รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาความแตกต่างในการแสดงออกของยีนในใบและ หัวมันสำปะหลัง

โดย ดร. ปัญจภัทร โสจิกุล

เดือน พฤษภาคม ปี 2552

สัญญาเลขที่ MRG5080119.

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สังกัด ภาควิชาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code: MRG5080119

Project Title: mRNA differentially display in leaf and storage root of *Manihot esculenta* Crantz

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Project Period: December 1, 2006 – November 31, 2008

Cassava (*Manihot esculenta* Crantz) is an important food crop in Thailand for exporting and utilizing in starch processing industries. The different starch compositions, granule size, and crystalline structure were observed in cassava root that harvested at different ages. Gaining information in the groups of gene expression at different stages of storage root development would lead to the thorough understanding of genes involving in controlling; the storage root formation & development, starch biosynthesis, and crystalline formation. To unravel genes associated with storage root formation, cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) was employed. The gene expression profiles were compared among fibrous roots and storage roots (cortex and parenchyma) of cassava cultivar Kasetsart 50 (KU 50) at different developmental stages and the expression profiles of leaf were used as a control. From 64 primer combinations, 100 transcript-derived fragments (TDFs) were sequenced, characterized, and then classified into 11 groups by their functions based on deduced amino acid sequences. The 3 main groups were: no similarity (26%), cellular metabolism and biosynthesis (18%) and hypothetical or unknown protein (15%). The differential expressions of 5 storage roots specific TDFs were confirmed by semi-quantitative RT-PCR on 12 week-old wet crop samples: TDFD82 (sulfite reductase), TDFD83 (calcium-dependent protein kinase), TDFD102 (mitotic checkpoint protein), TDFD106 (*ent*-kaurene synthase) and TDFD154 (hexose transporter). They exhibited significant expression in storage roots especially in the initiation and early developmental stages. TDFD106 and TDFD154 may involve in transiently induction of TDFD83 expression, which might play important role in signaling pathway of storage root initiation. TDFD82 potentially played role in cyanide detoxification, which may lead to biosynthesis of aspartate that involved in storage root development. TDFD102 might involve in maintaining genomic DNA integrity via mitotic checkpoint mechanisms during cell division especially at the storage root initiation stage. The full-length gene of these 5 TDFs will be cloned for further functional analysis.

KEY WORDS: CASSAVA / cDNA-AFLP / MANIHOT ESCULENTA / STORAGE ROOT DEVELOPMENT

Executive Summary

From this project, cDNA-AFLP was employed for visualizing cassava storage root's gene expression profiles at different developmental stages. 100 TDFs from 64 primer combinations were sequenced and characterized. Three major groups of differentially expressed TDFs found in this work are unique genes with no similarity (26%), genes encoded for proteins involved in cellular metabolism and biosynthesis (18%) and gene encoded for hypothetical or unknown proteins (15%). Our finding about unique genes and genes encoded for hypothetical or unknown proteins is quite similar to previous reports elsewhere on differentially expressed genes in other plants. The expression of 5 storage roots specific TDFs were confirmed by semi-quantitative RT-PCR on 12 week-old wet crop samples: TDFD82 (sulfite reductase), TDFD83 (calcium-dependent protein kinase), TDFD102 (mitotic checkpoint protein), TDFD106 (*ent*-kaurene synthase) and TDFD154 (hexose transporter). Since they exhibited significant expression in early developmental stages especially at 6 week-old, the results suggested their roles in storage root initiation. TDFD106 and TDFD154 may transiently induce TDFD83 expression, which might trigger storage root initiation signaling. TDFD82 potentially function in aspartate biosynthesis that involved in storage root development. TDFD102 might involve in maintaining genomic DNA integrity during rapid cell division especially at the storage root initiation stage. The full-length sequence of these 5 TDFs will be cloned for further functional analysis particularly effects on storage root development. Gaining information in storage root development would be beneficial in improving cassava storage root production and possibly applied for marker assisted selection in breeding program. In summary, the further knowledge obtained from this project would provide useful information for the variety improvement of cassava with early storage root production cultivar, high storage root yield, and consistent quality.

Acknowledgement

Thank to Thailand Research Fund (TRF-MRG5080119) for the financial support throughout this research.

Materials and Methods

Sample Collection & Processing

Cassava samples, cultivar Kasetsart 50 (KU50) were planting at the Rayong Field Crops Research Center, Ministry of Agriculture and Cooperatives, Rayong. For cDNA-AFLP, the samples: fibrous root, storage root, and leaf were collected at 3, 7, 10, and 12 months after plantation in dry season. The 12 weeks KU50 samples that used in expression pattern analysis were planted every week for 12 weeks in the next season (wet crop) and all of the samples (fibrous root and storage root) were harvested at week 13th and also at 6, 9, and 12 months. For each sample, 6 uniformity cassava plantlets were selected at random. The storage roots were stored at -80°C until used.

RNA Extraction and Quantification

Frozen phenol RNA extraction protocol was used for parenchyma tissue. Modified-CTAB RNA extraction protocol was used for cortex tissue. Concert™ Plant RNA Reagent (Invitrogen) was used for leaf tissue following the manufacture protocol.

Electrophoresis of RNA through Formaldehyde Gel

The method was modified from those of Lehrach et al. (1997), Goldberg (1980), and Seed (1982a). A 1% agarose gel was prepared in 1X MOPS, pH7.0 (200 mM MOPS buffer, 50mM sodium acetate, 20 mM EDTA, pH to 7.0 with NaOH) with 2.7 ml of 37% formaldehyde. RNA sample was mixed with 2 μl of 5X formaldehyde gel running buffer, 3.5 μl of formaldehyde, and 10 μl of formamide. The samples were incubated at 65°C for 15 minutes. The samples were run at 4-5 V/cm for at least 2 hours. To assess the RNA quality, the gel was stained briefly in 0.25-0.5 μg/ml ethidium bromide then examined under UV light.

cDNA Synthesis

First strand cDNA synthesis was carried out using the SuperScript^{TM}III First-Strand cDNA Synthesis Kit (Invitrogen). 2 μg of total RNA or 1 μg of mRNA were used in first strand cDNA synthesis with oligodT25(V) following the company protocol. In briefly, 60 units (U) of RNase OUT (Invitrogen) and 200U of SuperScript^{TM}III (Invitrogen) were added to the reaction and incubated at 50°C for 50 minutes. The enzymes were inactivated at 85°C for 5 minutes then proceeded to the second strand cDNA synthesis.

The first-strand cDNA was treated with 1 unit of RNase H to remove the mRNA template. Then, second-strand cDNA was synthesized with 30U of *E. coli* DNA polymerase I at 25°C for 30 minutes. The short second-strand cDNA fragments were then ligated together by 2.5U of *E. coli* DNA ligase at 16°C for 1 hour. Subsequently, 6U of T4 DNA polymerase were added and incubated at 12°C for 15 minutes to fill up the 3' end. The double-strand cDNA were purified by adding 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1). The upper aqueous phase was collected and purified with 1 volume of chloroform. The double-strand cDNA was then precipitated with 0.6 volume of isopropanol at room temperature for 30 minutes. The cDNA pellet was washed with 70% ethanol and dissolved in 11 μl of sterile-water. One microliter of the cDNA was subjected to PCR analysis and the least was subjected to cDNA-AFLP.

Restriction Enzyme Digestion

This step generates short double-strand cDNA fragments, which are necessary for AFLP adaptor ligation and fragment amplification. The cDNAs were digested with Mse *I*, a fourth-base-cutting restriction enzyme and Eco *RI*, a sixth-base-cutting restriction enzyme. 10U of Mse *I* (NEB) was used to digest the purified cDNA at 37°C for 1 hour. The Mse *I* was inactivated at 75°C for 5 minutes. Then 10U of Eco *RI* (NEB) was added and incubated at 37°C for 1 hour. The Eco *RI* was inactivated at 75°C for 5 minutes.

AFLP

The short double-strand cDNA fragments were ligated with 0.5 nM Eco RI adapters and 5 nM Mse I adapters in the present of 26U of T4 DNA ligase (NEB) at 16°C overnight. The ligase was inactivated at 65°C for 10 minutes. Pre-amplification was carried out using adaptor-specific primers (Eco RI adapter: 5'-GACTGCG TACCAATTCA-3' and Mse I adapter: 5'-GATGAGTCCTGAGTAAC-3'). The 2-3 μl of ligated-products (0.4-0.6 ng) was amplified by 0.2 pmoles/μl of both Eco RI and Mse I primers or

0.3 μM of each pre-selective primer (E-N vs M-N). PCR reaction composed of denaturing at 94°C for 30 seconds, annealing at 51°C for 1 minute and extension at 72°C for 1 minute at a total of 30 cycles.

The pre-amplification product was diluted 50-fold or 100-fold and PCR amplification was carried out on 3.3% of the diluted pre-amplification product using primers with two selective bases on one of each primer (EcoRI+2: 5'-GACTGCGTACCAATTCANN-3' and MseI+2: 5'-GATGAGTCCTGAG TAACNN-3'). PCR reaction composed of denaturing at 94°C for 10 seconds, annealing at 65°C for 30 seconds with 0.7°C temperature increment and extension at 72°C for 1 minute at a total of 13 cycles, following by, denaturing at 94°C for 10 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1 minute with 1 second time increment at a total of 25 cycles. The result was analyzed by running 5 μl of final-amplification fragments on 5% polyacrylamide sequencing gels with 0.5X TBE and visualized by silver staining.

Cloning of Transcription Derived Fragments (TDFs) and Sequence Analysis

The TDFs bands were cut and eluted from the acrylamide gel. The TDFs solution was subjected to PCR with primer combination as used in final amplification. The PCR products were cloned into pBlueScript SK+/- T-Vector. TDFs were subjected to sequence determination. The identity of each differentially expressed gene was determined by homology searches against the GenBank database using the BLAST program[2].

Expression Analysis by Semi-quantitative RT-PCR

TDFs that homologous to the interesting or unknown proteins were selected for expression analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The specific primers were designed according to the sequence of those TDFs. The 12 week-old KU50 samples were used in expression pattern determination. PCR reactions were performed using 60 ng of each cDNA sample. The appropriate cycle numbers of amplification was determined from the lowest cycles, which the PCR product in each tissue could be detected. The PCR condition consisted of 5 minutes at 95°C, n cycles of 30 seconds at 94°C, 30 seconds at Ta and 1 minute at 72°C, and one final extension step of 10 minutes at 72°C. The RT-PCR products were run on 5% acrylamide gel electrophoresis.

Results

Sample Collection & Processing

Cassava cultivar Kasetsart 50 (KU 50) samples planting in dry season at Rayong Agronomic Research Center were collected at 3, 7, 10, and 12 month-old. The samples from this experiment were separated into four groups. First, fibrous roots were collected only from 3 month-old samples since most of them developed into storage root after 3 month cultivation (Figure1). The second and the third types were storage roots, which were separated into two layers; cortex and parenchymatous tissues (Figure 1). The last group was leaf that was used as a control of the housekeeping profile.

For expression analysis, the samples were separated into three groups; fibrous root, intermediate root and storage root (Figure2). The Intermediate root is justified by a diameter at size 0.5-1cm and storage root is at the size more than 1cm. Intermediate 6th week root was the transition stage from fibrous to storage root. The 7th week storage root was the transition stage from intermediate root to storage root (Figure 2).

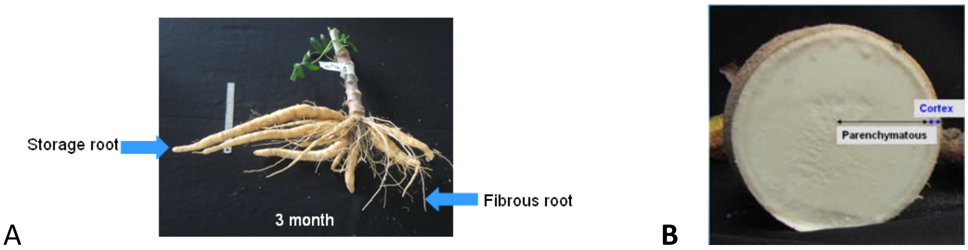
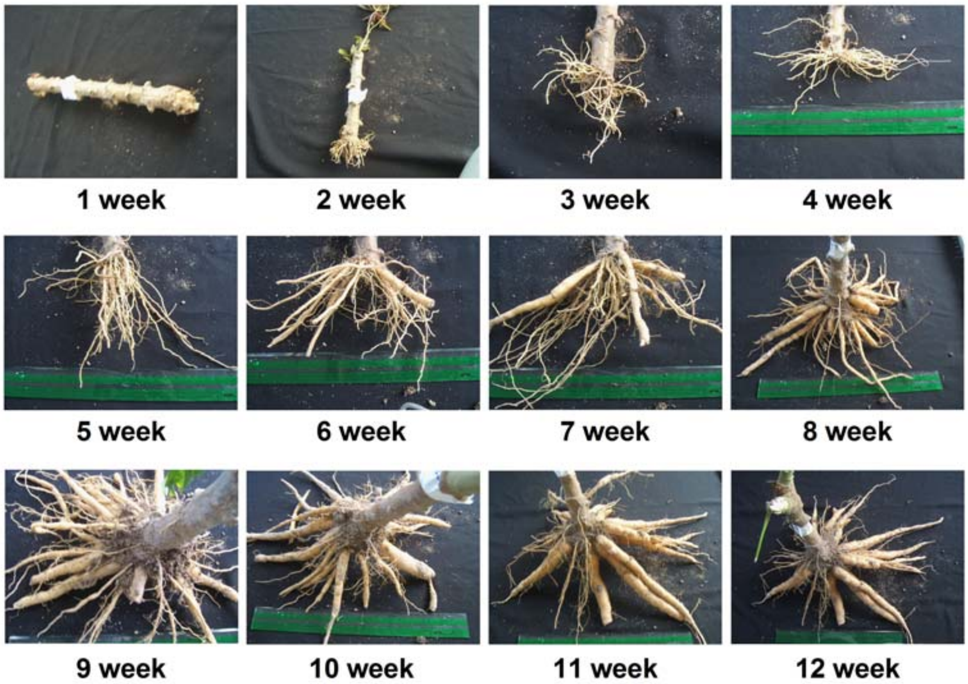


Figure 1 Cassava cultivar Kasetsart 50 (KU 50) A) at 3 month-old and B) two tissue layers of cassava's storage root

RNA Extraction

Cassava storage root tissues contain large amount of polysaccharides and polyphenolic compounds. These compounds have effect on quality of RNA. The RNA samples containing high amounts of polysaccharides and polyphenolic compounds are considered as a poor quality RNA [20, 29]. The polysaccharides and polyphenols are co-precipitated with RNA [32] and interfered with further experimental procedures such as reverse transcription (RT) and cDNA synthesis. 260/280 OD ratio, 260/230 OD ratio, and integrity of RNA bands in gel electrophoresis were used as indicators for high quality of nucleic acid. 260/280 OD ratio at 1.8-2.0 indicates low level of protein contamination in nucleic acid sample. 260/230 OD ratio at ≥ 2.00 indicates low level of chaotropic salt and polysaccharide contamination. By gel electrophoresis, intact RNA with prominent bands of 28S and 18S rRNA without smear bands indicates high quality of RNA without nucleic acid degradation. To overcome these problems, 5 RNA extraction methods were tested. The first method for RNA extraction from Stiekema *et al.,* (1988) was modified. This method is referred to as a "Frozen Phenol method". The second procedure is modified from the potato tuber RNA extraction protocol (The Institute for Genomic Research (TIGR) website (http://www.tigr.org/tdb/potato)). This method; the TIGR method, has been demonstrated to be effective in extracting RNA with minimum polysaccharide and polyphenolic compound contamination. However, the RNA from the TIGR method contained higher level of protein contamination than other tested methods. The third method, Hot Phenol method was developed in order to reduce protein, polysaccharide and polyphenolic compound co-precipitated in RNA.

The Concert^{TM} plant RNA reagent from Invitrogen was tested with all sample tissues. The last method was modified CTAB method.



1 week
2 week
3 week
4 week
5 week
6 week
7 week
8 week
9 week
10 week
11 week
12 week

Figure 2 The 12 week KU50 samples planted in wet season

Frozen Phenol method was found suitable for extracting of RNA from parenchymatous tissue of cassava storage root, since the RNA products contain low polyphenolic and polysaccharide contaminations as suggested by the 260/230 OD ratio (Table 1). Low protein contamination could be found in all methods as suggested by 260/280 OD ratio. Moreover, the Frozen Phenol approach gave approximately 4-fold and 2-fold as much parenchymatous RNA as obtained from the TIGR and Concert^{TM} method, respectively.

For leaf and fibrous root, RNA from Concert^{TM} method gave 260/280 OD ratio about 1.8 to 1.9, which represent low protein contamination. Both of them show 260/230 OD ratios higher than 2.0, the electrophoresis results showed sharp and clear RNA bands represented good quality of RNA (Figure 3A). The process is much less tedious and time-consuming. The Concert^{TM} method was chosen as a method of choice to isolate the RNA from leave and fibrous root samples.

Since high level of polysaccharide and polyphenolic compound, that co-precipitated with total RNA from cortex tissue as a gel-like precipitant or other contaminant from cortex tissue, might interfere with RT reaction and/or PCR. With this problem, various extraction methods were tested with cortex samples. Cortex RNA obtained from TIGR, modified-TIGR, frozen phenol, hot phenol, and Concert^{TM} plant RNA methods were considered as a poor quality RNA, even though in some cases the RNA had high 260/280 (~1.80) OD ratio. Since the cortex RNA could not be successfully amplified by RT-PCR with actin primer sets. Finally, Modified CTAB RNA Extraction method[36] was found amendable for high quality cortex RNA extraction. The gel electrophoresis showed intact RNA with prominent 28S and 18S rRNA bands similar to RNA from other tissues (Figure 3A) with high 260/280 (~1.80) and 260/230 (~2.0) OD ratio (table 1). The extraction buffer of modified

CTAB contains (2% (v/v) β-mercaptoethanol, 2% (w/v) hexadecyltrimethyl-ammonium bromide, 100 mM Tris [tris(hydroxymethyl)-aminomethane]-HCl (pH 7.5), 20 mM EDTA, 2 M NaCl, and 1% (w/v) polyvinylpyrrolidone). The 1% (w/v) polyvinylpyrrolidone in extraction buffer reduced co-precipitation of polyphenolic compound with RNA because polyvinylpyrrolidone formed complexes via hydrogen bonding with polyphenolic compound and was removed with cellular debris[21]. A high concentration of β-mercaptoethanol was used to inhibit RNase activity and prevent sample oxidation. Since phenolic compounds were oxidized and bound to nucleic acids, which caused irreversible damage to RNA[8]. Whereas 2M NaCl caused polysaccharide remained in supernatant while RNA was precipitated[9]. The actin gene and EF1-α gene could be amplified from cortex cDNA as from other tissues (Figure3B and 3C). The result suggested that Modified CTAB RNA Extraction method is suitable for cortex RNA preparation.

Table 1 Results of RNA extraction from various cassava tissues using the Modified CTAB, Frozen Phenol, and Concert^{TM} plant RNA reagent OD 260/280 and 260/230 ratio (in 10mM Tris pH 8.0) indicate quality of nucleic acid. The OD 260/280 ratio at ≥1.80 indicates low protein contamination. The OD 260/230 ratio ≥2.00 indicates low chaotropic salt and polysaccharide contamination.

Tissue parts	Extraction method	OD260/OD280	OD260/OD230	Yield(μg/g FW)
Cortex	Modified CTAB	2.04	2.09	103.91
Parenchyma	Frozen Phenol	1.80	2.30	21.30
Fibrous	Concert^{TM}	1.61	2.51	73.17
Leave	Concert^{TM}	1.80	2.50	450.0

Since RNA is prone to enzymatic degradation by RNase, it is very important to assess the integrity of the cassava RNA extracted. Thus after extraction, the RNA samples obtained were run on 1.2% formaldehyde agarose gels to check for their integrity and presence of degradation, if any. From the Formaldehyde gel electrophoresis shown in Figure 3A, there is no detectable degradation of the RNA isolated from any of those extraction systems. Thus the 28s rRNA bands are of similar intensity to the 18s rRNA bands in each sample run.

The abundant house-keeping actin gene and EF1-α gene were used as probes to monitor the success of the PCR amplification from cDNA. The results of the RT-PCR analysis on the extracted RNA samples are shown in Figure 3B and 3C. The gels clearly demonstrated successful amplification of actin and EF1-α cDNA fragments from the RNA samples tested. Band from genomic DNA showed bigger sized because the primer set was spanning upstream and downstream of intron. RT-PCR results suggested that these RNAs are of high quality, which allows for further downstream enzymatic treatments including the cDNA-AFLP analysis.

Template quality in cDNA-AFLP amplification

In order to validate suitable conditions for cassava cDNA-AFLP, quality of cDNA fragment template for AFLP was tested. From AFLP validation, the amplified fragments showed low number of amplified fragments and inconsistence band intensity (Figure4A). These problems were not caused by inadequate concentration or unequal cDNA template loading in AFLP reaction but because of the remnant of reagents from reverse transcription and second strand cDNA synthesis reaction. Since high-quality differentially expressed patterns with discrete and consistence band intensity (Figure4B) were achieved after apply phenol/chloroform/isoamylalcohol extraction step in addition to chloroform/isoamylalcohol extraction after second strand cDNA synthesis. The contaminated substances that could inhibit polymerase chain reaction in both pre-amplification and final-amplification were removed. Reliability on judgment of up regulated and down regulated TDFs were improved (Figure4B).

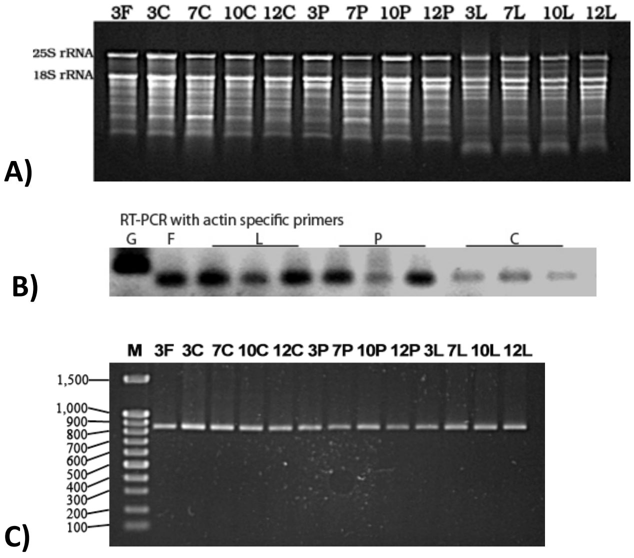


Figure 3 Electrophoresis of RNA and RT-PCR products A) RNA quality visualized on agarose gel electrophoresis; a formaldehyde gel electrophoresis of one-half microgram loading of DNase-treated total RNA samples for cDNA-AFLP from fibrous root, cortex, parenchyma, and leave at 3, 7, 10, and 12 month-old B) Gel electrophoresis showed result of RT-PCR analysis of α-actin cDNA from fibrous, cortex, parenchyma, and leave RNA samples. Genomic DNA was used as a positive control. C) Gel electrophoresis showed result of RT-PCR analysis of EF1-α cDNA; F: fibrous root, L: leave, P: parenchyma, C: cortex, and G: genomic DNA.

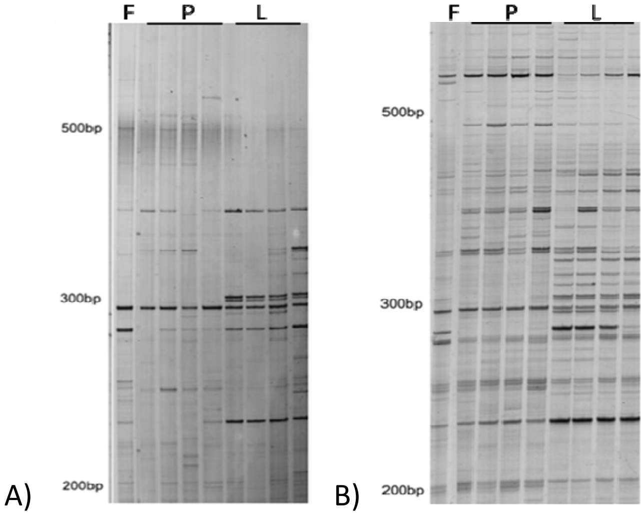


Figure 4 Cassava's cDNA-AFLP patterns amplified with ECA vs MCG. A) AFLP from untreated double-stranded cDNA B) AFLP from phenol/chloroform/isoamylalcohol treated double-stranded cDNA: Results showed discrete and consistence fragments in phenol/chloroform/isoamylalcohol treated double-stranded cDNA. F: fibrous, L: leave, P: parenchyma.

Template quantity in cDNA-AFLP amplification

In order to validate suitable conditions for cassava cDNA-AFLP, concentration of cDNA template for pre-amplification and concentration of pre-selective amplification for final amplification were tested. Difference from AFLP; cDNA-AFLP used RNA as a starting material. RNA was then used to generate cDNA following by AFLP. With lower quantity of starting RNA material than genomic DNA since RNA represented only expressed genes at specific time frame, cDNA fragments at 0.2, 0.6, and 1.0 ng were used in pre-amplification. Low stringent of selective amplification primers was used. *Eco*RI+1: 5'-GACTGCGTACCAATTCAN-3' and *Mse*I+1: 5'-GATGAGTCCTGAGTAACN-3' were used in pre-amplification. Pre-amplification of AFLP fragments was analyzed by running 3 μl of pre-amplification fragments on 1.2% agarose gel. The results showed that 0.6ng of cDNA fragments gave good distribution of pre-amplified fragments (Figure5). *Eco*RI+2: 5'-GACTGCGTACCAATTCANN-3' with *Mse*I+2: 5'-GATGAGTCCTGAGTAACNN-3' were used in final-amplification. The amount of selective amplified template in final amplification reaction is also essential. cDNA-AFLP fingerprints from final amplification with primer combination: E-CA vs. M-CN showed that the 0.6ng of cDNA templates in pre-amplification with 1:50 dilutions of pre-amplified fragments gave discrete differentially expressed band patterns especially at band sizes between 100 to1000 base pairs. Therefore, 0.6ng of cDNA in pre-amplification and 1:50 dilution of pre-amplified fragments in final amplification were used in further experiments.

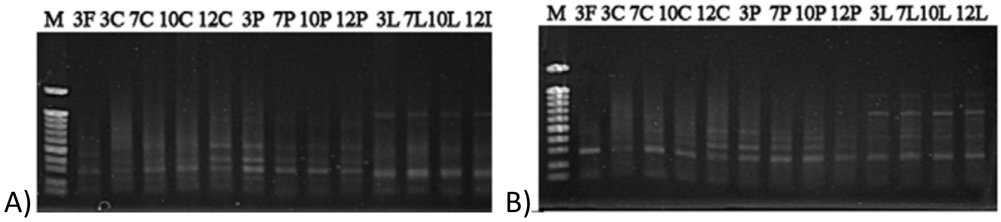


Figure 5 cDNA quality visualized on agarose gel electrophoresis; a gel electrophoresis of pre-amplification of 0.6 ng cDNA-AFLP samples amplified with A) E-C vs M-C B) E-C vs M-G primers from fibrous root, cortex, parenchyma, and leave at 3, 7, 10, and 12 month-old; F: fibrous root, C: cortex, P: parenchyma, and L: leaf

Selection of transcript derived fragment bands (TDFs)

From 32 selective primer combinations tested (Table 2), 31 selective primer combinations showed differentially expressed bands in various tissues (Figure 6). Only one combination showed no differentially expressed band. Expression profiles of cassava storage root at different developmental stages were compared on AFLP patterns from 31 selective primer combinations. TDFs were found expressed in various tissues at different time frame. About 100 TDFs were selected and grouped based on tissue specific expression and expression level as in Table3. For examples (Figure 7), TDFD77 (group 5) was expressed in all tissues. TDFD78 (group13: F-C-L) was down regulated in cortex from 3 to 12 months after planting and absented in parenchyma but had high expression in leaf. TDFD 82 (group 12: F-C-P) highly expressed in cortex and parenchyma but low expression in fibrous with non-detectable expression in leave. TDFD 84, 103, 104, and 112 (group 1: F) expressed only in fibrous root. TDFD 102 (group 2: C) was down regulated in cortex. TDFD 91 and 114 (group 9: C-P) expressed in cortex and parenchyma with non-detectable expression in fibrous root and leaf.

Table 2 The summary of the primer combinations /: primers combinations that showed differentially expressed profiles; X: primer combinations that showed no differentially expressed profile.

Primer combinations	M-CA	M-CC	M-CG	M-CT	M-GA	M-GC	M-GC	M-GT
E-CA	/	/	/	/	/	/	/	/
E-CC	/	/	/	/	/	/	/	/
E-CG	/	/	/	/	/	x	/	/
E-CT	/	/	/	/	/	/	/	/
E-GA	/	/	/	/	/	/	/	/
E-GC	/	/	/	/	/	/	/	/
E-GG	/	/	/	/	x	/	/	/
E-GT	/	/	x	x	/	/	/	/

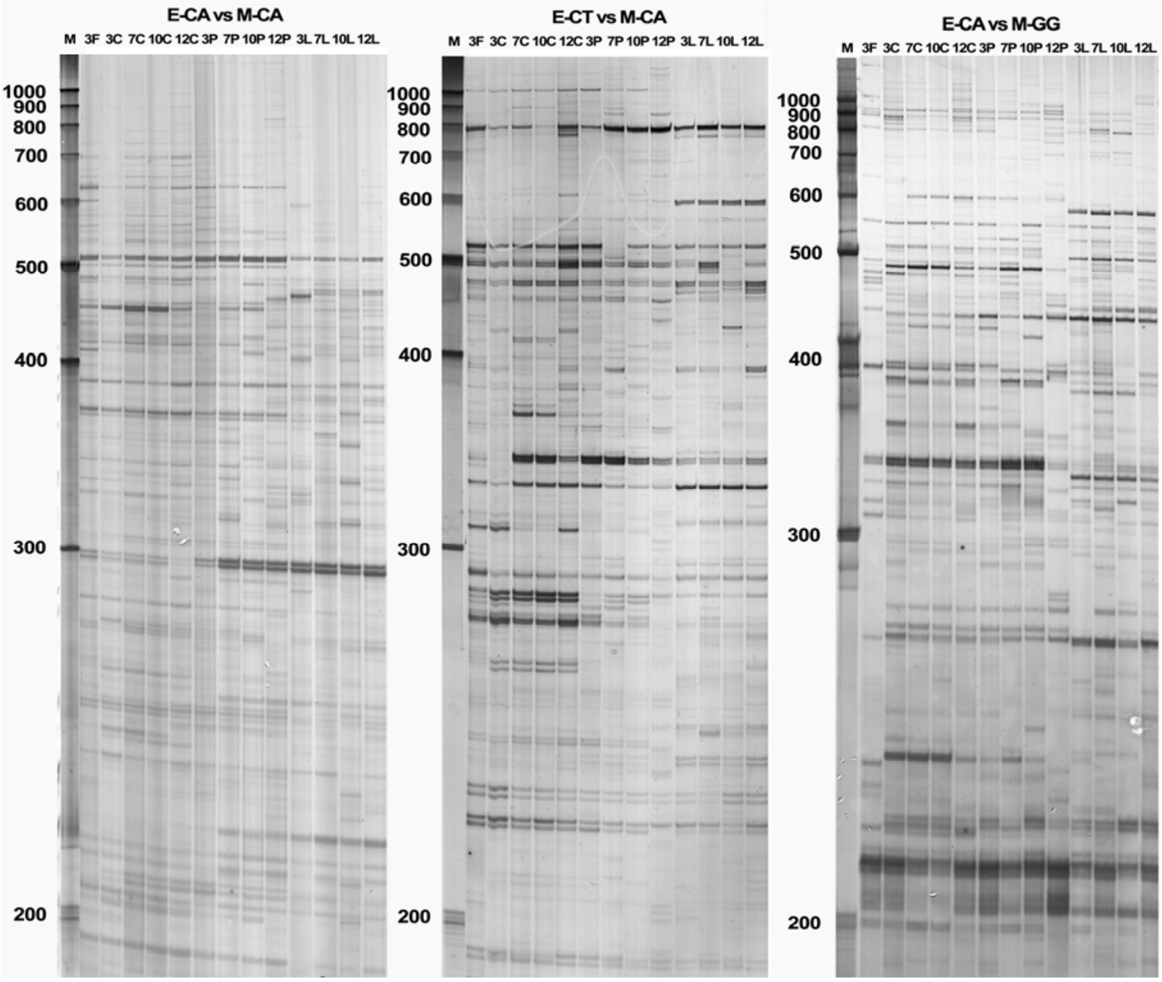
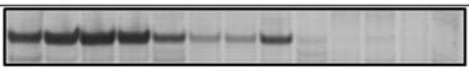
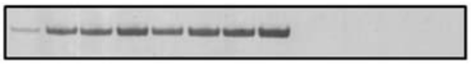


Figure 6 Differentially expressed gene patterns of cassava amplified with E-CA VS M-GA, E-CT VS M-CA, E-CA VS M-GG primer combinations. Pre-amplification template from 0.6 ng cDNA at dilution of 1:50 showed clear transcript derived fragments (TDFs) produced from all samples. (C: cortex, P: parenchyma, L: leaf, and F: fibrous)

Table 3 Classification of TDFs by expression pattern

Group	Expression pattern
	F C P L
1. Differential expressed genes	
2. Root specific genes	
3. Leaf specific genes	

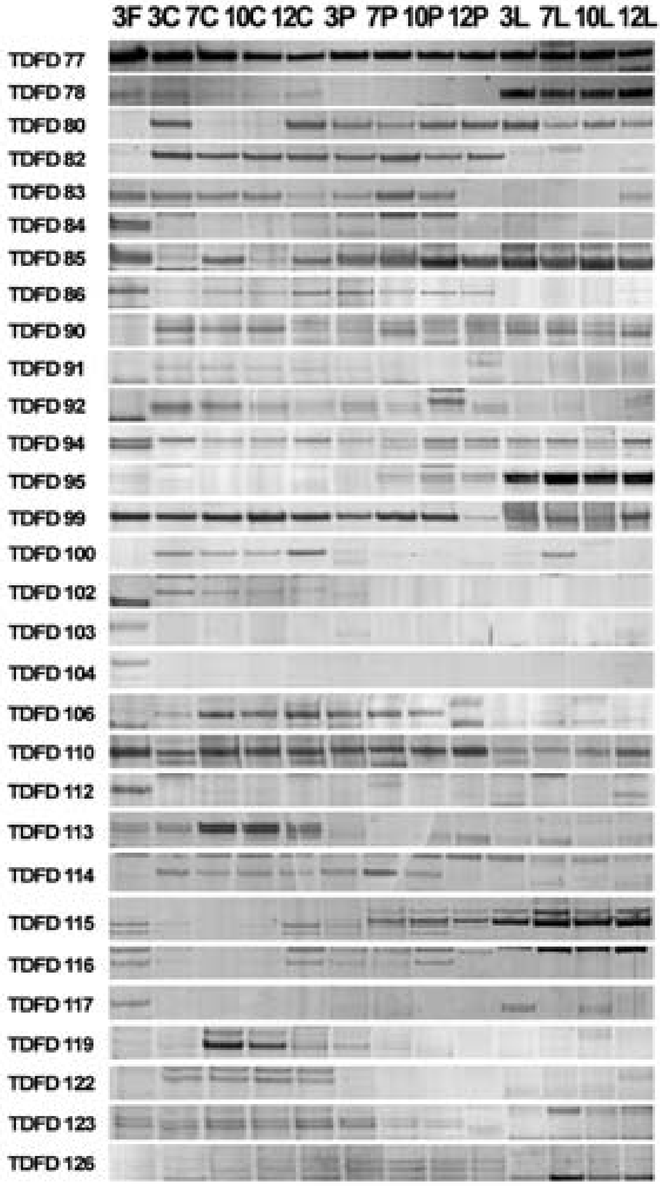


Figure 7 Expression patterns of selected transcription derived fragments (TDFs) from cDNA-AFLP

The selected TDFs were excised from 5% denaturing polyacrylamide gel and then re-amplified by PCR. Re-amplification products that showed PCR product at expected size were cloned into pBluescript T-vector. Then each clone was subjected to sequencing verification. The sequence of each TDF was analyzed by BlastX with NCBI database. One hundred TDFs with size in the range of 200 - 800 bp were identified. Of these, 50 TDFs were isolated from polyacrylamide gel, re-amplified, cloned, and subjected to sequence verification. The 39 TDFs could not be re-amplified. The sequence of each TDF was analyzed against Genbank[5], EMBL[31], TAIR[16], and *Me*EST (Chanvivattana *et al.,* pers. comm.) databases using BLAST analysis[3]. Descriptions of known genes and information from the Gene Ontology (GO)[4] were used to classify TDFs based on their functions (Figure 8).

Table 4 TDFs differentially expressed between leaves and roots identified by cDNA-AFLP (- = no significant homology)

Clone no.	Gene description	BLASTX or BLASTN			*Me*EST			Length
		Accession No.	E-value	Identity (%)	Accession No.	E-value	Identity (%)	(bp)
Cell growth and division								
TDFD-102	Mitotic checkpoint family protein	NP_199799	4E-26	81	-	-	-	217
Cellular metabolism and biosynthesis								
TDFD-17	C-4 sterol methyl oxidase	ABE87434	4E-41	83	C03639#3	700	99	345
TDFD-18	Nucleotide pyrophosphatase-like protein	ABE90599	6E-33	79	C01119#2	3E-73	98	303
TDFD-20	Purple acid phosphates-like protein	NP_188686	4E-69	84	-	-	-	421
TDFD-22	UDP-glucuronosyltransferase	AAB99950	7E-34	65	C08417#14	5E-147	97	292
TDFD-62	Cobalamin-independent methionine synthase	Q42699	1E-57	90	C09349#55	6E-166	92	433
TDFD-75	Anthocyanin 5-aromatic acyltransferase	ABE91268	4E-11	62	CAFI001_O06.b	2E-47	92	230
TDFD-77	Cytosolic phosphoglucomutase	Q9M4G4	3E-41	95	C07332#8	6E-160	98	312
TDFD-82	Sulfite reductase	AAC24584	6E-58	87	CAON012_P18.b	4E-90	99	362
TDFD-90	Catalytic/ methionine gamma-lyase	NP_176647	3E-31	81	C07905#10	5E-129	99	239
TDFD-94	Succinyl-CoA synthetase	CAN70448	1.E-05	92	C06581#6	5E-148	100	267
TDFD-99	Cinnamoyl CoA reductase	AAR83344	6E-42	93	C06053#5	5E-145	99	266
TDFD-100	Ferritin-1, chloroplast precursor	Q8RX97	2E-09	56	C07313#8	4E-107	97	217
TDFD-106	*ent*-Kaurene synthase	ABE87878	2E-29	52	-	-	-	349
Protein synthesis and processing								
TDFD-23	40S ribosomal protein S8-like protein	ABA46755	8E-40	86	C09173#34	5E-150	99	274
TDFD-24	40S ribosomal protein S8-like protein	ABB72814	8E-40	86	C09173#34	5E-145	98	272
TDFD-86	Ubiquitin	AAQ07453	3E-104	100	C09419#77	6E-165	96	588
TDFD-109	Polyubiquitin	ABU40645	7E-29	100	C08631#17	4E-107	100	212

Clone no.	Gene description	BLASTX or BLASTN			*Me*EST			Length (bp)
		Accession No.	E-value	Identity (%)	Accession No.	E-value	Identity (%)	
Energy utilization								
TDFD-56	NADH dehydrogenase	YP_173443	7E-81	86	CA6N003_O21.b	4E-98	97	199
Transcription factor								
TDFD-111	MYB transcription factor	AAM65540	3E-23	68	-	-	-	256
Cellular communication and signaling								
TDFD-30	Protein kinase	ABE82074	4E-67	73	C01119#2	5E-130	97	798
TDFD-63	Non-green plastid inner envelope membrane protein	AAA84891	4E-10	68	C08318#13	2E-45	87	199
TDFD-67	Rab GTPase activator (TBC domain)	NP_200289	3E-34	66	-	-	-	355
TDFD-83	Calcium-dependent protein kinase	CAC82998	8E-44	89	C05409#4	2E-32	81	290
Transport facilitation								
TDFD-78	H$^+$-transporting ATPase	CAN64375	6E-63	95	-	-	-	380
TDFD-89	Pigment Defective Embryo 135 (PDE135) permease	NP_180219	2E-08	72	-	-	-	160
TDFD-110	Ankyrin repeat family protein	AAM64309	2E-38	83	C02025#2	5E-148	100	301
Stress response and aging								
TDFD-29	Retrotransposon protein	BAD18986	4E-12	57	-	-	-	169
Hypothetical and unknown protein								
TDFD-68	Unknown protein	AM485932	2E-14	77	-	-	-	251
TDFD-69	Unknown protein	AM485932	2E-14	77	-	-	-	251
TDFD-72	Hypothetical protein	CAN72178	3E-49	91	-	-	-	332
TDFD-80	Hypothetical protein containing TPR domain	NP_197519	2E-32	75	C05544#4	3 E-65	100	404
No similarity								
TDFD-25	No similarity protein	-	-	-	-	-	-	272
TDFD-26	No similarity protein	-	-	-	-	-	-	268
TDFD-27	No similarity protein	-	-	-	-	-	-	268
TDFD-28	No similarity protein	-	-	-	-	-	-	264
TDFD-32	No similarity protein	-	-	-	-	-	-	264
TDFD-65	No similarity protein	-	-	-	C08318#13	2E-45	87	300
TDFD-66	No similarity protein	-	-	-	-	-	-	220

Clone no.	Gene description	BLASTX or BLASTN			*Me*EST			Length
		Accession No.	E-value	Identity (%)	Accession No.	E-value	Identity (%)	(bp)
TDFD-81	No similarity protein	-	-	-	-	-	-	220
TDFD-85	No similarity protein	-	-	-	C08818#20	5E-131	99	242
TDFD-88	No similarity protein	-	-	-	CAYD008_M09.b	3E-91	100	173
TDFD-91	No similarity protein	-	-	-	C04366#3	3E-85	99	166
TDFD-92	No similarity protein	-	-	-	C09246#40	2E-61	100	224
TDFD-95	No similarity protein	-	-	-	C09462#149	5E-130	100	236
TDFD-103	No similarity protein	-	-	-	C05702#4	5E-131	100	379
Contamination								
TDFD-76	18S small subunit ribosomal RNA	AY757873	6E-101	100	C08978#24	9E-15	94	207
TDFD-84	Transcriptional regulator, MerR family	ZP_01663348	6E-49	100	-	-	-	282
TDFD-104	Cytoplasmic asparaginase I	ABE87878	6E-14	95	-	-	-	283
TDFD-112	Hypothetical protein	ZP_00874766	9E-16	72	CAFI004_N11.b	2E-34	87	191

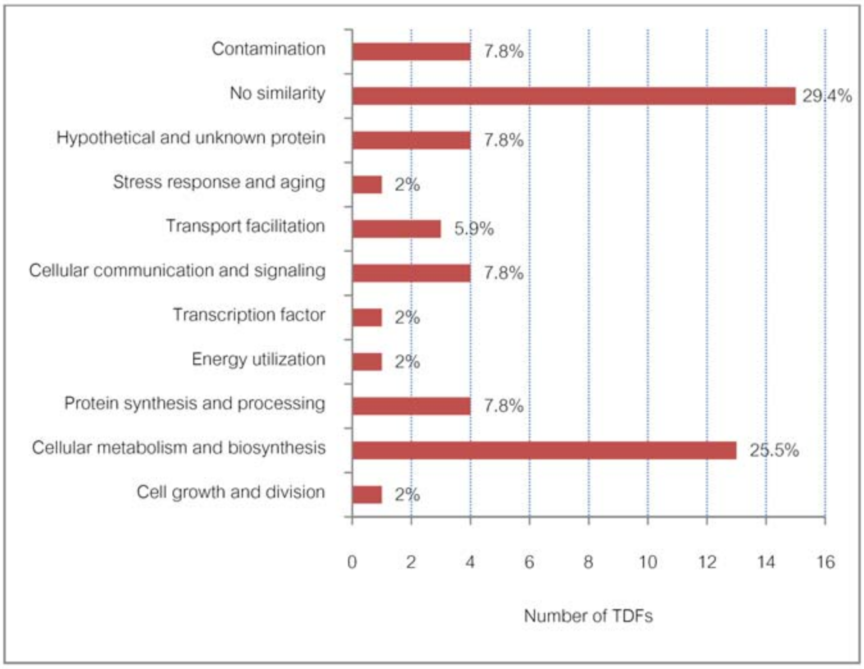


Figure 8 Functional classifications of TDFs from cDNA-AFLP of cassava dry crop.

TDFs that had sequence homology to known genes could be classified into 9 groups by their functions; 1 Cellular metabolism and biosynthesis, 2 Cellular communication and signaling, 3 cell growth and division, 4 stress response and aging, 5 Protein synthesis and processing, 6 Energy utilization, 7. Transport facilitation, 8 Transcription factors, and 9 Contamination (Table 4).

The contamination group comprised of TDFs that homologous to known genes and hypothetical protein from microorganisms, i.e., *Meloidogyne mayaguensis* (TDFD-76), *Ralstonia pickettii* (TDFD-84), *Salmonella enteric* (TDFD-104), and *Streptococcus suis* (TDFD-112). 28 TDFs (55% of sequenced TDFs) were found homologous to known genes or proteins (BLASTN or BLASTX) (Table 4). While 4 fragments (7.8% of sequenced TDFs) were matched to hypothetical or unknown genes, and the rest 14 fragments (29.4% of sequenced TDFs) represented no significant similarity to any proteins in the database (Figure 8).

5TDFs were chosen for further expression analysis by semi-quantitative RT-PCR

TDFD82 (Sulfite reductase), TDFD83 (Calcium-dependent protein kinase), and TDFD154 (Hexose transporter) were expressed predominantly in roots but rarely expressed in leaf. They were highly expressed in early storage root development from fibrous root to intermediate root and to early storage root (7 week-old). The expression was reduced more than 2.5 folds from 5 week fibrous to 12 week storage root. Then the expressions were dramatically decreased in 24 week-old (6 month) root that the root was fully developed into storage root (decrease 3.5 to 4 folds from 5 week fibrous and 1 to 1.5 folds from 12 week storage root) and continue expressed in low level after 24 weeks (Figure9).

TDFD106 (*ent*-Kaurene synthase) was highly expressed in 5 week-old fibrous and 6 week-old intermediate root. Then TDFD106 was dramatically decreased after 6 weeks that root became storage root (2 to 2.5 folds) and maintained in low level until 12 weeks. The expression was rarely detectable in storage root after 24 weeks (Figure9) and in 3-12 month-old leaf.

TDFD-102 (Mitotic checkpoint family protein) was up regulated at intermediate 6 week root and significantly decreased after storage root initiation (Figure 10).

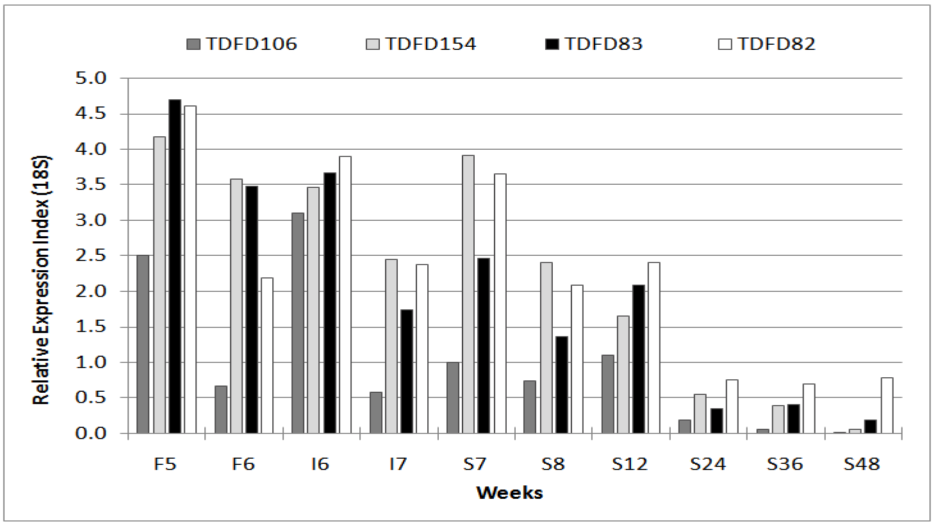


Figure 9 Expression analysis of TDFD106 (▓) similar to *ent*-Kaurene synthase, TDFD154 (░) similar to Hexose transporter, TDFD83 (■) similar to CDPK1, and TDFD82 (□) similar to sulfite reductase by RT-PCR: The graph shows relative expression index between TDFD and 18S in 12 week samples (F = fibrous roots, I = intermediate roots, S = storage roots)

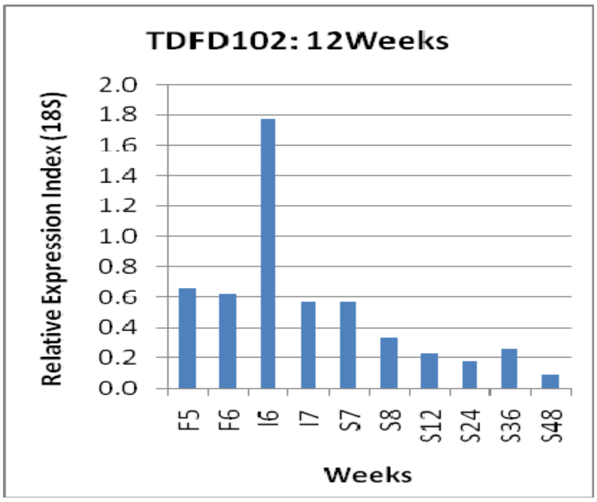


Figure 10 Expression analysis of TDFD-102 similar to mitotic check point gene family by RT-PCR. The graph shows relative expression index between TDFD-102 and 18S in 12 week samples (F = fibrous roots, I = intermediate roots, S= storage roots)

Discussion

The ent-kaurene synthase and CDPK like protein play role in the onset of cassava storage root development

TDFD-106 was homologous to *ent*-kaurene synthase (KS) from *Lactuca sativa*. The KS and copalyl diphosphate synthase (CPS) regulate biosynthesis of *ent*-kaurene from geranylgeranyl diphosphate (GGDP) in proplastids[13, 15]. The over-expression of either AtCPS or AtKS enhanced accumulation of *ent*-kaurene and *ent*-kaurenoic acid. The *ent*-kaurene is the first committed step of gibberellins (GAs) biosynthesis. GAs are a large family of diterpenoid compounds. Some of which are bioactive plant growth regulators that control diverse biological processes[18]. In wheat, pea, and pumpkin, CPS and KS are localized in proplastids of meristematic tissue in vegetative plant organs, in developing chloroplasts and in leucoplasts but CPS and KS are not found in mature chloroplasts[1]. The *ent*-Kaurene synthesis takes place in leucoplasts of the rapidly dividing endosperm nucellus[1, 18].

TDFD106 transcript was significantly up-regulated at the initiation stage of storage root development (F5 and I6) and extensively down-regulated to a low level during early developmental stage (S7-S12) and rarely expressed in late developmental stage (S24-S48; figure9). The TDFD106 expression show related pattern to the endogenous GA_1 level found in *in vitro* induced potato tuber. The GA_1 was up-regulated at the onset of induced stolon (2^{rd} day after tuber induction with 8% sucrose) in root meristem and sharply decreased during swelling stolon and tuber enlargement as the tuber growth was permitted at the low level of GA_1[34].

In potato, GA might induce production and transportation of tuber initiation signal[28] and also exogenous treatment of GA transiently induced stCDPK1 expression in swelling stolon[11, 23]. Expression pattern of TDFD83 (CDPK like protein) was consistent with that of TDFD106. TDFD83 showed high expression at the initiation stage of storage root development and gradually down-regulated during early developmental stage. Moreover, TDFD83 was dramatically decreased at S24 (6 months) when the root was fully developed into storage root and expressed in the minimal level through the late developmental stage as of the TDFD106. TDFD-83 may play an important role in signaling pathways involving in storage root initiation. *St*CDPK1 transcript is up-regulated at apical meristem of early swelling stolon in responded to 8% sucrose and down-regulated in the differentiated storage tissue where starch granules were accumulated[24]. StCDPK1 protein was accumulated at a high level in early swelling stolon and was strongly induced after 8% sucrose treatment[11]. Antisense of *St*CDPK1 showed early tuberization phenotype when treated with 8% sucrose[11].

Hexose transporter involves in initiation stage and early storage root developmental stage

Sugars play crucial role as cellular carbon sources in energy metabolism and signaling molecules. Sugar metabolism is a dynamic process. Metabolic fluxes and sugar concentrations dramatically alter both during development and in response to environmental signals[6]. In plants, sucrose is generated by photosynthesis and carbon metabolism in source and sink tissues. Sucrose is transported between source and sink via phloem. In sink tissues, sucrose is imported into cells through plasmodesmata (symplastic transport) or cell wall (apoplastic transport). Several sucrose are cleaved into monosaccharides, glucose and fructose, by an extracellular invertase[26, 27]. Extracellular monosaccharides are taken up by hexose transporters that are plasma membrane carriers that function as proton (H^+) / hexose symporters[7].

TDFD-154 was (91%) identical to hexose transporter from tomato (*Solanum lycopersicum*), grapevine (*Vitis vinifera*), and *A. thaliana*. TDFD-154 may function in the monosaccharide retrieval. TDFD-154 may gave further insight into cassava storage root development and starch biosynthesis since it showed high expression at initiation stage and early developmental stage.

There are 3 isoforms of hexose transporter (LeHT1, LeHT2, and LeHT3) in tomato. LeHT2 expressed at relatively high level in source leaves and in flowers whereas LeHT1 and LeHT3 expressed predominantly in sink tissues; young fruit and root tips[12]. In grapevine (*Vitis vinifera*), 5 hexose transporters (VvHT) were functional characterized coordinately to cell wall invertase gene (VvcwINV) between source and sink organs[14]. VvHT1, VvHT2, and VvHT3 were high expressed in berries. VvHT1 showed high expression in early berry development whereas VvHT2 and VvHT3 expression was elevated during sugar accumulation phase. In Arabidopsis, there are 14 genes encoding sugar transporters (AtSTPs). The AtSTPs were almost found in sink tissues with one exception; AtSTP3 that was expressed only in green tissues. AtSTP4 gene was expressed in root tips, pollen tubes, and leaves. AtSTP4 expression is, strongly enhanced in response to wounding and to pathogen attack. In addition, this gene showed coordinated expression with invertase gene in the host response to powdery mildew infection[10]. These observations suggested that hexose transporters play important role in sink organ development and express in correlated to cell wall invertase. In this experiment, RT-PCR showed that TDFD154 highly expressed at the initiation stage and early stage of storage root development, which conforms with the preferentially expression of this gene family in the sink organ of other plants. Thus, TDFD-154 is more likely involved in the monosaccharide upload needed for cell differentiation and starch accumulation. Interestingly, the expression pattern of TDFD154 was related to that of TDFD83 (Figure 10).

The result suggested that enhancing hexose transport into the root, thus increase sucrose, might enhance CDPK like protein expression in initiation stage of storage root development. This finding consistent with previous reports that sucrose could enhance StCDPK1 expression that induced stolon to tuber transition[11, 24] and tuber differentiation[34].

Sulfite reductase may play role in assimilation of inorganic sulfur into cyanide which is required for storage root formation in cassava

TDFD82 cDNA-AFLP pattern showed expression only in cassava root (Figure7) has sequence similarity to sulfite reductase (SiR) aligned within nitrite/sulfite reductase 4Fe-4S domain. TDFD 82 was highly expressed in early storage root development and reduced after fully developed into storage root and stably expressed over the 12 month storage. In higher plants, SiR localizes in plastids but not in mature chloroplast. Assimilation of sulfur is one of the most important metabolic processes. Sulfate ions are up-taked from soil by sulfate transporter(s). The sulfate ions are activated by ATP sulfurylase to form adenosine 5'-phosphosulfate (APS). Then APS is converted into 3'-phosphoadenosine-5'-phosphosulfate (PAPS) by APS kinase. APS are also reduced to sulfite by APS reductase. SiR transfers 6 electrons from ferredoxin to sulfite to produce sulfide (Figure 11). SiR assimilates inorganic sulfur into various sulfur-containing compounds, such as amino acids, lipids, and coenzymes. As the product of SiR, sulfide is further incorporated with O-acetylserine (OAS) to form cysteine[17, 35]. Cysteine acts as a sulfur donor for diverse molecules that play various roles in protein synthesis, in plant defense against biotic and abiotic stress, in sulfur transport and storage[19, 25]. Cyanogenic glucoside, a secondary metabolite in cassava, functions as a plant defense metabolite and as a nitrogen source for amino acid biosynthesis during root development. The assimilation of cyanide with cysteine into aspartate was required for storage root formation in cassava and this detoxification mechanism was needed when the amount of cyanide was excess (Figure 11)[30].

Mitotic checkpoint protein might play role during rapid lateral proliferation at initiation stage of storage root development

The cell cycle is tightly regulated by checkpoint mechanisms including the DNA damage checkpoint and the mitotic checkpoint, which arrest cell cycle progression in case of unwanted events such as DNA damage and defects in chromosome alignment. Defects in cell cycle checkpoint mechanisms cause genomic instability. DNA damage, in the form of single-stranded DNA, is created when the replication fork is physically blocked by DNA lesions or during the process of DNA repair (e.g., homologous recombination, nucleotide excision repair, and mismatch repair). These damage sensors then activate downstream checkpoint mechanisms to prevent progression into the next phase of the cell cycle as well as up regulate DNA repair[22]. TDFD 102 similar to mitotic checkpoint protein from Arabidopsis. This protein family consists of several eukaryotic mitotic checkpoint proteins (Mitotic Arrest Deficient; MAD). The mitotic spindle checkpoint monitors proper attachment of the bipolar spindle to the kinetochores of aligned sister chromatids and causes cell cycle arrest in pro-metaphase when failures occur. Therefore, mitotic checkpoint protein is crucial for maintaining genomic DNA integrity. TDFD-102 was up-regulated at intermediate 6 week root and dramatically decreased. It might involve in maintaining genomic DNA integrity via mitotic checkpoint mechanisms during rapid lateral proliferation at initiation stage of storage root development. This lateral meristematic, precursor of vascular parenchyma, undergoes DNA replication following by cell differentiation[33].

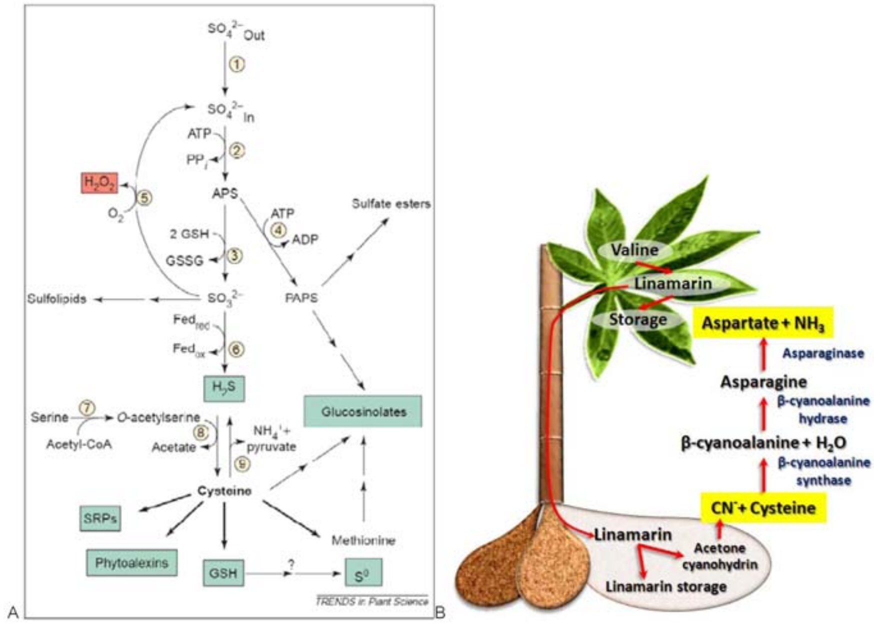


Figure 11 A.) Sulfur assimilation as a platform for the biosynthesis of sulfur-containing defence compounds (SDCs) SDCs are H_2S, glucosinolates, sulfur-rich proteins (SRPs), phytoalexins, glutathione (GSH), and elemental sulfur (S_0). 1: sulfur transporters, 2: ATP sulfurylase, 3: APS reductase, 4: APS kinase, 5: sulfite oxidase, 6: sulfite reductase, 7: serine acetyl transferase, 8: O-acetyl (thiol) lyase, and 9: desulfhydrases; GSH: glutathione, PAPS: 3'-phosphoadenylylsulfate[25]. B.) The transport of linamarin from leaves to roots and its metabolism to produce asparagines[30].

In conclusion, cDNA-AFLP could illustrate cassava's cDNA differentially expressed genes. The results from expression pattern analysis suggested that TDFD106 (*ent*-kaurene synthase) and TDFD154 (hexose transporter) may induce TDFD83 (CDPK) expression and then enhance storage root initiation and rapid cell proliferation at 6 weeks after plantation. Then TDFD102 (Mitotic checkpoint family protein) may assist in maintaining genomic DNA integrity during rapid lateral proliferation at storage root initiation stage. While TDFD82 (sulfite reductase) may involved in assimilates inorganic sulfur amino acids, lipids, and coenzymes to facilitate rapid cell growth and development. Full-length cDNA of TDFD82, TDFD83, TDFD106, TDFD102, and TDFD154 will be cloned and use in further function analysis. The TDFs that have sequence similarity to genes in starch biosynthesis pathway, hormone regulation, and transcription factors are of interest and their expression patterns will be further investigated. The function of interested genes would be further studied by over-expression and/or suppression. A better understanding of these genes should add some knowledge on storage root development in this important crop and later may extend to its application.

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Output

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   Sojikul, P., Thaiprasit, J., Intawong, B., Kongsawadworakul, P., Viboonjun, U., Triwitayakorn, K., Narangajavana, J., and Svasti, M.R. J Analysis of differentially expressed genes of cassava (KU50) during storage root development (in prep.)

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   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

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