HAYATI
Journal of Biosciences

Vol. 31 No. 1, January 2024 110-125
DOI:10.4308/hjb.31.1.110-125
ISSN: 1978-3019
EISSN: 2086-4094

Development of Few Significant SNP Markers from Transcriptomic Data for Selection of Sengon (*Falcataria falcata* (L.) Greuter & R. Rankin) Resistant to Boktor Stem Borer and Gall Rust Disease

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ARTICLE INFO

Article history:
Received May 2, 2023
Received in revised form September 24, 2023
Accepted September 29, 2023

KEYWORDS:
High-Resolution Melting,
Falcataria falcata,
Single Nucleotide Polymorphisms,
Pest and Disease Resistance,
Transcriptome

ABSTRACT

Sengon (*Falcataria falcata* (L.) Greuter & R. Rankin) plantations in Indonesia are threatened by attacks from Boktor stem borers and gall rust disease. Controlling pests and diseases is difficult; therefore, planting resistant trees obtained from tree selection programs is necessary. Currently, genomic breeding often incorporates GWAS, which uses thousands of SNP markers to identify markers with significant associations with the traits studied. This study aimed to bypass such expensive studies by identifying and developing SNP markers from sequences of putative resistance genes to Boktor stem borer and gall rust disease, identified from sengon transcriptomic data analysis. A total of 496,194 putative SNP sites were identified from transcriptomic sequences using the SAMtools and BFCtools programs, of which 119 SNP sites were associated with resistance genes. Of the 101 non-synonymous SNPs selected, only 12 were located in the conserved domain of each gene and were used for primer design. Of the 13 primers designed, only 10 were successfully amplified. Validation of 10 developed SNP markers on 100 sengon accessions using the HRM method confirmed a significant association between SNP markers and resistance traits, with a -log 10 (P-value) between 10.49 and 16.63. A few SNPs markers developed from putative resistance gene sequences are associated with resistance traits in sengon. Therefore, the SNP markers could be applied in selection programs for sengon trees resistant to Boktor stem borers and gall rust disease.

1. Introduction

Sengon (*Falcataria falcata* (L.) Greuter & R. Rankin) is a fast-growing legume tree that has become an economically important species in Indonesia. Currently, sengon wood production is 2,595,175.82 m^3, representing 54.87% of the total wood production of Java Island (BPS 2019). However, plantations face serious pest and disease issues that affect their productivity. The principal pests and diseases that have been observed to harm sengon plantations are the Boktor stem borer (*Xystrocera festiva*) and gall rust infection by Uromycladium falcatariae fungus. So far, there are no effective control methods, so planting resistant clones would be beneficial both economically and ecologically. A selection program to obtain resistant lines has been initiated; however, progress is slow. A molecular approach is required to accelerate the selection process, such as utilizing molecular markers to obtain more reliable findings.

Several studies have shown that several provenances were more tolerant to gall rust infections and Boktor attacks (Baskorowati *et al.* 2012; Setiadi *et al.* 2014; Darwiati and Anggraeni 2018). However, because of the outcrossing nature of this species, these superior provenances have not yet been genetically or molecularly verified. Lelana *et al.* (2018) studied sengon resistance to gall rust disease using RAPD markers. However, RAPD could not differentiate the resistant from the susceptible accessions. Siregar *et al.* (2019) used microsatellite markers to separate resistant sengon accessions from susceptible ones and implied differences in

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the two accessions' genetic backgrounds. However, many resistant and susceptible accessions were still clustered within the same group. Both studies reported that the markers used were less specific. Therefore, new and more powerful markers are required for fingerprinting sengon accessions in selection programs.

Single-nucleotide polymorphisms (SNPs) have recently become popular and are frequently used as molecular markers in various laboratories for practical applications (Lai *et al.* 2012). SNPs are particularly suitable for studying complex genetic traits and understanding genome evolution (Wellenreuther *et al.* 2019; Mageiros *et al.* 2021). The wide application of SNP markers is due to rapid advances in high-throughput sequencing technology, which is becoming cheaper to obtain high-throughput data sequences rapidly and, when combined with various bioinformatics tools, enables the analysis of many gene functions. Yuskianti and Shirashi (2017) developed SNP markers to study the genetic diversity and relationships of 8 sengon populations from 3 regions in Indonesia, namely Papua, Maluku, and Java, as well as from Mindanao, the Philippines. However, this study was limited to genetic diversity and relationships between provenances without considering the condition of plant resistance to pests and diseases. Therefore, specific SNP markers associated with resistance to Boktor stem borer pest and gall rust disease are required.

SNP markers associated with certain complex traits are usually derived from genome-wide association studies (GWAS) involving thousands of SNP and samples. However, such GWAS studies are often expensive. This study aimed to bypass such expensive studies by identifying and developing SNP markers using transcriptome data analysis of sengon accessions resistant and susceptible to gall rust disease (Shabrina *et al.* 2019) and sengon accessions resistant and susceptible to Boktor stem borer (Siregar *et al.* 2021). The identified and developed SNP primers were tested on small samples of sengon accessions displaying resistance to the pest and disease. By developing SNP markers from sequences of putative functional genes for resistance, it is expected that the markers will be significantly associated with the phenotypic traits studied without spending much on analyzing many unrelated SNPs.

2. Materials and Methods

2.1. Plant Material

The plant materials for SNP validation were collected from sengon tree plantations in Kediri Regency, East Java, Indonesia, under the management of the National Forest Estate (11 resistant and 13 susceptible accessions) and from private plantations in Bogor Regency, West Java, Indonesia (39 resistant and 37 susceptible accessions). The sample trees used were the same five years old and planted in the same plot. Their health was examined before being identified and classified as either resistant or susceptible to Boktor stem borer and gall rust disease. Resistant and susceptible accessions were selected from trees planted in the same plot to eliminate the possibility of environmental factors affecting resistance. Susceptible individuals are trees attacked by pests and diseases with an attack severity level of more than 50%. In contrast, resistant individuals are trees that do not show any symptoms of the pest or gall rust disease infestation. The number of samples used in this study corresponded to the minimum sample size for population genomic analysis by Li *et al.* (2020). Two upper leaves were collected from these trees in January 2018 and January 2019. The upper leaves were chosen because young leaves have more tender tissues that are easily macerated and have lower levels of polyphenolic, polysaccharides, and lignin compounds, which could potentially interfere with the DNA extraction process.

2.2. DNA Isolation

DNA was isolated from 0.1 g leaf samples using the modified CTAB method (Doyle 1991). The quantity and quality of the DNA were measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and visualized on 1% agarose gel (1st BASE, 41 Science Park Rd, Singapore) electrophoresis at 100 volts for 25 min.

2.3. Mapping Transcriptome Sequence

The assembled transcriptomic data of sengon susceptible and resistant to Boktor stem borer and gall rust disease were obtained from the Data Bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp) with accession numbers DRA008389 and DRA007983 published by Shabrina *et al.* (2019) and Siregar *et al.* (2021). The read data used were from eight individual trees, where reads of rust-resistant, rust-

susceptible, Boktor-resistant, and Boktor-susceptible were the results of sequencing using the BGISEQ-500 platform (Siregar *et al.* 2021). Reads of rust on leaves 1 and 2 and stems 1 and 2 were sequenced using the Illumina HiSeq4000 platform (Shabrina *et al.* 2019). The sequences were realigned using CAP3 software (Huang and Madan 1999) to reduce clusters of nearly identical transcripts and obtain more complete contigs. Then, they were clustered to remove redundant or ambiguous contigs using CD-HIT-EST software (Li and Godzik 2006), applying an identity threshold of 95%. FastQC software was used to check the quality of data reads.

Raw reads were trimmed and filtered using Trimmomatic to remove adapter sequences contamination, low-quality bases (>Q30), and 3'/5' bias positions. Clean reads were then mapped to the assembled transcriptome reference using Bowtie2 software (Langmead and Salzberg 2012). The SAM file was sorted according to the position of the reference sequence and then converted to BAM (Binary Alignment/BST) format using SAMtools software.

2.4. SNPs Calling

SNPs were called using the BCFtools program, which includes mpileup, call, and filter steps (Li *et al.* 2009) to identify nucleotide bases that are highly likely to be putative SNPs. The input file in the BAM format resulted in a VCF output that contains various information in the form of the occurrence position, variation quality, alternative bases, and other information related to the identified variations.

2.5. Designing SNPs Primer

Primers were designed to validate the SNP sites of the selected genes. The selected genes were related to resistance to Boktor stem borers and gall rust disease. The genes were identified based on DEG analysis, showing upregulated activity in the previous studies. The sequences were reannotated using BLAST (Basic Local Alignment Search Tools) to confirm that the sequences were highly related to the resistance genes. The target gene sequences were annotated or compared with the GenBank (NCBI) sequences and the results of the whole-genome sequencing of sengon (DRA012508) to ensure the gene sequences were correct before designing the primers. The conserved area was identified in the target gene sequences using the GenBank website's Conserved Domain Database (CDD) (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The sites of SNPs selected for primer preparation must be in this conservative region, non-synonymous, and there are no other adjacent SNP sites. A pair of primers were then designed using Primer3Plus (https://primer3plus.com).

A primer selection step was performed to obtain primers that could detect SNPs in the assayed samples. The selection was performed by testing several candidate primers under the same PCR conditions and using the same DNA sample to obtain optimum PCR conditions and the level of variation of the bands produced by each primer. The PCR mixture used GoTaq® PCR Core System I (Promega Corporation, Madison, Wisconsin, USA), and amplification was performed in an Applied Biosystems Veriti 96-Well Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) for 35 cycles with the following steps: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 56°C to 60°C for 30 s, extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min.

2.6. SNPs Genotyping and Validation

The 10 selected primers were used to validate SNPs in the DNA samples from resistant and susceptible plants using the StepOne™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). High-Resolution Melting (HRM) reaction was performed using the SensiFAST™ HRM Kit (Meridian Bioscience Inc, Cincinnati, Ohio, USA) in a final volume of 20 µl containing 2× SensiFAST HRM Mix, 6.4 µl H_2O, 10 µM of each primer, and 2 µl of DNA template (approx. 100 ng/µl). The qPCR condition followed: initial denaturation at 95°C for 1 min, denaturation at 95°C for 5 s, annealing/extension at 60°C for 25 s, and the PCR process was carried out as many as 40 cycles. This was followed by forming a melt curve process to determine the base variation that occurred with the following steps: denaturation at 95°C for 10 s, annealing at 60°C for 1 min, high-resolution melting at 95°C for 15 s, and annealing at 60°C for 15 s with a ramp rate of 0.3%.

Melt curve analysis was performed using HRM software with normalization and temperature shift of the fluorescence data, followed by fluorescence difference plots. A reference sample was used for observation. The present study used a wild-type sample based on transcriptomic data analysis as a reference. The software grouped data that were

similar and assigned a cluster number. The melt curve corresponding to each cluster was color-coded for ease of visualization. The cluster detection settings included melt curve shape sensitivity (50% cluster default value) and melting temperature difference threshold (Tm) (default: 0.15).

2.7. Chi-Square Test

Chi-square analysis was used to determine the correlation between genotypes based on the results of HRM analysis with phenotypes. The following chi-square test formula was used:

$$x^2 = \sum_{i=1}^{p} \frac{(|O_j - E_j| - 0.5)^2}{E_j}$$

O_j = value of observations in J class = 1, 2; E_j = expected value in J-class = 1, 2. Each primer's "-log10(p)" value was calculated and plotted against each contig to generate Manhattan plots using the ggplot2 package in RStudio. We declared SNPs significant at an FDR-adjusted p-value of less than 0.001 to minimize the risk of false positives, which can lead to spurious associations and waste resources for follow-up studies (Duggal *et al.* 2008).

3. Results

3.1. Reference Sequence Design

This study used reads from the results of transcriptomic data assembly of Boktor- and gall rust-resistant and susceptible sengon samples. Two assembled transcriptome references were reassembled using CAP3 and CH-HIT-EST, resulting in 150,197 contigs (Table 1).

3.2. Quality Control and Filtering of Raw Reads

Raw data may contain low-quality bases and adapters that can cause errors in the alignment process. Filtering and trimming were performed to remove the low-quality bases and adapters (Table S1). Reads of boktor-resistant, boktor-susceptible, rust-resistant, and rust-susceptible had high base quality (>Q30), as seen from the results of quality control before filtering (Figure S1), indicating that the number of reads did not decrease.

Table 1. Number of contigs obtained from each read used in this study

Reads	Number of contigs
Boktor resistant-susceptible	70,089
Gall rust resistant-susceptible	96,164
Reassembly	150,197

3.3. Mapping and Detection of SNPs

Eight samples with clean reads against the transcriptome reference had an overall alignment rate of 90.78%. Quality control and trimming were performed on the eight clean-read samples. The alignment results were then used for the SNP calling stage using SAMtools. There were 496,194 nucleotide site changes or putative SNPs predicted in the resulting sengon transcriptome contigs, with an estimated frequency of one SNP per 608 bp. The identified SNPs were dominated by transition changes of 323,370 (65.21%) bases, whereas transversion changes were only 172,824 (34.79%) bases (Table 2).

3.4. Development and Selection of SNP Primers

The annotated transcriptomic data from previous studies showed that some genes play a role in plant resistance to pests and diseases, such as alpha-amylase inhibitors, trypsin inhibitors, ubiquitin carboxyl-terminal hydrolase 13, NADH-ubiquinone oxidoreductase, Indole-3-acetic acid-amido synthetase, and the WRKY transcription factor. A total of 119 SNPs were identified from the sequences of these resistance genes and categorized as synonymous or non-synonymous; 18 synonymous and 101 non-synonymous SNPs were identified (Table 3).

Twelve non-synonymous SNP sites in the seven target genes were selected for developing SNP markers and designed into 13 primers. List of SNP primers generated for the target genes (Table S2). The selection of primers based on the PCR process showed that only ten primers could be amplified, whereas two were unamplified and discarded. The amplified product sizes ranged from 107 to 216 bp. The amplification results of each primer and the product size predictions are listed in Table S3. Meanwhile, primers NUOR-2, UB13-1, and UB13-2 were not successfully amplified, presumably because of the presence of introns in the DNA region flanked by the primer pairs.

Table 2. The number of identified putative SNPs in the transcriptomes of sengon used in this study

Alternative base	Reference base			
	C	G	A	T
C		18,372	19,216	90,788
G	13,482		72,081	26,583
A	25,472	93,277		29,593
T	67,224	18,630	21,476	
Total	496,194 SNPs			

C-G-A-T refers to cytosine (C), guanine (G), adenine (A), and thymine (T)

Table 3. Summary of SNPs distribution across the sequences of resistance-associated genes

Name of gene	Number of contigs	SNPs	
		Synonymous	Non-synonymous
Alpha-amylase inhibitor (AAI)	1	-	10
Trypsin inhibitor (TI)	3	6	28
Ubiquitin carboxyl-terminal hydrolase 13 (UB13)	5	2	9
NADH-ubiquinone oxidoreductase (NUOR)	16	3	19
Indole-3-acetic acid-amido synthetase (IAA)	3	6	30
Transcription factor WRKY 11	3	1	4
Transcription factor WRKY 40	3	-	1
Total	119		

3.5. SNPs Validation and Genotyping

The selected primers were validated on 100 samples of sengon DNA with the following details: 50 susceptible tree samples versus 50 resistant tree samples. Ten primers were used for amplification. Based on the correlation analysis, we observed that the samples susceptible to Boktor stem borer were also susceptible to gall rust disease and vice versa ($R = 0.69$, p-value = 2.2×10^{-8}). Thus, we combined the susceptibility and resistance of both attackers into one category (Figure S2).

Differential plots from HRM analysis in this study showed curve-shape differences between wild-type and mutant DNA. The proper plot shows a clear melt profile, a single peak, and grouping, as seen in the differential plots of the IAA-2 primer (Figure 1). The wild-type sample was a sample with a reference base from the identification of SNPs, and the mutant sample was a sample with changes in the nucleotide base according to the results of SNPs identification. The list of references and alternative bases for each primer is presented in Table S2.

DNA mutations can cause allele pairs to be either the same (homozygous) or different (heterozygous) (Table S4). For example, in the TI-2 primer, the allele pair could be AA or GA by changing the DNA base G (guanine) to A (adenine) at a position of 850 bp. Only 7 primers, i.e. TI-2, NUOR-1, NUOR-3, WRKY-11, WRKY-40, IAA-1, and IAA-2 primers used in the HRM analysis, successfully differentiated wild-types and mutant samples. However, the wild-types and mutant samples were not categorized according to their resistance level (resistant or susceptible) yet.

Furthermore, a chi-square test was performed using data from the SNP detection of 100 samples to determine the allele segregation pattern at each locus. As shown in Table S4, there were three expected genotypes for each locus, of which the expected Mendelian ratio of the genotypes was 1:2:1 (AA:AB:BB), where AA was the homozygous genotype for the wild-type allele, AB was the heterozygous genotype, and BB was the homozygous genotype for the mutant allele. If the calculated χ^2 value < χ^2 table, then the observed segregation pattern follows Mendelian law, meaning that the allele pair formed results from an independent segregation process.

In addition to the chi-square test, the p-value (-log 10) was calculated to determine the SNP markers association level with resistance properties. A higher p-value indicated that the association between SNP markers and resistance properties was more significant. All SNP markers showed high p-values, with WRKY 40 and NUOR 3 having the highest p-values of 16.63 and 14.65, respectively (Table 4) (Figure 2).

The dendrogram of grouping samples based on genotyping using seven SNP primers in 100 samples of *F. falcata* (Figure 3) showed that most samples

were grouped according to their phenotypes, namely resistant or susceptible. Some samples were still incorrectly clustered into the resistant or susceptible groups, presumably because these samples probably had intermediate resistance or susceptibility and required more precise phenotyping. Thus, the developed SNP marker can distinguish between susceptible and resistant samples.

4. Discussion

The observation of read quality follows that of Zhu *et al.* (2018), who stated that the BGISEQ-500 has a higher base and raw data quality than HiSeq4000. Library construction on the BGISEQ-500 using DNA nanoballs (DNB) technology can minimize replication errors. Quality control and trimming processes aimed to avoid sequence contamination, adapters, low-quality bases (<Q30), and 3'/5 end nucleotide position bias (Indriani *et al.* 2020).

The estimated frequency of SNPs in this study was lower than that in *Pinus pinaster* (1/192 bp) (Modesto *et al.* 2022), *Hevea brasiliensis* (1/308 bp) (Pootakham *et al.* 2015), and *Pisum sativum* (1/475 bp) (Leonforte *et al.* 2013). According to Pootakham *et al.* (2014), the number of samples studied impacts the frequency

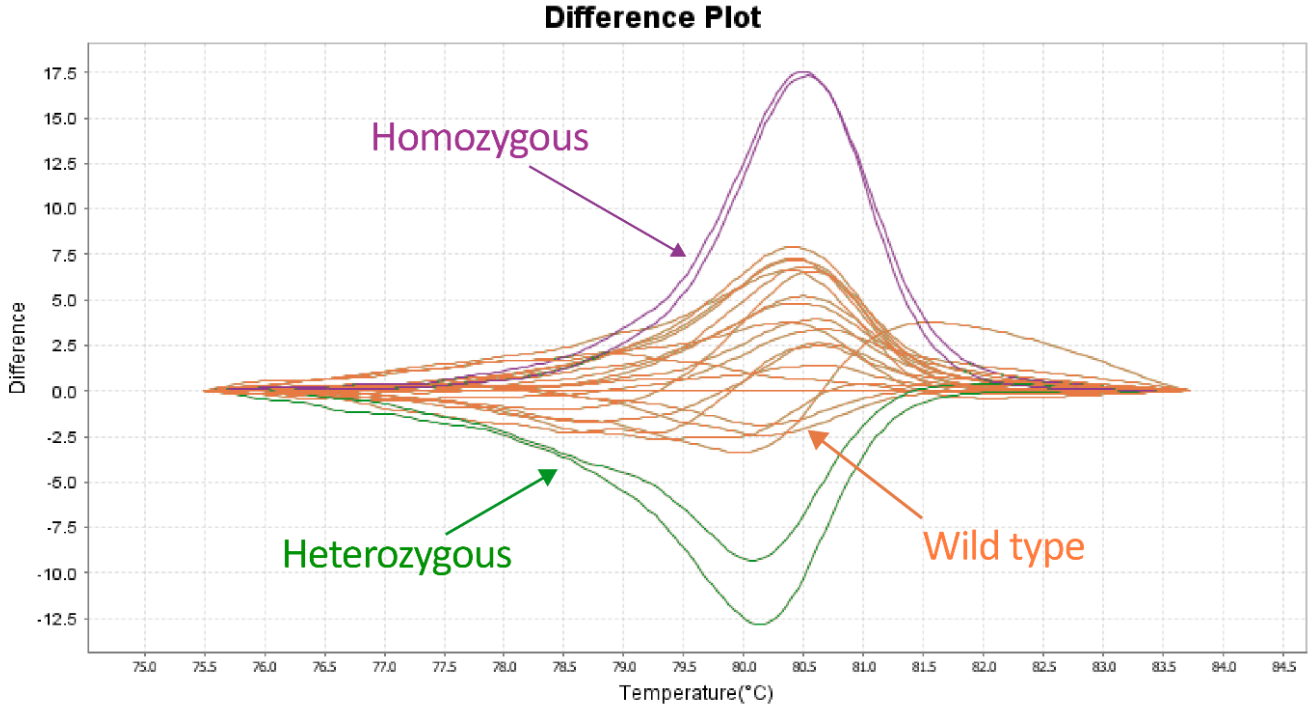

Figure 1. Differential plot of the IAA-2 locus according to the HRM melt curve

Table 4. Chi-square test of sengon trait resistance

Mendel's ratio	Primer	χ^2	-log 10 (P-value)	χ^2 table (0.05)
1:2:1	TI 2	48.32*	10.49	
	NUOR 1	60.12*	13.05	
	NUOR 3	67.48*	14.65	
	WRKY 11	63.48*	13.78	5.99
	WRKY 40	76.60*	16.63	
	IAA 1	61.56*	13.37	
	IAA_2	56.64*	12.29	

Degrees of freedom = 2. * = significant on p<0.05

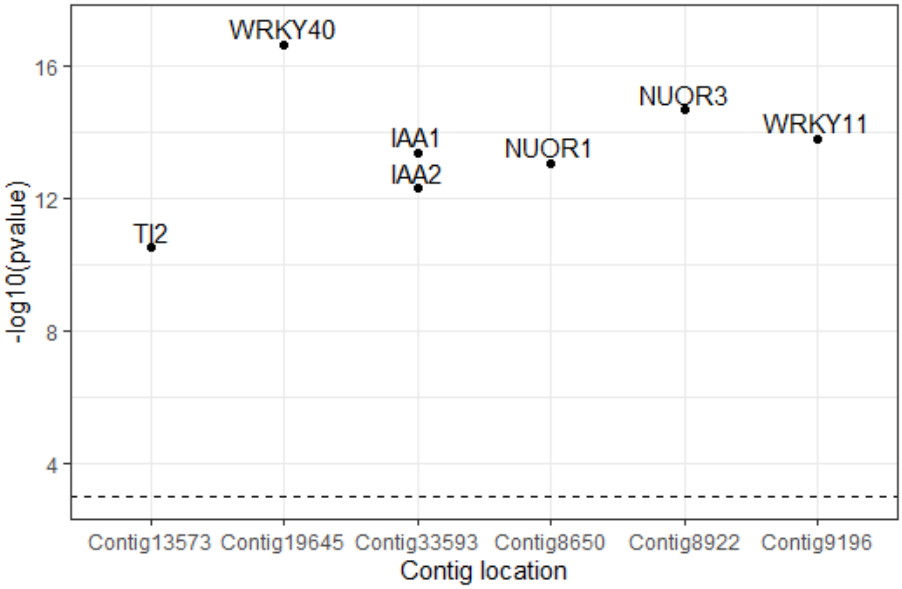

Figure 2. Manhattan plot of seven SNP loci from 100 samples showing either resistant or susceptible against boktor stem borer and gall rust disease in Sengon (*Falcataria falcata*) with threshold significance 0.05

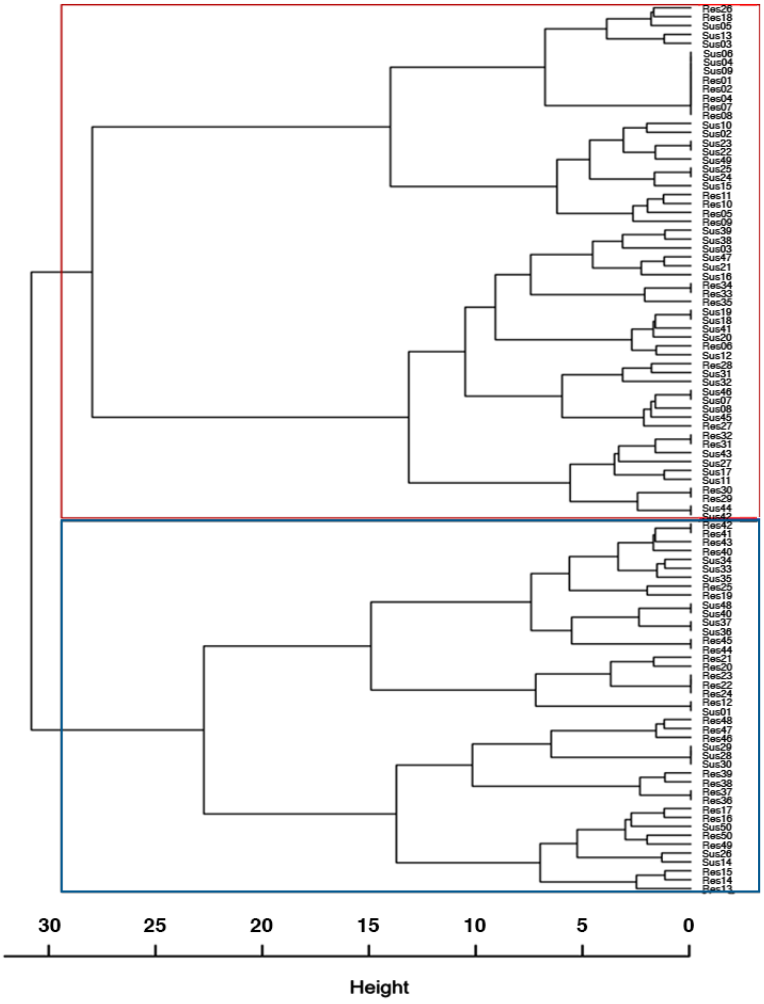

Figure 3. Dendrogram of 100 samples of sengon that are resistant and susceptible to boktor stem borer and gall rust. Res = resistant samples, Sus = susceptible samples. Red box = cluster one, blue box = cluster two

value of SNPs. The more samples used, the greater the chance to find new SNPs and, thus, would likely increase the frequency value of SNPs. Additionally, the frequency of SNPs in the previous transcriptome data, on which this study was based, was lower than that in the whole genome data (Anita *et al.* 2023) because the transcriptome consists of only part of the whole genome, that is, only expressed genes. Also, the coding region (exons) of an expressed gene used in this study to find the SNPs is relatively more conserved than other regions of the gene, i.e. introns (Wu *et al.* 2019).

SNPs can be classified as either transitions or transversions. Transition is a change in the nucleotide base adenine with guanine and cytosine with thymine, or vice versa. At the same time, transversion is a change in guanine and adenine with thymine and cytosine, or vice versa (Luo 2016). Based on these results, the transition-transversion ratio of 1.8 was observed. Similar findings have been reported for *Cryptomeria japonica* (Uchiyama *et al.* 2012), *Elaeis guineensis* (Pootakham *et al.* 2013) and *Hevea brasiliensis* (Pootakham *et al.* 2011, 2014; Shearman *et al.* 2015). Transition substitutions are more frequent than transversion and are thought to be due to the 5-methylcytosine reaction, which occurs frequently at CpG sites (Holliday and Grigg 1993; Zhang and Zhang 2005).

The advantage of detecting SNPs using transcriptome data is that the identified nucleotide base changes may be directly related to the traits to be observed, such as disease resistance and plant growth (Yu *et al.* 2014). Two important genes related to resistance to herbivore insects were AAI (alpha-amylase inhibitor) and TI (trypsin inhibitor) genes. Alpha-amylase inhibitors and trypsin inhibitors are inhibitory compounds that hinder the activity of amylase and proteinase enzymes in the digestive tract of insects; thus, they can inhibit the breakdown of starch into simpler carbohydrate molecules and proteins to be used in the insect metabolic system as an energy source (Siregar *et al.* 2021; Karray *et al.* 2022). In the case of resistance against gall rust disease, Ubiquitin carboxyl-terminal hydrolase 13 plays a role in the jasmonic acid signaling pathway (Jeong *et al.* 2017). Transcription factor genes are a group of genes that regulate the expression of other genes that are involved in resistance mechanisms. WRKY transcription factor 11 and WRKY transcription factor 40 respond positively to plant defense against pathogens (Jiang *et al.* 2016; Lee *et al.* 2018). NADH-ubiquinone oxidoreductase (Kant *et al.* 2019) and Indole-3-acetic acid-amido synthetase have been reported to increase plant susceptibility to pathogens. Indole-3-acetic acid-amido synthetase regulates auxin, stimulating excessive cell division and causing tumor swelling (Li *et al.* 2022). For the sengon tree improvement and breeding program, any genes involved in the resistance mechanisms against pests and disease are desirable. The SNP markers developed in this study are all needed and will be useful in fingerprinting and differentiating the resistant accessions from the susceptible ones; for example, the SNP markers related to AAI and TI genes will likely link to resistant accessions against the Boktor pest, while the SNP markers from IAA might be linked to susceptible accessions.

Synonymous SNPs are changes in one nucleotide base that do not change amino acids and are often called silent mutations. However, non-synonymous SNPs are changes in one nucleotide base that result in amino acid changes (Studer *et al.* 2013). Non-synonymous SNPs are thought to affect protein activity against genes directly (Yu *et al.* 2014), suggesting that these SNPs can be used as markers to help select resistant plants.

SNP markers were created based on changes in one nucleotide base (A, T, G, and C) in the sequences of target genes. SNP sites located in the conserved region were selected to prepare primers. For example, contig16297, detected as a trypsin inhibitor gene (accession number: cl11466), contains a conserved region between 360 and 875. Therefore, the SNP sites chosen for primer preparation for the trypsin inhibitor gene were at positions 771 and 850.

Even though it is already in conservative territory, the SNP site must be checked again using WGS data to ensure that the SNP site is located in the exon. One example is the results of ubiquitin carboxyl-terminal hydrolase 13 (UB13) gene sequence alignment with whole-genome sequencing (WGS) from Anita *et al.* (2023). It was observed that the Sengon WGS sequence had many introns and might cause the UB13 primer to be unsuccessfully amplified. Introns are DNA bases found between exons removed from the mRNA molecule to leave a series of exons that stick together so that the appropriate amino acid can be encoded (Shao *et al.* 2021).

Primer validation was performed using Real-Time Polymerase Chain Reaction (RT-PCR).

According to Kelly *et al.* (2019), the sample concentration greatly influences the amplification efficiency, resulting in a higher variation that is less favorable for forming a standard curve. In this study, the optimal concentration was in the range of 100-500 ng/µl. However, dilution is required if the DNA concentration is too high; higher dilutions are unsuitable for establishing a standard curve. The average DNA concentration used in this study was 100 ng/µl, per the SensiFAST HRM kit protocol (Bioline) for primer validation.

DNA amplicons were analyzed using HRM software to detect variations in the DNA base arrangement or mutations (Gomes *et al.* 2018). HRM can detect fluorescence when double-stranded DNA is denatured into single strands at high temperatures. The difference in one nucleotide base could give a different curve when the DNA fragment is denatured, so the HRM analysis results provide information about the sample with the target SNPs (Gupta *et al.* 2022), called a differential plot. This could explain the differences between wild-type DNA and DNA with mutations. The wild-type standard curve was used as the reference for the differential plot. The standard curve of the mutant was obtained by subtracting the normalized fluorescence values obtained from the normalized fluorescence standard curve of the wild-type DNA. The transformation of the fluorescence value into a differential plot was conducted and integrated into HRM software (Klafke *et al.* 2019).

According to Krypuy *et al.* (2006), the visualization of curves on different plots is strongly influenced by DNA isolation, DNA quality, amplicon length, primer design, and the reagent used. Moreover, unclear clustering and overlap may occur because of intronic SNPs (unexpected variations) located in the created primer sequence (Słomka *et al.* 2017).

The results of the Chi-Square test showed that the calculated χ^2 value for each primer was bigger than the χ^2 table of 5.99 (Table 4), so the distribution patterns of SNP genotypes regarding the resistance properties were deviated from the Mendelian ratio of 1:2:1. These results indicated that the alleles did not segregate freely. It is suspected that there is a linkage disequilibrium between the genes that control resistance traits. Linkage disequilibrium occurs when alleles of one genetic variant are inherited or correlated with a nearby allele of other genetic variants in the progeny population. The dendrogram (Figure 3) shows that 75% of the samples were separated. Cluster one (red box) was dominated by susceptible samples, and cluster two (black box) was dominated by resistant samples. Some samples that were not separated according to their clusters may have been caused by incorrect phenotyping, in which more phenotype groups or intermediates probably exist between the resistant and susceptible traits. These results were in line with the research of Shrestha *et al.* (2019), Liang *et al.* (2015), and Wanda *et al.* (2015), who reported that the genes for plant resistance to fungi, pests, and viruses were polygenic. This indicates that two or more genes can interact with each other during the formation of enzymes or proteins that affect the emergence of resistance traits.

Xu *et al.* (2006) stated that WRKY transcription factor 40 is essential in the signaling pathway of plant defense systems against pathogens. NUOR functions as a mitochondrial component in the ETC pathway, which plays a critical role in energy production and metabolism in plant cells. Compromised mitochondrial function can decrease the production of reactive oxygen species (ROS), important signaling molecules that play a key role in plant defense against pathogens (Cvetkovska *et al.* 2014). ROS production is typically induced in response to pathogen attacks. It helps to activate downstream signaling pathways that trigger various defense responses, such as programmed cell death and the production of antimicrobial compounds. NUOR also regulates programmed cell death, a key component of the plant immune response. When plant cells detect pathogen invasion, they often undergo programmed cell death to limit pathogen spread. Disruptions to the function of the ETC pathway can interfere with this process and impair the plant's ability to limit pathogen infection (Kant *et al.* 2019) effectively.

We discovered 496,194 SNPs in the transcriptomes of sengon-resistant and susceptible to Boktor stem borer and gall rust disease. SNP identification revealed putative 119 SNPs that may be linked to seven disease and pest-resistance genes. A few SNPs markers developed from putative resistance gene sequences are associated with resistance and susceptible traits in sengon. The bypass methodology of developing SNP markers for specific characters using the RNAseq data has proven desirable results. Those SNP markers are important for assisting in

fingerprinting resistant and susceptible accessions. Therefore, SNP markers could be applied in selection programs for sengon trees resistant to boktor stem borers and gall rust disease.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

This study was supported and funded by the Ministry of Education and Culture, Directorate General of Higher Education of the Republic of Indonesia with the scheme of Penelitian Pendidikan Magister menuju Doktor untuk Sarjana Unggul (PMDSU) based on contract number 200/SP2H/PMDSU/DRPM/2020. The authors are grateful to the National Forest Estate (Perum Perhutani) for giving access to their plantation for sampling and thankful to Ms. Aprilia Damayanti for helping with the collection of the sengon samples and Dr. M. Iqbal Maulana for his advice in the manuscript preparation.

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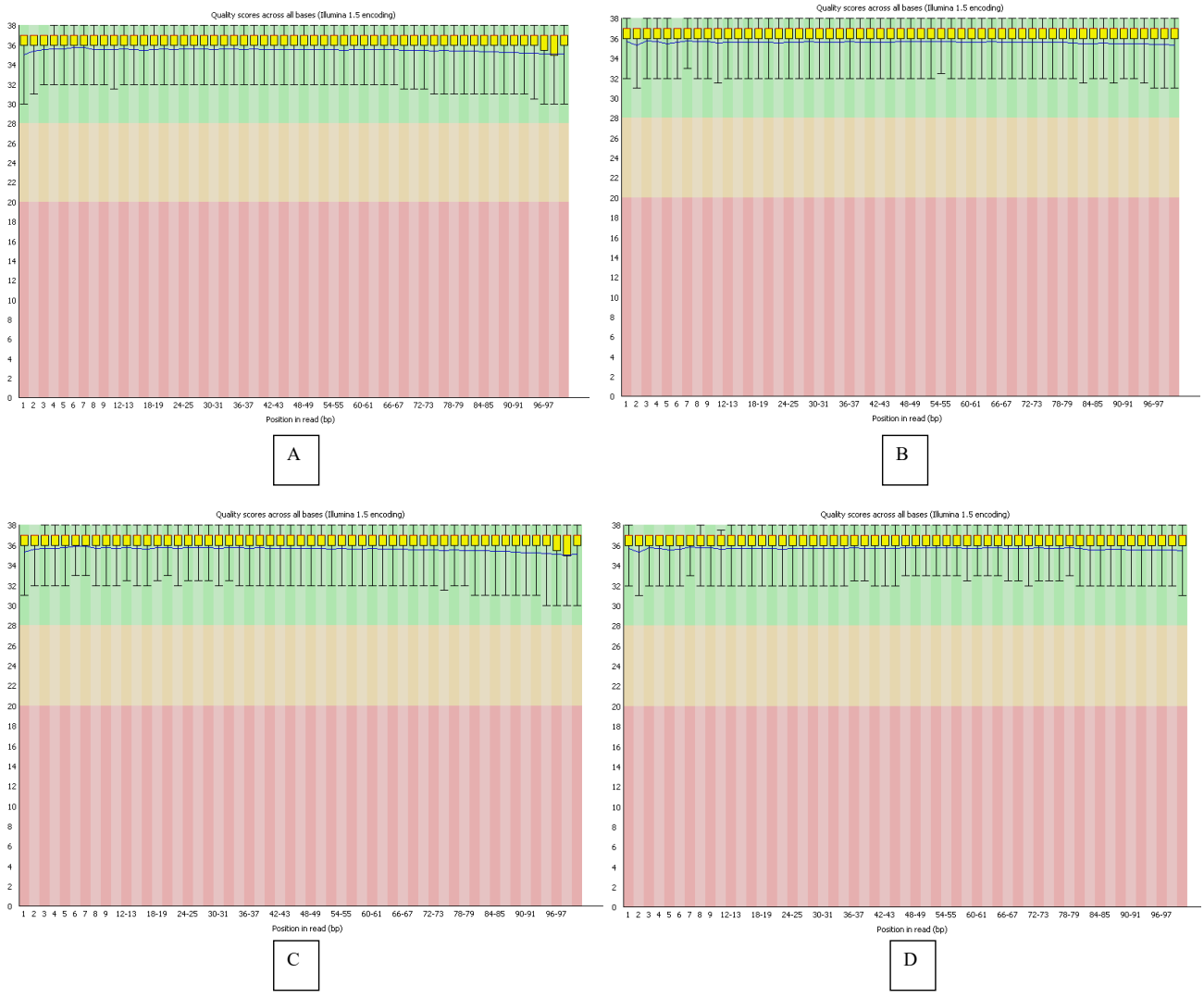
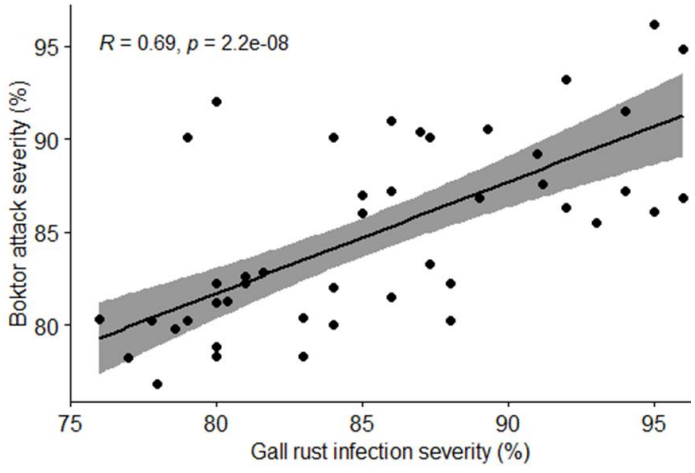
Supplementary Materials



Supplementary Figure 1. Base quality of reads (A) boktor susceptible, (B) boktor resistant, (C) gall rust susceptible, (D) gall rust resistant



Supplementary Figure 2. Correlation analysis between the percentage of boktor attacks and gall rust infection

Supplementary Table 1. Number of reads before and after filtering

Reads name	Number of reads	
	Before filtering	After filtering
Boktor resistant	Boktor resistant	77,634,868
Boktor susceptible	Boktor susceptible	79,315,980
Rust resistant	Rust resistant	79,037,484
Rust susceptible	Rust susceptible	79,054,112
Rust on leaves 1	Rust on leaves 1	60,259,198
Rust on leaves 2	Rust on leaves 2	64,269,442
Rust on stem 1	Rust on stem 1	64,100,912
Rust on stem 2	Rust on stem 2	58,235,308

Supplementary Table 2. Characteristics of SNP primer developed

Name of gene	Primer code	SNPs position	Nucleotides (5'-3')
Alpha-amylase inhibitor	AAI_1	575 (C>T)	F: TATCCCAATCCGACGACAA R: CGGCTGACGAGAGTGAAG
	AAI_2	944 (G>T)	F: GGTGTCCTACAGAAGTGTGTCC R: CGACAGGAAGAGCATTACCA
Trypsin inhibitor	TI_1	771 (A>G)	F: TTGTGACTTGTGTGTTGATGTG R: CCCATCGGTTCTCTCTCTCT
	TI_2	850 (G>A)	F: AGGTAAGTGTGGACATGTTGGT R: ATGCCCAGGGATGTTTATTC
Indole-3-acetic acid-amido synthetase	IAA_1	568 (C>A)	F: GGAGTTGTCCCATTGAGGTT R: TGCTGTCACCCATTTGTCTC
	IAA_2	568 (C>A)	F: AGTTGTCCCATTGAGGTTGAG R: TGTCACCCATTTGTCTCCTTT
NADH-ubiquinone oxidoreductase	NUOR_1	3392 (C>T)	F: CTTGCTTGCACTGAGAGACC R: GCACGGGACACAGAATAAGA
	NUOR_2	674 (A>G)	F: AGGACACGGATAGAACACAAGA R: TGCGCTTCAAAGGTAATAGG
	NUOR_3	1954 (A>C)	F: GCGATGAGAGGCGAAATAG R: CGACCGACTAAGGGCAAA
Transcription factor WRKY 11	WRKY_11	3272 (C>G)	F: CTTTGACTGTTGACCCTTCCT R: GGCACCCTCACTGTCTTCTT
Transcription factor WRKY 40	WRKY_40	1052 (A>T)	F: AATTGAAACATGGAACAGGAAA R: GATGGGTATCAATGGAGGAAA
Ubiquitin carboxyl-terminal hydrolase 13	UB13_1	4058 (T>C)	F:CCGACATATCTTAGCTCCTCTTTC R: CACCTTGAGGAGACCCAATC
	UB13_2	4114 (A>C)	F: CCGACATATCTTAGCTCCTCTTT R: CACCTTGAGGAGACCCAATC

letters in the SNPs position column are nucleotide thymine (T), cytosine (C), guanine (G), and adenine (A). Symbol > refers to the changes of the reference base to the alternative base. F = forward, R = reverse

Supplementary Table 3. Results of amplification of SNP primers

Primer code	Expected product (bp)	product size (bp)	Result
AAI_1	124	143	Amplified
AAI_2	111	126	Amplified
TI_1	107	107	Amplified
TI_2	189	189	Amplified
IAA_1	123	150	Amplified
IAA_2	118	118	Amplified
NUOR_1	160	160	Amplified
NUOR_2	198	-	Unamplified
NUOR_3	126	146	Amplified
WRKY 11	170	200	Amplified
WRKY 40	216	226	Amplified
UB13_1	197	-	multiple bands
UB13_2	197	-	unamplified

Supplementary Table 4. HRM analysis results on each sample

Sample	TI_2	NUOR_1	NUOR_3	WRKY40	WRKY11	IAA_1	IAA_2
SUS							
1	wt (GG)	wt (CC)	mt (AA)	mt (AA)	mt (CG)	wt (CC)	wt (CC)
2	wt (GG)	mt (TT)	mt (CC)	mt (AA)	wt (CC)	wt (CC)	mt (AA)
3	mt (AA)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
4	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
5	wt (GG)	wt (CC)	mt (CC)	mt (AA)	mt (GG)	mt (AA)	wt (CC)
6	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
7	mt (AA)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	mt (CA)
8	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	mt (CA)
9	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
10	wt (GG)	wt (CC)	wt (AC)	mt (AA)	wt (CC)	wt (CC)	mt (AA)
11	wt (GG)	wt (CC)	mt (AA)	wt (AT)	wt (CC)	mt (CA)	wt (CC)
12	wt (GG)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	wt (CC)
13	mt (GA)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
14	wt (GG)	wt (CC)	wt (AC)	mt (AA)	mt (CG)	mt (AA)	mt (CA)
15	wt (GG)	wt (CC)	wt (AC)	mt (AA)	wt (CC)	mt (CA)	mt (AA)
16	wt (GG)	wt (CC)	mt (AA)	mt (AA)	wt (CC)	mt (CA)	mt (AA)
17	wt (GG)	wt (CC)	mt (AA)	wt (AT)	wt (CC)	mt (CA)	mt (AA)
18	wt (GG)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	mt (AA)
19	wt (GG)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	mt (AA)
20	wt (GG)	mt (TT)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	mt (AA)
21	wt (GG)	mt (TT)	mt (AA)	mt (AA)	wt (CC)	mt (AA)	mt (AA)
22	wt (GG)	mt (TT)	wt (AC)	mt (AA)	wt (CC)	mt (AA)	mt (AA)
23	wt (GG)	mt (TT)	wt (AC)	mt (AA)	wt (CC)	mt (AA)	mt (AA)
24	wt (GG)	mt (TT)	wt (AC)	mt (AA)	wt (CC)	mt (CA)	mt (AA)
25	wt (GG)	mt (TT)	wt (AC)	mt (AA)	wt (CC)	mt (CA)	mt (AA)
26	wt (GG)	wt (CC)	wt (AC)	mt (AA)	mt (CG)	mt (CA)	mt (CA)
27	wt (GG)	wt (CC)	mt (AA)	wt (AT)	mt (CG)	mt (CA)	mt (CA)
28	mt (GA)	wt (CC)	mt (CC)	wt (AT)	mt (CG)	mt (AA)	mt (CA)
29	mt (GA)	wt (CC)	mt (CC)	wt (AT)	mt (CG)	mt (AA)	mt (CA)
30	mt (GA)	wt (CC)	mt (CC)	wt (AT)	mt (CG)	mt (AA)	mt (CA)
31	mt (GA)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	mt (CA)
32	mt (GA)	mt (CT)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	mt (CA)
33	mt (GA)	mt (CT)	mt (CC)	mt (AA)	mt (CG)	mt (AA)	mt (AA)
34	mt (GA)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	mt (AA)	mt (AA)
35	mt (AA)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	mt (AA)	mt (AA)
36	mt (AA)	mt (TT)	mt (CC)	mt (AA)	mt (GG)	mt (AA)	mt (AA)
37	mt (AA)	mt (TT)	mt (CC)	mt (AA)	mt (GG)	mt (AA)	mt (AA)
38	mt (AA)	mt (TT)	mt (AA)	wt (AT)	mt (GG)	mt (AA)	mt (AA)
39	mt (AA)	mt (TT)	mt (AA)	wt (AT)	wt (CC)	mt (AA)	mt (AA)
40	mt (AA)	mt (TT)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	mt (AA)
41	mt (AA)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	mt (AA)
42	mt (AA)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (CA)	mt (CA)
43	mt (AA)	wt (CC)	mt (AA)	wt (AT)	wt (CC)	mt (CA)	mt (CA)
44	mt (AA)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (CA)	mt (CA)
45	mt (AA)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (CA)	mt (CA)
46	mt (AA)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	mt (CA)
47	mt (AA)	mt (TT)	mt (AA)	mt (AA)	wt (CC)	mt (AA)	mt (AA)
48	mt (AA)	mt (TT)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	mt (AA)
49	mt (AA)	mt (TT)	wt (AC)	mt (AA)	wt (CC)	mt (AA)	mt (AA)
50	mt (AA)	mt (TT)	wt (AC)	wt (AT)	mt (GG)	mt (AA)	mt (AA)

SUS = susceptible, RES = resistant, wt = wild type, mt = mutant, C-G-A-T refers to Cytosine (C), Guanine (G), Adenine (A), and Thymine (T)

Supplementary Table 4. Continued

Sample	TI_2	NUOR_1	NUOR_3	WRKY40	WRKY11	IAA_1	IAA_2
RES							
1	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
2	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
3	wt (GG)	wt (CC)	mt (AA)	wt (AT)	wt (CC)	wt (CC)	wt (CC)
4	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
5	wt (GG)	mt (CT)	wt (AC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
6	wt (GG)	wt (CC)	wt (AC)	wt (AT)	wt (CC)	mt (AA)	wt (CC)
7	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
8	wt (GG)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
9	wt (GG)	mt (CT)	mt (CC)	wt (AT)	wt (CC)	mt (AA)	wt (CC)
10	wt (GG)	mt (CT)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
11	wt (GG)	mt (TT)	mt (CC)	mt (AA)	wt (CC)	mt (AA)	wt (CC)
12	wt (GG)	wt (CC)	mt (AA)	mt (AA)	mt (CG)	wt (CC)	wt (CC)
13	wt (GG)	wt (CC)	wt (AC)	mt (AA)	mt (CG)	wt (CC)	wt (CC)
14	wt (GG)	wt (CC)	wt (AC)	wt (AT)	mt (CG)	wt (CC)	wt (CC)
15	wt (GG)	wt (CC)	wt (AC)	wt (AT)	mt (GG)	wt (CC)	wt (CC)
16	wt (GG)	wt (CC)	wt (AC)	wt (AT)	mt (GG)	mt (CA)	wt (CC)
17	mt (AA)	wt (CC)	wt (AC)	wt (AT)	mt (GG)	mt (CA)	wt (CC)
18	mt (AA)	wt (CC)	mt (CC)	mt (AA)	mt (GG)	mt (CA)	wt (CC)
19	mt (AA)	mt (CT)	mt (AA)	mt (AA)	mt (CG)	mt (CA)	wt (CC)
20	mt (AA)	mt (CT)	mt (AA)	mt (AA)	mt (CG)	wt (CC)	wt (CC)
21	wt (GG)	mt (CT)	mt (AA)	mt (AA)	mt (GG)	wt (CC)	wt (CC)
22	wt (GG)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	wt (CC)	wt (CC)
23	wt (GG)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	wt (CC)	wt (CC)
24	wt (GG)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	wt (CC)	wt (CC)
25	wt (GG)	mt (CT)	mt (CC)	mt (AA)	mt (CG)	mt (CA)	wt (CC)
26	wt (GG)	wt (CC)	mt (CC)	mt (AA)	mt (CG)	mt (CA)	wt (CC)
27	mt (AA)	wt (CC)	mt (CC)	mt (AA)	wt (CC)	wt (CC)	mt (CA)
28	mt (AA)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	wt (CC)	mt (CA)
29	wt (GG)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (CA)	mt (CA)
30	wt (GG)	wt (CC)	mt (CC)	wt (AT)	wt (CC)	mt (CA)	mt (CA)
31	wt (GG)	wt (CC)	mt (AA)	wt (AT)	wt (CC)	mt (CA)	mt (CA)
32	wt (GG)	wt (CC)	mt (AA)	wt (AT)	wt (CC)	mt (CA)	mt (CA)
33	mt (GA)	mt (TT)	mt (AA)	wt (AT)	wt (CC)	wt (CC)	wt (CC)
34	mt (GA)	mt (TT)	mt (AA)	wt (AT)	wt (CC)	wt (CC)	wt (CC)
35	mt (GA)	mt (TT)	mt (CC)	wt (AT)	wt (CC)	wt (CC)	wt (CC)
36	mt (GA)	mt (TT)	mt (CC)	wt (AT)	mt (CG)	mt (CA)	wt (CC)
37	mt (GA)	mt (TT)	mt (CC)	wt (AT)	mt (CG)	mt (CA)	wt (CC)
38	mt (GA)	mt (TT)	mt (CC)	wt (AT)	mt (CG)	mt (CA)	mt (AA)
39	mt (GA)	mt (TT)	mt (CC)	wt (AT)	mt (GG)	mt (CA)	mt (AA)
40	mt (GA)	mt (TT)	mt (CC)	mt (AA)	mt (GG)	mt (CA)	mt (AA)
41	mt (GA)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	mt (CA)	mt (AA)
42	mt (GA)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	mt (CA)	mt (AA)
43	mt (GA)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	mt (CA)	mt (CA)
44	mt (AA)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	wt (CC)	mt (CA)
45	mt (AA)	mt (CT)	mt (CC)	mt (AA)	mt (GG)	wt (CC)	mt (CA)
46	mt (GA)	mt (TT)	mt (CC)	mt (AA)	mt (CG)	wt (CC)	mt (CA)
47	mt (GA)	wt (CC)	mt (CC)	mt (AA)	mt (CG)	wt (CC)	mt (CA)
48	mt (GA)	wt (CC)	mt (CC)	mt (AA)	mt (CG)	wt (CC)	mt (AA)
49	mt (AA)	wt (CC)	wt (AC)	mt (AA)	mt (CG)	mt (CA)	mt (AA)
50	mt (AA)	wt (CC)	wt (AC)	wt (AT)	mt (CG)	mt (CA)	mt (AA)

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