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cDNA Library Preparation from a Single Wheat Kernel

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A simplified cDNA cloning protocol was elaborated, easily scalable to very different sizes of plant tissue. The mRNA fraction was extracted from a single wheat kernel with oligo dT magnetic beads. The solid-phase mRNA was transcribed into single-strand cDNA by reverse transcription. Then DNA tags were incorporated into the ds cDNA fragments prior to PCR amplification. The amplified DNA was ligated to a T overhang cloning vector and some of the resulting clones were sequenced. These sequences were identified by BLASTN search in wheat EST databases.

Key words: Wheat kernel, cDNA library, solid-phase PCR, DNA sequencing, EST.

INTRODUCTION

Copy DNA cloning has been used as an effective method to isolate genes in past decades, despite its obvious disadvantages. cDNA sequences, being reverse transcripts of mRNA molecules, contain neither regulatory sequences (promoters, enhancers, terminators) nor introns. Many of them even carry partial coding sequences only, due to premature termination of reverse transcription (Ikeda et al., 2002). Unlike genomic libraries, cDNA banks contain only part of the total genetic information carried by the cell, representing the transcriptome of a given tissue. cDNA libraries allow easier isolation of genes, especially abundantly expressing genes (Zierold et al., 2005).

Random sequencing of cDNA fragments (EST databases) provides valuable information on the genetic constitution of species whose genome sequences are not available yet (Clarke et al., 2002). In many cases, however, the amount of target tissue is very small and the use of PCR amplification is unavoidable (Dresselhaus et al., 1994; McCarry et al., 1994). The most critical points of these procedures are the ethanol precipitation steps, in which the minute amount of DNA can be easily lost. The introduction of solid-phase nucleic acid isolation and PCR techniques has eliminated this problem, dramatically increasing the efficiency of small-scale cDNA cloning (Stamm and Brosius, 1991; Lambert and Williamson, 1993). Finally the procedure could be down-scaled to its absolute minimum, the single cell (Karrer et al., 1995). Since mRNA molecules contain unknown sequences at the 5' ends, an oligomer with a known sequence has to be incorporated at that position prior to PCR reactions. Several approaches employed to overcome this problem have met with obstacles. In this paper we describe a simpler and more effective procedure based on GMP tailing and single-strand linker ligation (Schmidt and Mueller, 1996; Shibata et al., 2001).

MATERIALS AND METHODS

PLANT MATERIALS

A single kernel was isolated from a field-grown plant of the wheat cultivar Lovászpatonai 407 16 days after anthesis.

mRNA ISOLATION

The kernel was crushed in liquid nitrogen with a mortar and pestle and dissolved in 400 µl Dynal lysis buffer (Invitrogen). The lysate was centrifuged in a benchtop microcentrifuge at 13,000 rpm for 10 min. The mRNA was extracted from the supernatant with 20 µl dynabeads according to the manufacturer's instructions. The purification procedure was repeated with a fresh aliquot of dynabeads.

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FIRST STRAND SYNTHESIS

First-strand cDNA was synthesized in 20 μl first-strand buffer with 200 U SuperScript II reverse transcriptase (Invitrogen) for 1 h at 45°C. The reaction was stopped with 1 μl 250 mM EDTA and the mRNA strand was hydrolyzed by adding 21 μl 0.1 N NaOH for 1 h at 65°C. The cDNA dynabead pellet was then washed with 2 × 300 μl sterile water and 50 μl 1 × terminal deoxynucleotidyl transferase (TdT) buffer.

rGTP TAILING

GMP addition was performed according to Schmidt and Mueller (1996). Dynabeads were resuspended in 20 μl volume containing 200 mM potassium cacodylate, 25 mM Tris-HCL, 0.25 mg/ml BSA, pH 6.6 (TdT buffer), 1.5 mM $CoCl_2$, 0.5 μM rGTP and 100 U TdT enzyme (Roche Applied Science). The reaction was incubated at 37°C for 1 h. The expected rate of reaction was 97.9%: +1 GMP (0.0%), +2 GMPs (68.7%), +3 GMPs (29.1%), +4 GMPs (0.1%). After finishing the reaction the sample was washed with 2 × 300 μl water and 20 μl ligation buffer.

ADAPTOR LIGATION

AD1 (5'-AATTCGCTCCGTTCAGCACC-3') and AD2P (5'-PHOS/-TGCTGAACGGAGCGAATT-3') oligomers were annealed to form a double-stranded adaptor with 2 C overhang. The ligation reaction was performed in 20 μl ligation buffer with 1 U T4 ligase (Invitrogen) at 1 nM adaptor concentration, at 9°C, for 16 h. The sample was washed again with 2 × 300 μl water and 2 × 100 μl Taq polymerase buffer.

SECOND-STRAND SYNTHESIS

The magnetic beads with the adaptor-tagged single-strand cDNA were resuspended in 50 μl standard PCR buffer containing 0.2 mM dNTP-s, 1.5 mM $MgCl_2$, and 1 U Platinum Taq polymerase (Invitrogen). The sample was incubated at 72°C for 3 min. After washing with 2 × 300 μl water the beads were resuspended in 50 μl Tris buffer (10 mM, pH 8). This suspension was heated to 94°C for 3 min, chilled on ice, and then the supernatant containing the second strand was transferred to a fresh tube. The beads with the first strand were overlaid with 50 μl 1% SDS and 10 mM Tris, pH 8, and stored at 4°C.

PCR AMPLIFICATION

Aliquots were taken from the second-strand solution and amplified with one forward (AD1) and two reverse primers: Not dT [5'- CTAGATCGCGAGCGGCCGCCCT(15)-3'] and Not 1 (5'-CTAGATCGCGAGCGGCCGCCC-3') at 0.2 μM concentration in 50 μl volume. Other components were identical to those of the second-strand synthesis (see above). The cycling parameters were as follows: 94°C for 3 min, 50°C for 1 min, 72°C for 3 min, 94°C for 0.5 min, 60°C for 0.5 min, 72°C for 3 min. The last three steps were repeated 10 times and the reaction was incubated for 20 min at 72°C. PCR products were analyzed on 1% agarose gel.

SIZE SELECTION

The amplified cDNA samples were ethanol-precipitated and loaded onto a Sephacryl-400 column (Amersham) according to the Stratagen ZAP cDNA Synthesis Kit protocol.

MOLECULAR CLONING

Approximately 20 ng cDNA was ligated to 50 ng pGEMT vector (Promega) in 10 μl volume with 1 U T_4 ligase (Invitrogen) at 4°C for 24 h. 1.5 μl aliquots were taken from this ligation mixture and electroporated into ElectroMax DH10B cells (Invitrogen) with a Biorad MicroPulser at 25 kV for 6 sec. The cells were spread on LB ampicillin (100 μg/ml) plates containing X-Gal indicator. White colonies were selected for further analysis.

DNA SEQUENCING

DNA minipreps were made with the Qiagen Plasmid Mini Kit and the sequencing reactions were performed with a BigDye Terminator 3.1 kit. Sequence data were read by an ABI Prism 3100 automated sequencer. The BLASTN search was performed at www.tigr.org

RESULTS AND DISCUSSION

mRNA ISOLATION

The mRNA fraction was directly extracted from the supernatant of the lysate without any pretreatment. It is worth noting that the traditional phenol-chloroform extraction, ethanol precipitation methods are not at all practical for use with immature kernels, due to the extremely high carbohydrate content of this tissue. After ethanol precipitation these carbohydrates form a compact insoluble pellet enclosing the nucleic acids as well. That is why the frozen tissue was immediately dissolved in the lysis buffer in the Dynal kit. The SDS and DTT content of this buffer proved to be enough to protect the RNA-s from the ribonucleases. Although the crudest debris was removed by centrifugation, the supernatant still remained very contaminated, so the mRNA was elut-

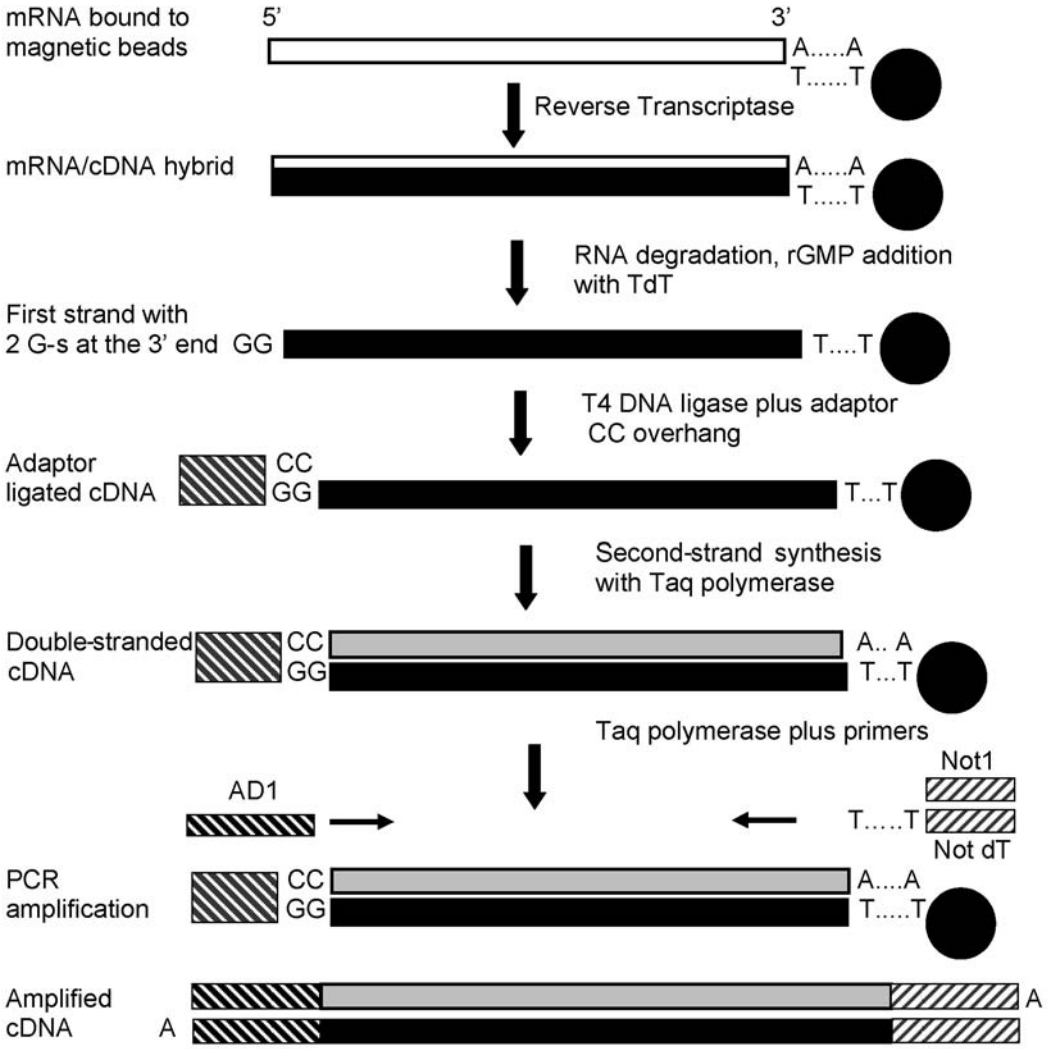

Fig. 1. Schematic representation of the cDNA synthesis and cloning procedure. Open box – mRNA; black box – first-strand cDNA; grey box – second-strand cDNA; striped boxes – primers and adaptors.

ed from the beads and the binding-washing procedure was repeated. In this way not only were the last traces of cell content removed, but the rRNA cross-contamination that can be detected in larger-scale experiments (Nagy et al., 2003) was reduced. We did not attempt to quantify the purified mRNA at this step because the amount was so small.

cDNA SYNTHESIS

First-strand cDNA synthesis can be easily accomplished with any kind of oligo dT primer, as the vast majority of eucaryotic mRNAs contain a 3' poly A tail. Initiation of the second strand is much more complicated, since the 5' ends of the mRNAs are generally unknown. In this work we chose (ribo)GTP tailing methods followed by single-strand adaptor ligation, because of its simplicity (Fig. 1).

In specific reaction conditions using rGTP as substrate, the terminal-deoxynucleotidyl-transferase reaction can be highly regulated. From two to four GMP residues can be added to the 5' ends of more than 90% of the single-strand cDNA molecules. Adaptors with two protruding cytosines were added to these G-tailed molecules at multiple molar excess to ensure efficient ligation. Tagging the 5' end of the cDNA with a known sequence solves three different problems in parallel: the tag serves as a primer for second-strand synthesis as well as for the subsequent PCR reaction, meanwhile incorporating an appropriate restriction site for molecular cloning. The 5' adaptor contains an incomplete Eco RI site,

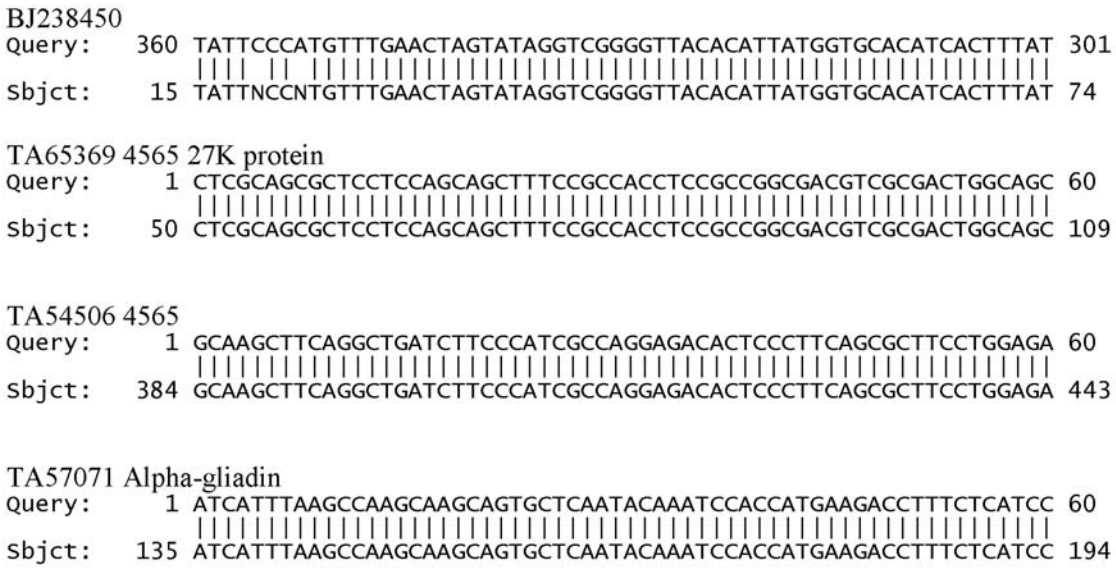

Fig. 2. Sequence alignments resulting from the BLASTN search. Highly homologous scores were identified in wheat EST databases. The first 60 bp at the N terminal end of each insert are shown.

TABLE 1. The most important characteristics of the cDNA library

Efficiency (cfu/ng cDNA)	% of recombinant clones	Average insert size (bp)	Total size of library (cfu)
3500	70	~800	3.5×10^7

which can be recovered with T4 polymerase treatment (Aslanidis and de Jong, 1990).

After performing the second-strand synthesis with Taq polymerase, the new strands were separated from the first strands and the beads, and subjected to PCR amplification (Fig. 1).

PCR AMPLIFICATION AND MOLECULAR CLONING

Not dT(15) primer-adaptor was used as a reverse primer in the first cycle introducing a Not I cloning site at the 3' end of cDNA. Subsequent cycles were initiated from the AD1 and Not 1 primers.

Pilot PCR reactions were conducted to determine the crucial experimental parameters in order to produce a sufficient amount of cDNA in the exponential phase of the amplification process, to avoid the PCR plateau effect (Sardelli 1993). Small aliquots were taken from the original cDNA pool representing 1%, 2%, 4% and 8% of the sample. These aliquots were amplified in 10 cycles. The products were analyzed on agarose gel along with Mbo I digested wheat genomic DNA of known quantity (data not shown). The distribution of this DNA is similar to the PCR product, allowing approximate quantification of the latter. According to our estimation, 0.5 µl template (1%) produced ~100 ng DNA. Calculating these data, 0.5 µl aliquots were finally amplified in four tubes to produce a substantial amount of DNA for subsequent cloning. This DNA was size-selected and purified on a Sephacryl 400 column, removing the short fragments (<500 bp) as well as the primers and unincorporated nucleotides.

The size-selected DNA was ligated to pGEMT plasmid vector, taking advantage of the adenine tailing specific to Taq polymerases. After electroporation the number of recombinant clones proved high enough (>10,000) to carry out large-scale macroarray screening, so preparation of cDNA fragments with Eco R I/Not I cohesive ends was not necessary (Tab. 1).

Four randomly selected inserts were sequenced to verify our library. The BLASTN search identified highly homologous wheat ESTs, of which three were isolated also from immature kernels (Fig. 2).

CONCLUDING REMARKS

The aim of this work was to compile a simple, fast and low-cost cDNA cloning procedure without sacrificing reliability. We think that this protocol can be applied to a very small amount of plant tissue as well as to large samples. We tried to minimize the number of experimental steps and enzymes in order to save time and costs. We anticipate that our protocol will be particularly useful to a researcher with lit-

tle experience in molecular biology and/or working in laboratories with limited facilities.

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