A novel sugary cassava (*Manihot esculenta* Crantz) accumulating phyto-glycogen in the storage root may be mutated in the gene coding for the branching enzyme[1]

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Depiction of cassava from Obelisk Tello representing food brought to humanity by the God Cayman of the Water.
Peruvian Chavin Culture in the Amazon.

ABSTRACT

A new class of cassava (*Manihot esculenta* Crantz) storage root, named sugary cassava, has been described with accumulation of a diverse starch type (Carvalho et al. 2000; Carvalho et al. 2004). A gene expression analyses for the major enzymes in the starch pathway is showed in the present report for the clone CAS36.1 which accumulates a glycogen like starch. cDNA clones for the genes coding for sucrose synthase, plastidic phosphoglucomutase, cytosolic phosphoglucomutase, ADPG pyrophosphorilase, starch synthase, branching enzyme and debranching enzyme (isoamilase I) were used as probe in gene expression analysis of the storage root of the sugary clone (CAS36.1) and farina type (IAC12-829) cassava. All the genes were expressed in both types of cassava except the gene coding for branching enzyme. This result strongly suggests that this gene is mutated, some how, altering the expression of the protein enzyme responsible for the formation of amylopectin in the sugary cassava CAS36.1. Sequence homology analysis of a cDNA fragment of this gene was carried out indicating that the non expressed branching enzyme gene in the storage root of the clone CAS36.1 coding to isoform I described for cassava. This gene is also not expressed in the leaf of this sugary cassava. Further research is underway in our lab to analyze the molecular genetics mechanism responsible for the differential expression of this gene.

INTRODUCTION

The possibility of designing starches for industrial use by genetic manipulation, including in cassava (Sivad and Preiss 1998; Munyikwa et al. 1997), implies the need to understand the biochemical mechanism of starch granule formation. Several mechanisms have already been proposed (Ball *et al.,* 1996; Zeeman *et al,* 1998). None of the proposed mechanisms have yet been shown to operate *in vivo*. Spontaneous mutations responsible for diversity in the starch structure present in a species of particular genetic background have been used with great success in *Arabidopsis* (Zeeman *et al.,* 1998), pea (Craig *et al.,* 1998), maize (Singletary *et al.,* 1997), and rice (Nakamura *et al.,* 1996) to understand such mechanisms. In the case of the storage root of cassava no mutation of any kind has been reported. Nevertheless, approaches using limited germplasm diversity analysis (Zakhia *et al.,* 1995) and the transgenic technology approach (Munyikwa 1997) are currently underway by distinct groups Worldwide. Results from the germplasm diversity analysis have indicated apparently low amylose content diversity, with values varying from 18 to 25%, a strong environmental effect on the amylose/amylopectin ratio in the accumulated starch, and cultivar dependency on the environmental effect during harvesting (Siroth *et al.,* 1999). Results from transgenic technology to generate mutant clones with antisense technology have indicated several limitations, including genetic background dependency to obtain success in the regulation of a starch phenotype, inefficient regeneration system of genetically transformed plants, lack of stable character expression, and non-existent genetic analysis of the transformed population of cassava. Sequences for the genes coding for the enzymes ADPG pyrophosphorilase (Munyikwa, 1997), Granule Bound Starch Synthase (Salehuzzaman *et al.,* 1993), and Starch Branching Enzyme (Salehuzzaman *et al*., 1992) from cassava storage roots, have been reported. Branching enzyme II (Baguma et al. 2003) have also been cloned and sequenced in cassava.

In this work we used the clone CAS36.1 from the previously unknown diversity in cassava with distinct starch structure and high free sugar accumulation (Carvalho et al 2000) to isolate gene in the starch synthesis pathway that could be related to the glycogen-like starch phenotype found in this particular clone. cDNA clones derived from a subtractive cDNA library and genes coding for the major enzymes of the starch synthesis pathway were evaluated. Gene expression analysis of the gene coding for the major enzymes in this pathway indicated the lack of a transcript for the gene coding for Branching enzyme I.

MATERIALS AND METHODS

Plant Material: Storage root samples from field grown plants of the clone CAS36.1 and the commercial variety IAC 12-829 were used in the present study. Uniform 25-35cm long storage roots were selected, washed, and the root peel removed. A 10 cm-long central part of the storage root with 2-4cm diameter was taken for the study. Freshly tissue sample was taken for total RNA and mRNA preparation.

cDNA Synthesis and Subtractive PCR from storage root of cassava: Total RNA was extracted from cassava plant storage roots as described by de Souza et al., 2002. Polyadenylated RNA was purified from 500 µg of total RNA using the mRNA Purification kit (Pharmacia). Double-stranded cDNAs were synthesized and subtractive PCR was conducted using the PCR-Select cDNA Subtraction Kit (Clontech). All procedure steps to obtain the subtracted cDNA were performed according to the manufacturer's protocol. In brief, Cas36.1 (tester) and IAC (driver) cDNAs were synthesized separately from 2 µg of polyA+ RNA. The tester and driver cDNAs were separately digested with *RsaI*. The tester sample then was divided into two populations and each one ligated with adaptor 1 or 2R. Each tester population was hybridized separately with excess driver cDNA (33-fold). The two hybridization reactions were combined (with additional driver cDNA) for a second subtractive hybridization. The hybridizations led to equalization and enrichment of differentially expressed sequences. Finally, the differentially expressed cDNAs were amplified by PCR and cloned into the pGEM-Teasy vector (Promega).

cDNA Amplification by PCR and gene cloning: Double-stranded cDNA from cv. IAC 12-829 obtained before was used as template. The primers specific for each enzyme are showed in table 1.

Tabele 1 - List of primers used in this work

- ADPGase	5'-TGGCGAGTATGGCGGCCATC-3' 5'-GATCACGGTTCCGCTGGGAATC-3';
- GBSS	5'-TGCCTTCTCAGACTTCCCACG-3' 5'-GACCACCAGTAGAGCAACAATGGG-3';
- Branching Enzyme isoform I	5'-TATCGTGAGTGGGCTCCTGC-3' 5'-TCCCATAGCTTATGGTAGCCTCG-3';
- Plastidic Phosphoglumutase	5'-GTATTTGATGCCATGCATGC-3' 5'-TTTCTATCACCATCTCCATC-3';
- Cytosolic Phosphoglucomutase	5'-GCAAATGGAGTAAGACGTGT-3' 5'-ACACCATGAAGTGCATCATA-3';
- Isoamilase	5'-ATTGTTGGCCCCCAATGGC-3' 5'-CACAATCCCATGCTTCAGC-3'.

The amplification protocol consisted of one cycle at 94°C for 5 minutes, 30 cycles at 94°C for 1 min, 45°C for 1 min and 72°C for 2 min and a final extension at 72°C for 15 min. The PCR products were cloned into the pGEM-Teasy vector (Promega) and sequenced. The clones obtained were named as pGEMcas4 for cytosolic and pGEMcas5 for plastidic phosphoglucomutase respectively, pGEMcas6 for ADPG-pyrophosphorilase, pGEMcas8 for branching enzyme, pGEMcas7 for starch synthase, pGEMcas9 for isoamilase were also and used as probes in the gene expression analysis.

DNA Sequencing and Analysis: Single bacterial colonies of the result of a previously prepared subtracted cDNA library were inoculated in 96-well microtiter plates containing LB and ampicillin (100 mg/L) and allowed to grow overnight with shaking at 37°C (18 hr at 320 RPM). The DNA was purified by standard alkaline lysis method with one modification at the end of the procedure, where the supernatant was passed through a multi-screen filter (Millipore) prior to DNA precipitation. The purified DNA was ressuspended in water. The sequencing reactions were performed with 200ng of DNA using standard protocols of the ThermoSequenase II dye terminator cycle sequencing kit (Amersham-Pharmacia Biotech) using universal M13 forward and reverse primers and the capillary sequencer MegaBACE 1000. The samples were electro injected by 2 KV for 100 seconds and the fragments were separated at 9 KV for 100 minutes. The electropherograms were submitted to the Phred, Phrap & Consed package for sequence quality evaluation. The sequences with Phred > 20 were automatically blasted against NCBI GenBank, the clone with insert for the sucrose synthase gene (pGEMcas3) was obtained from the subtractive cDNA library and used as probe.

Total RNA extraction and hybridization: Total RNA was extracted from storage root and leaf as described by de Souza et al., 2002. Samples containing 20mg of total RNA were separated on formaldehyde-agarose gel, transferred to Hybond XL membranes (Amersham) and hybridized with radiolabelled probes (Table 1). DNA fragments for clone pGEMcas8 was obtained by PCR amplification using forward and reverse primers and probe labeled with Ready-to-go DNA Labelling Beads (-dCTP) from Amersham. Hybridization was performed in 5X SSPE (1X SSPE is 150mM NaCl, 10mM NaH_2PO_4 H_2O, 1mM $Na_2EDTA_2H_2O$), 5X Denhardt's solution (1X Denhardt's Solution is 0.02% ficoll, 0.02% PVP, 0.02% BSA), 0.5% SDS and 0.1 mg/ml salmon sperm DNA at 60°C overnight. The filters were washed in 2X SSPE 0.1% SDS, 1X SSPE 0.1% SDS and 0.1X SSPE 0.1%

RESULTS AND DISCUSSIONS

CDNA cloning and sequence analysis:

Several cDNA clones were amplified by PCR and analyzed in agarose gel before sequencing to confirm predicted size. Figure 1 displays the selected cDNA clones and confirms the presence of a single expected fragment size for all genes according to Table 2. After nucleotide sequence the BLAST search confirmed the identity of each clone with the corresponding gene with a very high significant e-value (Table 2). The overall identity between clone pGEMcas8 and several plant species varied from 98% for the cassava gene CAA54308.1 to 72% for the wheat gene (CAA72154.1) at the NCBI data base.

Table 2 - Cassava storage root cDNA from variety IAC 12-829 deposited in Databank at EMBRAPA- Recursos Genéticos e Biotecnologia (Brasilia-DF, Brazil) used as probe.

CDNA databank code at EMBRAPA	Gene function at NCBI	Insert size (bp)	Reference at NCBI	E-value at NCBI databank
MAGL06G11	Sucrose synthase	508	BAA88905	4 e-65
pGEMas6	ADPGase small subunit	1066	A55317	5 e-39
pGEMcas4	Phosphoglucomutase citosolic	899	AAM55493.1	3 e-62
pGEMcas5	Phosphoglucomutase plastidial	1011	AAM55494.1	2 e-10
pGEMcas7	Granule bound starch synthase	692	CAA52273.1	2 e-92
pGeMcas8	Branching enzyme	586	CAA54308.1	2 e-73
pGEMcas9	Isoamilase	1053	AAN15317.1	5 e-90

The sequence for the cDNA clone pGEMcas8 was pairwise aligned with the sequence of branching enzyme I (BEI - CAA54308.1) and II (BEII -Baguma et al. 2003) from cassava to confirm its isoform identity. The pairwise identity was 98% for BEI and 35% for BEII. Figure 2 shows the pairwise sequence alignment for the BEI (CAA54308.1) and clone pGEMcas8 indicating its position between 1099 and 1716bp in the full length CDS.

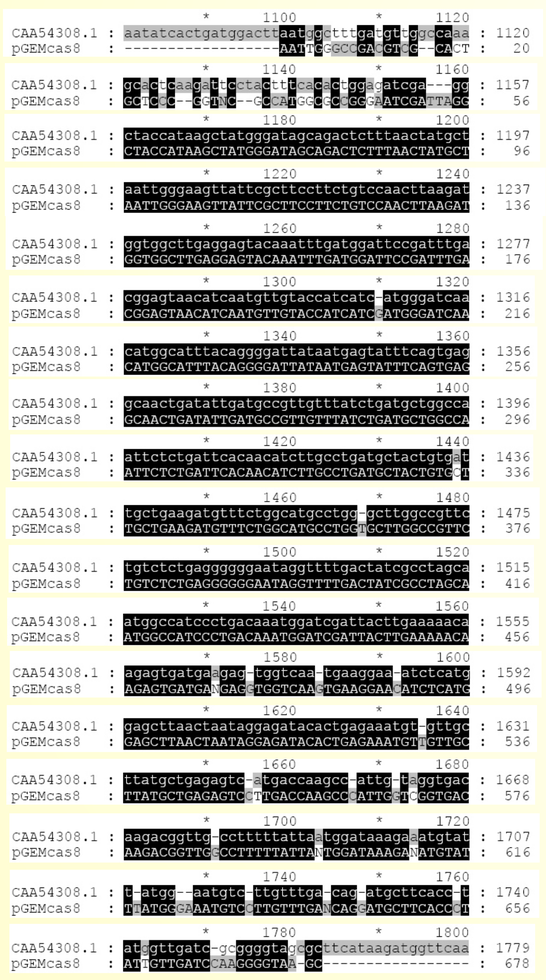

Figure 2 - Sequence analysis of the cDNA clone pGEMcas8 from IAC 12-829 in comparison to the gene sequence (CAA54308.1) of branching enzyme I from cassava.

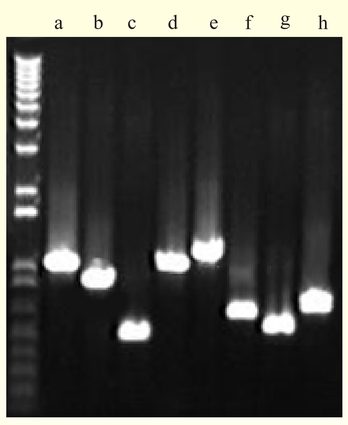

Figure 1 - Etidium bromide stained agarose gelshowing the PCR amplified fragments for the cDNA clones used as probe in this work.(a=pGEMcas9; b=pGEMcas4; c=MAGL06G11; d=pGEMcas5; e=pGEMcas6; f=pGEMcas8; g=control - Mec1; h=pGEMcas7).

Gene expression analysis:

The sucrose-starch conversion pathway was simplified and aligned with the RNA blot analysis to observe gene expression at the level of transcript by comparing farina and sugary cassava plants. Figure 3 displays the analytical results for several cDNA cloned from farina clone IAC 12-829 in this pathway that could help to explain the starch phenotype in the sugary cassava Cas36.1.

The enzyme ADPGppase turned out to be important candidate for the missing gene expression because our previous results indicated the missing protein at the level of protein blot analysis (Carvalho et al. 2004). However, the corresponding mRNA for ADPGase hybridized in both cassava types with the cDNA probe (pGEMcas6) which showed 96% identity with the gene coding for the small subunit of ADPGase from cassava reported by Munyikwa 1997.

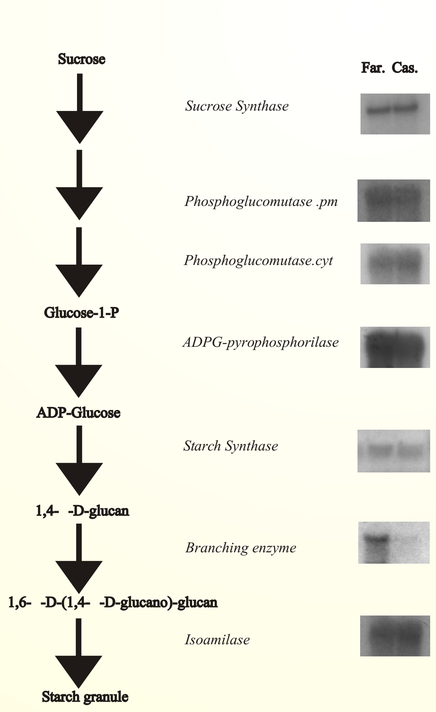

Figure 3 - Gene expression analysis for the gene coding for starch enzyme proteins in farina (IAC 12-829) and sugary (CAS36.1) cassava types. The result of the mRNA blots was aligned with the metabolic steps in which each gene product catalysis in a simplified starch pathway. In all autoradiogram the left line is the farina cassava (Far.) and the right line is the sugary (Cas). See the Material and Methods for the details on the cDNA generated and use.

The expression of the gene coding for sucrose synthase protein was included to test the possibility that this gene would drive the sucrose cleavage via the UDPglucose path, which would serve as the substrate for the UDPGppase in its production of glucose-1-phosphate to gain the glycogen pathway. However, the mRNA hybridization blots analysis with a cDNA clone (pGEMcas3), derived from our subtractive cDNA library showed that the expression of this gene was present in both cassava type.

Gene coding for two isoenzymes (cytosolic clone cGEMcas4 and plastidic pGEMcas5) of phosphoglucomutase was also tested for the occurrence of a starchless phenotype. The results, however, showed the expression of those two genes in both cassava tested.

Expression of the starch synthase responsible for step in the pathway leading the production of linear 1,4 glucan polymer was analyzed by using the probe pGEMcas7 derived from GBSS previously reported (Munyikwa, 1997). The expression of this gene was also present in both cassava type.

The debranching enzyme has been genetically shown to play roles in starch granule assembly. When the activity of this enzyme is missing in starch storage tissue there is a change in the balance of amylopectin and glycogen accumulation. We cloned a cDNA fragment (pGEMcas9) for the gene of the isoamylase isoform I and used as a probe. The results again showed the presence of the mRNA coding for this enzyme in both cassava type.

Finally, and somewhat unexpectedly, the expression of the gene coding for the cassava branching enzyme I, achieved by using a cDNA probe (pGEMcas8) derived from a previously known gene sequence (Salehuzzaman *et al., 1992) i*ndicated that this gene is not expressed at a detectable level in clone CAS36.1. These results suggest that the missing expression may contribute to the kind of starch accumulated in CAS36.1.

Organ-specific expression:

The expression of the cDNA clone pGEMcas8 was further analyzed in the sink and source starch organ in the sugary cassava plant. Expression in leaf and storage root of the sugary cassava Cas36.1 was compared with the storage root of the farina cassava IAC 12-829. Results displayed in Figure 4 indicated that this gene is not expressed in leaf of the sugary cassava. The missing expression of clone pGEMcas8 in leaf may be related to the regulation of starch synthesis in source organ by circadian clock. While for the case of storage root it is genetically regulated because this clone accumulate a glycogen like starch rather than normal starch granule (Carvalho et al., 2004), therefore it is not related to physiological regulation as observe in source organ like leaf.

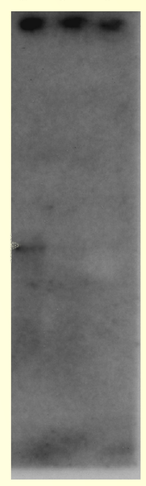

Figure 4 - Expression of the clone pGEMcas8 coding branching enzyme I in source and sink organ of Cas36.1. (a=root IAC 12-829; b=root cas 36.1; c=leaf cas 36.1)

CONCLUDING REMARKS

These results indicate, for the first time, that the natural mutation of the storage root in sugary cassava (clone Cas36.1) is due to a deficiency in the transcript for the gene coding for branching enzyme I. Further research is needed to elucidate the genetic mechanism responsible for the missing expression of the BEI gene in the clone Cas36.1.

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