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Discovery of an Amylose-free Starch Mutant in Cassava (*Manihot esculenta* Crantz)

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One of the objectives of the cassava-breeding project at CIAT is the identification of clones with special root quality characteristics. A large number of self-pollinations have been made in search of useful recessive traits. During 2006 harvests an S_1 plant produced roots that stained brownish-red when treated with an iodine solution, suggesting that it had lower-than-normal levels of amylose in its starch. Colorimetric and DSC measurements indicated low levels (3.4%) and an absence of amylose in the starch, respectively. SDS-PAGE demonstrated the absence of GBSS enzyme in the starch from these roots. Pasting behavior was analyzed with a rapid visco-analyzer and resulted in larger values for peak viscosity, gel breakdown, and setback in the mutant compared with normal cassava starch. Solubility was considerably reduced, while the swelling index and volume fraction of the dispersed phase were higher in the mutant. No change in starch granule size or shape was observed. This is the first report of a natural mutation in cassava that drastically reduces amylose content in root starch.

KEYWORDS: Amylose; genetic variation; germplasm collections; inbreeding; recessive traits

INTRODUCTION

Moorthy (*1*) mentioned that sources of starch include cereals, tree, fruit and vegetable crops, and, very relevant for tropical environments, the root and tuber crops. Commercial starch extraction, however, is carried out from a limited number of crops. Among the noncereal sources, the most important are the sago palm, potato, cassava, and sweet potato. About 73.7 to 84.9% of dry root weight of cassava is starch (*2*). A comprehensive revision of cassava starch properties has been published (*1*). The starch is easily extractable from the roots because they contain low levels of protein and fat.

Cassava starch, if properly extracted, is pure white and its low levels of fat and proteins imply that both the starch and its derivatives have a noncereal taste, which is very desirable in many food products. Compared with other root and tuber tropical crops, cassava starch and its biosynthesis have been better studied (*3*−*5*). 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.

Glucose seems to be the only monosaccharide detectable from cassava starch (*6*). Amylose content has been reported to range from 17.9 to 23.6% (*6*); 17 to 25% (*7*); 18 to 25% (*1*); or 13.6 to 23.8% (*2*). 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 2000 different genotypes was 16.6% ± 2.32 (CIAT, unpublished data). There is a clear genetic influence on the content of amylose in the starch, and neither the age of the plant nor environmental factors seem to play a major role in determining it. Swelling volume ranges between 25 and 30 mL/g, and digestibility is good. X-ray diffraction of cassava starch, follows an A and C pattern (*1*, *2*). 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*).

One of the earliest genes characterized in any organism is the waxy (*Wx*) locus of maize (*8*). Ample evidence later showed that it encodes the starch granule-bound glucosyl transferase (GBSS) in most if not all plants, an enzyme of about 58−60 kD (*9*). Early studies using the iodine test found that wild-type and mutants for the *Wx* function could be easily distinguished. Iodine solutions stain distinctively the *wx* starch because it lacks (or has drastically reduced levels of) amylose. This property has commercial advantages that have been extensively exploited.

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Starch composed exclusively of amylopectin is advantageous for several commercial purposes (*8*). Waxy maize starch shows properties similar to those of cassava starch (*10*).

Compared with the several mutations reported for starches from other crops like maize and potato, cassava offers comparatively very little variation (*11*, *12*). There are two reasons for this: cassava is seldom self-pollinated, and since most of these mutations are recessive in nature there has been little opportunity for them to express. But even if the mutations had the possibility of expressing it would be difficult to detect them because the root does not mature in the way the cereal kernel does. When the kernels from cereals like maize reach physiological maturity and dry down, different starch mutations became phenotypically distinctive and are easily detected visually (*13*).

The first report of transgenic cassava was made in 1995 (*14*). Work at Wagenningen University in The Netherlands produced the first transgenic amylose-free cassava (*15*, *16*). This genotype was produced using antisense technology to silence the GBSS-I gene.

CIAT has implemented several strategies to develop high-value cassava clones to take advantage of the new opportunities opened to cassava by the globalization of the economies in many tropical countries (*12*, *17*). The main objective is to develop not only clones with high and stable productivity, but also with root characteristics that better fit the needs of the different industries. For the feed industry high-protein clones have been identified (*18*). For the starch industry different approaches to develop and identify clones with novel starch properties have been gradually introduced in the cassava-breeding project (*17*). In addition, the identification of those genotypes where interesting starch quality variations are expressed requires the availability of special tests. CIAT has upgraded its laboratory capacity to be able to process as many as 15000 starch samples/year using rapid viscoanalyzers and measuring amylose/amylopectin ratio, total sugars, cyanogenic potential, and dry matter contents. DSC quantifications can also be made.

The objective of this article is to report the characteristics of one cassava clone (AM206-5) with very distinctive characteristics discovered in March 2006.

MATERIALS AND METHODS

As part of the project to introduce inbreeding in cassava (*Manihot esculenta* Crantz) genetic improvement, a large number of self-pollinations were performed in different genotypes from the cassava-breeding project at CIAT, as well as from the germplasm collection (*17*). More than 20 000 botanical seeds obtained from self-pollinations from 74 different parental clones have been obtained. These partially inbred genotypes were used for different purposes and carefully screened for root quality traits. Given the number of samples routinely analyzed, small flour and starch samples are taken from each genotype pooling different roots for sampling purposes.

Only one plant per genotype was available because the evaluations were made on individual plants obtained from botanical seed. Since genotypes included partially inbred plants, their vigor was somewhat affected and root productivity variable. At least one commercial size root was harvested per genotype. Whenever possible, up to five roots per plant and genotype were harvested. Roots were washed and peeled before samples were prepared for the different analyses performed.

Root and Starch Moisture Content. Up to five roots from the same plant were peeled and immediately cut into small pieces and mixed. Moisture content was determined after drying 50 g of sample (freshly cut pieces or starch) at 60 °C for 24 h (*19*).

Iodine Stained Field Evaluation of Roots and Stems. One slice from the central part of the each root and transversal cuts of the stems were sprayed with iodine solution 2% (2 g KI and 0.2 g I_2 in 100 cm^3 of distillated water). Reddish-brown staining is typical of amylose-free starch, whereas cassava starch with normal amylose-amylopectin mixture stains dark-blue.

Flour Extraction. Freshly cut pieces from the harvested root(s) were lyophilized during 24 h at −30 °C and then ground. A FreeZone stoppering tray drier (model 79480) and a 6 liter Freeze Dry System (model 77530) (Labconco Corporation, Kansas City, MO) were used. The flour thus obtained was stored in plastic bags for further analyses.

Starch Isolation. The freshly cut pieces were suspended in tap water and crushed in a 4 L capacity Waring commercial blender (New Hartford, CT). The slurry was filtered through a 100 μm sieve. The starch was allowed to settle, and the supernatant was decanted off and dried in an oven with fan-forced ventilation at 40 °C for 2 days (Thelco oven, model 28, Precision Scientific Subsidiary of GCA Corp., Chicago, IL).

Ash Content. Ash content was calculated following heating at 550 °C for 3 h (*20*).

Crude Fiber Content. The fiber content was determined for the loss on ignition of dried residue remaining after digestion of cassava flour (2 g) with 1.25% H_2SO_4 and 1.25% NaOH (*21*).

Total and Reducing Sugars. Content of total and reducing sugars were determined according to Cronin et al. (*22*). Sugars were extracted from 2 g of root flour using an 80% ethanol solution, a Fehling reagent, and a glucose standard curve. A Cecil spectrophotometer model CE 2021-series 2000 (Cambridge, U.K.) was used in the determination.

Determination of Starch Content. Starch was measured after incubation with thermostable α-amylase and then with amyloglucosidase. The released glucose was measured with a spectrophotometer after reaction with ABTS-reagent containing glucose oxidase and peroxidase (*23*). Starch content was calculated as 90% of glucose content.

Granule-Bound Starch Synthase Identification: SDS-PAGE. Fifty milligrams of dry starch were suspended in 0.35 cm^3 of loading buffer (3% SDS; 0.001 blue dye; 0.0625 M buffer pH 6.8; 1% B-mercaptoetanol), boiled for 10 min (*24*), and centrifuged at 10 000 rpm for 5 min. The resulting supernatant (crude extract) was subjected to SDS-PAGE denaturing electrophoresis on a 7.5% gel as described by Laemmli (*25*) with some modifications described in CIAT (*26*). Ten microliters of each sample were loaded per lane. Constant voltage of 100 V was applied for 1 h at 10 °C and increased to 150 V for the remaining duration of the run until the tracking dye reached the end of the gel. Gels were stained with silver.

Optical Microscopy. Starches from different clones were placed in a slide with a spatula, stained with iodine solution, 0.2%, and observed through an Olympus CX41 light microscope (Tokyo, Japan) using a 40× magnification lens.

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.

Paste Clarity. The methodology suggested by Craig et al. (*27*) was used. A 1% db aqueous dispersion of starch was boiled at 97 °C (1000 m above sea level) with thorough shaking every 5 min for 30 min. Transmittance was measured after cooling to room temperature at 650 nm.

Colorimetric Amylose Determination. Amylose content in the starch was measured following standard procedures (*28*). Starch granules were first dispersed with ethanol and then gelatinized with sodium hydroxide. An aliquot was then acidified 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 standard curves obtained using purified amylose and amylopectin extracted from potato tubers. Five different quantifications per starch sample were made, and mean values were then calculated.

Differential Scanning Calorimetry (DSC) and Amylose Content. The methodology reported by Mestres et al. (*29*) was used. DSC analyses were performed on a Perkin-Elmer DSC 7 device (Perkin-Elmer, Norwalk, VA) using sealed stainless-steel pans. The sample pan (10−11 mg of starch and 50 μL of lyso-phospholipid 2% *w*/*v* in

water) and the reference pan (empty) were heated from 25 to 160 °C at 10 °C min^{-1}, holding at 160 °C for 2 min, and then cooling to 60 °C at 10 °C min^{-1}. The onset temperatures (GT) of each sample were determined on the thermograms. Amylose content was also measured from the energy of amylose-lysophospholipid complex formation using the DSC. The analysis was performed in duplicate, and mean values were calculated.

Pasting Properties. Hot starch dispersion viscosity profiles were obtained with a Rapid Visco Analyzer model RVA-4 series (Newport Scientific, Australia). Starch (1.25 g db) was dispersed in distillated water (near 23 cm^3) to 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 °C at 6 °C min^{-1} with continuous stirring at 160 rpm. Four parameters were measured: pasting temperature (PT), peak viscosity (PV), hot paste viscosity at the end of the plateau at 90 °C (HPV), and the cool paste viscosity at 50 °C (CPV). With them, three additional parameters were calculated: breakdown (BD), estimated as PV − HPV; setback (SB), estimated as CPV − PV; and consistency (CS), estimated as CPV − HPV.

Swelling Power, Solubility, and Dispersed Volume Fraction Measurements. Swelling power and solubility patterns (*30*) were determined using 1.5% db (*w*/*w*) starch dispersions (0.42 g dm dispersed in 27.58 g of distilled water). Paste was prepared in Rapid Visco Analyzer (RVA) holding at 35 °C for 1 min, heating to 75 °C at 6 °C min^{-1} rate, holding at 75 °C for 2.5 min. The paste was immediately transferred to 50 cm^3 centrifuge tube. The supernatant and sediment after centrifugation for 5 min at 6000g at 25 °C were collected and weighed (W_{su} and W_{se}, respectively) then dried at 100 °C for 24 and 48 h, respectively, and weighed (D_{su} and D_{se}, respectively). Three parameters were calculated: concentration of soluble material in the supernatant (solubility), the swelling power, and the volume fraction of the dispersed phase (Φ):

$$\text{solubility (\%db)} = 100D_{su}/0.42$$

$$\text{swelling power} = (W_{se} - D_{se})/D_{se}$$

$$(\Phi) = (27.86 - (W_{su} - D_{su}))/27.86$$

where the factor 27.86 is calculated as total volume ($cm^{3)}$) of the paste. (Starch specific density is 1.5 g/cm^3.)

$$27.86 = 27.58 + (0.42/1.5)\ cm^3$$

Morphological Description of Cassava Germplasm. For the morphological description of different genotypes, descriptors mentioned in the literature (*31*) are used by the cassava-breeding project at CIAT.

Experimental Design. Stem cuttings can reproduce cassava asexually to produce cloned plants. Because the primary tap root system of a plant obtained from botanical seed ("seedling plant") is different than the adventitious roots of a plant obtained from stem cuttings ("cloned plants") some root quality traits may change from seedling to cloned plants (*31*). When roots from a seedling plant provide promising results during the routine screening of germplasm, several stem cuttings are obtained and that particular genotype is cloned for further evaluation. This study reports the characteristics of a particular genotype that showed promising results at the seedling plant stage (March 2006) and was thus multiplied to produce a total of 40 cloned plants. From these 40 cloned plants five of them were randomly chosen as the source of roots for further analyses (April 2007). Roots from each of these plants were harvested and processed independently to extract flour and starch. Analyses of flour and starch from AM206-5, therefore, are based on five independent replications. As a reference point results of two "normal" genotypes (MCOL 2208 and MPER 247) illustrate values that are typical for cassava flour and starch and help to highlight the uniqueness of some characteristics of AM206-5. Three independent aliquot analyses were made on the starch samples of pooled roots from MCOL 2208 and MPER 247. Flour samples for the two check genotypes, however, were too small to have replications.

Figure 1. Differential staining with iodine of roots (**A**) and stems (**B**) of a normal cassava clone (stained blue) and AM206-5 (stained reddish brown).

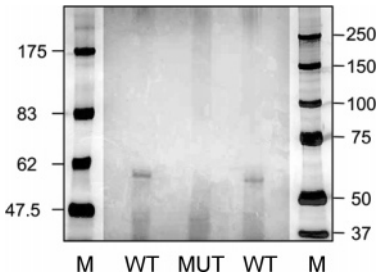

Figure 2. Comparison of granule-bound proteins. Starches were purified from the roots of two different wild type (WT) clones and from AM206-5 (MUT). Granule-bound proteins were extracted by gelatinizing the starches in gel loading buffer containing SDS, separated on SDS-PAGE gels and detected by silver staining. The molecular weights of marker proteins (M) in kDa are indicated.

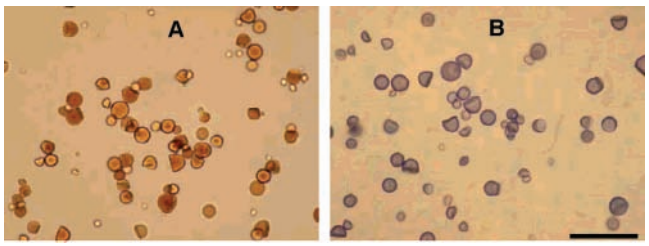

Figure 3. Differential staining with iodine of purified starches from the roots of abnormal cassava AM206-5 (**A**) and starch from a normal cassava clone (**B**). The bar is 50 μm.

Table 1. Proximal Analysis % (g/100 g db) from the Three Cassava Genotypes Analyzed

parameters[a]	AM206-5[b]	MCOL 2208	MPER 247
dry matter % (g/100 g wb)	31.5 (1.3)	34.8	35.7
ash content (%)	3.0 (0.2)	1.6	2.2
crude fiber content (%)	4.6 (0.7)	2.6	3.2
total sugars (%)	1.6 (1.1)	2.9	3.6
reducing sugars (%)	0.8 (0.8)	0.9	1.3
starch content (%)	86 (3.9)	88	86

[a] wb= wet basis. [b] The standard deviations based on the independent analyses of the roots from five different cloned plants of AM206-5 are given within parentheses.

RESULTS

In December 2004 several self-pollinations were made on a cultivated cassava genotype as part of the project to introduce inbreeding in cassava genetic enhancement at CIAT. The S_1 family AM206, one among many S_1 families produced and evaluated, included 79 seeds, which were germinated in a greenhouse on April 2005. Only 40 plants were viable and transplanted to the field in June 2005. Of those, 17 plants survived with a good development after 9 months. In March 2006, roots from the S_1 genotype AM206-5 showed a unique

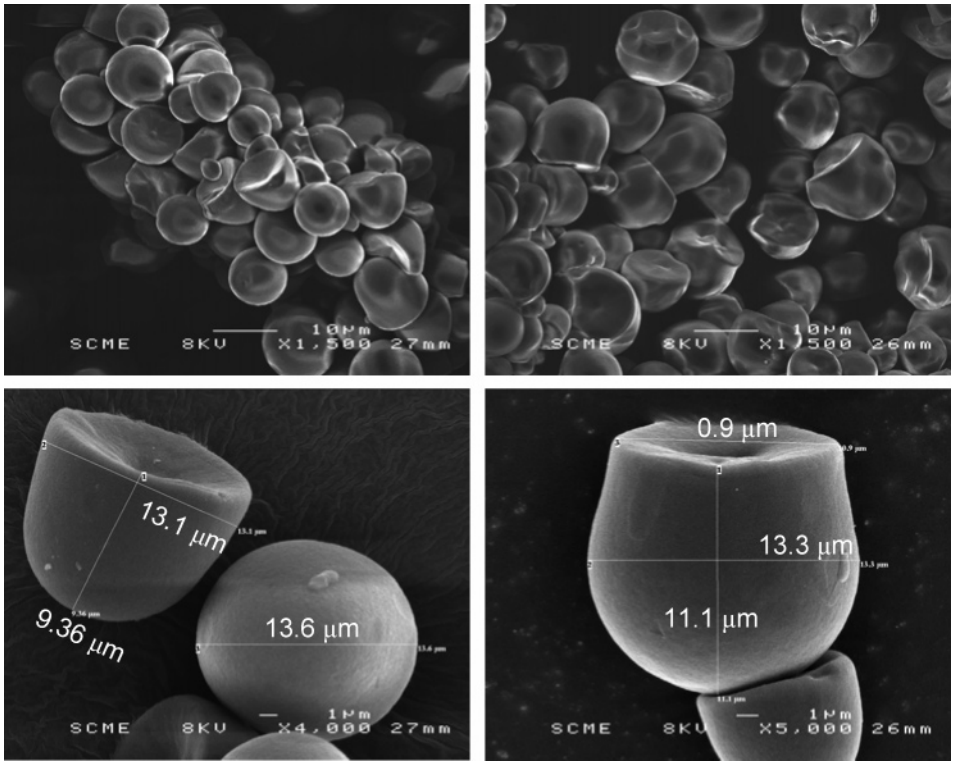

Figure 4. Scanning electron microphotographs (magnification specified in each microphotograph) of starch from genotype AM206-5 (left) and "normal" (right) cassava starch. Notice that magnifications for the photographs at the bottom are different.

and distinctive staining when treated with an iodine solution (**Figure 1**). There was a differential staining with the iodine solution on roots and stems from AM206-5 compared with those from other genotypes. Roots and stems from AM206-5 stained brown-reddish, while roots and stems from other genotypes showed the typical blue-dark staining (**Figure 1**). The differential staining prompted us to carry out other tests on AM206-5, and their results are presented herein.

Upon the discovery of the special characteristics of the single seedling plant representing AM206-5, up to forty stem cuttings were obtained to clone this genotype and planted at CIAT-Palmira on June 2006. Roots from five random cloned plants were then harvested in April 2007, and analyses were made to confirm the properties first identified in the seedling plant in March 2006.

Root and Starch Moisture Content. Table 1 presents results of the proximal analysis of root flour, including dry matter and starch contents of the three genotypes reported. Flour from AM206-5 was extracted from the five cloned plants and analyzed independently. Results from the two check genotypes were based on nonreplicated analyses and are included just as a reference for the reader. Dry matter content for the three genotypes fell within normal ranges for cassava, although that of AM206-5 was slightly lower than those of the reference genotypes. Ash content tended to be higher in AM206-5 than in the other genotypes (**Table 1**). The starch extraction procedure utilized left only traces of protein. In the case of AM206-5, for example, average protein content of the starch from the five plants sampled was 0.12% with a standard deviation of 0.037.

Granule-Bound Starch Synthase Identification: SDS-PAGE. Figure 2 shows the SDS-PAGE used to confirm the presence or absence of the GBSS enzyme in a preliminary electrophoresis. This study was made on the starch from the original AM206-5 seedling plant in March 2006. Starches from two different wild type genotypes were also analyzed. Results clearly suggested that the starch from AM206-5 lacks the GBSS enzyme (*9*), thus further indicating that it was, indeed, a waxy starch.

Table 2. Starch Physicochemical Properties of the Starches from the Three Cassava Genotypes Analyzed[a]

parameter	AM206-5	MCOL 2208	MPER 247
paste clarity (%)	57.6 (1.6)	56.2 (0.3)	50.3 (0.6)
colorimetric amylose content (%)	3.4 (0.2)	20.4 (0.3)	19.7 (0.4)
amylose content (%)	0.0 (0.0)	19.2 (0.0)	19.0 (0.5)
gelatinization onset temp (GT) in °C	63.1 (0.7)	60.4 (0.1)	61.8 (0.1)

[a] The standard deviations based on the analyses of roots from five AM206-5 plants or three aliquots from root samples of MCOL 2208 and MPER 247 are given within parentheses.

Optic Microscopy. Figure 3 presents contrasting photographs of starch from AM206-5 (obtained from the seedling plant in 2006) and that of a wild-type genotype stained with a 0.2% iodine solution. A clear differential staining could be observed between the starches from the two different genotypes. Starch from AM206-5 showed the typical morphology and size of a cassava starch granule but stained brown-reddish in comparison with normal starch, which stained dark blue.

Scanning Electron Microscopy (SEM). Figure 4 presents scanning electron microscope photographs of the starch from AM206-5 (obtained from the seedling plant in 2006) and a "normal" starch at different magnifications. This figure shows again the typical starch granule morphology of cassava and no visible difference in shape or size. Granules from AM206-5 and the "normal" starch are between 10 and 15 μm, and their shape showed the typical truncated morphology distinctive for cassava starch.

Table 2 presents starch physicochemical properties. Data from AM206-5 come from the starches of five random cloned

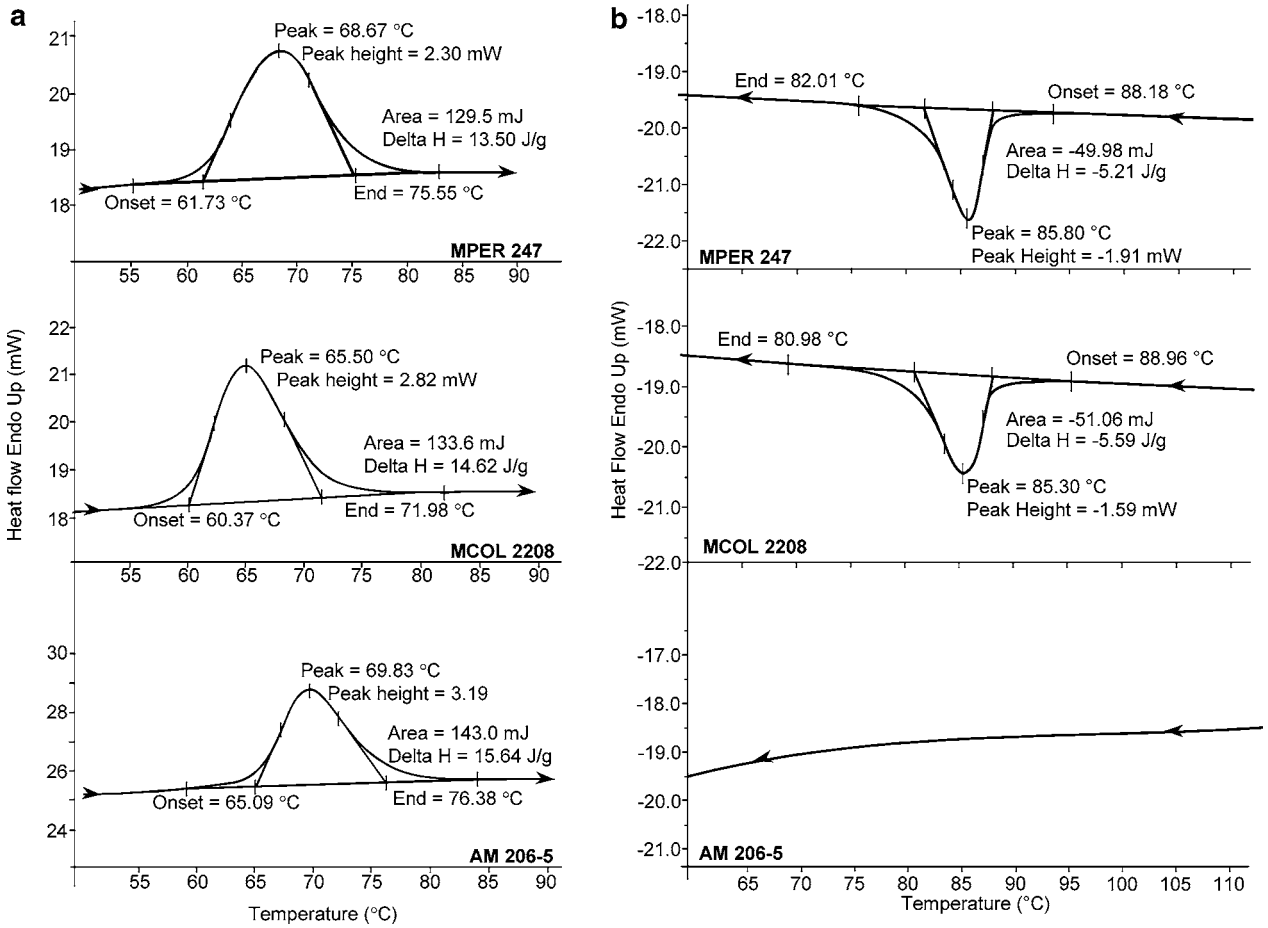

Figure 5. (**A**) Differential scanning calorimeter (DSC) results from AM206-5 (one of the five analyses made) and two cassava checks (one of the three analyses made) in the heating phase. (**B**) Differential scanning calorimeter (DSC) results from AM206-5 (one of the five analyses made) and two cassava checks (one of the three analyses made) in cooling phase.

plants harvested in April 2007 and analyzed individually. Data from MCOL 2208 and MPER 247 were based on three independent quantifications of a sample from pooled roots following the standard procedure at the cassava-breeding project of CIAT. Paste clarity was not different in the starch of AM206-5 compared with the other two genotypes. Average amylose content using the colorimetric method was 3.4%, compared with the averages of about 20% for the wild types, typical of cassava starch. Amylose content using the DSC indicated total absence of amylose in the starch. Both types of quantifications were statistically significant. These results confirm those obtained from the starch of the seedling plant in 2006 (**Figures 5A** and **5B**). The detection of a small amount of amylose using the colorimetric method can be due to lack of purity in the commercial potato amylopectin standard used in the analysis. Also, some long-chain amylopectin branches can bind like amylose, acting somewhat like amylose in colorimetric tests (*32*).

Figure 5 shows the DSC plots from the starches of the three genotypes evaluated. The differential curve of the AM206-5 starch (obtained in 2006 from the original plant) in the cooling phase was very striking. The mixture of amylose and amylopectin in the gels from MCOL2208 and MPER247 absorbed energy for a molecular re-organization which was proportional to the area depicted in **Figure 5** and has been demonstrated to be related to amylose concentration (*29*). The gel from AM206-5, on the other hand, did not show any energy absorption during the cooling phase further indicating absence of amylose in the starch.

Table 3. Pasting Behavior of Amylose-free and Normal Cassava Starch from the Three Genotypes Analyzed[a]

parameter[b]	AM206–5	MCOL 2208	MPER 247
pasting temperature (PT) in °C	68.3 (0.8)	65.4 (0.4)	67.5 (0.2)
peak viscosity (PV) in cP	890 (38)	577 (19)	746 (20)
hot paste viscosity (HPV) in cP	399 (18)	329 (12)	456 (16)
cool paste viscosity (CPV) in cP	490 (17)	416 (16)	580 (18)
breakdown (BD) in cP	491 (31)	249 (7)	290 (27)
setback (SB) in cP	−400 (32)	−161 (8)	−166 (24)
consistency (CS) in cP	91 (3)	88 (6)	124 (6)

[a] The standard deviations based on the analyses of roots from five AM206–5 plants or three aliquots from root samples of MCOL 2208 and MPER 247 are given in parentheses. [b] cP=centipoise.

Starch Functional Properties. Table 3 presents the most relevant results from the pasting behavior of waxy and normal cassava starch obtained from the amylograms presented in **Figure 6**. AM206-5 showed higher viscosity peak (890 cP) versus those from the other two genotypes (577−746 cP). Overall the amylograms show the typical performance of cassava profile: lack of resistance to high temperature and sensitivity to shearing stress. Breakdown was noticeably different in AM206-5 (491 cP) compared with typical cassava (249 and 290 cP), suggesting a reduced tolerance to shear stress in the mutant. The starch from AM206-5 also showed a distinctive setback value (−400 cP) compared with the starches from the two checks (−161 and −166 cP). There was no relevant difference for consistency.

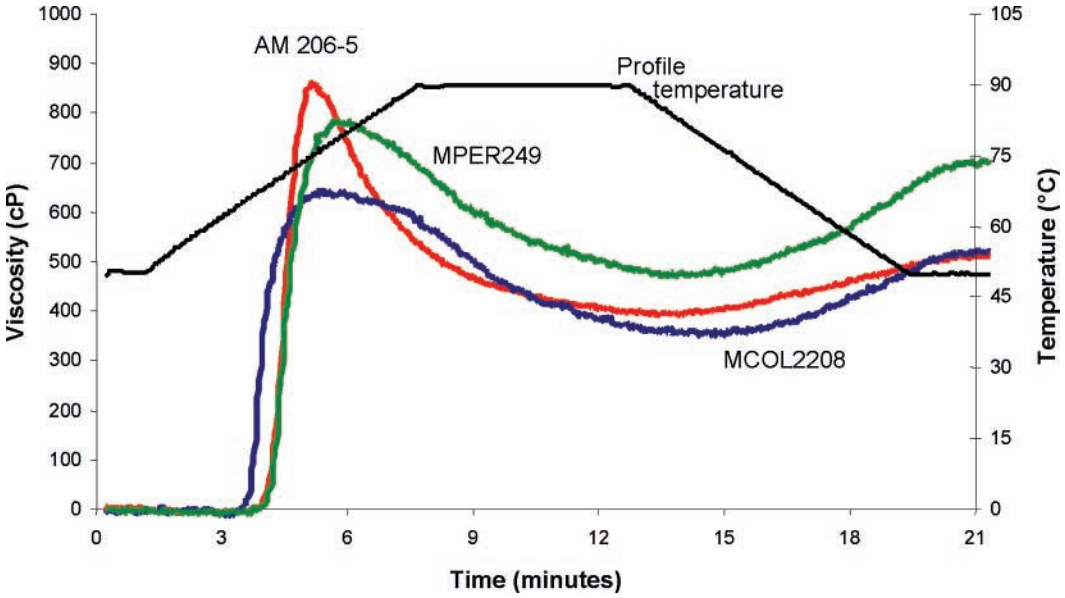

Figure 6. Amylograms from three cassava genotypes obtained using a rapid viscoanalyzer (RVA). The waxy starch from AM206-5 is depicted as a red line, which shows the averages for starches from five different roots. In the case of MPER 249 and MCOL 2208 three aliquots were used.

Table 4. Solubility and Swelling Values of Waxy and Normal Cassava Starch[a]

genotype	solubility (% db)[b]	swelling index ($g \cdot g^{-1}$)	volume fraction of dispersed phase (Φ)
AM206–5	6.0 (0.5)	55.7 (2.3)	0.50 (0.03)
MCOL 2208	14.1 (0.6)	32.3 (0.7)	0.45 (0.00)
MPER 247	13.4 (0.4)	30.8 (0.4)	0.41 (0.01)

[a] The standard deviations based on the analyses of roots from five AM206–5 plants or three aliquots from root samples of MCOL 2208 and MPER 247 are given within parentheses. [b] db= dry basis.

Solubility, swelling index, and dispersed volume fraction measurements for the starch from AM206-5 showed contrasting results in comparison with those from the other two "normal" genotypes (**Table 4**). Solubility of the starch from AM206-5 was about half of the values observed for MCOL 2208 and MPER 247. This is to be expected because amylose is more soluble than amylopectin. This behavior further suggests that the starch of AM206-5 had considerably lower amounts of amylose than normal cassava starches. Swelling index in AM206-5 was considerably higher (55.7 g g^{-1}) than for the other two starches (30.8−32.3 g g^{-1}). The volume fraction of dispersed phase was slightly higher in AM206-5 (0.5 Φ) compared with the starches from the other two genotypes (0.41−0.45 Φ).

Morphological Description of the AM206-5 Genotype. The morphology and plant architecture of AM206−5 (averages of five cloned plants) did not have any unique characteristic (except its starch) and can be considered typical of cassava. Plant height was 170 cm, and height for first branching was 56 cm. On average there were four branching levels, three branches per node (trichotomous), and a branching angle of 47.5 degrees. Branching and flowering occurred about 5 months after planting. Stem growth was straight, and plant type was compact. External color of the stem was light brown, whereas the color of the cortex was dark green. Internode length ranged from 8 to 15 cm. The color of apical leaf was light green, whereas fully expanded leaves were dark green. Terminal braches and leaf veins were green. Petioles were yellowish green with an average length of 12.5 cm and a horizontal position. The shape of the central leaf lobe was lanceloated with an average of five lobes per leaf. Central lobe was, on average, 3.2 cm wide and 13.0 cm long. Leaf scars were prominent. Apical pubescence was absent. Stipules were long. Root shape was cylindrical, without constrictions, dark brown external color and pink cortex. The parenchyma was white. The texture of the root epidermis was not smooth. Roots had peduncule. Root yield was not measured because it would have been based on single plant evaluation without replications. Yield of a partially inbred genotype was not as relevant as qualitative traits, such as starch characteristics. Root productivity, however, was adequate as revealed by the size of the roots (**Figure 1**) and other related data (**Table 1**).

DISCUSSION

All analyses converged to support the hypothesis that genotype AM206-5 has amylose-free (waxy) starch on the basis of data from the seedling plant derived from botanical seed (starch extracted and analyzed in 2006) and the five random cloned plants (starch extracted and analyzed in 2007). This is the first report of such discovery after thousands of evaluations made in different landraces and improved cassava germplasm. Differential iodine staining of roots, stems, and starch from AM206-5 was the first indication, which was then supported by the absence of the GBSS enzyme in the SDS-PAGE electrophoresis. Functional properties of starch from this genotype were also different and in agreement with the expectations for an amylose-free starch (high viscosity, high swelling index, and low solubility). Colorimetric and DSC results to quantify amylose content finally proved that the starch from AM206-5 has very low levels or absence of amylose, respectively. Granule morphology was not affected by this mutation.

The combined results reported in this study produce convincing evidence that AM206-5 has a naturally occurring mutation on the *Wx* locus which is the one codifying for the GBSS enzyme. Molecular and traditional genetic analyses are currently underway to further confirm this. These results are important not only because of the commercial applications of a cassava clone with waxy starch but also because they demonstrate that the self-pollination of cassava (particularly from genetically diverse germplasm) is likely to yield interesting results. This is

the first report of a naturally occurring waxy starch mutation in cassava. However, waxy cassava starch has been obtained through genetic transformation (*15*, *16*). Carvalho and co-workers reported in 2004 (*11*) a group of interesting "sugary" mutations in cassava that results in storage roots with high free sugars (mostly glucose) and a glycogen-like molecule. The roots from these genotypes have reduced levels of amylose.

Crosses of AM206-5 are underway to transfer the mutation to germplasm adapted to the most important cassava growing environments. Because of the heterozygous nature of parents used in cassava breeding, a traditional back-cross scheme cannot be properly implemented in cassava. Therefore, the strategy relies on making a first cycle of crosses between AM206-5 and elite germplasm. All the resulting F1 genotypes will be heterozygous (*Wx wx*) for the mutation and are, therefore, expected not to produce amylose-free starch. The F1 plants from the first cycle of crosses will be crossed among themselves to produce a second cycle of crosses. It is expected that about 25% of the segregating progenies will be homozygous (*wx wx*) for the GBSS locus and, therefore, will produce amylose-free starch. Because crosses will have been made among the genetically diverse germplasm from the first cycle of crosses, inbreeding in the second cycle of crosses will be minimized. It should be possible, therefore, to identify vigorous and productive genotypes with waxy starch in the second cycle of crosses.

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