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Induction and Identification of a Small-Granule, High-Amylose Mutant in Cassava (*Manihot esculenta* Crantz)

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Only two mutations have been described in the literature, so far, regarding starch and root quality traits in cassava. This article reports on an induced mutation in this crop, first identified in 2006. Botanical seed from five different cassava families were irradiated with γ rays. Seed was germinated, transplanted to the field (M_1 plants), and self-pollinated to produce the M_2 generation. Abnormal types regarding starch granule morphology were identified during the single plant evaluation of M_2 genotypes. To confirm these characteristics, selected genotypes were cloned and a second evaluation, based on cloned plants obtained from vegetative multiplication, was completed in September 2007. Two M_2 genotypes presented small starch granules, but only one could be fully characterized, presenting a granule size of 5.80 $\pm$ 0.33 μm compared with three commercial clones with granule sizes ranging from 13.97 $\pm$ 0.12 to 18.73 $\pm$ 0.10 μm and higher-than-normal amylose content (up to 30.1% in cloned plants harvested in 2007, as compared with the typical values for "normal" cassava starch of around 19.8%). The gels produced by the starch of these plants did not show any viscosity when analyzed with the rapid viscoanalyzers (5% suspension), and the gels had low clarity. Low viscosity could be observed at higher concentrations (8 or 10% suspensions). Preliminary results suggest that the mutation may be due to a lesion in a gene encoding one of the isoforms of isoamylase (probably *isa1* or *isa2*).

KEYWORDS: Amylose; amylopectin; granule size; mutation breeding; isoamylase

INTRODUCTION

Cassava is one of the most important sources of starch in tropical environments. Comprehensive reviews of cassava starch properties have been published (*1, 2*). The starch is easily extractable from the roots because they contain low levels of protein and fat (*3, 4*). The starch granules are generally round (oval), with a flat surface on one side (truncated), and range from 5 to about 40 μm in size. Sriroth and coworkers found starch granule size from four different varieties ranging from 8 to 22 μm, with an average of 15 μm (*3*). Several studies have reported differences in starch granule structure and chemical composition, which depend on the botanical origin of the starches (*5–7*). CIAT has conducted quantification of thousands of starch samples from improved clones as well as from clones of the germplasm collection. The average amylose content from 110 different genotypes (representative of the cassava world collection composed of more than 6000 accessions) was 19.8% with a standard deviation of $\pm$ 1.58 (CIAT, unpublished data). However, data based on smaller samples reported the amylose content varying from 18.6 to 23.6% (*5*). Recently, an amylose-free natural mutation has been identified and reported (*8*). Viscosity of cassava starch has been well studied and tends to be high compared with that of starch from other tubers and cereals (*1, 5*). There are clear genetic differences in viscosity. CIAT is currently screening the starch quality traits from the entire cassava germplasm collection (more than 6000 accessions). In most cases, pastes analyzed with the rapid viscoanalyzer show a single high-viscosity peak as a result of

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Table 1. Cassava Germplasm Irradiated with γ Rays (from Cobalt 60)[a]

	family	mother	father	no. seed
1	CM 9331	SM 1210-10	MNGA 1	150
2	SM 3015	MCOL 1505	unknown	150
3	SM 3045	HMC 1	unknown	150
4	GM 155	MTAI 1	SM 2102-34	158
5	C-4	TME3	TMS30555	787

[a] In full-sib families (CM 9331, GM 155, and C-4) both the female and male progenitors are known. In half-sib families (SM 3015 and SM 3045) only the female progenitor is known because the pollination in crossing nurseries is made by insects.

the high degree of swelling, which then generally breaks down sharply. In other cases, two adjacent peaks can be observed. In other cases, the peak is low and wide (CIAT, unpublished data). Cassava starch is one of the least resistant to enzymatic breakdown, among the noncereal starches, with hydrolysis curves similar to those of normal maize starch (*2*). In spite of the considerable variation in the physicochemical properties of cassava starch, there is little natural qualitative variation reported (*8–10*). Compared with the several mutations reported for starches from other crops such as maize, rice, barley, and potato, cassava offers comparatively very little variation. CIAT has therefore implemented several strategies to develop high-value cassava clones (*8, 10, 11*). For the feed industry, high-protein clones have been identified (*12*). For the starch and bioethanol industries, different approaches to develop and identify clones with novel starch properties have been gradually introduced in the cassava-breeding project, already with positive results (*8, 11*). In spite of their low frequency and the unpredictability of results, the induction of mutations has been a successful approach to generate new variability in other crops where natural genetic variation was limited and insufficient. As a result, several varieties have been developed after the induction of mutations (*13, 14*). CIAT and National University of Colombia have therefore implemented a joint mutation-breeding project in search of genetic modifications for useful traits, including starch quality. This article reports on two cassava genotypes identified in a mutagenized population that showed distinctive starch characteristics. They were initially discovered in March 2006 and further confirmed in September 2007.

MATERIALS AND METHODS

A project to induce mutations in botanical seed from five different families was initiated in 2003 (*11*). About 1400 botanical seeds from five different full- or half-sib families (**Table 1**) were irradiated with 200 Gy γ rays (from Cobalt 60). The dosage was based on previous experience at International Atomic Energy Agency (IAEA) with cassava as well as with other crops whose seeds have similar size as those of cassava. After transplanting the seedlings, normal cultural practices were used to maintain the crop in good growing conditions.

By August 2004, the plants from the M_1 generation (M_1 = first stage of mutagenesis) started to flower. Whenever possible (cassava plants do not always flower), the M_1 plants were self-pollinated to generate M_2 seed (M_2 = second stage in the mutation breeding process). This action is important to eliminate chimeras (typical in mutagenesis) and to allow recessive traits to express themselves.

M_2 seeds were germinated, seedlings transplanted to the field in May 2005, and plants harvested in March 2006. Only one plant per genotype was available because evaluations were made on individual plants obtained from botanical seed. At least one commercial-size root was harvested per genotype. Whenever possible, up to five roots per plant and genotype were harvested. Immediately after harvest, starch granule morphology was analyzed with an optical microscope. Roots were then washed and peeled before samples were prepared for the different analyses performed.

Optical Microscopy. A microscope slide was rubbed against the freshly cut section of the roots and stained with a drop of 0.2% iodine solution. The slide was observed through a light microscope (Olympus CX41) using a 40X magnification lens.

If any analysis resulted in unusual starch phenotypes (i.e., starch granule morphology that was different from those typical of cassava), vegetative cuttings were taken from the mother plant to clone the respective genotypes. Other tests, following the standard procedures of the root quality laboratory at CIAT (*15*), were conducted in the "seedling plants" (plants derived from botanical seed, not vegetative cuttings).

Starch Isolation. Freshly cut pieces from the harvested root(s) were lyophilized for 24 h at −30 °C. Cut pieces were then suspended in tap water and crushed in an Osterizer blender. The slurry was filtered through a 100 μm sieve. The starch was allowed to settle, and the supernatant decanted off and dried (at room temperature).

Scanning Electron Microscopy (SEM). Dehydrated starch granules were sprinkled on double-sided sticky tape, mounted on circular aluminum stubs, coated with 35 nm of gold−aluminum, and then photographed in a scanning electron microscope (JSM 820 Jeol, Tokyo, Japan) at an accelerating voltage of 20 kV. Granule size was measured.

Granule Size and Distribution. Starch granule size and distribution was determined with a laser diffraction particle size analyzer (SALD -3001-Shimadzu Jp). Starch samples were mixed with distilled water, and 1 drop of sodium hexametaphosphate (0.2%) was added. The suspension was mixed using a mechanical stirrer and sonicated to obtain a laser light obscuration level of ~30%. Refractive index of 1.600 ± 0.101 was set for the starch. Measurements were run in triplicate on two different starch samples per genotype at room temperature.

Paste Clarity. A 1% dry basis aqueous dispersion of starch was boiled at 97 °C (1000 m above sea level) and shaken thoroughly every 5 min for 30 min. Transmittance was measured after cooling to room temperature at 650 nm (*16*).

Colorimetric Amylose Determination. Amylose content in the starch was measured following standard procedures (*17*). Starch granules were first dispersed with ethanol and then gelatinized with sodium hydroxide. An aliquot was then neutralized with acid and treated with an iodine solution, which produces blue-black stain coloration. The color intensity, which is related to amylose content, was then measured with a spectrophotometer and compared with a standard curve obtained using purified amylose and amylopectin extracted from potato tubers. Five different quantifications per starch sample were made, and mean values were then calculated. The use of amylose and amylopectin from potato has proven to be also useful for a crop such as cassava, even though it belongs to a different family (*8*).

Pasting Properties. Hot starch dispersion viscosity profiles were obtained with a rapid viscoanalyzer model RVA-4 series (Newport Scientific, Australia). Starch (1.25 g db) was dispersed in distilled water (near 23 cm^3) to a 5% suspension. Viscosity was recorded using the temperature profile holding at 50 °C for 1 min, heating from 50 to 90 °C at 6 °C min^{-1}, holding at 90 °C for 5 min, and then cooling down to 50 at 6 °C min^{-1} with continuous stirring at 160 rpm. Pasting formation characteristics were pasting temperature (PT), peak viscosity (Vpeak), peak temperature (Tpeak), viscosity at start of 90 °C plateau (V90 °C/7.667 min), viscosity at the end of the plateau at 90 °C (V90 °C/12.667 min), and the viscosity at 50 °C (V50 °C/19.334 min).

Swelling Power, Solubility, and Dispersed Volume Fraction Measurements. Swelling power and solubility patterns were determined using 2.5% starch dispersions (w/w) (0.70 g of starch dry basis dispersed in 27.3 g of distilled water). Paste was prepared in a rapid viscoanalyzer (RVA) holding at 35 °C for 1 min, heating to 75 at 6 °C min^{-1} rate, and holding at 75 °C for 2.5 min. The paste was immediately transferred to a 50 cm^3 centrifuge tube. The supernatant and sediment after centrifugation for 5 min at 6000*g* at 25 °C were collected and weighed (Wsu and Wse, respectively), then dried at 100 °C for 24 and 48 h, respectively, and weighed (Dsu and Dse, respectively). Three parameters were calculated (*18*): concentration of soluble material in the supernatant (solubility), the swelling power, and the volume fraction of the dispersed phase (Φ).

$$\text{solubility (\% db)} = 100 \times \text{Dsu}/0.70$$

$$\text{swelling power} = (\text{Wse} - \text{Dse})/\text{Dse}$$

$$(\Phi) = (27.77 - (\text{Wsu} - \text{Dsu}))/27.77$$

Factor 27.77 is calculated as total volume (cm^3) of the paste. Starch specific density is 1.5 g/cm^3, and 27.77 = 27.30 + (0.70/1.5) cm^3.

Native Gel Electrophoresis. Roots from the small-granule mutant (clone 5G160-13) harvested in September 2007 were used for these experiments. To preserve enzyme activity, immediately after harvest in the field, the roots were sliced into 1 cm thick slices; each slice was cut into quarters and wrapped in aluminum foil, and these samples were frozen in liquid nitrogen and then stored at −80 °C. Samples of control roots with normal granules were prepared and stored in the same way. Two or three samples per root from five separate plants of each genotype were prepared. Enzyme activities were characterized using native (nondenaturing) polyacrylamide gels (PAG) as follows.

Isoamylase. Samples of the frozen roots (~0.5 g) were defrosted and homogenized in a mortar at 4 °C with ~50 mg of PVPP in 1 cm^3 extraction buffer containing 50 mM MOPS (pH 7.0), 1 mM EDTA, 5% (v/v) ethanediol, and 5 mM DTT. Extracts were centrifuged at 10 000*g* for 10 min at 4 °C, and the supernatants were mixed with 0.25 volumes of native gel sample buffer (300 mM Tris HCl (pH 6.8), 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue).

Samples were loaded onto discontinuous gels (80 mm × 60 mm × 1 mm). The main gel contained 7.5% polyacrylamide, 375 mM Tris HCl (pH 8.8), 0.2% potato amylopectin, and 0.1% (w/v) acarbose (Glucobay 100 Tablets: Bayer plc, Newbury, Berlshire, U.K.), 0.04% (w/v) ammonium persulphate, and 0.1% (v/v) TEMED. Gels were subjected to electrophoresis at 150 V and 4 °C in 25 mM Tris and 192 mM glycine. After electrophoresis, gels were rinsed twice in incubation buffer containing 100 mM MES (pH 6.0), 10 mM EDTA, 5% ethanediol, and 5 mM DTT and then incubated in this buffer for 3.5 h at 37 °C. Gels were rinsed briefly with water and then stained with Lugol's solution.

Starch Branching Enzyme. Samples of frozen roots (each ~0.3 g) were defrosted and homogenized in a mortar at 4 °C with ~30 mg of PVPP in 1.5 cm^3 extraction buffer containing 50 mM HEPES (pH 7.4), 2 mM $MgCl_2$, 12.5% (v/v) glycerol, and 50 mM 2-mercaptoethanol. Extracts were centrifuged at 20 000*g* for 30 min at 4 °C, and the supernatants were mixed with 0.05 volumes of native gel sample buffer (50% (v/v) glycerol, 0.2% (w/v) bromophenol blue).

Samples were loaded onto discontinuous gels (80 mm × 60 mm × 1 mm). The main gel contained 7.0% polyacrylamide, 375 mM Tris HCl (pH 8.8), 0.01% (w/v) phosphorylase a (rabbit muscle, 25 U/mg; Sigma, Dorset, U.K.), 0.2% (w/v) maltoheptaose, 0.1% (w/v) acarbose (Glucobay 100 Tablets: Bayer plc, Newbury, Berlshire, U.K.), 0.04% (w/v) ammonium persulphate, and 0.1% (v/v) TEMED. Gels were subjected to electrophoresis at 150 V and 4 °C in 25 mM Tris and 192 mM glycine. After electrophoresis, gels were rinsed twice in 20 mM MES (pH 6.6) and 100 mM Na citrate and then incubated for 2 h at 30 °C in 20 mM MES (pH 6.6), 100 mM Na citrate, 45 mM glucose 1-phosphate, 2.5 mM AMP, 1 mM EDTA, and 1 mM DTT. Gels were rinsed briefly with water and then stained with Lugol's solution. An additional assay to quantify starch-branching enzyme activity was conducted with extracts that were prepared as for SBE on native gels. The assays were performed as suggested in the literature (*19*).

RESULTS

During the harvest of approximately 1500 M_2 plants in March 2006, only about 800 genotypes produced commercial-size roots (M_2 plants were weak because natural inbreeding depression and the negative effects of the mutations), and 38 of them were selected because they were suspected to carry special characteristics (variation in starch granule morphology, capacity of the roots to withstand storage for up to three weeks, and roots that seemed to store compounds different from starch). Vegetative cuttings of these 38 genotypes were used to clone them. Cloned plants were then harvested in September 2007.

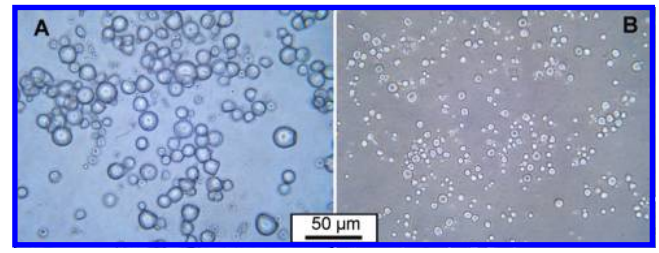

Figure 1. Photographs from light microscope (40X) of wild type cassava starch (**A**) and the small-granule mutation (**B**).

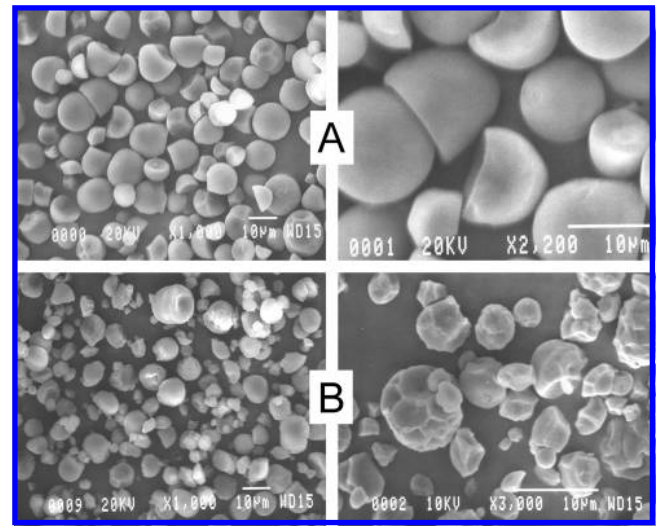

Figure 2. Photographs from a scanning electron microscope at different magnifications of typical cassava starch granules (**A**) and the small-granule mutant (**B**). The relative sizes of photographs on the right have been modified so that the 10 μm bars are of equal lengths.

One of the special characteristics observed was a starch whose granules were considerably smaller than those typical in cassava (**Figure 1**). This article describes the characteristics of this small-granule mutation, exclusively. This type of mutation was observed in self-pollinated progenies from two different M_1 plants (3G43 and 5G160) coming, respectively, from families SM 3015 and C-4 (**Table 1**). 3G43-1 was the only M_2 genotype from 3G43 that expressed the small-granule phenotype. On the other hand, there were three M_2 sister genotypes (5G160-13, 5G160-16, and 5G160-18) that trace back to a common M_1 progenitor plant (5G160) expressing this phenotype. The finding of three sister M_2 genotypes derived from the same M_1 mother was a strong indication that the special characteristic observed in these genotypes was indeed genetic in origin.

The first indication that the four M_2 genotypes mentioned above had a mutation that affected the starch-granule morphology was the visual observation of their small size through light microscopy (**Figure 1**). Although optical microscopy is a limited technique to determine granule size distribution, it proved to be adequate for detecting abnormal starch granule morphologies during the screening of a large number of genotypes (800) conducted during this study. This observation prompted a more careful analysis using scanning electron microscopy. **Figure 2** presents photographs at different magnifications of the small-granule phenotype compared with normal-sized cassava starch granules. In both figures, "wild type" (WT = normal) starch granules showing the typical morphology and size (around 15 μm) for cassava are provided. The small-granule mutation (MUT) however

Table 2. Analyses of the Starches from MUT and Three WT Genotypes (All from Cloned Plants) from Africa, Asia, and Latin America[a]

	seedling		cloned			
parameter genotype	5G 160-16	5G 160-13	5G 160-13	MTAI 8	MCOL 1505	MNGA 11
avg granule size (μm)	n.a.	n.a.	5.80 (0.327)	16.17 (0.086)	18.73 (0.099)	13.97 (0.118)
paste clarity (%)	17	26	13 (1.18)	63 (1.76)	64 (0.14)	59 (1.06)
amylose (%)	36.23	28.49	30.07 (0.87)	20.67 (1.62)	22.18 (0.76)	22.6 (0.60)
pasting temp (PT) in °C	[b]	[b]	60.17[b] (1.06)	64.23 (0.53)	61.60 (0.49)	63.55 (0.07)
peak viscosity (PV) in cP	[b]	[b]	22[b] (4.5)	976 (2.82)	1052 (6.36)	1080 (4.94)
solubility (% db)	33.83	37.17	36.84 (0.67)	10.15 (0.03)	12.20 (0.73)	8.34 (0.55)
swelling index (g g^{-1})	16.45	14.19	9.25 (0.26)	21.77 (0.44)	27.92 (0.57)	26.55 (0.66)
vol fraction of dispersed phase (Φ)	0.32	0.28	0.22 (0.008)	0.70 (0.002)	0.72 (0.002)	0.68 (0.009)

[a] Data from one MUT came from a seedling plant (March 2006 harvest) and five cloned plants (September 2007 harvest). Analyses for these five plants were made individually so standard deviations (within parenthesis) values could be provided. For the second MUT, only data from the seedling plant is available. Peak viscosity and pasting temperatures are based on 5% starch suspensions. [b] Values were very difficult to assess due to the almost flat amylogram.

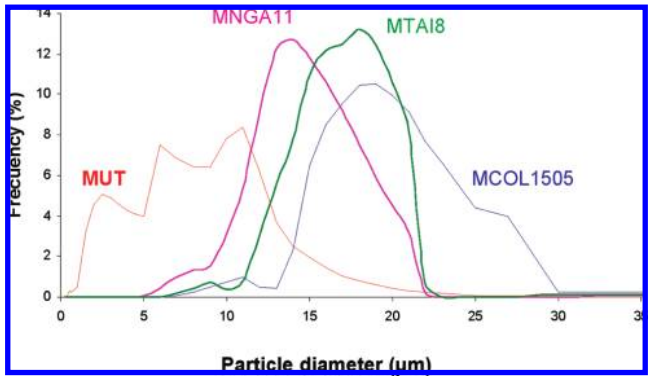

Figure 3. Granule size distribution of MUT (5G160-13) compared with three wild type cassava clones from Africa (MNGA11), Asia (MTAI 8), and Latin America (MCOL 1505) using a laser diffraction particle size analyzer.

showed a large proportion of granules ranging mostly from 5 to 8 μm and failing to show the truncated shape typical of cassava.

The observation of the MUT phenotype during the harvest of March 2006 (on plants derived from botanical seed) was confirmed again for all the four M_2 genotypes on the cloned plants whose roots were harvested in September 2007. This observation demonstrated that the mutation was stable and transmitted through vegetative multiplication. Further analyses were made on starches from five plants derived from 5G160-13, which was the most vigorous of the four MUT genotypes. For the other genotypes, plants were not harvested because they have been used to make crosses to start the transfer of the MUT trait into elite cassava germplasm (a root was taken without harvesting the plant to confirm the MUT phenotype) or because they failed to produce enough starch for complete analyses (3G43-1).

Table 2 presents results of the granule size, proximal, and reological analyses of the two MUT genotypes from which enough starch was available. Data from three WT genotypes of African (MNGA11), Asian (MTAI8 = Rayong 60), or Latin American (MCOL 1505) origin is also provided to facilitate comparison. Five plants from 5G160-13 were harvested, and the starch from each individual plant was extracted and analyzed separately.

Granule Size. Average granule size (independent starch samples from three 5G160-13 plants) was 5.80 ± 0.33 μm compared with typical cassava granule sizes ranging from 13.97 ± 0.12 (MNGA 11) to 16.17 ± 0.09 (MTAI 8) to 18.73 ± 0.10 μm (MCOL1505). **Figure 3** illustrates the differences in distribution of granule size in the mutant and the three wild type genotypes.

Figure 4. Paste clarity from a typical cassava starch (**A**) and from the small-granule mutation (**B**).

Paste Clarity. Paste clarity was 3−4 times lower in MUT compared with WT cassava genotypes (**Table 2**). The differences can also be appreciated in **Figure 4**.

Colorimetric Amylose Determination. Average amylose content using the colorimetric method ranged in MUT from 28 to 36%, a considerably higher value compared with the 20−22% values observed in the WT genotypes presented in **Table 2** or the average of 19.8% obtained after the analysis of 110 cassava genotypes (CIAT, unpublished data). Average amylose content from the cloned plants (5G160-13) was 30.03% (±0.87). Differential scanning calorimeter (DSC) determination of amylose, conducted at CIRAD's laboratories in Montpellier, France, corroborated the values obtained by the colorimetric method on starches from the seedling plants (data not presented).

Starch Functional Properties. Table 2 presents the most relevant results from the pasting behavior of MUT and WT cassava starch obtained from the amylograms presented in **Figures 5** and **6**. The most outstanding difference in the amylograms relates to the very low viscosity of MUT. This striking difference is clearly illustrated in **Figure 5**. Hot and cool paste viscosities, breakdown, setback, and consistency were very low and difficult to quantify in the almost flat amylograms of MUT (**Figure 5**). These results prompted the analysis of viscosity using 8 and 10% suspensions illustrated in **Figure 6**. Pasting temperature (PT) was difficult to assess with 5% starch suspension. However, a closer look at the amylograms using different concentrations demonstrated that PT was lower than

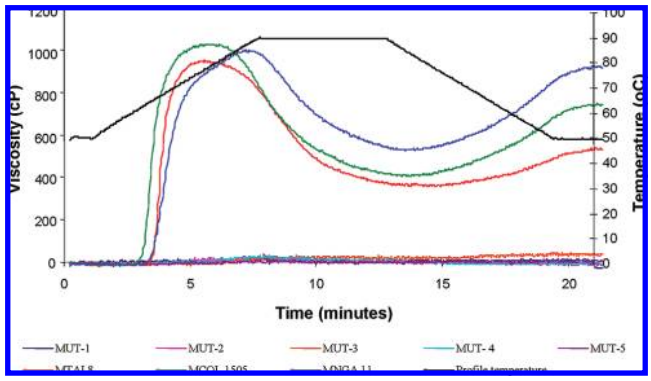

Figure 5. Amylograms from starch samples (5% suspension) of the five cloned plants from genotype 5G160-13 (MUT-1 to MUT-5) and three wild type cassava clones from Africa (MNGA11), Asia (MTAI 8), and Latin America (MCOL 1505) using a rapid viscoanalyzer (RVA). The amylograms from 5G160-13 are drastically different, with no increase in viscosity.

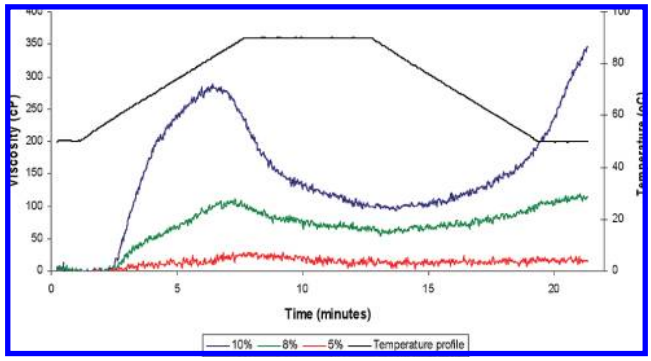

Figure 6. Amylograms from starch samples of genotype 5G160-13 in 5, 8, and 10% suspensions, using a rapid viscoanalyzer (RVA). The scale for the viscosity axis in this figure is different from that of **Figure 5**.

in WT cassava. PT values for amylograms using 8% suspensions were 58.06 and 62.15 °C, respectively, for MUT and WT. When 10% suspensions were used, PTs were 57.85 and 61.47 °C for the mutant and the average of the three WT genotypes, respectively.

Solubility and Swelling Properties. These properties were also distinctive for MUT. Solubility was about three times higher in those genotypes (34−37% db) compared with WT cassava (8−12% db). Swelling index was, on the other hand, lower in MUT (9−16 g g^{-1}) than in WT starches (22−28 g g^{-1}). Finally, volume fraction of the dispersed phase was significantly lower in MUT (0.22−0.32 Φ) compared with WT cassava (0.68−0.72 Φ).

Native Gel Electrophoresis and Enzyme Assays. Studies of starch mutants of other species suggest that small granules are characteristic of mutants lacking isoamylase (ISA) activity (*19, 20*). Small deformed granules have also been seen in mutants lacking starch synthase (SS) (*21*) or starch-branching enzyme (SBE) (*22*). High-amylose content is characteristic of mutants lacking starch-branching enzyme (SBE) or lacking one of the isoforms of SS that are responsible for amylopectin synthesis.

In a preliminary screen for the most likely cause of the cassava mutant phenotype, we chose to assay two of these candidate enzymes, SBE and ISA. The assays were performed on genotype 5G160-13, which provided roots from five cloned plants. The activities of SBE and ISA were assayed using native gels. This analysis was performed using roots from five and four separate plants for ISA and SBE, respectively. The same results were obtained for each plant, and representative results

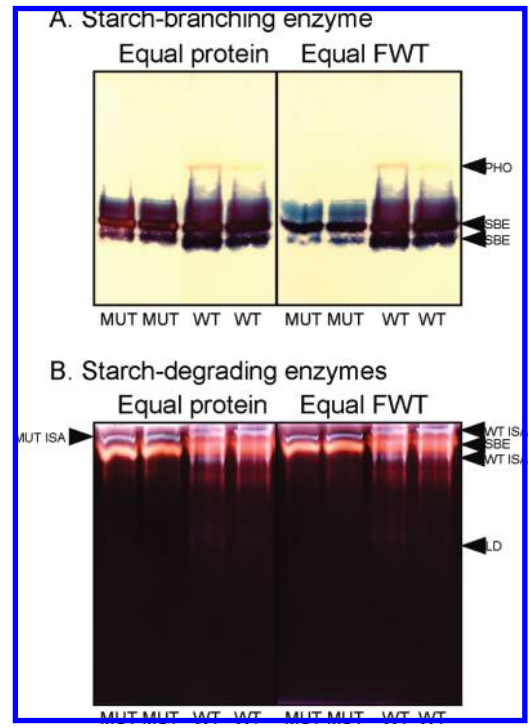

Figure 7. Native PAGs (zymograms) of starch metabolizing enzymes. Crude extracts of the roots of the 5G 160-13 MUT and WT cassava were subjected to native gel electrophoresis, incubated to allow enzyme activity, and then stained with Lugol's solution to reveal starch-branching enzyme (SBE), isoamylase (ISA), limit dextrinase (LD), and α-glucan phosphorylase (PHO) activity. Each track contains an extract from a different plant. FWT = fresh weight. (**A**) The gel was incubated in the presence of glucose 1-phosphate and α-glucan phosphorylase. Tracks were loaded with volumes of extract containing equal amounts of protein (8 μg/track) or equal amounts of root (2 mg of fresh weight/track). (**B**) The gel contains potato amylopectin. Tracks were loaded with volumes of extract containing equal amounts of protein (20 μg/track) or equal amounts of root (10 mg of fresh weight/track). Note that the ISA bands in the wild type cassava (WT ISA) and in the mutant (MUT ISA) differ in position and number.

are shown in **Figure 7**. The native gel analysis also gave information about the activities of PHO and limit dextrinase (LD). The bands corresponding to ISA, SBE, PHO, and LD activities were identified from previous knowledge of the positions and colors of these bands on native gels of extracts of other plants. For example, ISA activity typically appears as blue-staining bands running close to the top of native gels such as those used here (*19*).

The native gels showed that there was no qualitative difference between the MUT and WT samples with respect to the SBE activity. Since MUT roots had a lower dry matter content than WT roots, the extracts were compared in two ways: loading tracks on the gel with equal total fresh weight and with equal total protein content. MUT tended to have less total SBE activity than WT with both loading methods (**Figure 7A**). There were also no consistent differences between MUT and WT in LD or PHO activities (**Figure 7B** and **A**, respectively). With ISA, however, consistent differences in the pattern of bands could be observed (**Figure 7B**). In the WT, two ISA bands could be observed, above and below the SBE band. In the MUT, on the other hand, one ISA band in a different position from either of those in the WT was seen.

The activity of SBE was also determined using the same method described by Burton and coworkers (*19*), which measures the stimulation of exogenous α-glucan phosphorylase

(PHO) by SBE in the extract. There was no statistically significant difference between WT and MUT roots in SBE activity based on three or four separate extracts. The average activity for WT was 10.41, with a standard deviation of ±4.00 ($n = 4$), whereas for MUT the average and standard deviation were 12.50 ± 3.88 ($n = 3$). Each extract was from a different plant.

DISCUSSION

Only one starch mutant has been reported so far in cassava (*8*). A second mutation (*9*) completes all the variation so far reported in the literature on root quality traits for this crop. Because of the extremely limited variation on cassava root quality traits in general, and starch quality in particular, the mutation-breeding project described in this article was initiated. Although frequencies of mutation are very low and results unpredictable, two mutant genotypes with the same starch phenotype have been identified in an M_2 population. The most relevant characteristics of this starch mutation are the small granule size (about a third the normal size), higher-than-normal amylose content, and very low paste clarity and viscosity peaks. Pasting properties showed higher solubility and lower swelling index and volume fraction of the dispersed phase. Several of these characteristics offer potential commercial advantages. The addition of a third mutation that is clearly distinctive and different from those already reported (*8, 9*) and from WT cassava is a significant contribution, which has already generated the interest of the starch industry. In addition to these natural mutants, transgenic cassava has already been produced for different traits (*23, 24*), but so far, they have not been exploited commercially.

It is surprising to observe so many changes in biochemistry, granule morphology, and functional properties arising from just a single mutation. A similar situation has also been observed using transgenic sweet potato where SBE II activity was suppressed (*25*). Complicated structural changes including increased amylose content, increases in phosphate content, and alterations in the crystalline structure and in the length of chains have been reported elsewhere (*25, 26*).

The analysis of enzyme activity presented here is preliminary, but it suggests that the cassava mutant does not lack SBE, one of the common causes of a high-amylose phenotype in mutants of other species. No changes in the activity of PHO or LD were seen either, but we did see consistent differences between WT and MUT in the banding pattern of isoamylase on native gels. As in other species, there are likely to be three types of the isoamylase gene in cassava: *Isa1*, *Isa2*, and *Isa3*. Of these, *Isa1* and *Isa2* are thought to be required for normal starch synthesis in several plant species, including potato (*20*), whereas *Isa3* is thought to be involved in starch degradation (*27*). Previously identified mutants with small starch granules lack either *Isa1* or *Isa2* (*20, 28*). These ISA mutants also have low starch content, and often (e.g., maize sugary1 mutants) but not always (potato ISA transgenics), they accumulate soluble starch (also called phytoglycogen or water soluble polysaccharide). It has not been possible therefore to determine whether phytoglycogens are characteristic of isoamylase mutants or not, and this MUT may help to answer this question. Unfortunately, the accumulation of soluble starches is not part of the standard set of analyses performed at CIAT, and therefore, this quantification could not be conducted at this point. However, the methodology will be established, and analyses on the mutants will be made on the next harvest season. Whether the changes in ISA bands shown here for the cassava MUT are due to a lesion in an isoamylase gene or due to secondary effects remains to be determined. Other enzymes such as starch synthase are potential sites for the mutation in cassava and will also be investigated after the next harvest.

In addition to isoamylase, other enzymes (pullulanase, disproportionating enzyme, and α-glucan water dikinase) have also been related to amylopectin biosynthesis in different crop species (*29*) and are also potential site(s) of the observed mutation. Although on native gels we could not observe any effect of the mutant on SBE activity based on the native gels and the preliminary enzyme assay, this enzyme cannot be entirely ruled out at this stage. TILLING (*30*) using primers from as many as 16 different enzymes known to be related to starch biosynthesis is also underway. Most likely, therefore, the genetic origin of these mutations will be confirmed during 2008.

The factors affecting starch granule morphology and size within and between crops are not clearly understood (*26, 29*). How the lack of isoamylase activity reduces starch granule size, despite extensive work on mutants for this enzyme in other species, is not understood either. It is clear however that a decrease in isoamylase activity is always accompanied by an increase in the number of granules initiated, which results in a decrease in granule size. These new small-granule mutations in cassava may contribute for a better understanding of these factors, since they are clearly affecting simultaneously granule morphology and chemistry. According to Lindeboom and coworkers (*31*), the size of MUT granules should be classified as small (5−10 μm) compared with those of WT cassava, which should be classified as medium (10−25 μm).

The higher-than-normal level of amylose in MUT has important commercial implications. Increased amylose levels lead to slowly digestible and resistant starches (*26, 29, 32, 33*), which have a distinctive advantage in health, particularly in diabetes management. In addition, high-amylose starches in different crops offer advantages for the production of sweets, adhesives, corrugated boards, and in the paper industry and reduce the uptake of fat in certain fried products (*26, 29*). Very high levels of amylose result in "resistant" starches (maize starches with more than 50% and up to 90% can be produced commercially). Resistant starches cannot be digested, but rather, they are fermented in the large intestine, resulting in the production of butyrate that has been found to be beneficial to colon health (*29*).

Crosses between the two different MUT genotypes will be made. Since they are genetically unrelated, the resulting F_1 crosses will not have the negative effects of inbreeding observed in the M_2 cloned genoytpes currently available. Moreover, the F_1 crosses will be segregating for many loci, including those causing the small-granule phenotype (in case the mutations in 5G160 and 3G43 affected different loci). This segregation could allow for breeding for amylose content levels above the maximum level of 36% already observed in one of the quantifications made in this study.

Alternatively, the starch granule characteristics of these mutants may have other commercial applications. The reduced granule size and the obvious irregularities in their surface (**Figure 2**) would lead to a facilitated hydrolysis (*32*). If that were the case, these starches would offer important advantages for the bioethanol industries by requiring reduced quantities of enzymes or resulting in faster starch-degradation processes. It is acknowledged however that while the starch granule appearance would facilitate bioethanol production, the higher proportion of amylose would tend to make it less efficient. Only when proper fermentation studies are conducted, the relative importance of these contrasting and opposed trends would be clarified. A similar problem has already been analyzed for starches from other crops (*32*).

For the commercial exploitation of the special starch characteristics observed in MUT, it is necessary to combine the trait with a

competitive yield of fresh roots with acceptable levels of dry matter content. It is not possible to assess in the M_2 plants where MUT was identified the yield penalty (if any) of this special characteristic. A penalty in yield has been observed in high-amylose varieties from other crops (*29*). In addition, small granules may have problems for commercial exploitation related to the difficulties in purifications at the commercial level (*26, 31*). In spite of these problems, commercial exploitation of small-granule or high-amylose starch from other crops is a reality today.

In addition to crosses between the sources of MUT and elite WT germplasm with good agronomic performance, crosses between MUT and the amylose-free genotype already reported (*8*) and other unusual starch types (still in the process of characterization) have already been made. The resulting hybrids will be heterozygous for the two mutations they carry. Therefore, neither mutation will express in these hybrids, which need to be self-pollinated in order to obtain the double homozygotes. Hopefully, the simultaneous expression of two of these mutations combined will lead to different and new starch phenotypes. In addition, crosses between the two sources of MUT (originally derived from 3G43 and 5G160 M_1 plants) would help understanding if these two mutations are allelic or not.

Differences in the results from the seedling plant (March 2006 harvest) and the cloned plants (September 2007 harvest) of genotype 5G 160-13 are not surprising (**Table 2**). The root system of plants derived from germinating botanical seed (seedling plant) and those from cloned plants are different (seminal and adventicious tissue, respectively). Therefore, it is expected to find differences in the starch extracted from the two different types of plants. A similar situation was observed through the discovery of the amylose-free mutation (*8*). Important to emphasize is the fact that the main characteristics of MUT are preserved in the cloned plants therefore demonstrating that the mutation is stable.

The effect of irradiation overlaps and confounds with the normal genetic segregation that made each botanical seed unique and different from each other. Several (but not all) of these progenitors listed in **Table 1** have already been self-pollinated, and their progenies evaluated. In no case has the MUT phenotype been observed. Therefore, on the basis of the available information, our current working hypothesis is that two independent mutation events, with similar phenotypes, took place in four genotypes (belonging to two lineages from 3G43 and 5G160) out of about 800 evaluated. This is a high mutation frequency (whether or not the two mutations are allelic) and would suggest that mutations at the locus (or loci) controlling these characteristics are either easy to attain, or else, that the repairing mechanisms to revert the mutations are less efficient in this region of the genome. This information, in turn, is relevant for future mutation breeding work.

In conclusion, two different genotypes with the same MUT phenotype have been identified. Results conclusively demonstrate that these are indeed genetic mutations that are stable and therefore commercially exploitable. The biochemical and functional characteristics in these mutations have commercial relevance. Ongoing activities include further characterization of the small-granule starch, crosses with elite WT germplasm to place the mutation in a better genetic background, which in turn will result in a better agronomic performance required for commercial exploitation, and crosses with other starch mutants to produce double mutants and hopefully new starch phenotypes.

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