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Novel SNP Markers and Their Application in Low-Cyanide Cassava (*Manihot esculenta* crantz) Breeding Program

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Abstract

The major flaw of cassava is its indigenous cyanide. Traditional breeding program for diminishing cyanide in cassava take a long waiting time for harvesting cassava tubers to assess cyanide content. Genetic markers are useful for selection of good agricultural traits, which also applied in low-cyanide cassava breeding programs by detecting gene associate with cyanide content in cassava. Therefore, the objective of this study was to develop SNP markers to detect low cyanide trait in cassava. Genotype analysis was carried out using Genotyping by Sequencing (GBS) technology assembling with phenotype analysis. Then, the SNP markers associated with cyanide content were found. Afterward, the primers of SNP marker associated with cyanide content 2 positions were developed using tetra-primer ARMS-PCR techniques. S16_640082 and S16_735381 SNP markers on chromosome 16 showed potential ability of the selection of cassava varieties with cyanide content less than 250 mg HCN/kg fresh weight. The selection accuracy of S16_640082 and S16_735381 SNP markers was 73.33 and 76.64 %, respectively. These markers could be used in marker assisted selection (MAS) in cassava seedling. It substantially reduces cassava breeding programs cost and time.

Keywords: SNP detection, Tetra-primer ARMS-PCR, Cyanogenic glycoside, Hydrocyanic acid, Molecular breeding

Introduction

Cassava (Manihot esculenta Crantz) is an important staple crop for food and feed as the source of energy. Worldwide fresh cassava production in 2020 was ca. 37 billion USD [1]. However, the indigenous cyanide is a major flaw of cassava. There are several reports that cassava consumption associated with neurological disorders [2-4]. Thus, numerous breeding programs for diminishing cyanide content has been performed. Despite that, traditional breeding programs carried out about 1 year for harvesting cassava tubers to evaluate cyanide content [5]. Nowadays, modern plant breeding with the aid of molecular markers plays an important role in agriculture, especially for improving desired traits to meet the need of the market. Marker-assisted selection (MAS) is one of the most prominent techniques for selecting desirable traits. There was report that cyanogenic glucoside potential (CNP) traits were highly influenced by the environmental conditions and the genetic control (2 quantitative trait loci (QTL) on 2 different linkage groups) of these traits [6]. In the same manner, according to study of Whankaew *et al.* [7], QTL could provide useful markers to assist in cassava breeding and studying of genes affecting the trait. They found 5 new QTL affecting cyanogenic potential (CN) and CN09R1 was the most significantly associated with the CN trait [7]. This technique coupled with single nucleotide polymorphism (SNP) have been effectively used in many crops breeding such as rice [8], sugar beet [8,9] and corn [10]. SNP is a variation at a single position in a DNA sequence. It plays an important role in phenotypic variation among individuals. SNPs occur at a high frequency within cassava genes, and can be targeted as useful genetic markers [11]. Although, there were reports of using molecular markers in cassava breeding [12,13], however, they mainly focused in yield, nutrition and disease resistance. Recently, there was only few studies of SNP markers that link to cyanide content [12]. Up to our knowledge, there is rarely report on SNP marker related to cyanide content in Thai cassava population. The objective of this study was to develop SNP markers for detecting low cyanide trait in cassava.

Materials and methods

DNA extraction

DNA was extracted from cassava leaves using DNAsecure Plant Kit (TIANGEN, China). The quantity and quality of DNA were measured by using a UV-Visible spectrophotometer (Perkin Elmer). DNA was analyzed in 0.8 % (w/v) agarose gel electrophoresis.

Determination of the total cyanide content in cassava root

The cyanide content was evaluated in roots as mg HCN equivalents kg^{-1} fresh weight (ppm) using picrate method as described by Bradbury *et al*. [14]. The 3 roots of each plant were collected to analyze 3 replications. A hundred milligrams of the cassava root were weighed out into a plastic vial then 0.5 mL of 0.1 M phosphate buffer pH 7.0 was added. After grinding sample, the vial was immediately closed with a screw stopper containing picrate paper. Incubation was at room temperature for 16 h. Afterward, the picrate paper was removed and immersed into 5.0 mL water for 30 min. The absorbance was measured at 510 nm. The total cyanide content was expressed as mg HCN kg^{-1} fresh cassava tuber: ppm.

SNPs target information

The research was carried out with genotype analysis using Genotyping by Sequencing (GBS) technology and the phenotype of cyanide was examined to find out SNPs associated with cyanide content. DNA sequence containing the SNPs from Phytozome database was used in designing primers (https://phytozome-next.jgi.doe.gov). Two set primers were designed by using an accessible primer1 program (http://primer1.soton.ac.uk/primer1.html). The primers used for tetra primer Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) for S16_640082 and S16_735381 detections are provided in **Table 1**.

Table 1 Primers used in the tetra-primer ARMS-PCR for cyanide content detection in cassava.

SNP position		Primer set and sequences	product size
S16_640082	Outer 1 F	5’ AACTTTTTCCTGAATTGAATTGTTGGTG 3’	Control fragment: 265 bp
	Outer 1 R	5’ ACCATAAAGTCACTGACCAAATGGAACT 3’	
	Inner 1 F	5’ AAAGAAGCCATGAATCCAAAGCAATT 3’	T allele: 186 bp
	Inner 1 R	5’ ACCATACCTCACACTCTTTCAAGTTGCT 3’	A allele: 133 bp
S16_735381	Outer 2 F	5’ CTTGGCAAATTCTGAGGCTTATTTATGG 3’	Control fragment: 397 bp
	Outer 2 R	5’ TGGTGGTTCTTGAAATCATAGGAACAAA 3’	
	Inner 2 F	5’ GTGGACTCACAGAATCACAAGTCATTGTAC 3’	C allele: 207 bp
	Inner 2 R	5’ GAAGGGGAGGAATTATTTCTCACCCA 3’	T allele: 246 bp

Optimization of tetra-primer ARMS-PCR

The tetra-primer ARMS-PCR reaction was performed in total volume of 20 µL containing 40 ng of genomic DNA, 1x Colorless GoTaq® Reaction Buffer (Promega, USA), 0.2 µM of each inner primer and 0.2 µM of each outer primer. DNA amplification was applied by using GeneAmp® PCR System 9700 (Applied Biosystems). The optimum PCR cycling profile was 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 40 s, annealing for 40 s with different temperatures as followed at 60, 63 and 65 °C, 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.

Validation of SNP markers

To evaluate the accuracy of the novel SNP markers, tetra-primer ARMS–PCR was performed using the primer sets and tested with 109 cassava varieties from the genetic diversity collection of Rayong Field Crops Research Center, Thai Department of Agriculture (DOA).

Estimation of biological metrics

The ability of the markers to predict the response of genotypes to cyanide content phenotype was determine by using the accuracy (ACC, the proportion of correctly predicted genotypes, as either low or high cyanide) according to report of Ige *et al.* [13]. The statistics was calculated using the formula below:

Accuracy (ACC) = TP + TN /TP + FN + FP + TN

where FP = false positive, TN = true negative, FN = false negative, TP = true positive.

Results and discussion

Optimization of tetra-primer ARMS-PCR was carried out by using gradients of annealing temperature at 60, 63 and 65 °C. Tetra-primer ARMS-PCR optimization for S16_640082 detection was shown in **Figure 1**. The optimized annealing temperature for S16_640082 detection was 60 °C (**Figure 1A**). The best condition for S16_735381 was the annealing temperature of 60 °C (**Figure 1B**). For the ratio of outer and inner primer, the best condition was 1:1. This optimum condition was determined by the appearance of clear DNA fragment at expected size. Thus, optimum condition of tetra-primer ARMS-PCR to detect 2 SNPs in this study were successfully determined.

The product of tetra-primer ARMS-PCR specific to S16_640082 contained 265 bp as a control fragment, 133 bp as A allele, and 189 bp as T allele (**Figure 1A**); for S16_735381 contained 397 bp as a control fragment, 207 bp as C allele, and 246 bp as T allele (**Figure 1B**).

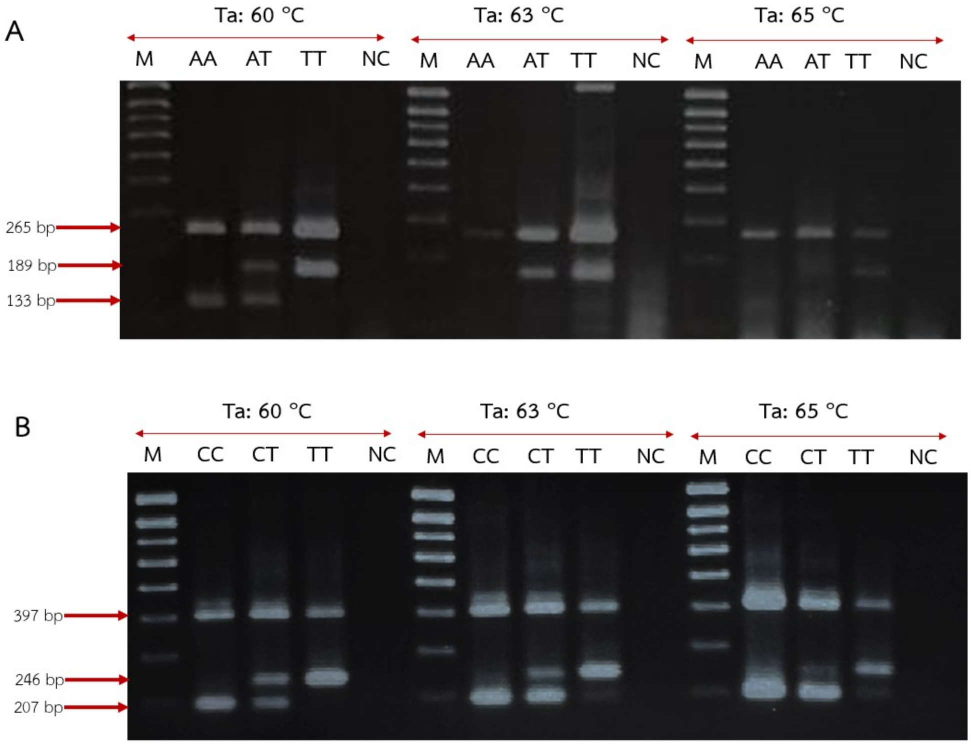

Figure 1 Tetra-primer ARMS–PCR optimization for A) S16_640082, B) S16_735381 detection with different annealing temperatures. The optimum PCR condition using outer and inner primer at the ratio of 1:1 and annealing temperature of 60 °C A) DNA fragments generated by S16_640082 include control fragment (265 bp), specific fragment of A allele (133 bp) and T allele (189 bp); B) DNA fragments generated by S16_735381 include control fragment (397 bp), specific fragment of C allele (207 bp) and T allele (246 bp) (M: DNA ladder 100 bp, Ta: annealing temperature, NC: negative control (water)).

Then, the 2 primer sets were tested on 109 cassava varieties from the genetic diversity collection of Rayong Field Crops Research Center, DOA for validating the accuracy of SNP using tetra-primer ARMS PCR. The genotyping results are given in Table 2 and an example of agarose gel electrophoresis of 14 cassava varieties genotyped by tetra-primer ARMS–PCR is shown in **Figure 2**.

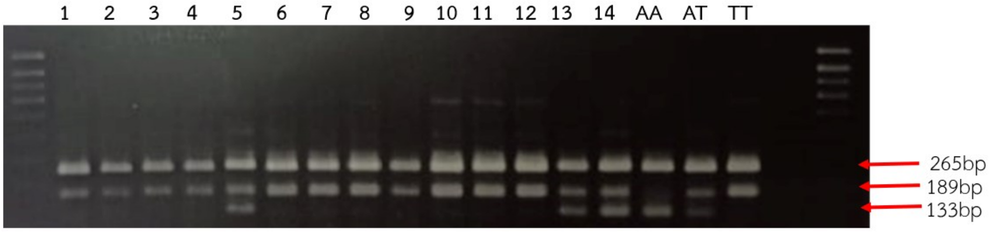

Figure 2 Agarose gel electrophoresis of 14 cassava varieties genotyped by tetra-primer ARMS-PCR using the primer set of S16_640082 (M: DNA Ladder 100 bp).

The SNP marker validation showed that the SNP markers S16_640082 and S16_735381 were effective in the selection of cassava varieties with cyanide content less than 250 mg HCN/kg fresh weight with 73.33 and 76.64 % accuracy, respectively. Cassava varieties with genotype TT (189 bp) by using S16_640082 (**Figure 3A**) and genotype CC (207 bp) by using S16_735381 (**Figure 3B**) showed low cyanide content. This result indicated that the 2 SNPs are effective marker (**Table 2**). Blair *et al.* [15] reported the result of validation for the marker to CMD resistance selection. The marker analysis and phenotypic evaluation of CMD resistance in the field revealed that the markers, RME1 and NS158 were excellent prediction tools for CMD resistance in some crosses (a prediction accuracy of 70 - 80 %).

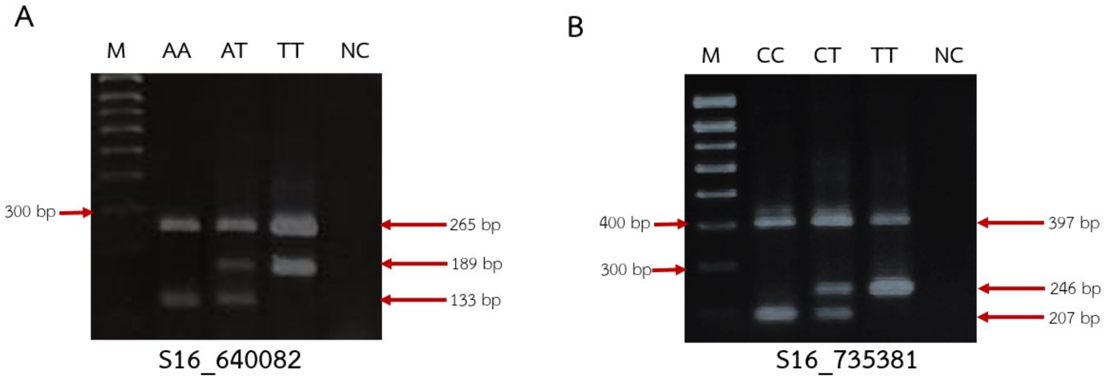

Figure 3 Genotyping results of cassava using SNP markers A) S16_640082 and B) S16_735381. The alleles driving low cyanide (< 250 mg HCN/kg fresh cassava tuber) at S16_640082 and S16_735381 loci show TT allele (189 bp) and CC allele (207 bp), respectively.

The criterion for decision is indicative of the presence or absence of a desired phenotype based on the banding patterns of SNP markers located in or near the genes controlling the interest phenotype. SNP marker S16_640082 is located on a chromosome 16 between a *glyoxalase I homolog gene* (*Manes.16G006100*) and *adenylyl-sulfate reductase (thioredoxin)/thioredoxin-dependent 5′-adenylylsulfate reductase gene* (*Manes.16G006000*). As reported by He *et al.* [16], glyoxalase and glutathione (GSH) same system function in the called glyoxalase system, which is a ubiquitous enzymatic network, which plays important roles in biological life. In addition to this, GSH is involved in the metabolism of cyanide [17]. Likewise, 5′-adenylylsulfate (APS) reductase generates sulfite an intermediate for cysteine generation essential for the biosynthesis of glutathione [18]. These facts are consistent with the positions of the SNPs identified in these genes. While, SNP marker S16_735381 is located on a chromosome 16 in intron of *calcium-dependent protein kinase 29 gene* (*Manes.16G007500*). This is consistent with the report that potent protein kinase C (PKC) plays important roles in cyanide poisoning [19] and MAP kinase activates cyanide constituents in the production of reactive oxygen species (ROS) and acute plant cell death. (hypersensitive cell death) [20]. Recently, Ogbonna *et al.* [12] reported the nonsynonymous SNP related to HCN content on Chromosome 16 (SNP S16_773999) within the Manes.16G007900 gene, which is annotated as a Multidrug and Toxic Compound Extrusion or Multi-Antimicrobial Extrusion protein (MATE). MATE transporters have been implicated directly or indirectly in mechanisms of detoxification of noxious compounds and ability to transport cyanogenic glucosides [21]. Besides, the second MATE gene

(Manes.16G008000) was also reported. It was in agree with this present study that found 2 SNPs were located on Chromosome 16 as well.

Validation of 2 SNP markers using together revealed that the accuracy is higher than the use of one SNP individual at 78.33 %. Therefore, the use of the marker together is recommended for more preciseness.

Table 2 The allele specific genotyping by tetra-primer ARMS-PCR of the 109 cassava varieties.

No.	Variety	Cyanide content (mgHCN/kg fresh cassava tuber)	S16_ 640082 allele	S16_ 735381 allele
1	Hanatee	178.89	T	C
2	*Huay Bong* 80	508.23	T	C
3	*Huay Bong* 60	389.58	T	C
4	Rayong 90	227.00	T	C
5	Rayong 9	324.97	A/T	C/T
6	Rayong 72	496.99	A/T	C/T
7	Rayong 7	191.96	A/T	C
8	Rayong 60	307.03	A	T
9	Rayong 5	221.15	T	C
10	Rayong 3	186.48	T	C
11	Rayong 2	120.63	T	C
12	Rayong 1	195.58	A/T	C/T
13	Yod Kum	344.17	A/T	C/T
14	Pirun 2	728.25	T	C
15	KU 50	567.82	A/T	C/T
16	Yolk	111.00	T	C
17	Wild 1	106.37	T	C
18	OMR 50-13-26	746.75	A	T
19	OMR 44-23-34	208.98	A/T	C/T
20	OMR 29-20-118	112.93	T	C
21	OMR 26-14-9	137.35	T	C
22	MVEN 67 B	208.56	T	C
23	MVEN 47	146.73	T	C
24	MVEN 297 A	220.16	A/T	T
25	MVEN 276	374.18	T	T
26	MVEN 204	169.31	T	C
27	MUSA 8	706.51	T	C/T
28	MUSA 5	140.02	A/T	C
29	MTAI 3	150.44	T	C
30	MTAI 1	155.05	T	C/T
31	MPTR 8	598.05	A/T	C
32	MPTR 26	249.19	T	C
33	MPER 613	142.17	T	C
34	MPER 349	247.34	T	C
35	MPER 283	151.48	T	C/T
36	MPER 281	242.57	A/T	C/T

No.	Variety	Cyanide content (mgHCN/kg fresh cassava tuber)	S16_ 640082 allele	S16_ 735381 allele
37	MPER 234	164.43	A	C
38	MPER 183	232.91	T	C
39	MPER 179	117.46	T	C
40	MPAR 4	132.09	T	C
41	MPAR 25	173.71	T	C
42	MPAN 127	253.78	T	C
43	MNGA 1	225.73	A/T	C/T
44	MMEX 65	497.40	T	C/T
45	MMAL 63	238.29	T	C
46	MENTEGA	223.92	T	C
47	MECU 72	123.55	T	C
48	MECU 71	111.75	T	C
49	MCOL 310	137.82	T	C
50	MCOL 2493	812.28	T	C/T
51	MCOL 2331	685.56	A/T	T
52	MCOL 2157	103.87	T	C
53	MCOL 2089	354.09	T	C/T
54	MCOL 1702	168.35	A	C/T
55	MCOL 1084 B	425.10	A/T	C
56	MBRA 931	297.24	A	C/T
57	MBRA 890	797.90	A	T
58	MBRA 885	346.39	A	C/T
59	MBRA 792	660.09	A/T	C/T
60	MBRA 781	196.03	A/T	C
61	MBRA 759	158.26	T	C
62	MBRA 534	777.94	T	T
63	MBRA 514	321.40	A/T	C/T
64	MBRA 403	474.53	A/T	C/T
65	MBRA 325	911.60	A/T	T
66	HL 23	208.28	T	T
67	Golden Yellow	268.20	A	C
68	CR 126	294.20	A/T	C
69	CR 1	146.30	T	C
70	CMR 51-23-14	396.74	A/T	C/T
71	CMR 51-13-14	320.56	A/T	C/T
72	CMR 51-04-42	412.60	T	C
73	CMR 50-73-6	205.17	T	C
74	CMR 50-41-1	136.52	A/T	C/T
75	CMR 50-30-23	211.79	A/T	C/T
76	CMR 50-20-2	662.95	A	T
77	CMR 50-20-114	312.85	A	T

No.	Variety	Cyanide content (mgHCN/kg fresh cassava tuber)	S16_ 640082 allele	S16_ 735381 allele
78	CMR 49-89-70	195.57	A/T	C/T
79	CMR 49-54-67	452.28	A	T
80	CMR 48-53-48	164.11	T	C
81	CMR 48-35-1	545.94	A/T	C/T
82	CMR 48-20-17	294.46	A/T	C/T
83	CMR 47-30-8	399.99	A/T	C/T
84	CMR 47-02-9	521.73	A/T	C/T
85	CMR 46-55-23	312.56	A/T	C/T
86	CMR 46-47-137	725.62	A	T
87	CMR 46-31-7	470.17	A/T	C/T
88	CMR 46-30-264	507.77	A/T	C/T
89	CMR 44-29-12	265.63	A/T	C/T
90	CMR 44-03-57	434.40	A	T
91	CMR 43-08-89	262.34	T	C
92	CMR 41-01-2	166.51	T	C
93	CMR 41-42-3	313.40	A/T	C/T
94	CMR 41-112-21	467.11	A/T	C/T
95	CMR 41-109-72	280.72	A/T	C/T
96	CMR 38-125-77	116.13	A/T	C/T
97	CMR 36-18-201	549.62	A/T	C/T
98	CMR 35-22-348	257.95	T	C
99	CMR 35-112-1	280.05	A/T	C/T
100	CMR 33-38-48	253.55	A/T	C/T
101	CMR 33-35-69	253.09	T	C/T
102	CMR 32-94-121	540.56	T	C
103	CMR 31-42-20	240.27	A/T	C/T
104	CMR 30-71-25	251.89	A/T	C/T
105	CMR 28-05-13	624.98	T	C
106	CMR 26-08-61	283.58	A/T	C/T
107	CMR 23-149-59	186.57	A/T	C/T
108	CM 4574-7	599.87	A/T	C/T
109	01-77-1	373.03	A/T	C/T

Conclusions

In this research, novel SNP markers associated with cyanide content 2 positions (S16_640082 and S16_735381) were identified and the primers to detect the SNP markers were developed using tetra-primer ARMS-PCR techniques. These SNP markers on chromosome 16 showed potential ability of the selection of cassava varieties with cyanide content less than 250 mg HCN/kg fresh weight. The selection accuracy of those SNP markers was 73.33 and 76.64 %, respectively. These markers could be used in marker assisted selection (MAS) in cassava seedling. This will be applied and useful in cassava breeding program with substantially reduces cost and time.

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