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A Standardized RNA Isolation Protocol for Yam (*Dioscorea alata* L) cDNA Library Construction

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ABSTRACT

For the purpose of constructing yam cDNA libraries, attempts to isolate high quality RNA using several previously reported protocols were unsuccessful. Therefore a protocol was standardized for yam total RNA isolation by using guanidium buffer at the Department of Biology, Virginia Sate University. The RNA isolated using this standardized protocol was high in quality and led to successful good quality cDNA library construction and identification of functional ESTs in yam.

INTRODUCTION

Yam, (*Dioscorea alata* L), is the main food source for over 100 million people in humid and sub-humid tropics. Its production is affected by several biotic and abiotic factors (Abang et al., 2003). Anthracnose, caused by *Colletotrichum gloeosporioides*, is the most severe foliar disease of water yam (*Dioscorea alata* L) and is a major hurdle in yam production. It is reported that anthracnose causes yield reduction up to 90% (http://annualreport.iita.org). There are no cost effective control measures and the long-term solution to the problem will be through the development of resistant genotypes (Mignouna et al., 2002. Very limited yam sequence information is available from public genome databases. A review of previous efforts to develop cDNAs towards EST development in yams revealed that housekeeping genes were prevalent in the libraries constructed using total RNA from male flowers (Mignouna et al., 2002a, b, c).

It is realized that obtaining high quality, intact RNA is the first and the most critical step in conducting cDNA library construction and for further analysis of gene of interest. After many attempts of total RNA isolations from yam leaf samples using standard plant RNA isolation protocols (Verwoerd et al,1989), only 6-10 ug of total RNA was extracted from the leaves and no colonies were observed when this RNA was used for cDNA library construction. The RNA appeared as a smear on 1.1% agarose gel (Fig. 1). The most likely reason for not getting good quality RNA is the mucilagenous tissue in yam plant parts like leaf, stem and tuber. This tissue causes problem because of polyphenols, polysaccharides and other secondary metabolites that are rich in yam plant parts and are not easily removed by conventional extraction methods. The aim of this study was to establish a protocol for RNA isolation from *Dioscorea alata* to get high quality and high quantity RNA that is suitable for generation of molecular markers, such as EST-SSRs and SNPs. Therefore, the following article discusses successful and reproducible method of RNA isolation

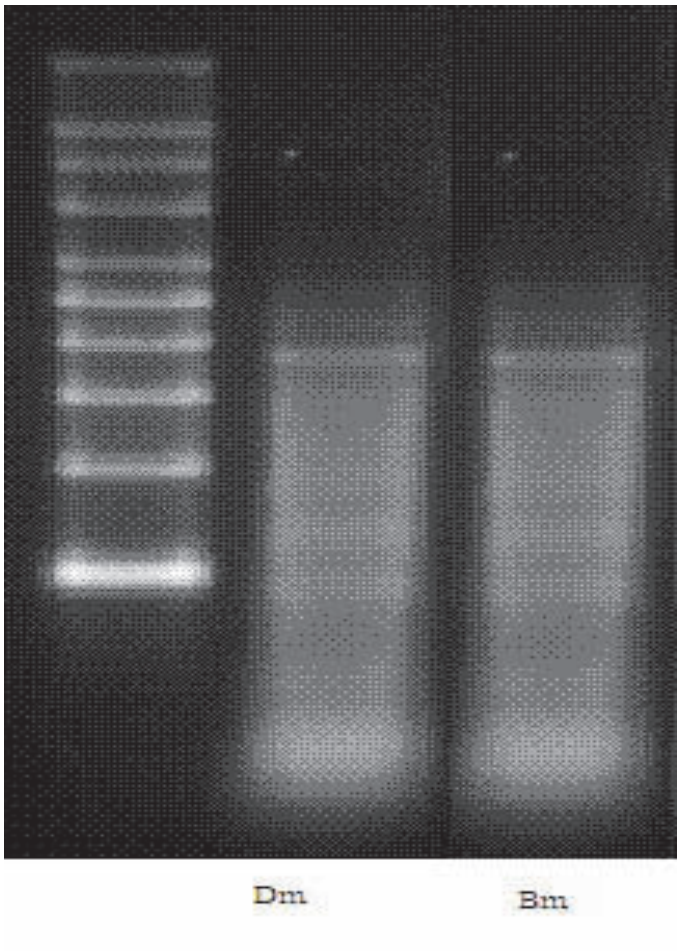

FIGURE 1. A smear of rRNA samples of Dm-Resistant yam genotype and Bm-Susceptible yam genotype isolated using standard protocols on 1.1% Formaldehyde agarose gel

procedure employed for yam cDNA library constrcution and ways of increasing RNA yields

MATERIALS AND METHODS

Tissue collection: In order to standardize the protocol for RNA isolation, the yam (source: local grocery store) were grown in the green house in pots. Fresh 1g leaf tissues are collected in 50ml BD Falcon tubes, frozen quickly in liquid nitrogen.

RNA isolation. Only the successful procedure of RNA isolation with the modifications to standard plant RNA isolation protocol is reported here.

Solutions and solvents used:

- *Extraction buffer* (100 ml stock): 76.424g of 8M Guanidium Hydrochloride + 425 mg of 20mM MES + 740mg of 20mMEDTA+ 35ml of DEPC water. Adjust the pHwith 10M NaOH, autoclave and store at 4°C. Add 1.38µl of β-mercaptoethanol (50mM) just before use.
- *Phenol:Cholorform:Isoamulalcohol* (24:23:1)

Procedure:

1. 1g tissue ground in liquid nitrogen was homogenized in 2ml extraction buffer + 2ml Ph:Chl:IAA. {The sample was homogenized using power operated mini grinder (the steel grinder part was pre-cooled in liquid nitrogen) that perfectly fits in to the falcon tube. It was necessary to maintain frozen conditions throughout the extraction to enhance the quality of the target RNA. }.
2. The sample was centrifuged for 10 min at 10,000rpm (at 0-2 °C).
3. To the Supernatant, Ph:Chl:IAA (equal volumes in 1:1 ratio) was added and the RNA was precipitated overnight in -20.
4. The next day the sample was centrifuged for 20 minutes at 10,000rpm (at 0-2 °C) and the pellet and was dissolved in Deionized water (Volume based on required concentration).
5. RNA was stored at -80°C.The quality of RNA was confirmed by using BIO-RAD Smartspec™ plus Spectrophotmeter and also by Formaldhyde agarose gel electrophoresis (Sambrook et al, 1989).

cDNA LIBRARY CONSTRUCTION

The freeze dried leaves of *D. alata* L genotypes, Tda 95/00328, resistant to the FGS strain of *C. gloeosporioides* but susceptible to the SGG strain and TDa 92-2, susceptible to the FGS and SGG strains of *C.gloeosporioides* were obtained from IITA, Ibadon, Nigeria. Leaves were ground in liquid nitrogen and total RNA was isolated using the standardized protocol. Total RNA thus isolated was used for the construction of cDNA library using The Creator smart cDNA library construction kit (BD Biosciences Clonetech). First strand cDNA was synthesized using SMART IV oligonucleotide followed by long distance PCR amplification to generate high yields of full-length ds cDNAs (~400 to >4000 bp) followed by Sfi I digestion and column fractionation. The cDNA fractions that match the desired size distribution (1-4kb) were selected. The Sfi I – digested cDNA was ligated to the Sfi I digested dephosphorylated pDNR-LIB Vector (Clonetech) and transformed into DH10B T1 Phase resistant bacterial cells. The chloramphenicol resistant colonies were picked and archived in 96 well plates. For preliminary round of sequencing, about 100 colonies from each library (resistant and susceptible) were randomly selected and subjected to single pass sequencing (Agencourt Biosciences).

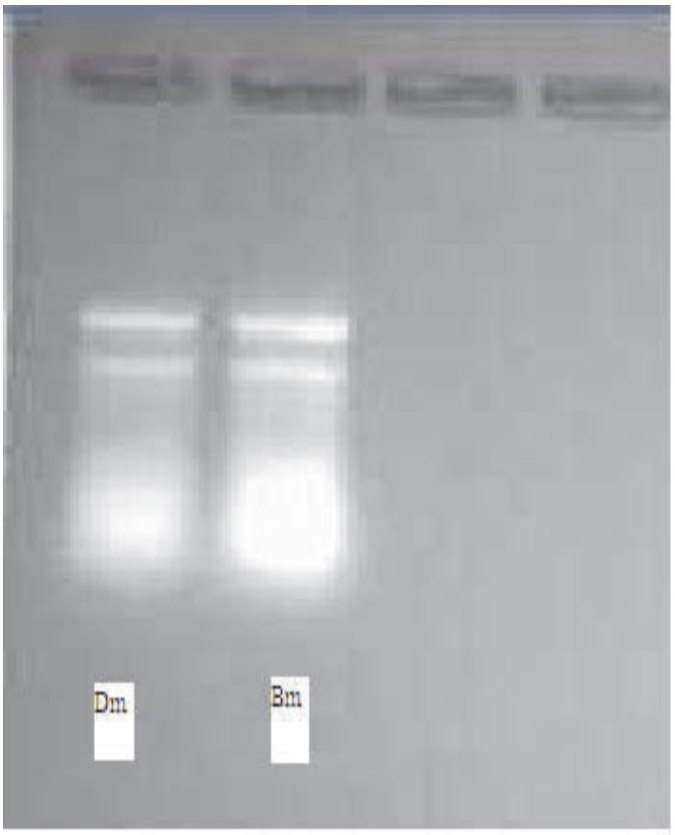

FIGURE 2. Intact yam rRNA samples using current protocol.

RESULTS AND DISCUSSION

The quantity of total RNA is between 250 to 500µg from 1g of yam leaf tissue. The 18S and 28S ribosomal RNA bands are clearly visible in the intact leaf RNA samples Dm and Bm of yam (Fig. 2) and the quality reading on spectrophotometer were presented in the Table 1.

Following quality check of the sequences, the pure quality sequences were checked for homology to sequences in GenBank using BLAST similarity search tool. Data obtained from the BLAST analysis of 100 clones from each resistant (Dm) and susceptible (Bm) accessions were compiled and interpreted with respect to the hits identified in other plant species (Table 2 and 3).

This preliminary data describes the initial efforts to develop tools to annotate EST's for anthracnose disease resistance genes by constructing good quality cDNA libraries for different accessions of *D.alata*. From each cDNA library 6000 colonies were arrayed into 96 well plates. A total of 100 clones randomly selected each from two

TABLE 1. Spectrophotometer readings of quality RNA samples from yam genotypes.

Sample ID	ng/μL	A260	A280	A260/A280	A260/A230	Constant	Cursor Pos	Cursor Abs	340 raw
Bm	257.6	6.438	2.997	2.15	1.5	40	230	4.287	0.082
Dm	309.6	7.741	3.646	2.12	1.15	40	230	6.703	0.022

distinct libraries namely Dm and Bm. Of the 100 cDNA clones from each yam genotype, 10 yielded no sequence and an additional 9 produced sequences of less than 100 bp and these were not used for sequence analysis. The average length of the remaining sequences was 762 bp.

Based on top Blast hits in plants, in yam type Bm, out of 100 sequences, 48 were distinct gave >400bp and were showing functional similarities. In Yam type Dm, out of 100 sequenced clones 48 were distinct, gave >400bp and 22 were duplicates of yam type 1 were observed. The genes putatively identified are shown in Table 2 and 3. The blast hits identified in different crops showed 88-100% identity and, in general, the homology of the insert sequence to the blast hit is about 400-500bp out of 700-800 bp length aligned. The genes (ESTs) identified based on sequence similarity are involved in various putative functions such as gene or protein expression, protein binding, ripening, cell wall and stress response, defense, photosynthesis, photoperiodic flowering response, cell division and proliferation, nodulation, and secondary metabolism etc. and some of them could not be classified into any of these categories.

The numbers of hits showing stress/defense related function were comparatively more in resistant genotype when compared to susceptible genotype (Satya et al, 2007). Of the distinct sequences there are sequences similar to unknown protein and unknown mRNA (1-2%) not presented here. The information on hits to clone sequences (10%) in different crop species and the top blast hits to mitochondrial genes and genes encoding for ribosomal protein genes (20%) were not listed in the table. By sequencing a large number of cDNAs, we can selectively avoid the clones that represent ribosomal and mitochondrial genes, and choose clones that represent genes that we wish to examine. This is a significant improvement compared to previous efforts where sequences coding for ribosomal proteins were predominant in the libraries. This achievement is attributed to quality RNA isolation.

CONCLUSION

Two cDNA libraries for yam, one each for resistant and susceptible genotypes, were constructed for the purpose of identifying clones that are differentially expressed in these two genotypes. Many new genes have been identified that can be useful for future studies. The sequences may also be a source of single-nucleotide polymorphisms or simple sequence repeats for molecular marker development.

Preliminary analysis of 200 clones revealed homologies to known genes in several related and distant plant species. Though the numbers of hits were comparatively more in resistant genotype compared to susceptible genotype, not much distinct differences were observed between the functional hits to sequences of these two genotypes.

TABLE 2. Blast hits from cDNA library of Yam accession Dm

Clone ID	NCBI Definition line for Putative function of the Blast hits	Crops in which hits were identified
Dm 3	mRNA, complete cds;	AC183495.1(Cabbage); gb|DQ903665.1|(Turnip); dbj|AP008209.1|(Rice)
Dm 4	genomic DNA, chromosome 1,10	dbj|AP008207.1|; dbj|AP001633.2|, dbj|AP008209.1(Rice)
Dm 7, 52	Metallothionein-like protein (MET,grip24,MWMT3) mRNA, complete	DQ202305.1(sago palm); AJ236913.1(African oil palm); AJ237990.1(grape); AY857933.1(Cotton); AF268393.1(Banana)
Dm 8	Solanum lycopersicum genomic DNA, chromosome 8, clone: C08HBa0323O07, complete sequence;Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 12	dbj|AP009293.1| (brinjal); NM_001073500.1, AP008218.1(Rice)
Dm 11, 32, 69, 59	chloroplast, complete genome	DQ887676.1(Drimys) AJ627251.1(Nymphaea alba); AY916449.1(Phalaenopsis aphrodite); DQ899947.1|(tulip)
Dm 12, 45	chloroplast mRNA for Tic62 protein;IbJ8 mRNA for JA-domain, complete cds; SrGLU5 mRNA for beta-1,3-glucanase, complete cds	AY437888.1(Sheperd's purse); AJ344551.2(Pea); DQ499754.2(Potato); AB246796.1(Sweet potato); AB242267.2(Sesbania); AB210846.1(Lemna)
Dm 15	Ribulose-bisphosphate carboxylase (AT5G38430) mRNA, complete cds;	NM_123204(Arabidopsis).3; V00458.1(Soybean); AY143814.1; AY142543.1; AY065026.1
Dm 18	aci-reductone dioxygenase-like protein (ARD) mRNA, complete cds	DQ244304.1; AY103746.1(Maize); CT831853.1, NM_001055581.1, AY955841.1(Rice); AB025597.1(Barley)
Dm 21	Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 2	dbj|AP008208.1|
Dm 24	mRNA sequence complete cds	NM_001032532.1

Dm 30	Oryza sativa microsatellite MRG5582 containing (GGA)X13, genomic	AY023257.1
Dm 34	Medicago truncatula clone mth2-13n2, complete sequence	AC136839.20
Dm 42	Full-length cDNA Complete sequence from clone	CR936947.2 , emb| CR931731.1|(Medicago); BX821860.1(Arabidopsis)
Dm 48	Glycine max mRNA for asparagine	gi|109940718|emb|AM158274.1|
Dm 53	cDNA clone:full insert, mRNA sequence,complete	NM_001066955.1; AK070897.1(Rice); AY107920.1(Maize)
Dm 54	Zea mays cultivar Mo17 locus 9008, complete sequence	AY664418.1
Dm 58	Sequence of BAC F15I1 from Arabidopsis thaliana chromosome 1; Prunus persica DNA, microsatellite marker MA035a	AC006577.2; AB077139.1
Dm 59	Croomia pauciflora large subunit ribosomal RNA gene, partial; Dioscorea sp. Qiu 94044 large subunit ribosomal RNA gene, partial	DQ629350.1(Nuttall); DQ629349.1(Yam)
Dm 61, Dm 80	Drosophila melanogaster chromosome 3L, complete sequence; Santalum austrocaledonicum microsatellite DNA, clone mSaCIRF04	AE014296.4(Drosophila); AJ831399.1(Santalum)
Dm 64	Lycopersicon esculentum BAC clone Clemson_ID 47I13, complete	AF411804.1
Dm 65	histidine ammonia-lyase-like mRNA, complete	EF051316.1(Gymnadenia); BT012683.1(Tomato); AC157536.31(Medicago)
Dm 70	Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 3	AP008209.1
Dm 79	Zea mays clone 92533 mRNA sequence	DQ245928.1
Dm 88	Brassica rapa subsp. pekinensis clone KBrB070J23, complete	AC189444.1

Dm 89	Sorghum bicolor clone SB_BBc0020O07, complete sequence	AC169375.4
Dm 91	Arabidopsis thaliana unknown protein (AT2G19830) mRNA, complete; Lycopersicon esculentum clone 134156F, mRNA sequence; Zea mays clone EK07D2310A10. c mRNA sequence	NM_127541.2; BT013152.1(Tomato); BT017005.1(Maize)

TABLE 3. Blast hits from cDNA library of Yam accession Bm

Clone ID	NCBI Definition line for Putative function of the Blast hits	Crops in which hits were identified
Bm 2, 13, 83	Metallothionein-like protein (MET) mRNA, complete	DQ202305.1(Sago palm), AJ236913.1(African oil palm), Grape, cotton, citrus, musa and rice
Bm 3, 5, 16, 17, 22, 23, 25, 26, 30, 45, 53, 66, 69, 76, 88, 91, 41, 52, 34, 38, 57, 73	Mitochondrial, chloroplast DNA, complete sequence; ribosomal RNA gene partial	DQ887676.1(Drimys granadensis); AJ627251.1(Lotus); AB240139.1(Tobacco); DQ629360.1(Dicentra Sp.); DQ340440.1(Pacific Dogwood), DQ923117.1(Heavenly Bamboo); AF205123.1; DQ629349.1, DQ629457.1(yam); DQ629350.1(Nuttall)
Bm 4	cDNA clone:OSIGCFA011A01, full insert sequence;	CT829335.1(Rice) ; AY224463.1(Rice)
Bm 12	mRNA for Mob1-like protein (mob1-B) complete cds	AY437888.1(shepherd's purse), AM161645.1(alfalfa)
Bm 20, 37	LpLHY H2 mRNA for LHY homologue2, complete	AB210846.1(Duckweed), DQ499754.2(Potato)
Bm 31	unknown protein (AT2G46100) mRNA, complete	NM_130173.3(Arabidopsis), BT012819.1(tomato)

Bm 35, 63	lipid transfer protein mRNA, complete cds	EF031153.1(Stevia); AY395741.1(Summer grape)
Bm 43	beta-1,3-glucanase, complete,cds	DQ499754.2(Potato); AB246796.1(Sweetpotato); AB242267.2(Sesbania)
Bm 47	Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1	AP008207.1(Rice)
Bm 48	aspartic proteinase 4, complete cds	CT829760.1(Rice); AB045894.1(Nepenthes); NM_001049320.1(Rice); AY103982.1(Rice)
Bm 55	putative terpene synthase, complete cds	AK227599.1(Arabidopsis); NM_001054333.1(Rice)
Bm 59	Full-length cDNA Complete sequence from clone	BX817199.1(Arabidopsis)
Bm 60	Brassica rapa subsp. pekinensis clone KBrB002G19, complete; Capsella bursa-pastoris ecotype CZ96 microsatellite ATCP70189	AC189190.1(Chinese cabbage); DQ144500.1(sheperd's purse)
Bm 32, 61, 62, 64	cDNA clone: full insert sequence;mRNA, complete sequence	AC137065.26(alfalfa), DQ244538.1, DQ245784.1, DQ244442.1(maize); CT830019.1(Rice); AK069033.1, CT829171.1, CT830462.1(Rice); AP006116.1(Lotus); BT014284.1(Tomato); AY085715.1(Arabidopsis);
Bm 75	Lotus japonicus genomic DNA, chromosome 3, clone: LjT13M14	AP004531.1(Lotus)
Bm 17, 39, 80	Nicotiana tabacum chloroplast pigment-binding protein CP29 (Lhcb4); Panax ginseng cab mRNA for chlorophyll a/b binding protein; Nicotiana tabacum chlorophyll a/b binding protein mRNA, complete	AB236867.1(Ginseng); DQ676843.1, AY219853.1(Tobacco); CT829715.1(Rice)
Bm 82	ubiquitin conjugating enzyme (UBC4), complete cds	L29077.1(Peas); CT833517.1(Rice); AY086109.1(Arabidopsis)
Bm 92	Populus trichocarpa clone Pop1-21I14, complete sequence	AC182669.2(Populus)
Bm 94	Glycine max mRNA for asparagine synthetase, type III (sas3 gene)	AM158274.1(Soybean)

Therefore this project is revised to continue cDNA library construction from challenged leaf tissues of these two genotypes besides including a third genotype resistant to SGG strain to identify the candidate genes to anthracnose resistance. The ESTs generated in this study also provide a good tool for more studies to understand the resistant and susceptible interactions of yam anthracnose.

Analysis of sequences from recently completed revised yam genomics project will generate more ESTs for differential expression analysis for the purpose of identifying candidate genes for anthracnose resistance, marker development and further yam QTL mapping studies.

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