Notes Plant Tissue Culture Letters, **10**(3), 307-313 (1993)

Subtractive Hybridization

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1. *Introduction*

A very useful method for identifying genes involved in biological events is subtracitive hybridization, which results in the isolation of mRNAs (in the form of cDNA) that occur in different amounts in two mRNA populations. Although several versions of the subtracitive hybridization method have been reported in recent molecular biology texts, we are particularly interested in establishing a reliable subtraction method that 1) requires only small amounts of starting material (*e. g.*, plant cells of *ca.* 100 mg fresh weight), 2) is capable of isolating genes that are expressed differentially but relatively weakly, and 3) does not use delicate separation techniques, such as hydroxyapatite chromatography. We have found that the subtractive hybridization method recently reported by Wang and Brown[1] is very suitable for our purpose and have adapted it to isolate genes involved in nicotine biosynthesis. This method, which was originally developed as a gene expression screen, utilizes the polymerase chain reaction (PCR) and a two-phase separation using biotinylated DNA and streptavidin.

In view of the growing interest among plant scientists in reliable and simple subtraction methods, we would like to introduce the method by Wang and Brown in this article. In this report we concentrate on the techniques that are described only briefly, or even omitted, in the original paper, and also add some practical tips that may be useful for scientists who are not familiar with this technique. We recommend that the original paper[1] be read first to obtain a better understanding of the protocols described here.

2. *Preparation of RNA and DNA*

Total RNA was isolated from cultured roots of wild-type tobacco (*Nicotiana tabacum* cv. Burley 21) and a low-nicotine tobacco mutant with the same genetic background (see ref. 2), by the SDS/phenol method as described in ref. 3, except that the final ultracentrifugation step was omitted. Poly $(A)^+$ RNA was selected with an mRNA Purification Kit (Pharmacia).

Five micrograms of poly $(A)^+$RNA from wild-type and mutant tobacco were used to construct double-stranded cDNA, using cDNA Synthesis System Plus (Amersham) (also see **Fig. 1**). A phagemid cDNA library was made separately from wild-type tobacco root, using Librarian II (bidirectional, electrocompetent system; Invitrogen).

3. *Restriction Enzyme Digestion, Linker Ligation, and PCR Amplification*

We essentially followed the procedures in the original paper[1] (**Fig. 1**). Two oligonucleotides (5′-CTCTTGCTTGAATTCGGACTA and 5′-TAGTCCGAATTCAAGCAAGAGCACA) were synthesized with 5′-dimethoxytrityl residues by 381A DNA Synthesizer (Applied Biosystems) and purified by HPLC using TOSOH TSK-GEL ODS-80TM (4.6×250 mm). The two oligonucleotides (50 μg each) were phosphorylated by T4 kinase (200 unit), mixed together, treated with phenol/chloroform, and concentrated to *ca.* 100 μl by butanol extraction. After the mixture was incubated at 45°C for 10

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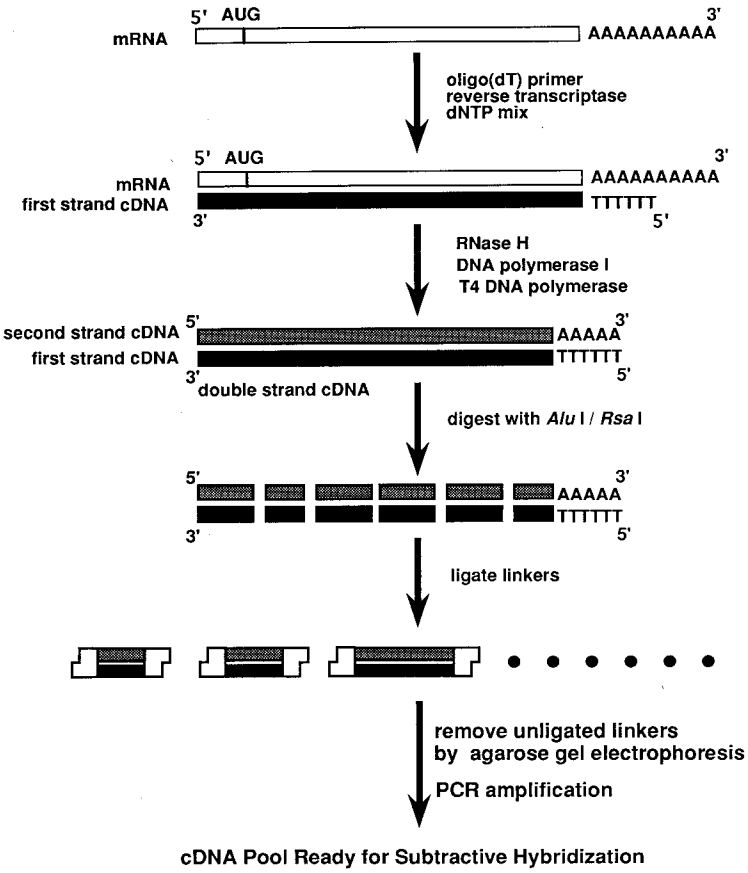


Fig. 1 Synthesis of cDNA for subtractive hybridization.

min, the phosphorylated linker was precipitated by ethanol and dissolved in 20 μl of 10 mM Tris buffer (pH 7.5).

Before using valuable cDNAs, a control experiment should be performed first to check the performance of the synthesized linker and to become familiar with the technique. To this end, we first used pBluescript II SK$^-$ (Stratagene) as a template DNA. The plasmid was digested either with *Alu* I alone, or by *Alu* I and *Rsa* I, to yield DNA fragments of smaller than 500 bp (see Appendix of the Stratagene catalog for the expected size of the fragments). The DNA fragments (0.25 μg for each digestion) were then ligated with the phosphorylated linker (5 μg) by T4 DNA ligase (5 unit). The unligated linkers were removed by electrophoresing the ligation mixtures through a 1.4% low-melting point agarose gel until bromophenol blue dye migrated 2 to 2.5 cm, and then by recovering the agarose portions corresponding to the DNA fragments of 0.2 to 2 kb (**Fig. 2-A**). PCR amplification was performed using 1 μl of the melted agarose as previously described[1], except that the autoextension time was reduced from 25 sec to 5 sec. Several DNA fragments of the expected size should be amplified (**Fig. 2-B**).

This control experiment should confirm that all the steps up to the first PCR amplification are performed as expected. In our work with tobacco root cDNAs, however, we noticed that PCR amplification often produced a smear of abberantly amplified DNA larger than 2 kb (**Fig. 3**). The high molecular weight smear did not diminish by changing the annealing temperature, but was effectively suppressed by using AmpliWax PCR Gem 100 (Takara) during PCR reactions (**Fig. 3**). Therefore, our tobacco cDNAs were PCR-amplified, and always "hot started" with AmpliWax.

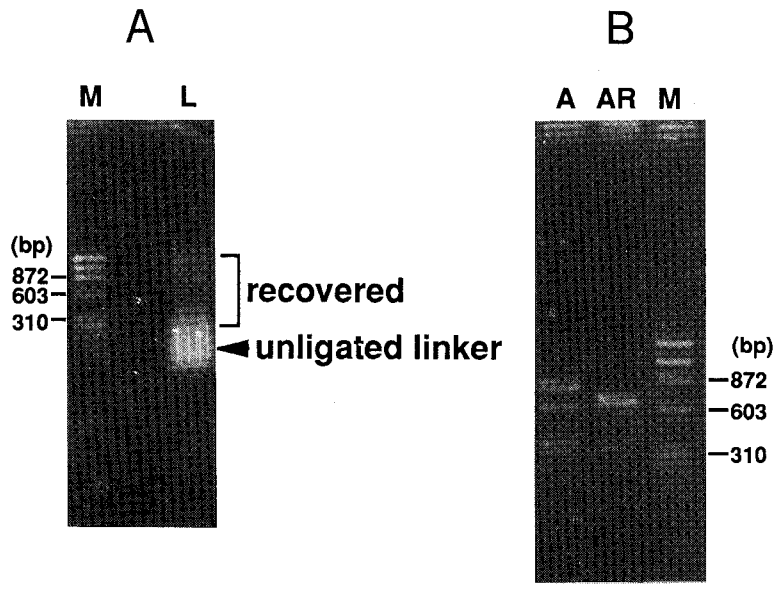


Fig. 2 PCR amplification of control DNA.
A: Gel electrophoresis of ligation mixture on low-melting point agarose. M, molecular size marker (ϕX174/*Hae* III); L, ligation mixture. B: Analysis of PCR products on 1.4% agarose. *Alu* I and *Rsa* I fragments of pBluescript II. M, molecular size marker (ϕX174/*Hae* III); A, *Alu* I-digested DNA as PCR template; AR, *Alu* I/*Rsa* I-digested DNA as PCR template.

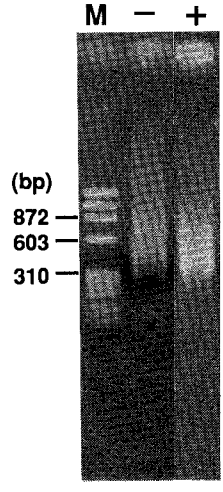


Fig. 3 Effect of "Hot start" on PCR.
M, molecular size marker (ϕX174/*Hae* III); −, wild-type cDNA amplified without "Hot start"; +, wild-type cDNA amplified with "Hot start".

4. *Subtractive Hybridization*

One hybridization cycle consists of photobiotinylation of the driver (mutant tobacco) DNA, hybridization of the driver and tracer (wild-type tobacco) DNAs for 20 hr, recovery of the non-hybridizing tracer DNA by streptavidin binding, hybridization of the driver and recovered tracer DNAs once again for just 2 hr, and finally PCR-amplification of the tracer DNA recovered from the

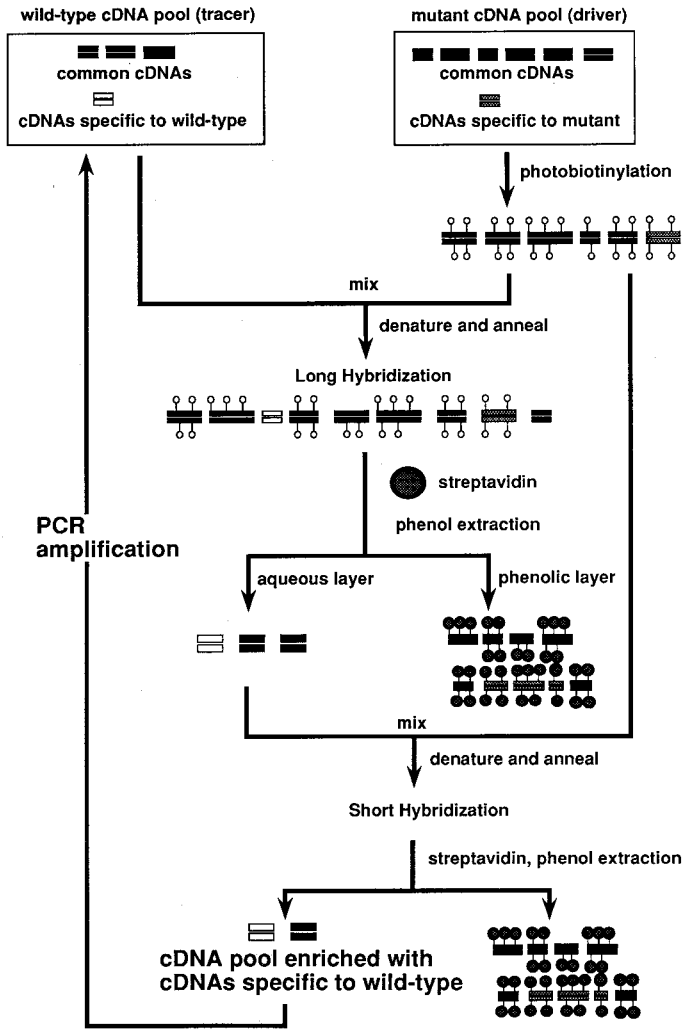


Fig. 4 Preparation of subtracted probe.

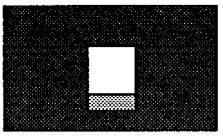
second biotin/streptavidin separation (**Fig. 4**). The long and short hybridization steps are needed to suppress the highly complex rare common cDNAs and the abundant common cDNAs, respectively. The photobiotinylation protocol is described in detail in **Fig. 5**. The hybridization cycle is repeated twice more, using driver DNAs made by an opposite protocol in which wild-type tobacco cDNA is used for the driver and mutant tobacco cDNA is used for the tracer (see also the **Fig. 1** of the original paper). The original protocols[1] were followed exactly.

After three cycles of subtractive hybridization, a few DNA fragments became predominant in the subtracted cDNA pools (**Fig. 6**). If discrete DNA bands are not observed when analyzed by agarose gel electrophoresis, or to check whether the subtraction procedure is working as expected, a small amount of a control DNA (*e. g.*, 15 ng of *Alu* I-digested pBluescript II) may be added to both pools of the initially synthesized cDNAs. After each hybridization cycle, subtracted cDNAs are analyzed by DNA blot hybridization using the control DNA as a labeled probe. Successful subtraction should result in decreasing hybridization signals from added DNA.

5. *Cloning of Differentially Expressed cDNAs*

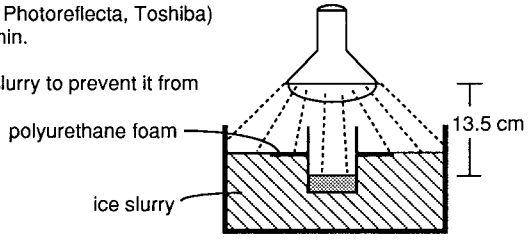
In the original protocol by Wang and Brown[1], the subtracted cDNA fragments after three cycles of enrichment are cloned into a plasmid vector to produce subtracted cDNA libraries, which are

1. Mix 50 μL of the driver DNA (1μg/μL) with 50 μL of Photoprobe biotin (Vector Laboratories; 1μg/μL) in a flat-bottom glass tube (e.g. 2 mL screw-cap vial, Funakoshi) in a dark room.



2. Irradiate with 500-W sunlamp (e.g. Photoreflecta, Toshiba) from a distance of 13.5 cm for 15 min.

   The sample should be kept on ice slurry to prevent it from overheating.



3. Add 30 μL of 1M Tris-HCl (pH 7.5) and 70 μL of water. Transfer the solution to an Eppendorf tube.
4. Add 300 μL of water-saturated 1-butanol, mix briefly by vortex and centrifuge at 15,000 rpm for 5 min. Remove the upper butanol layer . Repeat three times.
5. Add 30 μL of 3 M sodium acetate and 750 μL of ethanol and keep the tube at -80°C for 30 min.
6. Centrifuge and wash the pellet with 70% ethanol.
7. Dry the tube briefly.
8. Dissolve the biotinylated DNA pellet in 50 μL of TE buffer.
9. Repeat steps 1 to 8 once again to increase the density of biotin molecules on the driver DNA.

Fig. 5 Photobiotinylation of driver cDNA.

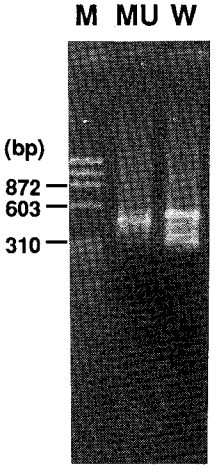


Fig. 6 Agarose gel electrophoresis of subtracted cDNAs using wild-type (W) and mutant (MU) as tracer DNAs.
M, molecular size marker (ϕX174/*Hae* III).

then screened with the same subtracted cDNA fragments. However, to clone full-length cDNAs, cDNA libraries containing high proportions of full-length cDNAs should be screened. Therefore, we screened replica filters made from a plasmid cDNA library of wild-type tobacco root (constructed as described above) with the subtracted wild-type probe and the initial PCR-amplified mutant probe. The probes were labeled by random priming (Random Primed DNA Labeling Kit; Boehringer Mannheim) to a specific activity of 6.3×10^8 cpm/μg of DNA. A total of 500 colonies that showed stronger hybridization signals with the subtracted wild-type probe than with the non-subtracted mutant probe were picked up for the second screening by dot-blot DNA hybridization

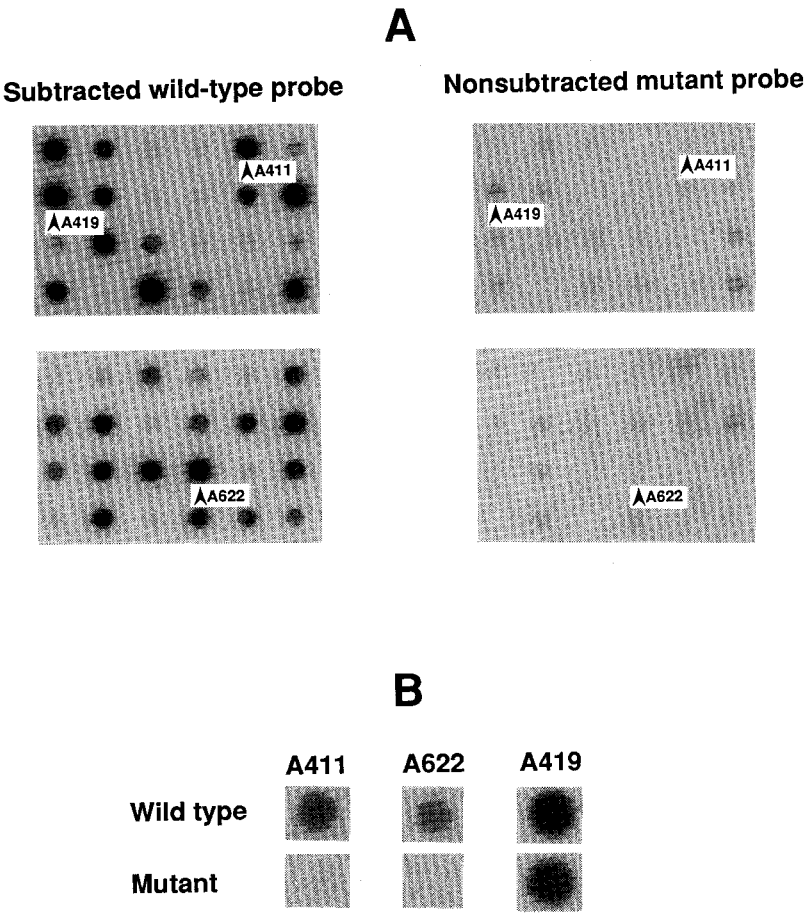


Fig. 7 Screening of differentially expressed cDNAs.
A: cDNA dot hybridization by subtracted wild-type probe and non-subtracted mutant probe. B: RNA dot hybridization by cDNA inserts of two differentially expressed clones (A411 and A622) and constitutively expressed clone (A419).

using the same probes (**Fig. 7-A**). The clones that hybridized to both probes (*e. g.*, A419 clone in **Fig. 7-A**) or to neither of the probes were eliminated. A total of 11 clones showed a clear difference in their hybridization intensity with the two probes, and plasmid DNAs were prepared from these positive clones. The inserts were excised with *Xba* I and *Hin*d III and purified by agarose gel electrophoresis. Several inserts were then labeled and used to hybridize the positive clones, which were then classified into two groups based on cross-hybridization patterns. The representative clones with the longest cDNA inserts in each group were A411 and A622 (**Fig. 7-A**). The inserts from these two clones, as well as the insert from A419, were labeled and used to probe RNA from cultured roots of wild-type and mutant tobacco (**Fig. 7-B**). RNA dot hybridization showed that A411 and A622 genes are both expressed strongly in the wild type, but scarcely in the mutant. Equally strong signals were detected in both RNA dots with the A419 probe, which confirmed that equal amounts of RNA were loaded onto each dot.

One of the advantages of this subtraction method is that subtraction (and thus the cloning of the other differentially regulated cDNAs) can be continued by driving out the abundant enriched DNA fragments from the subtracted probe. This can be accomplished, in brief, by short hybridization of the cloned differentially regulated cDNAs mixed with the non-subtracted mutant cDNA as a driver

to the subtracted wild-type cDNA pool. Details can be found in the original paper[1].

We are now applying the subtraction method to clone genes that are specifically expressed in the root cap cells of maize root. We hope that this sensitive and convenient subtraction method will be used by many scientists who study plant biology and development.

(Accepted June 5, 1993)

References

1) Wang, Z., D. D. Brown, 1991. Proc. Natl. Acad. Sci. USA, 88: 11505-11509.

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3) Watanabe, I., M. Sugiura, 1989. In "Cloning and Sequence", p. 14-73, Nohson-Bunka-shya, Tokyo.