Embryonic, Meiotic and Molecular Analysis of Apomictic Cassava (Manihot esculenta Crantz)

By

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Abstract

Apomixis genes have been successfully transferred to cassava by hybridizing it with the wild species, M. glaziovii. An interspecific hybrid of cassava and M. glaziovii was left for open pollination during three subsequent generations. Seven sibs and their maternal progenitor of the fourth generation were genotyped using five microsatellite loci previously developed for cassava. All sibs were identical with each other and with their maternal progenitor. Sibs from M. glaziovii itself proved to be identical when examined by the same microsatellite loci. Cytogenetic analysis shows the apomictic clone has chromosome constitution of 2n=38. The embryonic study revealed formation of multiembryonic aposporic embryo sacs.

Introduction

Cassava is the most important staple crop in the humid tropics, providing sustenance for more than 800 million people (FAO, 2001). Apomixis is the asexual production of seed whereby apomictic plants are clones of their mothers. It preserves heterosis vigour and maintains superior varieties without genetic segregation. In cassava, propagation is normally performed by cuttings, which accumulate bacterial germs and virus year after year, causing deterioration of productivity. The use of seeds to propagate this crop should avert this drawback. Thus, an advantage of introducing apomixis in cultivated cassava is that it will ensure preservation of superior clones since new stems emerging through apomixis will be free from viral and bacterial germs. Had apomixis been detected or introduced into excellent Brazilian clones like guaxupe and vassourinha, they would have been preserved for a long time. Apomixis could equally benefit international centres routinely exporting their germplasm, as destination countries need only to raise one plant and further propagate it vegetatively in order to maintain superior quality clones.

In earlier work by the first author, apomixis was discovered in the wild cassava Manihot glaziovii. Since this species is characterized by resistance to both mealy bug and bacteria blight, a decision was made to transfer genes for apomixis as well as resistance to mealy bug and bacteria blight by interspecific hybridization.

In 1996 hybrids of cassava and the wild species M. glaziovii were obtained by the first author. The detection of

aposporic embryo formation by a clearing method showed apomixis in these hybrids. Details of this method can be found elsewhere (Nassar et al., 1997; 2000; Nassar, 2001). The hybrid, however, formed fibrous roots, preventing its utilization for human consumption. It was thus left for open pollination among a population of cassava. Its progeny were then examined embryologically for apomixis, all the sibs but one displaying apomixis and sterility. Seeds from the only plant proving fertile were grown to form a progeny of 22 sibs. These plants were examined embryologically by the first author in 2000. One of them (clone 307) proved to be aposporic. It was left for open pollination, formed fruit, and seven plants from its progeny were raised in 2002 for genetic analysis to confirm apomixis.
Microsatellites (simple sequence repeats – SSR) are co-dominant multi-allelic markers that usually display high levels of polymorphism. They can be used to explore the presence of heterozygosity and/or identity (Morgante and Oliveri, 1993). Consequently, microsatellites are one of the most useful molecular markers for understanding detailed patterns of parentage composition and for individual discrimination in clone identification (see Dayanandan et al., 1998; Rita et al., 2002; Bekkaoui et al., 2003; James et al., 2003; Wyman et al., 2003). Additionally, microsatellite primers may be transferred between closely related species given the homology of flanking regions in simple sequence repeats.
In this report, we present the results of microsatellite screening of plant clone 307 and its progeny, and of the original progenitor plant M. glaziovii to confirm the occurrence of apomixis. We also present the result of our cytogenetic and histological study.

Material and Methods

For genetic analysis of the mother plants, clone 307 and M. glaziovii, and their progeny (seven sibs each), expanded leaves were collected and stored at –80ºC. Additionally, two individuals of M. esculenta were collected for amplification control. Genomic DNA was extracted following a standard CTAB procedure (Doyle and Doyle, 1987).
Fourteen microsatellite loci previously developed for M. esculenta (Chavarriaga-Aguirre et al., 1998) were tested for transferability to clone 307 and to M. glaziovii to perform the genetic analysis: GAGG-5, GA-12, GA-6, GA-21, GA-57, GA-126, GA-127, GA-131, GA-134, GA-136, GA-140, GA-161. To test this, DNA from the mother plants (clone 307 and M. glaziovii) and two sibs from each progeny array were used. Additionally, the DNA from two individuals of M. esculenta was used as positive control. Microsatellite amplifications were performed in a 15 ml volume containing 0.3 mM of each primer, 1 unit Taq DNA polymerase (Phoneutria, BR), 250 mM of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2), 0.25 mg BSA and 10.0 ng of template DNA. Amplifications were performed using a Gene Amp PCR System 9700 (Applied Biosystems, CA) under the following conditions: 96oC for 2 min (1 cycle), 94oC for 1 min, 45 or 55oC for 1 min (according to each locus), 72oC for 1 min (30 cycles); and a final elongation of 72oC for 10 min (1 cycle). The amplified products were separated in 4% denaturing polyacrylamide gels stained with silver nitrate (Bassam et al., 1991) and sized by comparison to a 10 bp DNA ladder standard (Invitrogen, MD).
Primers amplifying clear and interpretable products were used in our study. For this, the DNA of all individuals was amplified under the same PCR conditions described above, and fragments were visualized again in 4% denaturing polyacrylamide gels stained with silver nitrate (Bassam et al., 1991) and sized by comparison to a 10 bp DNA ladder standard (Invitrogen, MD).
For each locus the number of alleles and expected and observed heterozygosities was estimated by Hardy-Weinberg's equilibrium (NEI 1978). Further analysis of departure from Hardy-Weinberg, probability exclusion of the first and second parents and assignment test (Marshall et al. 1998) could not be performed because all the sibs, save one individual from the M. glaziovii progeny array, presented the same genotype as the mother plant. For the same reason, it was not possible to estimate the likelihood of observing at least n identical multilocus genotypes by simulation (Stenberg et al., 2003). The probability of genetic identity (Paetkau et al., 1995), i.e. the probability of two random individuals displaying the same genotype, was obtained for each locus and all loci taken en bloc in two ways. First, considering that the progeny array was obtained from a open pollinated population – without genetic drift and mutation, allele frequency should be maintained between generations – if the first parents were heterozygous for all loci, then it could be expected that the frequency of all alleles in the population be p=0.25. This method may represent a maximum value to be obtained if all alleles present the same frequency in the population (p=0.25). Second, in each generation mother plants were pollinated in a living cassava collection population; thus we estimated the probability using allele frequency obtained by Elias et al. (2001) from a genetic diversity study of 290 individuals among 29 varieties of M. esculenta. Although this method does not estimate the probability for the population of cassava used in our study, we nonetheless obtained an expected value for the battery of loci developed for M. esculenta.

A cytological study was carried out on individuals of the 307 progeny to examine their meiotic behaviour. Flower buds were fixed in acetic alcohol 3:1 for 24 h, preserved in 70% ethanol, smeared and stained with 1% acetocarmin.
For embryo sac analysis in these individuals, unpollinated pistillate buds were collected 2 hours before anthesis, fixed in acetic alcohol and dissected. Ovules were dehydrated and cleared overnight in Herr´s fluid (Young et al., 1979). Transparent ovules were then observed using a DIC contrast microscope.

Results

Of the 14 microsatellite loci tested, five (GA12, GA13, GA16, GA21, GA126) and six (GA12, GA13, GA16, GA21, GA126 and GA131) were amplified for 307 and for M. glaziovii, respectively. The two individuals of M. esculenta (positive control) presented clear amplification for all loci. For the five loci used for clone 307 and its progeny array, both individuals of M. esculenta presented the same genotype. For GA131 both presented genotype 98/116 bp, different from M. glaziovii (116/116).
All loci presented just one allele for the mother plant 307 and its progeny (Table 1). Thus, the difference between the observed and expected heterozygosity and the probability of exclusion of first and second parents was equal to zero. For M. glaziovii and its progeny all loci – GA21 and GA126 presented two alleles – presented just one allele (Table 2). For GA21 just one individual was heterozygous (GF25) and the difference between the observed and expected heterozygosity was equal to 0.125. For GA126 all individuals were heterozygous while observed and expected heterozygosity were 1.000 and 0.533, respectively. Hence, probability of exclusion of first and second parents for the battery of loci was very low (0.131008 and 0.232318, respectively). The probability of genetic identity (I), considering equal frequency for all alleles in all loci (p=0.25), was I=0.1094 for each loci, combined probability being IC=1.56527.10-5, for the five loci used for 307 and its progeny array, and IC=1.71202.10-6, for the six loci used for M. glaziovii and its progeny array. Considering the allele frequency obtained by Elias et al. (2001), genetic identity was possible to determine for only four loci: GA12 (I=0.2833), GA21 (I=0.4129), GA126 (I=0.1437) and GA131 (I=0.1684), resulting in a combined probability of IC=2.85124.10-3.

Discussion

Our results indicate that all the progeny from clone 307 may be the outcome of apomixis since all sibs presented the same genotype as the mother for the five loci (Table 1, Fig 1). Apomixis can be inferred for M. glaziovii as well, since only one sib presented a different genotype from the mother plant (Table 2, Fig. 1), which appears to be the result of cross pollination.
Considering that all progeny were submitted to open pollination in each generation, the low number of alleles may be the outcome of the low diversity of the original population used in the control breeding, the homozygous genotype of the parent used at the beginning of the breeding program, genetic drift during breeding research, or the occurrence of apomixis.
The loci used in our research presented a medium to high number of alleles (five to 15) for Chavarriaga-Aguirre et al. (1998) and for Elias et al. (2001) (four to nine alleles). The battery of loci used displayed a medium power of individual distinction, demonstrated by the probability of genetic identity. This may be the result of breeding design, resulting in a maximum value of 1.56527.10-5 and 1.711202.10-6.
The meiotic study of clone 307 individuals showed 2n=38 (fig.2). Chromosome configurations exhibit 36 bivalents and 2 trivalents in all 20 plates examined. Sterility was as high as 92% judged by pollen grain stainability.
The histological study revealed formation of aposporic embryos in all ovules examined. This apospory was identified with formation of embryos in unpollinated ovules. About 23 percent of the 90 ovules examined had 2 or 3 embryos (Fig.3)
The genetic uniformity of M. glaziovii and its progeny detected by microsatellite segregation demonstrated the apomictic nature of the maternal plant. It seems apomixis has played a major role in speciation for the entire Manihot genus. Polyploidy has apparently been providing broad genetic variability in the genus while apomixis has maintained genotypes that may have been favoured in certain niches. Facultative apomixis may then conserve genetic variability through sexual reproduction. These new genotypes may undergo another cycle of speciation in new environmental conditions. Rogers and Appan (1973) classified seven species in the subgenus Glazioviannae to which M. glaziovii belongs. The differences among these species are very narrow and confined to inflorescence size, petiole attachment and bracteole width. These species all hybridise in our living collection creating intermediate types.
The identical genotype of plant 307 and its progeny confirms their apomictic nature. The fact that all sibs investigated may be the outcome of apomixis showed the high level of apomixis attained in this clone. The first author has documented apomixis in the progeny of cassava hybrids with M. dichotoma (Nassar, 1995; Nassar et al., 1998a, 1998b) and in cassava hybrids with M. neusana (Nassar et al., 2000; Nassar, 2001), but it was detected only in less than 1% of the progeny, confirmed by RAPD technique. In further work by the same author, it was possible to select plants for a higher level of apomixis that reached 13% (Nassar and Santos, 2002). This is the first report where apomixis is as high as almost 100%. Apomixis was identified anatomically by the occurrence of one or more embryos in ovules of plants protected against pollination (Fig. 3). Genes controlling different levels of apomixis have been found in wild relatives of cultivated crops such as Panicum maximum (Warmke, 1954). Several F2 genotypes resulting from interspecific hybridization of cassava with wild relatives in our living collection have shown strong evidence of apomixis and abundant fruitfulness accompanied by sterility judged by stainability by acetocarmin. It is clear that recurrent selection has led to a very significant increase in apomixis level, to the point of making the present clone practical for use in cassava production, particularly in view of its high root productivity, attaining 6-7 kg per plant.
Some authors have questioned the existence of obligate apomixis (e.g. Askar, 1979), but certainly a very high level of

apomixis has been obtained in this research work. The presence of apomixis in the first generation of hybridization, and its absence in the following two generations, when plants were left for open pollination, clearly indicates two genetic facts with respect to apomixis inheritance in cassava: first, apomixis is determined by recessive alleles; second, it is controlled by more than one pair of genes, probably located in different chromosomes. One of the most important consequences of our results is the possibility of mapping and characterising apomixis genes by molecular markers and of isolating them in the future.

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APPENDIX:

Description of clone 307

The plant is 2 m. in height, roots from seed are swollen (not a tap root), the shape being ovate measuring 20-30cm, colour dark brown, stipule scars raised and prominent. Leaves are palmately lobed with seven lobes. Leaf lobes obovate measuring 8-10 cm; margin entire, petioles about 15 cm long, attached basally to the lamina, frequently 5-lobed, lamina dark green with red petioles, petioles 10 cm long, young foliage at stem apices green. Inflorescence a panicle about 6 cm long, bracts and bracteoles are caducous. Pistillate flowers green, staminate flowers green with stamens almost sterile exhibiting 10% pollen viability measured by carmine stain. Fruit winged, dark green. Seeds are caranculate and elongate and high brown in colour.

Table 1. Genotype of clone 307 and its progeny (seven sibs), based on five microsatellite loci transferred from M. esculenta. 307/M – clone 307 – mother plant; 307/2 to 307/9 – progeny; allele size in base pair.

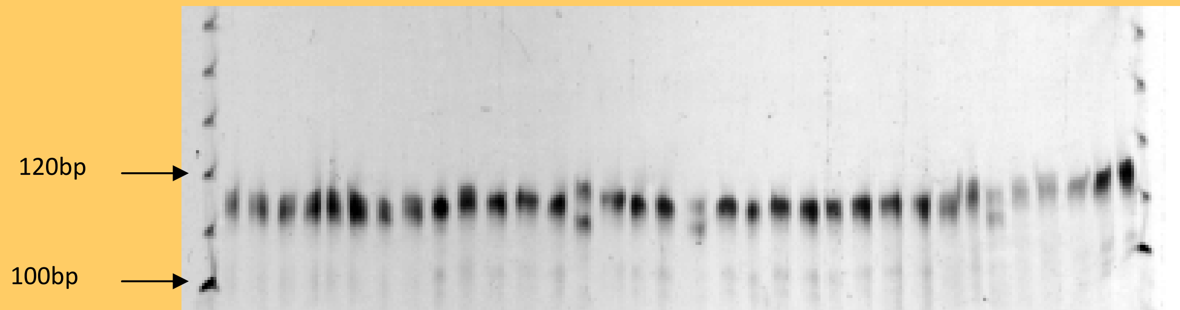
Individual	GA12	GA13	GA16	GA21	GA126
307/M	140/140	140/140	104/104	114/114	180/180
307/2	140/140	140/140	104/104	114/114	180/180
307/3	140/140	140/140	104/104	114/114	180/180
307/4	140/140	140/140	104/104	114/114	180/180
307/5	140/140	140/140	104/104	114/114	180/180
307/6	140/140	140/140	104/104	114/114	180/180
307/8	140/140	140/140	104/104	114/114	180/180
307/9	140/140	140/140	104/104	114/114	180/180

Table 2. Genotype of M. glaziovii and its progeny (seven sibs), based on six microsatellite loci transferred from M. esculenta. G/M – M. glaziovii mother plant; G/24 to G/32 – progeny; allele size in base pair.

Individual	GA12	GA13	GA16	GA21	GA126	GA131
G/M	140/140	140/140	104/104	114/114	176/206	116/116
G/24	140/140	140/140	104/104	114/114	176/206	116/116
G/25	140/140	140/140	104/104	110/114	176/206	116/116
G/26	140/140	140/140	104/104	114/114	176/206	116/116
G/29	140/140	140/140	104/104	114/114	176/206	116/116
G/30	140/140	140/140	104/104	114/114	176/206	116/116
G/31	140/140	140/140	104/104	114/114	176/206	116/116
G/32	140/140	140/140	104/104	114/114	176/206	116/116

Figure Captions

Fig. 1. Genotype of clone 307 and M. glaziovii, and their progeny for locus GA21, visualized in silver-stained denaturing polyacrylamide gel. First and last lanes are 10 bp DNA ladder standard (Invitrogen). Second and third lanes are positive controls (M. esculenta). Fourth to eleventh lanes are the hybrid and its progeny array. Twenty-eighth to thirty-fifth lanes are M. glaziovii and its progeny.



Caption
Fig.2. Meiotic metaphase I in apomictic clone 307 shows 2n=38 ; 16 bivalents and 2 trivalents

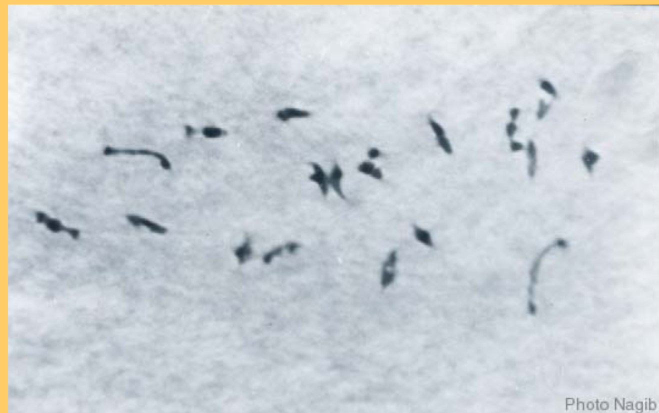


Fig.3. Multiembryonic sac of an ovule

Fig.3. Multiembryonic apospory sac in apomictic clone 307