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Improved cassava starch by antisense inhibition of granule-bound starch synthase I

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Abstract

Cassava is a poor man's crop which is mainly grown as a subsistence crop in many developing countries. Its commercial use was first as animal feed (also known as tapioca), but has shifted since the late sixties to a source of native starch. The availability of native starches, which on the one hand do not require substantial chemical derivatisation and on the other hand have improved properties, would make cassava also for small farmers a potentially attractive cash crop. Since breeding is difficult in this polyploid, vegetatively propagated, crop a transgenic approach would be ideal to improve certain characteristics. We have created a cassava genotype producing amylose-free starch by genetic modification. The absence of amylose increased the clarity and stability of gels made with the transgenic starch, without requiring treatment with environment-unfriendly chemicals such as epoxides (propylene oxide, ethylene oxide) and acetic anhydride, which are normally used to improve stability. The amylose-free starch showed no changes in particle size distribution, chain length distribution or phosphorous content when compared to amylose-containing starch, but the granule melting temperature was increased by almost 2 °C. Furthermore, the amylose-free cassava starch shows enhanced clarity and stability properties. These improved functionalities are desired in technical applications in paper and textile manufacturing, but also in the food industry for the production of sauces, dairy products and noodles.

Introduction

Manihot esculenta (Crantz) is a tuberous root crop grown in the sub-tropical regions of Africa, Asia and Latin America as a subsistence crop on which more than 500 million people rely (Thro et al. 1998). The tuberous roots have a dry matter content of 20% of which ~80% is starch. Yield of both tubers and starch can vary considerably and this can be attributed to the fact that it is grown by small scale farmers on poor soils, under adverse conditions (Thro et al. 1998). There are large differences in the use of cassava between the different regions of the world. In Africa it is mainly used for food production, whereas in Thailand half of the annual production of 20 million tons of roots is used in the Thai starch industry (Sriroth et al. 2001). With economic development, demand for starch for industrial purposes will increase in many developing countries. Although tropical countries have local sources of starch available such as cassava (but also sweet potato and yam), many of

them import expensive maize starch. It is expected that in many tropical countries the use of local sources of starch will shift from animal and human consumption to industrial purposes (Sriroth et al. 2001). This means that increasing emphasis will be placed on controlling the quantity and particularly the quality of the raw starch. Industry uses starch in paper, textile, medical and other applications. To this end, starch is modified in many different ways to obtain products which meet the criteria for their applications. If cassava starches with specific characteristics could be produced, this would give cassava starch a competitive advantage compared to other sources of starch. As a result, it could mean that small scale farmers would be able to grow cassava as a cash crop rather than a subsistence crop, and thus increase their income.

Cassava starch consists, as do other plant starches, of two glucan polymers: amylose and amylopectin. In cassava the amylose content ranges from 14 to 24% (Moorthy and Ramanujan 1986). Amylose is essentially a linear polymer of 100–1000 α(1–4) linked glucosyl residues with a molecular weight of $0.5–1 \times 10^6$. Amylopectin has a molecular weight of several millions and is much more branched (Hizukuri 1996). Many enzymes play a role in the biosynthesis of starch. The presence or absence of amylose is controlled by the action of one enzyme, granule-bound starch synthase I (GBSSI) (Ball et al. 1998; van de Wal et al. 1998; Denyer et al. 2001). This enzyme polymerises amylose from the donor substrate ADP–glucose and the acceptor substrates malto-oligosaccharides and/or amylopectin, inside the granular matrix, downstream of amylopectin. Inhibition of this enzyme has led to amylose-free starches in a variety of crop plants including maize, rice, wheat, and potato (Visser and Jacobsen 1993; Chakraborty et al. 2004); in cereals, these starches are usually referred to as *waxy*. It is known that amylose-free starches have different physicochemical properties compared to those containing amylose, and therefore they are used for the manufacturing of different products. For instance, amylose-free potato starch showed a higher granule melting temperature, less retrogradation (the alignment of amylose, leading to insoluble aggregates), and better adhesive properties than that containing amylose (Visser et al. 1997a, b). Moreover, the use of waxy wheat has been reported to extend the shelf-life of baked, refridgerated, and frozen wheat-based products (Chakraborty et al. 2004). Although retrogradation is mainly related to the presence of amylose, it is important to note that it is not only determined by the presence of amylose. It has been shown that waxy starches from different species (rice, maize, and barley) show different retrogradation behaviours, which appeared to be related to the chain-length distribution of the amylopectin side chains (Shi and Seib 1992).

Amylose-containing starches can be made less sensitive to retrogradation by chemical derivatisation, for instance by acetylation using acetic anhydride. Often, the use of such chemicals is considered unfriendly for the environment, and therefore amylose-free starch is regarded as a more attractive starting point for many applications. To this end, cassava plants producing an amylose-free starch are not available. If such plants could be made, a number of chemical modifications might become obsolete. This could result in a preferred raw material, with added value in different phases of production processes. Since cassava is an allotetraploid crop, which is vegetatively propagated, breeding is a difficult and time consuming process (Thro et al. 1998). Genetic modification of existing cultivars would thus seem a fast way of introducing such a trait in cassava. Since cassava is amenable to transformation (Li et al. 1996; Raemakers et al. 1996; Schöpke et al. 1996) and the gene encoding GBSSI from cassava is available (Salehuzzaman et al. 1993), we set out to produce amylose-free cassava plants by using antisense RNA inhibition of this gene. Here, we show that solutions and gels made with this amylose-free starch have an excellent stability and clarity, omitting the need for further chemical modification for food and industrial uses.

Materials and methods

Constructs

Standard molecular biological techniques were used in DNA manipulations. The constructs used were GBSS-as2 (Figure 1a) and GBSS-as7. Both plasmids contained the firefly *luciferase* gene as a reporter gene fused, between the CaMV 35S promoter and terminator (Guerineau and Mullineaux 1993), and the cassava *GBSSI* cDNA (partly) in

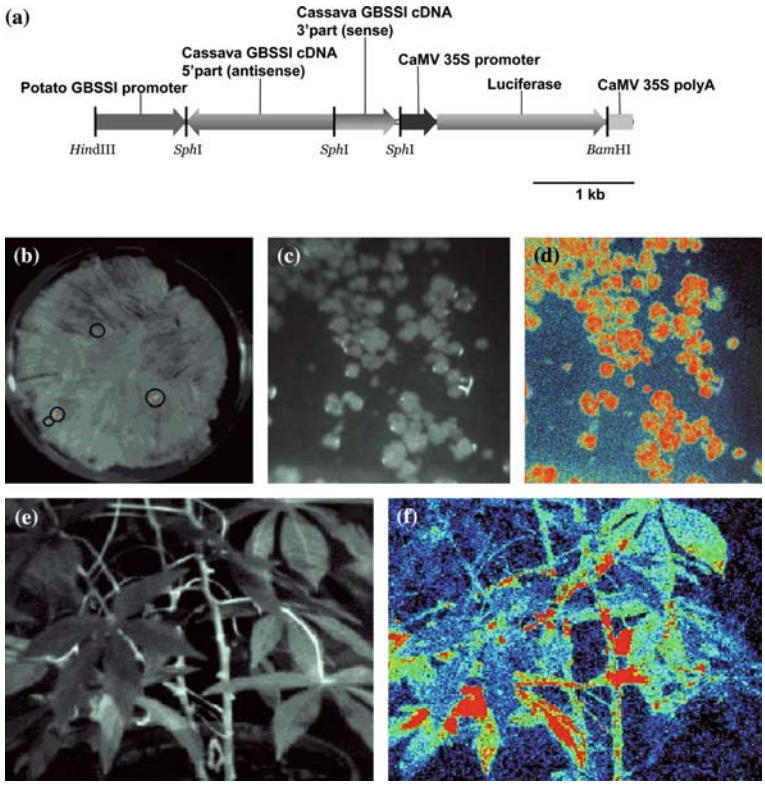

Figure 1. Map of the T-DNA region of vector GBSSas-2. The 1.5 kb 5′ part of the cassava *GBSSI* cDNA was merged in antisense orientation to the sense 0.6 kb 3′ part of the cDNA (a). Luciferase activity in FEC samples 2 weeks after bombardment (b), selected FEC lines, normal view (c), and after luciferase activity measurement (d). Transgenic cassava plants, normal image (e), and after luciferase measurement (f).

antisense orientation, fused between the potato GBSSI promoter and the nopaline synthase terminator (Salehuzzaman et al. 1993). The construct, used earlier for transformation of potato (pAG61; Salehuzzaman et al. 1993), contained the 5′ part (1.5 kb) of the full length cassava *GBSSI* cDNA in antisense orientation, merged to the 3′ part (0.6 kb) of the same cDNA in sense orientation, and was used for transformation. The difference between the two constructs GBSS-as2 and 7 was the orientation of the luciferase gene with respect to the *GBSSI* gene.

Transformation of cassava using particle bombardment

Friable embryogenic callus (FEC) cultures (Taylor et al. 1996) of genotype TMS60444 (International Institute of Tropical Agriculture) were used for particle bombardment essentially following the method described by Munyikwa et al. (1998). In short, FEC cultured for at least 5 weeks in liquid medium supplemented with Schenk and Hildebrandt (1972) salts and vitamins, 6% sucrose and 10 mg/l picloram (SH6), was sieved (mesh 1 mm) and collected on sheets. One hundred milligrams of FEC was spread on medium supplemented with (Greshoff and Doy 1974) salts and vitamins, 8 g/l Micro agar, 6% sucrose and 10 mg/l picloram (GD6) and bombarded using the BioRad (Hercules, USA) PDS-1000He biolistic device (helium pressures 1100 psi, 0.5 cm distance between rupture disc and the macrocarrier and between macrocarrier and the stopper plate, 5.0 cm distance between stopperplate and FEC, 27 in. Hg vacuum). After bombardment FEC was cultured in SH6 medium. In total 212 and 184 Petri dishes with FEC from TMS60444 were bombarded with construct GBSS-as2 or GBSS-as7, respectively. After bombardment the FEC was cultured in plastic pots filled with liquid SH6 medium. Two weeks later the

FEC was collected on solid GD6 medium and assayed for luciferase activity.

Luciferase positive FEC lines were cultured for plant regeneration on maturation medium consisting of Murashige and Skoog (1962) salts and vitamins, 2% sucrose, 8 g/l Micro agar (MS2) and 1 mg/l picloram. Torpedo shaped somatic embryos were isolated from the FEC and cultured on MS2, supplemented with 0.1 mg/l BAP, which allowed further maturation. Mature somatic embryos were cultured for regeneration of plants on solid germination medium (MS2 + 1 mg/l BAP). Plants were rooted on MS2 medium. The temperature in the growth chamber was 30 °C, the photoperiod 12 h and the irradiance was 40 μmol/m^2/s.

Luciferase assays

Plant tissues were sprayed (Douglas perfume dispenser) with a solution of 0.25 mg/ml luciferin (Promega, Madison, USA) in water, and luciferase activity was determined using a video-intensified microscopy (VIM) intensified CD camera and an Argus-50 photon counting image processor (Hamamatsu Photonic Systems, Hamamatsu City, Japan) at maximum sensitivity.

Starch staining

Thickened stems were induced by growing transgenic and non-transgenic cassava plants *in vitro* for 4 weeks on solid MS8 medium (Salehuzzaman et al. 1994). The presence or absence of amylose was visualised by iodine staining of cross sections of the *in vitro* thickened stems with a diluted (3:1 in water) Lugol's solution (L2:KI). The stained starch granules from the stem sections and from tuberous roots were visualised microscopically.

DNA and RNA analyses

DNA was isolated from leaves and RNA of tubers from transformed cassava plants and controls, blotted and assayed as described by Salehuzzaman et al. (1993). The Southern and northern blots were hybridised with the 32p dCTP labelled *GBSSI* cDNA from cassava. Equal amounts of RNA were applied in the northern blot as judged by concentration measurement and rRNA band identification verification (not shown).

Starch analysis

Tuber starch was isolated by peeling the fresh roots and grinding the central cylinder of the storage root in a laboratory blender in water. The slurry was sieved initially through a 250 μm screen. Starch granules sedimented rapidly, decanted several times using water and dried for several days at 20–30 °C. With this starch several analyses were performed. The amylose/amylopectin ratio (both from tuberous roots and thickened stems) was measured with a spectrophotometer using a protocol described by Hovenkamp-Hermelink et al. (1988). The spectrophotometer measures the absorption at 550 and 618 nm. The absorption at 618 nm divided by the absorption at 550 nm can be used to calculate the amount of amylose. When this apparent amylose value is below 3% (this value is obtained for amylose-free starch or pure amylopectin), it means that there is no amylose present, and thus the starch is termed amylose-free. All other starch analyses (granule size distribution, phosphorous content, differential scanning calorimetry (DSC), texture analysis) were determined as described (Visser et al. 1997a, b).

Viscometric profiles were determined by applying a small oscillating shear deformation at a frequency of 1 Hz on a 5% starch suspension, using a Thermo Haake RheoScope 1 (a rheometer combined with a microscope; Karlsruhe, Germany). The rheometer was equipped with parallel plate geometry (typ C70/1 Ti) and the gap size was 0.1 mm. The pasting profile of the 5% (w/v) starch–water suspension was obtained by heating the suspension from 40 to 90 °C at a rate of 2 °C/min, where it was kept for 15 min followed by cooling to 20 °C at a rate of 2 °C/min and held again for 15 min at 20 °C. The T_g (start gelatinisation temperature), T_p (peak temperature) with the corresponding peak and end viscosities were measured.

Gel stability after prolonged storage

Before performing this assay all used starches like normal cassava starch, amylose-free cassava starch, normal potato starch (Avebe Foodgrade

98w37), amylopectin potato starch (Avebe), corn starch (Meritena-A), and waxy corn starch (Meritena-300) were brought into the salt (NS-450) form. This was accomplished by resuspending 200 g of in 300–400 g demineralised water to which 10 g NaCl was added. After 30 min stirring at room temperature, the suspension was dewatered on a Büchner funnel and washed with demineralised water. Starch was dried at 30 °C. A 5% (w/v) starch suspension in demineralised water was placed in a Rapid Viscosity Analyser (Newport Scientific) and heated according to the following profile. The suspension was stirred at 50 °C during 1 min at 900 rpm, after which the stirring speed was reduced to 160 rpm. The suspension was heated at 0.2 °C/s until a temperature of 95 °C was reached. This temperature was maintained during 150 s, after which the suspension was cooled down at a rate of 0.2 °C/s, until a temperature of 50 °C was reached. This temperature was maintained during 120 s. After cooling to room temperature the samples were stored in a refrigerator for 5 days.

Freeze–thaw stability assay

The freeze–thaw stability of the starches was determined according to the method described by Jobling et al. (2003), with slight modifications. Screw tubes with 3 ml of a 5% starch solution were kept during 20 min at boiling temperature. After cooling to room temperature, the tubes were frozen overnight at −60 °C, then thawed for 4 h in an incubator at 22 °C, and centrifuged at 8000× *g* for 10 min at room temperature. The free liquid was decanted, and its weight was determined; this value, expressed as a percentage of the weight of the initial sample, was used as a measure of syneresis. The other tubes were placed back at −60 °C for additional freeze–thaw cycles and measurement.

Results

Three hundred ninety six Petri-dishes with FEC of the genotype TMS60444 were bombarded with the antisense *GBSSI* construct (Figure 1a). In this construct the cassava *GBSSI* antisense (partly antisense; 1.5 kb of the total 2.1 kb) gene is coupled to the luciferase reporter gene and selection of transgenic tissue was based on the activity of the *luc* gene as described previously (Munyikwa et al. 1998). A total of 84 luciferase positive FEC lines were obtained of which 58 lines gave rise to luciferase positive plants (Figure 1b–f). These plants were transferred to MS8 medium to accumulate starch in the stem tissue. After 2 months of growth on MS8 medium, 90% of the transgenic lines had formed thickened stems. One stem yielded between 1 and 6 mg of starch. After isolation and purification, this starch was stained with iodine, and analysed under a light microscope and by spectrophotometer. These starches were compared with those of wild-type cassava, wild-type potato, and the amylose-free mutant of potato. Figure 2a shows some typical results of the microscopic analysis. Some cassava plants had starch granules which stained brownish; when measured spectrophotometrically, they had amylose values which were comparable to those of the amylose-free potato mutant ($< 1.9\%$; Table 1). Fifty-five of the 58 plants yielded sufficient starch for analysis. Seven plants had values which made them potentially amylose-free ($< 3.5\%$, class I), 35 plants had values comparable to wild-type cassava starch ($> 12.9\%$, class III), and 14 plants had values in between these two extremes ($3.5\% < x < 12.9\%$; class II). A number of plants were chosen from all three classes and analysed for copy number by Southern blot analyses, for expression of the RNA by northern blot analyses, and for expression of the protein by western blot analysis. These analyses demonstrated that these plants were indeed transformed (between 2 and 8 inserts, results not shown) and that expression of the gene (Figure 2b) and protein (data not shown) was dramatically reduced in those plants that had a low amylose value. All plants were transferred to the greenhouse for tuberous root production. One plant (#45) failed to establish in the greenhouse. Starch was isolated from the tuberised roots of the surviving plants after 6 months of growth, and analysed for their amylose content. From this analysis it was obvious that all plants belonging to class III *in vitro* were also class III plants in the greenhouse. For class I and II plants, the picture was different in that most plants had changed to class III. Only 2 plants (#3 and #10) still belong to class I in the greenhouse (Table 1). To check the stability of the trait in a subsequent (vegetative) generation, eight transgenic plants and the control were multiplied

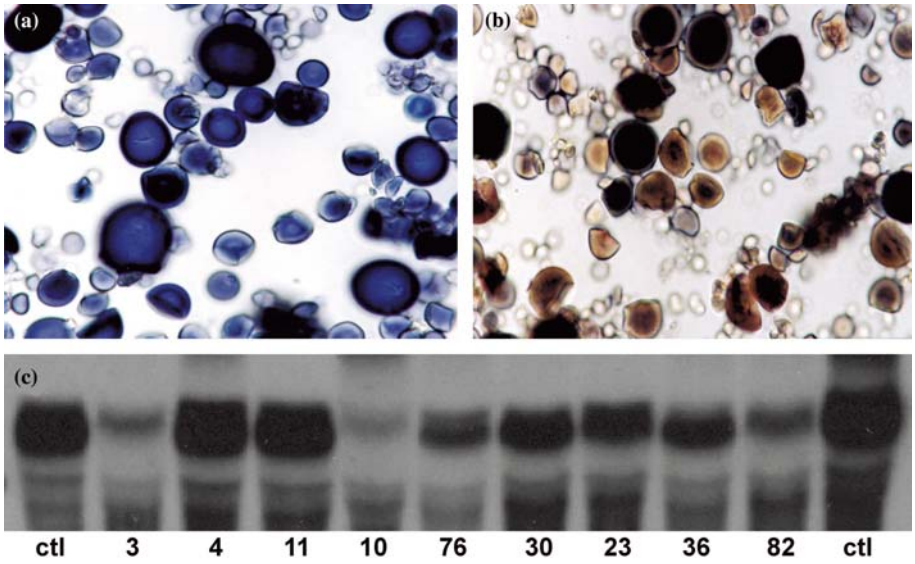

Figure 2. Phenotypic and molecular evidence for amylose-free cassava plants. Light microscopy of iodine stained starch granules from untransformed control plant (a) and a transgenic amylose-free plant clone #3 (b). Blue-staining granules indicate the presence of amylose. Northern blot analyses of RNA isolated from tuberous roots of transformed and control plants (c). The *GBSSI* cDNA from cassava was used as a probe. Plant numbers are indicated below the lanes. Ctl indicates the controls.

via stem cuttings in the greenhouse, and at least 30 cuttings per plant were grown for 8 months for the production of tuberous roots. Non-transgenic plants derived from FEC, and plants which never had undergone the process of somatic embryogenesis, were used as controls. It was obvious that most of the transgenic plants had a reduced vigour and produced less tuber material (only 75% fresh weight) than the control plants. This was also observed for the non-transgenic FEC regenerated plants.

Table 1. Amylose content of starches from different plants regenerated from independent FEC lines.

Clone number	% Amylose *in vitro*-grown	% Amylose greenhouse-grown
#3	1.9	2.2
#10	1.6	1.6
#11	1.6	18.4
#76	1.6	6.2
#82	1.6	4.6
#4	9.5	17.7
#45	1.6	Died
#23	18.5	19.2
#30	1.6	19.0
Control (wild-type)	19.2	19.2
FEC-derived plant	19.2	19.2

The amylose content was determined spectrophotometrically by measuring the absorbances of gelatinised starch/iodine solutions at 618 and 550 nm, from which the percentage of amylose can be calculated.

Again plants #3 and #10 were amylose-free, suggesting genetic stability of the trait. The other six plants (see Table 1) had starch with an amylose content comparable to that of the controls. The starches isolated from plants #3 and #10, as well as that of the control, were analysed further for their physico-chemical properties. Particle size distribution and phosphorous content were similar for the amylose-free and amylose-containing starches (Table 2). When measuring the gelatinisation characteristics of the starches using DSC, it became clear that amylose-free cassava starch has a higher *T*-onset than amylose-containing cassava starch (Table 2), indicating that the former melts at higher temperature than the latter. The viscosity of 5% starch gels was measured using a Thermo Haake Rheoscope and confirmed the higher gelatinisation temperature (T_g) as observed with DSC. Further analyses were done with a large-scale batch of starch, isolated from the two amylose-free clones #3 and #10. The results were similar for both clones, thus only the results for clone #3 are shown. Peak viscosity of amylose-free starches was considerably lower, and the end viscosity lacked the typical increase indicative for amylose (Figure 3a). The gel properties of the amylose-free

Table 2. Starch characteristics of selected cassava transformed plants and control plants.

	Median particle size (μm)	Phosphorous content (nmol/mg starch)	% Amylose	*T*-onset (°C)
Transformant #3	7.9 ± 0.1	30.7 ± 4.8	3.5 ± 0.2	60.7 ± 0.2
Transformant #10	8.2 ± 0.1	24.3 ± 0.9	3.0 ± 0.1	60.7 ± 0.1
Control	7.5 ± 0.1	25.9 ± 3.3	19.6 ± 0.7	59.1 ± 0.3

Data are the average of three independent measurements ± standard error.

starch were furthermore assessed using a Texture analyser, and by performing relative compression studies. This showed that 6% amylose-free gels were much weaker (breaking at a force of 1.3 N) than 6% amylose-containing gels (which broke at a force of 5.0 N).

The chain-length distribution of amylose-containing and amylose-free cassava starch, as measured by high-performance anion-exchange chromatography showed an identical pattern (Table 3). Compared with starch isolated from wild-type potato and maize, cassava starch has considerable more short chains ($Glc_{6-12)}$ and fewer longer chains (Glc_{13-24}).

To evaluate the stability of amylose-free cassava starch the visual appearance and syneresis was measured after prolonged storage at 4 °C and after repeated cycles of freeze–thaw. It was apparent that amylose-free cassava starch was superior over other amylose-free and normal starches (from potato and maize) as demonstrated by the clarity of 5% (w/w) gels after prolonged storage at 4 °C (Figure 3b), as well as the very limited amount of syneresis observed by these gels after 1 week of storage at 4 °C (data not shown).

A similar effect was also seen after repeated freeze–thaw cycles. After three cycles, the amylose-free cassava starch was still a clear gel, whereas the amylose-containing starches of cassava and potato were opaque (data not shown). Syneresis, which was 8% in the amylose-containing cassava starch after three cycles, was not even 1% in the amylose-free cassava starches (Figure 3c). These results showed that amylose-free cassava starch is extremely stable under different conditions, also when compared to other major amylose-free starch sources.

Discussion

Although the industrial use of cassava starch is increasing, it accounts for only a small percentage of the total starch on the world market. If cassava would be used more as an industrial crop, this would mean that tropical countries are less dependent on import of relatively expensive maize and potato starches, and that the excess starch could be sold on the world market. This could become possible if cassava starches with novel and superior properties are produced.

Starch is used in all kinds of applications and these require different types of starches or different modifications of existing starches. The native amylose-free cassava starch described here has the benefit that a number of desired functionalities can be achieved without chemical modifications. The selection of transgenic tissue was based solely on the activity of the luciferase gene (Figure 1b). This worked well as shown previously (Munyikwa et al. 1998). In total 36% of the *in vitro* plants had starch with reduced amylose content. The number of transformed plants showing a maximum inhibition of GBSSI activity, as measured by amylose content, was 11 % in the *in vitro* plants, which is in the same range as reported for other antisense constructs and systems (Kuipers et al. 1997). However, for the plants grown in the greenhouse this was only 4%. Measuring the amylose content of starch derived from stems of *in vitro* grown plants turned out to be a relatively fast (pre)-screening method. The reliability of this test for predicting the amylose content of the starch from tuberous roots grown in the greenhouse was, however, not good. Only plants, which were assigned as amylose-containing in the *in vitro* stems, were also amylose-containing in the tuberised roots of greenhouse grown plants. However, plants, which were amylose-free in the *in-vitro* stem test, are not necessarily amylose-free in the greenhouse. Although the reason for this apparent discrepancy is unclear, it might be that the starch in stems of *in vitro* plants is not so well comparable to that of tuberous roots as previously thought (Salehuzzaman et al. 1994). Different relative importance of the various isoforms of starch synthase might cause these differences. Nevertheless, the *in vitro*

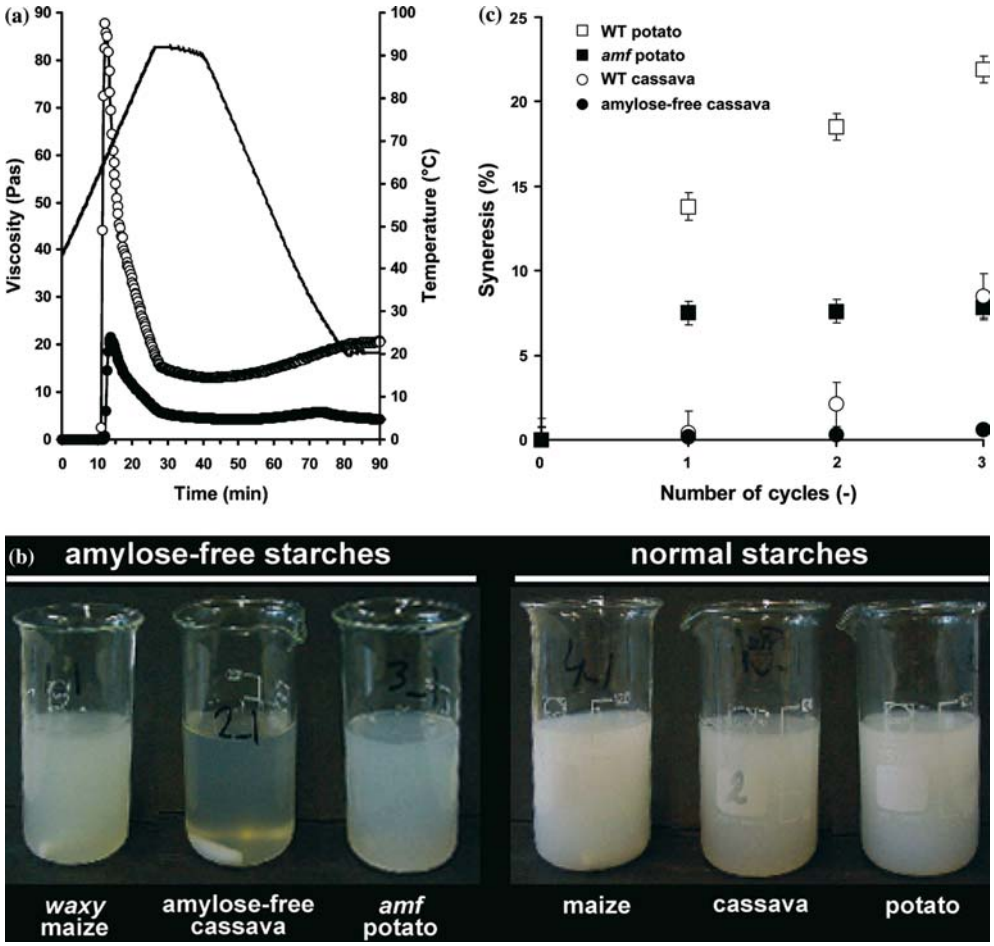

Figure 3. Compilation of some physical properties of starch. (a) Rheology measurement of 5% solutions of wild-type cassava starch (open circles) and amylose-free (closed circles) cassava starch from transformed clone #3. The temperature profile is indicated with a solid line without symbols. (b) Images of 5% (w/w) starch gels after 5 days storage in a refrigerator, using different amylose-free and amylose-containing starches. In this experiment the starch of the amylose-free cassava clone #3 was used. (c) Freeze–thaw stability of normal and amylose-free cassava and potato starches. For each cycle, the % syneresis was determined in triplicates; error bars in the figure represent standard error.

Table 3. Chain-length distribution of cassava starch compared to other starch sources (in % w/w).

Chain length	Cassava (wild-type)	Amylose-free cassava (clone #3)	Maize (wild-type)	Potato(wild-type)
DP 6–9	5.9	5.7	3.9	3.7
DP 10–12	21.7	20.4	18.2	13.5
DP 13–24	46.6	46.0	54.0	51.7
DP 25–40	20.4	21.8	21.1	22.3
DP >40	11.4	11.7	7.0	13.1

DP, degree of polymerisation.

screening method reduces the number of plants which have to be transferred to the greenhouse considerably.

There is a good correlation between the colour of the starch granules and the expression of the *GBSSI* mRNA (Figure 2b) and the protein con-

tent (not shown). However, although the level of mRNA was reduced dramatically, no plants which completely lacked the *GBSSI* transcript were observed, as was the case for amylose-free potato (Visser et al. 1997a, b). Also in potato it was reported that the values of mRNA in antisense and co-suppression transformants varied, and was different within one tuber and different tubers from the same clone. The only relation which could be found was that in all antisense plants the level of mRNA was always lower than in the controls (Kuipers et al. 1995; Flipse et al. 1996). This was also observed in cassava. The reduction in the amylose content was the most prominent alteration observed. Lack of amylose in turn leads to a range of differences in physico-chemical properties. The *T*-onset (or T_g) of the amylose-free cassava starch had shifted to a higher temperature like it was reported for potato. Because of the absence of amylose, also the typical retrogradation peak was absent in the viscosity profile (Figure 3a).

The potential applications of a starch depend on its stability and clarity after cooking and cooling. The amylose-free cassava starch differed significantly from amylose-containing cassava starch, and from other amylose-containing and amylose-free starches. The clarity and stability after heating at 95 °C and after prolonged storage at low temperature (4 °C) were superior. The amylose-free cassava gel was still transparent, whereas the other gels (including the amylose-free maize and potato starch gels) had become opaque (Figure 3b). Freeze–thaw stability showed that syneresis was already low in normal (amylose-containing) cassava starch, but was absent even after three freeze–thaw cycles in the amylose-free cassava starch samples (Figure 3c). It has been postulated that the chain-length distribution of the amylopectin molecule, besides the presence of amylose, is an important determinant of stability of starch (Shi and Seib 1992; Jobling et al. 2003). Cassava amylopectin has more short chains ($Glc_{6-12)}$ and fewer longer chains (Glc_{13-24}) compared to potato and maize amylopectin (Table 3). Jobling et al. (2003) showed that amylose-free, short chain amylopectin potato starch, produced by the simultaneous down-regulation of GBSSI and two soluble starch synthases, is much more freeze–thaw stable than amylopectin potato starch. Similarly, the waxy, sugary-2 maize mutant has freeze–thaw stable starch (Wurzburg and Fergason 1984). In both cases, the amount of short chains (Glc_{6-12}) of amylopectin has increased, whereas that of the longer chains (Glc_{13-24}) has decreased. It is tempting to speculate that the decrease of the amount of longer chains may reduce the association between the glucan chains, and subsequent retrogradation. Jobling et al. (2003) also suggested that the short chains may prevent re-association of the longer glucan chains, thus contributing to the improved stability. In the case of amylose-free cassava, we have a starch with a large percentage of naturally occuring short chains, without the need of down regulating two soluble starch synthases.

We have shown here the feasibility of creating a native starch with significantly improved stability properties. Stability is an important property for the use of starch pastes in numerous applications in the paper industry, for textile sizing and for use by the food industry in products like dairy, soup and sauces, and noodles. Currently, these solution stable starches are produced by chemical modification. The use of a starch source, which has this stability without chemical modification, can have great environmental and economical benefits, since it may replace conventional chemically modified starches in a variety of products. Since cassava can only be grown in tropical countries, this offers possibilities for developing countries to export this type of specialty starches.

At the moment field trials are being conducted to obtain more starch to be able to perform application tests. No doubt the superiority of this starch will lead to interesting new products.

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