Identification of polymorphisms in resistance gene candidates in cassava (*Manihot esculenta* Crantz)

Identificación de polimorfismos en genes candidatos de resistencia en yuca (*Manihot esculenta* Crantz)

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Abstract

Cassava production can be detrimentally affected by diseases caused by different pathogens. To defend against viral, bacterial and fungal diseases, plants have developed a group of resistance proteins (R), which are able to recognize pathogen's molecules. A wide repertoire of R proteins has been identified in a large group of plants. Even though conferring resistance to different pathogens, these R proteins have a few conserved domains. Taking advantage of the recent release of the complete cassava genome sequence, we identified cassava R-like proteins in this genome. With this information, primers were designed to amplify 13 genes showing similarity to known *R* genes. For 10 of them we obtained amplification in TMS30572 and CM2177-2 varieties, which represent the parents used in the construction of the cassava genetic map. After sequencing the obtained amplicons, we identified 37 SNPs (Single Nucleotide Polymorphisms) between these two cassava varieties, which represent 18 (48.6%) transitions and 19 (45.9%) transversions. The remaining are insertions/deletions (indels). This knowledge will help to develop appropriate strategies for the generation of CAPs (Cleaved Amplified Polymorphisms) markers to assess their segregation in the F1 population, allowing the localization of these markers on the cassava genetic map.

Key words: Cassava, genetic maps, genomes, *Manihot esculenta*, molecular markers, resistance.

Resumen

La yuca (*Manihot esculenta*) es la base de la alimentación para más de 1000 millones de personas en el mundo. La producción es severamente afectada por enfermedades ocasionadas por diferentes patógenos. Las plantas de yuca han desarrollado una serie de proteínas de resistencia R para defenderse de infecciones virales, bacterianas y fúngicas, las cuales son capaces de reconocer moléculas específicas de los patógenos. Un repertorio amplio de estas proteínas ha sido identificado en varias especies vegetales, no obstante, a pesar de conferir resistencia a patógenos diversos, presentan unos pocos dominios conservados. A partir de la reciente liberación de la secuencia completa del genoma de yuca se identificaron secuencias similares a proteínas R en este genoma. Con esta información se diseñaron cebadores para amplificar 13 genes *R*, logrando la amplificación de 10 de ellos en las variedades TMS30572 y CM2177-2, las cuales representan los parentales empleados en la construcción del mapa genético de yuca. A partir de la secuenciación de los amplicones obtenidos se identificaron 37 SNPs (Single Nucleotide Polymorphisms) de los cuales 18 (48.6%) corresponden a transiciones y 19 (45.9%) a transversiones. El restante corresponde a inserciones/deleciones. Este conocimiento permitirá desarrollar estrategias adecuadas para el desarrollo de marcadores moleculares tipo CAPs (del inglés Cleaved

Amplified Polymorphism) para posteriormente evaluar su segregación en la población F1, permitiendo, de esta manera, posicionar estos marcadores en el mapa genético de yuca.

Palabras clave: Genomas, *Manihot esculenta,* mapas genéticos, marcadores moleculares, resistencia a patógenos, yuca.

Introduction

Cassava (*Manihot esculenta*) is the carbohydrate source for more than 1000 millions of people that consume it daily, mainly in tropical regions of Latin America, Africa and Asia (FAO, 2009). Although it is a wild crop, highly tolerant to abiotic (salinity, drought, acid soils) and to biotic stress conditions, there are some diseases affecting its production considerably (Ceballos, 2002). Among the main fungal diseases there are leaf spots caused by *Cercosporidium henningsii* or by *Phaeoramularia manihotis* species. Viral disease are basically the cassava common mosaic (CsCMD) and the cassava vein mosaic (CVMD), which are present in South America but, generate important economic losses in the crop (Ceballos, 2002). One of the main limitants in the crop in Africa is the African mosaic virus (AMV) caused by a geminivirus (Ceballos, 2002). The main bacterial disease is the vascular bacteriosis produced by *Xanthomonas axonopodis* pv. manihotis, which is present in all the manihot cultivated regions (Lopez *et al.*, 2006). Although its importance, it has not been possible to identify the first gene for disease resistance in cassava. Having resistance genes allows strategy development for conventional or transformation genetic breeding in order to obtain varieties with longer resistance to a wide disease spectrum. The recent release of the cassava genome allows the identification of possible candidate genes for disease resistance in cassava.

Resistance proteins have the function of recognizing pathogens molecules and of activating a transduction signaling pathway to restrict growth and colonization by the pathogen (Zipfel, 2009). In the last 20 years, in different plant species more than 50 *R* genes have been cloned and characterized; they confer resistance to diverse pathogens like virus, bacteria, fungi, nematodes and insects (Hammond-Kosack and Kanyuka, 2007).

Most of the *R* proteins have a Nucleotide Binding Site (NBS) and Leucine Rich Repeats (LRR) with additional domains in the amino terminus (Meyers *et al.*, 2003). It is believed that the role of these domains is monitoring the plant protein state of the target proteins of the pathogen virulence factors. Most of these proteins lack of signal peptides or hydrophobic regions for tranmembral anchorage. Proteins with LRR domains are present in divergent beings as virus and eucariotes. It seems that the function of the amino-terminal part is associated with protein activity modulation, while the carboxy-terminal part is implicated in proteic interactions with another protein or ligand (Jones and Dangl, 2006). NBS-LLR proteins are subclassified in CC or TIR according to the presence of coiled-coil domains (CC) or domains that are similar to *Drosophila* Toll proteins and to the mammal interleukinas IL-1 (TIR domains) (McHale *et al.*, 2006). Another kind of R proteins is characterized by members with only one LRR extracelular domain, as the Cf protein family of tomato that give resistance to the fungus *Cladosporium fulvum* (Stergiopoulos *et al.*, 2010). Other resistance proteins present, together with the LRR extracellular domain, a transmembrane domain and can present a Ser/Tre kinase (STK) domain, this is the case of Xa21 in rice that confers a high spectrum resistance to different strains of *Xanthomonas oryzae* (Song *et al.*, 1995). Taking into account that until now resistance is the only function assigned to the NBS-LLR proteins type (McHale *et al.*, 2006) and taking as base the permanence of conserved domains in resistance proteins, similar proteins can be identified in low studied plant species.

Availability of complete sequenced genomes allows, by bioinformatic strategies, the identification of candidate *R* genes that code for proteins with these conserved domains. However, functional studies are needed to determine the specificity of these candidate

proteins. Alternatively, by mapping analysis is possible to locate these sequences in genetic maps to associate them with genetically identified loci associated with resistance to a particular pathogen or with QTLs (Quantitative Resistance Loci) (López *et al.*, 2007).

This work used the conserved nature of the R proteins to search, in the cassava genome, for homologs of previously described proteins on other plant species. These sequences were amplified and sequenced in the two parentals used in the construction of the genetic map to detect SNP (Single Nucleotide Polymorphism). It is hoped that the results substantiate the bases for strategy development to map these sequences in the cassava genetic map.

Materials and methods

Plant material and DNA extraction

Cassava cultivar used in the genome sequencing was AM560-2. In order to reduce the cassava heterogeneity and facilitate the genome sequencing, it was cross with MCOL1505 variety for three generations originating AM560-2. The sequence of the genome of this variety represents the reference sequence accessed from www.phytozome.com.

Cassava varieties, CM2177-2 and TMS30572, were supplied by the Cassava Germoplam Bank from CIAT in in vitro conditions. These varieties were propagated in the Molecular Biology Lab of the Department of Biology of the Universidad Nacional de Colombia. In vitro plant leaves were grinded in liquid nitrogen and DNA was extracted following the modified protocol previously described by Dellaporta *et al.* (1983). Both varieties are parentals of a F1 progeny composed of 150 individuals, which was previously developed at CIAT and used to develop the first genetic map of cassava (Fregene *et al.*, 1997).

Identification of R genes

A database was built including sequence information and structure of previously reported R proteins in different plant species. From this database some *R* genes were selected that have been widely characterized (Table 1). From the sequences of the coding proteins of the selected *R* genes (Table 1) a TBLASTN was performed against the *Manihot esculenta* genome (www.phytozome.com) in order to identify homolog proteins in cassava. TBLASTN standard parameters were used: expected E value threshold= -1, comparison matrix = Blosum62 and word size = 3. Based on the results obtained and from the respec-

Table 1. Resistance proteins used for alignment in the cassava genome and selected genic regions to be amplified by PCR.

Protein name	Plant	Pathogen	Structure	Primer design region
L6	Linen	*Melampsora lini*	TNL*	UTR 5'
M	Linen	*Melampsora lini*	TNL*	UTR 5'
RPP5	*Arabidopsis*	*Hyaloperonospora arabidopsidis*	TNL*	Intron
RPP1	*Arabidopsis*	*Hyaloperonospora arabidopsidis*	TNL*	Downstream
N	Tobacco	Tobacco mosaic virus (TMV)	TNL*	Intron
Xa1	Rice	*Xanthomonas oryzae* pv. *oryzae*	NL**	Downstream
RPS5	*Arabidopsis*	*Pseudomonas syringae*	NL**	Downstream
RPM1	*Arabidopsis*	*Pseudomonas syringae*	NL**	Downstream
RPS2	*Arabidopsis*	*Pseudomonas syringae*	NL**	Downstream
RGC2	Lectuce	*Bremia lactucae*	NL**	Intron
I2		*Fusarium oxysporum* sp licopersicum	NL**	Downstream
Cf2		*Cladosporium fulvum*	NL**	Downstream
Pto	Tomato	*Pseudomonas syringae* pv tomato	STK***	UTR 5' and UTR 3'

*TNL: TIR-NBS-LRR.   **NL: NoTIR-NBS-LRR.   ***STK: Serine/Treonine Kinase.

tive nucleotide sequences, primers were designed using Primer3 (Table 2) (Rozen and Skaletsky, 2000). Design parameters were modified to get amplification products from introns or UTRs (untranslated region) preferably, by using the target function of the program. Other used parameters were: product size = 200 – 1000, primer size = min. = 18 bp, optimal = 20 bp, max. = 27 bp, primer Tm: min. = 57, optimal = 60, max. = 63 and, %GC primer: min. = 20, max. = 80. Primers were used in amplifications from TMS30572 and CM2177-2 varieties DNA.

Amplificationa and sequencing

PCR reactions were performed on 10 µl final volume containing DNA 10 ng, and final concentrations of Buffer 1X (DreamTaq Buffer, Fermentas), MgCl$_2$ 2.5 mM, deoxynucleotides 0.2 mM each, primers 0.5 µM each and Taq polymerase 0.2 U (DreamTaq, Fermentas). Reactions were done on an iCycler thermocycler (BioRad). Reaction conditions were: initial denaturation 95 °C for 3 min; 45 cycles of 94 °C for 45 sec, 45 sec of the calculated primer annealing temperature for each primer

set and 1 min of extension at 72 °C; and final extension at 72 °C for 5 min. It was necessary to standardize the annealing temperature for each primer set. PCR products were subjected to electroforesis on 1.2% agarose gel and dyed with ethidium bromide. The size of the amplified fragment was confirmed using the molecular weight marker 1Kb Plus Ladder ® (Invitrogen, Carlsbad, CA, U.S.A.)

Amplification products were sent to a lab of certified services (Macrogen) for sequencing. Sequences from both varieties were edited and inspected using the program Sequencher (Genecodes, Inc) and aligned with the MUSCLE (MUltiple Sequence Comparison by Log- Expectation) program. From the alignments obtained SNPs (Single Nucleotide Polymorphisms) were manually identified.

Results

Amplification of homologous R genes in cassava

In total, 13 primer sets were designed that correspond to different *R* genes homologs (Table 2). For 12 primers (92.3%) amplification

Table 2. Primers designed from cassava genome sequences similar to resistance genes.

Name	Right primer	Left primer	Product size	Annealing temperature
S_L6	TTTCAGAGGTGGAGATACCCGCAA	AAGCTCGTCTAGGCACCATCTTGA	211	52°C
S_RPP5	AAGGCCTAGAAAGGCACTAAGCGA	TGTCGACACGGTTAAGGTATGGCA	493	50°C
S_RPP1	TTCCTTACCACAACACCTGGTGGA	ACGCCAACTCTCCATGATGTACCA	390	52°C
S_N	GAGCTAAGCACTTCGGAGCTTTCA	TCTGACGAGCTTGTTCGATATTGT	428	52°C
S_Xa1	TGGGCCAAGATTTCTCACATCCCT	GCTCGTATATGCAGTGCTCCACTT	666	54°C
S_RPS5	TGAAGCAGAGAAACACTGGTGGGA	AGGGTAGTGTAATGGGAGGAAATGGG	304	58°C
S_RPM1	AAATTGTAGTCCGCGTTGCACCAG	GCTTCTGCTTCTGGCTTGCTTGAT	420	
S_RPS2	AGACAGGCTTCCAACTCCAACTCA	TAAGCTCATTGGACATTGCCGTGC	480	54°C
S_RGC2	TGATGTGTTGATGTGCTTCGTCCC	GGTATTTCTATGGACTAGCCGTGC	253	50°C
S_I2	CTTTGCACAAGGCATGAGCAGGAT	TGACCATGCCAAGGCGACATGTAT	635	52°C
S_Cf2	GCCTGCATGGTTTGTGATGATGGT	CTCCCTTTCTTGTGGATGTTGTGC	772	60°C
S_Pto 3´	AGACTGTGGATCAAGCAGTGAAAC	AGGCGAATCCAATGTCTTCCAGGT	257	54°C
S_Pto 5´	GTTTGGACAAACAAAGAGAAGGTGT	CATCCAAATCAAGCAAGGATTGCC	222	54°C

products were obtained in both cassava varieties CM2177-1 and TMS30572 after different changes in annealing temperatures were performed, which varied between 50 °C and 60 °C (Figure 1). For the gen with similarity to *Cf2* there was no amplification product. In the case of amplifications obtained with primers designed from the *RPP1* gen an expected size amplicon was obtained but only in the CM2177-2 variety. For the primers designed from the sequence with similarity to the *RPM1* gen, different size fragments were amplified, which suggests the presence of various copies of this gene in the cassava genome.

In this way, the amplicons obtained from these genes were not analyzed by sequencing and, in consequence, high quality sequences were obtained for the other 10 amplicons (Table 1).

SNPs and indels identification

Comparison of obtained sequences for the 10 genes between the sequenced varieties and the reference genome, allowed the identification of a total of 50 SNPs present in four cassava genes, which are similar to the *RPP5*, *RPS5*, *RPS2* and *Xa1* genes. In the alignment of the obtained sequences for the other genes there were no polymorphisms. From these SNPs, 24 correspond to transitions and 26 to transversions. Additionally, five insertions /deletions (indels) were detected only in the alignment of the *Xa1* and *RPP5* homolog genes.

Intra-varietal SNPs

As cassava is a tetraploid and heterozygous crop that can present up to four different alleles in some genes (Fregene *et al.*, 1997),
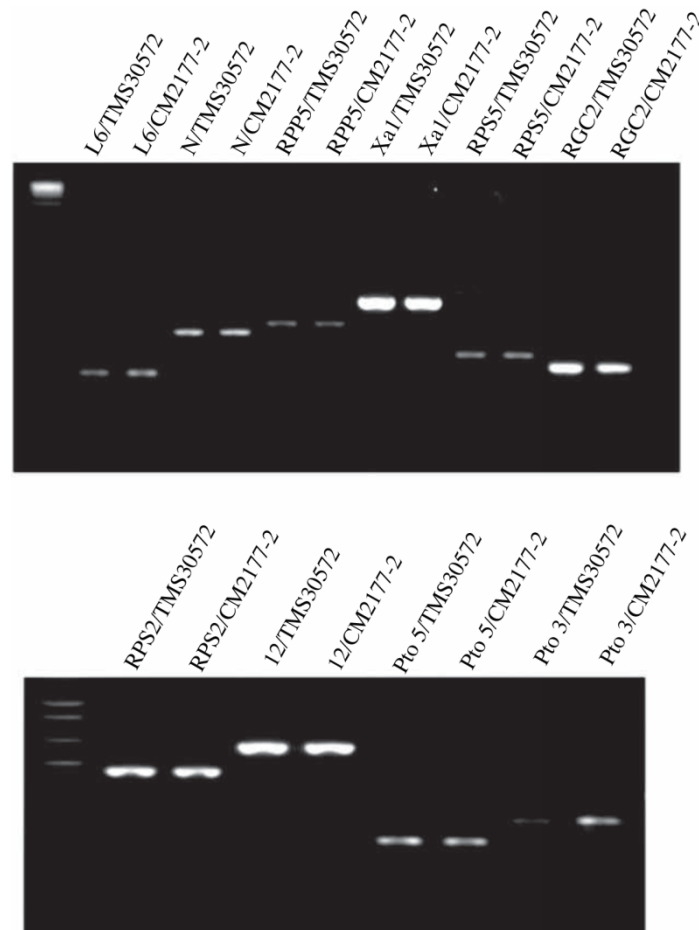
Figure 1. Amplification of homolog genes in CM2177-2 and
TMS30572 cassava varieties.

therefore SNPs can be presented in a variety. In this study 4 SNPs were identified in the CM2177-2 variety and, 10 in the TMS30572 variety (Table 3). For both varieties it was found that 50% of the SNPs were transitions and the other 50% were transversions.

SNPs between varieties

The number of SNPs for each gene between the three varieties is shown in Table 4. When the sequences of the varieties CM2177-2 and TMS30572 were compared there were 37 SNAps clearly identified, 48.6 % (18) correspond to transitions and 45.9% (19) are transversions. In Figure 2 there is an example of one of the SNPs detected in both varieties. Indels of one, two and four base pairs were also detected for the *RPP5* homolog gen and one indel of three base pairs for the *Xa1* homolog gene. These polymorphisms represent a valuable tool to be used in the strategy development for mapping these sequences on the genetic map of cassava. In general, a higher number of SNPs was detected in the AM560-2 variety with respect to the CM2177-2 and TMS30572 varieties than between them. Most of the differences between the last two varieties and the variety which genome was sequenced is found in the *RPP5* homolog gene where 71.4% of polymorphism were obtained.

Discussion

Genetic map development has been very useful to isolate genes (Keller *et al.*, 2005; Nagamura *et al.*, 1997). Most of the genetic maps has been build using molecular markers of anonymous genomic regions, which are regions where it is unknown if sequences belong to coding or non-coding regions (Fregene *et al.*, 1997; Mba *et al.*, 2001; van Os *et al.*, 2006). However in the last years, with the expressed sequences collections and the availability of full genomes, it has been included the use of markers developed in coding sequences (Blair *et al.*, 2011; Gujaria *et al.*, 2011). A strategy that has been used for identification and mapping of genes of interest is the approximation to candidate genes, which is based on conserved sequences present between species (Hu *et al.*, 2008; Muchero *et al.*, 2010). Conserved domain sequences in R proteins allow, through the candidate gene strategy, the identification of coding sequences for those domains in other plant species. However, their presence alone does not establish a partnership between a gene and the plant resistance to one species or particular strain of a pathogen. This could be possible through mapping of the candidate gene and their association with resistance loci or previously identified QTLs.

This work achieved the identification of cassava genes with high similarity to resistance proteins of different plant species. These genes were amplified and sequenced in both parentals used for the cassava genetic map development; this allows the identification of SNPs for some genes. This information will enable designing of strategies to map them. Direct sequencing of amplicons allows the direct polymorphism identification without the need of previous cloning and se

Table 3. Intravarietal SNPs determination in cassava varieties used in this study.

Protein	Cultivar	Total (no.)	Transicions	Transversions
RPP5	TMS30572	1	1	0
	CM2177-2	0	0	0
RPS2	TMS30572	4	2	2
	CM2177-2	0	0	0
	TMS30572	5	2	3
RPS5	CM2177-2	0	0	0
Xa1	TMS30572	0	0	0
	CM2177-2	4	2	2
Total		14	7	7

Table 4. Polymorphism determination in the different cassava varieties used in the study.

| Protein | Variety | Variety | | | | | | | |
| | | TMS30572 | | | | AM560-2 | | | |
		Total (no.)	Transicions	Transversions	Indels	Total (no.)	Transicions	Transversions	Indels
RPP5	CM2177-2	9	3	6	0	12	4	5	3
	TMS30572	-	-	-	-	13	4	6	3
RPS2	CM2177-2	1	0	1	0	1	0	1	0
	TMS30572	-	-	-	-	0	0	0	0
RPS5	CM2177-2	5	0	0	0	1	0	1	0
	TMS30572	-	-	-	-	0	0	0	0
Xa1	CM2177-2	5	2	3	0	7	3	3	1
	TMS30572	-	-	-	-	1	0	0	1

quencing of a number of clones. This is a significant fact, since cassava has high hete-rozygocity and it is tetraploid it requires the sequencing of a relatively high number of clones to increase the probability to cover all the possible allelic variants in a particular cassava variety. Genetic maps of *M. esculenta* are primarly based in anonymous markers like

RFLPs, RAPDs, SSRs (Fregene *et al.*, 1997; Mba *et al.*, 2001; López *et al.*, 2007). In this way, the inclusion of markers generated from candidate genes in genetic maps will contri-bute to associate markers with phenotypes. Additionally, since they are genes it is more probable to find their associations with parti-cular phenotypes and, can be a starting point to develop markers for breeding programs assisted by markers. However, these kinds of

strategies should be complemented with phe-notyping in response to different pathogens strains or species to increase the probability to associate genes with phenotype.

As an alternative to genetic mapping, strategies based on association mapping have been developed in the last years, from poly-morphisms in candidate genes among a group of particular individuals –not necessarily po-pulations from targeted crosses- an associa-tion with phenotype can be established (Hall *et al.*, 2010). This kind of alternative is valuable in plants like cassava that has a long life cycle, low seed production and, in consequence, any cross demands long periods of time. In this way, cassava genes with si-milarity to *RPP5*, *RPS2*, *RPP5* and *Xa*, for which a good number of candidate polymor-phism was obtained, comprise good candi-
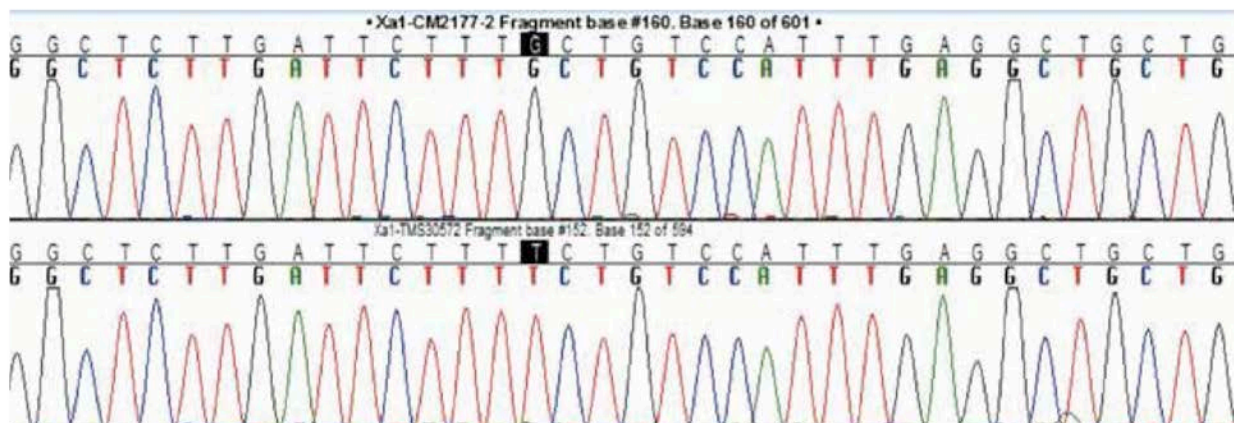
Figure 2. Example of a chromatogram for the Xa1 homologous gene amplified in CM2177-2 and TMS30572 cassava varieties. The highlighted position corresponds to one of the SNPs found between these varieties.

dates to start genotyping on a large group of varieties in order to perform association mapping.

Although there are characteristics controlled by various genes, it is possible to find SNP type markers associated with a phenotype of interest. For example, fragrance phenotype which is highly complex was associated to a unique SNP (Jin, *et al.*, 2003). Plant pathogens resistance is not an exception, and although there are many cases in which this phenotype is controlled by numerous genes, there are examples evidencing sequence changes of some genes that can explain differences between resistant and susceptible cultivars (Bryan *et al.*, 2000; Krattinger *et al.*, 2009). It is also important to note that other kind of polymorphisms, like indels, can explain phenotypical differences. For species like *M. esculenta* a low SNPs frequency is expected because of its endogamy and propagation, mainly asexual, by cuttings.

In order to increase the probability to find SNPs introns or downstream regions, probably 3'UTRs, of the selected genes were sequenced. These regions have shown that the polymorphism frequency could be up to three times higher than in other regions (Ganal *et al.*, 2009; Rafalski, 2002). The intron of the cassava gene similar to *RPP5* happened to be the sequence with more polymorphism, demonstrating that these regions have a high potential to search for markers.

Cassava SNPs frequency estimated in this work was 1 SNP each 164.14 bp. In *Arabidopsis*, for instance, there is an estimate of 1 SNP each 2.2 kb for intronic regions; while in exons it is 1 SNP each 3.1 kb (The Arabidopsis Genome Iniciative, 2000). In maize has been reported higher SNPs frequencies: 1 each 31 bp in no-coding sequences and 1 each 124 bp in coding sequences (Ching *et al.*, 2002). In soybean the approximate frequency is 1 SNP each 270 bp (Zhu *et al.*, 2003). Obviously SNP frequency changes among species and it is not homogeneous along the genome. In this sense, it is important to consider regions close to *R* genes that can have low selective pressures and consequently higher probabilities to find polymorphisms. Nowadays this possibility is a reality since the cassava genome is available. Identification of these kinds of markers, though not directly associated with *R* genes, will allow the reduction on the range where previously identified QTLs or resistance loci have been localized.

The methodology used in this work is proposed as a strategy to identify SNPs in a higher number of candidate genes and/or genomic regions closely located to homologs of resistance genes. Polymorphisms obtained in this work are a valuable resource, since from them, CAPs markers can be designed to genotype the F1 from CM2177-2 and TMS30572 crossing, in order to locate these genes in the cassava genetic map to establish associations with the regions imply in resistance.

Conclusions

Diverse cassava genes with similarity to previously reported *R* genes from plants were amplified. The sequences obtained from the cassava CM2177-2 and TMS30572 varieties allowed the detection of various SNPs and indels, which can be used to genotype the F1 population of this cross and to map them in the cassava genetic map.

Acknowledgments

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