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# DESIGN OF PRIMERS FOR RAPD ANALYSES OF CASSAVA, *MANIHOT ESCULENTA*

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**Key Word Index**—*Manihot esculenta*; Euphorbiaceae; RAPD markers; primer design; linamarin.

**Abstract**—Primers with higher G+C content showed better random amplified polymorphic DNA (RAPD) patterns among the cassava varieties. Clusters of Cs in the decamer primer sequence also seemed to influence banding patterns. RAPD patterns arising from 13 custom-made 10 and 12-base primers with varying lengths of successive Cs further revealed that strings of 4 to 6 Cs at the 5′ and 3′ end tended to provide stronger DNA amplification. Using a combination of RAPD patterns from several primers, it was possible to identify the eight cassava varieties used in this study. One custom-made primer also showed potential application as marker for cyanogenic potential in cassava. © 1997 Published by Elsevier Science Ltd

## INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important source of dietary carbohydrate for more than 500 million people in developing countries [1]. Many cultivated varieties of cassava vary in the levels of linamarin content/cyanogenic potential in the storage roots [2]. Thus, for the purpose of safety of cassava consumption, several analytical methods have been developed to determine the amount of cyanogenic potential in cassava roots and its products [2–8]. Food safety in cassava, however, could also be achieved through breeding of varieties with low linamarin content. In this respect, availability of biochemical and/or genetic markers related to linamarin accumulation in storage roots may be helpful. Biochemical and molecular approaches, including RAPD techniques, have been shown to be potentially useful in fingerprinting cassava germplasm collections [9–14]. However, they will be even more useful if markers for linamarin content/cyanogenic potential in cassava could be identified.

In this report, we focused on the design of primers for RAPD analyses of cassava. We tested 30 random primers with 60–80% G+C content on genomic DNA extracted from leaves of eight cassava varieties. Data from this work was then used in the design of thirteen 10 and 12-base primers with 50–90% G+C content. We also examined whether the RAPD patterns showed any correlation with the cyanogenic potential of the cassava varieties.

## RESULTS AND DISCUSSION

Thirty standard decamer random sequences with G+C content of 60, 70 and 80% were used as primers for RAPD analysis of eight cassava cultivars (Table 1). The results showed that under the conditions employed in this study, the robustness, complexity and reproducibility of the banding patterns improved with increasing G+C content of the primers. For example, only two out of 10 decamer sequences of 60% G+C content showed DNA amplification whereas for sequences with 70% G+C content, six out of 10 gave DNA amplification, and all decamers with 80% G+C content showed DNA amplification. An examination of these 30 random primer sequences also suggested that the distribution of C rather than its proportion could contribute to improved banding patterns.

Therefore 13 oligonucleotides ranging from 10 to 12 bases were custom-made to assess their usefulness as primers for RAPD analysis (Table 2). These primers also varied in their G+C content, proportion of Cs and arrangement within the sequence. All the 13 custom-made primers could be used for RAPD analyses; they differed in the number and intensity of the amplification of DNA bands. Primers with higher C content together with certain arrangements of Cs usually gave stronger banding patterns. For example, sequences with strings of 6 and 8 Cs produced weak

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Table 1. Number of reproducible fragments detected with random primers for eight cassava cultivars

| Random primer sequence | G+C content (%) | Proportion of C (%) | Number of reproducible fragments |
|---|---|---|---|
| GCAGACTGAG | 60 | 20 | —* |
| GTCCTCAGTG | 60 | 30 | 1 |
| GTCCTTAGCG | 60 | 30 | 0 |
| CGCAGTACTC | 60 | 40 | 2 |
| CTACACAGGC | 60 | 40 | 0 |
| GAGTCACTCG | 60 | 30 | 0 |
| GTCCTCAACG | 60 | 40 | 0 |
| GTCCTACTCG | 60 | 40 | 0 |
| CGTCGTTACC | 60 | 40 | 0 |
| CTACTACCGC | 60 | 50 | 0 |
| GTGTAGGGCG | 70 | 10 | 2 |
| GTCTCGTCGG | 70 | 30 | 3 |
| GGCCTTCAGG | 70 | 30 | **4** |
| TGCACGGACG | 70 | 30 | **4** |
| CGGGTCGATC | 70 | 30 | 0 |
| CAGGGGCATC | 70 | 30 | 0 |
| GGCCTACTCG | 70 | 40 | 4 |
| GAGACCTCCG | 70 | 40 | 0 |
| GCTCTCACCG | 70 | 50 | **1** |
| GCCCTCTTCG | 70 | 50 | 0 |
| GCAGGTCGCG | 80 | 30 | 2 |
| CGAGACGGGC | 80 | 30 | **3** |
| CGACGCGTGC | 80 | 40 | 5 |
| CGCGAACGGC | 80 | 40 | 1 |
| GCAGCTCCGG | 80 | 40 | 3 |
| GGCCACAGCG | 80 | 40 | **6** |
| GCAGCAGCCG | 80 | 40 | 2 |
| ACGCCCTGGC | 80 | 50 | 3 |
| ACCCGTCCCC | 80 | 70 | **6** |
| ACCGCCTCCC | 80 | 70 | **5**, 2 |

Bold print indicates strong DNA amplification while normal type face indicates weak DNA amplifications; * No amplification of cassava leaf DNA.

bands whereas those with 3–4 Cs either on the 5′ or 3′ end of the primer gave good banding patterns. Furthermore, it was observed that oligonucleotides with strings of Gs and no Cs (5′-GGGTAGGGG-3′,5′-GGGGATGGGG-3′ and 5′-GGGAGGGCG G-3′) gave weak DNA amplification. Overall, it appeared that in designing the primers, the number of Cs and their arrangement e.g. clusters of 3–5 Cs flanking the 5′ and 3′ end, must be considered. The results also showed that G in the sequences seemed unimportant. In fact, primers with strings of Gs gave weak DNA amplification.

RAPD patterns for cassava varieties arising from the use of primers that gave strong DNA amplification are given in Tables 3 and 4. It could be seen that no single primer could generate a RAPD profile that could be used to distinguish the different cassava varieties. The number of varieties these primers could distinguish varied from 2 to 6. However, if these patterns were used in combination, it was possible to characterise the different cassava varieties. In this respect, it should be useful for fingerprinting cassava germplasm collection or identification purposes, reaffirming the observations made by others [12, 13]. Using data from Tables 3 and 4, cluster analysis following the NTSYS-UPGMA algorithm [15] generated with the Jaccard coefficient grouped PRC2, PRC377 and PRC443 as one cluster whereas PRC60a, PRC329 and Putih were placed closer together, and PRC 476 and Green Twig formed a third group (Fig. 1). Within each group, varieties with high and low cyanogenic potentials could be found (see Table 5). However, with primer 5′-CCCTCCCGCC-3′, PRC2, PRC60a and PRC329 which had cyanogenic potential greater than 145 mg HCN $kg^{-1}$ fresh weight root could be distinguished from the other cassava varieties.

Overall, this study showed that primer design can be manipulated to produce more complex and repro-

Table 2. Number of reproducible fragments detected with custom-made primers for eight cassava cultivars

| Primer sequences | G+C content (%) | Proportion of C (%) | Number of reproducible fragments |
|---|---|---|---|
| CCCCCCATAATT | 50 | 50 | 1 |
| CCCACCCTATAA | 50 | 50 | 7 |
| CCCCTAATCCCC | 67 | 67 | 5 |
| CCCCCCATCCCT | 75 | 75 | **6** |
| GGGGTAGGGG | 80 | 0 | 7 |
| GGGGATGGGG | 80 | 0 | 5 |
| GGGAGGGCGG | 80 | 0 | 8 |
| GTGCCCCTGG | 80 | 40 | **7** |
| CCCCCCCCAT | 80 | 80 | 3 |
| CCCCCCATCC | 80 | 80 | 6 |
| CCCTCCCGCC | 80 | 80 | **9** |
| CCCCATCCCC | 80 | 80 | **8** |
| CCCCAGCCCC | 90 | 80 | **5** |

Bold print indicates strong DNA amplification while normal type face indicates weak DNA amplification.

ducible RAPD markers, thus saving time and effort in using commercially available random primers for cassava RAPD work. It was also interesting that one custom-made primer (5′-CCCTCCCGCC3′) might be potentially useful as a molecular marker for cyanogenic potential in cassava. In this respect, it might be worthwhile designing primers of different lengths and sequences to evaluate a wider collection of cassava germplasm.

## EXPERIMENTAL

*DNA extraction.* Cassava leaves (0.25 g) were ground to a powder in liquid $N_2$ in a chilled mortar and pestle. The powder was resuspended in 0.7 ml extraction medium containing 2.5% hexadecyltrimethylammonium chloride, 100 mM Tris–HCl (pH 8), 50 mM EDTA, 1.7 M NaCl, 10 mM 2-mercaptoethanol, followed by addition of 0.2 ml 10% PVP-10 and 75 ml 20% SDS. The content was thoroughly mixed, then heated for 30 min at 60°. This was followed by extraction ×2 with 0.5 ml $CHCl_3$–isoamyl OH (24:1). Cold *iso*-PrOH (2/3 vol) was added to the aq. phase to ppt the nucleic acids. The pellet was washed in 100% EtOH, followed by a 70% EtOH wash and vacuum drying. It was resuspended in 0.1 ml TE (10 mM Tris-HCl (pH 7.4), 1 mM EDTA) and digested with RNase A (10 mg $ml^{-1}$) for 30 min at 37°. PhOH–$CHCl_3$ (1:1) extraction was carried out and the nucleic acid was pptd with cold 100% EtOH, washed twice with cold 70% EtOH, vacuum dried and resuspended in 25 ml TE.

*PCR amplification.* Thirty standard decamer RAPD primers with G+C contents ranging from 60 to 80% were purchased from Genosys Biotechnologies, U.S.A. Thirteen 10 and 12-base primers with G+C contents from 50 to 90% were designed with various permutations of C and G clusters. Amplification reactions (total vol., 50 ml) containing 150 ng template DNA, 0.4 mM dNTP, 4 mM $MgCl_2$, 1 mM primer and 2.5 U Taq polymerase (Promega, U.S.A.) were performed using the Perkin–Elmer Thermocycler 480. Samples were subjected to a hot start at 95° for 10 min followed by 30 cycles of 1 min at 95°, 3 min at 32°, 2 min at 72° and ending at 72° for 10 min. Amplification was replicated at least ×3 to test reproducibility. A control reaction with sterile $H_2O$ instead of DNA was run for each primer to ensure fragments visualized were not false positives. Amplification products were resolved in 2% agarose gels in 1X TAE buffer [16] at 100 V for 90 min and visualized by staining in ethidium bromide. Fragment size was calculated using a computer program [17].

*Cyanogenic potential determination.* Roots were obtained from two plants for each variety and triplicate analyses were carried out for each root. The mid-region of freshly harvested cassava root (parenchyma tissue, 40 g) was homogenised in 200 ml of 0.1 M $H_3PO_4$. The homogenate was filtered through Whatman no. 1 filter paper and 0.4 ml of the filtrate was used for cyanogenic potential (linamarin) determination according to ref. [8] except that the pH of the root extract (0.4 ml) was adjusted to *ca* pH 7–7.2 by the addition of 0.1 ml 2 M $K_2HPO_4$. An enzyme-based dipstick was prepd using 10×20 mm alkaline picrate impregnated paper and samples were assayed in Na-Pi buffer. A standard curve was prepd with different concns of linamarin soln (up to 20 mg HCN equivalent, total vol. 0.4 ml).

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Table 3. RAPD patterns of cassava varieties obtained using random primers

| Primer sequence | Reproducible fragments (kb) | Cassava variety PRC 2 | PRC 60a | PRC 329 | PRC 377 | PRC 443 | PRC 476 | Green Twig | Putih |
|---|---|---|---|---|---|---|---|---|---|
| GGCCTTCAGG | 1.43 | + | + | + | + | + | + | + | + |
| | 1.00 | + | + | + | + | + | + | + | + |
| | 0.93 | + | 0 | + | 0 | + | + | + | + |
| | 0.45 | + | + | + | + | + | + | + | + |
| TGCACGGACG | 0.93 | + | + | + | + | + | + | + | + |
| | 0.86 | + | 0 | + | + | + | + | + | + |
| | 0.70 | + | + | + | 0 | + | + | + | + |
| | 0.46 | + | 0 | 0 | + | + | + | 0 | 0 |
| GCTCTCACCG | 0.57 | 0 | 0 | 0 | 0 | 0 | + | 0 | + |
| CGAGACGGGC | 1.28 | + | + | + | + | + | + | 0 | + |
| | 1.17 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.75 | 0 | + | + | 0 | 0 | 0 | 0 | + |
| GGCCACAGCG | 1.71 | + | + | + | + | + | + | + | + |
| | 1.60 | + | + | + | + | + | + | + | + |
| | 1.13 | + | 0 | + | + | + | 0 | 0 | 0 |
| | 1.20 | 0 | + | 0 | 0 | 0 | + | + | 0 |
| | 0.95 | + | + | + | + | + | 0 | 0 | 0 |
| | 0.70 | 0 | 0 | + | + | 0 | + | + | + |
| ACCCGTCCCC | 1.90 | + | + | + | + | + | + | + | + |
| | 1.00 | + | + | + | + | + | + | + | + |
| | 0.92 | 0 | + | + | + | + | + | + | + |
| | 0.85 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.72 | + | + | + | + | + | + | + | + |
| | 0.42 | + | + | + | + | + | + | + | + |
| ACCGCCTCCC | 1.16 | + | + | + | + | + | + | + | + |
| | 0.75 | 0 | 0 | + | 0 | + | 0 | 0 | 0 |
| | 0.90 | 0 | 0 | + | 0 | 0 | 0 | 0 | + |
| | 0.89* | 0 | + | + | 0 | 0 | 0 | 0 | + |
| | 0.85 | + | 0 | 0 | 0 | 0 | + | + | 0 |
| | 0.84 | + | + | + | + | + | + | + | + |
| | 0.73* | + | 0 | + | 0 | 0 | + | + | + |

* weakly amplified

Table 4. RAPD patterns of cassava varieties obtained with custom-made primers

| Primer sequence | Reproducible fragments (kb) | Cassava variety PRC 2 | PRC 60a | PRC 329 | PRC 377 | PRC 443 | PRC 476 | Green Twig | Putih |
|---|---|---|---|---|---|---|---|---|---|
| CCCCCCATCCCT | 1.28 | + | + | + | + | + | + | + | + |
| | 1.22 | 0 | 0 | 0 | + | + | + | + | 0 |
| | 0.89 | 0 | 0 | 0 | 0 | 0 | + | + | 0 |
| | 0.82 | 0 | 0 | 0 | + | + | 0 | 0 | 0 |
| | 0.71 | 0 | 0 | 0 | 0 | 0 | + | + | 0 |
| | 0.58 | 0 | + | + | 0 | 0 | 0 | 0 | 0 |
| GTGCCCCTGG | 1.28 | + | + | + | + | + | + | + | + |
| | 0.82 | 0 | + | + | + | 0 | + | + | + |
| | 0.93 | + | + | + | + | + | + | + | + |
| | 0.97 | + | + | + | + | + | + | + | + |
| | 0.67 | + | + | + | + | + | + | + | + |
| | 0.65 | + | + | + | + | + | + | + | + |
| | 0.60 | + | + | + | + | + | + | + | + |
| CCCTCCCGCC | 1.76 | + | + | + | + | + | 0 | 0 | + |
| | 1.40 | + | + | + | 0 | 0 | + | + | + |
| | 0.86 | + | 0 | 0 | 0 | 0 | + | + | 0 |
| | 0.78 | 0 | + | + | + | + | 0 | 0 | 0 |
| | 0.70 | 0 | + | + | + | + | 0 | 0 | + |
| | 0.68 | + | + | + | + | + | + | + | + |
| | 0.65 | + | + | + | + | + | + | + | + |
| | 0.63 | + | + | + | + | + | + | + | + |
| | 0.61 | + | + | + | + | + | + | + | + |
| CCCCATCCCC | 1.24 | + | + | + | + | 0 | 0 | 0 | + |
| | 1.17 | + | + | + | + | + | + | + | + |
| | 1.11 | + | + | + | + | 0 | + | + | + |
| | 0.78 | 0 | 0 | 0 | 0 | 0 | + | + | 0 |
| | 0.71 | + | + | + | + | + | + | + | + |
| | 0.64 | + | + | + | + | + | + | + | + |
| | 0.48 | 0 | + | 0 | 0 | 0 | + | + | + |
| | 0.46 | + | + | + | + | + | + | + | + |
| CCCCAGCCCC | 1.70 | + | + | + | + | + | + | + | + |
| | 1.54 | + | + | + | + | + | + | + | + |
| | 1.18 | 0 | 0 | 0 | 0 | + | + | 0 | 0 |
| | 1.11 | + | 0 | 0 | 0 | 0 | 0 | + | + |
| | 1.00 | + | 0 | 0 | + | + | + | + | 0 |

Table 5. Linamarin content in cassava storage roots

| Cassava variety | Linamarin content (mg $kg^{-1}$ fr. wt.) |
|---|---|
| PRC 443 | $30 \pm 11$ |
| Putih | $31 \pm 3$ |
| PRC 377 | $69 \pm 16$ |
| PRC 476 | $76 \pm 19$ |
| Green Twig | $104 \pm 19$ |
| PRC 2 | $145 \pm 13$ |
| PRC 60a | $210 \pm 19$ |
| PRC 329 | $261 \pm 9$ |

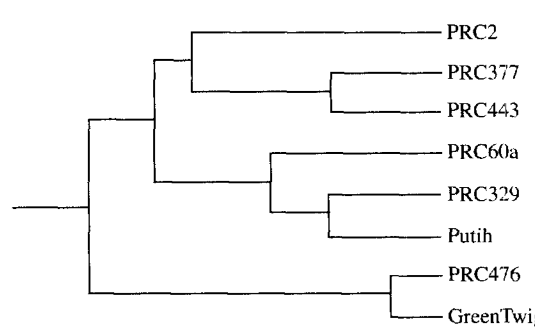


Fig. 1. Dendrogram showing the relationship between cassava varieties constructured using RAPD markers from Tables 3 and 4.

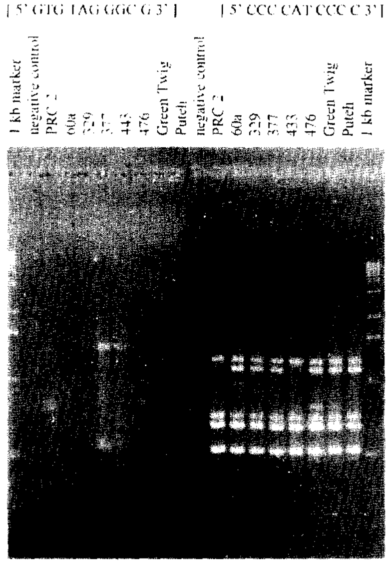


Fig. 2. RAPD profiles generated by random primer with 70% G+C content (5' GTGTAGGGCG 3') as opposed to RAPD profiles obtained by custom-made primer with 80% G+C content (5' CCCCATCCCC 3').


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