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Identification of Molecular Marker Based on *MYB* Transcription Factor for the Selection of Indonesian Fine Cacao (*Theobroma cacao* L.)

Lukita Devy[1,3], Indah Anita-Sari[2], Tengku Imam Saputra[3], Agung Wahyu Susilo[2*], Ade Wachjar[3], and Sobir[3]

[1]Agency for the Assessment and Application of Technology
[2]Indonesian Coffee and Cocoa Research Institute, Jl. PB. Sudirman 90, Jember
[3]Department of Agronomy and Horticulture, Bogor Agricultural University
*Corresponding author: soesiloiccri@yahoo.com
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Abstract

Indonesia is the third largest cacao (*Theobroma cacao* L.) producer in the world and also well-known for its fine cacao varieties (Java fine-flavor cacao). Indonesian fine cacao breeding program will be accelerated by early detection of its specific trait through the use of molecular marker. One of the traits that could differentiate fine and bulk cacao, in this case Criollo and Forastero, respectively, is the pod color. Previous research reported that *MYB* transcription factor gene regulated cacao pod color and was able to differentiate Criollo from Forastero. The gene involved in the control of plant-specific processes including primary and secondary metabolism, cell fate and identity, developmental processes and responses to biotic and abiotic stresses. This research aimed to identify the diversity of Indonesian fine and bulk cacao based on *MYB* nucleotide sequence fragments. Identification of the *MYB* nucleotide sequence was conducted by DNA isolation from cacao leaves and specific primer design based on two cacao *MYB* transcription factor gene accessions. These primers were used to evaluate the diversity of three Indonesian fine cacao (DRC 16, PNT 16, and ICCRI 01) and two bulk cacao (PA 191 and ICCRI 03) clones. The cluster analysis showed that this specific primer is similar to other *MYB* gene accessions in Malvaceae family (*Theobroma*, *Herrania*, *Gossypium*). It is also able to differentiate bulk and fine cacao in accordance to their pedigree. The primer developed in this study could be used for further analysis of Indonesian fine cacao molecular marker.

Keywords: Bulk cacao, java fine-flavor cacao, pod color, trinitario

INTRODUCTION

Indonesia as the third largest cacao producer in the world (ICCO, 2017) is also well-known for its specialty cacao, the Java A light breaking fine-flavor cocoa which were selected in 1900's at Djati Roenggo Plantation, Central Java (Susilo *et al*., 2013). These specialty cacao showed high economic value as premium cocoa raw material (Amores *et al*., 2007). However fine flavor cacao is more susceptible to biotic and abiotic stresses than bulk cacao. Therefore, many breeding efforts were conducted to maintain and improve fine cacao performance.

Because of its characteristic as a perennial tree, cacao breeding program required long period to produce superior clones. The breeding program mostly will be accomplished in 15-20 years (Susilo, 2007). Therefore, the

process required an accelerated and more precise breeding target achievement. In a breeding program, selection process conducted by the observation of certain selection criteria depended on the breeding objective. The criteria for fine flavor cacao used to be identified by its bean color at productive stage (Anita-Sari *et al.*, 2013). This stage started three years after planting so that the fine flavor cacao off spring could be detected after years of hybridization, which caused the slow rate of new fine flavor cacao superior clones availability.

Acceleration of fine flavor cocoa breeding could be conducted with the use of molecular marker. This method would be a preferred alternative to shorten and sharpen the selection process in a breeding program. Deoxyribonucleic acid (DNA) based marker is not influenced by environment and could be conducted on any part of the plant which make it is suitable for early detection of certain characteristics (Jiang, 2013). Information on specific gene that could differentiate fine from bulk cacao is urgently needed in specific molecular marker construction.

One of those genes that could be used as specific marker is R2R3-MYB transcription factor (Motamayor *et al.*, 2013). This gene is a member family of the MYB gene. The MYB family of proteins is large, functionally diverse and represented in all eukaryotes. Most MYB proteins function as transcription factors with varying numbers of MYB domain repeats conferring their ability to bind DNA. They are widely distributed in plants and also interact with other transcription factors. The large size of the MYB family in plants indicates their importance in the control of plant specific processes (Ambawat *et al.*, 2013).

The R2R3-MYB proteins are specific to plants and are also the most abundant type in plants, with more than 100 R2R3-MYB members in the genomes of dicots and monocots (Ambawat *et al.*, 2013). This gene was able to differentiate fine and bulk cacao based on their pod color, which the difference was due to their anthocyanin contents. The R2R3 MYB transcription factor is a key factor that involved in the control of plant-specific processes including primary and secondary metabolism, cell fate and identity, developmental processes and responses to biotic and abiotic stresses (Dubos *et al.*, 2010). This R2R3 MYB can act as transcriptional activators as well as repressors (Stracke *et al.*, 2001). Many researches have been conducted to identify the role of R2R3 MYB transcription factor in anthocyanin pathway such as in *Arabidopsis thaliana* (Gonzalez *et al.*, 2008), grapes (Cavallini *et al.*, 2015), pear (Pierantoni *et al.*, 2010), *Epimedium sagittatum* (Huang *et al.*, 2016) and Rosaceae (Lin-Wang *et al.*, 2010).

Previous research on the role R2R3 MYB transcription factor to differentiate Criollo and Forastero cacao used pure Criollo which has white cotyledon and green pod color as fine cocoa and pure Forastero which has purple cotyledon and red pod color as bulk cacao (Motamayor *et al.*, 2013). Meanwhile Java fine-flavor cacao cotyledon color are not pure white, they have whitish purple cotyledon and various pod color which were Trinitario, the hybrid product of Criollo and Forastero (Anita-Sari *et al.*, 2015). Assessment of *MYB* gene to differentiate fine and bulk Indonesian cacao is compulsory for accelerating the progress of Indonesian fine cacao breeding. The purpose of this research is to identify the diversity of Indonesian fine and bulk cacao based on *MYB* nucleotide sequence fragment.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

Five cacao clones were used in this experiment. Three clones were fine-flavor cacao (DRC 16, PNT 16, and ICCRI 01) while the other two were bulk cacao (PA 191 and ICCRI 03). These clones were breeding collection of Indonesian Coffee and Cacao Research Institute, Jember. The leaves were collected in the intermediate stage of maturation. In the field, these were placed in a plastic bag containing silica gel and stored at -20^{O}C at Laboratory of Plant Production Technology, Agency for the Assessment and Application of Technology, Serpong, for further analysis.

Cacao DNA was extracted from leaf tissue using the Genomic DNA Mini Kit (Plant) (GeneAid Biotech Ltd., Taiwan). The protocol was modified from the protocol provided in the microcentrifuge rotation speed and elution frequency. DNA quantifications were measured using Nanodrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific Inc.). The DNA concentrations were 50-200 ng/µL and A_{260}/A_{280} values were 1.8-2.0. These concentrations were standardized to 50 ng/µL by the addition of free nuclease water. Before subjected to thermal cycler (Takara Inc. TP-6000), 4.5 µL RNAse-A for 50 µL DNA solution was added and incubated at 37^{O}C for 1.5 hours. For quality and yield assessments, electrophoresis was conducted to all DNA samples in 1% agarose gel with TAE buffer 1x for 30 min at 100 V, stained with ethidium bromide and bands were observed.

PCR Amplification and Sequencing

Specific primer design was conducted by assessing R2R3-MYB complete DNA sequence (CDS) from cacao genome in NCBI. According to Motamayor *et al*. (2013), this gene could differentiate pod color of fine and bulk cacao from Criollo and Forastero groups. Gene accessions used were GU324346.1 and XM_007051442.2. These were aligned using Geneious ver. 8.1 (Biomatters, http://www.geneious.com) and the alignment consensus were used as the template for primer design using Primer3plus (http://www.bioinformatics. nl/cgi-bin/primer3plus/primer3plus.cgi) in order to generate forward and reverse primers.

Total mixed PCR volume of 10 µL reaction was used. The mixture consisted of Terra™ PCR Direct Polymerase Mix (Takara-Clontech Inc.), 0.3 µM of each primer (Table 1), 50 ng DNA template and free nuclease water. The PCR was performed in a thermal cycler (Takara Inc. TP-6000) initiated by one cycle of denaturation at 95^{O}C for 5 min, followed by 35 cycles which consist of denaturation at 95^{O}C for 30 sec, annealing at 58.5^{O}C for 30 sec and extension at 72^{O}C for 1 min. The final extension was carried out at 72^{O}C for 5 min. The amplified product was checked in 1% agarose gel electrophoresis with TAE buffer 1x and bands were observed.

Nucleotide sequence of single band PCR product were analyzed using DNA sequencer by 1st BASE (First BASE Laboratories Sdn Bhd, Malaysia). These sequences result were analyzed by online BLAST using Ensembl Plant (http://plant.ensembl.org) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in

Table 1. Specific primer sequence of MYB gene used to amplified five cacao clones

Primer	Sequence (5'-3')	Primer length (bp)	Tm (^{O}C)	GC content (%)
Forward	CTGGACTCCTAGAGAAGACA	20	55.0	50
Reverse	ATCCAACTGCACTTGATCAT	20	55.0	40

order to identified the similarity of DNA genomic fragment with other plants. Nucleotide sequence alignment, similarity analysis using Maximum Composite Likelihood and phylogeny analysis using Tamura-Nei method were conducted with MEGA6.

RESULTS AND DISCUSSIONS

DNA Amplification with Specific Marker

Specific MYB gene primer was able to amplify DNA sequence from 5 cacao clones. The primers produced clear single band at ~850 bp (Figure 1). Nucleotide sequence alignment of MYB gene fragment from this experiment and GeneBank were conducted with NCBI BLASTn algorithm (Zhang *et al.*, 2000). The result showed that nucleotide fragment of these 5 cacao clones had high identity (78-99%) with other MYB gene accessions deposited in GeneBank data (Table 2). This high similarity indicated that MYB gene fragment from 5 cacao clones had the same function with other MYB genes from other genus in Malvaceae family.

Amino acid sequence prediction alignment of these clones with MYB amino acid gene fragment were conducted with NCBI BLASTx algorithm (Altschul *et al.*, 1997). The result showed amino acid fragment of these 5 cacao clones had high identity (68-98%) with other accessions in GeneBank data (Table 3). The amino acid sequences have the highest similarity with myb-related protein 308 of cacao (XP_007051504.1).

The similar identity of nucleotide and amino acid sequence of five Indonesian cacao clones with those deposited in GeneBank showed that they performed the same function in anthocyanin biosynthesis. Motamayor *et al.* (2013) mentioned that R2R3 MYB gene was able to differentiate bulk and fine cacao based on their pod color. The difference is due to the absence of anthocyanin in green pods. Red color is superimposed over the base green color of the pod during development. The red pod color was originated from Criollo type. Martin & Paz-Ares (1997) stated that MYB transcription factors played an important role in transcriptional regulation of anthocyanins. Regarding color differentiation in cacao, Anita-Sari *et al*. (2016) reported that leaf flush anthocyanin content was able to differentiate Indonesian bulk and fine cacao, where its concentration in fine cocoa was lower than in bulk cocoa.

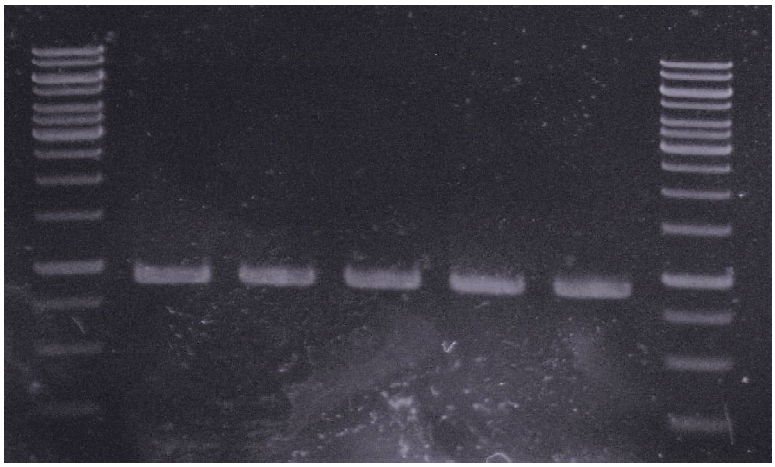

Figure 1. Specific primer from MYB gene was able to amplified DNA of 5 cacao clones at 850 bp (Bulk cacaos: 1. PA 191, 2. ICCRI 03 and fine cacaos: 3. DRC 16, 4. PNT 16 and 5. ICCRI 01)

Table 2. Sequence identity between MYB nucleotide sequence fragment from five cacao clones with other accessions in GeneBank

Accession	Description	Query coverage (%)	E value	Identity (%)
LT594788.1	*Theobroma cacao* genome assembly, chromosome: I	89	0	99
XM_007051442.2	Predicted: *Theobroma cacao* myb-related protein 308 (LOC18613948), mRNA	77	0	99
GU324346.1	*Theobroma cacao* cultivar Scavina 6 TT2 like MYB transcriptoin factor (MYBPA) mRNA, complete cds	77	0	96
XM_021424702.1	Predicted: *Herrania umbratica* myb-related protein 308-like (LOC110413757), mRNA	77	0	96
XM_012619612.1	Predicted: *Gossypium raimondii* myb-related protein 308-like (LOC105791527), mRNA	66	1.00E-154	83
XM_016825942.1	Predicted: *Gossypium hirsutum* myb-related protein 308-like (LOC107900270), mRNA	66	5.00E-152	82
XM_012619613.1	Predicted: *Gossypium raimondii* myb-related protein 308-like (LOC105791528), mRNA	77	2.00E-151	78

Table 3. Sequence identity between MYB amino acid sequence fragment from five cacao clones with other accessions in GeneBank

Accession	Description	Query coverage (%)	E value	Identity (%)
XP_007051504.1	Predicted: myb-related protein 308 [*Theobroma cacao*]	99	1.00E-147	98
ADD51352.1	TT2 like MYB transcriptoin factor [*Theobroma cacao*]	99	6.00E-146	97
XP_021280377.1	myb-related protein 308-like [*Herrania umbratica*]	99	5.00E-142	95
XP_012475067.1	Predicted: myb-related protein 308-like [*Gossypium raimondii*]	99	1.00E-92	68
XP_016724909.1	Predicted: myb-related protein 308-like [*Gossypium hirsutum*]	99	3.00E-92	70
XP_012475066.1	Predicted: myb-related protein 308-like [*Gossypium raimondii*]	99	5.00E-92	70
XP_017625585.1	Predicted: myb-related protein 308-like [*Gossypium arboreum*]	99	1.00E-91	68

Previous research reported that genes encoding R2R3 MYB transcription factor from apple was also induced the anthocyanin pathway when co-expressed with basic-helix-loop-helix (bHLH) proteins (Lin-Wang *et al*., 2010). The R2R3 MYB in Arabidopsis consisted of 23 subgroups and 3 of them (i.e. subgroup 4, 6 and 7) regulate the flavonoid biosynthetic pathway (Dubos *et al*., 2010). Gonzalez *et al*. (2008) reported that overexpression of R2R3 MYB transcription factor (MYB113 and MYB114) in Arabidopsis increased the pigment production and the analysis of MYB mutant seedlings indicated

that these MYBs were regulators of late anthocyanin structural genes which begin at F3'H. Dubos *et al*. (2008) stated that R2R3 MYB regulated flavonoid biosynthesis (proantho-cyanidins and anthocyanins) of Arabidopsis through control of the regulatory genes where PAP1, PAP2, MYB113 and MYB114 regulates anthocyanidins into anthocyanins and TT2 regulates flavan-3-ols into proantho-cyanidins.

Diversity Analysis of MYB Gene Fragment

The DNA sequence from MYB gene fragment of cacao clones showed exon and intron sites (Figure 2). The analysis of each clones conducted using BLAST algorithm from EnsemblPlant with cacao genome showed that these five cacao clones had 99-100% similarity with nucleotide sequence of *Theobroma cacao* "Matina 1-6" accession number TCM_005112 that encode MYB domain protein 7 in chromosome 1 (Kersey *et al*., 2013). The cross reference of this accession is NCBI cacao accession number XM_007051442.2 that encodes MYB-related protein 308.

The analysis of DNA sequence from MYB gene fragment showed that each genotype had two different exon sequences. From this analysis, the exon and intron sites were identified (Figure 3). The identification of the exon and intron sequence area will be useful for amino acid translation prediction. The alignment showed one intron area of 119 bp. This intron was deleted prior to amino acid translation prediction of five cacao clones.

The phylogenetic nucleotide sequence analysis from MYB gene fragment of five cacao clones with other accessions was conducted to reveal their similarity (Figure 4). The dendrogram showed three clusters based on maximum composite likelihood test with Tamura-Nei method, bootstrap value 500. The first cluster consisted of *Theobroma cacao* (DRC 16, PNT 16, ICCRI 01, ICCRI 03, PA181, accession LT594788, XM 007051442.2, GU 324346.1), the second was *Herrania umbratica* accession XM 021424702.1 and the third were *Gossypium raimondii* accession XM 012619612.1, *Gossypium hirsutum* accession XM 016825924.1, *Gossypium raimondii* accession XM 012619613.1 and *Gossypium hirsutum* accession XM 016874319.1. The genotypes used in this experiment were at the same group with the other *Theobroma cacao* accessions. Genetic distance between group 1 and 2 was 0.340, between group 1 and 3 was 0.122 and between group 2 and 3 was 0.205. This clustering analysis result also showed that MYB gene of each genus (*Herrania*, *Theobroma* and *Gossypium*) in Malvaceae family was segregated into different groups.

Grouping of five cacao clones (Figure 4) showed that fine cacao (PNT 16, ICCRI 01 and DRC 16) were clustered into the same group while the bulk cacao (ICCRI 03 and PA 191) were in different group. Ancestors of Java fine cacao, including DRC 16, ICCRI 01 and PNT 16, were clones from cacao selection by van Hall (1912). They were DR 1, DR 2, and DR 38 clones that released in 1948 and well-known as Java "A" light breaking fine-flavor, a famous specialty cacao from Indonesia (Susilo *et al*., 2013). Cacao DRC 16 was selected in 1950's and released in 1997 as resistant pod rot clone (Susilo, 2015). Meanwhile, ICCRI 01 and PNT 16 were selected from fine cacao population in Penataran Plantation, East Java. The first clone released in 2005 and the latter was selected in 2010 (Anita-Sari *et al*., 2015). They are the next generation of Java fine-flavor cocoa.

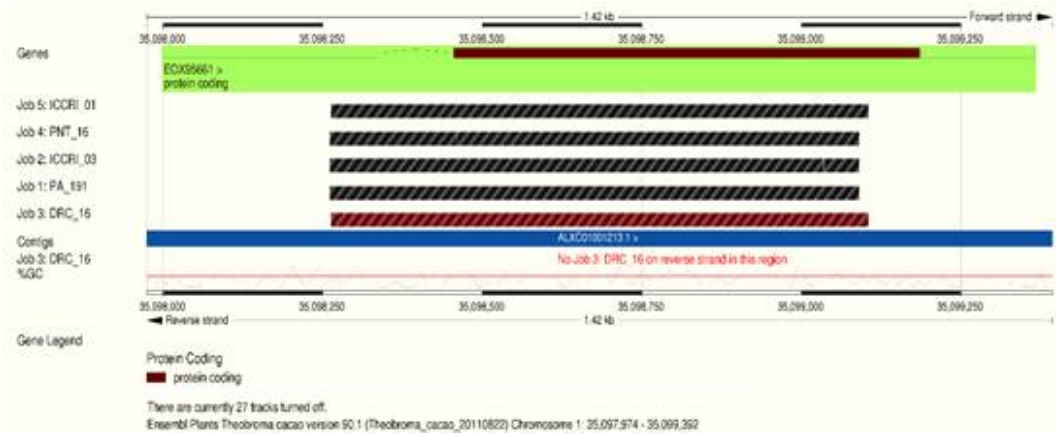

Figure 2. Five cacao clones nucleotide fragment were highly similar to MYB domain protein 7 sequence of *T. cacao* "Matina 1-6" (accession TCM_005112)

CTGGACTCCTAGAGAAGACACATTGCTTGTCAAGTACATTCAAGCTCAT
GGTGACGGTCACTGGAGATCACTTCCTAAGAAAGCCGgtaattcatttcaacttaat
taagacattccctagaagcatgattttgttttttcttgccaaatttggggtatgtcaaggttttttttttcttcattgtttgttaa
tggttttgcaGGCTTCTTAGGTGTGGAAAGAGTTGCAGGCTCAGATGGATGA
ACTATTTAAGACCAGATATAAAGAGAGGGAATATAACTCCCGATGAGG
ATGATCTTATCATCAGATTACATTCCCTCCTCGGCAATCGGTGGTCACT
CATTGCCGGAAGGCTTCCTGGTCGAACCGATAACGAGATTAAAAATTAC
TGGAACACCCATCTGAGTAAAAGACTTCTAAGCCAAGGGACTGACCCT
AACACCCACAAGAAACTATCAGAGCCCGCAGTTCAACAAGTGAAGAAG
AGAAAAAGCAGCAGAGGCAACAGCAACAAGAAGCAGAACAATAGCAAG
GGCAAAGGCGCAAAGGTTGAGCCAGAAAAGCCCAAAGTCCATCTCCCT
AAGCCCGTTAGAGTAACTTCTTTCTCTTTACCAAGAAACGACAGCTTTG
ACCAATGTAATACGTTTAGCACGGTGTCTTCAAGCCAAGGAGGAGAGG
GAGGATTGGGTACAGAGGTTGTACAAGGACCTTGGTCAGATAATGTCA
ACGATGATGAAAATGGGACCGGATTTCTTGCTGCTTATGATGATCATGG
TTTTGTTAACGGTTCAGATTTCGAGTGCCAGTCTCATGTACCAGCAAGT
GATGACGATAATTCTCTCGAGAAGCTTTACGAAGAGTATCTCCAGCTTC
TGAAGACAAACGATGATCAAGTGCAGTTGGAT

Notes: Nucleotide sequences of primers are underlined, exon (*coding region*) are capitalized while intron (*non-coding region*) are not

Figure 3. Example of DNA sequence from MYB gene fragment of cacao clone DRC 16

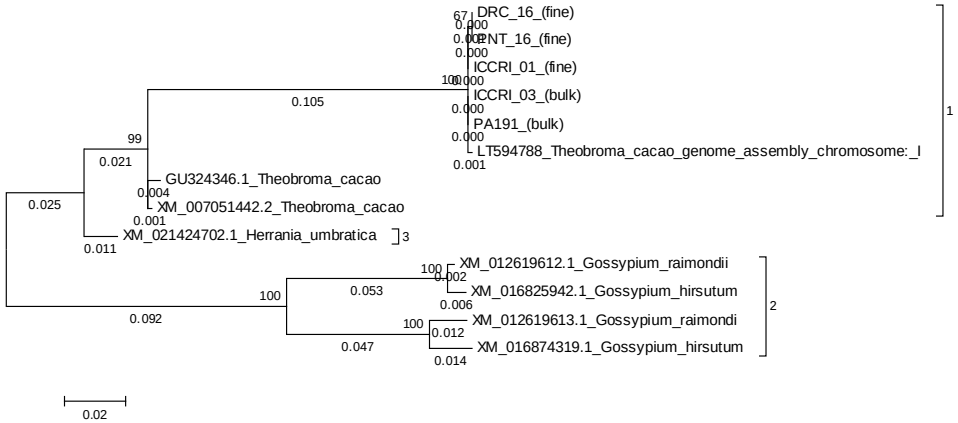

Figure 4. Nucleotide sequence of *MYB* gene in five cacao clones analyzed with UPGMA cluster analysis were at the same group with *T. cacao* while other genus clustered into different groups

CONCLUSIONS

This experiment result showed that specific primer of *MYB* gene could differentiate Indonesian fine and bulk cacao although Indonesian fine cacao are Trinitario, which were the hybrid of Criollo and Forastero. It is widely known that Criollo are fine cacao and Forastero are bulk cacao. The gene could be further analyzed to be used for molecular marker of Indonesian fine flavor cacao by identifying the single nucleotide difference between the two groups. A more precise marker could be developed from those nucleotide different information by the construction of single nucleotide polymorphism marker and evaluate them on a larger cacao population. It will be helpful for early detection of fine traits in Indonesian fine cacao breeding.

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