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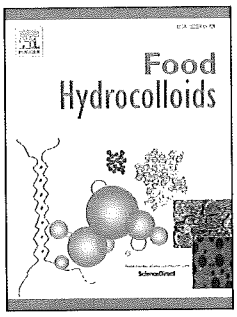

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Discovery of new spontaneous sources of amylose-free cassava starch and analysis of their structure and techno-functional properties

Nelson Morante[a], Hernán Ceballos[a,*], Teresa Sánchez[a], Agnès Rolland-Sabaté[b], Fernando Calle[a], Clair Hershey[a], Olivier Gibert[c,d,e] and Dominique Dufour[a,c,f]

a) Centro Internacional de Agricultura Tropical CIAT, Cali AA6713, Colombia.
b) UR1268 Biopolymères Interactions Assemblages, INRA, F-44300 Nantes, France.
c) Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), UMR Qualisud, 73 Rue Jean-François Breton, 34398 Montpellier, France.
d) ICAPRD - Kampus Penelitian Pertanian Cimanggu - Jl. Tentara Pelajar no 12A - Cimanggu - Bogor Jawa Barat, Indonesia.
e) CIRAD, UMR QUALISUD, Bogor, Indonesia.
f) CIRAD, UMR QUALISUD, Cali, Colombia.

* Corresponding Author e-mail: h.ceballos@cgiar.org.

Abstract

Waxy cassava starch (WS) from a spontaneous mutation offer enough advantages to encourage the development of commercial varieties. However, breeding work is limited because only one source of WS was available. This article reports the discovery of five new sources of WS which were compared with commercial (CC) and high amylose (HA) cassava starches (19.9 and 30.2% amylose, respectively). Waxy starch gels were considerably clearer and had higher solubility and swelling power than those from CC. Pasting temperature and peak viscosity in WS were higher (68.5 ℃ and 1149 cP) than in CC (64.2 ℃ and 993cP). No retrogradation or syneresis could be detected in refrigerated gels or after freeze/thaw cycles in any WS gel. Average λ_{max} for WS, CC and HA were 540.2, 591.3 nm, and 607.0 nm, respectively. Amylopectins from WS displayed $\overline{M}_w$ between 271×10^6 and 551×10^6 g mol^{-1}, $\overline{R}_G$ ranged between 229 and 298 nm and ν_G values ranged between 0.38 and 0.40 (between the sphere and the random coil). The apparent density distributions in all WS suggested close densities and branching structure. The corrected average number of glucosyl units in a linear chain averaged 21.2 in WS and 23.1 in CC. Amylopectins from WS showed a slightly more branched structure (average 4.8 %) than in CC (average 4.3 %), but lower than in HA (7.0 %). WS exhibited larger average granule size (15.9 μm) compared with CC (14.3 μm). New sources of WS will contribute the breeding efforts to develop successful commercial varieties.

Keywords: pasting properties; genetic resources; structural properties; freeze/thaw cycle;

refrigerated storage stability

Introduction

Starch is the most abundant storage reserve carbohydrate in plants. It is found in many different plant organs including seeds, fruits and many roots and tubers. Starch is widely employed in many applications for which the first step is generally a thermal dispersion, carried out in non-degradating conditions. The resulting pastes can be used for their thickening, gelling, and/or stabilizing properties. Starch owes much of its functionality to the characteristics and the conformation in solution of its constitutive polymers, as well as to their physical organization into the native semi-crystalline granules (Colonna & Buléon, 2010). Impacting biosynthetic pathways yields starches with different morphologies, amylose/amylopectin ratios, and amylopectin structures. The amylose/amylopectin ratio greatly impacts the starch functional properties.

Worldwide, cassava (*Manihot esculenta* Crantz) is the second most important source of commercial production of starch after maize (Stapleton, 2012). Cassava, therefore, is a very important crop for the starch industry, particularly for tropical and subtropical regions of the world (Davis, Supatcharee, Khandelwal, & Chibbar 2003; Moorthy, 2004). It is also the third most important source of calories in the tropics, after rice and maize.

Native starches from different crops show wide variation in their functional properties which can be widened by *in vitro* physico-chemical modifications. However, current

trends and consumer preferences for clean and less processed products favor modification *in planta* from spontaneous or induced mutations (Jobling 2004; Kaur, Ariffin, Bhat, & Karim, 2012; Copeland, Blazek, Salman, & Chiming Tang, 2009) and genetic transformation (Godwin, Williams, Pandit, & Laidlaw, 2009; Jobling, Westcott, Tayal, Jeffcoat, & Schwall, 2002; Zhao, Ceballos, Dufour, Sánchez, & Zhang, 2011; Koehorst-van Putten et al., 2012). Only recently, a spontaneous mutation of amylose-free starch in cassava has been reported (Ceballos et al., 2007), as well as an induced mutation for small-granule which has also been found to have higher-than normal levels of amylose starch (Ceballos et al., 2008). These mutations provided materials that have contributed to a new insight of starch functional properties, contrasts between cereals and root/tuber starches and promising commercial applications for cassava starch (Sánchez, Dufour, Moreno, & Ceballos, 2010; Rolland-Sabaté et al., 2012; 2013). Amylose-free starches have improved freeze-thaw stability compared with normal starches (Copeland et al., 2009Jobling, 2004; Koehorst-van Putten 2012; Sánchez, Dufour, Moreno, & Ceballos, 2010)

The discovery of a natural source of amylose-free cassava starch in 2007 quickly lead to the implementation of breeding projects to develop commercial varieties in Thailand and Colombia. However, the best materials so far developed, failed to reach a level of dry matter content in the roots comparable to that of commercial checks (Karlström, 2015). Finding new sources of amylose-free starch is highly desirable for the breeding work. It would offer alternatives for breaking undesirable genetic linkages of the

mutation locus with other loci controlling traits of agronomic relevance (such as dry matter content). It would also bring adaptation to new and different environmental conditions. These reasons encouraged CIAT to continue the work self-pollinating different accessions from the germplasm collection in search of new sources of novel starch types in cassava. The objective of this article is to describe the structure and the functional properties of new natural sources of amylose-free cassava starch. Special emphasis was placed on behavior of gels from the new sources of amylose-free cassava starch under freeze/-thaw and refrigerated conditions.

Materials and methods.

Germplasm.

As part of the project to introduce inbreeding in cassava (*Manihot esculenta* Crantz) genetic improvement, a large number of self-pollinations have been performed in different genotypes from the cassava-breeding project at CIAT, as well as from the germplasm collection (Ceballos, Hershey, & Becerra-López-Lavalle, 2012). About 1500 S_1 families (result of a self-pollination of a non-inbred progenitor) have been produced in the past decade from accessions of the germplasm collection as well as from improved germplasm. About 800 of those F1 families were developed for the specific purpose of identifying germplasm with novel starch properties. These partially inbred genotypes were used for different purposes and carefully screened for root quality traits such as carotenoids content or starch functional properties. 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 (seedling plant). Since genotypes included partially inbred plants, their vigor was somewhat affected and root productivity variable (Contreras Rojas et al., 2009). 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. Germplasm that offered interesting characteristics were selected and the seedling plant was cloned for multiplication and further evaluation.

Clones, roots production and cassava starch isolation.

Unless otherwise specified, cassava plants were grown at CIAT's Experimental Station at Palmira. Plants were grown following the standard recommended irrigation and fertilization procedures and roots harvested 11 months after planting (MAP), which is the standard age for harvesting cassava at Palmira.

Iodine stained field evaluation of roots and stems.

At harvest time, an exposed transversal section of the central part of the each root were sprayed with iodine solution 2% (2g KI and 0.2g I_2 in 100 cm^3 of distillated water) in the field. Reddish-brown staining is typical of amylose-free starch, whereas cassava starch with normal amylose-amylopectin mixture stains dark-blue.

Starch extraction and isolation

The freshly cut pieces were suspended in tap water and crushed in a 4 liters capacity Waring Commercial blender (New Hartford, CT, USA). The slurry was filtered through a 100 µm sieve. The starch was allowed to settle and the supernatant decanted off and dried in an oven with fan-forced ventilation at 40 ℃ for two days (Thelco Oven Model 28, Precision Scientific Subsidiary of GCA Corporation. Chicago, USA).

Macromolecular composition and structure features

Amylose determination

Amylose content was determined following three different procedures. The first approach was based on the ISO6647 standard colorimetric procedure (Morrison& Laignelet, 1983) with absorbance quantified at a wavelength of 620 nm. Amylose content was also evaluated by quantification of iodine binding capacity or IBC (Larson, 1953; Pérez et al., 2011). The wavelength at maximum absorption (λ_{max}) of the iodine complexes with native starches was determined after solubilization in 1 N KOH for 3 days at 4 ℃ under stirring, as previously describe d (Rolland-Sabaté, Colonna, Potocki-Véronèse, Monsan, & Planchot, 2004). Two measurements per sample were made to estimate this variable. The third amylose content procedure was based on the measurement of the energy of amylose/lyso-phospholipid complex formation by differential scanning calorimetry (DSC) using a DSC 7 device (Perkin-Elmer, Norwalk, CT, USA) from the cooling stage of the thermogram as per Mestres et al. (1996) revised

by Pérez et al. (2011).The sample pan (7-8 mg of starch and 40 µL of lyso-phospholipid 2% w/v in water) and the empty reference pan were heated from 25 to 160 ℃ at a scanning rate of 10 ℃ min^{-1}, held for 2 min at 160 ℃ and cooled to 50 ℃ at 1 0 ℃ min^{-1}. A pure amylose potato starch sample (Avebe, The Netherlands) was used as a standard (3-4 mg and 40 µL of lyso-phospholipid 2% w/v in water). The amylose/lyso-phospholipid complex enthalpy variation of samples and standard were determined, and the amylose content computed as the ratio of ΔH amylose to ΔH Standard in percentage. The analysis was performed in duplicate, and the mean values were calculated.

Molar mass and size distributions of starch macromolecules.

Macromolecular characteristics (molar mass, size, conformation and branching) were obtained using asymmetrical flow field flow fractionation (A4F) coupled with multi-angle laser light scattering (MALLS) using the same procedure and set up as per Rolland-Sabaté et al., (2012). A4F equipment, including the asymmetrical channel, Control-Box V3, Flow box P2.1 and the valve box, was obtained from Consenxus (Ober-Hilbersheim, Germany). The device, its configuration, the membrane and the flow method for A4F fractionation were similar to previously described (Rolland-Sabaté, Colonna, Mendez-Montealvo, & Planchot, 2007) except for the fixed elution flow rate set at 0.84 $mL.min^{-1}$. The detection of solutes was carried out using a Dawn® Heleos™ MALLS system fitted with a K5 flow cell and a GaAs laser, (λ=658 nm), supplied by Wyatt Technology Corporation (Santa Barbara, CA, USA) and a refractometer from Shimadzu (Tokyo,

Japan).

The samples were first dissolved in a DMSO/water (95/5) mixture and then solubilized in water by microwave heating under pressure (Rolland-Sabaté et al., 2007). Solutions were filtrated through 5 µm Durapore™ membranes (Waters, Bedford, MA, USA) before injection into the A4F-MALLS-QELS system. Before use, the mobile phase (Millipore water containing 0.2 g L^{-1} sodium azide) was carefully degassed and filtered through Durapore GV (0.1 µm) membranes from Millipore. Solubilization recovery was calculated from the ratio of the initial mass and the mass after filtration. Carbohydrate concentrations were determined using the sulfuric acid-orcinol colorimetric method (Planchot, Colonna, & Saulnier, 1997). Elution recovery was obtained from the ratio of the mass eluted from the A4F channel (integration of the differential refractive index (DRI) signal) and the injected mass (determined using the sulfuric acid-orcinol colorimetric method).

$\overline{M}_w$, $\overline{M}_n$, the dispersity index $\overline{M}_w$ / $\overline{M}_n$ and $\overline{R}_G$ (nm) were established using ASTRA® software from Wyatt Technology Corporation (version 6.0 for PC), as previously described (Rolland-Sabaté, Amani, Dufour, Guilois, & Colonna, 2003; Rolland-Sabaté, et al., 2007).

The average shrinking factor g_M ($g_M = \overline{R}^2_{Gw(br)}/\overline{R}^2_{Gw(lin)}$, where $\overline{R}_{Gw(br)}$ is the radius of gyration of the branched molecule and $\overline{R}_{Gw(lin)}$, the radius of gyration of its linear

equivalent at the same molar mass) and the apparent particle density (d_{Gappw} = $\overline{M}_w$ / $(4\pi/3)$ $\overline{R}_{Gw}^3$) were calculated from the A4F-MALLS data, as well as the average number of branching points per macromolecule ($\overline{B}$) and the average number of glucosyl units in a linear chain per branching point ($\overline{DP}_w/\overline{B}$) as previously described (Rolland-Sabaté et al., 2007). $\overline{R}_{Gw(lin)}$, was deduced from the equation linking molar mass and the radius of gyration established for strictly linear amyloses (Rolland-Sabaté, Colonna, Mendez-Montealvo, & Planchot, 2008).

Supramolecular properties

Starch granule size

The determination of starch granule size distribution was performed using a Malvern Mastersizer 3000 at room temperature. A small amount of native starch was suspended in water, and an aliquot of that suspension was directly fed into the mixing cell to reach a 2% about obscuration level. Volume distribution (%) was determined using the Fraunhofer scatting theory, while considering opaque starch granules. The granule size corresponded to the average granule diameter.

Thermal properties.

Thermal properties were determined using same DSC apparatus using stainless steel sealed pans as per Amani et al. (2004), revised by Pérez et al., (2011) with slight

modifications. The sample pan (7-8 mg of starch and 40 µL of pure deionized water) and the empty reference pan were heated from 25 to 140 °C at a scanning rate of 10 °C min^{-1}, held for 2 min at 160 °C and cooled to 60 at 10 °C min^{-1}. The gelatinization enthalpy variation (ΔH), the onset temperature (Onset, T_o), Peak temperature (T_p) and Endset gelatinization temperature (T_c) of each sample were determined on each thermogram within the 55-90 °C range of the linear baseline. The analysis was performed in duplicate, and mean values were calculated.

Crystallinity

X-ray diffraction was performed on native starches after adjustment of the water content at 20%. The samples (20 mg) were then sealed between two tape foils to prevent any significant change in water content during the measurement. The diffraction diagrams were recorded using a BRUKER (Karlsruhe, Germany) D8 Discover spectrometer. The X-ray radiation Cu $K\alpha_1$ (λCu Kα= 1.5405 Å), produced in a sealed tube at 40 *k*V and 40 *m*A was selected and parallelized using a double Gobël mirror parallel optics system, and collimated to produce a 500 μm beam diameter. Diffraction diagrams were collected with a two-dimensional GADDS detector and recording time was set to 600 sec. The distance from the sample to the detector was 100 *m*m and with an angle of 25°(2 θ).

Relative crystallinity was determined after normalization of all recorded diagrams at the same integrated scattering between 2θ values of 3° and 30°. A and B-type recrystallized amyloses were used as crystalline standards after scaled subtraction of an experimental

amorphous curve, in order to obtain null intensity in the regions without diffraction peaks. Dry extruded potato starch was used as the amorphous standard. The degree of crystallinity of the structures was determined using the method initially developed for cellulose (Wakelin, 1959). The percentage of crystallinity was taken as the slope of the line $(I_{sample} - I_{amor})2\theta = f\ (I_{crys} - I_{amor})2\theta$ where I_{sample}, I_{amor} and I_{crys} are the diffracted intensity of the sample, the amorphous, and the crystalline standards, respectively.

Functional properties determinations

Paste clarity (PC)

The methodology suggested by Craig, Maningat, Seib, & Hoseney (1989) was used. A 1% db aqueous dispersion of starch was boiled at 97 °C (1000 m above sea level) with shaking thoroughly every 5 min for 30 min. Transmittance was measured after cooling to room temperature at 650 nm. Three repetitions per genotype were used.

Swelling power, solubility and dispersed volume fraction measurements.

Swelling power (SWE) and solubility (SOL) patterns (Mestres, Nago, Akissoë, & Matencio, 1997) 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 ℃ for 1 min, heating to 75 ℃ at 6 ℃ min^{-1} rate, holding at 75 ℃ 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 ℃ were

collected and weighted (Wsu and Wse, respectively) then dried at 100 ℃ for 24 h and 48 h respectively and weighted (Dsu and Dse, respectively). Three parameters were calculated: concentration of soluble material in the supernatant (SOL), the SWE and the volume fraction of the dispersed phase (Φ).

Solubility (%db) = 100 * Dsu /0.42

Swelling Power = (Wse – Dse)/Dse

(Φ) = (27.86 – (Wsu – Dsu))/27.86

where 27.86 is calculated as total volume (cm^3) of the paste including the volume of water ($27.58cm^{-3}$) and the estimated volume of the starch (0.42/1.5) based on a starch specific density of 1.5 $g.cm^{-3}$. Three repetitions per genotype were used.

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 ℃ for 1 min, he ating from 50 ℃ to 90 ℃ at 6 ℃ min^{-1}, holding at 90 ℃ for 5 min, and then cooling down to 50℃ at 6℃ min^{-1} with continuous stirring at 160 rpm. Six parameters were measured: pasting temperature (PT), peak viscosity (PV), hot paste viscosity at the end of the plateau at 90 ℃ (HPV); cool paste viscosity at 50 ℃ (CPV); minimum viscos ity (MV) and final viscosity (FV). With these values, three additional parameters were calculated: breakdown (BD), estimated as PV-HPV; setback (SB), estimated as CPV-PV; and consistency (CS),

estimated as CPV-HPV.

Cold storage behavior

Gel preparation

Pastes were prepared and analyzed in RVA (5% db, w/w, containing 0.1% sodium azide) from 35 ℃, increasing temperatures at a 6 ° C min^{-1}rate to 93 ℃ (the boiling temperature of water at Cali, Colombia, is 97 ℃) a nd maintained during 2 min.

Syneresis after refrigeration

Ten centrifuge tubes were filled with approximately 6 g of gel (WG) and stored at 4 °C. The study lasted for five weeks. Every 7 days, two tubes were taken out of the refrigerator and held at room temperature during 1 hour. Tubes were centrifuged at 5000 rpm for 10 min and supernatant was separated and weighted (WS). Syneresis was calculated as WS/WG*100.

Syneresis after freezing

Ten centrifuge tubes were filled with approximately 6 g of gel (WG) and stored at -20 °C. The study lasted for five weeks. Every 7 days, two tubes were taken out of the freezer and held during 1.5 h in a water bath (30 °C). Samples were then centrifuged (5000 rpm for 10 min) and then supernatant was separated and weighted (WS). Syneresis was calculated using the following formula: WS/WG*100.

Syneresis after consecutive freeze/thaw cycles.

Ten centrifuge tubes were filled with approximately 6 g of gel (WG) and stored at -20 °C. The study lasted for five weeks. Every 7 days, tubes were taken out of the freezer and held during 1.5 h in a water bath (30 °C). Two random tubes were then centrifuged (5000 rpm for 10 min) and then supernatant was separated and weighted (WS). Syneresis was calculated using the following formula: WS/WG*100. The remaining tubes were frozen again for another freeze/thaw cycle. The last two tubes to be analyzed had undergone five freeze/thaw cycles (Baker & Rayas-Duarte, 1998; Zheng & Sosulski, 1998).

Evaluation of amylopectin retrogradation during cold storage and freezing by DSC measurements

Gels were prepared in capsules by putting starch samples (≈ 4mg dry matter) with ≈ 12 µL distilled water and hermetically sealing stainless-steel DSC pans then kept at room temperature for 2–3 h. Samples were scanned against an empty pan as the reference one, using a Perkin Elmer Pyris DSC 6 (Perkin-Elmer Co., Norwalk, CT) from 15 to 120 ℃ at a scanning rate of 10 ℃ min $^{-1}$.Each DSC endotherm was characterized by the T_o, T_p, T_c temperatures and gelatinization enthalpy (ΔH_0).

Retrogradation during one week refrigerated storage at 4 ℃: The methodology described by McPherson & Jane, 1999 was used. Three capsules per starch sample were prepared as previously mentioned. Capsules were then stored at 4 ℃ and

retrogradation quantified after 7 days. - 20 ℃ and retrogradation enthalpy change (ΔH7) and the T_o,T_p and T_c temperatures were recorded. Percent of retrogradation were calculated as retrograded starch enthalpy (ΔH7)/ starch gelatinization enthalpy (ΔH0). Enthalpy (ΔH) and temperatures were determined in triplicate and averaged.

Retrogradation after five freeze/thaw cycles at -20 °C: Nine capsules per starch sample were prepared as previously mentioned. After scanning, the pans were frozen at –20 ℃, after 22 h the samples were thawed for 1.5 hour in water bath (30 °C), and remaining capsules refrozen for 22 h (Baker & Rayas-Duarte, 1998). Thermal properties of the retrograded gels were analyzed between 15 ℃ and 120 ℃ at a rate of 10 ℃ min^{-1}after 1; 3; and 5 freeze-thaw cycles (three capsules per cycle, enthalpy (ΔH) as temperatures were determined as the average of the three replications). The enthalpy value (ΔHn) required to melt the retrograded starch was expressed as a percentage of (ΔH_0) required to gelatinize the starch sample.

Results

The continuous efforts to identify spontaneous mutations that affect starch functional properties in self-pollinated genotypes allowed for the identification of new cassava sources of amylose-free starches. A varying number of self-pollinated genotypes were derived from the same original mother genotype (**MG**) to generate S_1 families. Size of each S_1 family depends on the amount of flowers produced by the MG. It has been demonstrated that amylose-free starch in cassava (as in other crops) depends on a single recessive trait (Aiemnaka et al., 2012). Therefore, it is expected that 25% of plants in an S_1 family derived from a progenitor heterozygous for the waxy starch trait will produce amylose-free starch.

Five new sources of amylose-free starch (MG21, MG31, MG41, MG42, and MG44) were identified from their respective S_1 segregating families through the simple and reliable test spraying an iodine solution on the parenchyma of the roots (Table 1). These new sources are unrelated to each other. In the respective S_1 families from MG21 and MG44 one genotype with waxy starch were identified a CL21-5 and CL44-2, respectively, where CL stands for cassava line. The first number identifying the different lines, relates to the specific MG it was derived from. The number following a dash refers to the particular individual within the family that was found to produce amylose-free starch. Three genotypes (CL41-1, CL41-6, and CL41-7) with waxy starch were identified in the S_1 family derived from MG41. Two genotypes in the S_1 family from MG42 produced amylose free starch (CL42-3, and CL42-6). Similarly, two genotypes derived

from MG 31 (genotypes CL31-24, and CL31-32) produced waxy starch. This last family had up to 35 genotypes but only two of them were detected to produce amylose-free starch. This low ratio (2/35) is considerably lower than the expected segregations for a recessive trait from heterozygous progenitors (25%). The reason for this is that not all genotypes from these families were vigorous enough to produce roots due to inbreeding depression and other reasons (e.g. diseases).

In two cases, self-pollinations were made from S_1 plants producing amylose-free starch (CL21-5 and AM 206-5). Amylose-free cassava was first identified in AM 206-5 (Ceballos et al., 2007). Two S_2 genotypes (S_2 stands for second consecutive self-pollinated generation) were derived from CL21-5 (CL21-5-3, and CL21-5-5) and one from AM 206-5 (AM 206-5-6). These segregating materials were produced and analyzed to determine whether functional properties in related genotypes are shared or not.

Amylose content, iodine complexation and macromolecular characteristics

Amylose contents from the new waxy starch sources, the original source (reported by Ceballos et al. 2007), and from another starch mutation (5G 160-13, reported by Ceballos et al., 2008), with a small granule size and high levels of amylose (30.2%) are presented in Table 1. As a reference amylose contents from four wild type commercial starches are also presented and exhibit an average amylose content by DSC of 19.9%. The absence of amylose was determined initially by the iodine test and confirmed by

DSC and IBC measurements (Table 1). For non-waxy starches, amylose contents were quantified by the colorimetric approach, DSC and IBC. The average amylose contents obtained for the four wild type genotypes are 19.9, 19.9, and 17.2% respectively, while those for the high-amylose clone amylose contents were 30.2, 30.2, and 27.5%, respectively. These results agree with those reported by other authors (Gomand et al., 2010a; Gomand, Lamberts, Visser, & Delcour, 2010b; Rolland-Sabaté et al., 2012; 2013; Sánchez et al., 2009).

Table 1 also presents information regarding $\lambda_{max.}$ The absorption curves at different wavelengths are shown in Figure 1. Average λ_{max} for waxy starches was 540nm, whereas for the commercial wild types it was 591nm, and the high amylose 607nm. These differences are well illustrated in Figure 1. Curves for wild type starches are closer to each other than those from waxy starches. There is a clear relationship between amylose contents and absorbance. The increase in λ_{max} is indicative of longer α-(1,4) linear chains for an homogeneous media containing one type of macromolecule, i.e. it is related to the degree of polymerization between two α-(1,6) links (John, Schmidt, & Kneifel, 1983). The lowest λ_{max} observed from the strictly amylose-free cassava starches would then account for the shortest linear chain lengths that interact with iodine. However, the differences among waxy starches illustrated in Figure 1 as well as their small range of variation in λmax (from 536 to 544nm, Table 1) would suggest slight variations in linear chain lengths.

The macromolecular features of the cassava starch samples are analyzed by an A4F-MALLS set up with good solubilization (>94%) and elution recoveries (>94%), excepted for CL21-5-5, CL31-32 and AM206-5-6 for which the solubilization recoveries were around 85%. For all waxy starches, the elugrams (Figure 2a) display only one peak eluting at ~ 14-15 ml in line with the elution volume of the amylopectin population previously reported (Rolland-Sabaté, 2007, 2012, 2013). This confirms that the samples contain amylopectin only, and show slight differences in terms of molecular size among the waxy amylopectins. Moreover, the molar mass distributions obtained for the waxy cassava amylopectins are very similar to each other, even if small differences in absolute values are found.

Amylopectins from waxy cassava starches display $\overline{M}_w$ and $\overline{R}_G$ between 271×10^6 g mol^{-1} and 551×10^6 g mol^{-1} and 229 and 298 nm, for CL21-5 and CL21-5-3, respectively (Table 2). Their dispersities, $\overline{M}_w$ / $\overline{M}_n$, were low and ranged between 1.19 and 1.45 (data not presented). These values are consistent with those reported in the literature for transgenic and natural waxy cassava starches (Rolland-Sabaté et al., 2012; 2013) and for normal cassava and yam starches (Pérez et al., 2011; Rolland-Sabaté et al., 2003; 2007; 2012; Tetchi et al., 2007). Moreover $\overline{M}_w$ were generally smaller or in the same range than those reported for waxy maize amylopectins and higher than the ones reported for waxy potato amylopectins (Pérez et al., 2011; Rolland-Sabaté et al., 2003; 2007; 2012 Tetchi et al., 2007; Wahlund et al., 2011; Roger et al., 2011; Bello-Perez, 1998; Radosta et al., 2001; Aberle et al., 1994; Hanselman et al., 1996; Perez-Rea et

al., 2015). Amylopectins from normal cassava exhibit $\overline{M}_w$, $\overline{R}_G$ and $\overline{M}_w / \overline{M}_n$ in the same range whereas amylopectin from amylose rich cassava shows lower $\overline{M}_w$ and $\overline{R}_G$ and a higher dispersity. Among amylopectins from waxy cassava CL21-5-3, CL41-1 and CL42-3 are the largest $\overline{M}_w$, whereas CL21-5, CL31-32 and CL41-7 the smallest (Table 2) and no evident trend is observed depending on the source: in a same family one can found $\overline{M}_w$ values from 271×10^6 and 551×10^6 g mol^{-1} (MG21) or 366×10^6 and 516×10^6 g mol^{-1} (MG 41).

By plotting the radius of gyration versus molar mass for each fraction one can obtain structural data from the exponent ν_G in the equation: $R_G = K_G M^{\nu G}$. The ν_G parameter reflects the global conformation of macromolecules in solution. 0.33, 0.50, 0.60 and 1.00, are the theoretical values calculated for a sphere, a random coil in a θ solvent, a random coil in a good solvent or a rod conformation, respectively.. Amylopectins from waxy cassava show νG values between 0.38 and 0.40 that corresponds to a conformation between the sphere and the random coil, characteristic of branched polymers (Burchard, 1999; Burchard, 1983; Ioan et al., 1999, 2000; Hanselman et al., 1995; Galinsky et al., 1995) (Table 2). These values are consistent with previous data reported for waxy cassava, normal cassava and yam starches (Rolland-Sabaté et al., 2007; 2012, 2013; Tetchi et al., 2007) and corn, potato or other starches (Hanselman et al., 1996; Modig et al., 2006; Bello-Perez et al., 1998; Roger et al., 2001). No significant differences between the ν_G values of amylopectins from waxy cassava are observed.

Amylopectins from normal and amylose rich starches exhibit νG values in the same range as waxy varieties (0.35 to 0.40), and among them MCOL 1505 and MTAI-8 have the lowest ν_G suggesting they are more spherical.

Apparent particle density, d_{Gappw} informs also about the structure and conformation of a macromolecule. It is calculated on the basis of a uniform density in the particle considered as a homogeneous sphere. d_{Gappw} of waxy cassava amylopectins ranged from 6.0 g mol^{-1} nm^{-3} for AM 206-5 to 7.3 g mol^{-1} nm^{-3} for CL21-5 (Table 2). These densities agree with data reported by Rolland-Sabaté et al. (2012; 2013) for waxy and normal cassava starches. Moreover, these values fell in the same range as d_{Gappw} values obtained for normal cassava starches (6.1 to 6.5 g mol^{-1} nm^{-3}) but are lower than the molecular density obtained for amylose rich cassava amylopectin (12.3 g mol^{-1} nm^{-3}) as shown in Table 2. Among waxy amylopectins, the lowest d_{Gappw} are obtained for AM 206-5, CL21-5-5, CL21-5-3 and CL41-1 (from 6.0 to 6.1 g mol^{-1} nm^{-3}) and the highest for CL21-5, CL41-7 and CL31-32 (from 6.9 to 7.3 g mol^{-1} nm^{-3}). d_{Gappw} slightly decreased when the molar mass rose (Figure 2a). The apparent density distributions illustrated show that all the waxy cassava amylopectins exhibit very close densities and then probably very close branching structure. The Figure allows to point out the slight differences in density and to rank samples according to their molecular density. Then, by comparing $\overline{M}_w$ at the same d_{Gappw}, it could be concluded that CL21-5-3 and CL41-1 amylopectins could be denser (high molar mass), and probably more branched than CL21-5-5 and AM 206-5. Nevertheless, no clear relation is found between density and

waxy starch family.

Amylopectins branching features also are shown in Table 2. The gM value account for the contraction of a molecule due to branching, for a given molar mass it decreases when branching increases, and it decreases when molar mass increases (Yu & Rollings, 1987; Burchard, 1999, 1983). For waxy cassava amylopectins, the gM values are very similar and ranged from 0.021 for CL21-5-3 to 0.028 for CL21-5. The g_M data were analyzed using a polymer science theory, the ABC three-functional polycondensation model (ABC model) proposed by Burchard (1983). The amylopectin branching characteristics were approached using the theory of hyperbranched macromolecules which are considered to arise from a polycondensation reaction of monomers containing one, functional group called A and at least two other functional groups, called B and C. This statistical model consider that the branching points are not distributed in a random way within the macromolecule. Transposed to amylopectin, the reducing-end group establishes a focal-end group A, which due to the specificity of biosynthesis enzymes, can only react with the hydroxyl groups B and C in the C_4 and C_6 positions of the ring. A reaction with B is 25 times more frequent than with C. If both B and C groups react, a branching point is created. $\overline{B}$, the number of branching points of the macromolecule, could then be determined by the following relation (Burchard, 1983):

$$g_M = 4 \{[(1 + 2\overline{B})^{1/2}]/ [1 + (1 + 2\overline{B})^{1/2}]^2\}.$$

Yet, in the case of amylopectin, $\overline{B}$ does not correspond to the total number of branching points of the amylopectin molecule, but only to the anchorage points on the very long chains of amylopectin of B2 and B3 chains bearing clusters (i.e., A and B1 chains) (Rolland-Sabaté et al., 2007). The average chain length between two branching points, $\overline{DP_w}/\overline{B}$ determined using the ABC model is then overestimated for amylopectin (Rolland-Sabaté et al., 2007; Aberle, et al., 1994), with values ranging for waxy cassava amylopectins from 170.0 to 209.2 (Table 2). Therefore, the ABC model was modified to take account of the heterogeneity of amylopectin branching pattern (Rolland-Sabaté et al., 2007) using the most recent model proposed for the structure of cassava amylopectin (Laohaphatanaleart, Piyachomkwan, Sriroth, & Bertoft, 2010; Pérez & Bertoft, 2010), where each long B chain (B2+B3) carries on average 8.75 A and B1 chains (Rolland-Sabaté et al., 2013). The corrected average number of glucosyl units in a linear chain, $\overline{DP_w}/\overline{B}_{mC}$ is then $\overline{DP_w}/\overline{B}$ x 8.75 and ranged between 19.4-19.8 for CL41-7 and CL21-5, respectively and, 23.9-23.1 for AM 206-5 and CL21-5-5, respectively.

Based on the corrected branching degree (BD_{mC} =100 x $\overline{B}_{mC}/\overline{DP_w}$) CL41-7, CL21-5 and CL44-2 amylopectins appeared to be the most branched (5.0-5.1) whereas AM 206-5 and CL21-5-5 the least branched ones (4.2-4.3) (Table 2). These observations are consistent with literature data (Rolland-Sabaté et al., 2012; 2013) and with the d_{Gappw} values also presented in Table 2. Amylopectins from waxy varieties show generally a slightly more branched structure (average 4.8 %) than amylopectins from normal

cassava varieties (average 4.3 %). The starch from the small granule mutation (5G160-13), on the other hand showed high branching values (7.0 %). Branching parameter distributions of all amylopectins from waxy cassava (data not shown) are very close, thus confirming their very similar branching structure, despite the slight differences observed in terms of average branching degree.

To conclude on the macromolecular characteristics, natural waxy cassava starches consist exclusively of amylopectin. These amylopectins have very similar but slightly different macromolecular features that are not linked to their family source. They have a molar mass and a branching degree generally higher than amylopectins from normal cassava.

Starch granule size, thermal properties and crystallinity

Figure 3 presents the frequency distribution for different granule sizes <7µm, 7-20 µm, 20-40 µm, and >40 µm). The small granule mutation 5G160-13, illustrated on the right side of the graph had, as reported previously (Ceballos et al., 2008), small sizes with an average of 6.5 µm (53.7% of granules <7µm, 45.9% from 7-20 µm, 0.4% from 20-40 µm). The remaining samples exhibit larger granule average sizes ranging from 14.3 µm for wild type starches to 15.9 µm for waxy samples. Waxy starches from CL42-3 and CL21-5 showed the largest average granule size (18.0 and 16.6 µm respectively). In CL42-3, 60.2% of granules ranged from 7-20 µm, 39.6% from 20-40 µm and 0.2% > 40 µm, whereas in CL21-5: 7.3% of granules were <7 µm, 64.5% ranged 7-20 µm, and

28.3% from 20-40 µm.

Thermal properties of the different starches are presented in Table 3. Among waxy starches, the lowest enthalpy (ΔH) values (namely 17.3 and 17.4 J g^{-1}) correspond to CL31-32 and AM 206-5 starches, respectively. CL42-6 and CL21-5-3 starches have the highest ΔH values (namely 19.1 and 20.0 J g^{-1}, respectively). Wild type starches, however, presented a very large variation for ΔH values, ranging from 17.4 J g^{-1} (MPER 183) to 22.2 J g^{-1} (HMC-1). The starch from 5G160-13 showed the lowest ΔH value (13.3 J g^{-1}). ΔH reflects the organization of double helices of amylopectin and is known to be linked to the fusion of the crystalline structure and then it increases with crystallinity (Zobel, Young, & Rocca, 1988). In the literature, ΔH has been generally reported to be higher for waxy starches of maize and cassava (Zobel, et al., 1988; Gomand et al., 2010a; Rolland-Sabaté et al., 2012), except for transgenic cassava clones (Rolland-Sabaté et al., 2013). Here ΔH values from waxy starch samples tend to be lower than those obtained for normal cassava starches and there was no obvious relationship with crystallinity.

Average T_p values for waxy cassava starches was higher than that for normal ones (66.3 vs 62.1℃, Table 3). CL21-5-5 was the only waxy sample with T_p value comparable to normal starches. These data are consistent with the literature which reports higher T_p values for waxy starches of maize and cassava (Gomand et al., 2010a; Rolland-Sabaté et al., 2012; 2013). This could be related to the larger granule

size observed for waxy cassava starches or to less crystalline defects, as these latter are known to decrease the melting temperature of crystallites (Jane, Wong, & McPherson, 1997; Jane et al., 1999). Waxy cassava starches have generally a broader gelatinization peak (Table 3) than normal ones, except MPER 183. Yet, CL21-5-5, and CL31-24 exhibit a lower ΔT (namely 9.8 and 10.1 ℃). ΔT is linked to the crystalline organization and stability among the granules (Zobel, et al., 1988; Garcia, Colonna, Bouchet, & Gallant, 1997). Then, CL21-5-5, CL21-5-3 and CL31-24 probably have a more homogeneous crystallite size than the other waxy cassava starches, whereas CL41-7 (ΔT 14.7 ℃) would have the most heterogenous crystal lites sizes.

Differences in the averages of waxy versus wild type starches were all statistically significant at the 1% probability level. Except for CL21-5-5, amylose-free starches require a higher temperature for the initiation of the gelatinization. Except for MPER 183, most wild type starches require a lower energy to gelatinize than amylose-free starches. Similar trends were observed for T_p and T_c. However, ΔH was lower for waxy starches (18.2 J g^{-1}) than for wild types (19.8 J g^{-1}).

The crystallinity of wild type cassava starches was 35% for all samples (Table 3). On the other hand a broader diversity was observed for waxy starches ranging from 30% (CL41-7, CL41-1, CL42-3 and CL31-24) to 40% (AM 206-5). Taking account that the higher the water content in starch samples the higher the crystallinity is, one can conclude that CL41-7, CL41-1, CL42-3 have a lower crystallinity than the others. These

crystallinity values for wild type as well fell in the range of values reported generally for wild type cassava starches (38, 40 and 33-35% by Zobel et al. (1988), Gomand et al. (2010a) and Rolland-Sabaté et al. (2012; 2013), respectively). On the other hand, the crystallinity in most waxy starches was lower than previously reported for other amylose-free cassava starches (49% by Gomand et al., (2010a)). This could be partly due to a lower amount of water in the sample, as the crystallinity value is highly impacted by the amount of water in the sample. The average crystallinity value of 34% for waxy starches is in line with the previously reported crystallinity for waxy cassava transgenic clones (Rolland-Sabaté et al., 2013).

All waxy cassava starches exhibit a mixture of A- and B-type crystallites with a majority of A type (85-95%), in agreement with literature data (Gomand et al., 2010a; Rolland-Sabaté et al., 2012; 2013). CL42-6 exhibits the lowest amount of A-type crystals (85%) whereas CL31-34, CL31-32, CL21-5-5 and AM206 5-6 have the highest amount of A-type crystals (95%). This higher amount of A-type crystals observed in some of these samples may be due to a lower water content (based on data from CL31-24 with 17.6% moisture content).

Functional properties

Table 4 provides information regarding paste clarity (PC) and other related characteristics. Gels from amylose-free starch were considerably clearer (average PC 63.5%) than those from normal cassava (average PC 49.9 %) and even more in relation

to gels from 5G 160-13 (17.5%). The PC of the new sources ranged from 57.1 to 69.6% in CL41-6 and AM 206-5-6, respectively. Amylose-free starch results from a mutation in a single locus (*GBSS*) in different plant species (Hannah, 2000; Preiss, 2004; Aiemnaka et al., 2012). It is clear that gels from amylose-free starch have increased PC in cassava as well as in other crops (Sánchez et al., 2010). There is not enough evidence that the particular mutation in the GBSS locus present in the different sources of amylose-free starch result in gels with particularly high clarity. For example PC in the three genotypes from family CL41 ranged from 57.1 to 64.1%. Similarly, those from CL42 ranged from 57.6 to 63.2%. The two genotypes from family CL31, however, had relatively high paste clarity values (66.3 and 67.3%), according to the average cassava starch clarity reported (44.5 ± 10.7%), based on the characterization of 3272 cassava landraces (Sánchez et al., 2009). The number of samples from each source is too limited to draw definitive conclusions.

Solubility was higher in waxy starches compared with wild types. The highest SOL, however, was observed for 5G160-13 (24.2 %). Waxy starches had SOL ranging from 8.4 (CL41-6 and CL42-3) to 13.8% (AM 206-5-6), with an average of 11.1%. Average SOL of starches from commercial clones was 7.3%. As was the case for PC, considerable variation could be observed among clones derived from the same MG. This is the case of family CL41 with SOL ranging from 8.4 and 12.8%. The lowest SWE was observed in the starch from 5G160-13 (33 g g^{-1}), followed by the starches from commercial clones (average of 41.6 g g^{-1}) and the different waxy starches (average of

62.3 g g^{-1} and ranging from 54.7 to 67.2 g g^{-1}). A similar trend was observed for the volume fraction of the dispersed phase (Φ) which was lowest for 5G160-13 (0.15), intermediate in starches from commercial clones (average = 0.40) and highest in waxy starches (average = 0.57). Sánchez et al. (2010) also reported higher Φ for waxy compared with wild type cassava starches. The paste clarity for these cassava starches is related to the swelling of starch granules: the more the swelling the clearer the gel will be, as was already observed by Tetchi et al. (2007) for other sources of starches (yam,cocoyam, sweet potato and ginger). The starch from MTAI 8 exhibited the lowest SWE and PC among the different starches (except for the small granule mutation).

Table 5 provides a summary of the results from the viscoanalyzer. PT was higher in waxy starches compared with normal cassava: 68.5 vs 64.2 °C (Table 5). There was considerable variation for PT in waxy starches (ranging from 65.6 to 71.7 °C) but not so much in normal starches (64.0-65.0 °C). PV was also higher in waxy starches than in normal cassava (1149 vs. 993 cP). In waxy starches, it ranged from 1079 to 1285 cP, whereas in normal cassava it varied from 876 to 1105 cP. HPV was also higher in waxy starches (561 P) than in normal cassava (503 cP). This is in line with the higher swelling power and volume fraction of the dispersed phase observed in waxy starches. Average CPV was higher (716 cP) in wild type cassava starches compared with waxy starches (618 cP). The range of variation for this variable in normal starches was wider (538 to 816 cP) than in waxy starches (535 to 746 cP). And, the lowest CPV in normal starches found in MTAI 8 (538 cP) was below the average of waxy

starches. Considerable variation could be observed among related genotypes.

The starch from 5G 160-13 had a very low viscosity at the 5% suspensions used in this study (Ceballos et al., 2008). This behavior is linked to the small granule size of this sample (Table 3, Rolland-Sabate et al., 2012).

MV was higher in waxy starches with an average of 532 cP compared with normal starches (487 cP). The range of variation of MV among normal starches was from 388 (MTAI8) up to 577 cP (HMC1), whereas in waxy starches the lowest observed value was in CL21-5 (466 cP) and the highest in CL41-6 (677 cP). Variation in minimum viscosities do not seem to be associated with particular sources of waxy starches as considerable variation was observed in related genotypes (e.g. those derived from MG41).

As expected, average for FV was higher in starches with amylose (799 cP) than in waxy starches (623 cP). FV values varied widely for commercial cassava starches (from 576 to 961 cP for MTAI 8 and HMC 1, respectively), whereas waxy starches showed a narrower range of variation (544-746 cP). It should be noticed as well that the low viscosity values (PV, HPV, CPV, MV and FV) observed for MTAI 8 starch are in line with the lower swelling power of its granule.

Waxy starches had a higher average for BD (588 cP) and a wider range of variation

(489 to 714 cP) than wild type starches (average of 489 cP and ranging from 455 to 556 cP).

Setback was considerably larger in magnitude for waxy starches (average of -531 cP) than in normal starches (-277 cP). As for the other variables from the amylograms, variation for SB does not seem to be specifically associated with particular sources of waxy starch. CS was considerably higher in commercial starches (average 212 cP) than in waxy starches 56 cP). It also varied widely in the wild type starches (from 117 to 267 cP) but was relatively uniform in waxy starches (from 25 to 71 cP). The low CS values observed for waxy starches was expected because of the lack of amylose. The average cassava starch consistency reported in the literature is about 158 ± 59cP, based on the characterization of 3272 cassava landraces (Sánchez et al., 2009).

Gel stability in refrigeration, freezing and freeze/thaw cycles in tubes is presented in Table 6. No gel from waxy starch showed syneresis after five weeks of storage at 4 ℃, indicating a high tolerance to refrigerated storage. On the other hand, gels from two commercial starches (MPER 183 and MTAI 8) showed some syneresis starting at two weeks of storage. The other two wild types had no syneresis. A similar pattern was observed after freezing at -20 ℃, although syneres is in the two commercial clones was accentuated and always higher in MTAI 8 than in MPER 183. Syneresis in freeze/thaw cycles (in tubes) was absent in every sample except for HMC1, which showed some syneresis after the third cycle.

The starch from the small granule mutation showed a contrasting reaction to storage at low temperatures (Table 6). After one week of storage at 4 ℃ or -20 ℃, the gel presented syneresis values of 20% and 43%, respectively. Similarly, reaction to freeze/thaw cycles showed syneresis after the first cycle (33%). The high proportion of amylose in this starch is likely to be the reason for the high syneresis values shown by this starch.

There was no measurable retrogradation by DSC after storage at low temperatures (4 ℃ for one week, following the methodology suggeste d by McPherson & Jane, 1999) or freeze/thaw cycles at – 20 ℃ (Baker & Rayas-Duarte , 1998). These methods could not detect retrogradation in the gels from any of cassava starches studied.

Discussion

Several new sources of spontaneous mutations of the granule bound starch synthase – GBSS - gene (resulting in the production of amylose-free starch) in cassava have been identified. It is not clear at this point if the different genotypes where the mutation was identified carry the same mutation (e.g. they trace back to the same mutation event which was then transmitted by sexual reproduction to different segregating progenies) or else, if these sources of waxy starch are the result of new and independent mutation events. Regardless of the independent or common origin of the mutation the relatively high frequency would indicate that it has no negative impact on the performance and fitness of the accessions carrying it. It has to be acknowledged, however, that the

mutation was present in a heterozygous condition and, therefore, the accessions carrying it did not produce amylose-free starch themselves. There are ongoing efforts to sequence the DNA these new sources of waxy starch in cassava.

The different amylose-free starches shared the expected characteristics (e.g. higher paste temperature, peak viscosity and paste clarity) compared with wild type starches. But there was considerable variation among genotypes from the same MG. For example, PT ranged from 65.6 to 71.5 ℃ for CL21-5 and CL21-5-3, respectively. PV ranged from 1079 and 1285 cP for CL31-24 and CL31-32, respectively. Paste clarity was 60.5 % in CL21-5-3 and 68.3% in CL21-5-5. The variation observed among starches from genetically related clones would indicate that segregation in loci different from GBSS also influence considerably the functional properties of the resulting starches. This, in turn, would point out the potential of further improving starch properties by additional breeding work.

As expected, the new amylose-free cassava starches showed the known properties of waxy starches from different crops, including cassava (Sánchez et al., 2010; Gomand et al., 2010a;b; Jobling, 2004). A remarkable feature is their excellent performance after freeze-thaw and storage under refrigerated conditions (Table 6) in agreement with a previous report by Sánchez et al., 2010. In addition to data from Table 6, DSC assessment of stability of refrigerated gels and after freeze/thaw cycles could not detect any retrogradation or syneresis in waxy starch gels. In the current study syneresis

evaluation (in test tubes) was more accurate differentiating gel stability than retrogradation measured by DSC. As a matter of fact, there was no retrogradation measured by DSC (enthalpy = 0), even for wild type (amylose-containing) cassava starch. It should be pointed out, however, that the equipment used for the retrogradation analysis (DSC 6 with a single oven) may not be precise enough for detecting small variation for these parameters.

Viscosity peak was higher in all waxy cassava starches (Table 5) and their gels showed improved clarity (Table 4). These results agree with those reported in the literature on spontaneous and transgenic waxy cassava starches (Sánchez et al., 2010; Zhao et al., 2011; Gomand et al., 2010a; b). The best clarity measured in amylose-free starches was 70%.This value is considerably better than those for wild type starches used in this study (average of about 50%) and those for a large analysis in more than 4000 cassava starch samples (Sánchez et al., 2009) where the average was 45%. However, this value is still considerably lower than those for potato starches whose excellent paste clarity has been linked to phosphate monoester contents (Craig et al, 1989, Kasemsuwan & Jane 1996).

No association between enthalpy and granule size was observed in amylose-free and wild type cassava starches. Similarly, it was expected that higher crystallinity would be associated with higher enthalpy, but this association was not observed in the different waxy cassava starch samples analyzed herein.

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Table 1. Wavelength at maximum absorption (λ_{max}) of the iodine complexes and amylose content of wild type and mutant cassava starches[a].

Sample name	λ_{max} (nm)	Amylose content (%)		
		Colorimetric[b]	DSC[c]	IBC[d]
CL21-5	544	N.D.	0.0 (0.0)	0.0 (0.0)
CL21-5-3	539	N.D.	0.0 (0.0)	0.0 (0.0)
CL21-5-5	542	N.D.	0.0 (0.0)	0.0 (0.0)
CL41-1	540	N.D.	0.0 (0.0)	0.0 (0.0)
CL41-6	539	N.D.	0.0 (0.0)	0.0 (0.0)
CL41-7	541	N.D.	0.0 (0.0)	0.0 (0.0)
CL42-3	540	N.D.	0.0 (0.0)	0.0 (0.0)
CL42-6	540	N.D.	0.0 (0.0)	0.0 (0.0)
CL44-2	540	N.D.	0.0 (0.0)	0.0 (0.0)
CL31-24	539	N.D.	0.0 (0.0)	0.0 (0.0)
CL31-32	543	N.D.	0.0 (0.0)	0.0 (0.0)
AM 206-5	536	N.D.	0.0 (0.0)	0.0 (0.0)
AM 206-5-6	542	N.D.	0.0 (0.0)	0.0 (0.0)
Average	**540.2**	N.D.	0.0 (0.0)	0.0 (0.0)
MCOL 1505	592	20.3 (0.28)	20.3 (0.28)	17.7 (0.1)
MPER 183	590	19.6 (0.28)	19.7 (0.28)	ND
MTAI-8	592	19.5 (0.31)	19.5 (0.31)	17.0 (0.1)
HMC-1	592	20.1 (0.36)	20.1 (0.36)	16.8 (0.6)
Average	**591.3**	**19.9**	**19.9**	**17.2**
5G 160-13	607	30.2 (1.96)	30.2 (1.96)	27.5 (0.8)

[a] Results were obtained from two repetitions. Standard deviations are given within parenthesis and are based on two independent measurements.

ND: Not determined.

[b] Values obtained from colorimetric measurements.

[c] Values obtained from DSC measurements.

[d] Values obtained from IBC measurements.

Table 2. Macromolecular characteristics of cassava amylopectins determined by A4F-MALLS: weight-average molar mass ($\overline{M}_w$), z-average radius of gyration ($\overline{R}_{Gz}$), molecular density (d_{Gappw}), hydrodynamic coefficient (ν_G) and branching features[a].

Sample	Macromolecular characteristics							
	Global parameters[b]				Branching features[d]			
	$\overline{M}_w$x 10^{-6} (g mol^{-1})	$\overline{R}_{Gz}$ (nm)	d_{Gappw}[c] (g mol^{-1} nm^{-3})	ν_G	g_M	ABC model[e]	Modified ABC model[f]	
						$\overline{DP}_w/\overline{B}$	$\overline{DP}_w/\overline{B}_{mC}$	BD_{mC} (%)
CL21-5	271	229	7.3	0.40	0.028	173.5	19.8	5.0
CL21-5-3	551	298	6.1	0.38	0.021	189.7	21.7	4.6
CL21-5-5	404	269	6.1	0.39	0.025	202.3	23.1	4.3
CL41-1	516	286	6.1	ND	0.022	191.2	21.9	4.6
CL41-6	456	279	6.5	0.39	0.022	181.2	20.7	4.8
CL41-7	366	251	7.1	0.39	0.024	170.0	19.4	5.1
CL42-3	486	278	6.3	ND	0.022	186.3	21.3	4.7
CL42-6	415	272	6.6	0.39	0.023	180.5	20.6	4.8
CL44-2	421	266	6.7	0.38	0.023	176.0	20.1	5.0
CL31-24	393	264	6.5	0.39	0.024	187.5	21.4	4.7
CL31-32	334	246	6.9	0.40	0.026	180.5	20.6	4.8
AM 206-5[g]	408	277	6.0	0.38	0.025	209.2	23.9	4.2
AM 206-5-6	415	272	6.6	0.39	0.023	180.5	20.6	4.8
Average	418	268	6.5	0.39	0.024	185.3	21.2	4.8
MCOL 1505[g]	418	276	6.1	0.35	0.025	202.4	23.1	4.3
MTAI-8[g]	370	263	6.1	0.37	0.026	205.8	23.5	4.3
HMC-1[g]	306	247	6.5	0.40	0.028	198.3	22.7	4.4
Average	365	262	6.2	0.37	0.026	202.2	23.1	4.3
5G 160-13[g]	50	123	12.3	0.40	0.054	125.0	14.3	7.0

[a] Standard deviations are around 5%.

[b] These values are taken over the whole amylopectin peak.

[c] $d_{Gappw} = \overline{M}_w / (4\pi/3)\ \overline{R}_{Gw}^3$

[d] Branching parameters: Average branching parameter ($g_M = \overline{R}^2_{Gw(br)}/\overline{R}^2_{Gw(lin)}$, where $\overline{R}_{Gw(br)}$ is the radius of gyration of the branched molecule and $\overline{R}_{Gw(lin)}$, the radius of gyration of its linear equivalent at the same molar mass. Average number of glucosyl units in a linear chain per branching point ($\overline{DP}_w/\overline{B}$) and branching degree ($BD=100\,\overline{B}\,/\overline{DP}_w$) determined from:

[e] The simple ABC model (Burchard (1983), using the equation: $g_M = 4\{[(1+2\overline{B})^{1/2}]/[1+(1+2\overline{B})^{1/2}]^2\}$;

[f] Modified ABC model for cassava amylopectin (Laohaphatanaleart et al., 2010; Pérez & Bertoft, 2010) where each long B chain carries on average 8.75 A and B1 chains ($\overline{DP}_w/\overline{B}_{mC} = 8.75 \times \overline{DP}_w/\overline{B}$ and $BD_{mC} = 100\,\overline{B}\,/\overline{DP}_{wmC}$).

[g] Values reported from Rolland-Sabaté et al., 2012.

Table 3. Thermal properties and crystallinity of native dry starches.

Source	Thermal properties[a]					Crystallinity[b]			
	T_o	T_p	T_c	ΔH	ΔT	Crystallinity	A-type	B-type	Water content
	(°C)	(°C)	(°C)	(J g^{-1})	(°C)	(%)	(%)	(%)	(%)
CL21-5	58.7	63.6	71.5	18.4	12.8	35	90	10	18.4
CL21-5-3	62.7	66.4	72.9	20.0	10.1	35	90	10	N.A.
CL21-5-5	58.0	61.4	67.8	17.9	9.8	35	95	5	N.A.
CL41-1	60.5	66.0	74.0	17.9	13.5	30	90	10	18.5
CL41-6	61.4	68.8	74.8	18.1	13.4	35	90	10	N.A.
CL41-7	59.5	64.2	74.2	18.8	14.7	30	90	10	19.3
CL42-3	62.0	67.9	73.7	17.8	11.6	30	90	10	18.9
CL42-6	59.9	66.3	73.0	19.1	13.1	35	85	15	N.A.
CL44-2	59.2	65.1	72.0	18.5	12.8	35	90	10	18.0
CL31-24	61.6	65.8	71.8	17.6	10.1	30	95	5	17.6
CL31-32	63.9	68.4	75.1	17.3	11.2	35	95	5	N.A.
AM 206-5	61.1	68.0	72.7	17.4	11.6	40	85	15	19.0
AM 206-5-6	61.1	69.9	75.3	17.6	14.2	35	95	5	N.A.
Average	**60.7**	**66.3**	**73.0**	**18.2**	**12.2**	**34**	**91**	**9**	**18.5**
MCOL 1505	58.4	62.2	68.4	19.8	10.1	35	85	15	18.0
MPER 183	58.4	62.8	73.2	17.4	14.8	N.A.	N.A.	N.A.	N.A.
MTAI-8	58.2	62.4	69.1	19.9	10.9	35	85	15	18.0
HMC-1	57.2	61.2	66.7	22.2	9.6	35	85	15	19.0
Average	**58.0**	**62.1**	**69.4**	**19.8**	**11.3**	**35**	**85**	**15**	**18.3**
5G 160-13	53.9	59.0	66.3	13.3	12.4	25	90	10	19.0
SE	0.79	1.45	0.43	0.85	0.71	N.A.	N.A.	N.A.	N.A.

[a] Values obtained from DSC: T_o: Onset gel temperature; T_p: Peak gel temperature; T_c: Conclusion temperature. Water content 80%. Results are based on two independent measurements allowing the analysis of variance from which the standard error of the means (SE) was obtained.

[b] Values obtained from X-Ray diffraction, experimental uncertainties were 5% for A type or B type content and 3% for crystallinity. Water content 20%

N.A.: Not available

Table 4. Paste clarity (PC), solubility (SOL), swelling power (SWE) and volume fraction of dispersed phase (Φ) of gels derived from normal and waxy cassava starches as well as the small granule mutation[a].

Source	Paste clarity (%)	Solubility (% db)	SWE ($g\ g^{-1}$)	Φ
Amylose-free starches				
CL21-5	66.0	13.4	61.1	0.57
CL21-5-3	60.5	11.5	60.2	0.55
CL21-5-5	68.3	12.0	67.2	0.55
CL41-1	63.3	11.0	62.0	0.60
CL41-6	57.1	8.4	62.0	0.53
CL41-7	64.1	12.8	59.4	0.56
CL42-3	63.2	8.4	67.2	0.62
CL42-6	57.6	9.3	61.4	0.57
CL44-2	60.1	12.2	63.4	0.57
CL31-24	66.3	12.5	62.1	0.58
CL31-32	67.3	10.7	64.7	0.60
AM 206-5	61.5	8.7	54.7	0.48
AM 206-5-6	69.6	13.8	64.6	0.62
Average	**63.5**	**11.1**	**62.3**	**0.57**
Wild type commercial starches				
MCOL 1505	51.1	6.9	44.8	0.43
MPER 183	51.0	7.3	40.3	0.37
MTAI-8	46.8	7.6	37.0	0.37
HMC-1	50.8	7.2	44.2	0.42
Average	**49.9**	**7.3**	**41.6**	**0.40**
Small granule starch				
5G 160-13	17.5	24.2	33.9	0.15
SE	0.90	0.87	1.13	0.02

[a]. Averages of three replications. Standard errors (SE) are also provided in the bottom line.

1

2 **Table 5.** Functional properties of different cassava starches analyzed with the rapid

3 viscoanalyzer.

Source	PT (°C)	PV	HPV	CPV	MV	FV	BD	SB	CS
		(cP)							
Amylose-free starches									
CL21-5	65.6	1119	489	560	466	578	630	-559	71
CL21-5-3	71.5	1226	657	702	627	731	569	-524	45
CL21-5-5	68.2	1146	598	669	571	694	548	-478	71
CL41-1	68.6	1133	532	586	501	614	601	-548	54
CL41-6	71.7	1230	721	746	677	746	509	-485	25
CL41-7	67.0	1088	524	581	498	604	565	-508	57
CL42-3	68.6	1125	524	590	502	613	601	-535	67
CL42-6	70.9	1178	689	746	633	695	489	-432	57
CL44-2	66.1	1120	511	566	486	600	609	-554	55
CL31-24	66.9	1285	571	626	546	651	714	-659	55
CL31-32	69.2	1079	496	564	472	590	583	-515	68
AM 206-5	67.3	1119	488	535	470	544	631	-585	47
AM 206-5-6	69.4	1093	501	563	476	594	592	-530	63
Average	**68.5**	**1149**	**561**	**618**	**532**	**635**	**588**	**-531**	**56.2**
Wild type commercial starches									
MCOL 1505	64.4	1011	546	796	517	873	465	-215	250
MPER 183	64.9	979	497	712	469	785	482	-267	215
MTAI-8	63.7	876	421	538	388	576	455	-338	117
HMC-1	63.8	1105	549	816	577	961	556	-289	267
Average	**64.2**	**993**	**503**	**716**	**487**	**799**	**489**	**-277**	**212**
Small granule starch									
5G 160-13	At 5% suspension the small granule starch does not develop viscosity								
SE	**0.2**	**21.6**	**12.0**	**14.8**	**11.8**	**20.8**	**21.3**	**21.9**	**13.7**

4 Results are based on two independent measurements allowing the analysis of variance from which the
5 standard error of the means (SE) was obtained.
6 **PT**: pasting temperature; **PV**: peak viscosity; **HPV**: hot paste viscosity at the end of the plateau at 90 ℃;
7 **CPV**: cool paste viscosity at 50 ℃; **MV**: minimum viscosity; **FV**: final viscosity; **BD**: breakdown; **SB**:
8 setback and **CS**: consistency.
9

Table 6. Gel stability (%) in storage at 4 ℃ and - 20 ℃ a nd freeze/thaw cycles at - 20 ℃ in different native cassava starches.

Refrigeration (storage at 4 °C)						
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
MCOL1505	0	0	0	0	0	0
MPER 183	0	0	2	2	2	2
MTAI 8	0	0	1	4	7	9
HMC-1	0	0	0	0	0	0
5G 160-13	0	20	20	19	22	n.a.
All waxy	0	0	0	0	0	0
Freezing (storage at - 20 °C)						
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
MCOL 1505	0	0	0	0	0	0
MPER 183	0	0	2	5	8	10
MTAI 8	0	0	8	11	15	22
HMC-1	0	0	0	0	0	0
5G 160-13	0	43	36	34	31	33
All waxy	0	0	0	0	0	0
Freeze / thaw cycles (freezing at - 20 °C)						
	Native	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
MCOL 1505	0	0	0	0	0	0
MPER 183	0	0	0	0	0	0
MTAI 8	0	0	0	0	0	0
HMC-1	0	0	0	11	14	15
5G 160-13	0	33	40	40	39	41
All waxy	0	0	0	0	0	0

Figure 1. Iodine absorption spectra of the iodine complexes (iodine solution at 2% KI, 0.2% I_2) and starch solutions at 0.1%

Figure 2. A4F-MALLS. (**A**) Apparent molecular densities (d_{Gappw}) of waxy cassava amylopectins. CL21-5 (purple), CL21-5-5 (orange), CL21-5-3 (red), CL41-7 (blue), CL41-6 (yellow), CL42-6 (clear green), CL31-24 (green), CL31-32 (brown) and AM206-5-6 (black). (**B**) Elugrams (normalized DRI signals) and molar mass distributions of waxy cassava amylopectins. CL21-5 (purple), CL21-5-3 (red), CL41-6 (yellow) and CL31-32 (brown).

Figure 3. Granule size distribution (%) of different types and sources of cassava starch. SG stands for the small granule mutation. Granule sizes for starches from CL21-5-3, CL21-5-5 and CL42-6 are not available. Average granule size for SG, normal and waxy starches were 6.5, 14.3 and 15.9 μm, respectively

List of symbols

$\overline{B}$: average number of branching points in the ABC three-functional polycondensation model.

$\overline{B}_{mC}$: average number of branching points obtained using theoretical amylopectin structures as proposed by Laohaphatanaleart et al. (2010).

BD: branching degree.

BD_{mC}: branching degree obtained using theoretical amylopectin structures as proposed by Laohaphatanaleart et al. (2010).

$\overline{CL}$: average chain length.

d_{Gappw}: apparent particle density calculated on the basis of a smeared uniform density in the particle using the following equation: $d_{Gappw} = \overline{M}_w / (4\pi/3)\ \overline{R}_{Gw}^3$.

Ø: average granule diameter.

DP: degree of polymerization

$\overline{DP}_w$: weight average degree of polymerization.

$\overline{DP}_w/\overline{B}$: average number of glucosyl units in a linear chain per branching point.

$\overline{DP}_w/\overline{B}_{mC}$: average number of glucosyl units in a linear chain per branching point obtained using theoretical amylopectin structures as proposed by Laohaphatanaleart et al. (2010).

ΔH: gelatinization enthalpy.

DSC: differential scanning calorimetry.

$g_{M:}$ average branching parameter.

IBC: iodine binding capacity.

K_G: constant.

λ_{max}: wavelength at maximum absorption of the iodine complexes with starch polymers.

M: molar mass of a fraction.

$\overline{M}_n$: number average molar mass.

$\overline{M}_w$: weight average molar mass.

$\overline{M}_w/\overline{M}_n$: dispersity index.

$\nu_{G,}$: hydrodynamic coefficient.

R_G: radius of gyration of a fraction.

$\overline{R}_{Gw}$: weight average radius of gyration.

$\overline{R}_{Gw(br)}$: weight average radius of gyration of the branched polymer.

$\overline{R}_{Gw(lin)}$: weight average radius of gyration for the corresponding linear polymer.

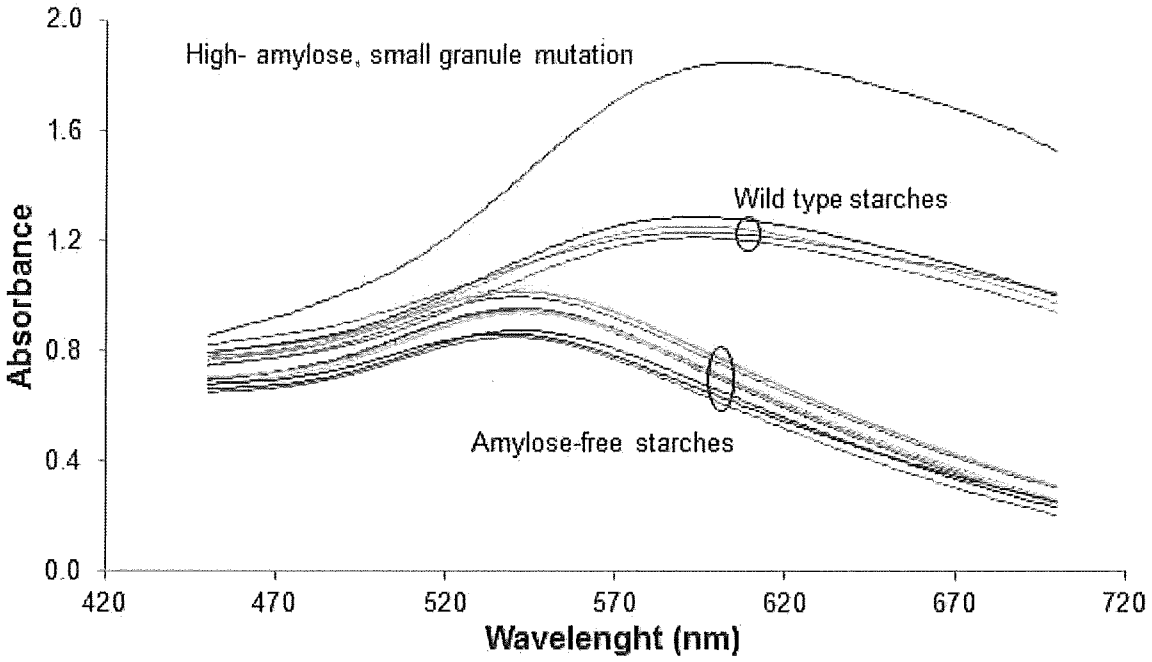
$\overline{R}_{Gz}$: z-average radius of gyration.

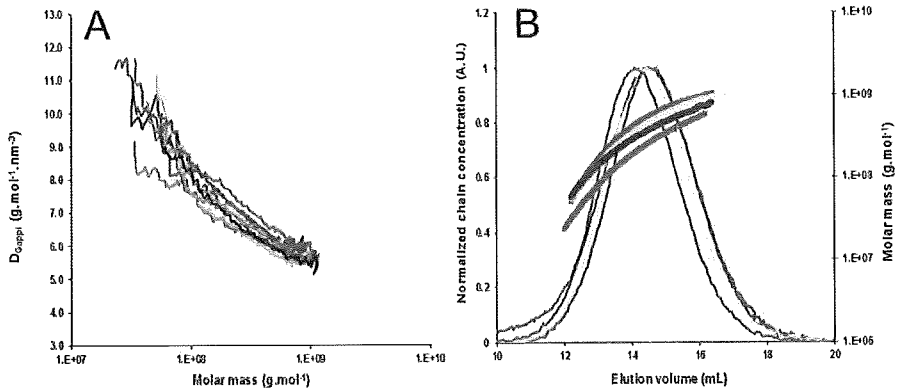
T_c: conclusion temperature.

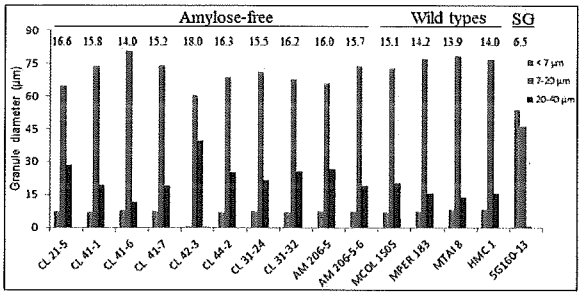
T_o: onset temperature gelatinization.

T_p: peak temperature gelatinization.

Φ = Volume fraction of dispersed phase


2.0
1.6
1.2
0.8
0.4
0.0
Absorbance
High- amylose, small granule mutation
Wild type starches
Amylose-free starches
420
470
520
570
620
670
720
Wavelenght (nm)




Amylose-free
Wild types
SG
16.6 15.8 14.0 15.2 18.0 16.3 15.5 16.2 16.0 15.7 15.1 14.2 13.9 14.0 6.5
Granule diameter (µm)
90
75
60
45
30
15
0
< 7 µm
7-20 µm
20-40 µm
CL 21-5
CL 41-1
CL 41-6
CL 41-7
CL 42-3
CL 44-2
CL 31-24
CL 31-32
AM 206-5
AM 206-5-6
MCOL 1505
MPER 183
MTAI 8
HMC 1
SG160-13

- Five new (non-transgenic) sources of amylose-free cassava discovered in CIAT.
- Functional and physical properties of these new waxy starches were characterized.
- Waxy starch gels were clearer and had higher solubility, swelling power and peak viscosity
- Excellent stability in refrigerated, frozen and freeze/thaw cycles from waxy starch gels.