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Nucleotide sequence diversities of Chitinase and PR1 genes from *Theobroma cacao* cv. MCC 02 and Sulawesi 1

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Abstract. *Setiowati A, Ardie SW, Santoso TJ, Sudarsono. 2024. Nucleotide sequence diversities of Chitinase and PR1 genes from* Theobroma cacao *cv. MCC 02 and Sulawesi 1. Biodiversitas 26: 1-13.* Cocoa (*Theobroma cacao*) faces vascular streak dieback (VSD) disease caused by *Ceratobasidium theobromae*, so disease control strategies are necessary, such as developing disease-resistant cocoa cultivars. Characterization of Chitinase (*Chi*) and Pathogenesis Related Protein 1 (*PR1*) could be the starting point for such purposes. This research aimed to characterize *Chi*-like and *PR1*-like genes from cocoa cv. MCC 02 (VSD resistant) and Sulawesi 1 (SUL 1, susceptible). The *Chi*-like and *PR1*-like amplicons were generated from cocoa cultivars, and sequencing of the amplicons generated 858 bp (*Chi*-like) and 569 bp (*PR1*-like). The multiple sequence alignment (MSA) of seven *Chi*-like fragments from two cocoa clones identifies four non-synonymous and two synonymous SNPs. The MSA of seven *PR1*-like fragments from two cocoa clones identifies three non-synonymous and two synonymous SNPs in the *PR1*-like coding and two SNPs in the 3'-non-coding regions. Phylogenetic tree construction among four *TcChi*-like accessions and 36 accessions from the NCBI Database indicates that all cocoa *Chi*-like genes are clustered in Clade II, separated from *Chi*-like of other species that belong to Clade I or Clade III. Phylogenetic tree construction among 24 accessions of *PR1*-like sequences from various plant species indicates that the cocoa *PR1*-like gene sequences are more closely related to *Herrania umbratica* and *Theobroma grandiflorum.*

Keywords: Multiple sequence alignment, pathogenesis-related protein 1, SNP identification, *Theobroma cacao*, vascular streak dieback resistant

INTRODUCTION

Indonesia was the world's third-largest cocoa exporter 2017 (Augustin et al. 2022). Four provinces in Sulawesi (i.e., Central, Southeast, South, and West Sulawesi) contributed the largest cocoa (*Theobroma cacao*) plantation and annual production (Central Bureau of Statistics 2023). However, cocoa plantations have continuously decreased from 2018 to 2022 by up to 11.8% (Central Bureau of Statistics 2023). The cocoa production in Indonesia decreased by 5.46% in 2022 (Central Bureau of Statistics 2023), partly because of the poor planting material qualities, pest and disease attacks, and non-inadequate cultivation practices (Farhanandi and Indah 2022). Most of the cocoa varieties are still vulnerable to pests and diseases (Rubiyo 2013), such as cocoa pod borer (CPB), cocoa pod rot (CPR) (Defitri 2017), and vascular streak dieback (VSD) (Defitri 2019).

Ceratobasidium theobromae is the causal agent of VSD, which become a significant threat to cocoa production in Indonesia (Hamdi and Lakani 2021). VSD infection blocks the xylem vessels, which leads to leaf chlorosis, abscission, and dieback in cocoa branches (McMahon et al. 2018). VSD infection in susceptible cocoa clones, such as cocoa cv. Sulawesi 1 (SUL 1) could result in mortality of more than 59% of the plantations (Taufik et al. 2021). Therefore, it is crucial to have VSD-resistant cocoa clones to keep cocoa productivity. Therefore, developing VSD-resistant cocoa clones is essential. Understanding morphological, anatomical, and other characteristics associated with cocoa responses to VSD is necessary (Farhanandi and Indah 2022).

Most cocoa clones are genetically heterozygous in most loci, controlling the phenotypic characteristics. Therefore, segregated progenies of selected high-yielding and VSD-resistant cocoa cultivars could be used to select desirable individuals (Anita-Sari and Susilo 2011). Superior cocoa cultivars can be developed using conventional breeding and biotechnological approaches (Rubiyo et al. 2010; Agung et al. 2016; Tasma 2017). Hybridizing superior cocoa cultivars has improved the yield of cocoa varieties in Indonesia. The conventional breeding approach needs extensive field evaluations (Farid et al. 2021) and requires 15-20 years for field assessment (Tasma 2017). On the other hand, non-conventional breeding incorporates molecular techniques to aid the identification of desirable individual progenies, which shortens the selection cycle (Rubiyo 2013). Moreover, the development of DNA marker technology could speed up the processes of conventional cocoa breeding approaches (Tasma 2017).

The association between DNA markers and the quantitative trait loci (QTL) for various essential phenotypes has previously been reported by Maximova et al. (2006), Rubiyo et al. (2010), Agung et al. (2016), and Fang et al. (2019). Disease resistance and responsive genes could be used to develop molecular markers to identify VSD-resistant cocoa cultivars. Once isolated and sequenced, the nucleotide sequence diversity of the genes could be used to develop molecular markers based on single nucleotide polymorphism (SNP) (Allegre et al. 2012) due to single-base DNA substitution mutations (Mathur and Jha 2020). SNP-based markers have been widely used as gene-specific markers in plant breeding (Jehan and Lakhanpaul 2006; Livingstone et al. 2011; de Wever et al. 2019; Carranza et al. 2020; Gutiérrez et al. 2021). The identified SNP sites could be converted into allele-specific single nucleotide amplified polymorphism (SNAP) markers and used as indirect indicators of VSD-resistant phenotype. This study aimed to characterize the nucleotide sequence diversity of Chitinase (*Chi*) and Pathogenesis-Related Protein 1 (*PR1*) genes from cocoa cv. MCC 02 (VSD resistant) and SUL 1 (VSD susceptible). This research was conducted by designing the *Chi* and *PR1* specific primers, isolating and sequencing the *Chi* and *PR1* specific amplicons, and analyzing their nucleotide sequences. Subsequently, the presence of SNPs was identified, and the phylogenetic tree was evaluated based on the nucleotide sequences of the *Chi* and *PR1* genes from cocoa and other related accessions in the NCBI GenBank DNA Database.

MATERIALS AND METHODS

Plant materials, VSD inoculation, and response characterization

The cocoa genetic materials used were clonally propagated of cocoa cv. MCC 02 and SUL 1 are maintained at the Industrial and Beverage Crops Research Institute field experimental station, Sukabumi, West Java, Indonesia. The cocoa cv. MCC 02 was also released as a superior cocoa cultivar by the Minister of Agriculture Decree No. 1082/Kpts/SR.120/10/2014. The MCC 02 is resistant to pests and diseases and has a cocoa bean yield of 3.1 tons per ha. The cocoa cv. SUL 1 was officially released as a superior cocoa cultivar by the Minister of Agriculture Decree No. 1082/Kpts/SR.120/10/2014. The SUL 1 cocoa bean yield is 1.8-2.5 tons per ha. Seed-derived planting materials were germinated and grown in polybags to evaluate the response of MCC 02 and SUL 1 cultivars. Before VSD inoculation, one-year-old planting materials were maintained in a screen house at the Industrial and Beverage Crops Research Institute field experimental station. Eight selected seedlings for each MCC 02 and SUL 1 cocoa clone were tested to determine their response to VSD infection.

Inoculation of the tested planting materials against VSD was conducted using the natural infection method by placing them under the VSD-infected cocoa cv. BL 50 in the field garden. Occurrences of VSD infection symptoms were observed and recorded every two weeks for four months after inoculation, including leaf symptoms (chlorosis of the leaf and necrosis of the leaf tip and lamina, and blackish-brown dots at the petiole) and branch symptoms (xylem discoloration and die back).

Procedures

DNA isolation

DNA isolation was carried out using the standard cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). Samples of fresh cocoa leaves of 0.3 g were cut and homogenized in 2.5 mL of extraction buffer, added with 0.05 g of polyvinyl pyrrolidone (PVP) and 5 µL of mercapto-ethanol. The ground tissues were transferred into a 2 mL tube, incubated for one hour in a water bath at 65°C, and centrifuged for 15 minutes at 13,500 rpm. The 800 µL supernatant was transferred into a new 2 mL tube, and 800 µL of chloroform isoamyl alcohol (CIA) was added. After gentle mixing, the solution was centrifuged for 15 minutes at 13,500 rpm. Next, 500 µL of the supernatant was transferred into a 1.5 mL tube, and 250 µL of isopropanol and 8 µL of 1% NaOAC were added. The mixture was mixed with gentle inversion until DNA strands occurred, and then the mixture was incubated at room temperature for 10 minutes. The DNA strands were centrifuged for 15 minutes at 13,500 rpm, the supernatant removed, and the DNA pellets dissolved in 250 µL TE buffer. After addition of 2.5 µL RNAse, the dissolved DNA was incubated at 37°C for 30 minutes, 25 µL of NaOAC, and 600 µL of cold 95% ethanol was added. The mixtures were incubated at -20°C for 45 minutes, centrifuged at 13,500 rpm for 10 minutes, the supernatant removed, and the pellet washed with 500 µL of cold 70% ethanol. The washed pellet was centrifuged at 13,500 rpm for 10 minutes, the ethanol was removed, and the DNA pellets were air-dried for 12 hours. Finally, the dried DNA pellets were dissolved in 200 µL TE buffer.

The DNA stock quality and quantity were figured out using a nanodrop. The DNA integrity was tested in an agarose gel 1% electrophoresis using 1× Tris-Acetate-EDTA (TAE) buffer, 90 V, for 30 minutes. After staining with GelRed, the DNA was visualized on a UV transilluminator and photographed for recording.

Primer design

Partial DNA of the gene encoding Chitinase (*Chi*) and PR1 proteins (*PR1*) was isolated by polymerase chain reaction (PCR) using gene-specific primers. The primers were designed based on the nucleotide sequences of the *Chi* (XM_018127106.1, predicted mRNA of *T. cacao* Chitinase 2-like [LOC108663365]) and *PR1* gene (XM_007041360.2, predicted mRNA of *T. cacao* Pathogenesis-Related Protein 1 [LOC18607284]), in the annotated *T. cacao* genome reference ASM3589663 v1 (GCA_035896635.1) available in the NCBI genome Database (https://www.ncbi.nlm.nih.gov/genome/browse). Primer design was conducted using the online Primer3 Plus software (https://primer3plus.com/). The sequences of the designed gene-specific primers for *chi* and *PR1* genes are presented in Table 1.

Table 1. Target genes, primer names, primer sequences, the expected amplicon sizes, and the primer combinations' annealing temperature (Ta), designed to amplify fragments of *Chitinase* and *PR1* genes

Genes	Primers	Primer sequences (5′-3′)	Expected amplicon size (bp)	Ta (°C)
Chitinase	*TcChi*1	F: TTTCCTCAACCCCTGCAGTA R: GTTGATCTGGCCAACAAGGT	700	50
Chitinase	*TcChi*2	F: GAGCTGAGGACAAGGGTGTT R: GTTGATCTGGCCAACAAGGT	800	50
PR1 Protein	*TcPR1*a	F: TGTCTCCTTGGCCTAGCAAT R: TTGCCTGGAGGATCGTAGTT	400	50
PR1 Protein	*TcPR1*b	F: TGTCTCCTTGGCCTAGCAAT R: AATGGTGTCGCCCACATAAC	500	50

DNA amplification

Chitinase and *PR1* genomic DNA fragments were amplified using the appropriate primers (Table 1) in a DNA thermal cycler (Bio-Rad, USA). The PCR reaction of a total volume of 20 µL comprised 20 ng of DNA template (2 µL of 10 ng/µL DNA stock), 2 µL of 10× buffer (Kapa Biosystems, USA), 10 mM of dNTP mix (Kapa Biosystems, USA), 0.5 µM of forward and reverse primers, and 0.5 U of *Taq* DNA polymerase (Kapa Biosystems, USA). Subsequently, ddH_2O was added to the mixture up to 20 µL. The PCR amplification was conducted using the following steps: initial denaturation at 94°C for 4 minutes, 35 cycles comprised of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and primer extension at 72°C for 45 seconds, and one cycle of final extension at 72°C for 7 minutes. The generated PCR amplicons were checked in 1% (w/v) agarose gel electrophoresis using 1× TBE buffer at a constant voltage of 100 V for 30 minutes. After staining with GelRed, the amplicons were visualized on a UV transilluminator and photographed for recording.

DNA sequencing

The PCR amplification shows that a single band was sent to the 1st Base Asia Malaysia for direct PCR DNA sequencing. Before DNA sequencing, PCR clean-up was conducted, the PCR product was electrophoresed in agarose gel, and the target amplicon was cut from the gel and purified using a GeneAid fragment extraction kit (https://www.geneaid.com/, Taiwan). The direct amplicon sequencing was conducted using the BigDye® Terminator v3.1 cycle sequencing and fractionated using automatic capillary electrophoresis on Applied Biosystems DNA sequencer. Each *Chi* or *PR1* gene amplicon was sequenced from both directions using the appropriate forward and reverse primers, respectively. Low-quality nucleotide sequences were trimmed and removed using Geneious Prime Software (https://www.geneious.com). Subsequently, the identity of the high-quality sequences was validated against all nucleotide accessions in the NCBI GenBank DNA Database (https://ncbi.nlm.nih.gov) using the nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, Ismail 20, Ismail 2022). The structure of the *Chi* or *PR1* gene was analyzed by comparing the determined sequences against the proper reference sequences (XM_018127106.1 and XM_007041360.2). Representative *Chi* and *PR1* accessions from various crops identified using BLAST analysis were downloaded from the NCBI GenBank DNA Database and used for the following multiple sequence alignment (MSA) analysis.

Data analysis

The basic sequence handling and MSA were done using Geneious Prime Software (https://www.genious.com). Only high-quality sequences of the *Chi* or *PR1* were used in the MSA and phylogenetic analysis. The MSA and phylogenetic analysis were conducted using global alignment with free end gaps in the Geneious Prime software (https://www.geneious.com), following the generic sequence analysis protocol for plants. The presence of single nucleotide polymorphisms (SNPs) due to substitution mutations was evaluated based on the MSA outputs. The SNPs were recorded in the coding sequences and the 3'-end non-coding region (NCR) of the genes being assessed.

The phylogenetic tree was constructed using Geneious Tree Builder of the Geneious Prime software (https://www.geneious.com), with the Tamura-Nei genetic distance and the Neighbour-Joining (NJ) tree-building method (Davidson and del Campo 2020; Clausen 2023). The *Chi* (XM050515411) and *PR1* (XM022906495) genes of *Durio zibethinus* were used as the outgroup for the tree construction. Genetic distance among accessions was calculated based on the Tamura - Nei genetic distance model (Tamura and Kumar 2013), and resampling analysis was conducted by bootstrap using a random seed and 1,000 replications.

RESULTS AND DISCUSSION

VSD inoculation and response characterization

The observed symptoms of VSD infection in the cocoa cv. BL 50 is presented in Figure 1. The symptoms include leaf chlorosis on the second or third flush (Figures 1.A and 1.B), necrosis at the leaf tip or lamina (Figure 1.C), the three blackish-brown dots in the petioles (Figures 1.D and 1.E), and blackish brown stripes in the xylem tissue (Figures 1.F and 1.G). When the younger cocoa leaves are infected, the symptoms spread throughout the young branches (Trisno et al. 2016). Trees of cocoa cv. BL 50,

showing the VSD symptoms of infection, was selected and used as an inoculum source for tested seedling inoculation. Natural VSD inoculation was done by placing the tested seedlings under VSD-infected cocoa cv. BL 50 tree.

The seedlings of cocoa cv. MCC 02, naturally infected in this study, showed no symptoms of VSD disease infection. None of this study's MCC 02 seedlings naturally infected with VSD disease showed any symptoms. The attached and detached leaves of cocoa cv. MCC 02 seedlings showed no chlorosis nor necrosis symptoms of VSD infection. Moreover, the absence of three blackish-brown dots at the base of the leaf petiole also indicates the absence of VSD infection. Representative photographs of the natural VSD inoculation results on MCC 02 seedlings were presented in Figures 2.A-2.C. In contrast, some cocoa cv. Sulawesi 1 (SUL 1) seedlings naturally infected in this study showed symptoms of VSD disease infection. The SUL 1 seedlings naturally infected with VSD disease in this study showed typical VSD disease infection symptoms in the attached and detached leaves. The attached and detached leaves of cocoa cv. SUL 1 seedlings showed chlorosis and necrosis symptoms of VSD infection. Moreover, three blackish-brown dots at the base of the leaf petiole confirm the presence of VSD infection. Representative photographs of the natural VSD inoculation results on SUL 1 seedlings were presented in Figures 2.D-2.F.

The VSD disease infection symptoms are the same as those observed by Rosmana et al. (2019), which indicate that the VSD disease infection dynamics are characterized by rapid chlorosis and leaf necrosis development, which eventually cause the branches dieback and plant death. Based on the occurrences of the VSD disease infection symptoms among the naturally inoculated seedlings, cocoa cv. MCC 02 is a VSD disease-resistant cultivar because the tested planting materials showed no symptoms of VSD disease infection. On the other hand, cocoa cv. SUL 1 is a VSD disease-susceptible because the tested planting materials showed symptoms of VSD disease infection. Based on the study conducted by Zakariyya and Yuliasmara (2015), top grafting of cocoa cv. MCC 02 and SUL 1, MCC 02 performs better than SUL 1. The MCC 02 has been previously reported as resistant to moderate resistance against VSD disease infection (Widiyani et al. 2022), while SUL 1 was reported as susceptible to VSD disease infection (Nurlaila et al. 2020). Therefore, MCC 02 and SUL 1 were suitable for comparing genes associated with VSD disease infection response between the resistant (MCC 02) and the susceptible (SUL 1) cocoa cultivars.

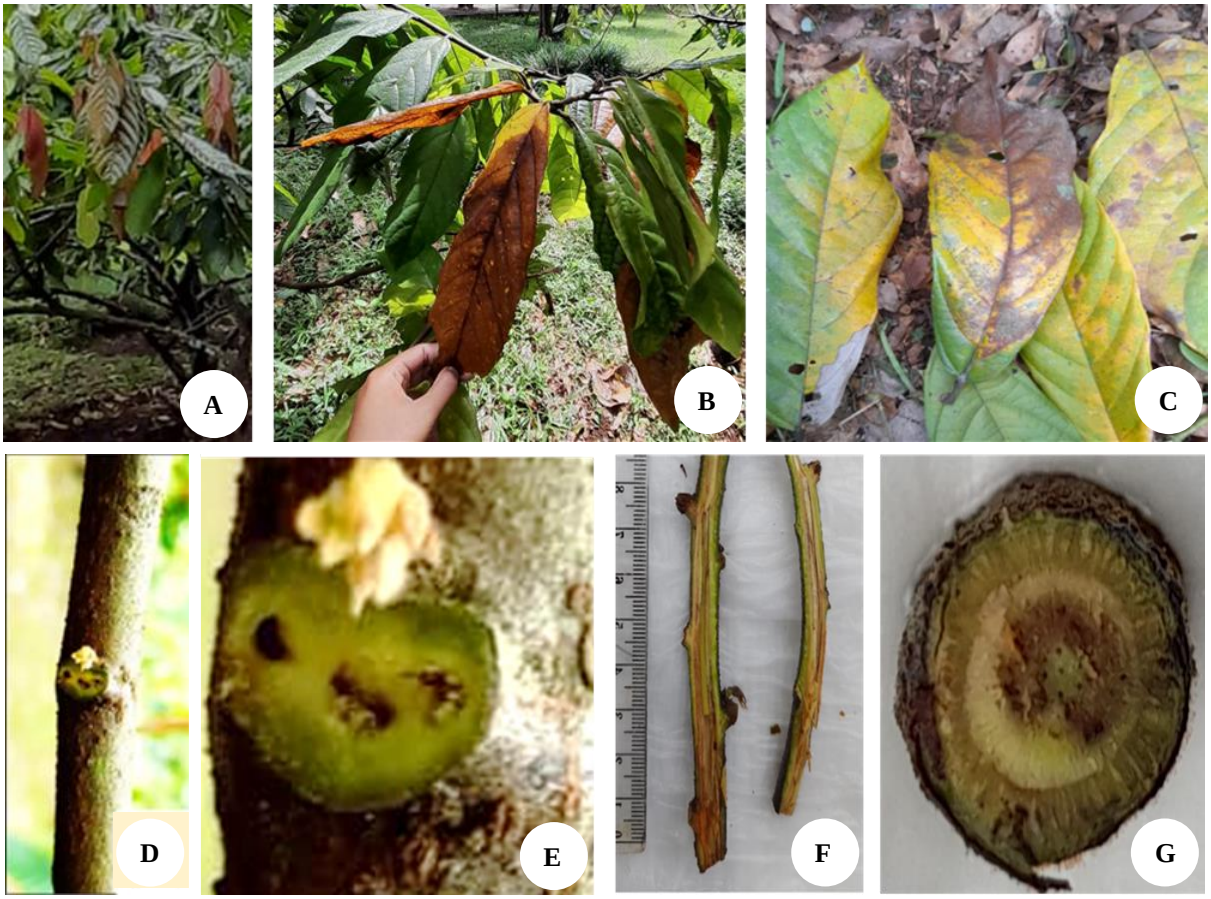


Figure 1. Symptoms of the vascular streak dieback (VSD) disease infection in the susceptible cocoa cv. BL 50 in the experimental garden of Industrial and Beverage Crops Research Institute, Sukabumi, West Java, Indonesia: A. Chlorosis on leaves of BL 50; B. Attached leaf with necrosis symptoms on the tip of leaf lamina; C. Detached leaf with necrosis symptoms; D and E. Three dots of blackish brown spots at the petiole base; F and G. Blackish brown stripes and necrosis tissue symptoms of the infected xylem

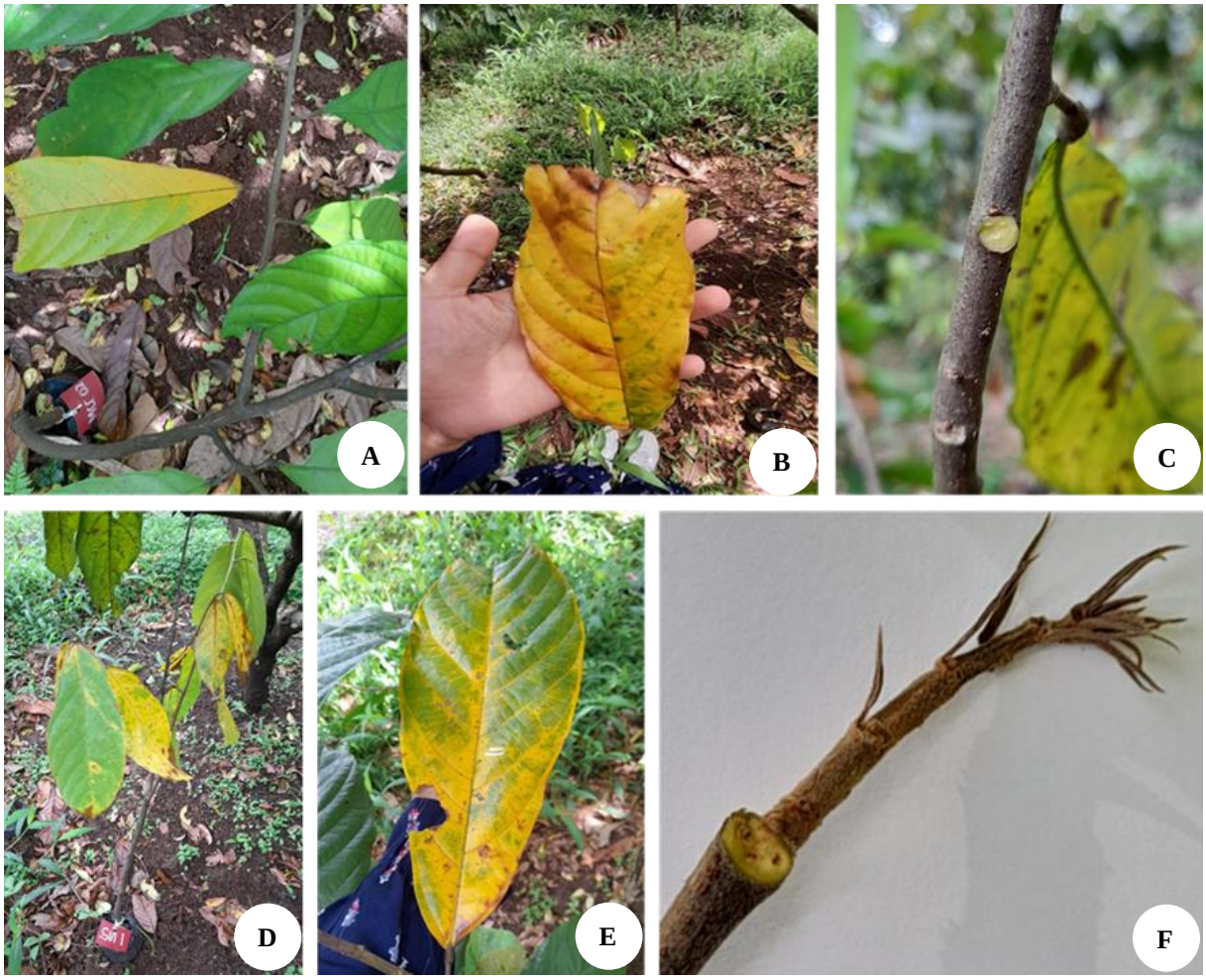


Figure 2. Representative of the tested cocoa cv. MCC 02 (A-C) and Sulawesi 1 (SUL 1) (D-F) after the vascular streak dieback (VSD) disease infection: A. Attached and B. Detached leaves of cocoa cv. MCC 02 without symptoms of VSD infection; C. The absence of three blackish-brown dots at the base of the leaf petiole - shows the absence of VSD infection; D. Attached and E. Detached leaves of cocoa cv. SUL 1 with leaf chlorosis and necrosis; F. Three dots of blackish brown spots at the petiole base of cocoa cv. SUL 1), indicating the presence of VSD disease symptoms

Primer design for *Chi* and *PR1* amplification

Two sets of primers have been developed using the *Chi* (XM_018127106.1, predicted mRNA of *T. cacao* Chitinase 2-like [LOC108663365]) and *PR1*-like gene (XM_007041360.2, predicted mRNA of *T. cacao* Pathogenesis-Related Protein 1 [LOC18607284]) as the reference sequences (Table 1). Positions of the designed *Chi*-like and *PR1*-like specific primers compared to their gene structure, the amplicon sizes, and the representative amplicon generated using the primers are presented in Figures 3.A-3.C. The expected amplicon sizes generated using *TcChi1* (F1 and R) are 25 bp shorter than that of *TcChi2* (F2 and R) primer pairs (Figure 3.A). However, the estimated amplicon sizes based on the 1% agarose gel electrophoresis gel photographed are similar (Figure 3.C). On the other hand, the expected amplicon sizes generated using *TcPR1* (F and R1) are 120-130 bp shorter than that of *TcPR1b* (F and R2) primer pairs (Figure 3.C). Hence, the estimated amplicon sizes based on the 1% agarose gel electrophoresis gel photographed differ in size (Figure 3.C). PCR amplification of DNA fragment of the target gene's DNA fragment has successfully been done using gene-specific primers (Syamsidi et al. 2021). This study used an in-silico approach to design *Chi*-like and *PR1*-like gene-specific primers and successfully used the primers to generate amplicons using template DNA of cocoa cv. MCC 02 and SUL 1.

The designed gene-specific primers developed in this study could successfully generate the expected size amplicons from the total nucleic acids isolated from cocoa cv. MCC 02 and SUL 1. Gene-specific primers have been used to generate DNA fragments of the target genes from *Phalaenopsis* orchid (Elina et al. 2017), coconut (Hatta et al. 2023), and snake fruit (*Salacca zalacca*) (Prihatini et al. 2023). The gene-specific primers usually range from 18-25 (Purwakasih and Achyar 2021).

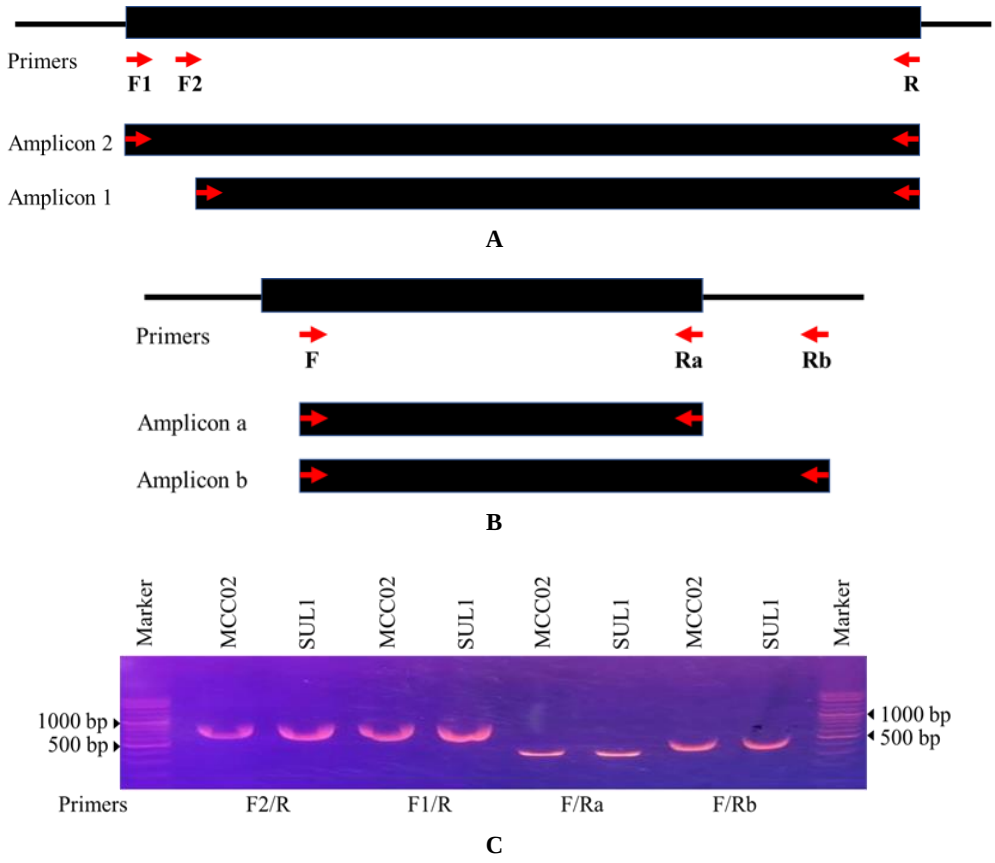


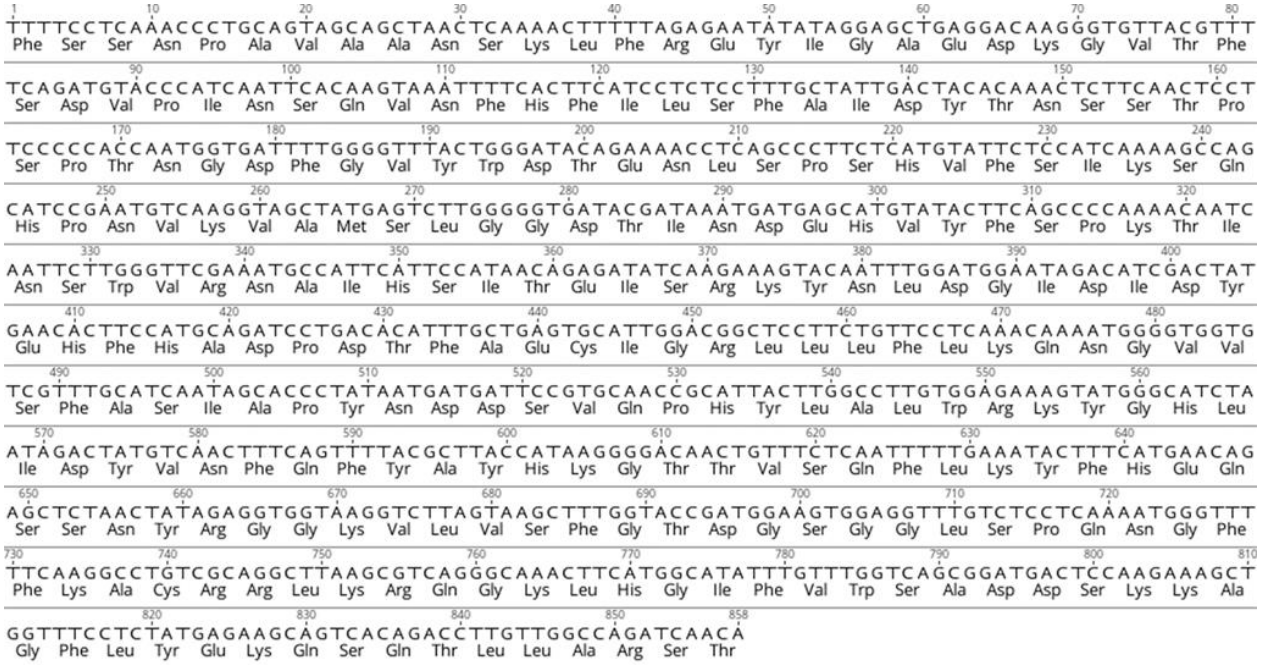
Figure 3. Structure of the Chitinase (*Chi*)-like and PR1 Protein (*PR1*) -like genes, the positions of gene-specific primers, and the generated amplicons: A. *Chi*-like gene structure, positions of the *TcChi1* (F1 and R) and *TcChi2* (F2 and R) primer pairs, and sizes of the expected amplicon 1 and 2; B. *PR1*-like gene structure, positions of the *TcPR1*a (F and Ra) and *TcPR1*b (F and Rb) primer pairs; C. agarose gel electrophoresis results of the generated amplicons by Chitinase-like specific F2/R and F1/R primers and PR1-like F/Ra and F/Rb specific primers using either MCC 02 or SUL

Partial *Chi*-like and *PR1*-like amplicon DNA sequences and their identity

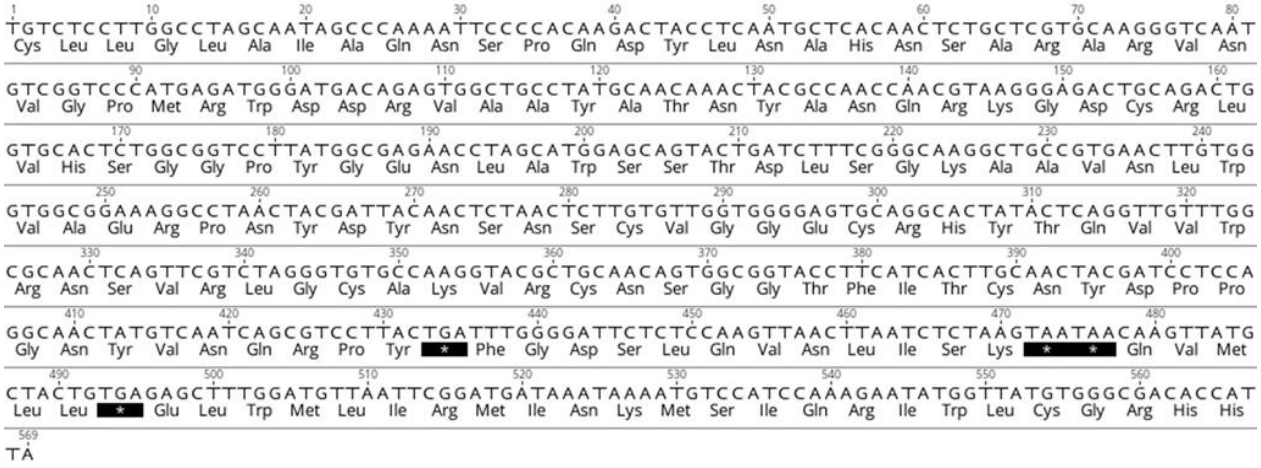
DNA sequencing of the two partial *Chi* amplicons showed 795 bp (F2/R amplicons) and 858 bp nucleotides (F1/R amplicons). The longest *Chi*-like amplicon DNA sequencing results identified sequences from nucleotide numbers 55 to 912, while the shortest identified sequences from nucleotide numbers 117 to 912 of the reference predicted *Chi*-like mRNA (XM_018127106.1). Translation of the partial *Chi* amplicon sequences results in 265-286 amino acid residues out of 304 total CHI proteins. Therefore, the identified nucleotide sequences for the *Chi*-like gene are the partial coding regions. Representative nucleotide sequences and their partial *Chi*-like gene translation products isolated from cocoa cv. MCC 02 is presented in Figure 4.A.

On the other hand, DNA sequencing of the two partial *PR1*-like amplicons showed 410 bp (the F/Ra amplicon) and 569 bp nucleotides (the F/Rb amplicon). The longest *PR1*-like amplicon DNA sequencing results identified sequences from 37 to 471, while the shortest identified sequences from 37 to 446 of the reference predicted *PR1*-like mRNA coding sequences (XM_007041360.2). Translation of the partial *PR1*-like amplicon sequences results in 136-144 amino acid residues out of 156 total amino acid residues of *PR1*-like proteins. Moreover, the longest *PR1*-like amplicons contain 134 bp of the 3'-end non-coding region. Therefore, the identified nucleotide sequences for the *PR1*-like genes are partial coding regions. Representative nucleotide sequences and their partial *PR1*-like gene translation products isolated from cocoa cv. MCC 02 is presented in Figure 4.B.

The nucleotide BLAST analysis results confirmed the identity of the generated putative *TcChi* and *TcPR1* gene sequences as Chitinase 2-like and PR1 Protein-like genes from cocoa. Representative of the BLAST analysis results using putative *Chi*-like and *PR1*-like genes isolated in this study are presented in Table 2. The percent identity of the putative partial *Chi* nucleotide sequences generated in this study ranges from 98.0 to 99.4% compared to the predicted chitinase of two mRNA sequences from *T. cacao* in the NCBI GenBank DNA Database (XM_018127106.1 and XM_007016547.2) (Table 2). Moreover, the percentage identity of the putative partial *Chi*-like nucleotide sequences generated in this study ranges from 75.8 to 89.2% compared to the predicted *Chi*-like mRNA of *Malus sylvestris* (XM_050255276.1), *Gossypium hirsutum* (XM_016889538.2), *G. arboreum* (XM_017781381), and *Durio zibethinus* (XM_022876628.1) (Table 2).



A



B

Figure 4. Representative nucleotide sequences and their translation products of A. partial Chitinase (*Chi*)-like gene (acc. no. PQ563280); B. partial PR1 Protein (*PR1*)-like gene (acc. no. PQ563284) isolated from cocoa cv. MCC 02. ▮ is a stop codon

Meanwhile, the percent identity of the putative partial *PR1* nucleotide sequences generated in this study ranges from 99.3-99.5% compared to the nucleotide positions range from nucleotide no. 1710323 to 1710891 (LT594789.1) and from nucleotide no. 41717712 to 41718280 (OY284459.1) in the assembled chromosome 2 of *Theobroma cacao* genome that were found using BLAST nucleotide as having sequence identities to the partial PR1-like gene in the NCBI GenBank DNA Database (Table 2). Moreover, the percent identity of the putative partial *PR1* nucleotide sequences generated in this study ranges from 72.6 to 95.7% compared to the predicted *PR1*-like mRNA of *Cucumis sativus* (XM_011660559.2), *Vitis riparia* (XM_034825915.1), *D. zibethinus* (XM_022906491.1), and *Herrania umbratica* (XM_021444586.1) (Table 2). The percent identity BLAST results show that the putative partial *Chi*-like and *PR1*-like genes isolated and sequenced in this study are indeed *Chi*-like and *PR1*-like genes from *T. cacao* cv. MCC 02 and SUL 1.

Chitinase is an enzyme that hydrolyzes chitin at the β-1,4-N-acetyl-glucosamine, becoming the N-acetyl-glucosamine monomer. Chitinase is also used in waste management, insect biocontrol (Pratiwi et al. 2015), and phytopathogen biocontrol (Dukariya and Kumar 2020). Meanwhile, *PR1* is involved in plant defense responses and increases resistance against plant pathogens (Han et al. 2023).

Table 2. Representative results of the Basic Local Alignment Sequence Tool (BLAST) using the nucleotide sequences of putative Chitinase (*Chi*) and PR1 Protein (*PR1*) from *Theobroma cacao* cv. MCC 02 and SUL 1 as queries and confirmation of their identities as *Chi* and *PR1* genes

Accession number	Predicted protein function	Plant species	E-value	Percent identity (%)
XM_018127106.1	*Chi*-like	*T. cacao*	0.0	99.4
XM_007016547.2	*Chi*-like	*T. cacao*	0.0	98.0
XM_022876628.1	*Chi*-like	*Durio zibethinus*	0.0	89.2
XM_017781381.2	*Chi*-like	*Gossypium arboreum*	0.0	86.7
XM_016889538.2	*Chi*-like	*G. hirsutum*	0.0	86.2
XM_050255276.1	*Chi*-like	*Malus sylvestris*	2e-159	75.8
OY284459.1*	*PR1*-like	*T. cacao*	0.0	99.5
LT594789.1*	*PR1*-like	*T. cacao*	0.0	99.3
XM_021444586.1	*PR1*-like	*Herrania umbratica*	0.0	95.7
XM_022906491.1	*PR1*-like	*D. zibethinus*	3e-143	88.8
XM_034825915.1	*PR1*-like	*Vitis riparia*	2e-46	75.8
XM_011660559.2	*PR1*-like	*Cucumis sativus*	1e-08	72.6

Multiple sequence alignment analysis and SNP site identification

The MSA analysis showed the presence of high sequence identity among *Chi*-like and *PR1*-like nucleotide sequences. Representative sequence identity among the isolated *Chi*-like (NCBI acc. No. PQ563280, PQ563281, PQ563282, and PQ563283) and *PR1*-like (NCBI acc. No. PQ563284, PQ563285, PQ563286, PQ563287) genes in this study was presented in Figures 5.A and 5.B. Six SNPs are shown in the isolated and sequenced *Chi*-like amplicons of cocoa (i.e., nucleotide position no. 55 to 912, Figure 5.A). The identified SNPs are all in the coding region, at the nucleotide position no. 163, 212, 228, 419, 510, and 670 from the start codon of the reference predicted *Chi*-like gene (XM_018127106.1) (Figure 5.A). Further analysis showed that among the six identified SNP loci in the partial *Chi*-like gene coding region, the substitution mutations have resulted in two synonymous SNPs and four non-synonymous SNPs. The existing SNP sites in the evaluated partial *Chi*-like nucleotide sequences from cocoa cv. MCC 02 and SUL 1 and their characteristics are presented in Table 3.

Seven SNPs in cocoa are isolated and sequenced partial *PR1*-like amplicons (i.e. nucleotide position no. 37-605) (Figure 5.B). The identified SNPs are both in the coding sequences (SNP1-5) and the 3'-end non-coding region (SNP 6-7) at the nucleotide position no. 176, 243-244, 260, 411, 478, and 525 from the start codon of the reference predicted *PR1*-like mRNA (XM_007041360.2). Further analysis showed that the substitution mutations have resulted in two synonymous and three non-synonymous SNPs among the five identified SNP loci in the coding region of the partial *PR1*-like gene. The existing SNP sites in the evaluated partial *PR1*-like nucleotide sequences from cocoa cv. MCC 02 and SUL 1 and their characteristics are presented in Table 3.

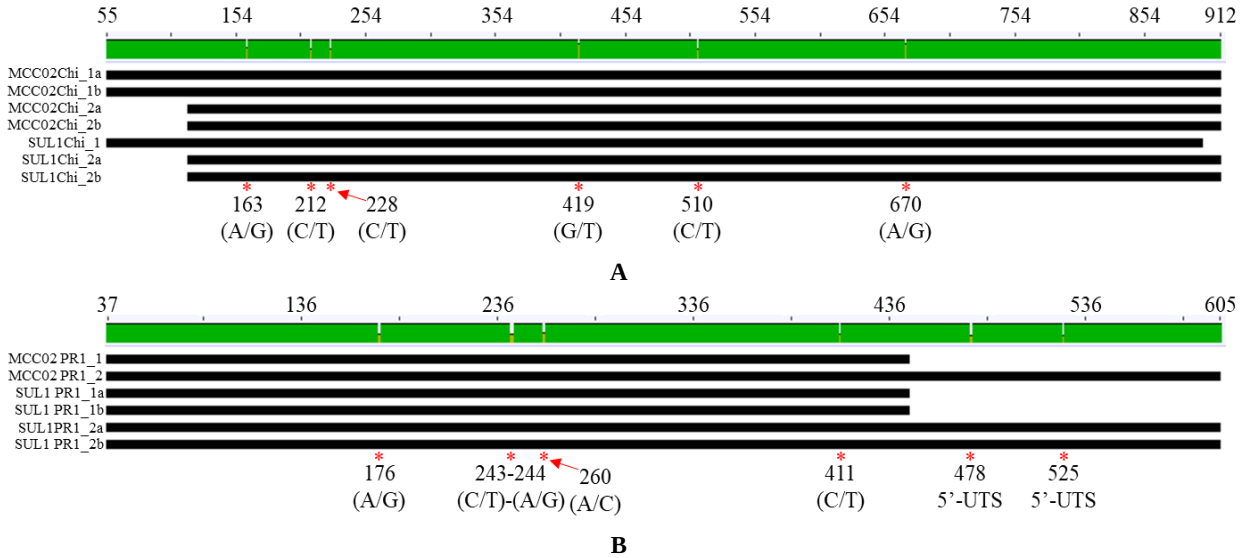


Figure 5. The identified single nucleotide polymorphism (SNP) sites among nucleotide sequences of A. partial Chitinase (*Chi*)-like; and B. PR1 Protein (*PR1*)-like genes isolated from cocoa cv. MCC 02 and SUL 1

Table 3. The identified single nucleotide polymorphism (SNP) sites among nucleotide sequences of the partial Chitinase (*Chi*)-like and PR1 Protein (*PR1*)-like genes isolated from cocoa cv. MCC 02 and SUL 1

Genes and SNPs	Positions	Nucleotide substitution	Amino acid changes	SNP types
Chitinase (*Chi*)-like gene				
SNP1	163	A/G	Asn → Asp	Non-synonymous
SNP2	212	C/T	Thr → Ile	Non-synonymous
SNP3	228	C/T	Asn → Asn	Synonymous
SNP4	419	G/T	Ile → Arg	Non-synonymous
SNP5	510	C/T	Leu → Leu	Synonymous
SNP6	670	A/G	Ile → Val	Non-synonymous
PR1 Protein (*PR1*)-like gene				
SNP1	163	A/G	Gln → Arg	Non-synonymous
SNP2	243	C/T	Ser → Ser	Synonymous
SNP3	244	A/G	Thr → Aln	Non-synonymous
SNP4	260	A/C	Lys → Thr	Non-synonymous
SNP5	411	C/T	Gly → Gly	Synonymous
SNP6	478	G/A	-	3'-UTR
SNP7	525	A/C	-	3'-UTR

Multiple sequence alignment (MSA) analysis finds SNP sites from a few DNA sequences. SNP identification has been studied in various accessions of the gene from *Phalaenopsis* orchids (Elina et al. 2017), coconut (Hatta et al. 2023), and snake fruit (*Salacca zalacca*) (Prihatini et al. 2023). Subsequently, the identified SNP loci were used to develop single nucleotide amplified polymorphism (SNAP) markers of the respective crops. Subsequently, the developed SNAP markers were used to study genetic diversity and mutant identification in orchids (Elina et al. 2017; Raynalta et al. 2018), sex phenotype in snake fruits (Prihatini et al. 2023), the specific markers for the yearly coconut height increment (Hatta et al. 2023).

Chi and *PR1* genes could be associated with VSD resistance in cocoa. In general, plants have developed complex defense mechanisms against phytopathogen infections. These defense response mechanisms depend on constitutive and inducible factors (dos Santos and Franco 2023). *Chi* also has a significant role in plant defense response to phytopathogens, especially ones with chitins in their cell wall. For example, the chitinase gene (*PbChi* a1) is a candidate gene for broad-spectrum disease-resistant mechanisms (Zhao et al. 2022). Moreover, chitinase-producing fungi have also been used to control aphids in cotton (Anwar et al. 2023). Meanwhile, Pathogenesis-Related protein 1 (*PR1*) is a plant protein associated with initial fungal infection. Furthermore, Han et al. (2023) reported that plant pathogenesis-related (PR) proteins were initially shown as proteins strongly induced by biotic and abiotic stresses. The identified SNPs in the *Chi* and *PR1* gene nucleotide sequences may be used to develop single nucleotide amplified polymorphism (SNAP). Such SNAP marker development from the shown SNP has been done in coconut (Pesik et al. 2017; Hatta et al. 2023), orchids (Raynalta 2018), and snake fruit (Prihatini et al. 2023). The developed SNAP marker could be used for somaclonal variant identification in orchids (Raynalta et al. 2018), sex phenotype identification in snake fruit (Prihatini et al. 2023), and tagging genes associated with yearly height increment in coconut (Hatta et al. 2023).

Phylogenetic tree of *Chi*-like and *PR1*-like

The partial gene sequences isolated from cocoa cv. MCC 02 (2 accessions) and SUL 1 (2 accessions), identified as Chitinase 2-like based on nucleotide BLAST analysis, were used to construct the Neighbor-Joining phylogenetic tree (Davidson and del Campo 2020; Clausen 2023). Subsequently, 34 accessions of representative *Chi*-like gene sequences from various plant species available in the NCBI GenBank DNA Database were also included in the study. Results of the phylogenetic tree construction using the predicted *Chi*-like mRNA of *Durio zibethinus* (XM_022876628.1) as the outgroup are presented in Figure 6. The results showed that the evaluated *Chi*-like accessions belong to three major clades (Clade I, II and III). Four members of the Clade I include the predicted *Chi*-like mRNA accessions of *Gossypium raimondii* (Gr, XM_012618669.2), *G. hirsutum* (Gh, XM_016865055.2, and XM_016889538.2), and *G. arboreum* (Ga, XM_017781381.2).

Phylogenetic analysis results showed that the six *Chi*-like accessions from *T. cacao* (accessions with red and blue colors) belong to Clade II. The two partial *Chi*-like accessions from cocoa cv. MCC 02 and two from SUL 1 belong to Clade II (Figure 6), along with the two predicted *Chi*-like mRNA accessions from *T. cacao* available in the NCBI GenBank DNA Database (XM_018127106.1 and XM_007016547.2). Meanwhile, the rest of the predicted *Chi*-like accessions (29 accessions) belong to Clade III, such as the predicted *Chi*-like mRNA accessions from *Argentina anserina* (Aa), *Cucumis sativus* (Csa), *Camellia sinensis* (Csi), *Euphorbia lathyris* (El), *Gossypium arboretum* (Ga), *G. hirsutum* (Gh), *G. raimondii* (Gr), *Helianthus annuus* (Ha), *Hevea brasiliensis* (Hb), *Herrania umbratica* (Hu), *Jatropha curcas* (Jc), *Malus domestica* (Md), *M. sylvestris* (Ms), *Manihot esculenta* (Me), *Mangifera indica* (Mi), *Nicotiana tabacum* (Nt), *Populus alba* (Pa), *P. euphratica* (Pu), *P. nigra* (Pn), *P. trichocarpa* (Pt), *Prunus dulcis* (Pd), *Ricinus communis* (Rc), Sesamum indicum (Si), *Solanum tuberosum* (St), *Vitis riparia* (Vr), and *V. vinifera* (Vf). Three accessions are identified as

DNA sequences from chromosome 11 (nucleotide no. 10852803 to 10853589 and 11 10713746 to 10714548) in the assembled genome of *Malus domestica* cultivar Golden Delicious (CP168775.1 [Md]) and from nucleotide no. 53184 to 54002 in the *Passiflora edulis* clone Pe69C7 (AC278163.1 [Ped]) were found using BLAST nucleotide as having sequence identities to the partial PR1 gene (Figure 6).

The partial gene sequences isolated from cocoa cv. MCC 02 (2 accessions) and SUL 1 (2 accessions) are identified as *PR1*-like based on the nucleotide BLAST analysis. The four *PR1*-like accessions from cocoa cv. MCC 02 and SUL 1, six *PR1* accessions from cocoa available in the NCBI GenBank DNA Database, were used to construct the Neighbor-Joining phylogenetic tree (Davidson and del Campo 2020; Clausen 2023). Moreover, 16 accessions of representative *PR1*-like gene sequences from various plant species were also included in the tree construction. Results of the phylogenetic tree construction using the predicted *PR1*-like mRNA of *D. zibethinus* (XM_022906495) as an outgroup are presented in Figure 7. The results showed that the evaluated *PR1*-like accessions belong to two major clades (Clade I and II).

The four partial *PR1*-like accessions from cocoa cv. MCC 02 and SUL 1 belong to Clade I (Figure 7). Moreover, five DNA sequences from chromosome 2 of the assembled genome of *T. cacao* (Tc, CP139292.1, LT594789.1, OY284359.1, OY284449.1, OY284459.1), one from chromosome 2 of the assembled genome of *Theobroma grandiflorum* isolate C1074 (CP142127.1 [Tg]) showing high sequence identities to the partial *PR1*-like gene (i.e. identified using BLAST nucleotide, accessions in brown), and one accession of predicted *PR1*-like mRNA of *T. cacao* (XM_007041360.2) that are available in the NCBI GenBank DNA Database are closely related to the *PR-1*-like from cocoa cv. MCC 02 and SUL 1 (Figure 7). The cocoa *PR1*-like accessions in this study are also closely related to the predicted *PR1-like* mRNA of *Herrania umbratica* (Hu, XM_021444586) (Figure 7).

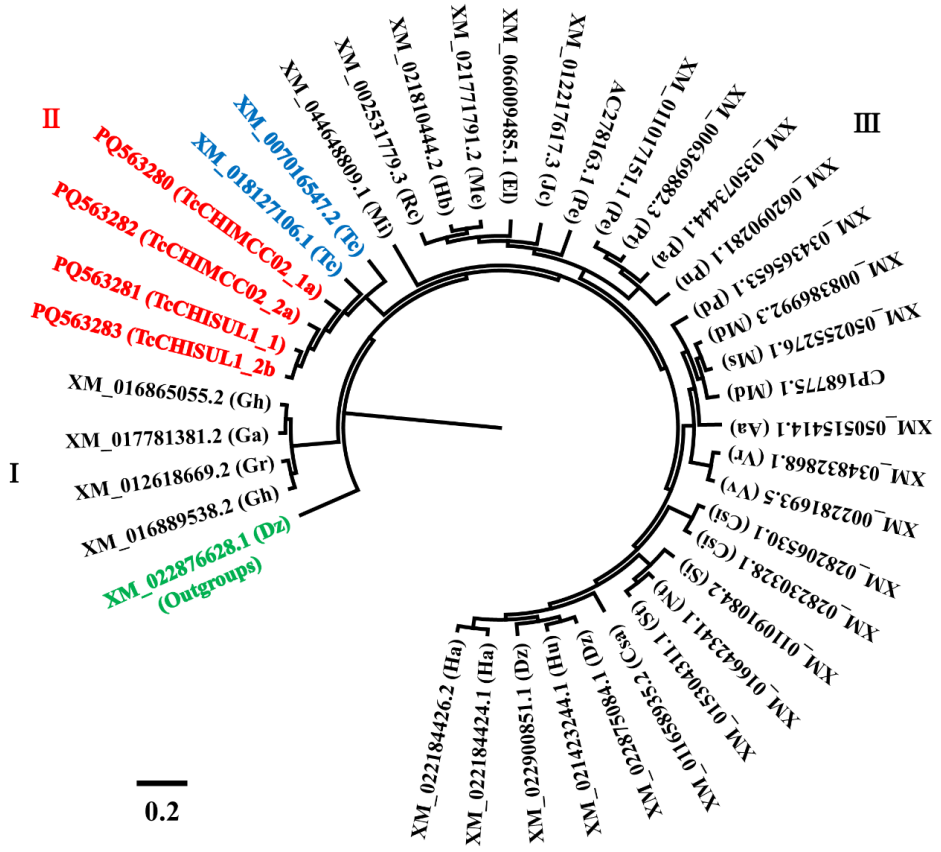


Figure 6. The results of the phylogenetic analysis were conducted using a partial Chitinase gene (*Chi*, accessions in red) isolated from cocoa cv. MCC 02 and SUL 1. The other predicted *Chi*-like mRNA accessions (accessions in black) and DNA sequences from the assembled genome of some species showing high sequence identities to the partial *Chi* gene (i.e. identified using BLAST nucleotide, accessions in brown) that are available in the NCBI GenBank DNA Database were also included in the analysis. The phylogenetic tree was constructed using Geneious Tree Builder of the Geneious Prime software (https://www.geneious.com), with the Tamura-Nei genetic distance model, neighbor-joining tree building method, and predicted *Durio zibethinus Chi*-like (LOC111286591), mRNA (acc. no. XM_022876628.1 [Dz]) as an outgroup (accession in green). All *Chi*-like accessions from the NCBI GenBank DNA Database were identified using BLAST nucleotide analysis, and BLAST outputs were downloaded. The NCBI accession numbers of the *Chi* genes isolated from cocoa cv. MCC 02 are PQ563280 and PQ563282, while those from cocoa cv. Sulawesi 1 are PQ563281 and PQ563283

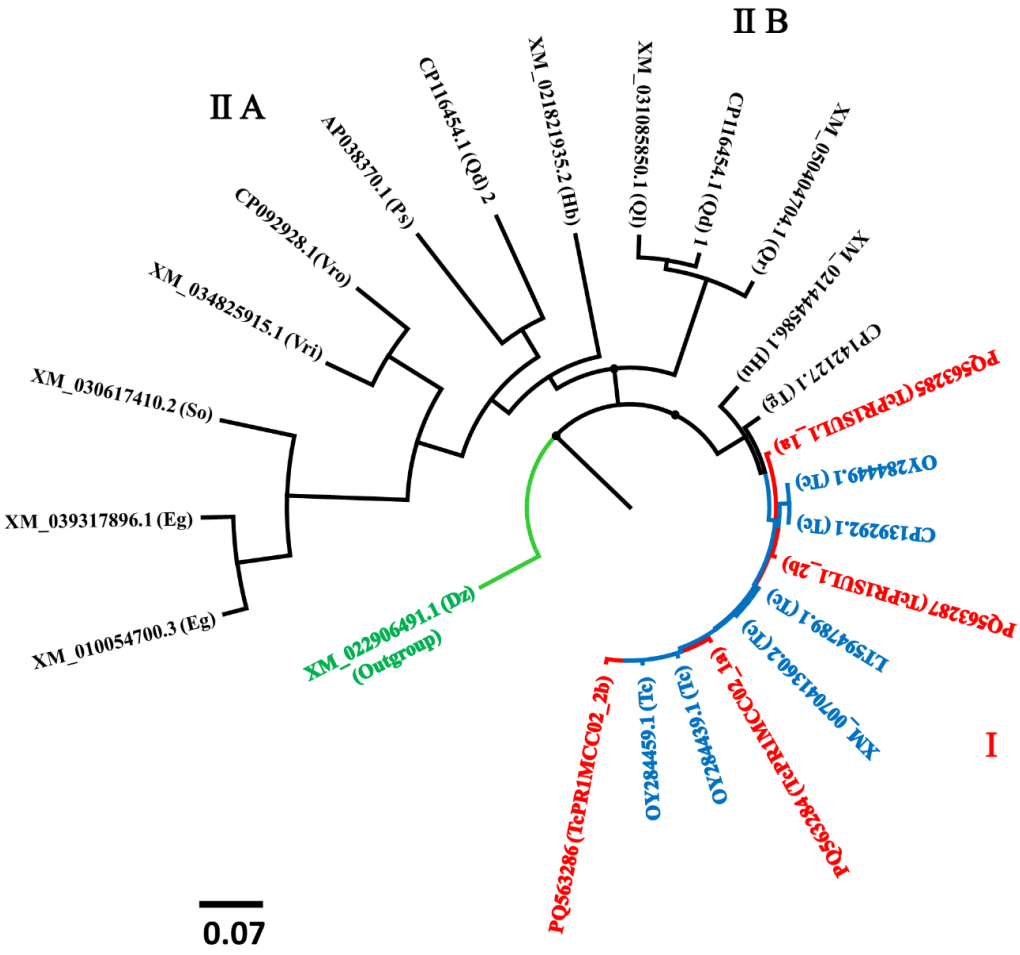


Figure 7. Results of the phylogenetic analysis were conducted using a partial PR1 protein gene (*PR1*, accessions in red) isolated from cocoa cv. MCC 02 and SUL 1. The other predicted *PR1*-like mRNA accessions (accessions in black) and DNA sequences from the assembled genome of some species showing high sequence identities to the partial PR1 gene (i.e. identified using BLAST nucleotide, accessions in brown) are available in the NCBI GenBank DNA Database were also included in the analysis. The phylogenetic tree was constructed using Geneious Tree Builder of the Geneious Prime software (https://www.geneious.com), with Tamura-Nei genetic distance model, neighbor-joining tree building method, and predicted *Durio zibethinus PR1*-like (LOC111308175), mRNA (acc. no. XM_022906491.1 [Dz]) as the outgroup (accession in green). All *PR1-like* accessions from the NCBI GenBank DNA Database were identified using BLAST nucleotide analysis, and BLAST outputs were downloaded. The NCBI accession numbers of the *PR1* genes isolated from cocoa cv. MCC 02 are PQ563284 and PQ563286, while those from cocoa cv. Sulawesi 1 (SUL1) are PQ563285 and PQ563287

Eleven members of Clade II include six predicted *PR1*-like mRNA accessions belonging to Clade IIA, such as from *Eucalyptus grandis* (Eg, XM_039317896.1 and XM_010054700.3), *Hevea brasiliensis* (Hb, XM_021821935.2), *Prunus speciosa* (Ps, AP038370.1), *Syzygium oleosum* (So, XM_030617410), *Vitis riparia* (Vri. XM_034825915.1), the nucleotide sequences found using BLAST nucleotide as having sequence identities to the partial *PR1*-like gene of the assembled chromosome 11 (nucleotides no. 34,802,093 to 34,802,499) of *Quercus dentata* isolate Qdleaves-2020 and the chromosome 3 (nucleotide no. 7937071 to 7937479) of *Vitis rotundifolia* cv. Noble (Figure 7). The predicted *PR1*-like mRNA of *Q lobata* (XM_031085850.1 [Ql]), *Q. robur* (XM_050404704.1 [Qr]), and one nucleotide sequences found using BLAST nucleotide as having sequence identities to the partial *PR1*-like gene from the assembled chromosome 11 (nucleotides no. 34699819 to 34700213) of *Quercus dentata* isolate Qdleaves-2020 are included in the Clade IIB.

In conclusion, gene-specific primers were successfully designed to reference two disease-responsive genes, Chitinase (*Chi*)-like and Pathogenesis-Related Protein 1 (*PR1*)-like, and used to generate amplicons from cocoa cv. MCC 02 (VSD resistant) and SUL 1 (VSD susceptible). Sequencing of the longest putative *Chi*-like amplicons generated 858 bp nucleotide sequences, and the BLAST analysis results confirmed its identity as a partial Chitinase gene, spanning from nucleotide number 55 to 912 of the reference *Chi* coding sequences. Multiple sequence alignment of four *Chi*-like accessions from cocoa cv. MCC 02 and SUL 1 find the presence of four non-synonymous

and two synonymous SNPs in the *Chi* coding region. Phylogenetic tree among *Chi*-like accessions from cocoa cv. MCC 02, SUL 1, and 36 accessions from the NCBI GenBank DNA Database show that the *Chi*-like from cocoa is more closely related to *Chi*-like from two cocoa accessions in the NCBI GenBank DNA Database than other *Chi*-like accessions. Sequencing of the longest putative *PR1*-like amplicons generated 569 bp nucleotide sequences, and the BLAST analysis results confirmed its identity as a partial *PR1*-like gene, spanning from nucleotide number 37 to 471 of the reference *PR1*-like coding sequences and 134 bp of the 3'-non-coding region. Multiple sequence alignment of four *PR1*-like accessions from cocoa cv. MCC 02 and SUL 1 identify the presence of three non-synonymous and two synonymous SNPs in the *PR1*-like coding regions and two SNPs in the 3'-non-coding regions. The phylogenetic tree among 24 accessions of *PR1*-like from various plant species shows that the *PR1*-like from cocoa is more closely related to *PR1*-like from *Herrania umbratica* and *Theobroma grandiflorum* than other accessions. Since the MCC 02 is VSD resistant and SUL 1 is susceptible, the finding of this research could be the starting point for developing VSD-resistant markers to support cocoa breeding.

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