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An improved method for generating subtracted cDNA libraries using phage lambda vectors

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We have used a biotin-based subtraction procedure to enrich a potato tuber cDNA library for sink-specific clones. The method uses single-stranded phagemids with directional inserts as both driver and target. We modified the λZAP II vector for the directional cDNA cloning and for subsequent subtractive hybridization. This improved method of subtractive hybridization circumvents many problems facing standard protocols. The present method uses the advantage of λ-phage cloning which makes it possible to establish libraries from limited amounts of tissue. *In vivo* excision of single-stranded phagemids containing the cDNA inserts was performed to provide an unlimited source of DNA for biotinylation, hybridization and subtraction. This ability to produce unlimited quantities of DNA for subtraction is advantageous over other methods which require large amounts of RNA as the starting point (2,7,8). The phage vector system is more convenient than a plasmid/colony system (3,6,9) when the original library is to be re-screened for additional clones. The λZAP II vector was modified by introducing an adaptor (EX1: 5′-AAT TAT CTC GAG GGC CCG ATC GGC CGA ATT CGT-3′ annealed to EX2: 5′-T CGA ACG AAT TCG GCC GAT CGGGCC CTC GAG AT-3′; TIB MolBiol, Germany, Berlin) that destroys the original *Eco*RI and *Xho*I sites but contains both restriction sites in inverted orientation flanking a central *Sfi*I site. The resulting vector was designated λPAZ II. Total RNA from sink (growing) and source (sprouting) tubers was isolated according to (4) and poly A^+ RNA was purified by chromatography on oligo-d(T)-cellulose (Pharmacia, Type 7). cDNA libraries were constructed using Uni-ZAP cDNA synthesis system (Stratagene). The cDNAs from sink and source tubers were cloned into λZAP II and λPAZ II, respectively (Fig. 1). Both libraries had a complexity of 3×10^6 p.f.u. with ~90% recombinant clones. The average size of the inserts were in the range of 1 kb. From both libraries, single stranded (ss) circular DNA were generated by *in vivo* excision and single-stranded DNA was isolated (6). Single-stranded DNA from source tubers was biotinylated with 1 μg/μl long-arm Photoprobe® biotin (Vector Laboratories, Burlingame, UK; 5). Typically, 2.5 μg ssDNA from the sink tuber library was hybridized to 20–30 μg of biotinylated ssDNA from source tuber, in 20 μl 0.75 M NaCl, 50 mM HEPES pH 7.6, 10 mM EDTA and 0.1% SDS at 65°C for 20 h under mineral oil (Sigma). Subsequently, the mineral oil was removed and the mixture was incubated with 100 μg vectrex-avidin (Vector Laboratories) for 5 min at room temperature. Streptavidin–biotin–DNA complexes were precipitated by ammonium acetate to the final concentration of 2.5 M on ice. Samples were extracted 3 times with avidin and ammonium acetate. The ssDNA in the unbound fraction (enriched for sink tuber specific ssDNA) was precipitated in the presence of glycogen. The subtracted ssDNA was converted to dsDNA prior to transfection into *Escherichia coli* using a poly A-tail specific oligo (XA: 5′-$(A)_{11}$CTC GAG-3′; TIB MolBiol) and 10 U DNA polymerase I, to increase the transformation efficiency. An aliquot of the double-strand DNA was used to electrotransform DH5α cells (BioRad Gene Pulser/Pulse Controller). The ratio of blue versus white colonies is increased in the subtracted library because of the simultaneous enrichment of non-insert containing ssDNA molecules. Fifty randomly-picked white colonies from the subtracted libraries were analysed by sequencing the C-terminus.

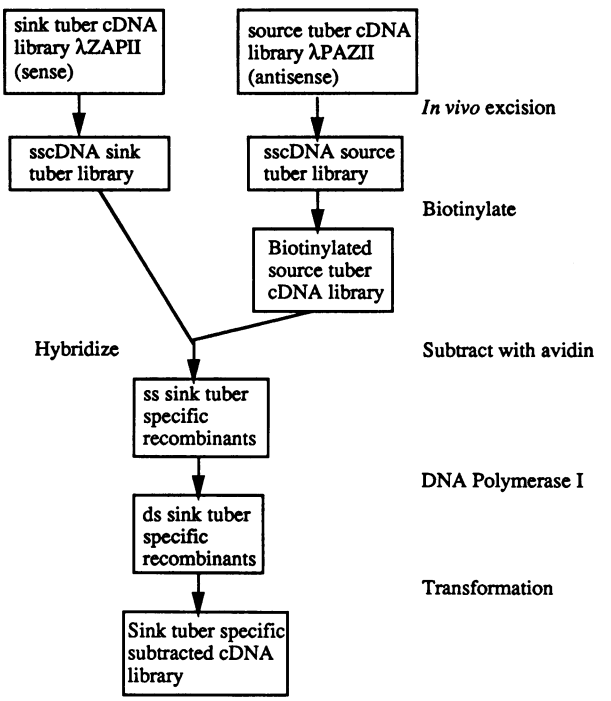

Figure 1. Schematic diagram of the library subtraction procedure. ss, single-stranded; ds, double-stranded.

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All analysed cDNAs contained a poly A-tail, indicating that the cDNAs derived from the sink tuber cDNA library. Colonies (~5000) were picked into microtiter plates and characterized by differential colony hybridization and, in some cases, were verified for differential expression by Northern analysis (Fig. 2). Hybridization with cDNA probes encoding potato tuber proteins, which were predominantly expressed in sink tubers (1), showed that <10% of the subtracted cDNA library are represented by these investigated genes. Almost 65% of the clones are not detectable while only 1% of the cDNAs presented exclusively expressed genes in source tubers (data not shown). This included several novel sink-tuber specific cDNAs. While this work was in progress, several reports (3,5,6,8,9) describing methods using photobiotinylated ssDNA species were published. However, the complementarity of the hybridizing species from two directional cDNA libraries is an improvement over conventional hybridization procedures. Use of different orientated phagemids is suitable for obtaining large amounts of biotinylated driver DNA and conversion of ssDNA obtained in the unbound fraction further improves the efficiency of the subtraction procedure described here. The method represents a powerful tool for studying gene expression even at the level of small cellular populations.

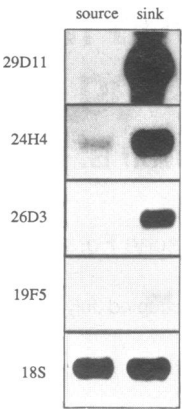

Figure 2. Examples of Northern blots for cDNA clones isolated by subtraction. Total RNA (50 µg) from sink and source tuber was fractionated by electrophoresis. The resultant Northern blots were hybridized with random primer labeled cDNA inserts from clones 29D11, 24H4, 26D3 and 19F5 and equilized with 18S rDNA probe.

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