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STUDY ON SENGON (*Falcataria moluccana*) RESISTANCE TO BOKTOR PEST (*Xystrocera festiva*) AND GALL RUST (*Uromycladium tepperianum*) PART III: OPTIMATION OF CLONING PROTOCOLS AND GENE EXPRESSION STUDY

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APPROVAL SHEET

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Abstract

Monoculture sengon plantation often suffers from pest and disease attack, however effective control measure of pest and diseases is not available yet, that tree improvement program by selecting resistant lines has much to offer. This study aimed at (a) Optimization of construction of genomic library library of sengon as materials to obtain full length sequence of resistance genes against pests and diseases, (b) Optimization of construction of cDNA library for identification of expressed resistance genes by PCR and sequencing, (c) Study the expression of some sequenced gene(s) by RT-PCR, (d). Development of new marker, e.g. SNiP, from sequence analysis. Various methods were tried in experiments started from isolating both genomic DNA and total RNA, synthesis of double strand cDNA, optimizing PCR process to obtain genes sequences encoding resistance to pests and diseases using various specific primers and annealing temperature, also gene expression analysis using RT-PCR. Similar experiments were done on agarwood plants (*Aquilaria malaccensis* dan *Gyrinops verstegii*) as control. Primer AcTI, TaACT, and AcACT using sengon DNA template produced fragment sequences of genes encoding TI and actin with sizes 228 bp, 92 bp, and 146 bp, respectively. Fragment sequences from primer AcTI had high similarity with TI gene sequences from *Acacia confuse.* Fragment sequences from primer TaACT and AcACT similar to actin genes from Fabaceae family, such as *Glycine max, Vigna radiata Lupinus angustifolius* and Poaceae family, i.e. *Oryza sativa*. Fragment sequences from primer TAAct with size 98bp had similarity with actin gene of *Sarocalamus faberi*. Sequence of putative α-amylase inhibitor gene was obtained from primer GMAAI at size 144bp and similar to LTP gene from *Rosa chinensis*, which had domain structure and function similar to α-amylase inhibitor. PCR using DNA template of *A. malaccensis* amplified fragment at 54.8^oC for HMGR gene and at 57^oC for actin gene. Using DNA template of *G. versteegii* amplified fragment at 54.8^oC for HMGR gene and 55.8^oC for actin gene. Using cDNA template of *A. malaccesnis* and *G. versteegii* amplification happened at 56^oC for HMGR and actin genes. Genes encoding HMGR from both agarwood plants were similar to *A. sinensis* gene, while their actin genes were similar to *A. microcarpa* gene in the Genebank.

Keywords: actin, α-amylase inhibitor, HMGR, trypsin inhibitor

1. INTRODUCTION

1.1. Background

Sengon is widely grown by farmers and community, either in monoculture or agroforestry, because it is multi-purpose tree species, easily cultivated, and belongs to legume species, which produce nitrogen (Budelman, 2005). The main pests and diseases, which are reported capable of destroying sengon plantation are Boktor stem borer (*Xystrocera festiva*) and gall rust disease (NFTA, 1989; Suharti *et al.*, 1998; Atmosuseno, 1999; Rimbawanto, 2006; Rahayu *et al.* 2009). Stem borer began attacking the 3-4 year old plants, and without adequate control of pests can ruin the entire crop. Meanwhile gall rust disease, caused by the fungus *Uromycladium tepperianum*, attacks plants at all ages, from seedlings in the nursery until mature plants in the field, also potentially could damage the entire crop. Observations in the field showed that both the boktor pests and gall rust disease could attack plants at the same time causing enormous economic losses. The main concern is that the effective control method both for pests and diseases is not yet available.

Although almost all sengon trees are susceptible to both the pest and disease mentioned above, observations in the field showed that some individual trees show signs of resistance, because they can survive among severely affected populations. This phenomenon is very interesting for tree breeding resistance to pests and diseases program. The existence of individuals who are resistant among the most vulnerable tress is highly expected, because the population of sengon in Indonesia generally, and Java particularly, are known to have a fairly high genetic diversity (Siregar *et al.*, 2005; Rahayu *et al.* 2009). Currently there are no fundamental information about biological processes and mechanisms of resistances in tropical tree species. Thus, research on Sengon resistant to boktor pests and gall rust disease is necessary, as the basis for the resistant Sengon tree breeding programs. Understanding mechanism of tree resistant to pests and diseases is not only important for managing the pests and diseases, but also for applying the mechanism to produce some secondary metabolites associated with the mechanism for other purposes, for example agarwood.

The results from Siregar et al. (2009) showed that sengon trees resistant to boktor pest have trypsin-inhibitor and alpha-amylase-inhibitor activities, which are significantly higher than the vulnerable ones. There is a possibility that the presence of inhibitors is a mechanism of resistance to these pests. For that, we need clarification by identifying the genes coding for these two inhibitors. Further research using

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microsatellite marker (Siregar 2016) showed that resistant sengon trees are different from susceptible ones, indicating a difference in the genetic background of the two accessions. Microsatellite markers thus could become guide for further research, for example locating the genes encoding those two inhibitors by PCR for cloning or directly for selection of resistant lines in a breeding and improvement program.

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Effort to identify and isolate an expressed gene usually started with isolation of RNA, since RNA is the product molecule of gene expression. The next step is production of cDNA from the mRNA, because RNA molecule is easily degraded, and keeping the cDNA molecule in library clones for further examination of their genes content. Cloning is thus a very basic and important step to overcome in molecular studies. Screening the cDNA library could be done using specific primer from the gene of interest in a PCR process.

Previous research showed that isolation of DNA and RNA from tropical tree species was much more difficult than any agricultural plants because of many interfering phenolic compounds in the tissue or presence of other inhibiting secondary metabolites. However, by modifying the extraction methods and protocols, eventually relatively good quality of DNA and RNA has been obtained (Siregar dan Hartati, 2014). In this research, effort to identify the gene encoding trypsin inhibitor and other resistance genes against diseases will be continued by construction of both genomic and cDNA library, as well as cloning of PCR products with primers derived from trypsin inhibitor and other resistance genes sequences. The identification of gene(s) encoding trypsin inhibitor and others in sengon would become important contribution to both plant science as new molecular protocol for tropical tree species, as well as tree improvement program and biotechnology of tropical tree species.

1.2. Objectives

a. Optimization of construction of genomic library library of sengon as materials to obtain full length sequence of resistance genes against pests and diseases

b. Optimization of construction of cDNA library for identification of expressed resistance genes by PCR and sequencing

c. Study the expression of some sequenced gene(s) by RT-PCR

d. Development of new marker, e.g. SNiP, from sequence analysis

1.3. Expected Output

a. Clones of cDNA and genomic library as material for further investigation of genes encoding resistant to boktor pest and gall rust fungi

b. Full-length sequences of gene(s) encoding resistance to boktor pest as well as gall rust diseases

c. New marker for sengon tree improvement and breeding

d. Publications in an international journal

2. STATE OR THE ART OF THE RESEARCH

Sengon (*Paraserianthes falcataria*) is one of fast growing species that is widely used in reforestation programs by Indonesian government, as well as on community forests. About 20% of national timber supply in Indonesia is served by community forests, which keeps increasing role of community forests at present, especially when natural forests continues to degrade. However many pest and disease have threatened community forests and forest plantation as a whole due to changing environment from mix forest into monoculture plantation. The main problem is that community does not have adequate resources to conduct investigations and breeding programs to mitigate them.

Pest and disease attacks can occur when there is compatibility between the tree host and the pest and disease, and the interaction takes place at the optimum environmental conditions. Resistance or susceptibility to pests and diseases, is often associated with certain natural substances such as, proteins, enzymes, extractive, or secondary metabolites produced by individual trees. According to Finkeldey (2005) often, that natural material is a product(s) of one or few gene loci, which can be

detected by molecular methods, or linked with several molecular genetic markers. Trees do not have immune system based on antibodies, such as found in animals, but they activate several defense mechanisms, such as the strengthening of cell walls, and accumulation of proteins that are anti-microbial, and some form of bonded small substances (phytoalexins) (Lamb & Dixon, 1997). Therefore, the usual approach to investigate resistance is to compare the chemical content of resistant and susceptible individuals, or genetic profile of those individuals.

Research on defense mechanisms against pests and diseases of tropical tree species is rare. Such research on sengon in particular has never been done before, that this research would be the first and only one in the world. Previous investigation on the chemical content of two sengon accessions of resistant and susceptible ones resulted in discovery that the two different accessions had different activity levels of enzyme inhibitors, i.e. trypsin- and α-amylase inhibitors. Resistant accessions had significantly higher activity levels than susceptible ones (Siregar 2010). This result was in accordance with other phenomena on other agricultural plants from temperate regions, of which trypsin- and α-amylase inhibitors are responsible for insect pest resistance. Previous project report also found similar results on resistance against gall rust diseases. Sengon accessions resistant to the diseases have stronger activity of secondary metabolite, i.e. triterpenoid, saponins and steroids (Siregar 2016). Electron Microscopic scanning also showed that wood tissue of resistant lines are free from invading hyphae of the disease.

In further research molecular markers can easily differentiate genetic profile or background or gene expressions encoding the phenotypes, in this case resistance to pest and diseases. Siregar (2015, 2016) employed RAPD and microsatellite markers to differentiate genetic background of two sengon accessions, i.e. resistant and susceptible. Results showed that while RAPD failed to detect differences, microsatellite could separate the two accessions in the constructed dendrogram (Siregar *et al*. 2009; 2016). Observation on microsatellite markers employed also pinpoint differences in genetic background of susceptible from resistant lines. These findings needs a follow up research whether the differences related to genes responsible for those resistances. Such investigation requires detection of specific genes, cloning the fragment for isolation and identification of those genes.

Genomic and proteomic approaches using cDNA libraries are emerging that link the molecular and phenotypic bases of plant defenses and pathogen infection.

Experiments to identify certain expressed gene started with isolating RNA which is a product molecule of an expressed gene. Due to unstable nature of RNA molecule, it is necessary to convert the RNA into cDNA and then keeping it in the form of cDNA library clones for further investigation on the differences of genes encoding protease inhibitors of interest. A cDNA library is a collection of individual DNA sequences derived by reverse transcription from messenger (m)RNA of expressed genes in pathogen-challenged and unchallenged hosts. To locate the responsible genes in the constructed cDNA library, selection and investigation of the clones are necessary. When a clone containing a putative gene is found, the expressed genes or expressed sequence tags (ESTs) are sequenced, followed by hybridization experiments to reveal the upregulation or downregulation of genes (increase or decrease in gene expression owing to change in sensitivity or number of receptors). When the structure of genes encoding resistance is elucidated, a marker for differentiating the resistant seedlings from susceptible ones could be designed. In such a way selection of only resistant seedlings for plantation is ascertained. The established method on finding different gene expressions will lead the path to finding another genes responsible for resistance to gall rust disease.

Plant disease resistance (R) genes are abundant in every plant species (Michelmore and Meyers 1998) and are characterized by a gene-for-gene interaction. They confer broad-spectrum resistance against pathogens, including viruses, bacteria, fungi, and nematodes (Dangl and Jones 2001; McDowell and Woffenden 2003). Five classes of disease-resistance genes have been defined according to the structural characteristics of their predicted protein products. The largest two classes of R genes encode putative intracellular receptors (Dangl and Jones 2001), and they contain either a coiled-coil (CC) or a Toll/Interleukin-1receptor (TIR) domain at their N-terminal end, followed by a nucleotide-binding site (NBS) having homology to eukaryotic cell death effectors.

In this continuation research cloning protocols developed last year will be optimized, both for genomic and cDNA library. Previously it seemed that only small fragments could be captured by plasmid vector in genomic library construction, while cDNA remained inaccessible. The roadmap of the research can be seen in Figure 1.
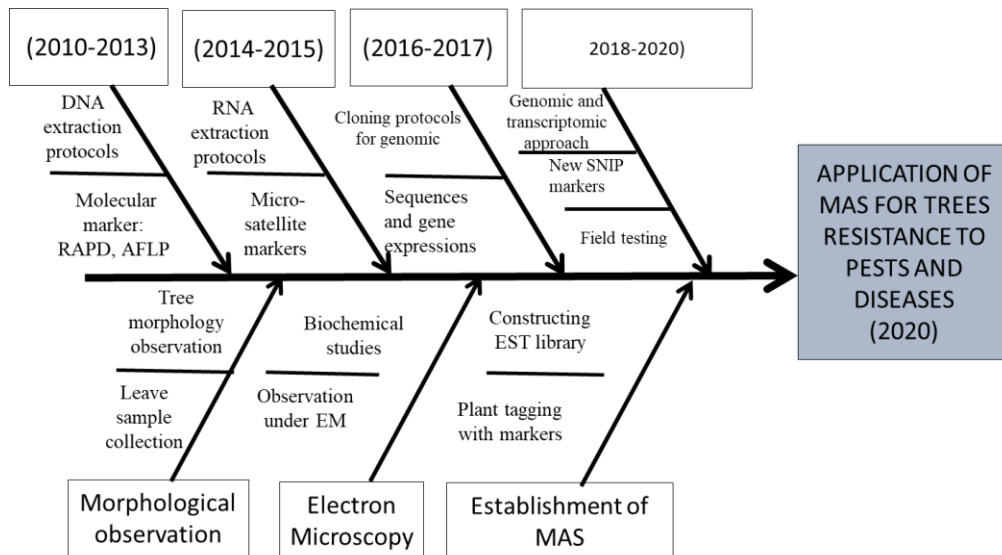
Figure 1. The research roadmap

3. METHODOLOGY

3.1 Plant Materials

Plants material was sengon (*Falcataria moluccana* Miq.) cambium from community forest in Kampung Cangkurawok, Babakan Village, Dramaga Sub-district, Bogor District. The tree accessions were 3 years old. The accessions were taken from accessions of resistant and susceptible to either/or both stem borer pest and gall rust. The accession object were obtained from incision on the sengon stem ofby using cutter sprayed RNAse away. The samples was put in the tube contained RNAse away. The tube then was stored in the liquid nitrogen.

3.2 DNA Genome Isolation

DNA genome isolation procedure used protocols from *Qiagen DNeasy plant mini kit* from Qiagen (2012). The first step was washing Sengon (*F. moluccana*) leaf samples thoroughly under running water and weighed for 100 mg. Using mortar and pestle leaf sample was ground with liquid nitrogen until powdered. Powdered sample was put into a 1.5 ml tube filled with 500 µl AP1 buffer, 4 µl RNAse A stock (100 mg/ml) and PVP 26%. The mixture was vortexed until homogeneous and then incubated at 60 ^{0}C for 10 minutes by inverting the tube for every 1 minute.

The next step was DNA separation from other mixtures, such as proteins, polysaccharides, and polyphenols by adding 130 µl P3 buffer, followed by incubation inside ice for 5 minutes. The solution later undergone centrifugation at speed of 8 000 rpm for 5 minutes. Supernatant was transferred to QIAshredder for centrifugation at speed 8 000 rpm for 2 minutes. The solution, which passed thru QIAshredder membrane, was transferred into new tube and added with 1.5 V AW1 buffer. The solution was homogenized using pipetting technique. About 650 µl solution was transferred into DNeasy mini spin column and undergone centrifugation at speed 6 000 rpm for 1 minute.

The solution was centrifuged at speed 11 000 rpm for 5 minutes. The supernatant was transferred to QIAshredder for centrifugation at speed 11 000 rpm for 2 minutes. The solution, which passed thru QIAshredder membrane, was transferred into new tube and added with 1.5 V AW1 buffer. The solution was homogenized using pipetting technique. About 650 µl solution was transferred into DNeasy mini spin column and undergone centrifugation at speed 8 000 rpm for 1 minute. The solution passed thru mini spin column membrane was discarded; the column was centrifuged again if any solution was still remained. The next step was adding 500 µl AW2 buffer into mini spin column followed by centrifugation at speed 8 000 rpm for 1 minute. The solution passed thru mini spin column membrane was discarded and into the mini spin column 500 µl AW2 buffer was added. The solution was centrifuged again at speed 10 000 rpm for 2 minutes. Collection tube was changed with new 1.5 ml micro tube and then warmed 100 µl AE buffer was added into mini spin column for DNA elution. Prior centrifugation the solution undergone incubation at room temperature for 5−10 minutes. The solution was then centrifuged at speed 11 000 rpm for 2 minutes. The elution step was repeated in order to obtain more DNA. Result of isolated DNA was electrophoresis in 1% (w/v) agarose gel with power supply was turned on at 75 V for 20 minutes. The result of electrophoresis was visualized using Kodak Gel Logic 200..

RNA Total Isolation

Total RNA isolation was done using Total RNA Mini Kit from Genaid and Trizol. Trizol method was using 0.1 gram cambium tissue of sengon tree was powdered using liquid nitrogen. Sample powder was put into 1 ml microtube which contained Trizol reagent, then it was incubated for 10 minutes at room temperature. Mixture of the solution and the sample was centrifuged at speed 12 000 rpm at temperature 4ºC for 5 minutes. The supernatant was transferred and added by chloroform, then it was vortexed for 15 seconds and centrifuged

again with speed 12 000 rpm at temperature 4° C for 15 minutes. The upper liquid was transferred and added 0.5 ml isopropanol, then centrifuged at speed 12 000 rpm at temperature 4^oC for 10 minutes. The pellet was separated from supernatant, then it was added to cold 1 ml ethanol 70%. Next, it was vortexed for 5 seconds and centrifuged with speed 12 000 rpm at temperature 4° C for 15 minutes. The pellet was dried by using vacuum for 10 minutes, then the pellet was dissolved by 25 µl RNAse free water.

Genaid method was using 0.1 gram cambium tissue of sengon tree was powdered using liquid nitrogen. Sample powder was put into 1 ml microtube which contained 500 µl PRB buffer and 5 µl ß-merkaptoetanol. The mixture was vortexed to mix it well, then it was transferred to Filter Column and centrifuged with speed 5 000 rpm at temperature 4° C for 10 minutes. The filtration result was transferred to a 1.5 ml tube and mixed with 1.5x volume of absolute ethanol by pipetting method. The solution mixture transferred onto RB Column and was again centrifuged for 2 minutes at 10000 rpm and temperature 4°C. The liquid was discarded and it was added 400 µl W1 buffer onto RB Column. It was centrifuged for 1 minute at 10000 rpm and temperature 4°C. Next, the liquid was removed and it was added 600 µl Wash Buffer. The addition of Wash Buffer is done twice with speed 10 000 rpm at temperature 4^oC for 1 minute for the first rinse and 2 minutes for the second rinse. The rinse solution was discarded and was centrifuged for 3 minutes at 10 000 rpm and temperature 4°C to dry the RB Column. The RB Column tube was moved into a new 1.5 ml tube, then it was added by 12.5 µl RNAse free water. It was incubated at room temperature for 5 minutes, and was centrifuged for 5 minutes at 10 000 rpm and temperature 4°C. The elution was done two times so that the total RNA 25 µl was produced. The isolated result was electrophoresis in 1% (w/v) agarose gel with power supply was turned on at 75 V for 45 minutes. The result of electrophoresis was visualized using Kodak Gel Logic 200.

3.4 Quantitative and qualitative analysis of total RNA

The isolated RNA total was carried out by two analysis, e.g. quantitative and qualitative. The quantitative analysis is a measurement of total RNA concentration (ng/µL) by measuring absorbance at 230, 260, and 280 nm. 1 µL of RNA sample was measured using a NanoDrop spectrophotometer. The absorbance ratio of A260/280 and A260/230 was calculated to determine the purity of isolated total RNA. Qualitative analysis of total RNA samples was analyzed by agarose gel electrophoresis technique, 2 µL of RNA samples were mixed with 1 µL loading buffer using micropipette on parafilm paper. The electrophoresis

was then processed at a voltage of 100 V for 45 minutes and the bands formed on the electrophoregram results was visualized with a Kodak Gel Logic 200.

3.5 cDNA Synthesis

Synthesis of cDNA was using the Reverse Transcript kit from Toyobo. The composition of cDNA synthesis materials is presented in Table 3. RNA template, 4x DN MM and NFW were mixed, then it was incubated at 37°C for 5 minutes by using PCR machine. After incubation, mix buffer was added to the solution mixture. Then it was incubated again with temperature of 37°C for 15 minutes, 50°C for 5 minutes, and 98°C for 5 minutes. It was stored at a temperature of -20°C.

Table 1 Material composition of cDNA synthesis

Bahan	Volume
RNA	15μl
4x DN MM	6 µl
NFW (*Nuclease Free Water*)	3 µl
5x RT Mmix buffer	6 µl
Total	30 µl

Table 2 Sequences of designed AAI and TI primers

No	Jenis	Primer F	Primer R	Product Size (bp)	Annealing T (°C)
1	*G. max* AAI	CTAAGCCAAACCCTATCCCATTCCC (25bp) Tm: 61.6 °C	GCAGAGGCAAACCGCAACTTCAA (23bp) Tm: 62.9 °C	200	56.6
2	*P.vulgaris* AAI	TTCTCAGCCACGCAAACTCAGCC (23bp) Tm: 64.5 °C	AGTAGCCTTTGGATGTGACGGTGG (24bp) Tm: 62.9 °C	108	57.9
3	*P. trichocarpa* AAI	AGGGAGTTCAAGTGCTGGAC (20bp) Tm: 58.9 °C	GCCAAACCATCTCCATCATCAG (22bp) Tm: 57.7 °C	132	52.7
4	*T. aestivum* AAI	AATGTTGGTGAGAGCGGC (18bp) Tm: 57.3 °C	GGCGACGAGTAATCTGACGAAG (22bp) Tm: 58.4 °C	81	52.3
5	*G. max* Actin	CCAGTTCTGTTGACGGAGGCT (21bp) Tm: 61.3 °C	CAGTGAAAGAACAGCCAGAACAGC (24bp) Tm: 60.3 °C	123	55.3

6	*P. trichocarpa* Actin	TTGTGCTGGATTCTG GTGATGGTGT (25bp) Tm: 62.0 °C	ATTTCCCGTTCAGC AGTGGTGGTG (24bp) Tm: 63.7 °C	172	57
7	*P. vulgaris* Actin	ACATCGTTCTCAGTG GTGGTTCTAC (25bp) Tm:59.9 °C	AGAGAAGCCAAGAT AGAGCCACCAA (25bp) Tm: 61.4 °C	163	54.9
8	*T. aestivum* Actin	CCAAGGCTAACAGGG AGAAGATGA (24bp) Tm:60.2 °C	CGATACCTGTTGTG CGTCCA (20bp) Tm:59.0 °C	120	54

3.6 Specific Primer Designation

Primer is a single short DNA or oligonucleotide, which is between 16-24 bases (Chawla 2002). Primer functions as an initiator of in vitro DNA polymerization reaction. The primer design is done with primer3plus web-based application (www.primer3plus.com), Thermofisher Tm calculator and OligoAnalyzer application. Several parameters are considered in the primer design in this research, there are primer length, temperature of melting (Tm) and primer composition.

3.7 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is an enzymatic method for DNA amplification by way of in vitro (Joseph 2010). Each cycle consists of three stages of denaturation, annealing and elongation. On the denaturation stage, the template DNA, which is double strands is made into single strands by an increase in temperature (94-96°C). The next stage is the primer attachment stage or annealing, i.e. with a lower temperature depending on the primer used. Primer attaches to the complementary DNA part of the base sequence. This attachment is specific to the target sequence. The sequence of primary nucleotides will be the determinant on which part of the primer will attach (anneal) to the genome. Incorrect temperature causes no attachment or primer attach in misplace. The next stage is the elongation stage. The temperature used is in accordance with the optimal temperature of the DNA polymerase activity. Generally, temperatures use between 65-72°C. The PCR reaction composition presented in Table 3.

Table 3 Material composition of specific gene PCR

Material	Volume
Template (50 ng/µl)	2 µl
Primer F 10µM	0.5 µl
Primer R 10µM	0.5 µl
Green Go Taq Mix	5 µl
NFW	2 µl
Total	10 µl

The mixture was in the vortex and was reacted into the PCR machine with reaction conditions as seen in Table 4. The PCR product was then electrophoresis in agarose 2% with marker of 50bp. Annealing temperature is also carried out optimization.

Table 4 The PCR steps

Stage	Temperature (^{0}C)	Time (minute)	∑ Cycle
Predenaturasi	95	2	1
Denaturasi	95	0.5	35
Annealing	*	1	35
Elongasi	72	2	35
Postelongasi	72	5	1

* annealing temperature based on specific primer used

3.8 Sequencing

Sequencing will be done to analyze the suitability of the primers used. Sequencing will be carried out by using Genetika Science, Jakarta.

4. RESULTS AND DISCUSSION

4.1. Genomic DNA isolation

Early important step of genes detection, including alpha-amylase inhibitor, trypsin inhibitor (*TI*), actin, HMGR dan guaiene genes, is DNA isolation. DNA or RNA isolation separate the DNA or RNA from other cell components, such as protein, fat, and

carbohydrate (Liana 2017). One example of electrophoregram from sengon and agarwood DNA as comparison is Figure 2. Good quality DNA appears as thick fragment indicating high concentration, very little contamination which appear as smear. DNA isolation steps include cell lysis, degrading cell walls, membranes and precipitation of cell debris (Faatih 2009). Sengon samples utilizes cambium, because very little secondary metabolites present rather than leaves. Sengon leaves contain many secondary metabolites such as alkaloid, flavonoid, polyphenol, saponins, quinone, steroid-triterpenoid, and mono-sesquiterpenes (Faramayuda *et al.* 2015), while the cambium has only flavonoid, saponin, phenol, tannin, triterpenoid, and steroid (Rahmawati 2017).
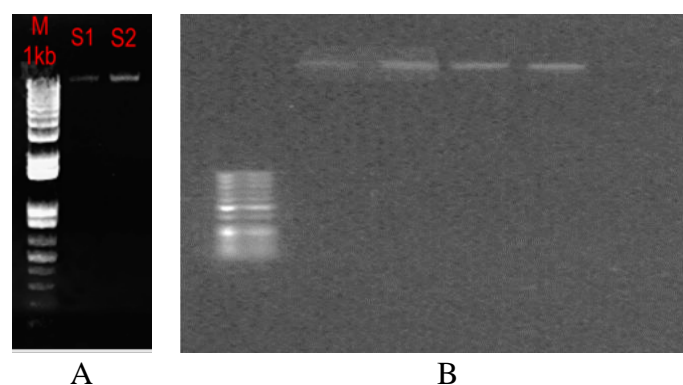
Figure 2. Elektrophoregram of isolated DNA A. Healthy sengon cambium free from boktor pest. B. Leaves of *A. malaccensis* (1,3) and *G. versteegii* (2,4).

4.2. Total RNA Isolation
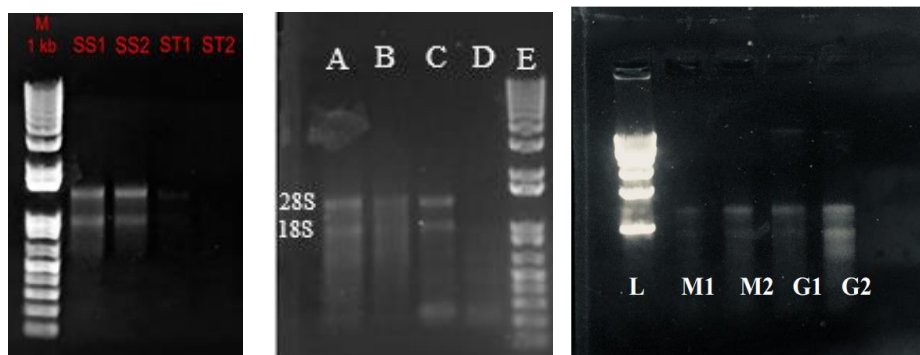
Figure 3 Elektrophoregram of sengon total RNA. Left : (SS) healthy tree; (ST) Tree infested by boktor pest. Middle: A = Isolated total RNA of SS1; B = Isolated total RNA of SS2; C = Isolated total RNA of ST1; D = Isolated total RNA of ST2; E = 50bp ladder. Right: Isolated total RNA of agarwood.

Basically RNA isolation is similar to DNA isolation, however since RNA is easily degraded more careful handlings is necessary. Total RNA isolation was successful as shown

in the visualization by 1% agarose gel electrophoresis as double bands (Figure 3). According to Rapley dan Manning (1998), the two bands indicated 18S ribosomal RNA (rRNA) and 28S rRNA which constitute the most fraction of total RNA. Thickness of the bands indicated the RNA concentration. The thicker the bands, the higher the concentration. Total RNA isolation is very important step determining success of cDNA synthesis for gene expression analysis. Results of electrophoregram showed that isolated sengon and gaharu RNA had smears, meaning had rather low integrity (Figure 3). Smear could meant there were mRNA and tRNA component (Farrell 2005). Contained mRNA could appear as smear because the amount was very little (1-4% of total RNA) and due to high heterogeneity of mRNAsizes. Meanwhile the tRNA and rRNA are small molecules of RNA which could appear as thin smear under 18S rRNA fragment. However long smear along gel column indicated degraded RNA due to RNAse contamination in the isolation process, of which high activity of RNAse present in the tissues (Amanda and Cartealy 2015). ST2 sample did not give a band indicating failure of RNA isolation, due to degraded plant tissues. Supriatna et al. (2017) stated that boktor pest attacks could cause plat tissue to dry-up and cracked that it contains much less RNA.

Table 5 showed concentration of isolated RNA from the ratio of A260/A280 absorbances measured in the Nanodrop. Highest total RNA concentration belonged to SS1 (424,84 ng/µL). Good total RNA has absorbance at $A_{260/280} \geq 1,8$, as well as ranges from 2,0 – 2,4 at $A_{260/230}$ (Sambrook dan Russel 2001, Farrel 2005). Total RNA purity is related to the condition of the plant, i.e intensity of gene expression in question as well as health of the tissue, that related to the amount of gene products needed by the plant. The SS1 dan SS2 samples had $A_{260/280}$ value around ≥ 1.8, however based on absorbance $A_{260/230}$ the value were not high enough. Sample ST1 has low value on both absorbance ($A_{260/280}$ and $A_{260/230}$), maybe due to protein or polysaccharide contaminations (Rapley and Heptinstall 1998). Further RNA purification could solve the problems to eliminate effects of RNAse. The molecule structure of RNA makes it prone to RNAse rather than DNA (Chandrawijaya *et al.* 2013).

Table 5. Quantification and purity of total RNA using NanoDrop

Sample	RNA Consentration (ng/µl)	Purity (A260/A280)
SS1	223.32	2.133
SS2	251.00	1.730
ST1	106.30	2.080

ST2	235.52	1.797
SS1 (2)	424.84	1.400
SS2 (2)	169.08	2.148
ST1 (2)	149.84	1.461
ST2 (2)	-	-

4.3   cDNA Synthesis

RNA is very sensitive to temperature changes, which makes it easily degraded, that immediately after isolation the RNA must undergo transformation into cDNA, in order to make it more stable. The synthesis of cDNA starts with a single strand, which is derived from mRNA strand in the total RNA, using PCR with the aid of a reverse transcriptase enzyme (Yunbi 2010). The primer oligo dT aligns to poly A tails, which is characteristics of a mRNA. According to Allison (2007), poly A tails of mRNA is resulted from polyadenylation process, which doesn't happen to other kind of RNA.

Synthesis of cDNA is an important step to identify certain gene; it requires a good quality and quantity of total RNA. The synthesis comprised of two steps, i.e. first strand using reverse transcription, followed by second strand. Figure 4 showed elektrophoregram of synthesized cDNA. Sample from sengon only showed smear, which might be due to the amount of cDNA were too small, that it was difficult to detect using electrophoresis (Figure 4A). Figure 3B showed results of cDNA synthesis from agarwood. The synthesized cDNA should be amplified first using housekeeping gene primers, before electrophoresis. In this research the housekeeping gene was *Act*.

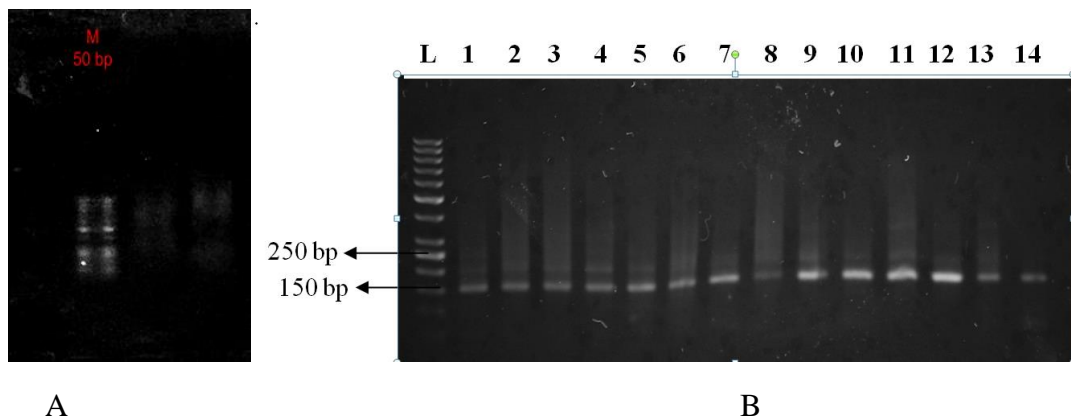A   B

Figure 4. Electrophoregram of cDNA. A. Using TI primer on sengon B. Using HMGR
        primer 1 on agarwood; 1,2,5,6,13 and 14 = *A. malaccensis*; 3,4,7,8,9,10,11 and
        12 = *G. versteegii.*

Effort to estimate the amount of sengon cDNA using NanoDrop (Table 6) was not aqqurate, because the NanoDrop could not differentiate various kinds of DNA in the solutions. The measurements result only showed the total amount of nucleotides in the solutions, including cDNA, unsynthesized RNA molecules, other DNA, or remaining dNTP.

Table 6   Concentration of sengon cDNA based on NanoDrap measurements

Sample Code	Concentration (ng/µL)
SS1 (2)	1498,40
SS2 (2)	629,63
ST1 (2)	815,37

The cDNA synthesis was applied only on sample SS1 and ST2 due to their god quality of total RNA as template for reverse transcription. Testing the synthesized cDNA quality was not done using electrophoresis, because the result would be just a smear, therefore testing would be done using PCR with primers of certain gene, such as housekeeping gene. In this case since sengon housekeeping genes are not known yet, the testing was done using primers from actin encoding genes from other species.

4.4 Optimization Primers Annealing Temperature for Primers of Actin Encoding Genes in Sengon dan HMGR also Guaiene Encoding Genes from Agarwood.

4.4.1. Optimization of Primers Annealing Temperature using Sengon and Agarwood DNA as Template

Optimization of annealing temperatures for primers of actin encoding genes was conducted in order to obtain targeted PCR products. Recommended annealing temperature is $\pm 5^{\circ}$C away from Tm (melting temperature), that the utilized temperature ranged from, 55°C - 60°C.

Results showed that SoyAct and PVAct primers formed dimers at those temperature, while TAAct formed fragments about ± 80 bp, and PTAct formed ± 110bp fragments (Figure 5). Optimum annealing temperature for primer PTAct and TAAct were 55°C and 56°C respectively, with clear and big bands. These 55°C dan 56°C temperatures were used to obtain much more PCR products for sequencing purposes

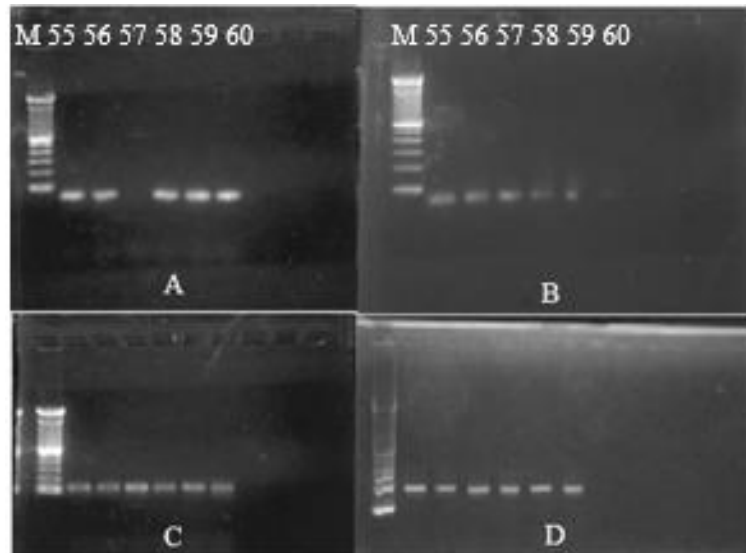Figure 5. Optimizing annealing temperature (°C) for PCR using sengon DNA with actin primers. M= 50bp ladder; A = SoyAct; B = PVAct; C = TAAct; D = PTAct.

A   B
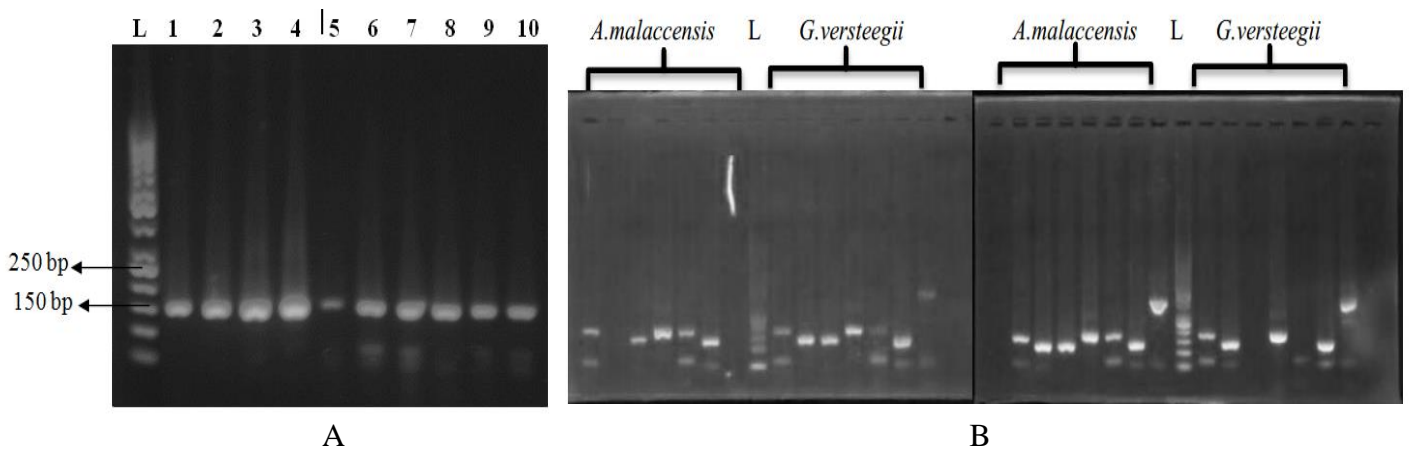
Figure 6. Optimizing annealing temperature (°C) for PCR using agarwood DNA with 1_HMGR primer; L = Ladder 50 bp gen ruller; 1,2,3,4, dan 5 = A. malaccensis ; 6,7,8,9, and 10 = G. versteegii. B. guaiene primers using annealing temperature of 56.5°C and 57°C

Figure 6A showed PCR products from DNA of *A. malaccensis* and *G. versteegii* with HMGR target gene successfully amplified at annealing temperature of 54.8°C. The fragments were visualized on 2% agarose gel electrophoresis. Similar results obtained for Actin gene, which was amplified at 55.8°C for *G. versteegii* and 57°C for *A. malaccensis*. Those PCR products were sequenced to confirm the results.

On *A.malaccensis* samples amplification took place at 56.5°C for primers Am1 -δG, Am3-δG, Am4-δG, AC-δG, and Amic-δG, of which the best results were from primers

Am3-δG and Am4-δG, appeared as single bands, which means the annealing is specific. At 57°C using *A.malaccensis* DNA as template amplification took place, however several primers showed smear and multiple bands, e.g. primers Am1-δG, AC-δG, and Amic-δG. Results of PCR process using *G.versteegii* DNA as template at two annealing temperatures, i.e. 56.5°C and 57°C is shown in Figure 6B. PCR at 56.5°C amplified all DNA samples; however several samples showed multiple bands and smear. Primers gave multiple bands were Am1-δG, AC-δG, and Amic-δG. Amplification at 57°C dusing *G.versteegii* DNA as template was successful except primers Am3-δG dan AC-δG. The two temperatures used still gave double bands in several samples, that more optimization are still needed. According to Priambodo (2011) low annealing temperature caused primers attached to non-specific sites, resulting in double or multiple bands. However too high annealing temperature could cause primers failure to attached to the template and thus no amplification takes place. The results indicated that specific primer for *A.malaccensis* DNA at 56.5°C dan 57°C annealing temperature were Am3-δG and Am4-δG, with PCR products size about 150-200bp, while for *G.versteegii* DNA at annealing temperature of 56.5°C were Am3-δG and Am4-δG, while at 57°C was Am4-δG.

4.4.2 Primers Annealing Temperature Optimization Using Sengon and Agarwood cDNA

Annealing temperature used for optimization of cDNA template ranged 50°C - 60°C (Figure 7). Primers SoyACT and PvACT gave products as single bands with size <100 bp (Figure 7 left). Most probably those bands were dimeric fragments and not a PCR products. As shown previously this research used primers size 20-26 bp. Due to amplification failure SoyACT and PvACT primers will not be used anymore. Meanwhile primer TaACT could amplify a single band at all temperatures with fragment size ±100 bp; especially at temperature 56°C the band is very clear. Primer PtACT also generated single band at six temperatures tested with product size ±150 bp. Thus primers TaACT and PtACT would be used for further experiments to detect actin gene in sengon.
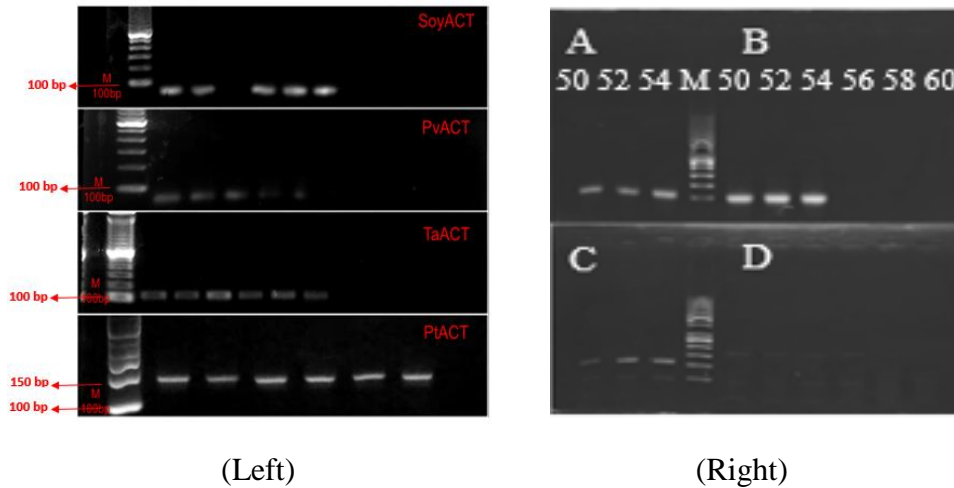
(Left)   (Right)

Figure 7. Optimization of annealing temperature (°C) for PCR of sengon cDNA using several primers. (Left) Actin primers at temperature 54,5°C, 55°C, 56°C, 57°C, 58°C, dan 59°C, (Right) A = SoyAct; B = PVAct; C = TAAct; D = PTAct.

Primer SoyAct and PVAct generated dimer at all temperatures, while primer TAAct gave double band at 50°C, 52°C and 54°C, primer PTAct gave thin band at all six temperatures. The most suited temperature, which gave best band using primer PTAct were 50°C, 52°C, and 56°C. Best annealing temperature for primer TAAct was 52 °C, while for primer PTAct was at 50°C (Figure 7).
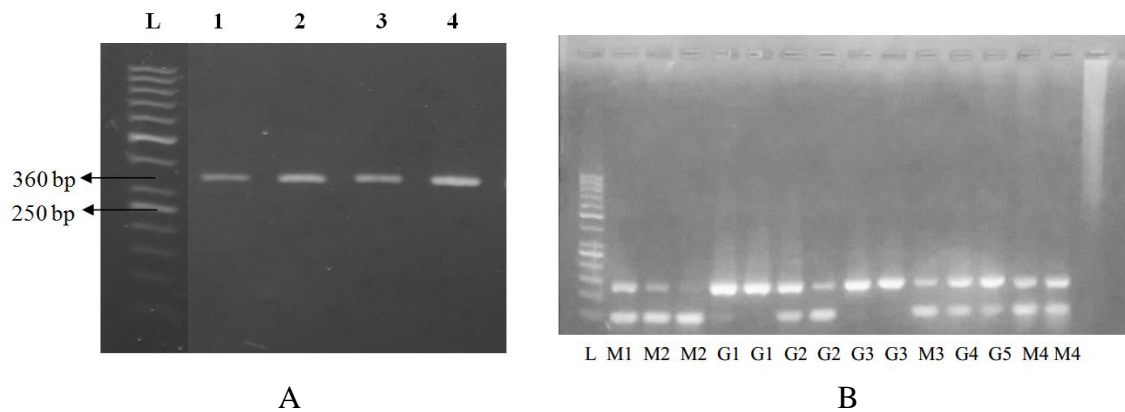
Figure 8. Optimization of annealing temperature (°C) for PCR with agarwood cDNA using various primers. A. primer QAct_1 Actin: L= Ladder 50 bp gene ruller; 1 and 2 = *A. malaccensis* ; 3 and 4 = *G. versteegii*; B. primer guaiene (δG): M= *A. malaccensis;* G= *G. versteegii*

Optimization of cDNA amplification by PCR was done following the results of DNA optimization. The best DNA amplification products were obtained at 56.5°C or 57°C using primers Am3-δG and Am4-δG. Optimization of cDNA amplification was done with only

one primer, i.e. Am3-δG, because of limited amount of isolated RNA, and the results is showed in Figure 8. Sample visualization was arranged based on the time of cDNA isolation. Primer Am3-δG at annealing temperature 56.5°C still generated double bands with almost similar sizes. The similar band sizes obtained might be due to similar primer used. Although the result implied that further optimization was still needed, several samples such as G1, G2, G3, and G4 could be used for following experiments, which was sequencing.
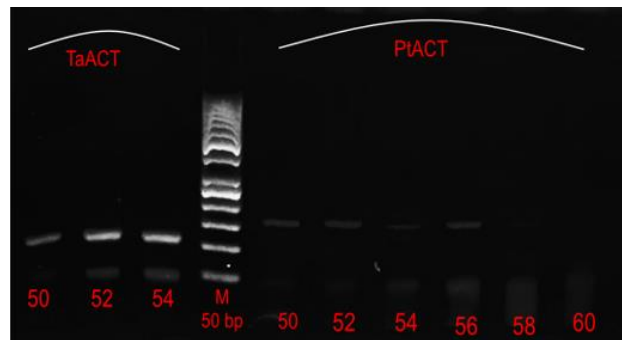
Figure 9. Annealing temperature optimization of TaACT and PtACT primers for cDNA amplification to analyzed sengon TI gene

Figure 10 PCR products for sequencing of actin gene from DNA and cDNA as templates. M= 50bp ladder; A = PTAct of cDNA; B = TAAct of cDNA; C = PTAct of DNA; D = TAAct of DNA

Optimum annealing temperature from experiments with DNA template was then used to optimize cDNA amplification using gradient temperatures at PCR machine. Figure 8 shows cDNA amplification optimization generated single band at temperature range 50°C, 52°C, and 54°C with primer TaACT, and the best result was at 52°C. The amplicon obtained have size ±100 bp. The amplicon sizes were similar with the sizes of previous amplicon using DNA as template. However, this optimization using TaACT on cDNA still generated another bands, which might be dimers, because of their sizes ±50bp. Primer PtACT

generated single band using cDNA template at 50ºC, 52ºC, 54ºC, and 56ºC, especially at 50ºC, 52ºC, and 56ºC the bands were very clear with little smear. Thus primer PtACT successfully amplified cDNA and generated band with size ± 150 bp (Figure 9).

The optimum annealing temperature subsequently was used to generate PCR products for sequencing purposes, amounted at 33 μl (Figure 10).

4.4.1.1 Optimization of Annealing Temperature for Primers of Genes Encoding Alpha-amylase Inhibitor of Sengon

Optimization Using DNA Template

The annealing temperature that were applied to optimize PCR condition using primer AAI gene ranged from 55ºC - 60ºC (Figure 11).
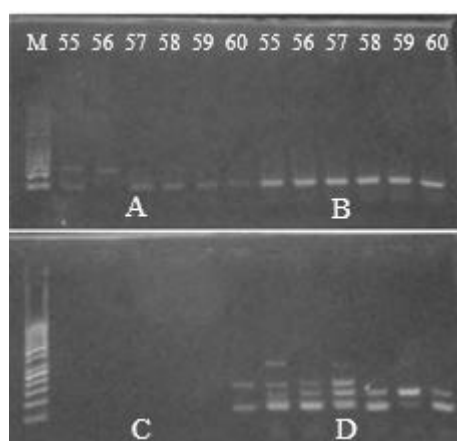

Figure 11. PCR products from optimization of annealing temperature (ºC) using DNA template with primer AAI. M= 50bp ladder; A = PTAAI; B = PVAAI; C = GMAAI; D = TAAAI

Figure 11 showed that primer PTAAI generated single band only at 56ºC, while at 55ºC gave double band, and at other temperatures,i.e. 57 ºC, 58 ºC, 59 ºC and 60 ºC produced dimers. Meanwhile primer GMAAI only at 60ºC produced double band while at other temperatures, i.e. 55ºC, 56ºC, 57ºC, 58ºC and 59ºC did not produce bands. Bands were not appear probably because primers failed to attach to DNA strand due to unsuitable temperature. Primer TAAAI on the other hand generated multiple bands at all temperatures.

Figure 12 showed primer GMAAI produced double band at three temperatures, while primer PTAAI gave smear at 55ºC and dimer at 60ºC. Based on this results subsequent experiments, which are sequencing and gene expression analysis, utilized primer GMAAI with optimum annealing temperature of 55ºC to detect sengon AAI gene. The reason was at

that temperature PCR process gave clearer bands compared to other temperatures, e.g. 56°C and 58°C, even though the bands were still double band.
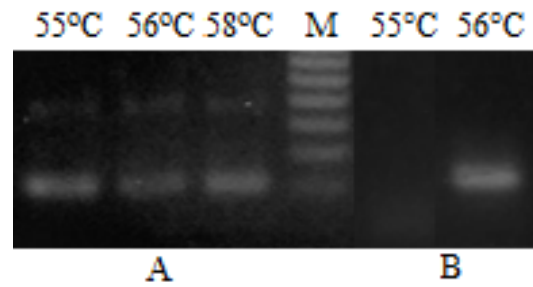

Figure 12 Optimization of cDNA PCR annealing temperature (°C).
M= 50bp ladder; A = GMAAI; B = PTAAI

4.4.1.2 Optimization of Annealing Temperature for Sengon TI Encoding Gene Primers
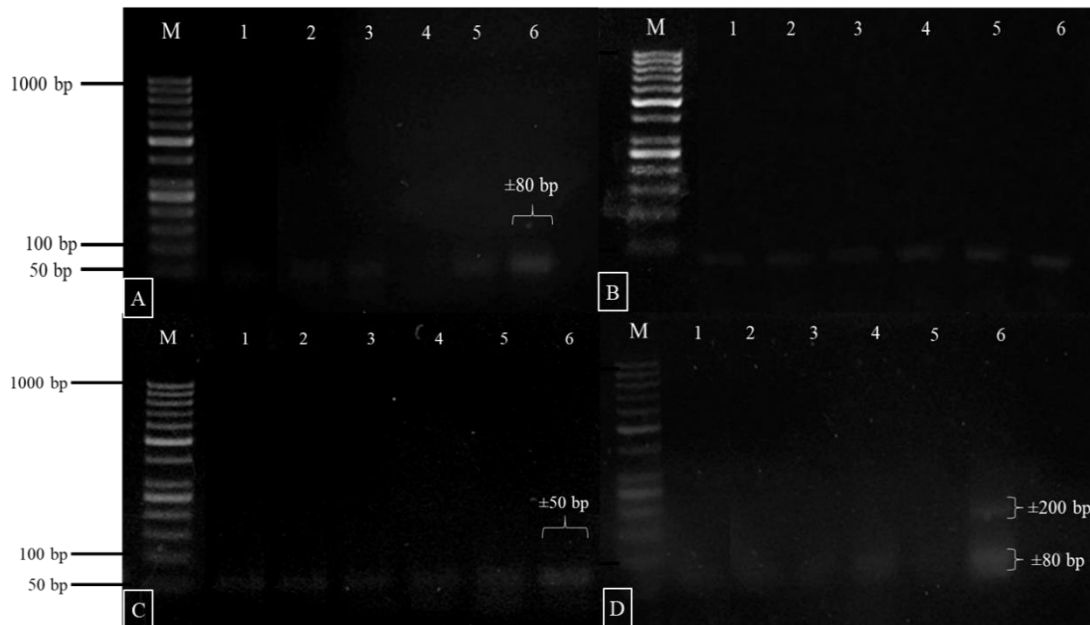
Figure 13 Amplification from annealing temperature optimization using TI specific primer: TinTI (A), VuTI (B), DrTI (C), GmTI (D). M: DNA *ladder* 50 bp; S-1: 50 °C; S-2: 52 °C; S-3: 54 °C; S-4: 56°C; S-5: 58°C; S-6: 60 °C

Amplification of sengon genes encoding actin and *TI* showed amplicons with various sizes. Optimization using those primers was also done at six different temperatures using DNA tempale (Figure 13). Primer TinTI successfully gave amplicon with size about 80bp at 60°C as optimum annealing temperature. Primer VuTI generated amplikon below marker size <50 bp, while primer DrTI produced amplicon size 50 bp at all temperature tested. Amplification using primer GmTI gave double band at 60 °C with sizes about 200bp and 80bp each. The fragments sizes were far from predicted sizes, and the band were not very

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clear with smear. Amplification using sengon DNA template by primer AcT I also used those six different temperature ranges. Figure 14 showed that primer AcTI produced single band at all annealing temperature tested. The AcTI fragment size was about 250bp, with relatively visible intensity. Lane 6 with annealing temperature 60^0C gave band with best intensity. Amplification of those five primers from candidate gene encoding *TI* showed that only primer AcTI gave fragment with satisfying criteria.
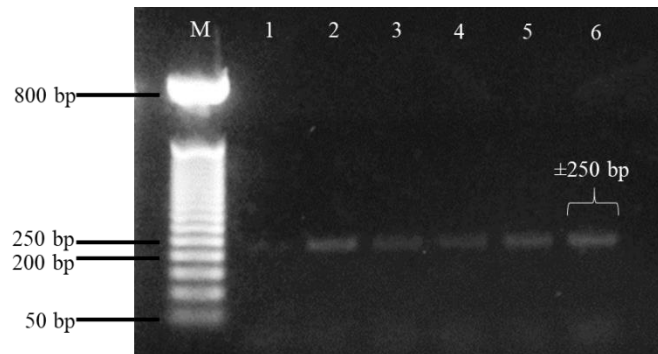
Figure 14 Amplification from Ta optimization using primer AcTI. M: DNA *ladder* 50 bp; S-1: 50 ^{0}C; S-2: 52 ^{0}C; S-3: 54 ^{0}C; S-4: 56 ^{0}C; S-5: 58 ^{0}C; S-6: 60 ^{0}C

Figure 15 Amplification from Ta optimization using Actin primer: SoyACT (A), PvACT (B), TaACT (C), AcACT (D). M: DNA *ladder* 100 bp; S-1: 50 ^{0}C; S-2: 52 ^{0}C; S-3: 54 ^{0}C; S-4: 56 ^{0}C; S-5: 58 ^{0}C

Fragments from amplification with primer SoyAct and PvAct had sizes below marker (<100 bp) at five different temperatures (Figure 15). Meanwhile amplification of TaACT and AcACT both produced single at all temperature intervals. The TaACT amplicon

had size 120 bp, while AcACT amplicon had size 160 bp, of which nearly similar with the predicted sizes. The intensity of AcACT band was stronger than AcACT amplicon, maybe due to different concentration of template or gene copy numbers, of which binding sites of primer TaACT was more than AcACT primer. Amplification results showed that only primers TaACT and AcACT fulfilled the product criteria than the other four specific actin primers. According to Margawati and Ridwan (2010), selected optimum annealing temperature was related to the amplicon produced, whether it has the right size and clear band. The optimum temperature could be applied for further experiments using qPCR. Thornton and Basu (2011) stated that annealing temperature for qPCR analysis ranges from 59 – 60°C.

4. 5 Sequencing and Homology Analysis

4. 5. 1 Sequencing and Homology Analysis in Sengon

Sequencing was performed to validate the suitability of gene isolation results with the desired target genes (Actin and AAI gene) and determined the homology of species and genes used. The samples which sequenced were the amplicon of TAAct primer with annealing temperature is 52 °C and and the GMAAI primer with annealing temperature is 55 °C in cDNA template. Results of the sequencing the TAAct and GMAAI primer with cDNA template are shown in Table 7.

Table 7 PCR products sequences using sengon DNA and cDNA template for actin encoding genes

Primer	Sequence (5' – 3')	Insert size (bp)
TAAct cDNA	CCCATTGTTGAACTTCATGTGCCTGCTATGTATGTCGCTATCC AGGCCGTCTT--- GTCTCTCTATGCTAGTGGACGCACAACAGGTATCGAAACATA CAT	98
TAAct DNA	CTCGGAGTTCTGGTTTGAGACTTTCA- TGTGCCTGCTATGTATGTCGCTATCCAGGCCGTCTTGTCT	66
GMAAI cDNA	NNNNNNGNNNTGGNGNAGTGGCACATGACCCTAGGGATGCC TTAAAACTTGGGGTGTGTGCTAATGTTCTTAACGGTTTGCTTA ACGTGACCCTTGGACAGCCACCAGTGACCCCTTGCTGCACTC TGATTCAAGGACTCGCCG	144
FMAAI DNA	NNNNNTNTANNANNAGAGCCAGGAA- GTTACAGAAACACCAATGGGACTTCTGTGTTGCCACTCAGAG	141

GATCAAGAACAACCATCTCATGAAATTTCCGATCACCTTCATT
CTGAATTATCAAATGCTTCTTCCCTTTCTTC

The results of sequencing then analized in BLASTn on NCBI (*National Center for Biotechnologi Infomation*) database and obtained information about the similarity (homology) of the DNA sequence with nucleotide sequence which contained in NCBI database. The results of the homology analysis of TAAct primer with DNA and cDNA template are shown at table 8.

Table 8 Sequence homology from primer TAAct using BLASTn

Primer	Description	Score max	Identity (%)	E value
TAAct cDNA	*Sarocalamus faberi* actin Mrna, complete cds	87.8	87%	4e-14
TAAct DNA	PREDICTED:Glycine max actin-3-like (LOC100807341), transcript variant X	56.5	87%	6e-05
GMAAI cDNA	PREDICTED:*Rosa chinensis* lipid transfer protein EARLI 1-like (LOC112180515) Mrna	89.8	75%	2e-14
FMAAI DNA	PREDICTED: Eucalyptus grandis glucan endo-1,3-beta-glucosidase 4 (LOC104433781), transcript variant X2, mRNA	41.1	90%	9.1

The results in Table 8 showed that TAAct primer with cDNA template or DNA template have the similarity sequence with species that coding actin. Although *Sarocalamus faberi* (bamboo china) not including to the Fabaceae family, but actually there are several species in the result which have common with TAAct cDNA sequences and dominated by Fabaceae family, one of that is *Glycine max*. So as TAAct DNA, the sequences show the highest similarity with *Glycine max*. This means that the sequences are formed resemblance to sequences of Fabaceae family though the primer used was not including that family (Poaceae family). Therefore, this primer can be used for further analysis, the gene expression analysis despite it has low identity value. The results of the AAI gene sequence by using GMAAI primer show the similarity sequences with lipid transfer proteins gene of *Rosa chinensis* with a maximum score is 89, identity 81% and E values of 2e-14 that shows the sequence has high enough homologous with that gene and the result by using FMAAI primer

show the similarity with the beta-glucosidase gene of Eucalyptus grandis with a maximum score is 41.1, identity of 90% and E values of 9.1 which indicates that sequences has quite low homologous with that gene because it has low the maximum score value and high value of E values. According to Oda (1997) and Gourinath (2000), lipid transfer proteins or LTP is one part of protein that has high conservation. One of the function of lipid transfer proteins is could be a response by pathogen attack and can inhibit the action of enzyme (enzyme inhibitor) like alpha-amylase inhibitor. The similarity of lipid transfer protein and alpha-amylase inhibitor is the domain structure that have superhelix, four helix tied by disulfide and contains cavity inside. According to Silva (2018), a recombinant of lipid transfer protein in Vigna unguiculata has a function to inhibit α-amylase in insects as well as α-amylase inhibitor. While beta-glucosidase also have the same functions with alpha amylase inhibitors that can inhibit the formation of glucose. Differences domain structure of lipid transfer protein inhibitor and an alpha amylase are shown at Figure 16.
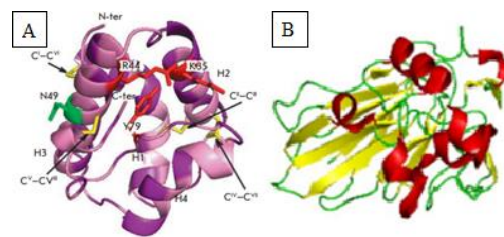

Figure 16 Domain structure A = lipid transfer protein pada *Oryza sativa*; B = alfa amylase inhibitor at kunits type (beans)

There are several parameters that must be considered in the results of homology analysis using BLAST, which are Max Score, Query Cover, E-value, and Identity. The results of homology analysis of TaACT primer with cDNA template showing the sequencing results have sufficiently high homology with the percent similarity of 87% of the Chinese bamboo actin mRNA (*Sarocalamus faberi*). The BLAST results of cDNA amplicon of TaACT primer also quite specific because it has common only to the 13 sequences that contained in the NCBI database. *Glycine max* or soy, which is one family (Fabaceae) with sengon also detected had similar BLAST results with cDNA amplicons of TaACT primer (Figure 1). From the four parameters, the most accurate is seen from the E-value produced. E-value or ecpectacy value, a level of statistical probability of an item. According Nugraha

et al. (2014), if the E-value has small value, the homology is high. In contrast, in Max score and Identity, if it have high value, the homology is high too.

The results of BLAST on TaACT-DNA amplicon indicates the low level of homology to sengon when compared with TaACT-cDNA amplicons. It can be seen from the Max score and also the value of the E-value which has quite big different. The results of BLAST hits DNA amplicons of TaACT primer can be seen from three sequences in the database that have the same sufficient homology with the sequences generated. However, as well known that TaACT primer has been designed from wheat, but it does not appear in the list of BLAST hits od DNA amplicon of TaACT primer (Figure 17).

Figure 17 BLAST analysis of TaACT-cDNA amplicon on NCBI

Figure 18 Hasil analisis BLAST amplikon TaACT-DNA pada database NCBI

Characterization of Actin and TI Genes Encoding amplicon sequences

Bases Nucleotide Sequence and homology of Actin and IT Gene Encoding

The sequencing result that have been processed by SeqTrace and SnapGene showed that the consensus sequence of ACTI, TaACT, and AcACT primers consecutive have bases size with length of 228 bp, 92 bp and 146 bp. The three amplicon sequences are homology analyzed using the BlastN tools (megablast) at NCBI. The analysis results showed that the homology of AcTI amplicon has very high homology level (score bit ≥200) against the only entry in the database which is marked in red (Figure 18). Table 9 showed the AcTI amplicon sequences have 94% similarity to the partial actin gene from *Acacia confusa*, with the E-value is very small (1e-92).
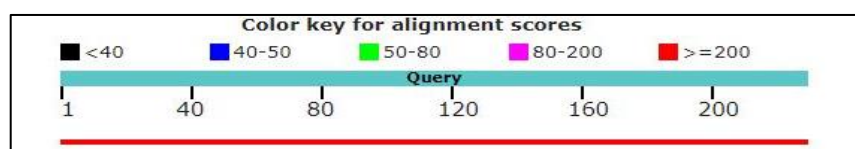
Figure 19. Homology of AcTI amplicon sequence with BLASTn

Table 9 The alignment of closest BLASTn AcTI amplicon sequence

Deskripsi	Max Score	Total Score	Query Cover	E-value	Identity	No. Aksesi
Acacia confusa trypsin inhibitor (acti) mRNA, partial cds	350	350	100%	1e-92	94%	M92852.1

BlastN analysis of TaACT amplicon sequences produce 101 entries, with high homology level that marked by the pink line (Figure 20i). The BlastN results of AcACT amplicon sequences produced the similarity with 100 entries, with very high homology level (red line) and high level (the pink line). The very high homology level (score bit ≥200) owned by thirteen top entry actin genes derived from varied organisms (Figure 20ii). According to Saputri et al. (2017), the high homology level indicated that most of the genetic material of individual that aligned contain the individual genetic material reference at database. AcTI primer used is derived from partial trypsin inhibitor gene sequences of *Acacia confusa*.

Table 10 shows the results of the closest alignment with TaACT amplicon actin genes from several organisms such as *Glycine max*, *Vigna radiata*, *Sacrolamus faberi*, *Oryza*

sativa, and so on. Identity percent or similarity values of the alignment results has a range of 85-91%. The alignment result of nine top entry indicates the same value of bit score, E-value, and similarity value, respectively is 158, 4e-35, and the similarity value of 91%. Table 11 shows that the top ten entries with the bit highest score is dominated by actin gene predictions derived from *Glycine max* and *Juglans regia*, with the similarity value in the range of 91-92%. In comparison, E-value on AcACT amplicon sequences is smaller than the TaACT amplicon sequences. According to Nugraha et al. (2014), if E-value is small, the homology will higher. In contrast, in Max score and Identity value, if the value is high, the homology level will high too. According to Narita et al. (2012), if the value of the E-value closer to zero, then the result will be more reliable. It indicates that the AcACT primer area predicted can amplify the actin gene area more specific than TaACT primer.

(i)   (ii)
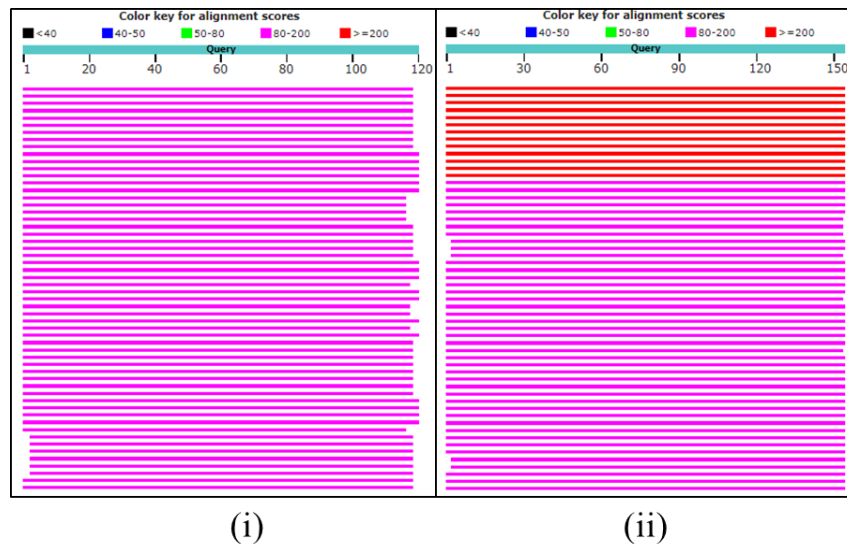
Figure 20 Homology of amplicon sequence (i) TaACT dan (ii) AcACT

Table 10 The alignment of closest BLASTn TaACT amplicon sequence

No	Deskripsi	Max Score	Total Score	Query cover	E value	Identity
1	*Glycine max* actin-101 (ACTIN), mRNA	158	158	98%	4e-35	91%
2	PREDICTED: *Vigna radiata* var, radiata actin-7 (LOC106775698), mRNA	158	158	98%	4e-35	91%

No	Deskripsi	Max Score	Total Score	Query cover	E-value	Identity
3	PREDICTED: Vigna radiata var, radiata actin-101(LOC106775699), mRNA	158	158	98%	4e-35	91%
4	Saroclamus faberi actin mRNA, complete cds	158	158	98%	4e-35	91%
5	PREDICTED: Lupinus angustifolius actin-2-like (LOC109329864), mRNA	158	158	98%	4e-35	91%
6	PREDICTED: Vigna angularis actin-101-like (LOC108341772), mRNA	158	158	98%	4e-35	91%
7	Crotalaria spectabilis, partial mRNA for actin (act gene)	158	158	98%	4e-35	91%
8	Phaseolus vulgaris clone BE3517 actin-101-like mRNA, complete cds	158	158	98%	4e-35	91%
9	Glycine max cDNA, clone: GMFL01-06C11	158	158	98%	4e-35	91%
10	PREDICTED: Oryza sativa Japonica Group actin-7 (LOC4349863), transcript variant X2, mRNA	156	156	100%	4e-34	90%

Table 11 The alignment of closest BLASTn AcACT amplicon sequence

No	Deskripsi	Max Score	Total Score	Query cover	E-value	Identity
1	PREDICTED: *Glycine mas* actin (LOC100777705), transcript variant X3, mRNA	211	211	100%	4e-51	92%

2	PREDICTED: *Glycine mas* actin (LOC100777705), transcript variant X2, mRNA	211	211	100%	4e-51	92%
3	PREDICTED: *Juglans regia* actin (LOC109016780), mRNA	211	211	100%	4e-51	92%
4	PREDICTED: Glycine mas actin (LOC100777705), transcript variant X1, mRNA	211	211	100%	4e-51	92%
5	PREDICTED: Glycine mas actin (LOC100813210), transcript variant X3, mRNA	206	206	100%	2e-49	92%
6	PREDICTED: Glycine mas actin (LOC100813210), transcript variant X1, mRNA	206	206	100%	2e-49	92%
7	PREDICTED: Juglans regia actin (LOC109011951), mRNA	206	206	100%	2e-49	92%
8	PREDICTED: Glycine mas actin (LOC100813210), transcript variant X2, mRNA	206	206	100%	2e-49	92%
9	PREDICTED: Juglans regia actin-like (LOC109011951), mRNA	206	206	100%	9e-48	91%
10	Cicer arietinum mRNA for actin, partial	200	200	100%	9e-48	91%

The homology of amino acid prediction

The homology of three amino acid amplicon sequences to the database of amino acid sequences predicted using Blastx tools at NCBI. The Blastx analysis results of the AcTI amplicon sequences show various homology levels to the trypsin inhibitor proteins from various organisms in the database. There are proteins with high homology level (pink line), medium (green line), and low (blue) (Figure 20). Table 12 shows the entry with the highest similarity is occupied by the A chain protein crystal structure of trypsin inhibitor derived from *Enterolobium contortisiliquum* (EcTI) with similarity of 95% and the E-value is 7e-43. The results also indicate the presence of a protein alignment trypsin inhibitor derived from other plants, from *Acacia confusa* and *Tamarindus indica*. In addition, the Blastx analysis also showed regional domain conserve of AcTI amplicon nucleotide sequence, which is a superfamily of Kunitz STI (Soybean Trypsin Inhibitor) (Figure 21). The alignment results which done on the research from Zhou et al. (2013) also shows the amino acid sequence EcTI have 87% similarity to sequences of trypsin inhibitors from Acacia confusa (ACTI), which is the origin of the design sequence primer in this study. E. contortisiliquum otherwise known as karo-karo is a flowering plant that comes from the same family as sengon, namely Fabaceae. (2013) also shows the amino acid sequence EcTI have 87% similarity to sequences of trypsin inhibitors from Acacia confusa (ACTI), which is the origin of the design sequence primer in this study. *E. contortisiliquum* otherwise known as karo-karo is a flowering plant that comes from the same family with sengon, namely Fabaceae.
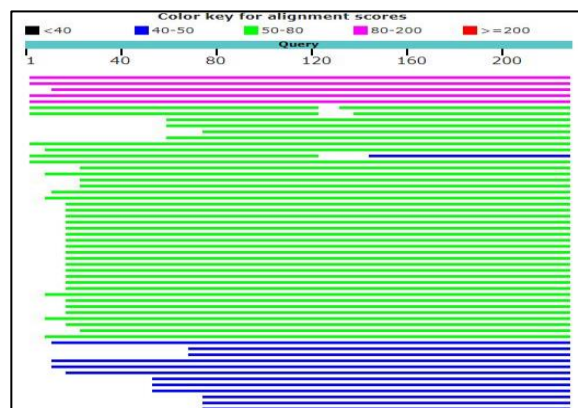
Figure 21 Homology of AcTI amplicon sequence with Blastx

Table 12 The alignment of closest AcTI amplicon sequence

No	Deskripsi	Max Score	Total Score	Query cover	E-value	Identity
1	Chain A, Crystal Structure Of A Plant Trypsin Inhibitor Ecti	145	145	98%	7e-43	95%
2	RecName: Full=Trypsin inhibitor; Contains: RecName: Full=Trypsin inhibitor chain A; Contains: RecName: Full=Trypsin inhibitor chain B; Flags: Precursor	135	135	98%	1e-38	88%
3	RecName: Full=Trypsin inhibitor; Short=EcTI; Contains: RecName: Full=Trypsin inhibitor alpha chain; Contains: RecName: Full=Trypsin inhibitor beta chain	109	109	94%	2e-28	76%
4	trypsin isoinhibitor DE5	106	106	98%	2e-27	75%
5	RecName: Full=Trypsin inhibitor; Short=LTI; Short=LlTI; AltName: Full=Kunitz-type trypsin inhibitor LlTI; Contains: RecName: Full=Trypsin inhibitor alpha chain; Contains: Kunitz-type trypsin inhibitor A chain,	103	103	98%	5e-26	66%
6	ACTI-A [Acacia confusa, seeds, Peptide, 136 aa]	78.06.00	78.06.00	52%	1e-16	93%
7	RecName: Full=Trypsin inhibitor DE5 alpha chain	67.08.00	67.08.00	52%	2e-12	88%
8	Kunitz-type trypsin inhibitor B chain, ACTI-B [Acacia confusa, seeds, Peptide, 39 aa]	59.07.00	59.07.00	42%	2e-10	88%

9	Chain A, Kuntiz Type Trypsin Inhibitor With Factor Xa Inhibitory Activity	62.00.00	62.00.00	73%	6e-10	54%
10	trypsin inhibitor [Tamarindus indica]	62.00.00	62.00.00	73%	7e-10	54%
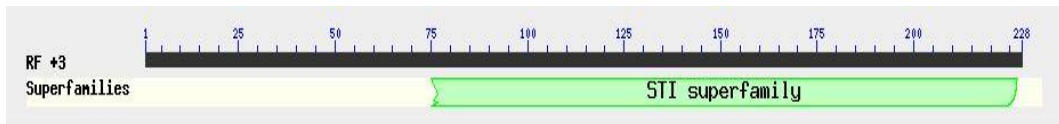
Figure 22 Prediction of conserved region AcTI amplicon sequence of Blastx

TaACT amplicon sequence homology with Blastx analysis showed high similarity level (the pink line) on the majority of the protein actin in the database. The AcACT amplicon sequences have various similarity level with the actin protein in the database, from low level (black line) to moderate level (green line) (Figure 22). Table 13 shows the ten results of the nearest alignment has similarity value of 100%, with the E-value is in the range 10^{-19} - 10^{-20} to various organisms. The highest similarity value for TaACT amplicon sequences indicated by the actin protein that derived from *Buglossoides arvensis*. Results top alignment AcACT amplicon sequences in Table 7 shows similarities to the dominant actin protein derived from non crops, such as *Scyliorhinus torazame* at the top (67% similarity; E-value 4e-10) are comes from the Chordata phylum in the Animalia kingdom.

(i)   (ii)
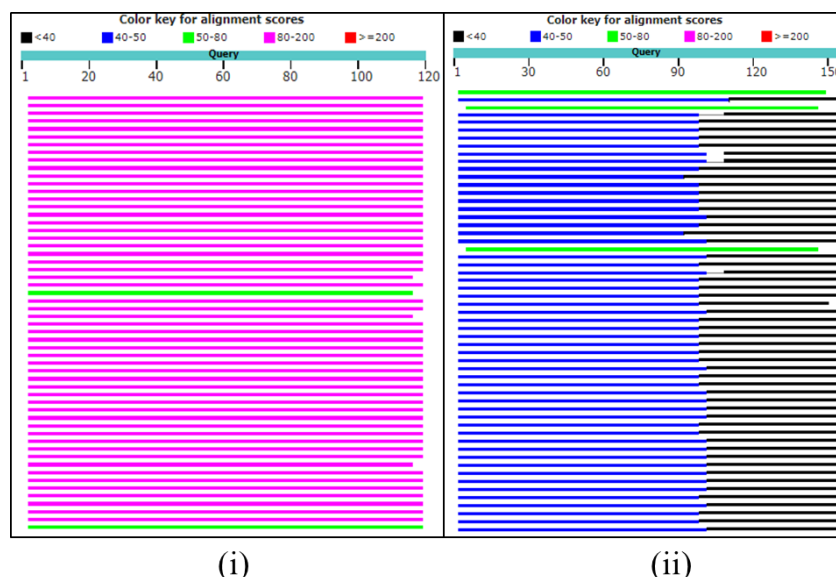
Figure 23 Homology of amplicon sequence i) TaACT dan ii) AcACT dengan Blastx

Table 13 The alignment of closest TaACT amplicon sequence

No	Deskripsi	Max Score	Total Score	Query cover	E-value	Identity
1	Alpha-actin (*Buglossoides arvensis*)	82.0	82.0	97%	8e-20	100%
2	Actin (*Tinca tinca*)	82.8	82.8	97%	9e-20	100%
3	Actin (*Cucurbita pepo*)	82.4	82.4	97%	1e-19	100%
4	Actin (*Lotus corniculatus*)	82.8	82.8	97%	1e-19	100%
5	actin, cystoplasmic 2 (*Acinetobacter baumannii*)	82.4	82.4	97%	1e-19	100%
6	Alpha-actin (*Pterophyllum scalare*)	82.4	82.4	97%	1e-19	100%
7	RecName: Full=Actin	82.4	82.4	97%	2e-19	100%
8	Actin (*Elaeis gineensis*)	82.4	82.4	97%	2e-19	100%
9	Actin (*Brassica rapa* subsp. Pekinensis)	82.4	82.4	97%	2e-19	100%
10	Alpha-actin 2 (*Xiphias gladius*)	82.0	82.0	97%	2e-19	100%

Table 14 The alignment of closest AcACT amplicon sequence

No	Deskripsi	Max Score	Total Score	Query cover	E value	Identity
1	Actin (*Scyliorhinus torazame*)	60.1	60.1	95%	4e-10	67%
2	Actin, aortic smooth muscle-like (*Scleropages formosus*)	48.5	84.7	98%	6e-08	61%
3	Actin-7 (*Zea mays*)	56.2	56.2	91%	9e-08	60%
4	Actin-100-like (*Durio zibethinus*)	47.8	83.6	91%	1e-07	72%
5	RecName: Full=Actin-1	47.0	83.2	98%	1e-07	72%

6	Actin and related proteins (*Klebsormidium nitens*)	47.4	82.8	98%	2e-07	69%
7	Actin-2-like isoform X3 (*Mizuhopecten yessoensis*)	46.6	82.8	98%	2e-07	69%
8	Unnamed protein product (*Homo sapiens*)	46.6	82.4	98%	2e-07	69%
9	Actin (*Mucor moelleri*)	47.0	82.4	93%	3e-07	67%
10	ACTG1 (synthetic construct)	46.6	82.4	93%	3e-07	67%

The Blastx analysis result also shows a prediction domain conserved region in the sequence of TaACT and AcACT amplicons when translated into amino acid sequences, namely Nucleotide Binding Domain (NBD) in the actin superfamily, sugar kinases, and Hsp70 protein and actin superfamily (Figure 24). The value of E-value from TaACT amplicon sequences to the NBD region and actin superfamily and is 7.92e-12 8.68e-13, while the high E-value of AcACT amplicon sequences obtained in the two regions superfamily is 0E + 00.

(i)

(ii)
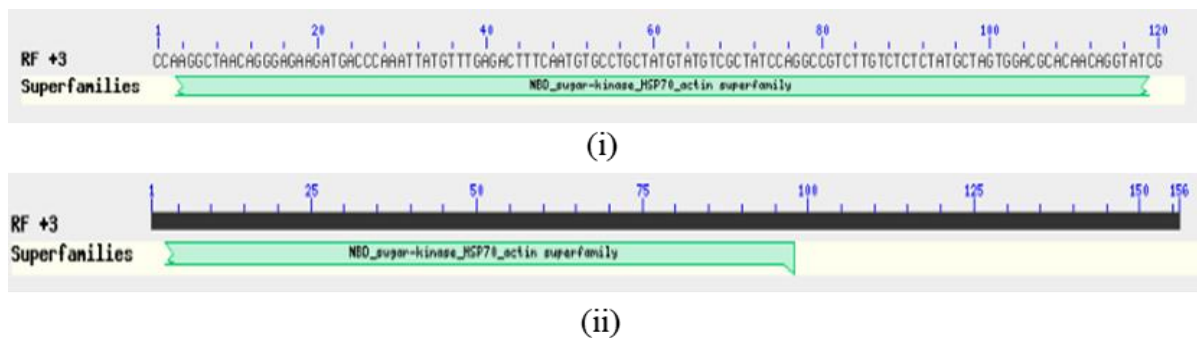
Figure 24 Prediction of conserved region amplicon sequence i)TaACT dan ii)AcACT

4. 5. 2 Sequence and Homology Analysis of Agarwood

Agarwood PCR products were sequenced to confirm its results. Table 15 showed the sequence edited results.

Table 15 DNA and cDNA sequences of HMGR and Actin genes from *A. malaccensis* and *G. versteegii* that aligned with *A. sinensis* sequence for HMGR and *A. microcarpa* for Actin

Gene	Sequence	Sequence Position
HMGR *Aquilaria* *malaccensis* 1743 bp	ATGGAGAGAGACACTACCATCTCAGCCCCCTCAA ACTTTTCCAAACAATTGGCGCTAGACGAAGGATCC ACTAAAGCTTCCGATGCCTTGCCGCTTCCCTTGCA CGTCACAAACACAGTCTCCTTCACGCTGCTGTTCT GGGTCGTCTATTTTCTGCTCACCCGGTGGCGGGAG AAGATCCGGAACTCCACCCCTCTCCATATGATCAG CTTCTCCGAGATGCTCGCCATTTTCGCCCTCTTAGT TTCCTTAATCTATCTCTTGGGCTTTTTCGGGATCGA ATTCGTCCAGTCTATCATCCGCCCTTCTTCAGACAT GTGGGCCGATGATGGAGAGGAGGATGACATCGCA CTGCAAAAGGAGGACGCCCGGAAAGTTCCTTGTG GACAGGC**GCTTGATTGCTCTCTTCCCGCT**ᵃ**CCTG TTGCTG**C**TCCCTCTCCCGAAACAGAAAAATTGC CCACCCCGA**A**GGTGTCCGATGAAATGCCTGATC CAGTTACAACGGAGGAAGACGAGGAGATCATT AAGGG**TGTTGTGGCTGGGACGACTC**ᵇCCTCTTAC TCTCTTGAGGCGAAACTGGGCAACTGCAAGAGGG CGGCTGCTATCAGGCGCGAGGCCTTGCAGAGGTT GACTGGGAAGTCCTTGTCTGGGCTTCCATTGGAGG GTTTCGATTACGAGTCCATACTGGGGCAGTGCTGC GAAATGCCTGTTGGGTACGTGCAGATCCCGGTGG GAATTGCTGGCCCGCTTTTCCTTATGGGAGAGAGT ATTCTGTGCCCAATGGCGACGACAGAGGGATGCTT GGTGGCCAGCACAAACAGAGGTTGCAAGGCCATT AACTTATCCGGTGGCGCCACCAGCGTTCTATTGAG AGATGGGATGACCAGAGCCCCTGTTGTGAGGTTTG GCACCGCGAAAAGGGCTGCTGATTTGAAGCAGTA CTTGGAGGATCCCATGAACTTCGAGACCGTGTCTG CTGCCTTCAACAAGTCCAGCAGATTTGGCAGGCTT CAGAGCATCAAATGCGCCATTGCTGGGAAGAATC TCTACGTGAGATTCTCTTGTAGCACTGGTGATGCC ATGGGGATGAACATGGTTTCTAAAGGAGTTCAGA ACGTGTTGGATTTCCTGCAGCACGATTTCCCTGAC ATGGATGTCCTTGGAATTTCTGGCAACTATTGTTC TGACAAGAAGCCCGCTGCTGTCAACTGGATCGAA GGACGTGGCAAGTCTGTTGTTTGCGAAGCCATCAT TAAGGGTGACGTGGTGAGGAAGGTTTTGAAGACT AATGTTGCGTCTTTGGTTGAACTTAACATGCTCAA GAACCTTACTGGTTCCGCCATGGCTGGGGCTCTTG GTGGCTTCAATGCCCATGCCAGTAACATCGTGAGT GCAGTCTACATTGCCACCGGCCAAGATCCTGCTCA AAACATAGAGAGTTCTCACTGCATCACAATGATG GAGGCCATCAATGATGGACAGGACCTCCACGTCT CTGTGACTATGCCTTCCATCGAGGTTGGAACTGTT	414-521

36

Gene	Sequence	Sequence Position
	GGTGGCGGAACACAGCTAGCATCTCAGTCGGCAT GTTTGAACCTGCTTGGTGTGAAGGGTGCAGACAA AGAGTCTCCTGGTTCGAACGCAAGGCTGTTGGCCA GGGTTGTTGCCGGTGCCGTTCTTGCTGGAGAGCTC TCACTCATGTCTGCTATTTCAGCAGGACAGCTTGT AAAGAGTCATATGAAGTACAACAGATCCAACAAG GATGCTGCCAAGGCTTCTCCATGA	
HMGR *Gyrinops versteegii* 1743 bp	ATGGAGAGAGACACTACCATCTCAGCCCCCTCAA ACTTTTCCAAACAATTGGCGCTAGACGAAGGATCC ACTAAAGCTTCCGATGCCTTGCCGCTTCCCTTGCA CGTCACAAACACAGTCTCCTTCACGCTGCTGTTCT GGGTCGTCTATTTTCTGCTCACCCGGTGGCGGGAG AAGATCCGGAACTCCACCCCTCTCCATATGATCAG CTTCTCCGAGATGCTCGCCATTTTCGCCCTCTTAGT TTCCTTAATCTATCTCTTGGGCTTTTTCGGGATCGA ATTCGTCCAGTCTATCATCCGCCCTTCTTCAGACAT GTGGGCCGATGATGGAGAGGAGGATGACATCGCA CTGCAAAAGGAGGACGCCCGGAAAGTTCCTTGTG GACAGGC**GCTTGATTGCTCTCTTCCCGCT**[a]**CCTG TTGCTG**C**TCCCTCTCCC**G**AA**A**CAGAAAAATTG**C CC**A**CCCCGAAGGTGTCCGATGAAATGCCTGATC CAGTT**A**CAACGGAGGAAGACGAGGAGATCATT AAGGG**TGTTGTGGCTGGGACGACTC**[b]CCTCTTAC TCTCTTGAGGCGAAACTGGGCAACTGCAAGAGGG CGGCTGCTATCAGGCGCGAGGCCTTGCAGAGGTT GACTGGGAAGTCCTTGTCTGGGCTTCCATTGGAGG GTTTCGATTACGAGTCCATACTGGGGCAGTGCTGC GAAATGCCTGTTGGGTACGTGCAGATCCCGGTGG GAATTGCTGGCCCGCTTTTCCTTATGGGAGAGAGT ATTCTGTGCCCAATGGCGACGACAGAGGGATGCTT GGTGGCCAGCACAAACAGAGGTTGCAAGGCCATT AACTTATCCGGTGGCGCCACCAGCGTTCTATTGAG AGATGGGATGACCAGAGCCCCTGTTGTGAGGTTTG GCACCGCGAAAAGGGCTGCTGATTTGAAGCAGTA CTTGGAGGATCCCATGAACTTCGAGACCGTGTCTG CTGCCTTCAACAAGTCCAGCAGATTTGGCAGGCTT CAGAGCATCAAATGCGCCATTGCTGGGAAGAATC TCTACGTGAGATTCTCTTGTAGCACTGGTGATGCC ATGGGGATGAACATGGTTTCTAAAGGAGTTCAGA ACGTGTTGGATTTCCTGCAGCACGATTTCCCTGAC ATGGATGTCCTTGGAATTTCTGGCAACTATTGTTC TGACAAGAAGCCCGCTGCTGTCAACTGGATCGAA GGACGTGGCAAGTCTGTTGTTTGCGAAGCCATCAT TAAGGGTGACGTGGTGAGGAAGGTTTTGAAGACT AATGTTGCGTCTTTGGTTGAACTTAACATGCTCAA GAACCTTACTGGTTCCGCCATGGCTGGGGCTCTTG GTGGCTTCAATGCCCATGCCAGTAACATCGTGAGT GCAGTCTACATTGCCACCGGCCAAGATCCTGCTCA	414-521

Gene	Sequence	Sequence Position
	AAACATAGAGAGTTCTCACTGCATCACAATGATG GAGGCCATCAATGATGGACAGGACCTCCACGTCT CTGTGACTATGCCTTCCATCGAGGTTGGAACTGTT GGTGGCGGAACACAGCTAGCATCTCAGTCGGCAT GTTTGAACCTGCTTGGTGTGAAGGGTGCAGACAA AGAGTCTCCTGGTTCGAACGCAAGGCTGTTGGCCA GGGTTGTTGCCGGTGCCGTTCTTGCTGGAGAGCTC TCACTCATGTCTGCTATTTCAGCAGGACAGCTTGT AAAGAGTCATATGAAGTACAACAGATCCAACAAG GATGCTGCCAAGGCTTCTCCATGA	
HMGR *Aquilaria malaccensis* cDNA 1743 bp	ATGGAGAGAGACACTACCATCTCAGCCCCCTCAA ACTTTTCCAAACAATTGGCGCTAGACGAAGGATCC ACTAAAGCTTCCGATGCCTTGCCGCTTCCCTTGCA CGTCACAAACACAGTCTCCTTCACGCTGCTGTTCT GGGTCGTCTATTTTCTGCTCACCCGGTGGCGGGAG AAGATCCGGAACTCCACCCCTCTCCATATGATCAG CTTCTCCGAGATGCTCGCCATTTTCGCCCTCTTAGT TTCCTTAATCTATCTCTTGGGCTTTTTCGGGATCGA ATTCGTCCAGTCTATCATCCGCCCTTCTTCAGACAT GTGGGCCGATGATGGAGAGGAGGATGACATCGCA CTGCAAAAGGAGGACGCCCGGAAAGTTCCTTGTG GACAGGC**GCTTGATTGCTCTCTTCCCGCT**[a]**CCTG TTGCTG**C**TCCCTCTCCCGAAACAGAAAAATTG**C **CCACCCCGAAGGTGTCCGATGAAATGC**CTGATC CAGTTACAACGGAGGAAGACGAGGAGATCATTAA GGG**TGTTGTGGCTGGGACGACTC**[b]CCTCTTACTC TCTTGAGGCGAAACTGGGCAACTGCAAGAGGGCG GCTGCTATCAGGCGCGAGGCCTTGCAGAGGTTGA CTGGGAAGTCCTTGTCTGGGCTTCCATTGGAGGGT TTCGATTACGAGTCCATACTGGGGCAGTGCTGCGA AATGCCTGTTGGGTACGTGCAGATCCCGGTGGGA ATTGCTGGCCCGCTTTTCCTTATGGGAGAGAGTAT TCTGTGCCCAATGGCGACGACAGAGGGATGCTTG GTGGCCAGCACAAACAGAGGTTGCAAGGCCATTA ACTTATCCGGTGGCGCCACCAGCGTTCTATTGAGA GATGGGATGACCAGAGCCCCTGTTGTGAGGTTTGG CACCGCGAAAAGGGCTGCTGATTTGAAGCAGTAC TTGGAGGATCCCATGAACTTCGAGACCGTGTCTGC TGCCTTCAACAAGTCCAGCAGATTTGGCAGGCTTC AGAGCATCAAATGCGCCATTGCTGGGAAGAATCT CTACGTGAGATTCTCTTGTAGCACTGGTGATGCCA TGGGGATGAACATGGTTTCTAAAGGAGTTCAGAA CGTGTTGGATTTCCTGCAGCACGATTTCCCTGACA TGGATGTCCTTGGAATTTCTGGCAACTATTGTTCT GACAAGAAGCCCGCTGCTGTCAACTGGATCGAAG GACGTGGCAAGTCTGTTGTTTGCGAAGCCATCATT AAGGGTGACGTGGTGAGGAAGGTTTTGAAGACTA ATGTTGCGTCTTTGGTTGAACTTAACATGCTCAAG	414-521

Gene	Sequence	Sequence Position
	AACCTTACTGGTTCCGCCATGGCTGGGGCTCTTGG TGGCTTCAATGCCCATGCCAGTAACATCGTGAGTG CAGTCTACATTGCCACCGGCCAAGATCCTGCTCAA AACATAGAGAGTTCTCACTGCATCACAATGATGG AGGCCATCAATGATGGACAGGACCTCCACGTCTCT GTGACTATGCCTTCCATCGAGGTTGGAACTGTTGG TGGCGGAACACAGCTAGCATCTCAGTCGGCATGTT TGAACCTGCTTGGTGTGAAGGGTGCAGACAAAGA GTCTCCTGGTTCGAACGCAAGGCTGTTGGCCAGGG TTGTTGCCGGTGCCGTTCTTGCTGGAGAGCTCTCA CTCATGTCTGCTATTTCAGCAGGACAGCTTGTAAA GAGTCATATGAAGTACAACAGATCCAACAAGGAT GCTGCCAAGGCTTCTCCATGA	
HMGR *Gyrinops versteegii* cDNA 1743 bp	ATGGAGAGAGACACTACCATCTCAGCCCCCTCAA ACTTTTCCAAACAATTGGCGCTAGACGAAGGATCC ACTAAAGCTTCCGATGCCTTGCCGCTTCCCTTGCA CGTCACAAACACAGTCTCCTTCACGCTGCTGTTCT GGGTCGTCTATTTTCTGCTCACCCGGTGGCGGGAG AAGATCCGGAACTCCACCCCTCTCCATATGATCAG CTTCTCCGAGATGCTCGCCATTTTCGCCCTCTTAGT TTCCTTAATCTATCTCTTGGGCTTTTTCGGGATCGA ATTCGTCCAGTCTATCATCCGCCCTTCTTCAGACAT GTGGGCCGATGATGGAGAGGAGGATGACATCGCA CTGCAAAAGGAGGACGCCCGGAAAGTTCCTTGTG GACAGGC**GCTTGATTGCTCTCTTCCCGCT**[a]**CCTG TTGCTG**C**TCCCTCTCCCGAA**A**CAGAAAAATTGC CC**A**CCCCGAAGGTGTCCGATGAAATGCCTGATC CAGTTACAACGGAGGAAGACGAGGAGATCATT AAGGG**TGTTGTGGCTGGGACGACTC**[b]CCTCTTAC TCTCTTGAGGCGAAACTGGGCAACTGCAAGAGGG CGGCTGCTATCAGGCGCGAGGCCTTGCAGAGGTT GACTGGGAAGTCCTTGTCTGGGCTTCCATTGGAGG GTTTCGATTACGAGTCCATACTGGGGCAGTGCTGC GAAATGCCTGTTGGGTACGTGCAGATCCCGGTGG GAATTGCTGGCCCGCTTTTCCTTATGGGAGAGAGT ATTCTGTGCCCAATGGCGACGACAGAGGGATGCTT GGTGGCCAGCACAAACAGAGGTTGCAAGGCCATT AACTTATCCGGTGGCGCCACCAGCGTTCTATTGAG AGATGGGATGACCAGAGCCCCTGTTGTGAGGTTTG GCACCGCGAAAAGGGCTGCTGATTTGAAGCAGTA CTTGGAGGATCCCATGAACTTCGAGACCGTGTCTG CTGCCTTCAACAAGTCCAGCAGATTTGGCAGGCTT CAGAGCATCAAATGCGCCATTGCTGGGAAGAATC TCTACGTGAGATTCTCTTGTAGCACTGGTGATGCC ATGGGGATGAACATGGTTTCTAAAGGAGTTCAGA ACGTGTTGGATTTCCTGCAGCACGATTTCCCTGAC ATGGATGTCCTTGGAATTTCTGGCAACTATTGTTC TGACAAGAAGCCCGCTGCTGTCAACTGGATCGAA	414-521

Gene	Sequence	Sequence Position
	GGACGTGGCAAGTCTGTTGTTTGCGAAGCCATCAT TAAGGGTGACGTGGTGAGGAAGGTTTTGAAGACT AATGTTGCGTCTTTGGTTGAACTTAACATGCTCAA GAACCTTACTGGTTCCGCCATGGCTGGGGCTCTTG GTGGCTTCAATGCCCATGCCAGTAACATCGTGAGT GCAGTCTACATTGCCACCGGCCAAGATCCTGCTCA AAACATAGAGAGTTCTCACTGCATCACAATGATG GAGGCCATCAATGATGGACAGGACCTCCACGTCT CTGTGACTATGCCTTCCATCGAGGTTGGAACTGTT GGTGGCGGAACACAGCTAGCATCTCAGTCGGCAT GTTTGAACCTGCTTGGTGTGAAGGGTGCAGACAA AGAGTCTCCTGGTTCGAACGCAAGGCTGTTGGCCA GGGTTGTTGCCGGTGCCGTTCTTGCTGGAGAGCTC TCACTCATGTCTGCTATTTCAGCAGGACAGCTTGT AAAGAGTCATATGAAGTACAACAGATCCAACAAG GATGCTGCCAAGGCTTCTCCATGA	
Aktin *Aquilaria* *malaccensis* 632 bp	ATGGAGAAGATTTGGCACCATACTTTCTACAATGA ACTTCGTGTTGCCCCTGAAGAGCATCCAGTTCTTC TGACTGAAGCACCTCTCAACCCTAAAGCTAACAG GGAGAAAATGACCCAAATAATGTTTGAAACTTTC AATGTGCCTGCAATGTA**TGTTGCCATACAGGCTG** **TTC**[c]**TTTCACTCTATGCCAGTGGTCGTA**CGACAG GT**ATTGTTTTGGATTCTGGTGATGGTGTGAGCC** **ACACTGTACCTATCTATGAAGGCTATGCCCTCC** **CGCATGCCATTCTTAG**A**TTGGATCTTGCC**GGTC **GTGACCTTACTGATGCCCTGATGAAGATCTTGA** **CTGAGAG**A**GGGTACTCCTTCACCACTACT**GCAG AGCGGGAAATTGTTAG[d]GGACATCAAAGAGAAG CTTGCTTATGTTGCTCTTGATTATGAACAAGAGCT TGAGACTGCCAAAAGTAGCTCTGCTATCGAAAAG AGCTATGAGCTGCCTGATGGGCAAGTAATCACAA TTGGAGCAGAAAGGTTCCGATGCCCTGAAGTTCTC TTCCAACCATCCTTGATAGGAATGGAAGCTGGTGG AATCCACGAGACAACGTACAACTCCATCATGAAG TGTGATGCCATATTTTCTCCATA	175-366
Aktin *Gyrinops* *versteegii* 632 bp	ATGGAGAAGATTTGGCACCATACTTTCTACAATGA ACTTCGTGTTGCCCCTGAAGAGCATCCAGTTCTTC TGACTGAAGCACCTCTCAACCCTAAAGCTAACAG GGAGAAAATGACCCAAATAATGTTTGAAACTTTC AATGTGCCTGCAATGTA**TGTTGCCATACAGGCTG** **TTC**[c]**TTTCACTCTATGCCAGTGGTCGTAC**GACAG GT**ATTGTTTTGGATTCTGGTGATGGTGTGAGCC** **ACACTGTACCTATCTATGAAGGCTATGCCCTCC** **CGCATGCCATTCTTAG**A**TTGGATCTTGCC**GGTC **GTGACCTTACTGATGCCCTGATGAAGATCTTGA** **CTGAGAG**A**GGGTACTCC**TTCACCACTACT**GCAG** **AGCGGGAAATTGTTAG**[d]GGACATCAAAGAGAAG CTTGCTTATGTTGCTCTTGATTATGAACAAGAGCT TGAGACTGCCAAAAGTAGCTCTGCTATCGAAAAG AGCTATGAGCTGCCTGATGGGCAAGTAATCACAA	175-366

Gene	Sequence	Sequence Position
Aktin *Aquilaria* *malaccensis* cDNA 632 bp	TTGGAGCAGAAAGGTTCCGATGCCCTGAAGTTCTC TTCCAACCATCCTTGATAGGAATGGAAGCTGGTGG AATCCACGAGACAACGTACAACTCCATCATGAAG TGTGATGCCATATTTTCTCCATA ATGGAGAAGATTTGGCACCATACTTTCTACAATGA ACTTCGTGTTGCCCCTGAAGAGCATCCAGTTCTTC TGACTGAAGCACCTCTCAACCCTAAAGCTAACAG GGAGAAAATGACCCAAATAATGTTTGAAACTTTC AATGTGCCTGCAATGTA**TGTTGCCATACAGGCTG TTC**ᶜTTTCACTCTATGCCAGTGGTCGTACGACAGGT ATTGTTTTGGATT**CTGG**T**GATGG**TG**TGAGCCACA CTGTACCTATCTATGAAGGCTATGCCCTCCCGC ATGCCATTCTTAG**ATTGGATCTTGCC**GGTCGTG ACCTTACTGATGCCCTGATGAAGATCTTGACTG AGAG**AGGGTACTCCTTCACCACTACT**GCAGAGC GGGAAATTGTTAG**ᵈGGACATCAAAGAGAAGCTTG CTTATGTTGCTCTTGATTATGAACAAGAGCTTGAG ACTGCCAAAAGTAGCTCTGCTATCGAAAAGAGCT ATGAGCTGCCTGATGGGCAAGTAATCACAATTGG AGCAGAAAGGTTCCGATGCCCTGAAGTTCTCTTCC AACCATCCTTGATAGGAATGGAAGCTGGTGGAAT CCACGAGACAACGTACAACTCCATCATGAAGTGT GATGCCATATTTTCTCCATA	175-366

Note:

a = primer Forward of HMGR

b = primer Reverse of HMGR

c = primer Forward of actin

d = primer Reverse of actin

After the editing process, the sequence was then verified into gene bank on NCBI website. Basic Local Alignment Search Tools (BLAST) with BLAST nucleotide (*blastn*) program showed candidate of encoding gene of HMGR2 and Act-1 detected from *A. sinensis* and *A. microcarpa*, the information showed in Table 16. Parameters used in this research included query cover, identity, dan E-value. Query cover showed the percentage of nucleotide sequence length that aligned with nucleotide sequence provided on gene bank, NCBI website (Aqmarina 2018).

Based on Table 16, Query cover value of HMGR gene for *A. malaccensis* and *G. versteegii* was 100 %. Meanwhile the Actin gene value was 53% for *A. malaccensis* and 64 % for *G vertigii*. The same thing was shown in Aqmarina's research (2018) that showed the Query cover of *A. malaccensis* was greater than *G. versteegii*. It might cause by the product size sequencing result was not same as the primary designed. Miller *et al*. (1990) stated the Identity values describe percentage of Query cover samples that were appropriate with the gene bank database. The Identity value was quite high, i.e. 95-98 %. The last parameter was

E-value. Claverie dan Notredame (2007) stated that E-values describe the homology sequence with data gene bank database. The homology will be greater as it closer to zero. The actin gene from *A. malaccensis* had the smallest E-value was 2e-42. Therefore the greatest E-value was HMGR gene from *A. malaccensis* as much as 1e-78. The identification of HMGR gene was done by using cDNA samples. Based on BLAST results, HMGR gene from cDNA samples were identified in *A. sinensis* on gene bank with 97% of Query cover, 98% of Identity and 2e-64 E-value for *G. versteegii*. Therefore *A. malaccensis* had 66% of Query cover, 97% of Identity and 3e-32 of E-value. Query cover in *G. versteegii* cDNA was greater due to the peak quality resulted from sequence was better than *A. malaccesnsis*.

Table 16. BLAST results of HMGR2 and Act-1 sequences from agarwood

Sample	BLAST Results	Query Cover (%)	Identity (%)	E-value	No. Aksesi
A.malaccensis HMGR (DNA)	*A. sinensis* HMGR2 mRNA, complete cds	100	95	2e-42	KC140287.1
G.versteegii HMGR (DNA)	*A. sinensis* HMGR2 mRNA, complete cds	100	98	1e-50	KC140287.1
A.malaccensis Aktin (DNA)	*A. microcarpa* putative actin 1 (Act-1) mRNA, partial cds	53	98	2e-78	GU128950.1
G.versteegii Aktin (DNA)	*A. microcarpa* putative actin 1 (Act-1) mRNA, partial cds	64	98	3e-76	GU128950.1
A.malaccensis HMGR (cDNA)	*A. sinensis* HMGR2 mRNA, complete cds	66	97	3e-32	KC140287.1
G.versteegii HMGR (cDNA)	*A. sinensis* HMGR2 mRNA, complete cds	97	98	2e-64	KC140287.1
A.malaccensis Aktin (cDNA)	*A. microcarpa* putative actin 1 (Act-1) mRNA, partial cds	75	92	2e-61	GU128950.1

Table 17. BLAST results of Guaiene sequence from agarwood DNA

Sample	Hasil BLAST	Query Cover (%)	Identity (%)	E-value
A.malaccensis (guaiene)	*A.crassna Delta guaiene synthase gene complete*	79	100	1e-22
G.versteegii (guaiene)	*A.crassna Delta guaiene syntesis gen complite*	76	100	9e-24

BLAST results in *A. malaccensis* and *G. versteegii* showed similarities with *A. crassna*. The parameters used in sequencing analysis were *query cover, identity,* and *e-value*. Query cover showed the suitability percentage between the nucleotide length of the sample and database (Wehantouw 2017). The similarity of it will be increase as the increasing number of Query cover. The low quality of isolated DNA might affected the low number of query cover, it was also happen on Aqmarina's research (2018). The other parameter was Identity, it showed the percentage of similarity between sequence sample and aligned sequence (Miller *et al.* 1990). The high value of Identity showed the closest similarity with database. The Identity of sequence results ranged between 98-100%, which showed the primer which carried encoding sequence had 98-100% similarity. The third parameter was e-value, it was a homology between DNA sequence and database. The low E-value indicated that the sample has a high homology of the sequences found in Gene Bank (Aqmarina 2018). According to Claveri *et al.* (2003) in Budiani *et al.* (2009) E-value ≤ e-04 indicated a high level of similarity. *Aqualira malaccensis* and *Gyrinops versteegii* in guaiene primer had a low e-value, it indicated that the four samples were identical to the sequence in the Gene Bank. It also indicated that the primer used in both types of samples was sufficient to be able to detect the presence of expected encoding gene.

Amplified cDNA then was sequenced, the result was showed in Table 18. The samples used were *A.malaccensis* dan *G.versteegii* with Am3-δG primer resulted from PCR optimization.

Table 18 BLAST results of Guaiene sequence from agarwood cDNA

Sample	BLAST Result	Query Cover (%)	Identity (%)	E-value
A.malaccensis (guaiene)	*A.crassna* strain n6 delta-guaiene synthase 1 pseudogene, partial sequence	87	99	6e-27
G.versteegii (guaiene)	*A.microcarpa* isolate deltaGS4Delta20 delta-guaiene	85	100	2e-28

The sequencing results in Table 18 showed *A.malaccensis* sample had a high similarity with *A.crassna* strain n6 delta-guaiene synthase 1 pseudogene, partial sequence with an identity value of 99%, cover query 87, and E-value 6e-27. E-value had a lower value than ≤ e-04 and indicated it had the similarity. A high identity value of 99% showed

a similarity between *A.malaccensis* and database was 99%. The similarity of *A. malaccensis* with *A. chassna* delta-guaiene synthase showed that Am3-δG primer could detected genes contained in the isolated cDNA of *A.malaccensis*.

Sequence of *G.versteegii* showed the high similarity with *A.microcarpa* isolate deltaGS4Delta20 delta-guaiene synthase mRNA, partial cds with identity of 100%, query cover of 85%, and *E-value* of 2e-28. The similarity of *G.versteegii* and *A.microcarpa* delta-guaiene synthase mRNA showed the primer used can detect guaiene gene that contained in *G.versteegii*. The high parameter value such as identity showed that the primer can sort the similarity of sequence as 100% with database. The e-value which was equal to 2e-28 pr lower than e-04 also showed a high specification for the primer detecting guaiene genes.

4. 6. Optimization of *Real-Time* PCR

Optimization of qPCR conditions is important to obtain a test technique that has high sensitivity and specificity. The low reproducibility and precision values between inefficient test replications is an indication of bad optimization. In some initial experiments, cDNA amplification with TaACT primer on qPCR machine showed that two peaks were formed on the melt-curve and there was amplification on the NTC (non template) amplification curve. NTC is a control tube that does not contains cDNA template. The amplification on the tube indicates the presence of contaminants, both from outside contaminants which are technical errors or contaminants by the presence of non-target products. That contaminants can be seen from the melt curve formed. Double peak that formed showed that the amplification of two products during PCR process takes place. The results of confirmation of qPCR products with 2% agarosa electrophoresis showed that the dimer in all samples (Figure 25). Therefore, the further optimization aims to enhance sensitifity of target products and eliminate non target products. The optimization performed include the optimization of primers concentration and cDNA template concentration.

Figure 25 The confirmation of the qPCR results of cDNA template sengon SS1 and ST1 with two replicates.

One of the causes of the occurence of primer dimer was the high concentration of primer that were used. The previous experiment used primer concentration in accordane with the protocol, which was 0,4 µM. Furthermore, the amplification performed using a primer with a concentration 0,1 µM and 0,2 µM. The results of amplification was double peak at both primer concentration used, either in the first or the second replicates (Figure 25). The peak that formed in each amplification have values of Temperature melting (Tm) at 75 ^{0}C and 80 ^{0}C. This indicated that the primer concentration used still produces the not spesific product, where should the target product has a size of 120 bp. The presence of double peak in the melt curve can eventually affect the reading of Ct on real time PCR, so the interpretation of the data will be disrupted. The amplicon of qPCR product was also validated with 2% agarosa, where the most of the amplicon that there is a dimer which measures around 50 bp (Figure 26).
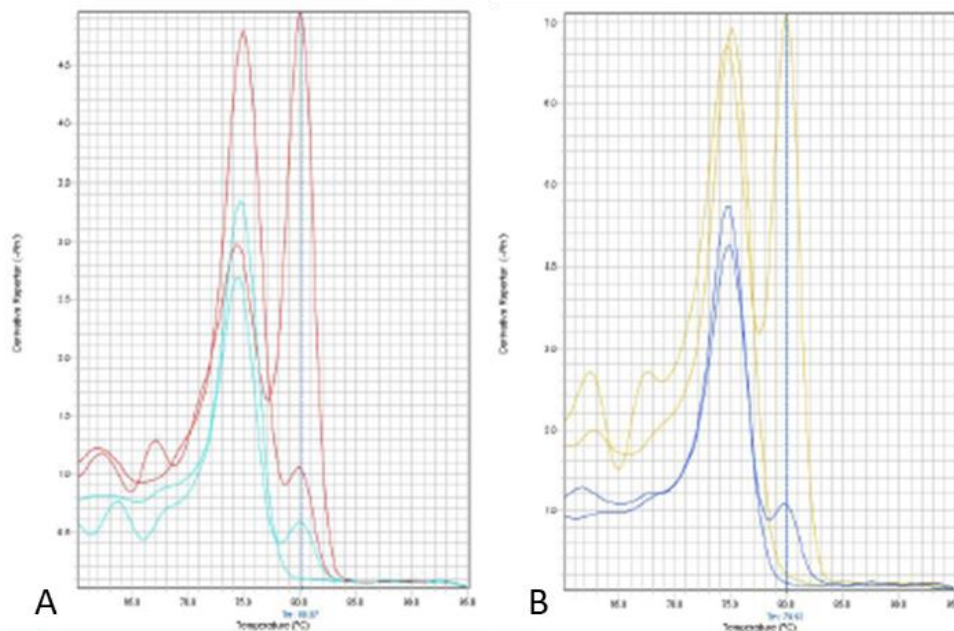
Ficture 26 The m*elt-curve* of SS1 sample amplification on TI gene with a concentration A) 0,1 µM and B) 0,2 µM
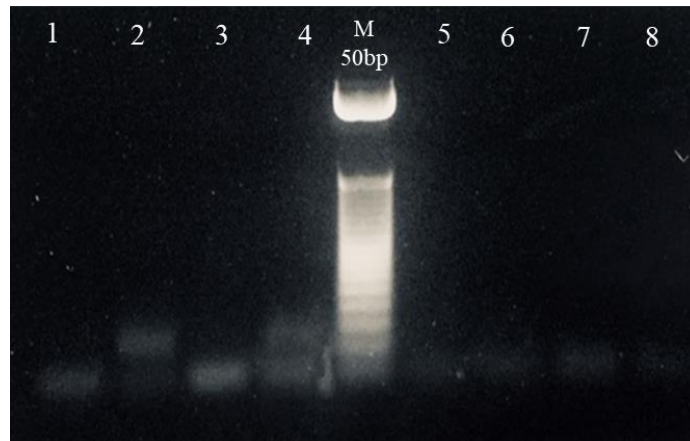
Figure 27 The results of electrophoresis of qPCR product on optimization of primer concentration TI, 1-2: 0,1 µM; 3-4: NTC 0,1 µM; 5-6: 0,2 µM; 7-8: NTC 0,2 µM

Optimization of qPCR amplification was also performed on cDNA template concentration. Experiments conducted with template dilution to a concentration to 50 ng / mL and 100 ng / mL at the four cDNA samples (SSI, SS2, ST1, and ST2). It is seen that the melt-curve of the fourth sample has a single peak that is at Tm 75 0C, except SS1 peak at second repeat (Figure 28). However, validation using 2% agarose electrophoresis showed that the four samples, both with a concentration of 50 ng / mL or 100 ng / mL indicate that the amplicon formed a primer dimers (Figure 27). In addition, at the NTC wells also contained the presence of a primer dimer. Primer dimer itself is usually a product with a Tm of 75 ᵒC. This indicates that the template dilution has no effect on the product formed, where the product is not target product, but a primer dimer.
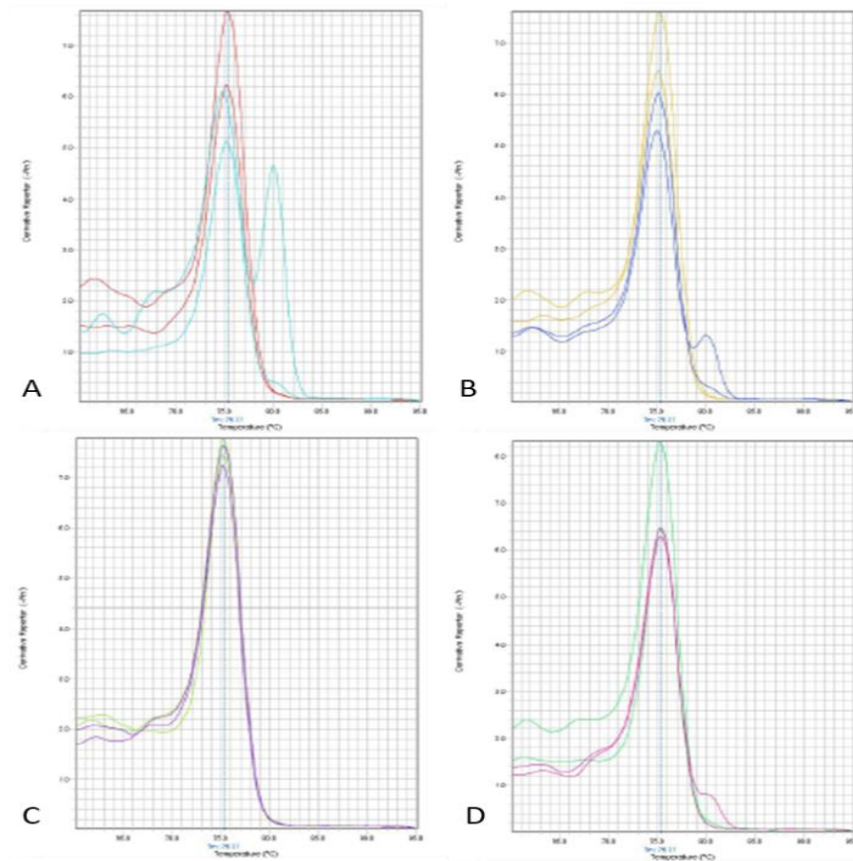
Figure 28 Melt curve with cDNA concentration of 50 ng / mL and 100 ng / mL in sample
A) SS1, B) SS2, C) ST1, and D) ST2.
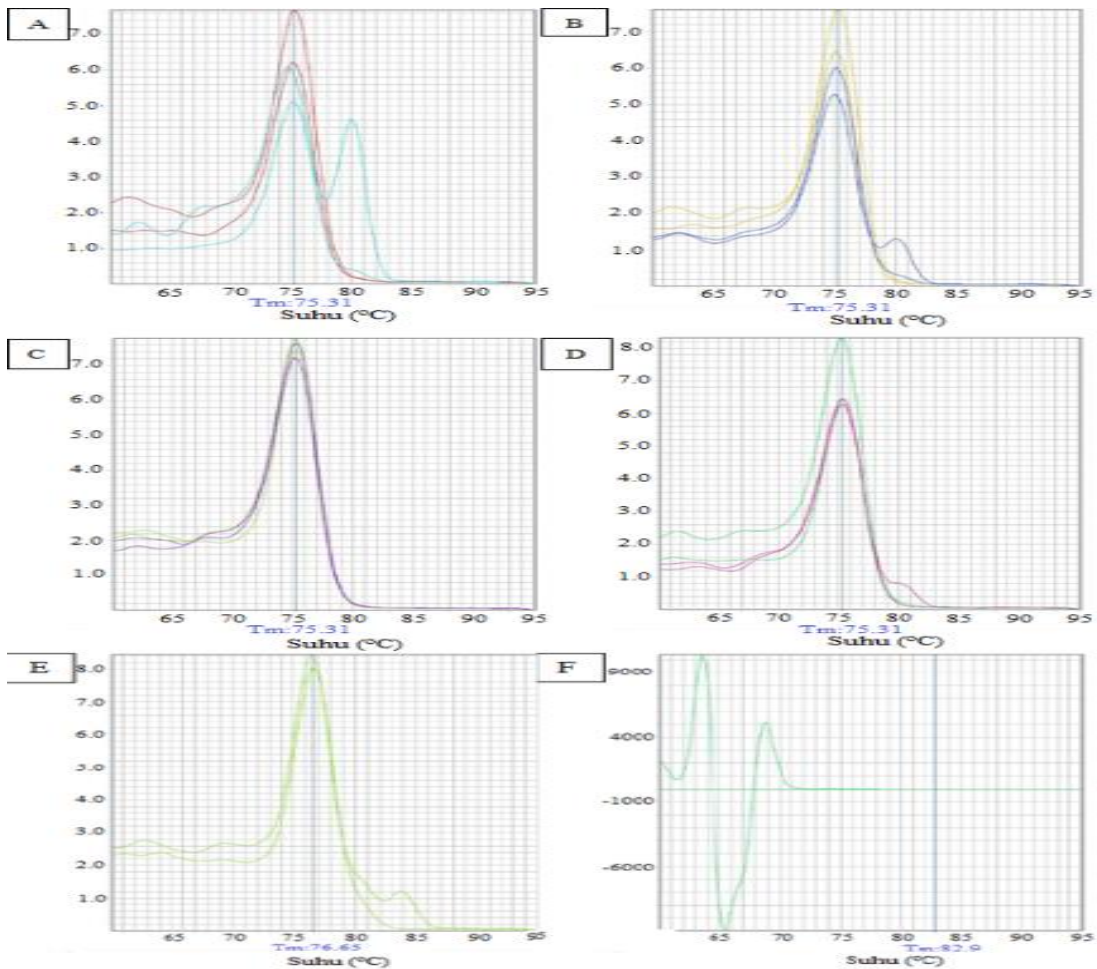
Figure 29 Melt peak curve A = TAAct SS1 50 ng/µl and 100 ng/µl ; B = TAAct SS2 50 ng/µl and 100 ng/µl ; C = TAAct ST1 50 ng/µl and 100 ng/µl ; D = TAAct ST2 50 ng/µl and 100 ng/µl ; E = GMAAI SS1 100 ng/µl and F = GMAAI ST1 100 ng/µl.

The results obtained from Real Time PCR was amplification curve, melt curve and melt peak curve. The optimization of various aspects need to be done on the process of Real Time PCR, so that the results of amplification can be passes the threshold (threshold line that described the flourocense generated by DNA amplification) and a melt peak curve that has peak formed either a single peak as well as the results of electrophoresis that form the band in accordane to the size of the target. Besides that, the optimization in the process of Real Time PCR is performed in order to eliminate the dimer formed in conventional PCR process before. Optimization can be done by concentration of cDNA sample aspect. It is because the cDNA sample with a high concentration potentially having a high concentration of contaminant samples, so that the recommended concentration of cDNA have ranges between 1-100 ng/µl (Applied Biosystem 2010). Treatment to

concentration of the cDNA sample used for optimization process was 50 ng / mL and 100 ng / ml at the four samples of cDNA (SS1, SS2, ST1 and ST2) with replicated twice conducted in both primer namely TAAct and GMAAI primer using temperatures annealing the previous optimization results (52 $^{\circ}$C and 55 $^{\circ}$C). The negative control is also included in order to be aware of any contaminants in the material. Results of the both concentration of cDNA samples with TAAct and GMAAI primer at melt peak curve on showed at Figure 29.

Meltpeak curve is the peak melting curve to show the specificity of the product formed. The results in Figure 28 show that meltpeak formed on all samples, concentrations, and replications apart SS1 sample replicates both the TAAct primer was good enough for forming a peak that shows the specificity primer with value of temperature melting (Tm) uniform (75.31 °C). While meltpeak curve on SS1 concentration of 100 ng / mL (blue) on the second replicates forming a double peak with a Tm of 75.31 °C and 80 °C. This shows that the TAAct primer on these samples was less specific because the primer attached to the two targets in a single cDNA. This could be due to the unappropriate annealing temperature (Kartika 2018). While meltpeak curve generated from the sample SS1 with GMAAI primer is good enough, but in ST1 samples not form a regular curve. This can be caused due to an error in the handling of reagents for amplification and mixing ingredients for Real Time PCR method is very sensitive and easily damaged.

Amplification curve showed that the amplification in a thermal cycler. Amplification curve of TAAct and GMAAI is shown in Figure 29. The results in Figure 29 showed that amplification curve is formed quite well from all the samples on the TAAct and GMAAI primer seen from the peak of constant and parallel on both replicates except ST1 samples with GMAAI primer not form a stable curve. It is related to the results of meltpeak curve that is not well formed. Based on the value of CT (threshold cycle) and ΔRn (baseline-corrected normalized reporter), amplification curve on the TAAct primer with a concentration of 50 ng / mL has a value of CT is lower and ΔRn higher than the concentration of 100 ng / ml with a value of CT an average of 23.7 at a concentration of 50 ng / ml and 25. 6 at a concentration of 100 ng / ml. It can be showed that the lower concentration of the sample then results obtained are better characterized than the value of CT is lower and ΔRn higher and the curve amplification positive control (gene actin) that is good, that which has the value of CT and ΔRn lower than the curve amplification of target genes (gene AAI).

TAAct primer on amplification curve has an average CT value of 24.6, while the GMAAI primer (only SS1 sample, the amplification curve of ST1 is not formed so that CT value can not be determined) at 30. Meanwhile, there were no significantly difference of CT

values average of samples were not attacked by boktor (SS1 and SS2) and samples of were attacked by boktor (ST1 and ST2). This could be due to sample with boktor were not express AAI gene at the time of sample collection or boktor already so long that AAI gene is less expressed. According to Christopher et al. (2004), the resistance gene against insects on plants poplar (*Populus trichocarpa*) can be expressed maximum within 24 hours after the attack. Based on these results, the yield on the TAAct primer was good enough so that it can be used for gene expression analysis, however, for the GMAAI primer especially on ST1 samples need to be re-optimization in a wide range of sample concentration, annealing temperature, the concentration of primer and concentration of fluorescent dye so that the results obtained the better of amplification and meltpeak curve.

Control is important thing in an experiment that can be used as a reference for the success of a trial, as well as in the Real Time PCR. There are two types of the controls that were used, positive controls and negative controls. Positive controls that were used that actin gene which is a housekeeping genes and compared with the target gene to obtain its gene expression data, while the negative control used is the NTC (no template control) which is an indicator of contaminants in Real Time PCR reaction. Meltpeak curve and amplification curve of the NTC samples is shown in Figure 30.
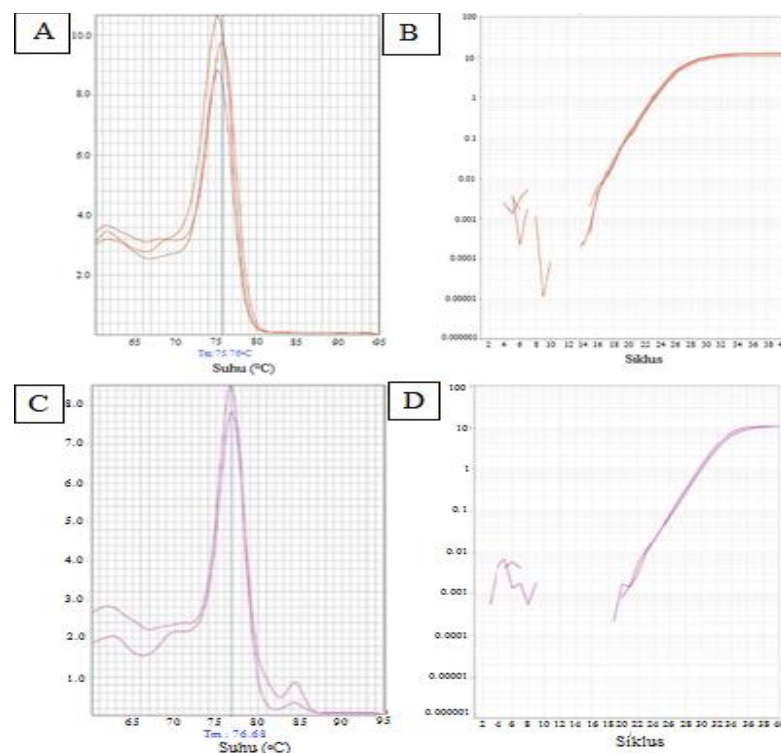

Figure 20 NTC at A = meltpeak curve of NTC with TAAct primer; B = amplification curve of NTC with TAAct primer ; C = meltpeak curve of NTC with GMAAI primer and D = amplification curve of NTC with GMAAI primer.

Validation of the results of real time PCR was performed by electrophoresis to knew the suitability of products with the size of the target size. It also aims to determine the specificity of the primers used. The results of electrophoresis of the four samples is shown in Figure 31.
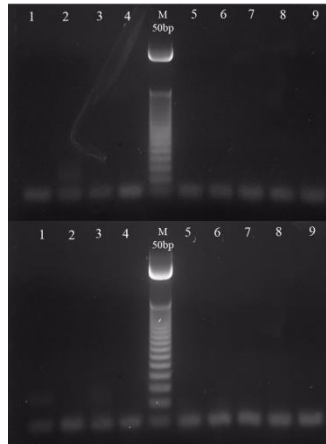

Figure 31 The results of elektrophoresis of qPCR product optimization of cDNA concentration of 50 ng/µL (upper) and 100 ng/µL (lower): 1-2: SS1; 2-4: SS2; 5-6: ST1; 7-8: ST2; and 9: NTC.

Electrophoresis results showed that the concentration of TAAct primer of 50 ng / mL and 100 ng / ml to form a dimer. Both optimizations performed on Real Time PCR showed that it is possible that the primer dimer formed has a strong bond, which is possibility of cDNA samples degraded, so that actin gene can not be amplified; or because of the primers used (TaACT) was less specific in amplify the actin range on sengon. The cause of the dimer, double band or smear may be caused by the annealing temperature that is not right, the primer was less specific or the quality cDNA sample is not good. Therefore, the TaACT primer re-amplified using DNA sengon template to see whether or not primer dimers are formed.

Amplification was again conducted using three stock of DNA sengon isolated at different times with the dilution of 10x, 20x, up to 50x. But the results of the amplification of TaACT primer at three concentrations of DNA stock resulted target product, ie, with a size of 120 bp and accompanied by a dimer with a very bright bands intensity (Figure 31). This is different from the DNA amplification with TaACT primer performed at early research, which the amplification of TaACT product is not followed by the formation of dimers. Primer dimer is likely to be caused by incorrect primer storage, primer design errors, or errors in primer elution. The previou elution of primer used NFW (Nuclease free water). When compared with the elution process using TE buffer, The buffer can prevent primer dimer with chelating Mg 2+ ions, so that the primer is stored more stable.

The homology of the amplicon sequence 18s rRNA, AcACT, and AcTI

The result of the sequence alignment of 18s rRNA amplicons obtained red line (score bit ≥200) at 100 entries contained in the NCBI database (Figure 31). The highest similarity value obtained by alignment of the 18s RNA gene prediction of Senegalia Laeta or Acacia Laeta or derived from the family Fabeceae with the similarity value reaches 99% and E-value of 5e-65. This indicates that the primer use are predicted to amplify RNA sengon 18s. However, the results of the chromatogram at 18s RNA amplicon itself has peak is less good with a lot of noise going on (Figure 32). The low quality of the resulting peak may be caused by sequencing samples that still contained a contaminant, so that could harm in reading the nucleotides sequence. Therefore, for a more reliable results can be done by resequencing with focuses on quality samples that want to be included.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Senegalia laeta isolate In Zbib 8-II 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 26S ribosomal R	257	257	99%	5e-65	99%	KY316159.1
Senegalia laeta isolate In Zbib 8-I 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 26S ribosomal RI	257	257	99%	5e-65	99%	KY316158.1
Faidherbia albida isolate In Zbib 5 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 26S ribosomal RI	257	257	99%	5e-65	99%	KY316157.1
Vachellia seyal isolate Mazoliet 36 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 26S ribosomal R	257	257	99%	5e-65	99%	KY316156.1
Vachellia tortilis subsp. raddiana isolate Nezmet 23 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and	257	257	99%	5e-65	99%	KY316155.1
Vachellia tortilis subsp. raddiana isolate Nezmet 19 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and	257	257	99%	5e-65	99%	KY316154.1
Vachellia nilotica isolate Amezgin 20 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 26S ribosomal	257	257	99%	5e-65	99%	KY316153.1
Vachellia flava isolate Adriana 1 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 26S ribosomal RN/	257	257	99%	5e-65	99%	KY316152.1
Albizia lebbeck isolate MDN2 18S ribosomal RNA gene, partial sequence	257	257	99%	5e-65	99%	HQ116781.1
Albizia lebbeck isolate MDN1 18S ribosomal RNA gene, partial sequence	257	257	99%	5e-65	99%	HQ116780.1

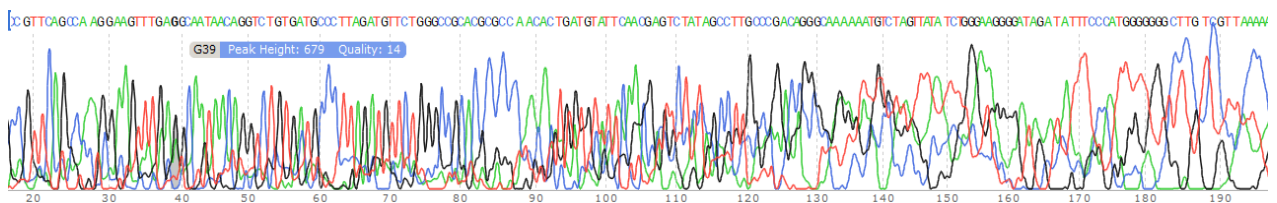Figure 32 The closest alignment of 18s rRNA amplicon sequence

Figure 33 Chromatogram of *forward* sequence in 18s rRNA

The alignment results of AcACT amplicon showed similarity with 103 entries in the NCBI database, with a very high homology values indicated by the red line (score ≥200 bits) and high homology indicated by the pink line (score of bits 80-200). The highest similarity values obtained from the aligning of the soybean actin gene soybean (Glycine max) with a similarity of 92% and an e-value of 3e-50 (Figure 33). In addition the results of the alignment also shows similarities to the actin genes derived from other organisms, such as Cajanus cajan, Phaseolus vulgaris, and so on. These values indicate that the amplicon sequences

AcACT have a significantly similarity with the other of sequences of actin genes contained in the NCBI.

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	PREDICTED: Glycine max actin (LOC100777705), transcript variant X3, mRNA	208	208	100%	3e-50	92%	XM_006587148.3
☐	PREDICTED: Glycine max actin (LOC100777705), transcript variant X2, mRNA	208	208	100%	3e-50	92%	XM_006587147.3
☐	PREDICTED: Glycine max actin (LOC100777705), transcript variant X1, mRNA	208	208	100%	3e-50	92%	XM_003533867.3
☐	PREDICTED: Glycine max actin (LOC100813210), transcript variant X3, mRNA	204	204	100%	1e-48	92%	XM_006575146.3
☐	PREDICTED: Glycine max actin (LOC100813210), transcript variant X1, mRNA	204	204	100%	1e-48	92%	XM_003518989.4
☐	PREDICTED: Cajanus cajan actin-like (LOC109792253), transcript variant X3, mRNA	204	204	100%	1e-48	92%	XM_020351653.1
☐	PREDICTED: Cajanus cajan actin-like (LOC109792253), transcript variant X2, mRNA	204	204	100%	1e-48	92%	XM_020351652.1
☐	PREDICTED: Cajanus cajan actin-like (LOC109792253), transcript variant X1, mRNA	204	204	100%	1e-48	92%	XM_020351651.1
☐	PREDICTED: Juglans regia actin (LOC109016780), mRNA	204	204	100%	1e-48	92%	XM_018999157.1
☐	PREDICTED: Glycine max actin (LOC100813210), transcript variant X2, mRNA	204	204	100%	1e-48	92%	XM_006575145.2

Figure 34 The closest alignment of AcACT sequence

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Acacia confusa trypsin inhibitor (acti) mRNA, partial cds	344	344	100%	7e-91	94%	M92852.1

Figure 35 The closest alignment of AcTI sequence

The alignment of AcTI's results amplicon sequences using the megablast BLASTn program on NCBI showed a very high similarity which was pointed by a red line (bit score ≥200). In addition, the resulting entries were only from organisms, i.e. trypsin inhibitor gene from *Acacia confusa* with a similarity value of 94% and an E-value of 7e-91 (Figure 35). Trypsin inhibitor sequence from *Acacia confusa* sequence is the basic for primer design. Those indicated that AcTI primer was predicted to be able to amplify trypsin inhibitor region in DNA template of sengon.

5. CONCLUSION

AcTI, TaACT, and AcACT primers were able to amplify the TI and actin gene encoding regions in sengon DNA templates based on the high level of similarity in the alignment results. The Blastx results in the three primary amplicons also had predictions of the conserved area of TI superfamily and actin in DNA fragments of sengon. The sequence of actin-specific genes was successfully obtained from TAAct primer of 98bp and showed similarities with actin gene of *Sarocalamus faberi*.

A. malaccensis DNA was amplified at 54.8 °C of annealing temperature for HMGR gene and 57 °C for Actin gene. *G. versteegii* DNA samples were amplified at 54.8 °C of

annealing temperature for HMGR gene and 55.8 °C for Actin gene. BLAST results showed HMGR encoding genes in *A. malaccensis* and *G. versteegii* were same as *A. sinensis*. While the Actin gene in *A. malaccensis* and *G. versteegii* were same as *A. microcarpa* in the gene bank. cDNA samples of *A. malaccesnis* dan *G. versteegii* were amplified at 56 °C of annealing temperature for HMGR and Actin genes. HMGR genes of cDNA samples were identified in *A. sinensis*, whereas the Actin genes were identified in *A. microcarpa*. The results of *A.malaccensis* DNA PCR with annealing temperature of 57˚C showed successes in all amplification results, while at annealing temperature of 56.5˚C, Am1-δG, Am3-δG, Am4-δG, AC-δG, and Amic-δG were able to detect the existence of guaiene gene. The results of the G.versteegii DNA PCR with annealing temperature of 56.5˚C were successfully amplified throughout the primers. Primer Am3-δG can detect guaiene genes in cDNA samples of *A.malaccensis* and *G.versteegii*.

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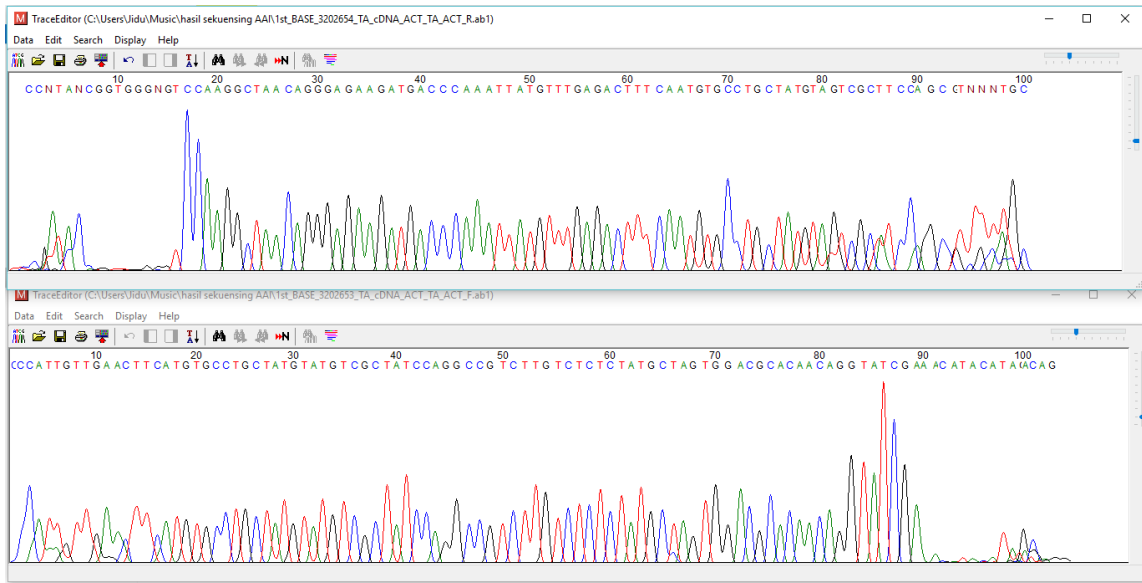
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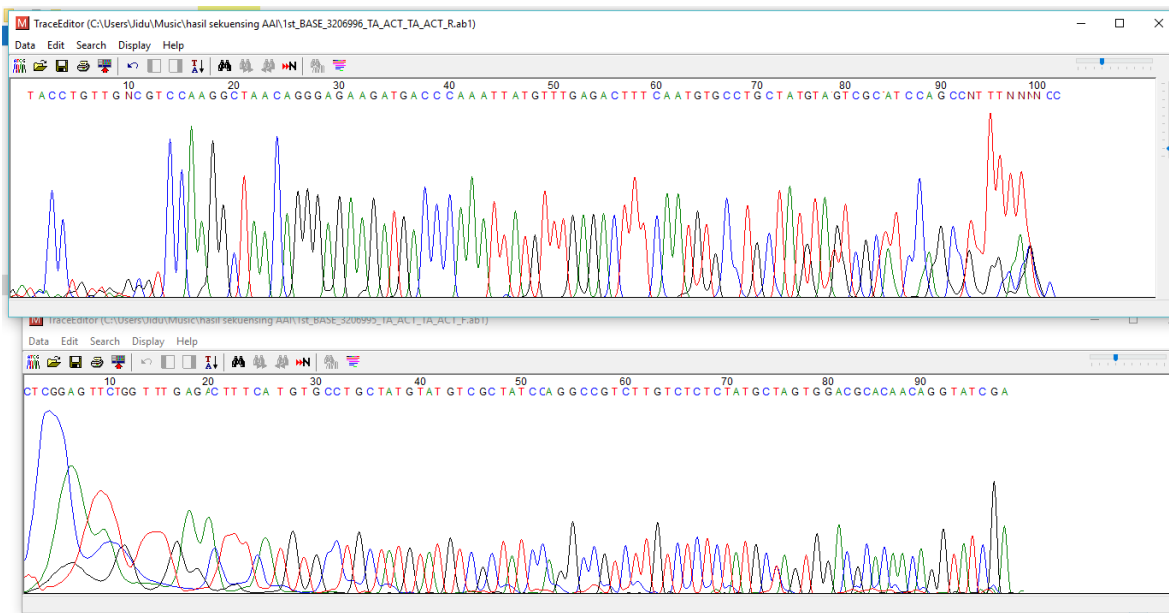Yunbi X. 2010. *Molecular Plant Breeding*. Oxford (UK): CABI.

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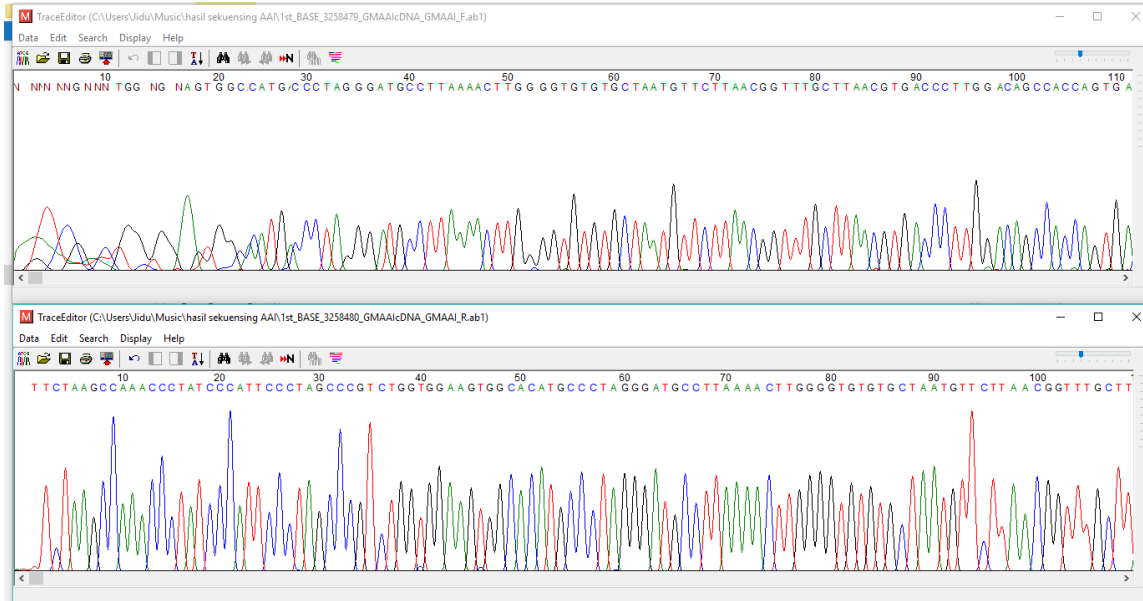
7. APPENDICES

cDNA act

DNA act

cDNA AAI

cDNA TI