An efficient method for generating cDNAs in *Brassica carinata* A. Br.

Supriya, Neelam R. Yadav, Ram C. Yadav, Dhiraj Singh[1]

Department of Biotechnology and Molecular Biology Email: rcyadav@hau.ernet.in
[1] Department of Plant Breeding, CCS Haryana Agricultural University, Hisar-125004 INDIA

Abstract

Recombinant DNA technology makes it possible to identify and isolate genes from any biological system. Previously genes were isolated using genomic/cDNA libraries. Dealing with low abundance transcripts can be frustrating as conventional cDNA library screening is labour intensive and there are chances of loosing low abundance transcript during sample handling, poor efficiency of first and second strand cDNA synthesis and the proportion of recombinant clones to be screened. The development of PCR to detect rare transcripts has revolutionized the sensitivity of gene expression analysis as well as identification of these transcripts/ genes. Therefore, RT-PCR approach is relatively simple and efficient in identification and isolation of cDNA of a particular gene, large scale functional genomics and sequencing projects. An efficient method was developed for large scale RNA isolation and RT-PCR to generate cDNAs for isolation of Myb transcription factor genes related to drought in *Brassica species.* The total RNA was isolated from drought exposed two weeks old seedlings grown *in vitro* on MS medium. Lyophilized and liquid nitrogen frozen seedlings were used in RNA isolation. Trizol method, traditional Guanidine isothiocyanate and commercial kits were used for RNA isolation. It was found that Trizol method was quite efficient and yielded 2.25 mg per g fresh weight of the plant tissue. Electrophoretic analysis on formaldehyde gel revealed two distinct ribosomal bands. The mRNA was isolated from total RNA using Genelute mRNA miniprep kit and yielded 150-200 µg per g fresh weight of the plant tissue. Two primer sets were used to generate cDNA from isolated mRNA/ total RNA. Total RNA did not yield amplified product, however, mRNA showed amplification in all the three sets of primers which were designed on the basis of Atmyb2 conserved domain sequence. The optimized procedure gives good quality and quantity of RNA for generating cDNAs in *Brassica carinata* which is crucial in gene isolation and transcript level studies.

Key Words: *B. carinata, B. tournefortii,* RNA isolation, cDNA, Gene isolation.

Introduction

Drought is a quantitative trait involving action of many genes with small additive effects. Conventional breeding could not achieve significantly to improve stress tolerance due to the complexity of trait while genetic engineering is attractive because of its potential to improve abiotic stress tolerance more rapidly (Pellegrineschi *et al*., 2004). Plant transformation coupled with recombinant DNA technology offers attractive opportunity to improve crops in a targeted manner. Recombinant DNA technology makes it possible to identify and isolate plant genes using cDNA libraries. Recently, PCR based cloning has gained momentum as it forgoes the preparation of genomic/cDNA libraries or molecular marker analysis of a large number of plants in a suitable dihaploid or recombinant inbred lines. The development of PCR to detect rare transcripts has revolutionized the sensitivity of gene expression analysis as well as identification of these transcripts/genes. RT-PCR offers a rapid, versatile and extremely sensitive way of analyzing whether a target gene is being expressed since the technique has a virtual capability to amplify one single RNA molecule. It requires the use of specific primers on the basis of known DNA sequences of identified similar gene in other plant species (Chen *et al*., 2005; Maxfield *et al*., 2005) or degenerate primers when gene is completely unknown. Reverse transcription of RNA into cDNA is an invaluable method for gene expression analysis (Aslanian *et al*., 2004; Maxfield *et al*., 2005; Yang *et al*., 2002).

Considerable effort has been made to identify the drought and salinity inducible genes and to establish whether they have role in stress tolerance. Identification of novel transcription factor genes and their role in regulating the expression of stress induced genes will help in understanding signaling pathways leading to stress tolerance. The Myb gene family is represented by 190 genes in *Arabidopsis* (Riechmann *et al*., 2000) and 80 members in maize (Rabinowicz *et al*., 1999) but there are no reports in *Brassica* so far. Atmyb2 gene has been reported to have role in drought tolerance. So, here we describe the generation of cDNAs in an attempt to isolate Myb gene from highly heat and drought tolerant *Brassica carinata.*

Materials and Methods

The seeds of *Brassica carinata* (variety HC212) were germinated on hormone free MS (Murashige and Skoog, 1962) medium at 25^0C with a photoperiod of 16 hours light and 8 hours dark. Two weeks old seedlings were uprooted and kept on filter paper in the air at room temperature for 1hour/2hours for drought treatment. The total RNA was isolated using Trizol reagent and quantified on the Biophotometer (Eppendorf). mRNA was isolated from total RNA using Genelute mRNA miniprep kit (Sigma). RT-PCR was carried out using Qiagen One Step RT-PCR Kit and mRNA/ total RNA as template. Two sets of primers (Set I, II) were used. Primer 3 software was used to design the Set I and Set II primers covering the conserved domain of AtMyb2 gene sequence which has been found to have role in drought tolerance. PCR was carried out to further amplify the cDNA obtained after RT-PCR and the amplified product was analyzed on agarose gel. The amplified fragments were eluted from agarose gel using GenElute™ Agarose Spin Columns kit and cloned in Stratagene's pPCR-Script™ Amp

SK(+) cloning vector in XL-Blue strain of *E.coli*. Plasmid DNA was isolated from transformed clones using alkaline lysis method to study the plasmid profile.

Results

The total RNA concentration was 4500.0 µg per ml and yielded 2250.0 µg per g fresh weight of seedlings in *Brassica carinata* (Table 1). Total RNA yield was poor when it was extracted after lyophilization of seedlings. The concentration of RNA was 1212.5 µg per ml for lyophilized seedlings and yielded 606.3 µg per g fresh weight of *Brassica carinata* seedlings. The total RNA was analysed on both formaldehyde and agarose gels and two distinct ribosomal bands of 28S and 18S were observed. mRNA concentration ranged from 297.5-423.3 µg/ml yielding 148.8-211.7 µg/g fresh wt. in *Brassica carinata* (HC212) and the $A_{260/280}$ ratio varied from 1.56-1.65 (Table 2.) thus confirming the isolation of high purity of mRNA. An amplified product of around 500 bp was obtained with Set I and Set II (Fig.1) primers. Band at same position was observed in Set I and Set II as these both primers were designed from the conserved domain of AtMyb2 sequence. The gel eluted cDNA fragment was transformed using Stratagene kit. After incubation of 15-17 hrs, blue (untransformed) and white (transformed) were observed on selection plates. White colonies were putatively transformed cells with cloned insert. The transformation percentage varied from 14.8 to 20.0. The isolated plasmid DNA analyzed on agarose gel showed that the plasmid DNA was higher than 3.0 Kb (size of pPCR Script Amp SK(+) vector, Stratagene) confirming the presence of insert (Fig.2). PCR amplification of isolated plasmid DNA was carried out using designed primers and amplification of around 500 bp product was observed using Set I and Set II primers. Thus, plasmid DNA amplified the desired cDNA in PCR reactions. The PCR-Script^{TM} Amp cloning kit of Stratagene gave efficient cloning of PCR fragments with high yield and a low rate of false positives because out of ten clones screened, insert was present in seven clones. Here, we have isolated and cloned partial cDNA of Myb transcription factor gene confirmed by above experiments. The plasmid DNA band of higher molecular wt. than vector DNA was evident in the gel.

Discussion

Total RNA isolation by Trizol reagent method is very effective as it is easy, efficient and less time consuming method and total RNA yield was 2.25 mg per g fresh weight of the plant tissue. The commercial kits may be efficient but are not cost effective when compared to Trizol and other methods. In our experiments, 148.8 to 211.7µg/g fresh wt. of mRNA was recovered from 2250-2862.5 µg/g fresh wt. of total RNA in *Brassica carinata* The mRNA was, therefore, one-tenth part of total RNA. Stangegaard *et al*. (2006) isolated mRNA using oligo dT Dynabeads and from 100 µg total RNA, 2-3 µg mRNA was obtained. The use of purified mRNA is recommended by several workers since it gives higher concentration of first strand cDNA. The failure of total RNA to generate detectable cDNA also supports this view rather than the absence of Myb transcripts in total RNA. We performed RT-PCR reactions using total RNA as well as mRNA as template. We successfully, used mRNA as template for RT-PCR but no amplification was observed using total RNA as template. The myb cDNA was successfully cloned using pPCRscript vector. Transformation efficiency obtained using this kit was approximately 14.2 to 20.0 per cent. However, further confirmation will come from sequence analysis of cloned cDNA. Rapid amplification of cDNA ends (RACE) is an efficient approach for obtaining full length cDNA when only partial sequences are available (Frohman et al., 1988; Eyal *et al*., 1999; Park *et al*., 2003). Availability of the sequence will enable us to design primers for RACE or PCR walking to isolate the full length cDNA of drought inducible Myb transcription factor gene in *Brassica carinata*.

Conclusions

The research paper describes the efficient method for total RNA isolation in *Brassica carinata*. mRNA purified from total RNA preparations were successfully used for producing myb cDNAs related to drought tolerance in B.carinata. It can also be used to generate cDNAs in other Brassica species.

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Table 1. Spectrophotometric analysis of total RNA of two weeks old *Brassica carinata* A. Br. seedlings extracted by Trizol reagent

Sample	Concentration (µg/ml)	$A_{260/280}$	Concentration (µg/g fresh wt.)
	Liquid Nitrogen		
Control	4500.0	1.54	2250.0
1hr dehydration	5725.0	1.52	2862.5
2hr dehydration	4650.0	1.61	2325.0
	Lyophilization		
Control	1212.5	1.63	606.3
1hr dehydration	1189.1	1.60	594.5
2hr dehydration	1350.0	1.52	675.0

Table 2. Spectrophotometric analysis of mRNA of *B. carinata* A.Br. variety HC212

Sample	Concentration (µg/ml)	$A_{260/280}$	Concentration (µg/g fresh wt.)
Control	297.5	1.59	148.8
1hr dehydration	423.3	1.56	211.7
2hr dehydration	355.0	1.65	177.5

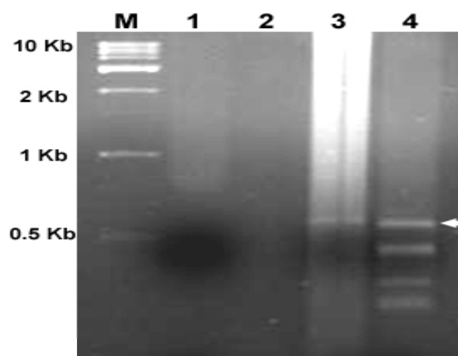
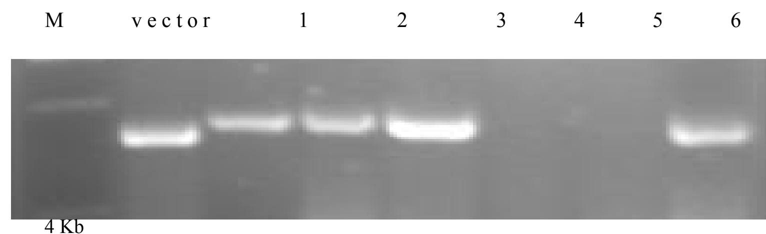


Fig. 1 Electrophoretic pattern of cDNA obtained from RT-PCR of mRNA isolated from two weeks old seedlings of *Brassica carinata* var. HC212 using Set I and Set II primers M- 1Kb Ladder, 1 - Negative control (water); 2 – Control; 3- Set I Myb primers (1hr dehydration); 4 – Set II Myb primers (1hr dehydration)

M vector 1 2 3 4 5 6



4 Kb

Fig.2. Plasmid DNA isolated from transformed colonies showing pPCR Script amp SK+ with *myb* insert. M -1 Kb Ladder; Vector DNA; 1 – 6- Plasmid DNA from transformed clones