Development of Tetra-primer ARMS-PCR for Selection and Discrimination of CMD-Resistant Cassava Varieties Used in Thailand

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ABSTRACT

Cassava mosaic disease (CMD) is a major disease that causes damage to cassava production worldwide. CMD-resistant cassava varieties obtained from the International Center for Tropical Agriculture (CIAT) and the International Institute of Tropical Agriculture (IITA), including 7 varieties, namely TME 3, C 33, IITA-TMS-IBA 920057, IITA-TMS-IBA 972205, IITA-TMS-IBA 980505, IITA-TMS-IBA 980581 and TME B 419, are currently being used as parent lines to create hybrids resistant to CMD suitable for cultivation in Thailand. The tetra-primer ARMS-PCR is a technique for single-nucleotide polymorphism (SNP) detection. It has the advantages of amplifying multiple alleles in a single reaction with the inclusion of a control fragment, cost-effectiveness as well as simplicity in operation. The purposes of this research were to develop primer sets using the tetra-primer ARMS-PCR technique to detect SNP markers associated with CMD resistance for the selection of resistant varieties and to develop a primer set for discrimination of variety within 7 CMD-resistant varieties being used in Thailand. The resultant primer sets have been readily developed and optimized condition to detect SNP markers Ex2-78, Ex2-157, Ex3-128, and S12_7926132 on chromosome 12, and can be used to select CMD-resistant varieties. For discrimination of CMD-resistant varieties, a primer set was developed to detect the SNP marker S12_8910428 in the *DNA polymerase δ subunit 1 gene*, and could distinguish TME B 419 from the remaining six resistant varieties. To our knowledge, this is the first report of using tetra-primer ARMS-PCR, which is cost-effective and simple to operate, in SNP detection for CMD-resistance selection in cassava.

Keywords: cassava; cassava mosaic disease; detection; molecular marker; single-nucleotide polymorphism (SNP)

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INTRODUCTION

Cassava is one of the important economic crops of Thailand with an area of 1.59 million hectares of plantations throughout the country. Thailand's cassava production amount was ranked third in the world after Nigeria and Congo. In 2022, Thailand has exported cassava products worth 172,593 million baht (Office of Agricultural Economics, 2022).

Cassava mosaic disease (CMD) is caused by the *Cassava mosaic virus* (CMV). It can infect cassava at all growth stages, causing a decrease in yield by more than 80%. Cassava plants infected with this disease show symptoms such as spotted leaves to yellow and deformed leaves, short trees and stunted stems. The disease epidemic is carried by the tobacco whitefly, *Bemisia tabaci*, and can be transmitted by infected cassava cuttings. The disease is endemic in many countries in Africa and Asia. The first outbreak of CMD in Thailand was reported in 2018 (Maneechoat et al., 2018). The causal virus was *Sri Lankan cassava mosaic virus* (SLCMV) (Rakkrai et al., 2022).

Until now, three loci or genes related to CMD resistance have been identified, namely *CMD1*, *CMD2* and *CMD3*. *CMD1* is a recessive gene originating from the wild cassava, *Manihot glaziovii* Muell. Arg. (Fregene and Puonti-Kaerlas, 2002). *CMD2* is the major dominant locus controlling CMD resistance and is called the qualitative resistance source (Akano et al., 2002). It was identified in the cassava variety TME3 and is commonly found in West African landraces (Lim et al., 2022). *CMD3* is a minor gene that was identified in TMS 972205 (Okogbenin et al., 2012).

Molecular markers associated with the *CMD2* locus were reported. Sequence Characterized Amplified Region (SCAR) marker, namely RME1 and Simple Sequence Repeat (SSR) markers, namely NS158, SSRY28 and NS169, are flanking near the *CMD2* locus with the distances 4, 7, 9 and 16 centimorgan, respectively (Carmo et al., 2015).

Wolfe et al. (2016) reported 3 SNP markers in the *peroxidase* gene (Cassava4.1_029175) differentiating between the CMD-resistant cassava genotypes having the qualitative resistance source *CMD2* (TMEB3, TMEB7 and I011412) and the CMD-tolerant cassava genotypes without *CMD2* (I30572 and TMEB1), namely Ex2-78 (the 78th nucleotide of Exon2), Ex2-157 and Ex3-128. The CMD-resistant cassava genotypes have alleles homozygous GG, TT and TT at SNPs Ex2-78, Ex2-157 and Ex3-128, respectively. Rabbi et al. (2020) conducted a Genome-Wide Association Study (GWAS) using a cassava population of 5,130 clones developed at the International Institute of Tropical

Agriculture (IITA) in Nigeria and found an additional SNP marker associated with CMD resistance at position S12_7926132 in the CMD2 locus on Chromosome 12 in *Manihot esculenta* v6.1 of the Phytozome database (https://phytozome-next.jgi.doe.gov). Ige et al. (2021) and Codjia et al. (2022) used the Kompetitive Allele-Specific PCR (KASP) assay to evaluate the effectiveness of the SNP S12_7926132 in selection for CMD resistance and found that the SNP S12_7926132 provided 77 – 80% accuracy.

Recently, Lim et al. (2022) discovered a gene involved in the loss of CMD2 resistance (LCR) after regeneration through de novo morphogenesis in multiple landraces but not in varieties developed through a breeding program. The gene was *DNA polymerase δ subunit 1* (*MePOLD1*). The SNP marker that co-segregates with CMD resistance was also identified. The SNP is in the coding sequence of *MePOLD1* and changes amino acid from valine to leucine at position 528 (V528L) (Lim et al., 2022), which is nucleotide position 8910428 on chromosome 12 (S12_8910428) in *Manihot esculenta* v8.1 of the Phytozome database, changing nucleotide from G to C. The resistant allele of SNP in *MePOLD1* was found in cassava varieties TME204 and TME B 419 (Lim et al., 2022), which are genetically close to each other (Raji et al., 2009). TME B 419 is one of the CMD-resistant varieties that were used in breeding programs in Thailand.

There are several methods for the detection of SNP markers such as restriction fragment length polymorphism (RFLP), cleaved amplified polymorphic sequence marker (CAPS), pyrosequencing, real-time PCR and tetra-primer amplification refractory mutation system PCR (tetra-primer ARMS–PCR). Among these methods, the tetra-primer ARMS–PCR has the advantage of speed and cost-effective, simple to operate and requires basic instrumentation (Lajin et al., 2013). Therefore, in this research, primer sets used for the tetra-primer ARMS-PCR technique were developed to detect SNP markers associated with CMD resistance for the selection of resistant varieties and to discriminate variety within the 7 CMD-resistant varieties used in cassava breeding programs in Thailand.

MATERIALS AND METHODS

Sample collection and DNA extraction

Thailand Department of Agriculture (DOA) received 7 CMD-resistant varieties from the International Center for Tropical Agriculture (CIAT) and the International Institute of Tropical Agriculture (IITA) including TME 3, C 33, IITA-TMS-IBA 920057, TMS-IBA 972205, IITA-TMS-IBA 980505, IITA-TMS-IBA 980581 and TME B 419 (Rakkrai et al., 2022). These varieties were

used as parents to develop varieties resistant to CMD and suitable for cultivation in Thailand. The cassava breeding program has been carried out at Rayong Field Crops Research Center, DOA, Ministry of Agriculture and Cooperatives, Thailand.

Seven introduced CMD-resistant varieties and a Thai-landrace CMR43-08-89 susceptible variety were collected from the Rayong Field Crops Research Center. Leaves were taken from 6-month-old cassava from the top of the plant for DNA extraction using hexadecyltrimethylammonium bromide or the CTAB method (Devi et al., 2013). The quantity and quality of DNA were measured by using a spectrophotometer (Biodrop, UK).

Primer Design

To design primer sets for the tetra-primer ARMS-PCR technique to detect SNP markers Ex2-78, Ex2-157, Ex3-128, S12_7926132, and S12_8910428, genome sequences adjacent to each SNP obtained from the Phytozome database (https://phytozome-next.jgi.doe.gov) were used. A primer set for each SNP detection was designed by using an accessible primer1 program (http://primer1.soton.ac.uk/primer1.html) (Ye et al., 2001). The sequences of primer sets for Ex2-78, Ex2-157, Ex3-128, S12_7926132, and S12_8910428 detection are provided in Table 1.

Table 1 Primers used in the tetra-primer ARMS-PCR for selection and discrimination of CMD-resistant cassava varieties

SNP position	Primer set and sequence (5' – 3')		Product size
Ex2-78	Outer forward	CCTTTGTTGAGAATGCATTTCCATGATT	Control fragment: 416 bp
	Outer reverse	GTCCAGTTTTAACATCCCAAAATGGTCC	
	Inner forward	AAAGAAGCAATCCCAAACCAAACCGTA	A allele: 253 bp
	Inner reverse	TTCACAGCATCAATCACATTGAATCCTATC	G allele: 220 bp
Ex2-157	Outer forward	TTCAAAGAGAGGCAATCAAGCTGAGAAA	Control fragment:296 bp
	Outer reverse	GACACTCTTCCATCTCTCCGTCCAGTTT	
	Inner forward	TGGTGTGGTTTCTTGCGCTGATATCT	T allele: 192 bp
	Inner reverse	AAACTGCATCTCGAGCTACTAAGGCCAC	G allele: 158 bp
Ex3-128	Outer forward	GTAGCTCGAGATGCAGTTTCAATGGTAA	Control fragment:434 bp
	Outer reverse	CTTTGCCTGTGAAATTGTATAAGCGGTT	
	Inner forward	TAAGTGAGCTTAAACAAAATTTTGCGGC	C allele: 231 bp
	Inner reverse	AAGGTCCTTAACATTTAAACCCCGGA	T allele: 257 bp
S12_7926132	Outer forward	CCAAGTTTTATGGACCTTTCGTGGTTCT	Control fragments:291,240 bp
	Outer reverse	CCAATGGACTAAAATCTCAAGTTGTCCG	
	Inner forward	TATTTTCCATGTTTCCACCCTCAAACGT	T allele: 204 bp
	Inner reverse	GGAGTACAAGAATCTTGTCCTTTGTGACAC	G allele: 145 bp
S12_8910428	Outer forward	ATTTCTTCTTTGAAACAAGGATTTGCAT	Control fragment:398 bp
	Outer reverse	TAGCCTTCCTCAGGAGTTGAGAAAGTAC	
	Inner forward	TTATTTATAATTATGTAGAAATGGCCCCTG	G allele: 196 bp
	Inner reverse	AGGAAAGATAAAGGGACGCCTGTCAG	C allele: 258 bp

PCR amplification

For DNA amplification by single PCR for RME1 marker detection, PCR was performed using primers designed by Fregene et al. (2006): F: 5'-ATGTTAATGTAATGAAAGAGC-3' and R: 5'-AGAAGAGGGTAGGAGTTATGT-3'. The reaction was assembled in a total volume of 20 µl containing 100 ng of genomic DNA, 1X Taq buffer with $(NH_4)_2SO_4$ (Thermo Scientific, USA), 0.2 mM for each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, 1.5 mM $MgCl_2$ and 1 U Taq DNA Polymerase (Thermo Scientific, USA). The PCR cycling profile was 94° C for 5 min, followed by 35 cycles of denaturation at 94° C for 40 s, annealing at 56° C for 40 s, extension at 72° C for 1 min, and final extension at 72° C for 5 min PCR products were analyzed by 2% (w/v) agarose gel electrophoresis.

For DNA amplification using the tetra-primer ARMS-PCR technique for Ex2-78, Ex2-157 and Ex3-128 SNP detection, PCR was performed in a total volume of 20 µl containing 100 ng of genomic DNA, 1X Taq buffer with $(NH_4)_2SO_4$ (Thermo Scientific, USA), 0.2 mM for each dNTP, 0.2 µM of each outer primer, 0.2 µM of each inner primer, 1.5 mM $MgCl_2$, 1.5 U Taq DNA Polymerase (Thermo Scientific, USA). In addition, 1% dimethyl sulfoxide (DMSO) was added for Ex2-157 detection. The mixture was then applied to DNA amplification using the GeneAmp® PCR System 9700 (Applied Biosystems). The optimum PCR cycling profile was 94° C for 5 min followed by 35 cycles of denaturation at 94° C for 40 s, annealing for 40 s at 58° C for Ex2-78 and at 60° C for Ex2-157 and Ex3-128, extension at 72° C for 30 s, and final extension at 72° C for 5 min PCR products were analyzed by 2.5% (w/v) agarose gel electrophoresis.

For DNA amplification using the tetra-primer ARMS-PCR technique for S12_7926132 and S12_8910428 SNP detection, PCR was performed in a total volume of 20 µl containing 50 ng of genomic DNA, 1X GoTaq® Green Master Mix (Promega, USA), 0.25 µM of each outer primer and 0.25 µM of each inner primer. The mixture was then applied to DNA amplification using the GeneAmp® PCR System 9700 (Applied Biosystems). The optimum PCR cycling profile was 95° C for 2 min, followed by 35 cycles of step for S12_7926132 and 38 cycles of step for S12_8910428, consisting of denaturation at 94° C for 40 s, annealing for 40 s at 58° C for S12_7926132 and at 59° C for S12_8910428, extension at 72° C for 30 s, and final extension at 72° C for 5 min PCR products were analyzed by 2.5% (w/v) agarose gel electrophoresis.

For DNA amplification by single PCR for SSRY75 marker detection, PCR was performed by using primers designed by Mba

et al. (2001): F: 5'-TCTGGTAAACCTACTAGTGCTCCA-3' and R: 5'-TTCATGCACGTCCTGATACA-3'. The reactions were assembled in a total volume of 20 µl containing 50 ng of genomic DNA, 1X GoTaq® Green Master Mix (Promega, USA), 0.25 µM forward primer and 0.25 µM reverse primer. The mixture was then applied to DNA amplification using the GeneAmp® PCR System 9700 (Applied Biosystems). The optimum PCR cycling profile was 95° C for 2 min, followed by 35 cycles of denaturation at 94° C for 40 s, annealing at 55° C for 40 s, extension at 72° C for 30 s, and final extension at 72° C for 5 min PCR products were analyzed by 3% (w/v) agarose gel electrophoresis.

RESULTS AND DISCUSSION

CMD-resistant varieties used in the cassava breeding program in Thailand

Most of the resistant varieties have red leaf petioles, except TMS-IBA 972205 and TME B 419 which have green leaf petioles. All varieties have similar leaf shapes with an average of 7 lobes. The genotype analysis using a SCAR marker named RME1 associated with the CMD2 locus revealed that all CMD-resistant varieties showed a DNA band approximately 700 bp in size (Figure 1 lane 1 - 7), consistent with the expected size found in CMD-resistant varieties as reported previously (Bi et al., 2010; Carmo et al., 2015; Houngue et al., 2019). While the 700 bp band did not appear by CMR43-08-89, a CMD-susceptible variety, and distilled water control samples, (Figure 1 lane 8 and 9, respectively).

Development of primer sets for detection of SNP markers related to CMD resistance

The cassava varieties with different genotypes of SNP Ex2-78 were used in the optimization of tetra-primer ARMS-PCR, including TME 3 (GG), Rayong 5 (AG) and Rayong 3 (AA). The optimization for SNP Ex2-78 detection was carried out by using gradients of annealing temperature at 58, 60, 64 and 66° C, the addition of dimethyl sulfoxide (DMSO) and increasing of inner reverse (IR) primer concentration (Figure 2). A suitable DMSO concentration improve PCR amplification (Kang et al., 2005). The best condition for SNP Ex2-78 detection was the annealing temperature of 58° C without DMSO (0% DMSO) and without changing the primer concentration (Figure 2 panel D), which provided clear DNA fragments at the expected size as described in Table 1. The increase of IR primer from 0.2 µm to 0.4 µm was tried in optimization, but this condition did not provide the expected bands (Figure 2 panel B). The product of tetra-primer ARMS-PCR specific to SNP Ex2-78 contained 416 bp as a control fragment, 220 bp as the G allele and 253 bp as the A allele (Figure 2).

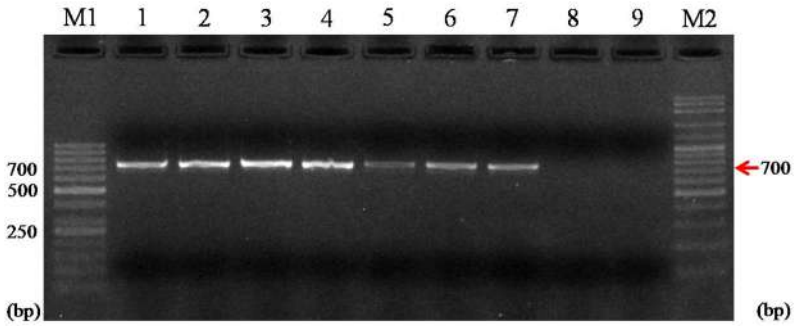

Figure 1 Genotype analysis of cassava varieties using SCAR marker RME1, M1: 50 bp DNA Ladder; 1: TME 3; 2: C 33; 3: IITA-TMS-IBA 920057; 4: TMS-IBA 972205; 5: IITA-TMS-IBA 980505; 6: IITA-TMS-IBA 980581; 7: TME B 419; 8: CMR43-08-89 (CMD-susceptible variety); 9: ddH_2O; M2: 100 bp DNA Ladder plus

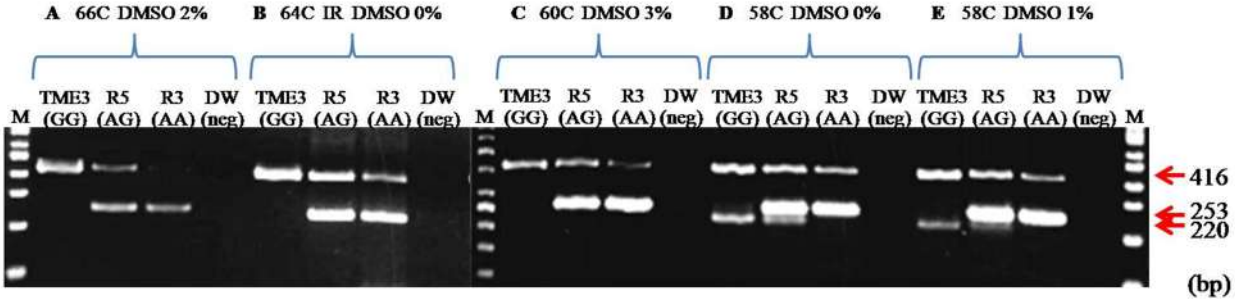

Figure 2 Tetra-primer ARMS–PCR optimization for SNP Ex2-78 detection with different annealing temperatures and conditions, A: Annealing temperature of 66° C and addition of 2% DMSO in PCR reagents; B: Annealing temperature of 64° C and increasing of IR primer without the addition of DMSO; C: Annealing temperature of 60° C and addition of 3% DMSO; D: Annealing temperature of 58° C without the addition of DMSO, which was the optimum PCR condition for SNP Ex2-78 detection; E: Annealing temperature of 58° C and addition of 1% DMSO; Lane M: DNA ladder. DNA fragments generated by primers of SNP Ex2-78 include a control fragment (416 bp), a specific fragment of the A allele (253 bp) and the G allele (220 bp)

The SNP Ex2-78 genotype analysis showed that all CMD-resistant varieties used in this study have genotype GG (Figure 3A lane 1 - 7), consistent with the report of Wolfe et al. (2016) that CMD-resistant varieties (TMEB3, TMEB7 and I011412) have alleles homozygous GG at SNP Ex2-78. While CMR 43-08-89 had a genotype of heterozygous GA (Figure 3A lane 8).

The optimization of tetra-primer ARMS-PCR for SNP Ex2-157 and Ex3-128 detections revealed the best conditions for SNP Ex2-157 and Ex3-128 detections by the annealing temperature of 60° C with 1%

DMSO and the annealing temperature of 60° C without DMSO, respectively. The genotype analysis of SNP Ex2-157 is shown in Figure 3B. The 296-bp band was a control fragment and the 192-bp band represented the T allele fragment. The result showed that all CMD-resistant varieties used in this study and a susceptible variety, CMR43-08-89, have a homozygous TT genotype (Figure 3B). The genotype analysis result of SNP Ex3-128 is shown in Figure 3C. The 434-bp band was a control fragment and the 257-bp band represented the T allele fragment. In this marker, all 7 CMD-resistant varieties and CMR43-08-89 have similar genotypes, which are homozygous TT (Figure 3C). According to Wolfe et al. (2016), CMD-resistant varieties have genotype homozygous TT at SNPs Ex2-157 and Ex3-128. However, CMR43-08-89 also has the same genotype with resistant varieties at SNPs Ex2-157 and Ex3-128. Therefore, these two SNP markers may not be as effective as SNP Ex2-78 for selecting varieties resistant to CMD.

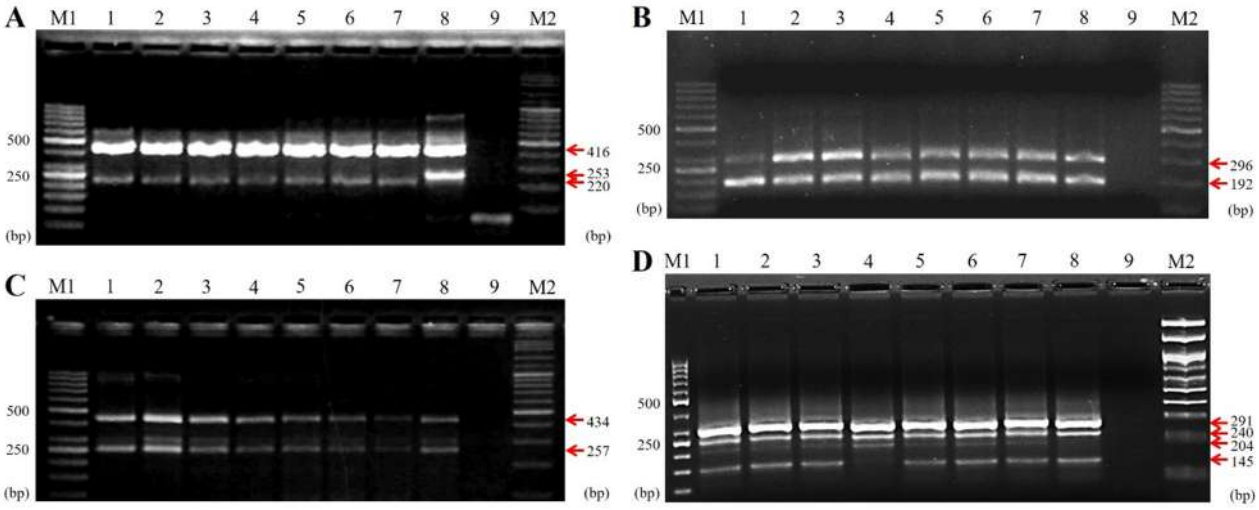

Figure 3 Genotype analysis of cassava varieties using SNP Ex2-78 (A), Ex2-157 (B), Ex3-128 (C) and S12_7926132 (D). M1: 50 bp DNA Ladder; 1: TME 3; 2: C 33; 3: IITA-TMS-IBA 920057; 4: TMS-IBA 972205; 5: IITA-TMS-IBA 980505; 6: IITA-TMS-IBA 980581; 7: TME B 419; 8: CMR43-08-89 (CMD-susceptible variety); 9: ddH_2O; M2: 100 bp DNA Ladder plus

For the detection of SNP S12_7926132, the optimum condition was an annealing temperature of 58° C without DMSO. The genotype analysis of SNP S12_7926132 is shown in Figure 3D. The 291-bp band and 240-bp band were control fragments. The 204-bp band and 145-bp band represented the T allele and the G allele, respectively (Figure 3D). Rabbi et al. (2020) reported that the T allele is a favorable genotype found in CMD-resistant varieties. Similar to this study, the T allele

was found in all 7 CMD-resistant varieties. Cassava varieties TME 3, C 33, IITA-TMS-IBA 920057, IITA-TMS-IBA 980505, IITA-TMS-IBA 980581, and TME B 419 have genotype heterozygous TG (Figure 3D lane 1 - 3 and 5 - 7), while TMS-IBA 972205 has genotype homozygous TT (Figure 3D lane 4). The susceptible variety CMR43-08-89 has the genotype homozygous GG (Figure 3D lane 8).

Development of a primer set for discrimination of each CMD-resistant cassava variety

Because the shape and number of lobes of 7 CMD resistant varieties were similar, it was difficult to differentiate these varieties, especially among TME 3, C 33, IITA-TMS-IBA 920057, IITA-TMS-IBA 980505 and IITA-TMS-IBA 980581 with similar red leaf petioles and TMS-IBA 972205 and TME B 419 with green leaf petioles. To identify the cassava variety TME B 419, a primer set for detection of the SNP S12_8910428 was developed.

The optimum condition was an annealing temperature of 59° C without DMSO. The genotype analysis of SNP S12_8910428 is shown in Figure 4. The 398-bp band was the control fragment. The 258-bp band and 196-bp band represented the C allele and the G allele, respectively (Figure 4). The C allele was uniquely observed in TME B 419, which had genotype heterozygous CG (Figure 4 lane 1), while the other CMD-resistant varieties had genotype homozygous GG (Figure 4 lane 2 -7).

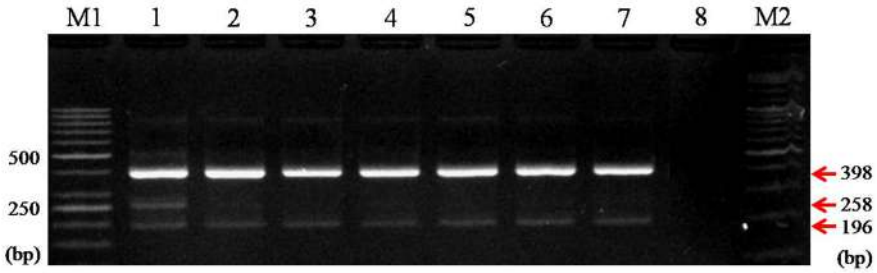

Figure 4 Genotype analysis of cassava varieties using S12_8910428, M1: 50 bp DNA Ladder; 1: TME B 419; 2: TME 3; 3: C 33; 4: IITA-TMS-IBA 920057; 5: TMS-IBA 972205; 6: IITA-TMS-IBA 980505; 7: IITA-TMS-IBA 980581; 8: ddH_2O; M2: 100 bp DNA Ladder plus

This result was consistent with the report of Lim et al. (2022) that the C allele is found in TME B 419 and TME 204 but not in other resistant varieties examined in the research, such as TME 3, TME 8, TME 14, NASE 12, NASE 14 and TMS-9102324. Therefore, SNP S12_8910428 can differentiate TME B 419 from the remaining six CMD-resistant varieties. Moreover, the SNP S12_7926132 can distinguish TMS-IBA 972205 from the others because only this variety has a

homozygous TT genotype at the SNP position (Figure 3D lane 4).

In addition, using the SSR marker, SSRY75, could differentiate IITA-TMS-IBA 980505 and IITA-TMS-IBA 980581 from the remaining varieties. Analysis of the genotype using the SSRY75 marker revealed that TME 3, C 33, IITA-TMS-IBA 920057, IITA-TMS-IBA 972205 and TME B 419 showed only 1 band with the approximate size of 280 bp (Figure 5 lane 1 - 4 and 7), while IITA-TMS-IBA 980505 and IITA-TMS-IBA 980581 showed 3 and 2 bands, respectively (Figure 5 lane 5 and 6).

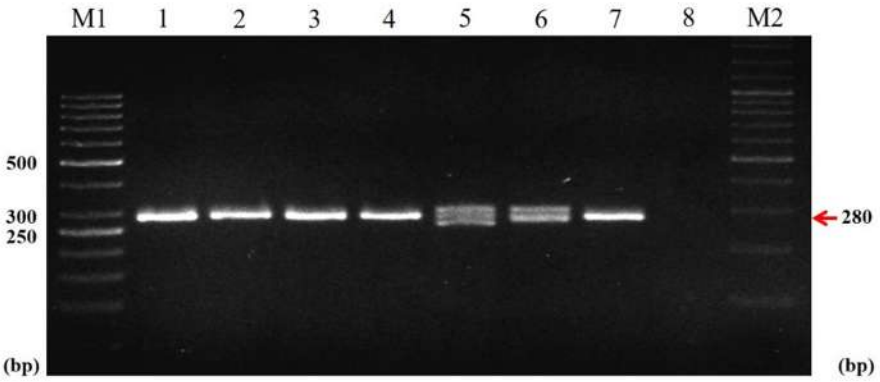

Figure 5 Genotype analysis of cassava varieties using SSR marker SSRY75, M1: 50 bp DNA Ladder; 1: TME 3; 2: C 33; 3: IITA-TMS-IBA 920057; 4: TMS-IBA 972205; 5: IITA-TMS-IBA 980505; 6: IITA-TMS-IBA 980581; 7: TME B 419; 8: ddH_2O; M2: 100 bp DNA Ladder plus

CONCLUSION

Primer sets used for the tetra-primer ARMS-PCR technique to detect SNP markers associated with CMD resistance for the selection of resistant varieties and to discriminate variety within the 7 CMD-resistant varieties used in cassava breeding programs in Thailand were developed. Four primer sets for the detection of SNP markers Ex2-78, Ex2-157, Ex3-128 and S12_7926132 were successfully developed for selecting CMD-resistance trait. Another SNP marker, S12_8910428 was developed for discriminating TME B 419 variety. The tetra-primer ARMS-PCR technique is specific, sensitive, speedy, cost-saving and convenient to carry out in a molecular laboratory for cassava breeding programs in Thailand with basic equipment.

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