Comparative gene expression study to identify genes possibly related to storage root formation in cassava (*Manihot Esculenta* Crantz)[1].

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

Cláudia Regina Batista de Souza[2,3], Luiz Joaquim Castelo Branco Carvalho[2*] and Julio Cesar de Mattos Cascardo[4]

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ABSTRACT

Cassava storage roots result from swelling of adventitious roots by secondary growth. Storage root formation involves continuous elongation (primary growth) and radial growths. The cells of vascular cambium, a meristematic tissue, are rapidly divided and expanded resulting in secondary growth that gives an increased diameter, giving secondary phloem to the outside and secondary xylem to the inside with parenchymatic cells packed with starch. In the present work we aimed to gain insight into molecular processes occurring during cassava storage root formation. Identifying genes expressed during secondary growth, will be possible to elucidate molecular processes related to parenchyma cells development (starch, protein and carotenoid accumulations) and prospect specific promoter and enhancers able to drive efficient storage root specific expression. In this work, we report a comparative gene expression study by Northern blot analysis in adventitious and storage roots to identify such genes. Cassava genes clones from our cDNA library of storage root, available in the Cassava Databank at the Biochemistry and Biophysics of EMBRAPA Genetic Resources and Biotechnology, were sorted by molecular and cellular processes and used as probes in this study. Results, so far, revealed four classes expressed genes based on the intensity of the hybridization signal in the northern blot analysis.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important food crops in the tropics and rates with respect to furnished calories, on the fourth place, after rice, maize and sugarcane (Puonti-Kaerlas, 1998). In developing countries cassava roots are very often the sole source of calories. Cassava roots contain about 85% of starch and only about 1-2% of proteins (Cock, 1985).

The cassava storage root results from swelling of adventitious roots by secondary growth. Anatomical studies of the cassava storage root differentiate three major tissue systems (Rateaver, 1951; Castilloa *et al*., 1997) and a model of storage root tissue organization, suitable for gene expression analyses, was established (Cabral *et al*., 2000; de Souza, 2001). This model describes three tissue systems: System I is composed of phellogen and phelloderm, System II of phloem and vascular cambium, and System III of secondary xylem with its highly specialized parenchyma cells packed with starch granules (Cabral *et al*., 2000; de Souza, 2001). Protein polymorphism of adventitious and storage roots of cassava have been studied in 2-D gel system and the results showed over 260 proteins unique to the storage root and possibly related to secondary growth (Cabral and Carvalho, 2001). In addition storage root protein genes as well as expressed genes were also initiated (de Souza, 2001; de Souza *et al*., 2002; Carvalho *et al*., 2002; de Souza *et al*., 2003; accompany posters). In these studies were identified an 18 kDa protein with high identity with the small heat shock protein class and an alcohol-soluble protein similar to allergenic Hev b5 from rubber tree, designed Pt1L4 and Pt2L4, respectively (de Souza, 2001; de Souza *et al*., 2002; Carvalho *et al*., 2002). Gene expression analyses suggested that Pt2L4 is possibly related to the secondary growth pattern of cassava storage root since the level of its coding gene (Mec1) transcripts increases during the maturation of the secondary xylem and its parenchyma derived cells (de Souza, 2001; de Souza *et al*., 2002; Carvalho *et al*., 2002). Recently a cassava promoter related to Pt2L4 protein was isolated by Zhang *et al*. (2003). The expression pattern in transgenic plants showed that this promoter is related to vascular expression and storage root formation since it is active in phloem, cambium and xylem vessels (Zhang *et al*., 2003). In sweet potato, another underground storage organ, genes possibly related to storage root induction were recenly identified by You *et al*. (2003).

Here we report a study with comparative gene expression analyses in adventitious and storage roots of cassava in order to identify genes related to storage root formation. Clones from our storage root cDNA library were sorted by molecular and cellular processes possibly present during storage root formation (cell division and expansion, gene regulation, signal transduction, tumorigenesis, programmed cell death) and used as probes in the present study.

MATERIALS AND METHODS

Plant material: Adventitious and storage root were harvested from variety IAC 12-829 after sixteen days and six month old plants respectivelly and stored at -80°C until use.
Comparative sequence analysis and functional identification:
Cassava gene sequences obtained previously from our subtractive cDNA library (see accompany poster) were aligned to the GeneBank nucleotide sequence databank using the BlastX algorithm with E-value cut off at 0.027 or lower. Based on the BlastX comparison results, cassava genes were identified according to their predicted function and used as probes in comparative gene expression analysis. Predicted protein sequences were analyzed by Conserved Domain Search program from NCBI.
Northern blot analysis: Total RNA was extracted as described by de Souza *et al*. (2002). Samples containing 20 ig of total RNA were separated on formaldehyde-agarose gel, transferred to Hybond XL membranes (Amersham) and hybridized with radiolabelled probes (Table 1). DNA fragments were obtained from several clones by PCR amplification using forward and reverse primers and probes were labeled with Ready-to-go DNA Labelling Beads (-dCTP) from Amersham. Hybridization was performed in 5X SSPE (1X SSPE is 150mM NaCl, 10mM NaH2PO4 H_2O, 1mM $Na_2EDTA2H_2O$), 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% SDS. All washings were performed at 60°C for 45 minutes each.

RESULTS AND DISCUSSIONS

cDNA clone sequences analyses and selection: Based on the BLASTX homology analyses, cassava genes predicted function were selected based on different cellular and molecular processes possibly related to storage root formation. The cDNA clones used as probes in this comparative Northen Blot analysis are listed in Table 1. The Mec1 gene (AY101376) was also included in this study, since previous results indicated that this gene to be related to secondary growth in storage root (de Souza 2001, de Souza *et al*. 2002, Carvalho *et al*. 2002).

Table 1: Cassava storage root genes from Databank at EMBRAPA- Recursos Genéticos e Biotecnologia (Brasilia-DF, Brazil) used as probes in gene expression analysis.

CDNA library code	Putative function	Species	Reference at NCBI	E-valueb
Mec1	Pt2L4 Secondary growth	*Manihot esculenta*	AAM55492	-----
MALC01-D06	RING Zinc Finger transcription factor protein (RZF)	*Arabidopsis thaliana*	AAM67030.1	1e-25
MALC04-C04	Protein induced upon tuberization (TUB)	*Solanum demissum*	CAA66948.1	8e-14
MALC01-F09	Translationally Controlled Tumor Protein (TCTP)	*Hevea brasiliensis*	Q9ZSW9	1e-34
MALC02-F04	Calmodulin	*Daucus carota*	AAQ63461.1	1e-79
MALC01-A10	Extensin	*Brassica napus*	T09546	0.027
MAGL02-H05	MYB transcription factor	*Malus xiaojinensis*	AAO45179.1	6e-49
MAGL02-B05	14,3,3 protein	*Nicotiana tabacum*	O49997	7e-45
MALC01-B02	Gh1 Auxin Regulated protein (ARP)	*Glycine max*	T05726	2e-31

Northern blot analyses: The mRNA blot anlyses of the nine selected genes displayed in Figure 1 shows four types gene expression distinction between storage and adventicious root. Genes expressed only in storage root (probes Mec1, MALC01-D6 and MALC04-C04) but not in adventicious root. Gene with higher expression in storage root (MALC01-F09 and MALC02-F04) in comparison to adventicious root. Genes with lower expression in storage root (MALC01-A10) in relation to adventicious root. Finally, gene with equivalent level of expresion (MAGL02-H05, MAGL02-B05, and MALC01-B02) in both type of roots.

In the first class of signal, the expression of Mec1 gene confirms our previous result (Souza, 2001; de Souza *et al*., 2002; Carvalho *et al*., 2002), indicating its role in differentiating secondary growth root from non-secondary growth roots. The hybridization signal of clones MALC01-D6 (RZV gene) and MALC04-C04 (TUB gene) may indicate their possible role in the development of tissue originated from cambium (gene RZV) and dehydration of it during maturation (gene TUB) as oberved in potato respectivelly. In addition the TUB gene with its LEA domain may also be involved in ABA induced processes.

The second class of hybridization signal indicates that the differential level of expression of the TCTP gene (clone MALC01-F09) could be related to processes such as cell death program (Bommer and Thiele, 2003) of vessels and laticiferis vessels formation in both type of roots as observed in stem wood formation of poplar (Aterky *et al*., 1998) and the TCTP expression in latex of rubber tree (Gachet *et al*., 1999; Kim *et al*., 2000) respectivelly. Both processes occurs in both type of root tested in our experiment with major differences in their intensities. Therefore these results contribute to understand the expression of genes closelly related to storage root formation. Nevertheless the differential levels of expression of the gene coding for calmodulin (clone MALC02-F04) in both type of roots may be related to the regulation of Ca+ flux in adventicious root as wells as to developmental processes in the tuber formation as have been reported in other plants (Poovaiah *et al*., 1996; Reddy *et al*., 2002).

The third classes of transcription signal observed with the gene coding for extensin (clone MALC01-A10) indicates a dow regulation. Similar result has also been observed in analyses of storage root induction in sweet potato (You *et al*., 2003). Extensins are proteins involved in diverse cellular processes, including cell wall formation. The significance of transcriptional down-regulation of this gene in secondary xylem of storage roots needs to be further examined.

Finally the identical level of expression of the genes Myb transcrition factor (clone MAGL02-H05), 14.3.3 protein (clone MAGL02-B05) and GH1 auxin regulated protein (clone MALC01-B02). It is not know what kind of functions in both type of roots neither if these functions are the same.

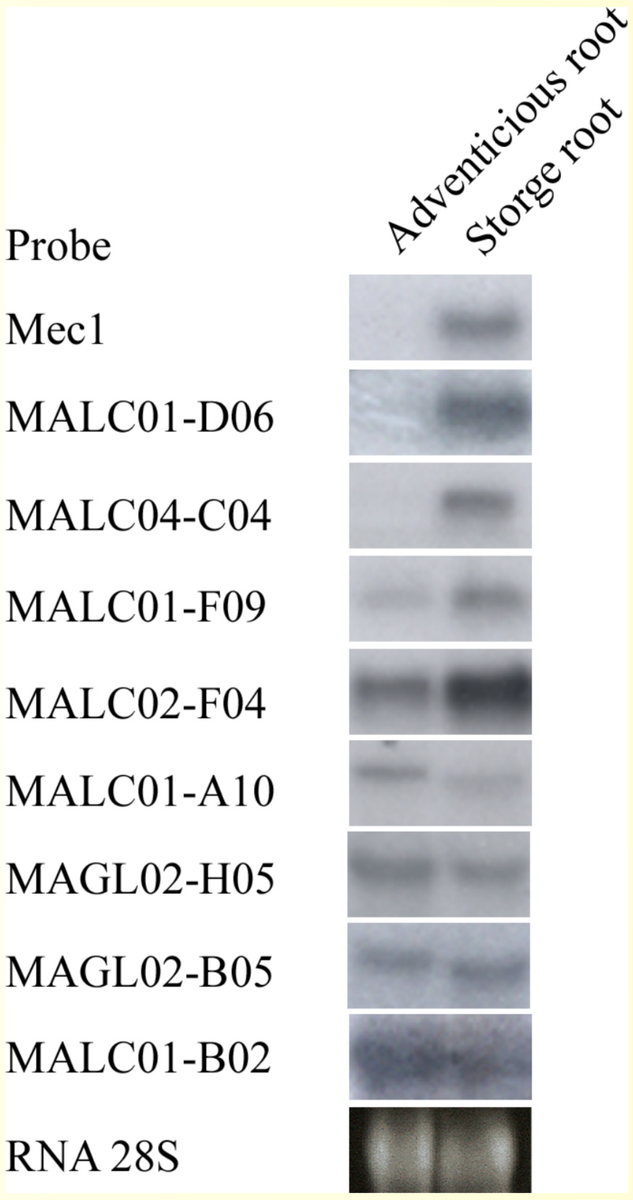

Figure 1: Comparative gene expression study of adventitious (AR) and secondary xylem tissues from storage roots (SR) of cassava by Northern blot analysis. Samples containing about 10 g of total RNA were separated on formaldehyde-agarose gel, transferred to membranes and hybridized with radiolabeled probes as in Table 1. Ethidium bromide stained agarose gel with a ribosomal RNA sample was included as total RNA quantitative control.

CONCLUDING REMARKS

In this work we further screened our cDNA library and identified potential cassava genes related to storage root formation that were transcriptionally regulated. The elucidation of the function of these differentially expressed genes is need, before final conclusion. However, these genes possibly play different roles in the storage organ formation and are under studies toward our intention of isolating specific storage root promoter and enhancers. Our focus is now oriented to isolate of genomic clones and functional analysis.

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