

Application of the mRNA Capture Kit in cDNA-AFLP

Richard Feron*, Celestina Mariani, and Wim Hendrik Vriezen

Department of Experimental Botany, University of Nijmegen, Nijmegen, The Netherlands

*Corresponding author: richardf@sci.kun.nl

Introduction

Since its introduction in 1996, cDNA-AFLP (Amplified Fragment Length Polymorphisms) has become a preferred tool for the study of differential genome-wide gene expression in both plant and animal systems [1]. cDNA-AFLP is a highly sensitive and reproducible PCR-based technique that can be used without prior knowledge of DNA sequences; it is therefore the favored method for gene expression analysis in species of which the genome has not been sequenced. The cDNA-AFLP method consists of:

- Conversion of mRNA to cDNA
- Digestion of the cDNA using a rare cutting (hexacutter, e.g., *Bst*Y) and a frequent cutting (tetracutter) endorestriction nuclease (e.g., *Mse*I) (Figure 1)
- Ligation of adapters
- Pre-amplification of the ligated fragments using adapter-specific primers
- Amplification of small fragment pools using the adapter-specific primers complemented with one or more selective nucleotides on each primer

Most cDNA-AFLP protocols are based on that of Bachem et al. and describe the use of streptavidin-coated paramagnetic beads to immobilize biotinylated cDNA during the digestion steps [1]. Here we describe a procedure using the mRNA Capture Kit from Roche Applied Science ("one-tube method") and the advantages of using streptavidin-coated PCR tubes instead of streptavidin-coated paramagnetic beads. Messenger RNA reverse transcription and cDNA restriction can now be performed efficiently in a one-tube format eliminating several time-consuming steps such as washing (Figure 1). In addition, all incubation steps can be performed in a thermocycler.

In the work presented here, the effect of gibberellic acid (GA_3) treatment or pollination on tomato ovules and ovary wall development was studied.

Materials and Methods

Total RNA was isolated from ovules and ovary walls of untreated ovaries, from GA_3 -treated ovaries, and from ovaries of flowers 1, 2, and 3 days after pollination using a RNA isolation kit. Five micrograms of total RNA in a maximum of 10 μ l demineralized RNase-free water was mixed with 40 μ l lysis buffer and 0.05 μ l biotinylated oligo

dT (both included in the mRNA Capture Kit). This mix was transferred to streptavidin-coated PCR tubes (mRNA Capture Kit), incubated at 37°C for 5 minutes in a thermocycler, allowed to cool to room temperature, and stored on ice. During this step, the polyA tails of the mRNA hybridize to the biotinylated oligo dT that binds with its biotin molecule to the streptavidin-coated PCR tube wall. The liquid was discarded and the bound mRNA washed twice with 100 μ l wash buffer (mRNA Capture Kit).

Reverse transcription was started by adding 50 μ l of first-strand cDNA synthesis mixture (31.3 μ l water, 10 μ l 5x first-strand buffer, 5 μ l 0.1-M dithiothreitol [DTT], 2.5 μ l 10-mM dNTPs, and 1.2 μ l RNase H-reverse transcriptase [200 units/ μ l]) and the reaction mix was incubated for 2 hours at 42°C. Thereafter, tubes were stored on ice and 10 μ l of the reaction volume was discarded to obtain a total volume of 40 μ l. A mixture containing 87 μ l demineralized water, 16 μ l 10x *E. coli* ligase buffer, 6 μ l 0.1-M DTT, 3 μ l 10-mM dNTPs, 1.5 μ l *E. coli* ligase, 4.25 μ l (5 units)



Richard Feron

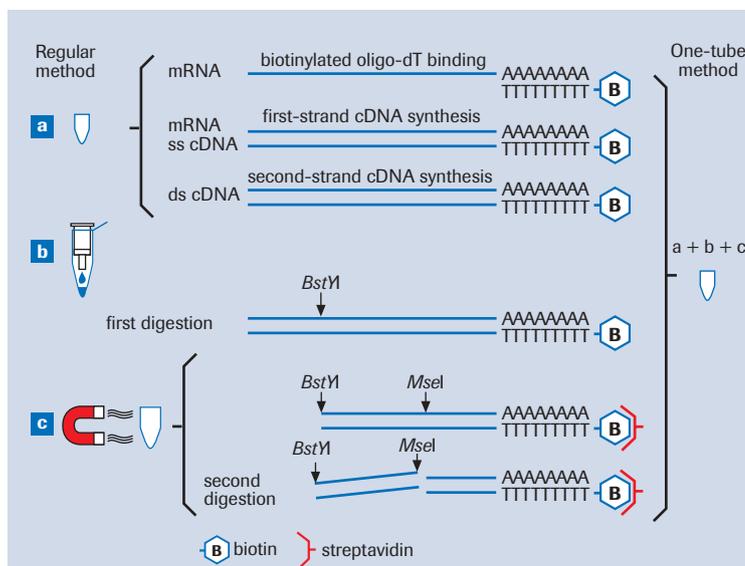


Figure 1: First steps of the regular cDNA-AFLP procedure to obtain gene-specific cDNA fragments.

(a) Double-stranded (ds) cDNA is synthesized in a 200- μ l PCR tube.

(b) The obtained cDNA is purified using a column and quantified. An aliquot of the cDNA is digested.

(c) Subsequently, the 3' ends of the cDNAs are captured with streptavidin-coated paramagnetic beads (red). Washing and capturing occurs in several steps. With the novel one-tube method all these steps are performed in one streptavidin-coated PCR tube.

E. coli DNA polymerase I, and 1.6 μ l RNase H was added and incubated for 1 hour at 12°C, and subsequently for 1 hour at 22°C in the thermocycler. After incubation, the reaction mixture was discarded while the double-stranded (ds) cDNA was still attached to the tube wall. The cDNA was washed three times with 200 μ l wash buffer (mRNA Capture Kit) by simply incubating the sample for 2–5 minutes at room temperature and subsequent removal of the liquid. The first cDNA digestion was started by adding a mixture containing 38.8 μ l water, 10 μ l 5x restriction-ligation (RL) buffer (50 mM Tris-HAc pH 7.5, 50 mM MgAc₂, 250 mM KAc, 25 mM DTT) and 1.2 μ l *Bst*YI (10 units/ μ l). After incubation for 2 hours at 60°C, tubes were washed three times with 100 μ l wash buffer (mRNA Capture Kit). The second digestion was initiated by adding a mixture of

38.8 μ l water, 10 μ l RL buffer, and 1.2 μ l *Mse*I (10 units/ μ l) followed by incubation for 2 hours at 37°C.

Results and Discussion

Liberated *Bst*YI-*Mse*I fragments were used for the next steps of the cDNA-AFLP procedure, which was carried out essentially as described by Breyne et al. [2]. Figure 2a shows that the cDNA concentration after pre-amplification of the cDNA fragments isolated with the one-tube method is within the same range for nine reactions performed with different RNA samples. Furthermore, the expression patterns of genes obtained after selective amplification and displayed on an acrylamide gel were distinct (Figure 2) and reproducible in two independent experiments (data not shown).

The advantages of the one-tube method are manifold. Wash steps are easier, quicker, and more efficient, as the void volume of the PCR tubes is smaller than that of a certain amount of paramagnetic beads. Total RNA can be used, but only the mRNA is bound to the tube wall and ribosomal and transfer RNA are washed away prior to the reverse transcription reaction. For the same reason, it is also unnecessary to remove traces of genomic DNA by DNase treatment. Two hundred DNA fragments that were isolated from gel and analyzed corresponded to tomato expressed sequence tags (EST), and none of them contained an intron. Streptavidin-coated PCR tubes are also usable in other applications that include capturing biotinylated fragments; e.g., we successfully used these tubes in a transposon display experiment. As in AFLP [3], genomic DNA is digested with a hexacutter and a tetra-cutter to generate fragments that are captured after ligation of biotinylated adapters. Subsequent procedural details are described by Van Den Broeck et al. [4]. ■

Acknowledgements

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References

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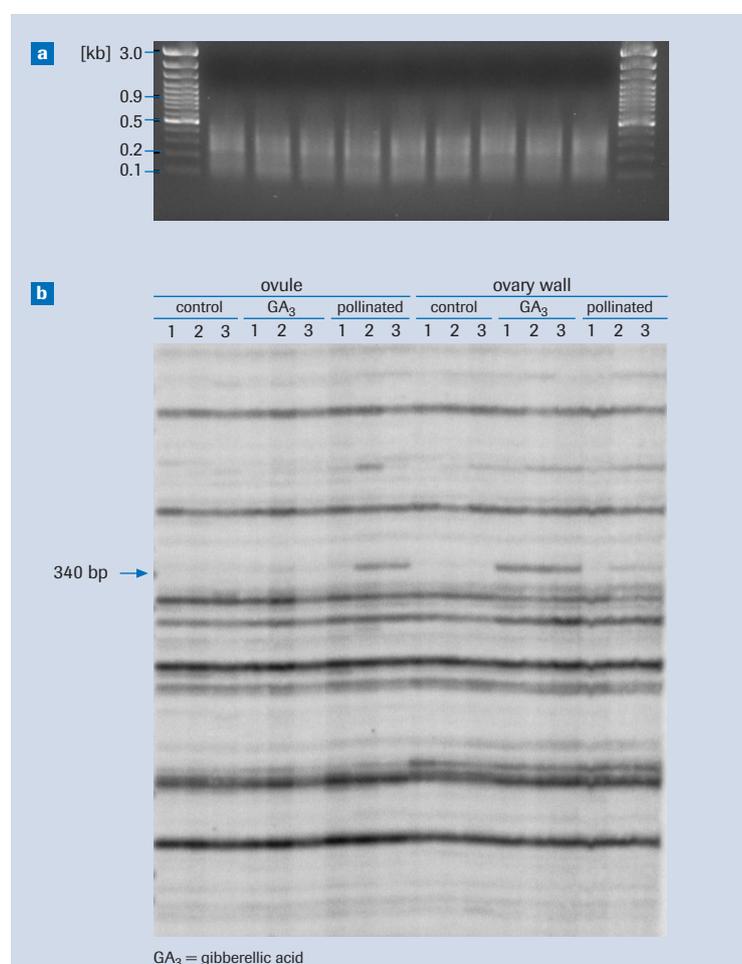


Figure 2: Pre-amplification and selective amplification products from cDNA of tomato mRNA.

(a) Ethidium bromide-stained agarose gel loaded with cDNA obtained after a pre-amplification reaction with nonselective primers on cDNA fragments. A 100-bp DNA ladder is loaded on both sides.

(b) Acrylamide gel displaying a representative result obtained after amplification of a part of the cDNA fragments using primers with two selective nucleotides each. The arrow indicates a fragment (340 bp) of a gene whose mRNA level is induced two and three days after pollination in the ovules and by gibberellic acid (GA₃) treatment in the carpel wall.

Product	Pack Size	Cat. No.
mRNA Capture Kit	1 kit (192 reactions)	11 787 896 001

