Plant Molecular Biology Reporter **15:** 255–262, 1997.

© 1997 *Kluwer Academic Publishers. Printed in Belgium.*

Protocols

Amplified Fragment Length Polymorphisms (AFLPs) detected with non-radioactive digoxigenine labelled primers in three plant species

Klaas Vrieling[1], Jenny Peters[1] and Hans Sandbrink[2]

[1]Institute of Evolutionary and Ecological Sciences, Van der Klaauw Laboratory, PO Box 9516, 2300 RA Leiden, The Netherlands, Email Vrieling@RULSFB.Leidenuniv.nl; [2]DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), PO Box 16, NL-6700 AA Wageningen, The Netherlands.

Key Words: AFLP, digoxigenine labelling, non-radioactive labelling

Abstract: A protocol for the detection of AFLPs with the non radioactive digoxigenine labelling is presented. The protocol has been tested on DNA samples from three different plant species. The AFLP technique was used for the first time in these species. The sensitivity and reliability of the digoxigenine labelled primers in the AFLP technique was of the same order as the sensitivity and reliability of the radioactive assay. No major adjustments of the current standard AFLP protocols is necessary to use the digoxigenine labelled primers.

Introduction

Recently a new technique called amplified fragment length polymorphism (AFLP) was introduced as a new tool for genetic analysis (Vos et al., 1995). The technique is an elegant combination of restriction enzyme digestion and the PCR. DNA is digested with restriction enzymes producing sticky ends. Specific adaptors with a fixed sequence are ligated to these sticky ends. Subsequently these fragments are amplified with PCR technique using the adaptor sequences as primer sites. By extending the primer three 'selective' basepairs at the 3′-end , a selection of all restriction fragments in a reaction is amplified. Generally an AFLP pattern produces 30–100 amplified fragments (Vos et al., 1995). The technique

is very powerful because it produces a dense but reliable banding pattern without the necessity of probe development. Although the technique has not reached its full application yet, it is already widely used in genetic studies in plants (Meksem et al., 1996; Van Eck et al., 1995), animals (Otsen et al., 1996), fungi (Majer et al., 1996), nematodes (Folkertsma et al., 1996) and bacteria (Keim et al., 1997). AFLP markers will also be useful for the analysis and recognition of individuals (paternity analysis, selfing rates, identification of clones, etc.).

Until now radioactive labelled and non-radioactive fluorescent labelled primers were used for the detection of AFLPs. However, radioactive labelling is not feasible in every laboratory. The detection of fluorescent labels requires expensive equipment. Alternatives to these methods are desirable. One possible way to visualize AFLP fragments non radioactively is the silver staining method (Chalhoub et al., 1997; Cho et al., 1996). In this commentary we present the protocol for a new alternative method based on the digoxigenine labelling of AFLP primers. The method proved to be reliable and cheap and in addition no major rearrangements of original protocols are required.

Materials and Solutions

Radioactive analysis

- AFLP^{TM} kit (GIBCO BRL Life Technology)
- ^{33}P-dATP (Amersham)
- Taq polymerase (Amplitaq, Perkin Elmer)
- Slab gel dryer (BioRad)
- BioRad sequencing system (BioRad)
- Kodak X-Omat AR film (Kodak)

Non-radioactive assay

- AFLP^{TM} kit (GIBCO BRL Life Technology)
- Taq polymerase (5 μ/μl) (SphaeroQ, Leiden)
- Nylon membrane (Dupont Nemours)
- 5$'$ digoxigenine labelled primers (Isogen Bioscience BV)
- Dig antibody Fab fragments (Boehringer Mannheim)
- Blocking reagent (Boehringer Mannheim)
- Buffer I (0.1 M Tris, 0.15 M NaCl, pH=7.0 with HCl)

- Buffer II (0.1 M Tris, 0.1 M NaCl, pH=9.5, just before use add $MgCl_2$ up to 50 mM)
- CSPD™ (Tropix)
- Reflection Film NEF 983 (Dupont-Nemours)

Protocol

DNA extraction

DNA of three genotypes of three plant species *(Senecio jacobaea, Echium vulgare and Hippophae rhamnoides)* was extracted from circa 10 mg fresh or frozen leaf material following a slightly modified protocol of Cheung et al. (1993).

- To the extraction buffer 2% PVP 40 and 1% Beta mercaptoethanol was added.

AFLP reactions

The AFLP reactions were carried out following the instructions supplied with the GIBCO BRL Life Technology AFLP™ kit.

- Approximately 5 μl of the extracted DNA (circa 30 ng/μl) was used for restriction with *Mse*I and *Eco*RI (2 h) and followed by ligation of the adaptors for the *Mse*I and *Eco*RI sites (2 h).
- Five μl of the restriction-ligation solution was used for the preamplification PCR with primers for the *Mse*I and *Eco*RI with one ‘selective’ basepair at the 3′-end. For the *Eco*RI site and *Mse*I site A and C were used as the selective base respectively. The PCR temperature programme as indicated in the AFLP kit was followed.
- The preamplification mixture was diluted 25 times and 5 μl of the diluted mix was used for the selective amplification. The selective amplification was performed with three selective bases (including the one selective base of the preamplification). For the *Eco*RI site and *Mse*I site AAC and CAC were used as the selective bases respectively. In a second selective amplification ACA and CTG were used as selective bases. In the selective amplification labelled primers of the *Eco*RI site were added.
- The radioactive primers were prepared by labelling them with ^{33}P (ATP) by a T4 polynuclotide kinase according to the protocol.

- For the digoxigenine labelling, the following PCR mix was prepared: 1.1 μl (27.4 ng/μl) digoxigenine labelled *Eco*RI primer, 4.5 μl *Mse*I primer with dNTPs, 2 μl 10$\times$ PCR buffer, 0.1 μl Taq polymerase (5 U/μl), 5 μl DNA template from the preamplification step (1:25 diluted) and 7.3 μl water. The PCR temperature programme as indicated in the AFLP kit was followed.

Electrophoresis

- After PCR 20 μl formamide dye was added. The samples were heated for 3 min at 90 °C and electrophoresed on a 0.4 mm thick 6% polyacrylamide (PA) gel (19:1 acrylamide:bisacryl; 7.5 M urea; 1$\times$ TBE buffer) with sharktooth combs.
- The gel was prerun at circa 110 W until the gel had reached a temperature of 45-50 °C. After loading the gel was run for approximately 2 h at 110 W.
- For the radioactive assay the PA gel was removed from the glass plates with the aid of a 3 MM Whatmann paper. The gels were dried for 30–50 min in a heated (70 °C) vacuum slab gel dryer. Gels were exposed for two days at room temperature to Kodak X-OMAT AR films.

Southern blotting and detection of the digoxigenine labelled AFLP fragments

- For the digoxigenine labelled assay, the DNA was transferred to a nylon filter by capillary blotting for 30 min.
- The fragments were crosslinked to the filter by UV light for three minutes. The detection of the digoxigenine labelled fragments was as described by Epplen (1992).
- The filter (15 $\times$ 40 cm) was blocked for 30 min in a bag with 50 ml buffer I with 1% blocking reagent.
- The blocking solution was replaced by 50 ml 1% blocking solution with 8 μl Fab fragments and incubated for 30 min.
- After washing two times 15 min with buffer I and then incubated for 30 min in the dark at 4 °C with 30 ml buffer II and 25 μl CSPD in a plastic bag. After removal of the buffer solution the filter was exposed overnight at room temperature to a reflection film NEF 983.

Table I. Number of scorable polymorphic (P) and monomorphic (M) bands in AFLP pattern in three genotypes of ***S. jacobaea, H. rhamnoides*** **and** ***E. vulgare*****.** Data are given for primer combination AAC on the *Eco*RI side and CAC on the *MSE*I side of the fragment and for primer combination ACA on the *Eco*RI side and CTG on the *Mse*I side of the fragment.

Primer extension	E-AAC/M-CAC		E-ACA/M-CTG	
bands	P	M	P	M
S. jacobaea	36	40	20	46
H. rhamnoides	6	47	5	27
E. vulgare	20	24	28	28

Results and Discussion

For all three, widely different, plant species, ragwort, *S. jacobaea*, vipers bugloss, *E. vulgare* and sea buckthorn, *H. rhamnoides* AFLP patterns were established for two sets of selective primers (Fig. 1). The digoxigenine labelling yielded the same results as the radioactive labelling for both sets of selective primers (Fig 1). However the background of the digoxigenine labelling was clearly higher compared to the radioactive labelling. In general faint bands in the radioactive method became more pronounced in the non-radioactive method as was reported earlier for digoxigenine labelling of minisatellites marker fragments (Weising et al., 1991; Vrieling et al., 1997).

The same method was equally successful applied to a snail species (*Albinaria*) and a lepidopteran species (*Bicylcus anyana*) (data not shown). The use of digoxigenine labelled dUTPs in the PCR did not yield reliable results. Bands became more fuzzy and many shadowbands appeared. The shadowbands are caused by the differential incorporation of the digoxigenine labelled dUTPs into the fragments, causing differences in the electrophoretic mobility of fragments, depending on the number of digoxigenine labels incorporated in a fragment.

Although only three individuals per species were sampled, genetic variation was ubiquitous (Table I). The number of scorable bands was highest in *S. jacobaea*, followed by *E. vulgare* and *H. rhamnoides* having the lowest number. The percentage of polymorphic bands was much higher in the two monocarpic perennial species (*S. jacobaea* and

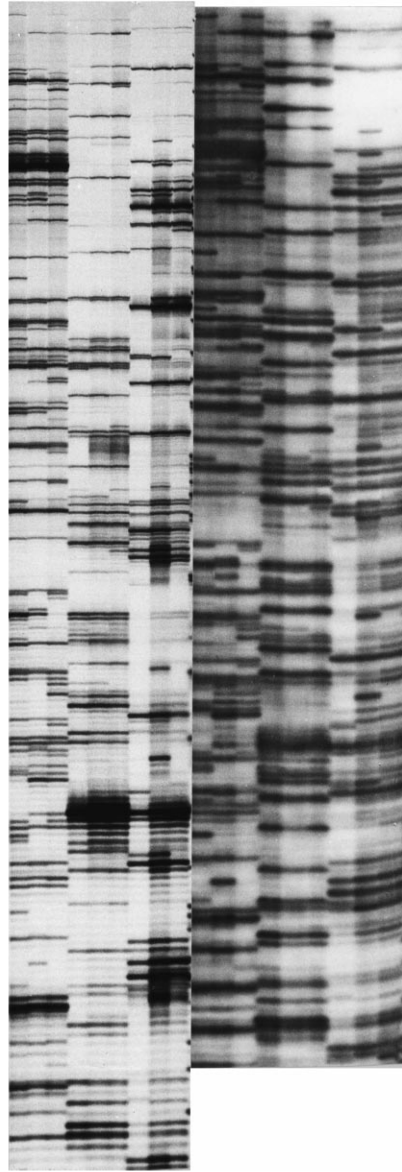

Fig 1. **AFLP pattern from three genotypes of three plant species using the selective primer combination AAC on the Eco RI side and CAC on the MSE I side of the fragment.** On the left the radioactive labelling, on the right hand side the digoxigenine labelling. Lanes 1–3: *S. jacobaea*, lanes 4–6: *H. rhamnoides*, lanes 7–9: *E. vulgare.*

E. vulgare) then in the woody perennial *H. rhamnoides*. For all three plant species the method offers a good opportunity for detecting genetic variation.

In general the digoxigenine labelling offers a good alternative to radioactive- and to the expensive fluorescent labelling and can be carried out with standard molecular biology laboratory equipment.

Acknowledgements. KV was supported by the Royal Netherlands Academy of Arts and Sciences.

References

Chalhoub, B.A., S. Thibault, V. Laucou, C. Rameau, H. Hofte and R. Cousin. 1997. Silver staining and recovery of AFLP amplification products on large denaturing polyacrylamide gels. Biotechniques 22:216.

Cheung, W.Y, N. Hubert, and B.S. Landry. 1993. A simple and rapid microextraction method for plant, animal and insect DNA suitable for RAPD and other PCR analyses. PCR Methods and Applications 3:69–70.

Cho, Y.G., M.W. Blair, O. Panaud and S.R. McCouch. 1996. Cloning and mapping of variety-specific rice genomic DNA sequences: Amplified fragment length polymorphisms (AFLP) from silver-stained polyacrylamide gels. Genome 39:373–378.

Epplen, J.T. 1992. The methodology of multilocus DNA fingerprinting using radioactive or non radioactive oligonucleotide probes specific for simple repeat motifs. In: Advances in electrophoresis, Vol. 5 (Eds. Crambach, A., M.J. Dunn and B.J. Radola) pp. 59–112. VCH, Weinheim.

Folkertsma R.T., J.N.A.M.R. van der Voort, K.E. de Groot, P.M. van Zandvoort, A. Schots, F.J. Gommers, J. Helder and J. Bakker. 1996. Gene pool similarities of potato cyst nematode populations assessed by AFLP analysis. Molecular Plant Microbe Interactions 9:47–54.

Keim, P., J. Schupp, K. Hill, S.E. Travis, K. Richmond, D.M. Adair, M. Hughjones, C.R. Kuske and P. Jackson. 1997. Molecular evolution and diversity in Bacillus anthracis as detected by Amplified fragment length polymorphism markers. J. Bact. 179:818–824

Majer, D., R. Mithen, B.G. Lewis, P. Vos and R.P. Oliver. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 100:1107–1111.

Meksem, K., D. Leister, J. Peleman, M. Zabeau, F. Salamini and C. Gebhardt. 1995. A high resolution map of the vicinity of the R1 locus on chromosome V of potato based on RFLP and AFLP markers. Mol. Gen. Genet. 249:74–81.

Otsen, M., M. den Bieman, M.T.R. Kuiper, M. Pravenec, V. Kren, T.W. Kurtz, H.J. Jacob, A.E. Lankhorst and B.F.M. van Zutphen. 1996. Use of AFLP markers for gene mapping and QTL mapping in rat. Genomics 37:289–294

Van Eck, H.J., J.R. van der Voort, J. Draaistra, P. van Zandvoort, E. van Enckevort, B. Segers, J. Peleman, E. Jacobsen, J. Helder and J. Bakker. 1995. The inheritance and

chromosomal localization of AFLP markers in a non-inbred potato offspring. Mol. Breeding 1:397–410.

Vos, P., R. Hogers, M. Bleeker, M Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nuc. Acid Res. 23:4407–4414.

Vrieling, K., P. Saumitou-Laprade, E. Meelis and J.T. Epplen. 1997. Multilocus fingerprints in the plant Cynoglossum officinale L. and their use in the estimation of selfing. Mol. Ecol. 6:587–593.

Weising, K., B. Beyermann, J. Ramser and G. Kahl. 1991. Plant DNA fingerprinting with radioactive and digoxigenated oligonucleotide probes complementary to simple repetitive DNA sequences. Electrophor. 12:159–168.