of laticifer cells in rubber tree undergoing successive mechanical wounding. BMC Plant Biology. 19(1): 1-13.

Stanford, A., M. Bevan, and D. Northcote. 1989. Differential expression within a family of novel wound-induced genes in potato. Molecular and General Genetics. 215(2): 200-208.

Sugimoto, K., S. Takeda, and H. Hirochika. 2003. Transcriptional activation mediated by binding of a plant GATA-type zinc finger protein AGP1 to the AG-motif (AGATCCAA) of the wound-inducible *Myb* gene *NtMyb2*. The Plant Journal. 36(4): 550-564.

Tangphatsornruang, S., P. Uthaipaisanwong, D. Sangsrakru, J. Chanprasert, T. Yoocha, N. Jomchai, and S. Tragoonrung. 2011. Characterization of the complete chloroplast genome of *Hevea brasiliensis* reveals genome rearrangement, RNA editing sites and phylogenetic relationships. Gene. 475(2): 104-112.

Thanseem, I., A. Joseph, and A. Thulaseedharan. 2005. Induction and differential expression of β-1,3-glucanase mRNAs in tolerant and susceptible *Hevea* clones in response to infection by *Phytophthora meadii*. Tree Physiology. 25(11): 1361-1368.

Thomma, B. P. H. J., K. Eggermont, K. F. M.-J. Tierens, and W. F. Broekaert. 1999. Requirement of functional ethylene insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiology. 121: 1093-1101.

Van Parijs, J., W. F. Broekaert, I. J. Goldstein, and W. J. Peumans. 1991. Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta. 183(2): 258-264.

Van Welzen, P. C., and K. Chayamarit. 2007. Euphorbiaceae. P. 331-333. In: T. Santisuk, and K. Larsen. Flora of Thailand 8 (2). Prachachon Co. Ltd., Bangkok.

Whelan, S., and N. Goldman. 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Molecular Biology and Evolution. 18: 691-699.

Xu, L., D. Chen, X. Zhu, P. Huang, Z. Wei, R. Sa, D. Zhang, B. Bao, D. Wu, H. Sun, X. Gao, Y. Liu, Z. Chang, J. Li, M. Zhang, D. Podlech, H. Ohashi, K. Larsen, S. L. Welsh, M. A. Vincent, M. G. Gilbert, L. Pedley, B. D. Schrire, G. P. Yakovlev, M. Thulin, I. C. Nielsen, B. H. Choi, N. J. Turland, R. M. Polhill, S. S. Larsen, H. Ding, I. Yu, C. M. Wilmot-Dear, G. Kenicer, T. Nemoto, J. M. Lock, A. D. Salinas, T. E. Kramina, A. R. Brach, B. Bartholomew, and D. D. Sokoloft. 2010. P. 1-577. In: Z. Y. Wu, P. H. Raven, and D. Hong. Flora of China 10. Missouri Botanical Garden Press, St. Louis.

Yang, S. G., J. H. Yu, M. J. Shi, Y. Y. Chen, and W. M. Tian. 2008. Effects of tapping, mechanical wounding and exogenous jasmonates on the expression of genes encoding for defense proteins in the latex of *Hevea brasiliensis*. Chinese Journal of Tropical Crops. 29(5): 535-540.

Yang, Y. X., G. J Ahammed, C. Wu, S. Y. Fan, and Y. H. Zhou. 2015. Crosstalk among jasmonate, salicylate and ethylene signaling pathways in plant disease and immune responses. Current Protein and Peptide Science. 16(5): 450-461.

Zhang, R., F. Zheng, S. Wei, S. Zhang, G. Li, P. Cao, and S. Zhao. 2019. Evolution of disease defense genes and their regulators in plants. International Journal of Molecular Sciences. 20(2): 1-25.