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Temporal Expression of a Putative Homogentisate Solanesyltransferase cDNA in Wounded *Aquilaria malaccensis*, an Endangered Tropical Tree

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ABSTRACT

Homogentisate prenytransferase (HPT) generally catalyses prenylation reactions in tocochromanol and plastoquinone-9 biosynthesis, while homogentisate solanesyltransferase (HST) is specific to reaction leading to plastoquinone, an essential component in the synthesis of carotenoid, a powerful antioxidant and precursor to vitamin A. In *Aquilaria* spp. abiotic stress in the form of wounding is the main trigger for the production of a highly-valued terpene-rich wood known as agarwood. Putative HST cDNA, *AmHST1* was cloned from total RNA of callus tissue of *Aquilaria malaccensis* using reverse transcription approach. Based on a partial HST sequence, specific primers were initially designed to amplify the internal open reading frame region followed by RACE, which successfully amplified the cDNA. The partial length *AmHST1* cDNA measured about 1182 bp nucleotides and encodes a polypeptide of 392 amino acid. Sequence alignment revealed that AmHST1 shares 74% - 77% similarity with HPT from Arabidopsis and *Theobroma cacao*. Gene expression analysis indicated that the *AmHST1* expression was suppressed in wounded tissues. Results suggest that there should be a potential trade-off between genes involved in plastoquinone and terpenoid synthesis as they both share similar upstream genes and precursors. When facing a major abiotic stress such as wounding, the latter is favoured.

Keywords: *AmHST1,* prenyltransferase, terpenoid, Thymelaeaceae, Tocopherol

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INTRODUCTION

Aquilaria malaccensis Lam. is one of the main agarwood-producing species in the world. The genus *Aquilaria* belongs to the family Thymelaeaceae and is found across South East Asia and tropical regions of China and India (Oldfield, 1998). Agarwood, also known as gaharu, eaglewood and aloeswood, is a dark fragrant resin used as medicine to treat asthma, diarrhoea, body ache, and other ailments (Barden et al. 2000), as incense for use in religious rituals, and also as an ingredient in perfumes (Persoon, 2008; Jayachandran et al. 2014). In Asia, huge pieces of agarwood are highly sought for use in sculpturing idols or decorative items. All these make agarwood an extremely prized forest product presently traded internationally. Because of its high demand and prices of agarwood, *Aquilaria* spp. in the wild have been over-exploited resulting in each of its species being listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013).

Agarwood is a resinous substance produced by the tree in response to 'wound' caused by many natural factors such as insect and microbe attack, lightning strikes and wind. However, the accumulation is rather slow and may take multiple years to yield a substantial amount. The main chemical components of agarwood from various *Aquilaria* species have been identified as sesquiterpenes and phenylethyl chromones (Naef, 2011). Based on plant model systems, the biosynthesis of terpenoids, which are required for cellular growth and survival, involves prenyltransferases (PTs) which catalyse sequential condensation of basic 5-carbon building blocks, isopentenyl diphospate (IPP; C_5) and dimethylallyl diphosphate (DMAPP; C_5) into three intermediate isoprenoid molecules, geranyl diphospate (GPP ; C_{10}), farnesyl diphosphate (FPP ; C_{15}) and geranylgeranyl diphosphate (GGPP ; C_{20}), which are the basis of other longer chain isoprenoid molecules (Aubourg et al. 2002; Dudavera et al. 2013). The terpene synthases (TPSs) then convert the three prenyl diphosphate intermediates into cyclic and acyclic terpenoid skeletons, yielding 10-carbon monoterpenes (monoterpene synthase), 15-carbon sesquiterpenes (sesquiterpene synthase) and 20-carbon diterpene (diterpene synthase) (Lange & Ahkami, 2013). In *Aquilaria*, the PT, also known as farnesyl pyrophosphate synthase (FPP synthase), is a critical enzyme because it catalyses formation of the intermediate FPP isoprenoids to serve as the substrate for subsequent TPS reaction leading to final sesquiterpene product. In previous studies, *Aquilaria* prenyltransferases in the terpenoid pathway were proven to be closely-related to agarwood synthesis (Kenmotsu et al. 2013; Yang et al., 2013).

In its natural state, the FPP is grouped under the general group of enzyme called homogentisate prenyltransferase (HPT) that plays an important role in the biosynthesis of various secondary metabolites such as tocochromanol, tocotrienol, flavonoid, terpenoid and plastoquinone-9. Similar to FPP, the homogentisate solanesyltransferase

(HST), which is specific to plastoquinone (PQ) biosynthesis, is also grouped as HPT. The HST is an important enzyme that catalyses formation of several compounds in the plant, such as tyrosine-derived aromatic compounds, which leads to multiple functions such as biosynthesis in vitamin E, photosystem II (PSII) mobile electron transport co-factor, PQ, and carotenoid (Norris et al., 1995; Sadre et al., 2006; Yang et al., 2011). Many genes encoding HST or its homologs have been isolated and identified from Arabidopsis and other plants (Venkatesh et al., 2006; Soderlund et al., 2009). However, the first HST gene, VTE2, was isolated from *Glycine max* (Venkatesh et al., 2006) and enzyme assay results for cell expression for HST gene in *Escherichia coli* put forward the contribution of HST in catalysing the first step in PQ biosynthesis (Sandre et al., 2006). Unusual expression in HST gene may result in growth abnormality, in which improvement in prenyl lipid, PQ and tocopherol levels was observed in transgenic Arabidopsis when HST gene was overexpressed; disruption of this gene may cause albino phenotype that leads to PQ and tocopherol synthesis deficiency (Norris et al., 1995).

In this study, using mRNA data sequence from *A. malaccensis* transcriptome (Siah et al. 2016), a gene with putative function as homogentisate solanesyltransferase (*AmHST1*) was cloned. Its amino acid sequence shares some similarity with FPP synthase, an important enzyme in agarwood synthesis, but it shares higher homology with HST of other plant origins. *HST* genes are involved in the synthesis of plastoquinone, an antioxidant substance that protects against stress. The temporal expression of the gene was characterised in a time-course wounding experiment and the expression patterns revealed the gene perhaps is not directly involved in agarwood synthesis.

MATERIALS AND METHODS

Plant Materials

In vitro plants were grown from seeds that were sterilised and introduced into half strength MS medium (Murashige & Skoog, 1962) as reported by Daud et al. (2012). Seeds were collected from an *A. malaccensis* mother tree growing at the Sungai Buloh Forest Reserve, Kepong in May 2011. Germinated shoots were cut and transferred into MS medium supplemented with 1.3 μM 6-benzylaminopurine (BAP) as described by He et al. (2005). Plants were sub-cultured onto a fresh medium every 4 weeks until they reach a height of 5cm. Plantlets were grown under long-day conditions (16 hours of light, 8 hours of darkness) with temperature of 25°C.

To initiate callus culture, fresh leaves were collected from four-year-old *A. malaccensis* trees maintained in polybags in the shade house of the Faculty of Forestry, Universiti Putra Malaysia, Serdang. The sterilisation protocol was adopted from Daud et al. (2012). The leaves were washed for 15 minutes under slow running tap water. Then, the leaves were dipped in 70% alcohol and rinsed twice with sterile distilled water. The leaves were surface sterilised in 0.1%

$HgCl_2$ (Sigma Aldrich, USA) for 1 minute; they were later washed four or five times with sterile distilled water. After surface sterilisation, the entire mid rib of the leaf was removed aseptically to produce leaf strip. Leaf explants were prepared by cutting the leaf strips into small squares of 10 mm x 10 mm. Then, they were soaked in 0.5 mg/L ascorbic acid (Sigma Aldrich, USA) for 30 minutes. The explants were dabbed on sterile tissue paper and placed on petri dishes containing MS with 30 g/L sucrose and 2.75 g/L gelrite (Duchefa, Netherlands) without hormone. The Petri dishes were incubated at 25°C in total darkness and observed for occurrence of contamination. After 2 days of observation, the contamination-free explants were transferred onto MS medium supplemented with 2.2 µM BAP and 1.1 µM naphthaleneacetic acid (NAA) following He et al. (2005) and Jayaraman et al. (2014). Calli were sub-cultured every other week in the same fresh media, but in magenta jars, for the next five months. The jars were incubated at 25°C in total darkness.

For gene expression study, wood samples were collected as described in Wong et al. (2013). Briefly, a three-year old *A. malaccensis* tree was drilled with a 3.5 mm diameter drill bit into a depth of 1 to 2 cm. Wounding proceeded in two straight lines with each wound spaced at approximately 10cm. Samples representing 0, 6, 12, 18 and 24 hour post-wounding were collected and stored at -80°C. For callus treatment, using a sharp scalpel, three clumps of calli were cut separately into tiny pieces about 1 – 2 mm and returned to the culturing medium. A portion of the cut callus was collected to represent samples at 0, 6, 12, and 24 hours after wounding treatment. All samples were kept at -80°C for RNA extraction.

RNA Extraction for cDNA Cloning

Stem tissues of *in vitro* plantlets were used for cDNA isolations. Total RNA was extracted from 0.5 g starting material using the RNeasy® Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. DNase treatment was carried out using the DNA-*free*™ Kit (Ambion, USA), according to the manufacturer's instructions. The RNA samples were quantified by measuring the absorbance at 260 nm and 280 nm using nanophotometer (IMPLEN, Germany). The integrity of the RNA samples was measured by 1% agarose gel electrophoresis. First-strand cDNA was synthesised by reverse transcription (RT) from 1µg of DNase-treated total RNA using SuperScript® III First-Strand Synthesis System (Invitrogen, USA) according to the manufacturer's instructions.

Isolation of *AmHST1*

Primers were designed using the Beacon Designer™ 7 software (PREMIER Biosoft, USA) for prenyltransferase gene obtained from our own transcriptome (Siah et al. 2016). All Polymerase Chain Reaction (PCR) experiments were conducted on a SpeedCycler^2 (Analytik Jena, Germany). The specific primers employed were designed to isolate a 760 bp fragment containing the internal sequence (Table

1). The cycle parameters were: 94°C for 5 minutes; 40 cycles at 94°C for 30 seconds, annealing at 60°C for 45 seconds, 72°C for 1 minute; and a final elongation at 72°C for 10 minutes. The PCR product was gel-electrophoresed, the desired fragment cut and cloned into the pGEM®–T Easy Vector (Promega, USA) and sent for sequencing at a commercial lab. Then, the 3'-cDNA end was amplified from RT reaction using the FirstChoice® RLM-RACE (Ambion, USA) according to the manufacturer's protocol. Based on the predicted open reading frame of the partial sequence of *AmHST1*, a Rapid Amplification cDNA Ends (RACE) primer was designed as shown in Table 1. The cycle parameters were: 94°C for 3 minutes; 35 cycles at 94°C for 30 seconds, annealing at the respective temperatures for 30 seconds, 72°C for 1 minute; and a final elongation at 72°C for 7 minutes.

Sequence Verification and Phylogenetic Analysis

A specific pair of primers were designed to verify the gene *AmHST1* using High Fidelity KOD Hot Start Mastermix (Favorgen, USA). The reaction was setup as follows: 10 µl (0.04 U/µl) of KOD Hot Start, both forward and reverse primers at 10 µM each, and 100 ng of cDNA in a final volume of

Table 1
Specific primers used in PCR analysis to obtain AmHST1 cDNA and the primers used in qRT-PCR

Amplification/ Gene	Primer Name	Sequence (5' to 3')	Amplicon size (bp)
Internal	*AmHST1*-F	5'-TCCTCACACCGTCGCCTCTCC-3'	760
	AmHST1-R	5'-CACAGGAGGACTCCACAAGAAAGG-3'	
3' RACE	*AmHST1*-3'O	5'-GGTCATCTCCTTTGCAGTGGCT-3'	749
ORF	*AmHST1*-F-full	5'-ATGGAGCACTCAATCTCTGTTTT-3'	1182
	AmHST1-R-full	5'-CTAAACGAATGGAAATATAGC-3'	
qRT-PCR/	RT-*AmHST1*-F	5'-GCTTCTGAATTATGTTGCTGCCATC-3'	224
AmHST1[1]	RT-*AmHST1*-R	5'-TACCCTAAACGAATGGAAATATAGCG-3'	
qRT-PCR/	TUA-F	5'-GCCAAGTGACACAAGCGTAGGT-3'	183
TUA[2]	TUA-R	5'-TCCTTGCCAGAAATAAGTTGCTC-3'	
qRT-PCR/	PAL-F	5'- GCCTTGCATGGTGGGAACTTTCAG -3'	192
AmPAL[3]	PAL-R	5'- GCCCTTGAAGCCGTAGTCCAG -3'	
qRT-PCR/	PD-F	5'-GAAGTGGCCTTCCTAAGATTTTCACA -3'	216
AmPD[1]	PD-R	5'- ATCGTGACAAATGAAGGTATGCGTC-3'	
qRT-PCR/	GAPD-F	5'-CCG GTC TTT TGG TAT CAG ACG C-3'	251
AmRPL[2]	GAPD-R	5'-CCC GAT AAC CAG GAC GTT CAA G-3'	
qRT-PCR/	WRKY-F	5'-CAACCGACCTAACAACAAC-3'	106
AmWRKY[3]	WRKY-R	5'-TAAATTGTGACCTGGGTTAC-3'	

[1]Primer sequences were designed in this study
[2]Primer sequences derived from Gao et al. (2012)
[3]Primer sequences derived from Wong et al. (2013)

20 µl. The cycling conditions: 95°C for 2 minutes; 40 cycles at 95°C for 20 seconds, annealing at 42°C for 10 seconds, 72°C for 40 seconds; and a final elongation at 72°C for 7 minutes. The PCR product was cloned into the Perfectly Blunt® Cloning Kits (Novagen, USA) and sequenced. The percentage of *AmHST1* nucleotides was predicted using the Bioedit software version 7.2.5 (www.bioedit.software.com). The sequence was searched against the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm to identify similar sequences. The molecular weight and theoretical isoelectric point for the deduced amino acid were calculated using ExPASy online software (http://web.expasy.org/compute_pi/). To identify the conserved domains present in the deduced amino acid, a comparison was made with conserved domain alignments found in the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The prenyltransferase-like sequences from the GenBank were aligned using ClustalW and phylogenetic analysis was performed using MEGA version 6 (Tamura et al. 2013). Bootstrap analysis was carried out with 1000 datasets.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR study, total RNA was isolated from 1g of wood tissue samples derived from the wounded tree using the RNeasy® Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. Similar isolation protocol was conducted for the fresh cut callus. Then, 1µg of total RNA was used to synthesise the first-strand cDNA using the QuantiTech® Reverse Transcription Kit (Qiagen, Germany). The parameters analysed that were used for qRT-PCR (Wong et al., 2013). Briefly, it was conducted in triplicate assays and each assay contained 10µl of 2× Sensifast™ SYBR Lo-ROX Kit (Bioline, UK), 10 µM of forward and reverse primers, and 100 ng of cDNA template in a final volume of 20 µl. The PCR parameters were: 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds, annealing at 60°C for 10 seconds, 72°C for 5 seconds, and the process continued with 95°C for 2 minutes, 60°C for 5 seconds and 95°C for 5 seconds, using a MX3005P™ instrument. It was analysed using MxPro™ QPCR (Software) (Agilent Technologies, USA). The calculation of normalised gene of interest (GOI) expression level was done by dividing the raw GOI quantities for each sample to appropriate normalisation factor. The error propagation rules for independent variables were applied to calculate the standard deviation (SD) on the normalised gene of interest (GO_{Inorm}) expression level.

For expression analysis of *AmHST1* gene, the RT-*AmHST1*-F and RT-*AmHST1*-R primers were utilised. Three other genes were included as comparison and to provide evidence for the function of *AmHST1*: 1) phenylalanine ammonia-lyase (*AmPAL*, GenBank Accession No. KT357522) and *AmWRKY* (GenBank Accession No. KT357521) (Wong et al. 2013), and 2) pyruvate dehydrogenase (PD). The

latter sequence was obtained from our transcriptome (Siah et al. 2016). The *Aquilaria* housekeeping genes, α-tubulin (TUA) and ribosomal gene (RPL), were used as reference genes for data normalisation (Gao et al. 2012). All primers sequences are listed in Table 1.

RESULTS

Identification of a Putative Homogentisate Solanesyltransferase cDNA

From our transcriptome data (Siah et al. 2016), several sequences similar to prenyltransferases were selected. Using reverse transcriptase-PCR amplification and specific primers designed to amplify the internal region of the transcriptomic sequence, the partial sequence from first-strand cDNA template of *in vitro* plant stems were amplified. The sequence length was 760 bp and matched with the original transcriptome sequence. Using the verified sequence, a near full-length sequence was cloned by 3' RACE using oligo (dT)-primed cDNA. Because the start codon was only 50 bp upstream from the N terminal portion of this sequence, the open reading frame (ORF) was amplified using a forward primer designed from the start codon. The resulting cloned cDNA sequence was 1312 bp and it contained an ORF of 1182 bp, and a 130 bp 3' un-translated region including a poly (A) tail (Figure 1). The sequence has been given a GenBank accession number, KT380852.

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   1 ATGGAGCACT CAATCTCTGT TTTctcccca tctcgaattt tagctctagc tcctcacacc
  61 gtcgcctctc cttctctact aaagatgggt ttggctccca ataagcccag ttgtagttct
 121 tgtcatttgc tctcgaaatg gtccaatcac ctcccgccaa ccggattctt cagcacgaga
 181 agttgctcga agctcgtttc tgttcgccgg ttcaagctaa attctataac ggcctcttca
 241 caagttggtg ctgctgattc tgatccgata ttgagcaaaa tttcgaattt caaagatgca
 301 tgctggagat ttttgaggcc tcatacaata aggggaacag ctctaggatc tgttgctttg
 361 gttacaagag ctttgattga gaatccacat ctaataaagt ggtctctagt gctcaaggca
 421 ttctctggcc taatagctct catatgcgga aatggttata tagttggcat caatcagatc
 481 tacgatattg ggatagacaa ggtaaacaaa ccttatttac ctatagctgc aggggaccta
 541 tccgttcaat ctgcctggat cttggtcatc tcctttgcag tggctggtct tttaattgtc
 601 ggaaccaact ttggtccatt catcacttcc ctttattctt ttggtctact tctgggcaca
 661 atctattctg ttcctccgct taggatgaag agattccctg ttgcagcatt tcttataatt
 721 gccacggtgc gcggcttcct tcttaatttt ggggtatatt atgccacgag ggctgctctt
 781 ggacttcctt tcttgtggag tcctcctgtg gcttttatca caactttcgt gactttgttt
 841 gcgcttgtta ttgccataac taaggatctt ccagatgtag aaggagatcg caagtttcag
 901 atatcaacct ttgcaacaaa gcttggagtt agaaacattg cattccttgg atctggactt
 961 ttgcttctga attatgttgc tgccatctg gctgcaatat acatgcctca ggcattcagg
1021 cataatgtga tgctacctgc acatttagtc ctggctatat gcttgatctt ccagacatgg
1081 gtgctggaac gagcaaatta cacccaggga gcaatctcag aattctaccg cttcatttgg
1141 aatcttttct atgcggagta cGCTATATTT CCATTCGTTT AGgtaacat ttttgtcttt
1201 tttgcttctc ttttgctttt tatctttgca tggtcattat gaggatcata gaaagttgta
1261 tatgtaaagc atttcatctc taaaatgtat atattcgaga caaaaaaaaa aa
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Figure 1. cDNA sequence of *AmHST1* and primer locations used in PCR, RACE and qRT-PCR. Forward primers are underlined. Primers used in PCR amplication of the 760 bp internal length are in brackets. Primer used in 3'RACE is underlined and in italic. Primers used for amplication of the 1182 bp ORF are indicated in uppercase letters. Primers used in qRT-PCR are boxed

The deduced amino acid sequence of *AmHST1* was used as query to search the GenBank protein databases. The predicted *AmHST1* encodes a protein of 392 amino acids and shares 77% and 74% identities with homogentisate prenyltransferase from *Theobroma cacao* (GenBank accession no. CM001883) and *Arabidopsis thaliana* (GenBank accession no. DQ231060) respectively, indicating it is involved in prenylation catalysis. The *AmHST1* protein had a molecular weight of 43.126 kDa and a theoretical isoelectric point (pI) of 9.79. From the comparison made with conserved domain alignments found in the Conserved Domain Database (CDD), it was predicted that the amino acid contained two D-rich sequence motifs in the active site (Figure 2). The two motifs, NQxxDxxxD and KD(I/L) xDx(E/D), are consistent with other known homogentisate group of prenyltransferases and are responsible for prenyldiphosphate recognition (Venkatesh et al. 2006; Sasaki et al. 2008; Shen et al. 2012). Using the prediction software ChloroP 1.1 indicated that *AmHST1* contained a chloroplast targeting peptide of 77 amino acids in length, while TMHMM 2.0 suggested that the protein has six putative transmembrane domains (http://www.cbs.dtu.dk). These predictions suggested that AmHST1 is a plastidic membrane protein.

To identify similar known HPT proteins from the GenBank, the *AmHST1* was searched against the non-redundant amino acid database using BLAST. A phylogenetic

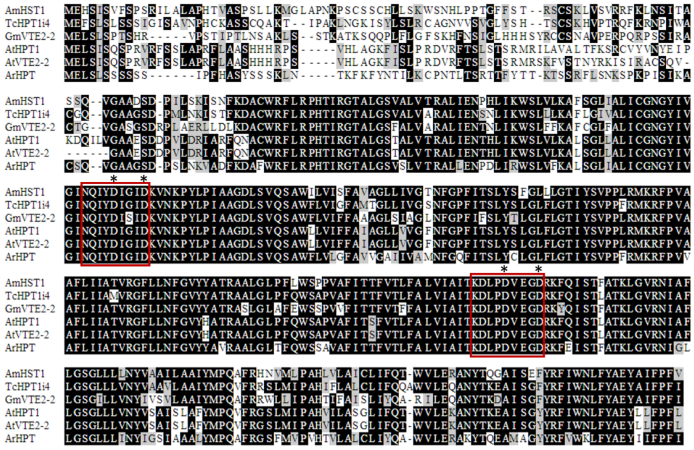

Figure 2. Multiple sequence alignment of homogentisate prenyltransferases. Sequence comparisons made between *Aquilaria malaccensis AmHST1* and five other homogentisate prenyltransferases from plant origins from the GenBank, *Theobroma cacao* TcHPT1i4 (XP_007029130), *Glycine max* GmVTE2-2 (DQ231061), *Arabidopsis thaliana* AtHPT1 (NP_001154609), *A. thaliana* AtVTE2-2 (DQ231060) and *Artemisia sphaerocephala* ArHPT (ACS34774) using ClustalW version 2.1. Identical and similar residues were shaded in black and in grey, respectively, using the Boxshade 3.3.1 program. Two D-rich motifs are marked with asterisks. The two conserved motifs are boxed

tree was constructed to examine the relatedness between these proteins (Figure 3). The *AmHST1* is grouped together with related prenyltransferases involved in the biosynthesis of plastoquinone-9 (PQ-9) and tocochromanols, also known as vitamin

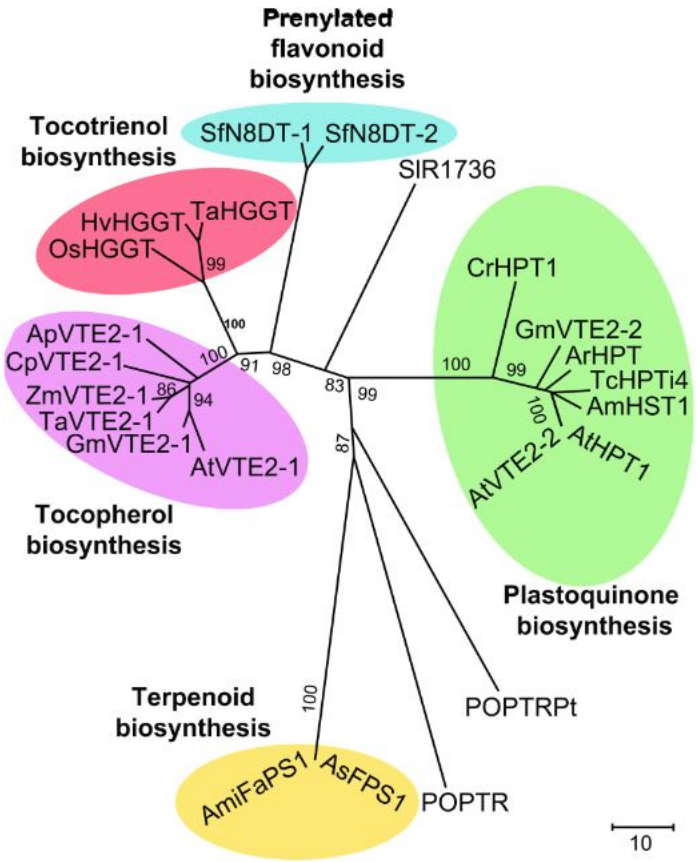

Figure 3. Neighbour-joining phylogenetic relationship of *AmHST1*. An unrooted phylogram was generated using MEGA6 (Tamura et al. 2013). Homogentisate phytyltransferase (VTE2-2) and homogentisate prenyltransferase (HPT) are involved in plastoquinone biosynthesis, putative farnesyl diphosphate synthase (FaPS1) and farnesyl pyrophosphate synthase 1 (AsFPS1) are involved in terpenoid biosynthesis, homogentisate phytyltransferase (VTE2-1) are involved in tocopherol biosynthesis, homogentisic acid geranylgeranyl transferase (HGGT) are involved in tocotrienol biosynthesis and naringenin 8-dimethylallyltransferase (DT-1 and DT-2) are involved in prenylated flavonoid biosynthesis. Accession numbers: *Arabidopsis thaliana* AtHPT1 (NP_001154609), *A. thaliana* AtVTE2-2 (DQ231060), *Artemisia sphaerocephala* ArHPT (ACS34774), *Chlamydomonas reinhardtii* CrHPT1 (CAL01105), *G. max* GmVTE2-2 (DQ231061); Aquilaria microcarpa AmiFaPS1 (ADH95185), Aquilaria sinensis AsFPS1 (AHG54251); *Allium porrum* ApVTE2-1 (DQ231057), *A. thaliana* AtVTE2-1 (AY089963), *Cuphea pulcherrima* CpVTE2-1 (DQ231058), *Glycine max* GmVTE2-1 (DQ231059), *Triticum aestivum* TaVTE2-1 (DQ231056), *Zea mays* ZmVTE2-1(DQ231055); *Hordeum vulgare* HvHGGT (AY222860), *Oryza sativa* OsHGGT (AY222862), *T. aestivum* TaHGGT (AY222861); *Sophora flavescens* SfN8Dt-1 (AB325579), *S. flavescens* SfN8DT-2 (AB370330)

E. It is clearly separated from the clade of *Aquilaria* prenyltransferases (*AmiFaPS1* and *AsFPS1*) in the terpenoid pathway (Yang et al., 2013; Kenmotsu et al., 2013), indicating it has no direct relationship to agarwood synthesis.

qRT-PCR Expression

We investigated expression of this gene to determine if it is wound inducible. In addition, we tested the expression in callus tissue after five months of growing in the dark. The qRT-PCR analysis indicated that *AmHST1* transcripts were expressed in callus tissue, but it was low compared with unwounded wood stem, which was 20-fold higher (Figure 4). Interestingly, when the stem was wounded, the expression levels in 6- to 24-hour post-wounding samples dropped to between 2- to 10-fold lower than unwounded stem (Figure 4). This clearly shows that *AmHST1* is down-regulated by wounding treatment. In a different experiment, so as to avoid compounding effects from natural surroundings such as from microorganisms and herbivores, *in vitro* callus was cut to mimic wounding treatment. The *AmHST1* expression was compared with several other genes from *A. malaccensis*. *AmPAL* and *AmWRKY*, two wound inducible genes had increased expressions of between 9- to 15-folds, respectively, at 24 hours after cutting, when compared with control callus, while *AmPD*,

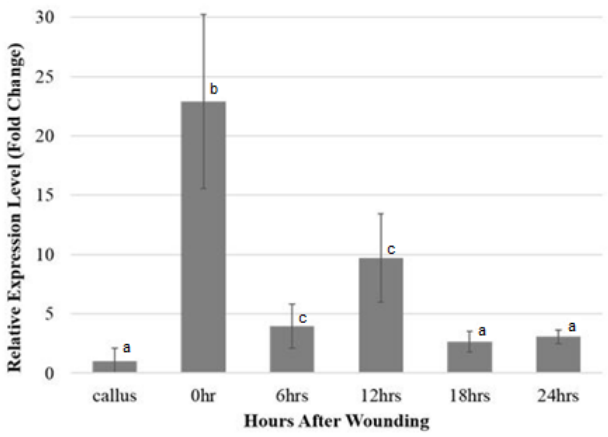

Figure 4. Relative gene expression of *AmHST1* in callus and wounded stem of *Aquilaria malaccensis* at time 0 (untreated control) and 6, 12, 18, and 24 hours after wounding. Callus was used as the calibrator. Bars in the chart represent fold changes in relative expression and the error bars represent standard deviations. Different alphabets indicate significant difference while same alphabets indicate no significant difference between sampling time, using Tukey's test ($p<0.05$)

a gene involved in basic metabolism and *AmHST1* expressions, was not perturbed (Figure 5). This suggests that *AmHST1* was highly expressed in stem tissue but not in callus.

DISCUSSION

This study reports the first putative homogentisate solanesyltransferase cDNA, *AmHST1*, cloned from *A. malaccensis*, a tropical tree widely known for its agarwood. Sequence and prediction analyses using multiple software suggest that the protein has a putative role in PQ-9 biosynthesis, a pathway closely related to vitamin E biosynthesis. Homogentisate prenyltransferases are enzymes involved in the biosynthesis of vitamin E and quinones (Collakova & DellaPenna, 2001). Neighbour-joining phylogenetic tree (Figure 3) reveals that members of HPT are divided into three main groups (Mène-Saffrané & DellaPenna, 2010): homogentisate phytyltransferases involve in tocopherol biosynthesis, homogentisate geranylgeranyltransferases involve in tocotrienol biosynthesis, and HST responsible for PQ-9 biosynthesis, of which *AmHST1* is most related to. Not much is known about this enzyme, except that it is located in the inner membrane of chloroplast and is hardly active with phytyl diphosphate. However, it catalyses the decarboxylation and prenylation of homogentisate with solanesyl diphosphate, leading to the formation of 2-methyl-6-solanesyl-benzoquinol (MSBQ), the immediate precursor of PQ-9 (Soll et al., 1985). An *AmHST1* homolog in Arabidopsis, when expressed in *E. coli* has been shown to react actively with its substrate, solanesyl diphosphate, consistent with its function in PQ-9 biosynthesis (Sadre et al., 2006). When constitutively over-expressed in *A. thaliana*, the transgenic plants have higher

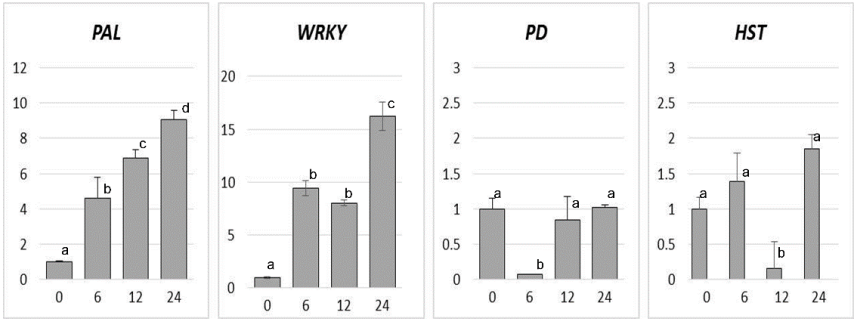

Figure 5. Relative gene expression of several *Aquilaria malaccensis* genes in callus at time 0 (untreated control) and 6, 12, and 24 hours after wounding. Untreated callus was used as the calibrator. Bars in the chart represent fold changes in relative expression and the error bars represent standard deviations. Y-axes are on different scales. (*PAL*=phenylalanine ammonia lyase; *WRKY*=*WRKY* transcription factor; *PD*=phytyl diphosphate; *HST*=homogentisate solanesyltransferase). Different alphabets indicate significant difference while same alphabets indicate no significant difference between sampling time, using Tukey's test (p<0.05).

PQ-9 level compared with control group. Interestingly there is also evidence that suggest higher levels of tocochromanols in the leaves of the transgenics (Sadre et al., 2006). It has been suggested that HST-mediated prenylation may have as many as two possible alternatives resulting with different intermediate molecules (Sadre et al., 2010). This is not surprising as HST and other homogentisate prenyltransferases share similar substrates in the pathway synthesis of PQ-9 and tocochromanols, therefore, they may over-lap in functions. The latter is a group of vitamin E precursors that are synthesised by photosynthetic organisms and possess antioxidants activity. In plants, vitamin E is believed to protect the cellular components from oxidative stress as significant increase of tocopherol levels are observed in response to various abiotic stresses including exposures to high light, saline, cold and drought conditions (Havaux et al. 2000). High-light stress has been shown to escalate total tocopherol levels in *Arabidopsis* leaves and increase the expression of related genes in tocopherol biosynthesis (Collakova & DellaPenna 2003a). This supports our observations on *AmHST1*, which was suppressed in tissues grown in prolonged darkness such as callus, while being expressed in non-stressed tissue, the 0-hour unwounded tree stem (Figure 4). Wounding induces several genes in defence response, such as the transcription factor *AmWRKY* and *AmPAL* of the phenylpropanoid pathway both in stem (Wong et al., 2013) and callus tissues (Figure 5). However, *AmHST1* did not respond to callus wounding (Figure 5), suggesting the long period in darkness might have suppressed the gene.

In tocopherol biosynthesis (reviewed in Mène-Saffrané & DellaPenna, 2010), HPT activity catalyses the committed step, where homogentisic acid (HGA) and phythyl diphosphate (PDP) are condensed into tocopherol. The PDP is generated from reduced form of GDPP by action of GGDP reductase. In an experiment that applies high-light stress to Arabidopsis, it was shown that genes in the tocopherol pathway have positive relationship with expressions and tocopherol accumulation, while the GGDP showed negative response (Collakova & DellaPenna 2003b). In addition, related downstream genes from GGDP reductase are also downregulated. GGDP is synthesised from IPP and DMAPP. Both are precursors in the biosynthesis pathways of tocochromanols, PQ-9 as well as terpenoids. Many important intermediate isoprenoid molecules including FPP and GGDP, which form the basis of other longer chain isoprenoid molecules originated from IPP and DMAPP (Dudavera et al. 2013).

Wounding is an abiotic stress that plays a major role in terpenoid-rich agarwood induction. The fact that the expression of a gene related to PQ-9 and vitamin E biosynthesis is suppressed when a terpenoid inducing situation emerged suggests that there could be precedence in the activation of controlling genes. It is speculated that genes controlling the committing steps

in PQ-9 and tocochromanol synthesis are down-regulated as to allow the formation of isoprenoids important in defence response against abiotic as well as biotic stresses. The up-regulation of several terpenoid synthesis genes from *Aquilaria* by wounding treatment and addition of biochemical elicitors further support our conclusion.

CONCLUSION

This study cloned the gene *AmHST1*, whose expression is down-regulated in the stems of *A. malaccensis* experiencing wounding and in callus grown in the dark. While reports have shown that abiotic stresses have positive relationships with tocopherol accumulation, the mechanisms that regulate its synthesis pathway and related pathways, such as the isoprenoid pathway that shares identical precursor building blocks in making end products of similar defence functions, remain poorly understood and need further investigation.

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ABBREVIATIONS

BAP	6-benzylaminopurine
DMAPP	dimethylallyl diphosphate
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
GOI	gene of interest
GPP	geranyl diphospate
HGA	homogentisic acid
HPT	homogentisate prenyltransferase
HST	homogentisate solanesyltransferase
IPP	isopentenyl diphospate
NAA	naphthaleneacetic acid
PCR	Polymerase Chain Reaction
PDP	phythyl diphosphate
PT	prenyltransferase
RACE	Rapid Amplification of cDNA Ends
TPS	terpene synthase
TUA	tubulin