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Inter-retrotransposon-amplified polymorphism markers for germplasm characterization in *Manihot esculenta* (Euphorbiaceae)

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ABSTRACT. Manioc, *Manihot esculenta*, is economically important in many tropical and subtropical countries. The genetic variability of the species has not been fully explored, and new information may help expand its use. Molecular markers based on retrotransposons have good potential for analysis of genetic diversity given their abundance in the genome. Eight long terminal repeat retrotransposons were selected for the development of inter-retrotransposon-amplified polymorphism markers. To test these primers, we analyzed 32 varieties from Anori, 30 from Manicoré and 10 Mandiocabas from the Manioc Germplasm Bank at Embrapa

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Western Amazonia. The six informative primer pairs yielded 20-60 polymorphic bands, averaging 92% polymorphism (51.7-98.4) and 0.37 heterozygosity (0.17 to 0.40), with a Shannon information index of 0.54 (0.26-0.59). These markers can be used to explore the genetic diversity of manioc.

Key words: Inter-retrotransposon-amplified polymorphism; Polymorphism; Genetic diversity; Varietal discrimination

INTRODUCTION

Manioc (*Manihot esculenta* Crantz) is a perennial shrub in the Euphorbiaceae that is a major carbohydrate crop in tropical and subtropical countries, where it is mainly used for the production of flour, pure starch, fresh consumption, and assorted industrial uses. Although propagated vegetatively, manioc has great genetic variability because sexual reproduction continues, often resulting in polyclonal varieties (Silva et al., 2001). These local varieties, grown mostly by small-holder farmers, represent the genetic resources conserved and used in breeding programs.

Molecular markers are used to increase the discriminatory power of genetic variability analyses among manioc varieties. Although there are numerous published markers, there is still a need for new and more variable genetic markers, given the polyclonal nature of manioc varieties. Markers based on retrotransposons (IRAP - inter-retrotransposon-amplified polymorphism) generate great quantities of information, making them good tools for detecting genomic changes associated with their activity, because they create large and stable insertions in the genome; they are highly reproducible, show abundant polymorphism, and are easily viewed in a single gel (Kalendar et al., 2011). Retrotransposon polymorphisms are detected using marker systems that rely on PCR amplification between long terminal repeat (LTR) ends and some components of flanking genomic DNA. The IRAP products are generated with one or two primers matching either the 5'- or 3'-end of the LTR using outward-facing primers (Kalendar and Schulman, 2006).

Several families of transposable elements have been reported in manioc (Gbadegesin et al., 2008). This study describes the development of eight sets of IRAP primers based on retrotransposons to discriminate between manioc clones and varieties.

MATERIAL AND METHODS

Sequences of LTR retrotransposons were located in Phytozome (http://www.phytozome.net/). LTRs were confirmed with the LTR_Finder software (http://tlife.fudan.edu.cn/ltr_finder/) and cross-checked in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Eight LTR retrotransposons were selected and the outward-facing primers of the LTRs were designed using Primer3 (Rozen and Skaletsky, 2000) for amplification of the members of a retrotransposon family in head-to-head, head-to-tail and tail-to-tail orientation. Seventy-two manioc plants (32 from Anori, Amazonas, 30 from Manicoré, Amazonas, and 10 “mandiocabas”, a very sweet variety) maintained in the Manioc Germplasm Bank at Embrapa Western Amazônia, Manaus, Amazonas, were analyzed to test the information

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content of these IRAP markers.

Total DNA was extracted with 2% CTAB in the Molecular Biology Laboratory at Embrapa. The 20-µL reaction mixture consisted of 0.2 mM dNTPs, 0.5 µM forward and reverse primers, 2 mM $MgCl_2$, 1X Taq buffer, 1.5 U Taq GoTaq® DNA polymerase (Promega, USA) and 50 ng template DNA. PCR was performed using the following program: 2 min at 92°C; 40 cycles of 15 s at 92°C, 1 min at 40-60°C (Table 1), 2 min at 72°C; and final extension at 72°C for 10 min. Amplifications were carried out in a Veriti Thermal Cycler (Applied Biosystems, USA). PCR products were resolved by electrophoresis on 1.5% agarose gels in 0.5X TBE buffer stained with ethidium bromide and photographed in a transilluminator (Loccus Biotecnologia, Brazil). Polymorphism was detected by the presence (1) or absence (0) of the PCR product. The percentage of polymorphic loci, expected heterozygosity (Nei's genetic diversity) and Shannon information index were calculated with PopGene 1.31 (Yeh et al., 1999), for each population and overall.

RESULTS AND DISCUSSION

Two primer pairs (ME_1 and ME_3) produced only six bands and were excluded from further analysis. The others generated 20-60 polymorphic bands with sizes between 100 and 12,000 bp (Tables 1 and 2). With these primers, the two populations and mandiocaba samples showed a mean polymorphism of 92% (range = 51.7 to 98.4%), 0.37 expected heterozygosity (0.17 to 0.40) and a Shannon information index of 0.54 (0.26 to 0.59) (Table 2). Guo et al. (2006) found 86% polymorphism with IRAP and REMAP primers in persimmon (*Diospyros kaki* Thunb.), very similar to the results found here.

In manioc, 69% polymorphism was previously found with AFLP markers and 56% with RAPD markers in estimating the genetic variability of 54 varieties (Mühlen et al., 2000), demonstrating that these IRAP markers are more informative than other dominant markers.

Table 1. Characteristics of IRAP primers for *Manihot esculenta*.

Primer name	Sequence (5'-3')	Length (nt)	Ta (°C)	Amplicon size (bp)	Scaffold location Phytozome v.9.0
ME_1	CTGCATTTGAAGTTTGGTCCA[a]	21	60	150-1500	00579:8924..18923
	TTTCCAGCTTATTGCTTGGG[b]	20			
ME_2	GGTGATGATGTGTCCCTTCC[a]	20	49	200-4000	06700:127431..132763
	CTAGTGTATACCCAATATGCCC[b]	22			
ME_3	TCCATCAAATGGGTCTCTCA[a]	20	50	200-2000	03122:396999..406998
	ACCCAGCATTTCAAGTCTCG[b]	20			
ME_4	TGGAGCTTGAGGGTGTAAGG[a]	20	48	100-5000	00077:42599..52000
	TTCGATTGCTTCTCTCCTGC[b]	20			
ME_5	GCAAGGAGGGGGAATAAAAG[a]	20	40	100-4000	03413:6000..16000
	GCTTCTTTCTTACCGGGCTT[b]	20			
ME_6	TTTTTCATTTTCTTACTTTCTGTGTAA[a]	27	42	150-1800	09428:1..12686
	CCTATGATTATGCTATCAATTATCAC[b]	26			
ME_7	TTTCTTGATTCCCAAGGGTG[a]	20	48	200-3000	01259:102333..122332
	CCTCTCCATATTCTCTTCTCC[b]	21			
ME_8	GGTGAATTTCGGTTATTGAA[a]	20	56	100-12000	03481:28000..36000
	CCAGAGAATGATGTTGAAGG[b]	20			

[a]Primer designed from 5'-LTR end; [b]primer designed from 3'-LTR end; Ta = annealing temperature; nt = nucleotide.

Table 2. Genetic information of each IRAP primer pair in 72 *Manihot esculenta* varieties in the Manioc Germplasm Bank at Embrapa Western Amazonia.

Primer	Samples	No. of plants	No. polymorphic bands	h ± SD	I ± SD	%
ME_2	Anori	32	25	0.38 ± 0.17	0.55 ± 0.23	89.3
	Manicoré	30	24	0.32 ± 0.18	0.47 ± 0.24	85.7
	Mandiocaba	10	19	0.26 ± 0.20	0.38 ± 0.28	67.8
	Total	72	25	0.36 ± 0.16	0.52 ± 0.22	89.2
ME_4	Anori	32	26	0.30 ± 0.18	0.45 ± 0.24	89.6
	Manicoré	30	24	0.30 ± 0.19	0.44 ± 0.26	82.7
	Mandiocaba	10	15	0.17 ± 0.19	0.26 ± 0.27	51.7
	Total	72	26	0.32 ± 0.18	0.47 ± 0.26	89.6
ME_5	Anori	32	33	0.34 ± 0.16	0.50 ± 0.22	89.2
	Manicoré	30	32	0.31 ± 0.17	0.46 ± 0.24	86.5
	Mandiocaba	10	24	0.25 ± 0.21	0.37 ± 0.30	64.8
	Total	72	33	0.36 ± 0.15	0.52 ± 0.21	89.2
ME_6	Anori	32	21	0.36 ± 0.17	0.52 ± 0.23	87.5
	Manicoré	30	22	0.34 ± 0.18	0.50 ± 0.24	91.6
	Mandiocaba	10	18	0.27 ± 0.19	0.41 ± 0.27	75.0
	Total	72	22	0.39 ± 0.17	0.55 ± 0.22	91.6
ME_7	Anori	32	19	0.30 ± 0.17	0.45 ± 0.24	82.6
	Manicoré	30	19	0.31 ± 0.19	0.45 ± 0.26	82.6
	Mandiocaba	10	13	0.23 ± 0.22	0.33 ± 0.31	56.5
	Total	72	20	0.32 ± 0.18	0.48 ± 0.24	90.0
ME_8	Anori	32	59	0.39 ± 0.12	0.57 ± 0.15	96.7
	Manicoré	30	60	0.40 ± 0.11	0.59 ± 0.14	98.4
	Mandiocaba	10	52	0.31 ± 0.17	0.46 ± 0.23	85.2
	Total	72	60	0.42 ± 0.09	0.61 ± 0.11	98.4
Mean				0.37 ± 0.15	0.54 ± 0.20	92.0

h = Nei's genetic diversity ± standard deviation (SD); I = Shannon information index ± SD; % = percentage of polymorphic loci.

CONCLUSIONS

This is the first report of IRAP markers to characterize manioc varieties. They proved to be efficient in estimating the percentage of polymorphism and genetic diversity, and will likely permit good variety discrimination.

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