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Application of a Modified cDNA-AFLP Technique to Screen Drought-Stress Induced Genes in Cassava (*Manihot esculenta* Crantz)

Xin C[1,2*], Yuyang W[1,2], Xuelin Q[1,2], Changying Z[1,2], Zhiqiang X[1,2], Cheng L[1,2] and Wenquan W [1,2*]

[1]*The Institute of Tropical Bioscience and Biotechnology (ITBB), Chinese Academy of Tropical Agricultural Sciences (CATAS), Haikou, PR China*
[2]*Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Haikou, PR China*

Abstract

We applied a modified cDNA-AFLP technique by using cassava leaf samples under drought treatment, and obtained over 100 transcript-derived fragments (TDFs) which showed significant differentially expressed during the process of drought treatment and 63 TDFs of them were cloned, sequenced and annotated. The results suggested that the recognized site of *Mme*I enzyme was integrated into double-strand cDNAs, and the 5' ends of TDFs that got by the modified technique were nearer to the transcript start site comparing with the previous method. Furthermore, most differentially expressed TDFs (DE-TDFs) really up-regulated or down-regulated through qRT-PCR validation and the homologies of part TDFs involved in or responded to drought tolerance. In general, the modified cDNA-AFLP technique has been established preliminary, and the genetic information of these TDFs needs further mining.

Keywords: Cassava; Drought; cDNA amplified fragment length polymorphism; Transcript-derived fragment

Abbreviations

cDNA-AFLP: cDNA Amplified Fragment Length Polymorphism; TDF: Transcript-Derived Fragment; Qrt-PCR: Quantitative Reverse Transcript Polymerase Chain Reaction; TSS: Transcription Start Sites; M: *Mme*i; E: *Eco*ri; B: *Bsty*i; ME, MM, MB, BE, EE: The Primers Pairs Basing On The Adapters Of Different Enzyme Combination

Introduction

Cassava (*Manihot esculenta* Crantz) was a woody shrub of the Euphorbiaceae, originated from tropical America, called one of the three biggest tubers along with potato and sweet potato. It was an important staple crop worldwide and consumed by 600 million people. It could grow on very poor soils under prolonged drought for more than six months, reduced its leaf canopy and transpiration water loss, but its attached leaves remained photosynthetically active, though at greatly reduced rates and this drought tolerance mechanism led to high crop water use efficiency values [1]. The cDNA amplified fragment length polymorphism (cDNA-AFLP) technique was a large-scale detected technology of differential gene expression, and didn't need the information of genome sequence [2,3]. Since its appearance, it was widely applied in disease resistance, nutrition stress, specific developmental stage and organ, etc, obtained many valued transcript-derived fragments (TDFs), and they had highly homologous to functional genes or unknown genes [4-7]. In order to get more information by cDNA-AFLP, some researcher optimized its enzyme combinations by AFLP inSilico, and found that *Bst*YI/*Mse*I was optimal restriction enzyme combination, could obtain more than 60% of all transcripts in tobacco [8]. In this study, a modified cDNA-AFLP technique would be established and used to screen the drought-stress induced genes in cassava. We applied the SMART™ technology to synthesize single- and double-strand cDNA, and integrated the recognized sequence (TCCRAC) of restriction enzyme *Mme*I in the 5'end of the synthesized double-strand cDNA, anchored a cutting site at the transcription start sites (TSS). Then, most TDFs that drought stress induced would start at the TSS and easy to obtain their full-length cDNAs in cassava.

Materials and methods

Plant materials

The cassava varieties SC124 and KU50 were preserved in cassava germ plasma of Chinese Academy of Tropical Agricultural Sciences in Wenchang City of Hainan Province, China. The potted seedling of SC124 and KU50 were placed in greenhouse with soil maximum moisture capacity of 90% and fertilized with Hoagland's solution [9]. After 2 months of planting, the uniform potted seedlings were subjected to drought stress treatment. The about 40 uniform potted seedlings were subjected to two types of drought treatment: 1) Drought treatment (DT): no water until an obvious symptom of drought appearing, total RNAs were collected at 6d, 8d, 10d and 12d after beginning drought treatment; 2) normal condition (NC): watering to soil maximum moisture capacity of 90% every day, total RNA was collected at 0d, 6d and 12d. All RNA samples were extracted from the leaves two individuals and used for cDNA-AFLP analysis, and further quantitative RT-PCR assays.

Modified cDNA-AFLP technique

Double-strand cDNA synthesis: The restriction enzyme *Mme*I was a key player in this modified cDNA-AFLP technique, and its

***Corresponding author:** Chen Xin, The Institute of Tropical Bioscience and Biotechnology (ITBB), Chinese Academy of Tropical Agricultural Sciences (CATAS), Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Haikou, PR China, Tel:+86-898-66890978; Fax: +86-898-66890978; E-mail: chenxin@itbb.org.cn.

Wang Wenquan, The Institute of Tropical Bioscience and Biotechnology (ITBB), Chinese Academy of Tropical Agricultural Sciences (CATAS), Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Haikou, PR China, Tel: +86-898-66890978; Fax: +86-898-66890978; E-mail: wangwenquan@itbb.org.cn.

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cutting site was back 20bp to C of the recognized site on forward strand, and back 18bp on reverse strand (Figure 1A). Refer to SMART™ cDNA Library Construction Kit (Clontech, BD, USA), its 5' SMART IV™ Oligonucleotide primer was changed and introduced a *Mme* I Recognized site TCCGAC in it, now its sequence was AAGCAGTTCCGACTGGTATCAACGCAGAGTggg-3' (Figure 1B), and the SMART CDS III/3' PCR primer was simplified to $dT_{30}N_1N_2$ (N_1=A, G, C; N_2=A, T, C, G). Then the double-strand cDNA was synthesized with these two primers (Figure 1C and D).

Double-strand cDNA digest

Three restriction enzymes, *Mme*I, *Eco*RI and *Bst*YI, were used to digest double-strand cDNA, and the reaction system was follow: double-strand cDNA 10μl, NEB buffer4 2.0 μl, *Mme*I 1.0 μl, *Eco*RI 1.0 μl, *Bst*YI 0.5 μl, ddH_2O 5.5 μl, total 20.0 μl. The reaction system was incubated at 37°C for 2 h, 60°C for 1h, and 80°C for 20min to inactivate enzyme activity in water-bath.

Adapter ligation, pre- and selective amplify

Digested double-strand cDNA 10.0 μl, 10 × T4 DNA ligase buffer 2.0 μl, T4 DNA ligase 1.0 μl, *Mme*I adapter (50 μM) 1.0 μl, *Eco*RI adapter (25 μM) 0.5 μl, *Bst*YI adapter (25 μM) 0.5 μl, and ddH_2O 3.0 μl. The ligation system was incubated at 16°C overnight in water-bath (Figure 1E).

Mme I adapter: 5'-GTCCTCACAACGATTCCACAGG-3'

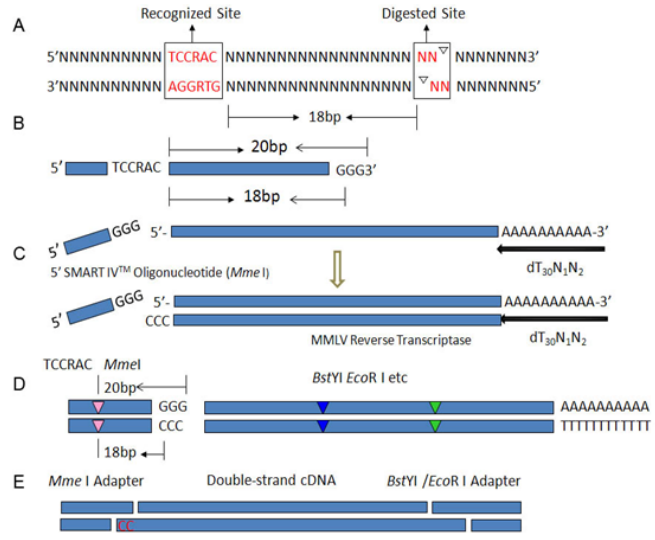
3'-CAGGAGTGTTGCTAAGGTGT-5'

EcoR I adapter: 5'-CTCGTAGACTGCGTACC-3'

3'-CATCTGACGCATGGTTAA-5'

BstY I adapter: 5'-CCGCGTTAACCGAGAT Pu-3'

3'-GGCGCAATTGGCTCTAPyCTAG-5'



A: the recognized site and digest site of *Mme*I; B: the recognized site of *Mme*I in 5'SMART™ Oligonucleotide primer; C: synthesize the single-strand cDNA with $dT_{30}N_1N_2$, 5' SMART IV™ Oligonucleotide (*Mme*I) and MMLV Reverse Transcriptase; D: the full-length double-strand cDNA has the recognized site of *Mme* I at its 5'end; E: The cohesive terminus double-strand cDNA links to its three corresponding adapters.

Figure 1: The key steps of modified cDNA-AFLP technique.

The ligation products were diluted 50 times as template for pre-amplification. The pre-amplification with non-selective nucleotide primer pairs was performed for 26 cycles with the cycle profile: a 30 s DNA denature step at 94°C, a 30 s annealing step at 56°C, and a 60 s extension step at 72°C. The selective amplification with three selective nucleotides prime pairs was performed for 36 cycles with the cycle profile: a 30 s DNA denature step at 94°C, a 30 s annealing step, and a 60 s extension step at 72°C. The anneal temperature at the first cycle was 57°C, was subsequently reduced each cycle by 0.3°C for the next 10 cycles, and was continued at 54°C for the remaining 26 cycles. All amplification reactions were performed in a Biometra Thermocycler (Biometra Corp, Göttingen, GER). The primers of the three enzymes for pre- and selective PCR amplification were list in Table 1.

Cloning and sequencing of drought stress induced TDFs: The selective amplification PCR products were analyzed on a 6% denaturing polyacrylamide gel, and were silver stained following the manufacturer's instructions for sequencing kit Q4310 (Promega Corporation, USA). The interested TDFs were excised from the gel and eluted in 50 μl ddH_2O overnight. The eluted DNA was amplified by using its corresponding selective-amplification primer pairs. The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced by Augct Company (Beijing, China) using an ABI377

c	Sequence (5'-3')	Primers Code	Sequence (5'-3')
	ACAACGATTCCACAGG		
	ACAACGATTCCACAGGtgc		
	ACAACGATTCCACAGGtga		
	ACAACGATTCCACAGGtac		
M	ACAACGATTCCACAGGtag	Egcg	GACTGCGTACCAATTCgcg
Mtgc	ACAACGATTCCACAGGtat	Egac	GACTGCGTACCAATTCgac
Mtga	ACAACGATTCCACAGGttg	Ecat	GACTGCGTACCAATTCcat
Mtac	ACAACGATTCCACAGGtta	Ecag	GACTGCGTACCAATTCcag
Mtag	ACAACGATTCCACAGGtcg	Ecaa	GACTGCGTACCAATTCcaa
Mtat	ACAACGATTCCACAGGtca	Eccg	GACTGCGTACCAATTCccg
Mttg	ACAACGATTCCACAGGcta	Eccc	GACTGCGTACCAATTCccc
Mtta	ACAACGATTCCACAGGcat	Ecct	GACTGCGTACCAATTCcct
Mtcg	ACAACGATTCCACAGGatc	Ecca	GACTGCGTACCAATTCcca
Mtca	ACAACGATTCCACAGGact	Ecgg	GACTGCGTACCAATTCcgg
Mcta	CGTTAACCGAGATPu GATCPy	Ecgc	GACTGCGTACCAATTCcgc
Mcat	CGTTAACCGAGAT(a/t)GATC(t/a)	Ectt	GACTGCGTACCAATTCctt
Matc	AGT	Ecta	GACTGCGTACCAATTCcta
Mact	CGTTAACCGAGAT(a/t)GATC(t/a)	Ectg	GACTGCGTACCAATTCctg
B	AGC	Ectc	GACTGCGTACCAATTCctc
Baagt	CGTTAACCGAGAT(a/t)GATC(t/a)	Ecga	GACTGCGTACCAATTCcga
Baagc	GAC	Eata	GACTGCGTACCAATTCata
Bagac	CGTTAACCGAGAT(a/t)GATC(t/a)	Etat	GACTGCGTACCAATTCtat
Bagca	GCA	Eact	GACTGCGTACCAATTCact
Bgagc	CGTTAACCGAGAT(a/t)GATC(t/a)	Eaca	GACTGCGTACCAATTCaca
Bgagt	AGC	Eagt	GACTGCGTACCAATTCagt
Bggac	CGTTAACCGAGAT(a/t)GATC(t/a)	Eatc	GACTGCGTACCAATTCatc
Bggca	AGT	Eatg	GACTGCGTACCAATTCatg
E	CGTTAACCGAGAT(a/t)GATC(t/a)	Eaga	GACTGCGTACCAATTCaga
Egtc	GAC	Egat	GACTGCGTACCAATTCgat
Egtg	CGTTAACCGAGAT(a/t)GATC(t/a)	Egta	GACTGCGTACCAATTCgta
Eacg	GCA	Etac	GACTGCGTACCAATTCtac
Etgc	GACTGCGTACC AATTC	Etag	GACTGCGTACCAATTCtag
Egag	GACTGCGTACCAATTCgtc	Etga	GACTGCGTACCAATTCtga
Egca	GACTGCGTACCAATTCgtg	Etgt	GACTGCGTACCAATTCtgt
Ecgt	GACTGCGTACCAATTCacg	Eagc	GACTGCGTACCAATTCagc
Ectc	GACTGCGTACCAATTCtgc	Egct	GACTGCGTACCAATTCgct
	GACTGCGTACCAATTCgag		
	GACTGCGTACCAATTCgca		
	GACTGCGTACCAATTCcgt		
	GACTGCGTACCAATTCctc		

Note: M, E and B represent the primers of pre-amplification which were designed basing on the adapters of *Mme*I, *Eco*RI and *Bst*YI enzyme, and the lower-case letters are the added selective nucleotides.

Table 1: The pre- and selective primers used in this study.

automated DNA Sequencer (Perkin-Elmer corporation, MA, USA).

qRT-PCR validation of drought stress induced TDFS: The primer pairs for qRT-PCR were design basing on the sequences of TDFs. If the TDF's length was less than 300bp, designed primer pair with its highest homologous gene, and one primer must locate in the TDF sequence. The RNA samples were converted to single-strand cDNA by using a polyT with reverse transcriptase, then PCR amplification with qRT-PCR primer pair and SYBR Premix Ex Taq™ kit (Takara, Dalian, China) using Rotor-Gene6000 machine (Corbett Robotics, Australia), the beta-actin gene as control, and all samples replicated three times. The relative expression quantification of mRNAs was calculated using by the $2^{\Delta\Delta}$CT method, and the primer pairs for qRT-PCR were list in Table 2.

Results

Establishment of the modified cDNA-AFLP technique

According to the modified cDNA-AFLP technical route, the double-strand cDNAs that contained the recognized site of *Mme*I were obtained and digested with three restricted enzymes (*Mme*I, *Eco*RI and *Bst*YI), then the digested double-strand cDNAs linked with their corresponding adapters for pre- and selective amplification. Totally, 483 TDFs were got by 63 selective primer pairs with their lengths varying from 50 bp to 1500 bp, mainly at 100 bp to 400 bp, and about seven TDFs per primer pair on average (Figure 2). Sixty-three TDFs were reused and sequenced, 45 TDFs of them were ME, and ME represented that TDF had M and E primer at its both ends, five of them were MM, nine of them were MB, and the rest four ones were EE and BE (Table S1). Most TDFs had M primer, suggested that the recognized site of *Mme*I was integrated to double-strand cDNA successfully.

Many TDFs were induced by drought stress

The cDNA-AFLP gene differential expression display technique was considered as a semi-quantitative detection method, many TDFs showed differential expression under drought condition. Totally, 106 out of 483 TDFs showed differential expression, and could be divided into four groups according to their expression change trend during the drought treatment: 1) up-regulate, 32 TDFs; 2) down-regulate, 23 TDFs; 3) down-regulate after up-regulating, 44 TDFs; 4) up-regulate after down-regulating, 7 TDFs (Figure 2 and Table 3). In general, the group of down-regulate after up-regulating was the largest one, followed by up-regulate group and down-regulate group, and the group of up-regulate after down-regulating was the smallest one. Furthermore, the number of down-regulated DE-TDFs (67) was more than that of up-regulated ones (39) at the later period of drought stress.

The 5'end of DE-TDFs nearer to transcription start site

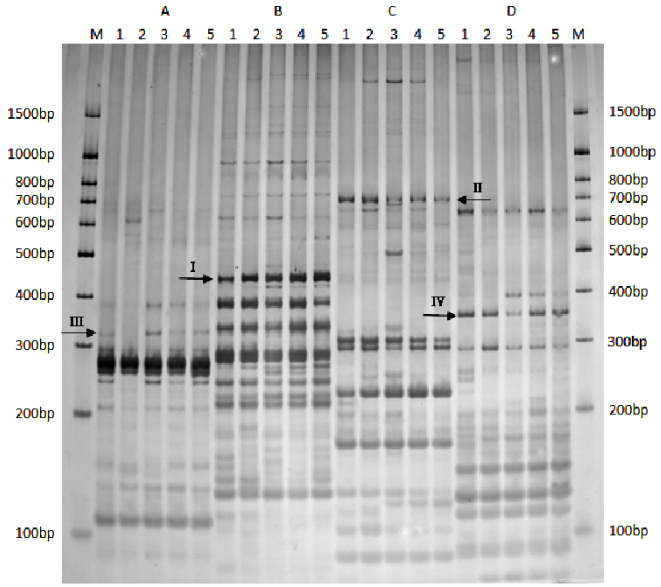
Sixty-three out of these 106 differentially expressed TDFs (DE-TDF) were reused, cloned and sequenced. After BLAST in http://www.phytozome.net/cassava, the positions of thirty-one DE-TDFs in their corresponding transcripts were determined in AM560 genome draft. The 5'end of four DE-TDFs began at transcription start site (TSS), two of them in 5'untranslated region (5'UTR), and the rest 16 ones located at coding sequence (CDS) regions, including two ones were less than 200bp, and 14 ones were more than 200bp far away from ATG. In addition, 35 DE-TDFs were got by the previous method with the same materials (Table S2), and among of them, the 5'end of only one DE-TDF began at TSS, other 34 ones all positioned in CDS region and over 90% of them were more than 200bp far away from ATG (Table 4). It suggested that there were much more 5'ends of DE-TDFs which began at TSS or nearer to TSS in the modified cDNA-AFLP technique.

Homologies of many DE-TDFs involved in or responded to drought stress tolerance

The sequences of 67 DE-TDFs were annotated in http://www.phytozome.net/cassava and NCBI database, 32 of them were homologous with known functional genes, 20 of them were homologous with unknown genes, and the rest 15 ones which had no homologous gene, maybe novel genes that response to drought stress in cassava. These 32 annotated DE-TDFs involved in 11 biological processes, and majority of them were ascribed into five functional categories, including cell growth and apoptosis, signal transduction, transcription regulation, genetic information processing and peptide synthesis and metabolism (Table 5). Among of them, their homologies of several DE-TDFs involved in drought tolerance or responded to drought in previous reports. For example, Eukaryotic translation initiation factor 3 subunit (MactEctc183) imparted stress tolerance and could be a potential candidate gene for developing crop plants tolerant

TDF Code	Primers Code	Primer(5'-3')	TDF Size	Amplification Size
Actin	ActinS	CCTTCGTCTGGACCTTGCTG		180 bp
	ActinF	CAAGGGCAACATATGCAAGC		
MtatBgagt376	SEC14S	ATGGTGTGGATAAAGAAGGGAGAC	376 bp	161 bp
	SEC14F	AGCAATGGTACAAGCAGGAAATT		
EatgEatg367	Psb-S	GAAATAGGCACAAGGAAAGAGCA	367 bp	159 bp
	Psb-F	TTGAAGTAGTTGAATAGGAGGATCG		
MtatBgagt141	Fas-S	ATCAGCAAGAGTTCTGGCAAG	141 bp	150 bp
	Fas-F	GAGATTCCTCCTCCGGTTAAA		
EctcMatc183	EIF3-S	AGGAAAGGAGACAACAAAGAAACT	183 bp	162 bp
	EIF3-F	CGGACAGGATTAACTGTAAGCATT		
MtgaEcga189	FBPase-S	GAATTGCAGCTCTAGTAGCGTCTC	189 bp	186 bp
	FBPase-F	CATCATCTTCTTCTGAAGCCATGA		
EcttMtat281	WD40-S	GCTCTGATGGAAGTTGCGTTAT	281 bp	194 bp
	WD40-F	AATAGTGCGTGAAATCTTCTCTCC		
MtgaEgag310	UN1-S	AAGTTCTGAGCGGGACAGTAAAG	310 bp	198 bp
	UN1-F	CGTGCCATCATAGCTAGGATAGG		
MtcgEccc215	UN2-S	ACAATCGTTCGGACTTGGTAAA	215 bp	169 bp
	UN2-F	GGCTTCGGGATCGAGGTATC		

Table 2: Primer pairs for quantitative RT-PCR.



1: SC124 DC, 2-5: SC124 DT 6d, 8d, 10d, 12d; A-D: four selective primer combinations, I-IV: Four differentially expressed TDF groups.

Figure 2: The electrophoretogram of selective amplification by using the modified cDNA-AFLP in SC124 under drought treatment (part results).

DE-TDF Groups	DE-TDF number	Percentage
Up-regulate	32	30.2%
Down-regulate	23	21.7%
Down-regulate after up-regulating	44	41.5%
Up-regulate after down-regulating	7	6.6%
Total	106	100%

Table 3: Four groups of differentially expressed TDFs (DE-TDFs).

Position		Modified cDNA-AFLP		Previous cDNA-AFLP[a]	
		No. of DE-TDF	Percentage	No. of DE-TDF	Percentage
TSS/ATG[b]		4	18.2%	1	2.9%
5'UTR		2	9.1%	0	0
CDS	≤ 200bp[c]	2	9.1%	1	2.9%
	>200bp	14	63.6%	33	94.3%
Total		22	100%	35	100%

[a]cDNA-AFLP technique followed with Bachem et al. [2-3];
[b]transcript has no 5'UTR, then its ATG is considered as the transcription start site.
[c]5'ends of DE-TDFs were more than 200 bp far away from ATG.

Table 4: The 5'ends of DE-TDFs in their corresponding transcripts.

to abiotic stress in *Arabidopsis* [10]; Cellulose synthesis (MtgaEcga125) was important for drought and osmotic stress responses including drought induction of gene expression [11]; The enzyme activity of Ribulose-bisphosphate carboxylase (MtgaEcga58) were 10 to 30% lower in drought stress as compared to normal control in soybean [12]; Over-expression of a maize E3 ubiquitin ligase gene (MtagMtag277) enhanced drought tolerance through regulating stomatal aperture and antioxidant system in transgenic tobacco [13]; five maize Cystain family members (MtagMtag292) were down-regulated in response to water starvation [14]. Moreover, part of them responded to other abiotic stresses, such as LRR receptor-like Serine/threonine protein kinase (EcagMtat337), Phosphatidylinositol transfer protein (MtatBgagt376), Fructose-1,6-bisphosphatase (MtgaEcga189), etc [15-17].

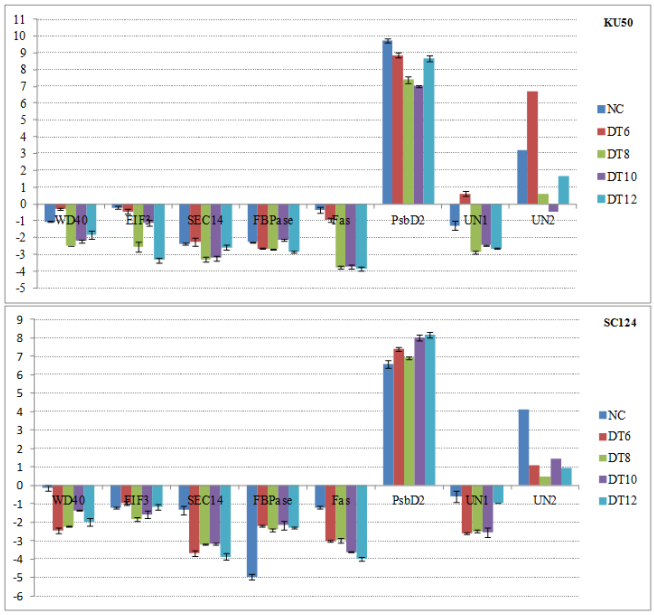
Part of DE-TDFs were validated by qRT-PCR

Eight DE-TDFs were randomly selected to confirm their differential expression under drought treatment by using qRT-PCR, included WD domain (WD40), Eukaryotic translation initiation factor 3 (EIF3), Fasciclin (Fas), Phosphatidylinositol transfer protein (SEC14), Fructose-1,6-bisphosphatase (FBPase), Photosystem II P680 reaction center D2 protein (PsbD2), and two unknown DE-TDFs (UN1 and UN2). Five of them showed significantly differential expression (NC-DT ≥ 1 or ≤ -1) in both KU50 and SC124 leaves under drought stress except for EIF3, FBPase and SEC14 (Figure 3). For example, Fas down-regulated in both KU50 and SC124 consistently under drought stress; PsbD2 highly expressed in KU50 and SC124 leaves, but it's up- or down-regulation was opposite in these two varieties; WD40, UN1 and UN2 down-regulated in SC124, but down-regulated after up-regulating at DT6 in KU50. It suggested that majority of DE-TDFs really responded to drought stress and cDNA-AFLP technique was an effective tool to screen differentially expressed genes.

Discussion

In this study, the modified cDNA-AFLP technique was established successfully, and over four hundred TDFs were got. The synthesis of single-strand and double-strand cDNA followed with the SMART technique in our modified technique. The MMLV reverse transcriptase that we used could add three C at the 3'end of single-strand cDNA when it arrived on the 5' cap of mRNA, and this was the key point for our double-strand cDNA synthesis. The double-strand cDNA was synthesized with the modified 5'SMART IV™ Oligonucleotide primer and the simplified CDSIII/3'PCR primer, so a recognized site of *Mme*I was integrated to 5'end of the double-strand cDNA, and its cutting site was anchored at the transcription start site in theory.

In previous cDNA-AFLP technique, the low frequency of restriction enzyme *EcoR*I and the high frequency of restriction enzyme *Mse*I were



Note: The relative expression quantifications of DE-TDFs were calculated by using the $^{\Delta\Delta}$CT method, *beta*-actin gene as references for DE-TDFs, the Y-axis=$^{\Delta}$CT (*beta*-actin)-$^{\Delta}$CT (DE-TDF).

Figure 3: The qRT-PCR validation of eight differentially expressed TDFs in KU50 and SC124.

DE-TDFs	Size(bp)	Functional Description	Homologous Gene Number	Homology
Cell growth and apoptosis (6)				
MtagMtag292[a]	292	Ceramidases	cassava4.1_002110m XP_002520446.1	E=2e-48 ID=78/95(82%)
MtgaEgca176	176	Prolyl 4-hydroxylase alpha subunit	cassava4.1_014646m DB948318.1	E=4.2E-52 ID=(117/119)98.3%
MtgaEcga93	93	Leukemia Virus Rnase H Domain	2HB5_A	E=5e-27 ID=69/69 (100%)
MtatEctc281	281	WD domain, G-beta repeat	cassava4.1_000071m XP_002515073.1	E=5e-40 ID=70/73(96%)
MtagMtag277	277	E3 ubiquitin ligase	cassava4.1_000529m XP_004307862.1	E=2e-56 ID=86/91(95%)
MtgaEcga92	92	Ribosomal protein S10	XP_003588337.1	E=2.60E-06 ID=(37/42)88.1%
Signal transduction (5)				
EcagMtat337	337	LRR receptor-like serine/threonine-protein kinase	cassava4.1_000537m XP_003618726.1	E=1e-34 ID=64/105(61%)
BgagcEctc346	163	Nucleotide binding protein	XP_002522890.1	E= 4e-28 ID= 52/54(96%)
MactEctc183	180	Eukaryotic translation initiation factor 3 subunit	cassava4.1_000145m XP_002528386.1	E= 8e-19 ID= 42/52(81%)
EgtgMtga131	131	Cystatin family member	cassava4.1_019939m AAF72202.1	E=1e-21 ID=42/42(100%)
MtgaEgcg439	439	SH3 domain and tetratricopeptide repeats 1	EDL37493.1	E= 2.7 ID= 20/62(32%)
Transcription regulation (5)				
MtcgEccc215	215	Mitochondrial ribosomal protein L9	cassava4.1_016718m YP_005090474.1	E=7e-27 ID=68/71 (96%)
EcatMtag293	293	Thiopurine S-methyltransferase	DB939009.1	E=4.3 ID=20/49(41%)
MtcgBaagc141	141	Fasciclin and related adhesion glycoproteins	cassava4.1_007317m XP_002309262.1	E=1e-15 ID=33/45(73%)
MtgaEgag281	281	Protein coding	XP_002518871.1	E=0.059 ID=21/56 (38%)
MtgaEgca213	213	Protein coding	cassava4.1_000297m EMJ00881.1	E=1.7E-102 ID=(210/212)99.1%
Genetic information processing (3)				
BaagcMtcg132	132	Retrotransposon protein	ABA94145.1	E=3E-56 ID=128/131 (98%)
MtatBgagt376	376	Phosphatidylinositol transfer protein SEC14	cassava4.1_006270m XP_003603969.1	E=3E-69 ID=103/113(91%)
MtgaEgca115	115	Splicing factor 3b, subunit 4	cassava4.1_024763m DB937609.1	E=3.10E-36 ID=(84/84)100.0%
Peptide synthesis and metabolism (3)				
MtatEctt234	234	Cell wall-associated hydrolase	XP_003638717.1	E=2e-18 ID=41/41 (100%)
EccaMtga93	93	Putative aminodeoxychorismate lyase	ZP_14386624.1	E=2.00E-09 ID=27/28 (96%)
BaagcMtcg133	132	Polyprotein	DB937177.1	E=1E-54 ID=127/131 (97%)
Transcription factor (2)				
MactEctc330	331	Transcription regulator AraC N-terminal arabinose-binding Domain	ZP_18864282.1	E=2e-54 ID=99/109 (91%)
MtgaEagt266	266	RNA-binding translational regulator IRP	cassava4.1_001348m XP_002530635.1	E=3e-94 ID=141/154(92%)
Energy metabolism (2)				
MtagEatc248	248	ATP-dependent RNA helicase	cassava4.1_001726m XP_004306326.1	E=2e-45 ID=75/84(89%)
MtgaEcga58	58	Ribulose-bisphosphate carboxylase	cassava4.1_017243m Q42915.1	E=9.7E-21 ID=(55/55)100.0%
Transport (2)				
EatcEatc527	527	Sugar transporter	cassava4.1_034097m XP_002517103.1	E=7e-128 ID=199/208(96%)
EcgaMtga87	87	Membrane-associated apoptosis protein	cassava4.1_012967m	E=1.9E-26 ID=(66/66)100.0%
Sugar metabolism (2)				

MtgaEcga189	189	Fructose-1,6-bisphosphatase	cassava4.1_008978m XP_002532766.1	E=1e-21 ID=51/62(82%)
MtgaEcga125	125	Cellulose synthase	cassava4.1_008148m	E=7.9E-53 ID=(117/118)99.2%
Photosynthesis (1)				
EatgEatg367	367	Photosystem II P680 reaction center D2 protein	cassava4.1_031110m YP_003330957.1	E=2e-28 ID= 66/68(97%)
Lipid metabolism (1)				
MtgaEgca99	99	Triacylglycerol lipase	cassava4.1_004098m	E=3.4E-43 ID=(97/97)100.0%

"a" is a code of DE-TDF, M, E and B represent the primers of pre-amplification which were designed basing on the adapters of *Mme*I, *Eco*RI and *Bst*YI enzyme, and the lower-case letters are the added selective nucleotides.

Table 5: Homology of 32 differentially expressed TDFs identified in modified cDNA-AFLP analysis.

often used to digest the double-strand cDNA [18-19], and two high frequency of restriction enzymes-*Taq*I and *Mse*I, were used for small genome-size microorganism occasionally [7]. In this study, because the cutting site of *Mme*I was anchored in the 5'end of double-strand cDNA, so another two enzymes were used to digest our double-strand cDNAs together. One was the frequently-used *Eco*RI, the other one was a novel enzyme *Bst*YI, because the *Bst*YI/*Mse*I restriction enzyme combination could obtain more than 60% of all transcripts in tobacco by AFLP in Silico [8]. The tri-enzyme system resulted in 7.2 TDFs for each selective primer pairs on average, and the number of TDFs was less than that in the previous method. One cutting site was anchored at 5'end, the *Eco*RI and *Bst*YI was low frequency enzyme, maybe three selective nucleotides should reduce to two ones for increasing the number of TDFs. Possibly, a high frequency enzyme with high GC content, e.g. *Cfo*I (GCG$^{\nabla}$C), *Taq*I (T$^{\nabla}$CGA), etc, should be used to replace one of these two low frequency enzymes, because the GC content of CDS region was higher than that of other regions [20].

Although the 5'ends of DE-TDFs were nearer to the TSS than that in previous method, but only three 5'end out of 31 DE-TDFs started at TSS, and *Mme*I had recognized site in more than 10% of all transcripts in cassava. The MMLV transcriptase could add three C at the 3'end of single-strand cDNA when it arrived on the 5' cap of mRNA, but if transcription interrupting by incomplete or complex second-structural mRNA, it also could add three C at the 3'end of single-strand cDNA with lower activity. Therefore, it might be the reason that many DE-TDFs didn't start at the TSS.

Many homologies of DE-TDFs involved in or responded to drought stress, and five of eight randomly selective DE-TDFs up- or down-regulated in two varieties SC124 and KU50 significantly and consistency, the other three ones showed differentially expressed in at least one variety under drought stress. Especially, two unknown DE-TDFs responded to drought stress, suggested that possibly many novel drought resistance related genes existed in cassava genome.

In conclusion, the modified cDNA-AFLP method was not very perfect at this stage, had many details need to be optimized. High-quality RNA, the usage of high frequency enzyme and the reduction of selective nucleotide, maybe can make the modified technique to have more practicability. At present, although more and more gene differential expression analysis relied on the next generation sequencing technique, while our modified method gave a new light to cDNA-AFLP technique, possibly could prolong its usage life in some low-cost research activities or minor crops.

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