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Use of non-radioactive digoxigenin-labelled DNA probes for RFLP analysis in coffee

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Introduction

Restriction endonuclease digestion of total genomic DNA followed by hybridization with a labelled probe reveals differently sized hybridising fragments. This form of polymorphism, termed Restriction Fragment Length Polymorphism (RFLP), has been used extensively for genetic study. However, cost and time requirements of such molecular marker technique, coupled with the widespread use of short-lived radioisotopes in the detection method has limited its application in large-scale programmes, especially for laboratories in developing country. Improved technology would be particularly useful for genetic study on tree tropical species such as *Coffea* species.

As an alternative to the use of radioactive element, several methods have been reported that describe non radioactive labelling of DNA for use as hybridization probes. Most techniques until recently were time-consuming and presented a low sensitivity. However, new labelling compounds and detection systems have greatly improved the reliability and sensitivity of this technology. In particular, system based on digoxigenin labelling (Boehringer Mannheim) and DNA probe detection after hybridization with an antidigoxigenin alkaline phosphatase antibody conjugate has been recommended for several plant species (Allefs et al., 1990 ; Ishii et al., 1990 ; Hoisington D.A., 1992) The steroid hapten digoxigenin (Dig) is an artificial hapten that is not present in most relevant tissues and, therefore, does not lead to unspecific signals, which can occur with natural labels like biotin. In addition, chemiluminescent alkaline phosphatase substrates have replaced the previous colorimetric procedures and allow reused of the membrane more times.

In this report, we describe a adapted protocol for dig-DNA labelling and detection of single-copy probe in genomic DNA blots of *Coffea* species. Results and potentialities of this procedure are discussed, especially chemiluminescent-based RFLP (c-RFLP) is compared with radio-activity-based RFLP (r-RFLP).

Protocol

Preparation of membranes

Genomic DNA is isolated from lyophilised leaf as previously reported (Lashermes et al., 1993) except that CTAB is replaced by MATAB (mixed alkyltri-methylammonium bromide) in the extraction buffer. Extracted DNA is digested using 2.5 units of restriction enzyme per microgram of DNA, and electrophoresed in agarose gels (8 μg per well). Following electrophoresis, the DNA is denatured and blotted onto a nylon membrane by capillarity transfer using 10X SSC. Both Hybond-N (Amersham) and positively charged (Boehringer) membranes can be used. The DNA is bound by UV cross-linking for 2 min (transilluminator) and baking at 100°C for 3 hours.

Amplification and probe labelling

Single-copy random *C. arabica* genomic clones are used as probe. Probes (plasmid inserts) are PCR-labelled by incorporation of digoxigenin-11-dUTP (Boehringer Mannheim). A dig-dUTP/dTTP ratio of 1/10 is commonly used but the ratio can be increased to 3/10 in order to obtain a stronger signal. Unincorporated dig-dUTP can be removed by either ethanol precipitation or Sephadex G-50 spin columns. Yield of labelled probe is estimated by gel electrophoresis followed by ethidium staining. The successful incorporation of dig-dUTP can be checked by comparing the molecular weight of dig-labelled probe and the original template since the molecular weights of dig-dUTP and dUTP are 1090.7 and 468.2 respectively. The greater the weight difference, the more efficient the labelling.

Hybridization

Hybridization and washing conditions for dig-labelled probes do not differ from those of radiolabelled probes. The membrane are prehybridised (5X SSC, 0.1% Sarkosyl, 0.01% SDS, 1% blocking reagent) for 5 hours at 65°C in bottles in hybridization oven. The prehybridization solution is replaced by 15 ml of hybridization solution containing 300 ng of denatured probe ; higher probe concentration may lead to high background. Hybridization proceed for 16-20 h at 65°C. Filters are washed two times for 5 min each at room temperature with 2X SSC, 0.1% SDS, followed by two times for 15 min at 65°C with 0.5X SSC, 0.1% SDS in plastic boxes. Hybridization solutions containing labelled probe may be stored frozen and reused several time (up to five times in our hand). For reuse, the probe-hybridization solution is denatured by heating to +95°C for 10 min.

Detection of Dig-labelled probe

Our protocol for signal detection does not show important modification from the one given by the supplier (Boehringer Mannheim). The Dig label is detected by polyclonal anti-digoxigenin Fab fragments, which are conjugated to alkaline phosphatase. Lumigen PPD (Boehringer Mannheim) as well as AMPPD or CSPD (Tropix) can be used as

chemiluminescent substrate. Substrates are supplied as 25 mM concentrates in aqueous buffer and diluted 300 fold in the recommended buffer. Diluted substrate solution can be stored at 4°C in the dark and reused. The membrane are exposed to standard X-ray film to record the chemiluminescent signal. The exposure time required varies with the concentration of probe used and the number of re-use of the membrane, but in our experimental conditions, the average time exposure is 3 hours.

Probe removal and re-use of the membrane

For reprobing, special care should be given to keep the membranes wet during all steps of the protocol. After exposure, the membrane are washed two times for 10 min each with 0.1 % SDS at 80°C and rinsed in 2X SSC. Membrane can be stored in a plastic-wrap at 4°C.

Results and discussion

The protocol given in this paper is used routinely in our laboratory for the development of a genetic linkage map in *Coffea canephora* and for a genetic diversity study including a large number of coffee accessions representing the cultivated species (*Coffea arabica* and *Coffea canephora*) as well as the major wild species. As estimated by laser flow cytometry, DNA contents per nucleus (2C value) of *Coffea* species are rather small and vary from 0.9 to 1.9 pg (Cros et al., 1993). Only the unique tetraploid species *Coffea arabica* has a DNA content higher than 2 pg per nucleus. Since 8 μg of digested DNA per lane is loaded on the gel , the amount of target DNA transferred to the membranes is expected to be high.

Sensitivity of the digoxigenin labelling procedure appeared comparable to that of radiolabelling. Detection of a single copy gene in our genomic DNA blots is obtained without trouble. Membrane can be re-uses up to 8 times. When compared to r-RFLP, hands-on time requirements appear slightly longer using the Dig-system due to revelation steps. Nevertheless, since a much shorter exposure time is required, result is obtained more rapidly with the Dig-system than by r-RFLP.

Amounts of supplies and their associated cost were calculated for each step of the protocol. r-RFLP cost was also estimated based on a protocol presented elsewhere (Cros et al., 1993). DNA extraction, membrane preparation, probe labelling as well as hybridization and detection of RFLP were taken in consideration when estimating either c-RFLP or r-RFLP costs. Characteristics of membrane included restriction digests of 36 DNA samples per membrane with enzyme such Hind III or Eco RI which are relatively cheap. Estimated costs were 150 FF for membrane constitution, 70 and 60 FF for probe labelling and detection in c-RFLP and r-RFLP respectively. However, based on five re-uses of the chemiluminescent substrate solution, cost associated with c-RFLP dropped to only 40 FF.

RFLP costs, whatever the method, is greatly affected by membrane usage. Figure-1 shows the cost of c-RFLP for one membrane (analysis of 36 DNA samples) when membrane usage varied from one to ten re-uses. Total cost decreased from 190 to 55 FF. Based on a average of eight membrane uses, total cost per DNA sample was 1.60 FF. Additional and important savings can be realized if the labelled probes are used several times. In case each pro-be is used three times, cost per DNA sample dropped to 1.10 FF.

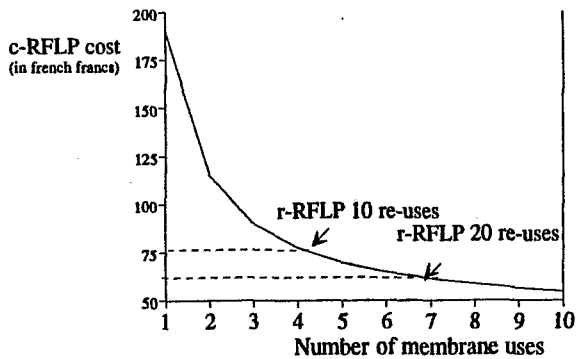

Figure 1: c-RFLP cost per probe/membrane hybridization in relation to the number of membrane uses. Cost of r-RFLP based on ten and twenty re-uses are also indicated.

Those results clearly demonstrated that RFLP cost is reduced when using cRFLP. Even in the case where the number of membranes uses with c-RFLP may appear as a limiting factor. To become more cost efficient, re-uses of membrane with r-RFLP should be more than twice than with c-RFLP. Ragot et al. (1993) comparing three classes of molecular markers: r-RFLP, c-RFLP and RAPD, also reported that genotyping would be performed at lowest cost with c-RFLP for most studies. Moreover, the protocol reported here could be certainly modified with the aim of reducing cost. Means to achieve this goal include utilisation of alternative reagents, more general re-uses of solutions and plastic ware.

Beside the performance and reduced cost, one great advantage of c-RFLP over r-RFLP is flexibility. No time constraint due to the short-lived of radioisotopes. Probe can be re-used any times. In addition, non-radioactive labelling of nucleic acids such as digoxigenin labelling procedure avoid disadvantages (e.g. safety requirements, waste disposal, special laboratory) that are associated with the use of radioactivity.

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