Research in Biotechnology, 3(4): 47-51, 2012 ISSN: 2229-791X
www.researchinbiotechnology.com

Short Communication

Synthesis of good quality double-stranded cDNA from the bark tissue of robusta coffee (*Coffea canephora*) plants

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Quality RNA in large quantity is often required in the analysis of gene expression. RNA extraction from samples collected from woody plants is generally complex and becomes the main limitation to study gene expression particularly in perennial crops like coffee. Standard RNA extraction protocols are time consuming laborious and cannot be adapted for high throughput functional analysis. A simple and effective protocol for extraction of high quality total RNA from bark tissue of woody stem was achieved using the RNeasy plant mini kit (Qiagen, USA). The extracted RNA was successfully converted into double-stranded cDNA using the SMATer cDNA synthesis kit (Clontech, USA) which is based on the Switching Mechanism At 5′ End of RNA Transcript (SMART) technology. The integrity of the total RNA used for synthesizing double stranded cDNA was assessed by amplifying a 1282 bp product targeting the glyceraldehyde 3-phospho dehydrogenase (GAPDH) gene by PCR. As expected, the PCR product contained the full coding sequence plus 69 and 196 bp of 5′ and 3′ UTRs respectively. The double-stranded cDNA was used successfully for creating a SSH cDNA library (results not reported here). The cDNA could also be useful for a number of other applications like cDNA library construction, EST analysis, RACE and Next Generation Sequencing (NGS).

Keywords: Bark tissue, cDNA, *Coffea canephora*, GAPDH, RNA.

Coffee (*Coffea arabica* L and *Coffea canephora*) is a popular beverage crop of the world and India produces about 3.5% of the world's coffee (Anonymous 1996). Undegraded intact RNA is the important starting material for most of the functional analysis. Double stranded cDNA from the coffee tissues such as leaf, berries and root (Fernandez 2004, Fernandez 2012, Mondego 2005, Idárraga 2012) were obtained from and used for the analysis of biotic and abiotic stress. However the cDNA from the woody bark tissue is yet to be studied. The study of the *C. canephora* bark transcriptome is of special interest especially in understanding the molecular mechanism underlying the resistance mechanism of the pest coffee white stem borer (*Xylotrechus quadripes),* which is a serious pest of arabica coffee confined to the Asian countries (Anonymous 1998; Seetharama *et al*., 2005; Sreenath and Prakash 2006). In this report we describe a simple and effective protocol for the extraction of high quality RNA from bark tissue of woody stem of robusta coffee plant and the method for converting this RNA to double strand cDNA.

This cDNA is useful in an array of applications including gene expression analysis, construction of subtractive cDNA library (Diatchenko 1996), qRT-PCR, RACE and for next generation sequencing.

Materials and methods

Plant Material

Bark tissue from the woody stem of a 12 year-old *C.canephora* cv CxR plant was used. The tissue was collected using a sterile scalpel blade and was immediately frozen and stored in liquid nitrogen in readiness for RNA extraction.

Total RNA isolation

Total RNA was isolated using RNeasy plant mini kit (Qiagen, USA) following the procedure given in the user manual with slight modifications. All solutions used for total RNA isolations were prepared using Diethyl PyroCarbonate (Sigma, USA) treated MilliQ-water (Millipore, USA). All non-disposable plastic materials were treated with DEPC and autoclaved. The bark tissue frozen in liquid nitrogen was transferred to a prechilled pestle and mortar and immediately ground to a fine powder. Around 100 mg of the frozen ground tissue were transferred to 950 µl of RLT buffer (Lysis buffer) in a microcentrifuge tube. The tissue was homogenized using a tissue homogenizer (Polytron PT 1200 E, Kinematica AG) for 2 min at full speed. The homogenized tissue lysate was passed through the silica membrane spin column provided with the RNeasy Plant Mini Kit (Qiagen, USA) and subsequently treated with DNase I (Qiagen, USA) to remove genomic DNA, according to the manufacturer's instructions. The integrity of the total RNA was analyzed using Ethidium bromide stained 1.2% native agarose gel electrophoresis. The concentration and purity of the total RNA were determined using a microvolume UV spectrophotometer (NanoDrop Technologies, USA).

cDNA Synthesis

First-strand cDNA was synthesized according to the SMARTer PCR cDNA synthesis kit. For this purpose 2.5 µl of total RNA sample (about 500 ng), 3' SMART CDS primer II A (5'-AAG CAG TGG TAT CAA CGC AGA GTA $CT_{(30)}N_{-1}N$–3' where N = A, C, G, or T; N_{-1} = A, G, or C) were mixed and incubated first at 72^{0}C for 3 min and then lowered to 42^{0}C for 2 min. 5x first strand buffer, DTT, dNTP mix and SMARTScribe Reverse Transcriptase were incubated at 42^{0}C for 90 min. The first strand reaction was terminated by heating the tubes at 70^{0}C for ten minutes.

Amplification of cDNA by long distance polymerase chain reaction (LD-PCR)

2µl of the first strand cDNA, deionized H_2O, Advantage 2 PCR buffer, dNTP mix, 5' PCR primer II A, and Advantage 2 polymerase mix were added into a pre-chilled microcentrifuge tube and Amplification of cDNA was done by long distance polymerase chain reaction (LD-PCR). Amplification was then done using the following program; 95^{0}C 1 min 24 cycles of 95^{0}C 15 sec, 65^{0}C 30 sec, 68^{0}C 6 min. 5µl of the PCR product was analyzed on Ethidium bromide stained 1.2% agarose gel electrophoresis. The cDNA was size fractionated and small DNA contaminants and unincorporated nucleotides were removed from the cDNA using CHROMA SPIN-1000 columns (Clontech) as per the manufacturer's instruction.

Analysis of cDNA integrity

2 µl purified SMARTer cDNA was amplified using deionized H_2O, 10x Advantage 2 PCR buffer, 50x dNTP mix, 5 pmol of forward and reverse primers, which were designed based on sequence of *Coffea canephora* SGN-U347734 from the SOL database (www.solgenomics.net), 50x Advantage 2 polymerase mix using the following program: 94^{0}C 4 min 30 cycles of

94^0C 30 sec, 55^0C 1 min, 72^0C 1 min. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, USA). The purified PCR product was ligated into pGEMT-Easy (Promega, USA) vector and transformed into competent JM109 *E.coli* cells. The transformed cells were spread on LB agar containing Xgal/IPTG/Amplicilin and incubated at 37^0C overnight. Pure white colonies were randomly picked and inoculated into 2 ml of LB broth with 100 µg/ml concentration of ampicilin and grown at 37^0C overnight in incubator shaker. Plasmid DNA was isolated from the overnight grown culture using Qiaprep Spin mini prep kit (Qiagen, USA). The purified plasmids were sequenced from the M13 region of the plasmids using Sanger's dideoxy method. The sequence of the GAPDH gene and deduced amino acid sequence was analyzed using DNASTAR (Madison, USA) and the homology search was performed using the BLAST program in the GenBank database.

Results and discussion

Isolation of total RNA

The ratio of the A_{260} /A_{280} for total RNA was 1.9 and around 20 µg of total RNA was obtained from 100 mg of the bark tissue. The total RNA showed two distinct bands corresponding to 28S and 18S ribosomal RNA bands on ethidium bromide stained 1.2% agarose gel and the brightness of 28S was twice as that of 18S RNA (Figure 1). The results indicate that minimal if any degradation or contamination occurred during isolation and the total RNA isolated from *Coffea canephora* bark tissue was pure, integrated and suitable for cDNA synthesis.

cDNA synthesis and amplification of cDNA by long distance Polymerase Chain Reaction (LD-PCR)

SMART technique is a novel and useful method for full length cDNA synthesis. Its unique characteristic is that it provides full-length cDNAs that preserve the complete 5' terminal sequence of the mRNA. In this method, as the superiority of LD-PCR in cDNA synthesis, the amount of available RNA starting material needed is very small, which is significant for situations where the original sample supply is limited by difficult gaining samples. Using the SMARTer cDNA synthesis and long-distance PCR (LD-PCR) amplification strategy (Barnes 1994), it was possible to synthesize high quality cDNA from RNA of the woody bark tissue of *Coffea canephora.* Majority of the cDNA produced from LD-PCR was analyzed on a 1.2% agarose gel and a homogenous smear of PCR product ranging from 0.2 to 4Kb in size was observed (Figure 2).

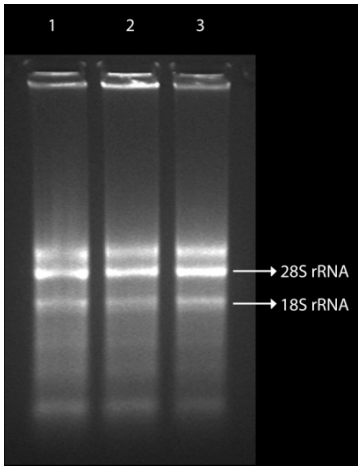

Figure 1: Total RNA isolated from 3 independent bark tissue of *Coffea canephora* using the Qiagen RNeasy plant mini kit. Lane 1, 2 and 3: Total RNA with 2:1 ratio of 28S rRNA and 18S rRNA.

Subsequently the synthesized cDNA was purified by CHROMA SPIN 1000 column to remove short DNAs. Full length representation of cDNA population is the key for most of the functional genomic applications and in the present study we provide the efficient method of total RNA from bark tissue using the RNeasy plant mini kit. Synthesis of double stranded cDNA was

achieved from minimum quantity of the starting material and the quality of the cDNA was assessed by cloning full length of coding region plus UTRs at terminal of the GAPDH gene (Figure 3). The Insilco analysis of the GAPDH gene revealed a complete coding region of 1015 bp starting from the ATG start codon to TGA stop codon. In addition to this 69 bp and 196 bp of 5′ and 3′ UTRs was found. The analysis of the GAPDH gene confirms that intact RNA was obtained and the cDNA synthesized contains the complete representation of the genes expressed.

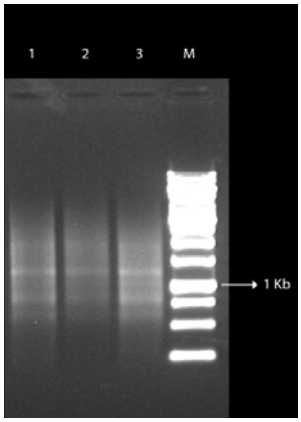

Figure 2: Uniform smearing pattern of double stranded cDNA. Lane M: 1 Kb marker (Fermentas). Lane 1, 2 and 3: 5µl of the ds cDNA showing a smear ranging from 0.2 to 4 Kb.

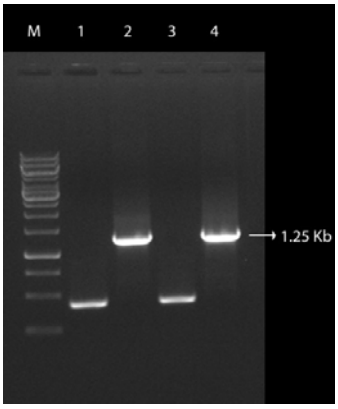

Figure 3: Analysis of the ds cDNA by PCR amplification. Lane M: 1Kb marker (Fermentas) Lane 1, 3 and 2, 4: replicates of the partial glucanase gene (0.4Kb) and full length of GAPDH gene (1.25Kb).

Conclusion

For the first time we describe an effective protocol for total RNA isolation from bark tissue of *Coffea canephora* and synthesis of full length double stranded cDNA. We successfully used the cDNA for construction of a subtractive cDNA library by SSH technique (results not reported here). This cDNA could be useful for an array of applications including gene expression analysis, isolation of full length genes, qRT-PCR, RACE and for Next Generation Sequencing (NGS).

Acknowledgement

The fellowship offered to P. Santosh by Department of Biotechnology, Government of India, New Delhi in the Project 'Indian Coffee Genome Research Programme' is gratefully acknowledged.

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