Plant Molecular Biology Reporter **18:** 235–241, 2000

Commentary

Modified Protocols for Rapid Carrot Genomic DNA Extraction and AFLP™ Analysis using Silver Stain or Radioisotopes

M. BRIARD[1,*] V. LE CLERC[1], D. GRZEBELUS[2], D. SENALIK[3] and P.W. SIMON[3]

[1]*Institut National d'Horticulture, Angers, France;* [2]*Krakow Agricultural University, Krakow, Poland;* [3]*USDA, Agricultural Research Service, University of Wisconsin, Madison, WI, USA*

Abstract. AFLP™ is one of the most frequently used techniques for identification of molecular markers. We have modified the procedures for genomic DNA extraction, AFLP product generation and silver staining in order to speed up analyses and screen large numbers of plant samples. Using this protocol, we were able to achieve an 82% reduction of costs without compromising the reliability and quality of data gathered.

Key words: AFLP, DNA extraction, electrophoresis, molecular markers, polyacrylamide, radioactivity, silver staining

Introduction

Molecular genetic markers are important in plant breeding and genetic resources management. However, for evaluation of large numbers of samples, standard methods are often time-consuming and expensive. The AFLP™ technique is frequently used for the identification of molecular markers, owing to certain advantages over other techniques, i.e. high level of identified polymorphism, high reproducibility, and relative technical simplicity. The use of radioactive isotopes to visualize AFLP™ products renders the technique relatively expensive and makes it inaccessible to researchers not equipped for use of radioactivity. It is also hazardous to health (Chalhoub et al., 1997). The technique we describe involves a simple, reliable extraction of carrot DNA, which has been problematic (Boiteux et al., 1999). This is followed by AFLP™ generation and visualization equally well with radioisotope or silver stain. The modifications described below are especially useful when large numbers of plant samples are to be studied, as they can speed up the analyses and minimize the expenses.

*Author for correspondence. Present address: Institut National d'Horticulture, 2 Rue Le Nôtre, 49045 Angers Cedex 01, France; e-mail: briard@angers.inra.fr; fax: +33 241 225515; ph: +33 241 225463.

Materials and Methods

DNA extraction

Lyophilized carrot leaf, root, or inflorescence were utilized as a source of DNA. To lyophilize samples, fresh tissue was wrapped in cheesecloth, frozen in liquid nitrogen, and held at -80°C until transfer to a Virtis 12XL lyophilizer for 3-4 d. Dried samples were then reduced to powder by grinding with a mortar and pestle in liquid nitrogen. The powder was then collected on glassine paper and transferred to 2 mL microcentrifuge tubes. Samples in this condition were held for up to 13 months at room temperature. For comparison, fresh tissue was analyzed by grinding with liquid nitrogen, transferring to a microcentrifuge tube, and DNA was extracted immediately.

To approximately 200 µL (~50 mg) of powder (visually estimated from markings on the tubes), 800 µL of sterile extraction buffer (50 mM pH 8 Tris, 0.7 M NaCl, 10 mM EDTA, 1% CTAB (hexadecyltrimethylammonium bromide), and 0.1% β-mercaptoethanol) were added. These samples were mixed and incubated at 60°C for 45 to 60 min. An equal volume of chloroform:isoamylalcohol (24:1) was added and mixed gently for a few min. Samples were centrifuged for 10 min at 8000 rpm, and 700 µL of the supernatant was transferred to a fresh tube, 700 µL of isopropanol was added and the contents of the tubes mixed gently for a few min. The DNA was collected by centrifugation for 10 min at 8000 rpm and the pellet was washed with 70% ethanol for 15 min without agitation. The ethanol was decanted and the pellet was dried 6 to 8 min under vacuum or 10 to 15 min at 50°C. The pellet was dissolved for 10 to 15 min at 60°C in 200 µL 1x TE. Fresh tissue samples were prepared by the same procedure except 500 mg of tissue was initially mixed with 80 µL of 10x extraction buffer. Extracted DNA was stored at 4°C for several days or at -20°C for long term storage.

AFLP

The AFLP reaction was performed as described by Vos et al. (1995) but with highly reduced quantities of enzymes and primers provided in the manufacturer's (GIBCO-BRL, Life Technologies) kit. We used 3 µL (~ 30 to 180 ng) of DNA double restriction-digested for 3 h at 37°C with 0.625 units each of *Eco*R I and *Mse* I in a 6.25 µL volume (10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate and 50 M K-acetate). After heat-inactivation for 15 min at 70°C, restriction-site derived adapters were ligated for 2 h at 20°C in a 12.5 µL reaction volume (0.25 µM *Eco*R I adapter, 2.5 µM *Mse* I adapter, 0.2 mM ATP, 4.8 mM Tris-HCl pH 7.5, 4.8 mM magnesium acetate, 24 mM potassium acetate, 0.25 units T4 DNA ligase). An aliquot of this ligation mix was diluted 10x with TE buffer and preamplified with adapter-derived primers having one additional (+1) nucleotide at the 3' end. Each 5.1 µL preamplification volume contained 0.5 µL diluted ligated DNA, 0.196 mM dNTPs, 3.76 ng each primer and 0.11 unit *Taq* polymerase in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl. This reaction was performed for 20 cycles with the following cycling profile: (i) 30 s denaturation at 94°C; (ii) 1 min annealing at 56°C; and (iii) 1 min extension at 72°C. After 50-fold dilution in TE buffer, this stock was used for all further selective amplifications.

For the radioactive AFLP procedures, the *Eco*R I primer was radioactively labeled as follows: 1.25 ng of an *Eco*R I primer with a (+3) extension [3 random nucleotides at the 3' end] was 5' end-labeled in 0.125 µL using 0.025 µL of -^{33}P ATP (2000 Ci/mmol, Amersham) and 0.0875 units T4 polynucleotide kinase in 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl and 1 mM 2-mercaptoethanol. The mix was incubated for 1 h at 37°C and heat-inactivated for 10 min at 70°C.

AFLP reactions for silver staining were performed with the same quantity of *Eco*R I primer, but without radioactive label. This differs from the conditions of Chalhoub et al. (1997) who recommended 6-fold more *Eco*R I primer for samples destined for silver staining than those for radioactive procedures.

Diluted preamplified DNA stock (1.25 µL) was PCR amplified in a 5 µL reaction volume with 1.25 ng *Eco*R I primer (end-labeled for the radionuclide-visualized system) in combination with 7.54 ng of *Mse* I primer also with (+3) extension in the same buffer as above, and 0.0437 units *Taq* polymerase. The touch down cycling conditions were: (a) 1 cycle at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s; (b) 13 cycles starting with an annealing temperature of 65°C and lowered by 0.7°C every subsequent cycle; (c) 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

These reaction products were stored at -20°C up to 10 months for those destined to be silver stained, or for as long as the radioactivity was effective.

Electrophoresis and visualization

A denaturing 6% polyacrylamide (29:1 acrylamide:bisacrylamide) gel containing 7.5 M urea in 1x Tris-Borate (TBE) buffer was prepared according to Sambrook et al. (1989). The gels for radioactivity or silver staining were poured in the same way, with one exception. For silver staining, the long glass plate was pre-treated with Silane® A-174 (Sigma chemical) in order to bind the gel to the glass plate for easy handling. For this procedure, the long plate was well washed, dried and coated with a 1 mL ethanol solution containing 0.5% acetic acid and 3.5 µL silane. This mixture was applied with a lint-free tissue and left to dry for 5 min. The resulting white film was removed with 3 to 4 rinses in 100% ethanol and by wiping (not enough to remove the silane). We cast 0.4 mm gels using model S2 sequencing apparatus (Life Technologies) which were pre-run at 60 W for 20-30 min in 1x TBE.

Before loading, an equal volume (5 µL) of formamide dye (98% formamide, 10 mM EDTA, 1 mg/mL of each bromophenol blue and xylene cyanol) was added to the reaction mixture. The reaction was heated for 3 min at 90°C and immediately placed on ice. Then 4 or 6 µL (for radioactive or silver stained gels, respectively) of denatured selectively amplified DNA was loaded and the gel was run at 60W for 2 h. *Msp* I-cut pBR322 was used as a size marker. Radio-labeled gels were briefly cooled on ice, transferred onto Whatman 3MM filter paper and vacuum dried using a model SE 1160 dryer (Hoefer) at 80°C for 50 min. These dried gels were then exposed to Kodak Biomax MR film for 1-3 d.

For non-radioactive visualization we used the improved photochemical derived procedures for protein silver staining proposed by Bassam et al. (1991) with slight modification. All procedures were performed at room temperature. Constant agitation of the bath during all the staining steps is essential. The gel bound to the

long plate was fixed in a stop solution, (10% acetic acid) for 40 min then rinsed 3 times in deionised or permuted water. Pre-treatment steps were omitted. The impregnation was performed for 45 min (not 30 min as recommended) in the coloring solution: $AgNO_3$ (1 g/L), 1.5 mL 37% HCOH/L (silver nitrate is from Sigma, S0139, purity ≥ 99%). The plate was then removed, vertically drained, rinsed 8 s in deionised or permuted water and immediately submerged into the developing bath. In case of a delay exceeding 20 s between rinse and developer, the coloring step was repeated. The developer consisted of sodium carbonate, formaldehyde and thiosulfate (30 g Na_2CO_3/L, 1.5 mL 37% HCOH/L, 2 mg $Na_2S_2O_3 5H_2O$/L) as recommended but we kept the developer refrigerated (4°C) before use (Sodium Carbonate is from Fischer, S0620, purity: 99.5%). Thus, the sodium carbonate solution was prepared, refrigerated overnight, and added just before use to the formaldehyde and thiosulfate. This increased the time to achieve proper staining, allowing repeatable image development. Removing the gel from the bath during developing is strongly ill-advised. When the optimal staining was reached (5 to 10 min) the reaction was stopped with the same stop solution used in the first step (5 to 10 min until effervescence stopped). The gel was rinsed twice in water over 10 min and left to dry vertically overnight. The dried gel was either preserved at room temperature, a copy made with photographic film (APC Promega), or scanned (Plustek A3I. Optic Pro) using the Adobe Photoshop™ 2.5.1. LE program (Adobe Systems, Mountain View, Ca, USA). Gels could be preserved for months without any quality loss as noted by Bassam et al. (1991) but scanning was preferred since it provides a computerized record of the results and keeps the glass plates available. The Adobe Photoshop program was used as needed to increase the contrast between bands and background staining. For photographic record, the gel was placed in contact with film and exposed for 10 to 60 s to white light and developed. Silver nitrate wastes were treated by storing the staining and developer solutions together in the same bottle until a complete precipitation of silver was obtained (~2 weeks). Then solid wastes were filtered before being sent to an appropriate recycling facility.

Results and Discussion

The procedures described for evaluating AFLP markers in carrot included several distinct improvements over previous methods (Vivek and Simon, 1999). Once lyophilized, the samples could be held over a year at room temperature. Furthermore, the entire reaction volume was reduced to fit into microcentrifuge tubes. From 10 to 60 ng DNA/μL were generally obtained. With this simple DNA extraction method we have obtained very clear AFLP banding profiles using both visualization methods. This has allowed us to compare over 250 different cultivars of carrot and other *Apiaceae* (other *Daucus, Chaerophyllum*, and *Conium*). DNA extracted in this way was successfully used not only for AFLP but also for RAPD, microsatellite and ISSR studies. Samples of 3 μL are sufficient for an AFLP reaction. For PCR, RAPD or ISSR, samples are first diluted 10-fold in TE. To demonstrate that a 32-fold variation in concentration (from 5 to 160 ng/μL) could give the same quality of AFLP profiles, we performed the reaction with 4 DNA samples, 2 concentrations each. Figure 1 demonstrates the high level of

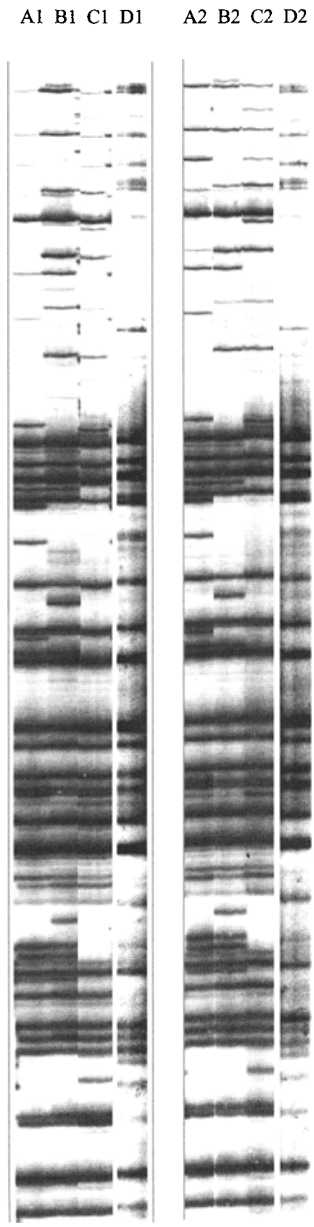

Figure 1: Autoradiograms illustrating the reproducibility of AFLP profiles obtained with divergent DNA concentrations for four carrot lines. A1: line 29775 [15 ng / µL]; A2: line 29775 [5 ng / µL]; B1: line 30809 [31 ng / µL]; B2: line 30809 [10 ng / µL]; C1: line 30415 [135 ng / µL]; C2: line 30415 [45 ng / µL]; D1: line 7149 [160 ng / µL]; D2: line 7149 [16 ng / µL].

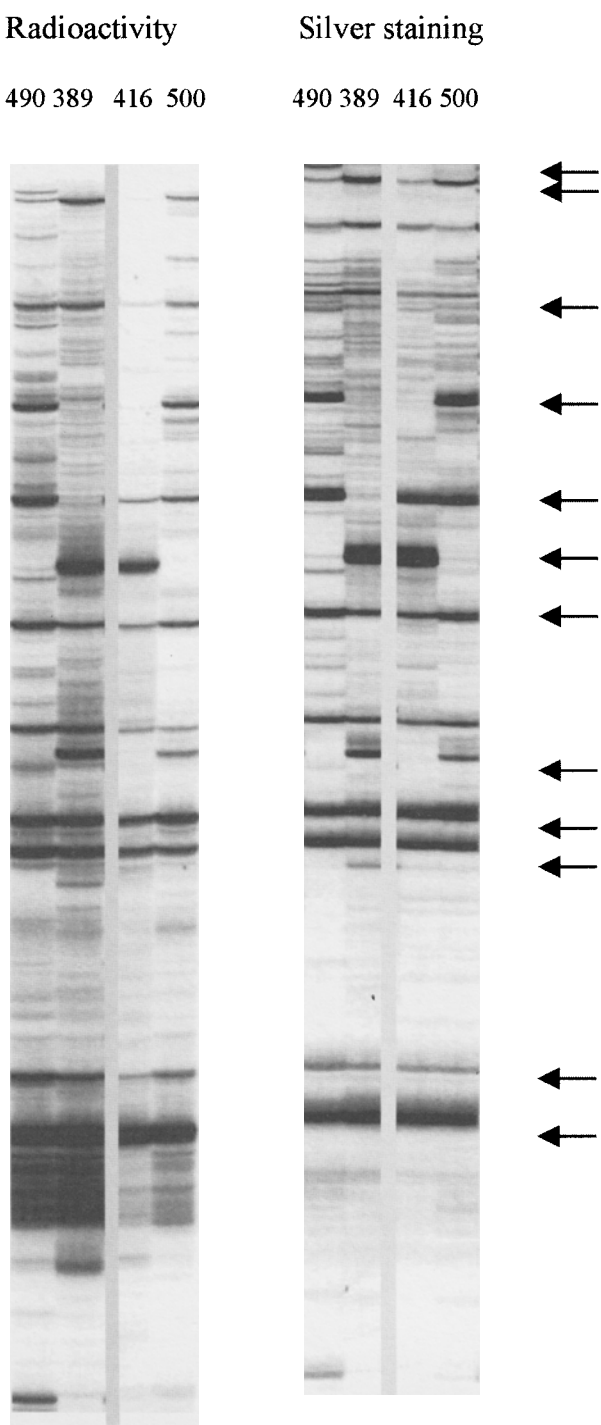

Figure 2: Comparison of radioactive and silver-stained AFLP profiles for four different carrot lines: 490, 389, 416 and 500. Arrows indicate major bands evaluated.

similarity among pairs of profiles. Because this large variation in concentration gave the same AFLP profile, we now omit the DNA quantification step, which saves time.

Band visualization with radionuclide and silver staining gave very similar results. To illustrate this, the DNA of more than one hundred individuals belonging to two different F_2 carrot populations was extracted from freeze-dried leaf or root tissue. 3 µL of each DNA extract were digested with *Eco*R I and *Mse* I restriction enzymes. The AFLP selective amplification was performed with primers using a (+3) extension: *Eco*+AAG / *Mse*+CTC. Results of AFLP fingerprinting visualized by autoradiography and the silver stained polyacrylamide gel are presented for 4 individuals in Figure 2. Silver staining of the polyacrylamide gels was a very useful alternative to radioactivity. Even if the sensitivity was sometimes slightly less than with radioactivity, only the faint bands, not included in the data analysis, failed to appear. Moreover, results were available within 1.5 to 2 h after electrophoresis with silver staining compared to the usual 1 to 2 d for the radioactive method. Furthermore, the stop solution could be reused two or three times and waste management was much easier.

The use of reduced quantities for AFLP reactions allowed a savings of at least 10 units of enzymes (polymerase, ligase, kinase) per complete reaction compared to the quantities used in the original protocol described by Vos et al. (1995). For radiolabelling, only 25% of the prescribed radionuclide was used (0.25 µCi instead of 1 µCi of radioactivity per gel). Furthermore, radioactivity can be replaced by silver staining and consequently the cost per sample in reagents can be reduced by 82%, from 625 FF or 93 USD per 100 samples with the Vos et al. (1995) protocol to 115 FF or 17 USD per 100 samples with our protocol using silver staining.

Acknowledgements

This work was granted in part by the city of Angers, France. The authors thank Delphine Melayah for her technical advice.

References

Bassam BJ, Caetano-Anolles G and Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196: 80-83.

Boiteux LS, Fonseca MEN and Simon PW (1999) Effects of plant tissue, and DNA purification method on randomly amplified polymorphic DNA-based genetic fingerprinting analysis in carrot. J Amer Soc Hort Sci 124: 32-38.

Chalhoub BA, Thibault S, Laucou V, Rameau C, Hofte H and Cousin R (1997) Silver staining and recovery of AFLP amplification products on large denaturing polyacrylamide gels. BioTechniques 22: 216-220.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd/Ed. CSH Laboratory Press, Cold Spring Harbor, New York.

Vivek BS, and Simon PW (1999) Linkage analysis among molecular markers and storage root traits of carrot (*Daucus carota* L. ssp. *sativus*). Theor Appl Genet 99: 58-64.

Vos P, Hogers R and Bleeker M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407-4414.