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Plant breeding

A comparative analysis between [^{32}P] and digoxigenin-labelled single-copy probes for RFLP detection in wheat

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Summary – The RFLP technique is widely used in plant-genome mapping and other genetic analyses. While it has great potential as a tool for plant breeders, the requirement for radioisotopes has limited its routine use. Here, we have used a commercially available nonisotopic method, the digoxigenin-labelling and detection system (Boehringer Mannheim), with modifications, to analyse a complex polyploid genome such as wheat, and have obtained results equivalent to the isotopic method. The detailed experimental procedures are described. This nonisotopic method will facilitate various RFLP-based genome analyses.

RFLP / digoxigenin / nonradioactive method / chemiluminescence / *Triticum aestivum* = wheat

Résumé – Analyse comparative entre les sondes mono-copie marquées au [^{32}P] et à la digoxigénine pour la détection de PLFR chez le blé. *Le polymorphisme de longueur des fragments de restriction (PLFR) a été largement utilisé pour l'établissement de cartes génétiques des plantes et pour diverses études génétiques. Bien qu'elle présente d'intéressantes potentialités et perspectives pour les sélectionneurs, l'utilisation des éléments radioactifs dans cette technique a limité son application en routine. Nous avons modifié et amélioré une des méthodes non radioactives commercialement disponibles, le marquage à la digoxigénine (Boehringer Mannheim), pour l'analyse d'un génome polyploïde tel que le blé. Les résultats obtenus sont comparables à ceux de la méthode radioactive. Les procédures expérimentales sont décrites en détail. Cette méthode non radioactive devrait faciliter tous les types d'analyses des génomes par la méthode PLFR.*

PLFR / digoxigénine* / Triticum aestivum = *blé

INTRODUCTION

Restriction fragment length polymorphism (RFLP) analyses have numerous applications in genetics and plant improvement (Burr *et al*, 1983; Beckmann and Soller, 1988; Tanksley *et al*, 1989). In the past few years, the genomes of many plant species have been extensively mapped using RFLP markers. These saturated maps can be used to mark useful agricultural genes, to analyse genetic control of qualitative or quantitative traits, and to reveal intervarietal or intergenomic relationships.

Despite the low level of polymorphism (Liu *et al*, 1990), the hexaploid status and an extremely large genome with a haploid DNA content of 16 billion base pairs, the construction of the genetic map of the cultivated hexaploid bread wheat (*Triticum aestivum* L em Thell) has advanced (Liu and Tsunewaki, 1991; Devos and Gale, 1993; Hart *et al*, 1993).

While radioactively [^{32}P]-labelled probes represent a general approach in plant RFLP analyses, their limited storage life and specialized requirements for their handling have limited their routine use. Nonradioactive techniques for plant-DNA analysis have now become routine in many laboratories, as they provide a good alternative to the use of radioactive agents such as [^{32}P], for rice

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(Ishii *et al*, 1990), potato (Kreike *et al*, 1990), tomato (Panaud *et al*, 1993) and tobacco (Neuhaus-Url and Neuhaus, 1993).

In this paper we describe the detailed procedures necessary for an efficient, economical, nonradioactive experimental protocol to analyse a complex polyploid genome such as wheat. The protocol uses digoxigenin-11-dUTP labelled probes (DIG-11-dUTP, provided by Boehringer Mannheim (Lanzillo, 1991; Höltke *et al*, 1992)), two-step detection by antibody conjugates (anti-DIG-alkaline-phosphatase) and chemiluminescent reaction (Beck and Köster, 1990), and we emphasize cost-saving features of the nonradioactive system.

MATERIALS AND METHODS

Plant materials and DNA probes

Two wheat lines, Chinese spring (CS) and Courtot (CT), were used in this study. The wheat single-copy probes were kindly provided by Dr MD Gale, Norwich, UK (Sharp *et al*, 1989). These probes (PSR) had pUC18 as the vector. Only the results with 1 cDNA (PSR162 on homoeologous chromosome group 1) and two genomic DNA (PSR463 on homoeologous chromosome group 6 and PSR543 on homoeologous chromosome group 3) probes are presented here, although we have used the procedure with many single-copy probes.

Genomic DNA extraction

The genomic DNA was extracted from leaves of greenhouse-grown plants using a modification (Staskawicz, personal communication, John Innes Centre, UK) of the procedure of Tai and Tanksley (1991). About 3 g tissue was ground in liquid nitrogen to a very fine powder and incubated with 15 ml extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 0.5 M NaCl, 1.25% SDS, 8.3 mM NaOH and 3.8 mg/ml sodium bisulfite, pH 8.0) at 65°C for 15 min. Potassium acetate (5 M, 5 mL) was then added and the mix was incubated on ice for 20 min. After 10 min centrifugation at 9 000 rpm (9 800 *g*, rotor JA-20, Beckman), the supernatant was filtered through Kimwipes paper into a second centrifugation tube containing 0.6 volumes of isopropanol. After 10 min at −20°C, the DNA pellet was recovered with the hooked tip of a Pasteur pipette, rinsed twice by soaking in 70% ethanol, and resuspended in 800 µl of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 500 µg/ml RNase A. The DNA solution was incubated at 37°C for 30 min. Ammonium acetate (7.4 M, 530 µl) was then added. The mix was transferred to a 1.5 ml Eppendorf tube, and centrifuged at 12 500 *g* in a bench microfuge for 5 min. The supernatant was transferred to a 5-ml hemolysis tube containing 1 ml cold (−20°C) isopropanol. The DNA pellet was recovered and rinsed with 70% ethanol, and then resuspended in 700 µl of 50 mM Tris-HCl pH 8.0, 10 mM EDTA by incubation for 5 min at 65°C. Sodium acetate (3 M, 70 µl) was then added. The DNA was precipitated by isopropanol, washed with 70% ethanol, and redissolved in 300 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). This procedure yielded more than 100 µg DNA/g fresh tissue.

Restriction digests, electrophoresis, and Southern blots

DNA from each sample (10 µg) was digested for 2 h with 20 units of restriction enzyme (*Eco* RI or *Hind*/III) in the buffer provided by the supplier (Boehringer Mannheim). Following digestion, some of the DNA samples were purified by a phenol/chloroform extraction followed by ethanol precipitation. The DNA fragments were separated in a 0.8% horizontal agarose gel by electrophoresis at 30 V overnight in TAE buffer (40 mM Tris-HCl pH 7.5, 40 mM acetic acid, 1 mM EDTA). The gels were then capillary blotted onto nylon membrane (Hybond-N^+, Amersham) with alkaline buffer (0.4 N NaOH) using a 1% agarose (low cost) gel at the bottom and a sponge at the top.

Probe labelling, prehybridization, hybridization, and detection

All the steps of prehybridization, hybridization, washing, and detection were performed in sealed glass bottles (15 × 3.5 cm) fixed on a rotor rotated at a constant speed in a thermostat oven (Hybaid, Schleicher & Schuell). All incubation steps were carried out in 10 ml of solution and all washing steps in 50 ml solution.

Radioactive method

The plasmid inserts were cut from low melting point agarose gels. About 30 ng DNA probe was radioactively labelled with 40 µCi [^{32}P]-dCTP using a random primer labelling kit (Amersham). The purification of inserts after labelling was not necessary.

The DNA blots were prehybridized for at least 3 h at 65°C in a buffer of 0.6 M NaCl, 20 mM Pipes pH 6.8, 4 mM EDTA, 0.2% Ficoll 400, 0.2% PVP, 1% SDS. They were hybridized overnight at 65°C in a prehybridization mix containing denatured (boiled for 5 min) radiolabelled probes. The filters were successively washed twice for 5 min at room temperature and twice for 15 min at 65°C in 0.5 × SSC (20 × SSC: 0.3 M sodium citrate, 3 M NaCl, pH 7.0), 0.1% SDS. Finally, they were autoradiographed for 3 d at –70°C using Amersham MP X-ray film and 2 intensifying screens.

Nonradioactive method

For nonradioactive RFLP analyses, the procedure of labelling of the probe, hybridization and detection of the hybridized probe was a modification of the supplier's manual (DIG Labelling and Chemiluminescent Detection Kit, Boehringer Mannheim).

Digoxigenin-11-dUTP was incorporated into DNA by PCR (Polymerase Chain Reaction) (Saiki *et al*, 1985). Two pairs of oligonucleotide sequences, homologous to pUC18 sequences flanking the inserts, were used as primers: 5′-ATTCGAGCTCGGTACC-3′ and 5′-AGGTCGACTCTAGAGG-3′ for *Sma* I site insert, PSR162, and for *Pst* I site insert, PSR543; 5′-AACAGCTATGACCATG-3′ and 5′-GTAAAACGACGGCCAGT-3′, for *Hind* III site insert, PSR463. The reaction mixtures (50 µl) consisted of 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatine, 50 µM dATP, 50 µM dCTP, 50 µM dGTP, 40 µM dTTP, 4 µM DIG-11-dUTP, 0.25 µM of each primer, 1.25 U *Taq* polymerase (Boehringer Mannheim), and 50 ng template plasmid DNA. The labelling reaction was carried out with GeneAmp PCR system 9600 (Perkin-Elmer Cetus) by a programmed thermal cycling consisted of a 1 min predenaturation at 94°C, 35 cycles of 15 s denaturation at 94°C, 30 s annealing at 48°C, and 3 min extension at 72°C, and a final extension for 7 min at 72°C. After completion of the reaction, 3 µl samples of the PCR products, accompanied by a known quantity of a standard reference DNA fragment, were analysed by 0.8% agarose gel electrophoresis and stained with ethidium bromide. The concentration of labelled DNA was then estimated.

The DNA blots were prehybridized for at least 3 h at 65°C in a buffer of 5 × SSC, 0.5% SDS, 0.1% *N*-lauroylsarcosine, 1% blocking reagent (Boehringer Mannheim), 100 µg/ml denatured herring sperm DNA. The filters were hybridized overnight at 65°C in prehybridization mix containing 20 ng/ml denatured digoxigenin-labelled probes (boiled for 5 min). The removal of unincorporated digoxigenin-11-dUTP was not necessary. The hybridization solution was recovered and stored at 4°C or –20°C for a long period. For subsequent probe reuse, the recovered hybridization solution was boiled for 10 min. The filters were successively washed twice for 5 min at room temperature and twice for 15 min at 65°C in 0.1 × SSC, 0.1% SDS.

For chemiluminescent detection, all the following steps were performed at room temperature. The filters were first washed for 5 min in buffer 1 (100 mM Tris-HCl pH 8.0, 150 mM NaCl), then preincubated for 30 min in buffer 2 (buffer 1 + 0.5% blocking reagent) and incubated for 30 min in buffer 2 containing 37.5 mU/ml (1:20 000, v:v) anti-digoxigenin-AP (Boehringer Mannheim). The unbound antibody conjugate was removed by two 20 min washes with buffer 1. The filters were incubated for 5 min in buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and for 5 min in buffer 3 containing 50 µg/ml (1:200, v:v) Lumigen-PPD (Boehringer Mannheim). The diluted substrate solution was recovered and stored at –20°C. The filters were enclosed in a transparent plastic folder and kept overnight at room temperature. On the next day (12–16 h later), the filters were exposed to Amersham MP X-ray film for 4–6 h at room temperature in a vinyl-covered exposure cassette (Sigma). If the hybridization signals were too strong with high background, or too weak, a secondary shorter or longer exposure might be made. The rate of signal production reaches its plateau 12–16 h after exposure to Lumigen-PPD and remains constant for several days.

To remove the hybridized probe, the filters were incubated in 0.2 N NaOH, 0.5% SDS at 45°C for 30 min, then rinsed in 2 × SSC. If the Southern blot is not reused immediately, it is essential to keep it in 0.1 × TE buffer and store it at 4°C.

RESULTS AND DISCUSSION

The hybridization patterns obtained by the nonradioactive method in comparison with those by the radioactive method are presented in figure 1. For each of the 3 representative wheat single-copy probes, PSR162, PSR463, and PSR543, the same hybridization profiles were obtained by using radioactive (fig 1A) or nonradioactive (fig 1B and 1C) labelling methods. Moreover, almost equally strong hybridization signals were obtained with the nonradioactive method (fig 1B) as with the radioactive method (fig 1A), although some background was observed with the former. The digoxigenin-labelled probes can be reused 5 times and still give usable results (fig 1C). A treatment of phenol/chloroform fol-

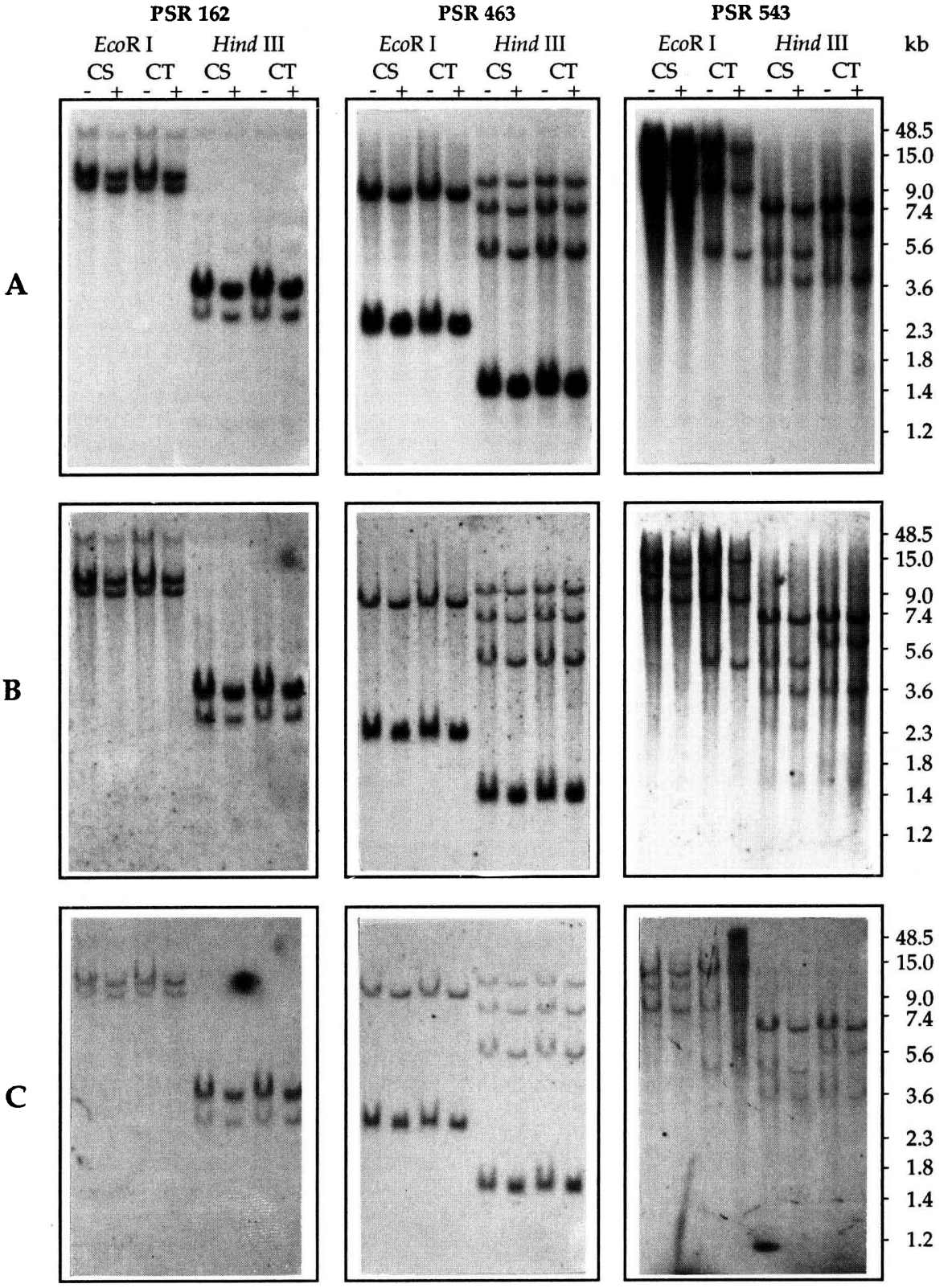

Fig 1. Autoradiographs of genomic DNA of Chinese Spring (CS) and Courtot (CT) hybridized with [^{32}P]-dCTP (A) and digoxigenin-11-dUTP (B and C) labelled probes. Following the enzyme digestions, the DNAs of the wheat lines were purified by phenol/chloroform extraction (track +) or not purified (track –). The DNA membranes were probed for the first (A), second (B) and sixth (C) times. The hybridization buffers containing digoxigenin-11-dUTP labelled probes used in the case of B were recovered and reused for their fifth times in that of C. The probe/blot combinations were different between the cycles of reutilization of the blots and of the recovered hybridization buffer. The exposure times were 3 d (A), 4 h (B) and 6 h (C).

lowing the enzyme restriction (track +) seems to improve the quality of hybridization bands.

Several major modifications and simplifications have been made to 2 recent protocols published on rice and tomato (Panaud *et al*, 1993) and on tobacco (Neuhaus-Url and Neuhaus, 1993), and the protocol given by the supplier (Boehringer Mannheim).

Isolation of genomic DNA

Unfortunately, it is not possible to apply the procedure described by Tai and Tanksley (1991) to wheat. In particular, it is essential to grind fresh wheat leaves with liquid nitrogen to avoid any DNA degradation. In addition, 3 modifications have been made (Staskawicz, personal communication, John Innes Centre, UK): (i) use of 8.3 mM NaOH in the extraction buffer; (ii) use of 7.4 M ammonium acetate followed by a centrifugation at 2 500 *g* before an isopropanol precipitation; and (iii) recovery of DNA pellet by the hooked tip of a Pasteur pipette instead of centrifugation.

Southern blot

Considering the procedure of Neuhaus-Url and Neuhaus (1993) and/or Panaud *et al* (1993), some parameters have also been simplified or eliminated for the preparation of the Southern blot: (i) 10 µg of wheat digested total DNA per track are necessary to obtain a good signal with wheat; (ii) the digested DNA is purified by phenol/chloroform extraction before agarose-gel electrophoresis; (iii) the gel is soaked 30 min in 0.4 N NaOH and then directly capillarily transferred using an agarose (lowcost) gel below it, and no buffer is necessary; (iv) no filter treatments (UV cross-linking or baking in a vacuum oven) are necessary.

PCR-mediated DNA probe labelling

The PCR reaction to label DNA probes has several advantages over other methods, such as nick-translation and random primer labelling, for which it is generally necessary to isolate the insert from its plasmid vector. For PCR labelling, only a small amount of plasmid DNA is required (50 ng) and is used as a template to produce a large quantity of efficiently labelled vector-free probe (2–5 µg in our case). Four major modifications were introduced for PCR amplification in comparison with the procedure of Panaud *et al* (1993). Firstly, use of a lower concentration of dNTP (50 µM dATP, dCTP, dGTP, 40 µM dTTP) primers (0.25 µM) and digoxigenin-11-dUTP (4 µM) in the reaction mixture. Secondly, addition of 10% glycerol in the reaction mixture, which improves the efficiency and specificity of PCR amplification (Lu and Nègre, 1993). Third, use of a prolonged extension time (3 min) and a reduced denaturation and annealing time for each thermal cycle. As the digoxigenin-labelled DNA is synthesized approximately half as efficiently as unlabelled DNA, a longer extension time for each thermal cycle is required for the labelling reaction (data not shown). Finally, removal of unincorporated digoxigenin-11-dUTP from the labelled probe is not necessary prior to hybridization to prevent non-specific hybridization (fig 1B and 1C). We have also used the solution resulting from a failed PCR-labelling reaction (no labelled DNA has been amplified as it has been verified by agarose-gel electrophoresis) as probe, parallel with that of a successful reaction. Neither an increased background nor the expected hybridization band were observed by using the failed PCR sample as a probe (data not shown).

Currently, we label our DNA probes directly from their bacterial hosts (Lu *et al*, 1993).

Nonradioactive hybridization and chemiluminescent detection

The major problem encountered in using this nonradioactive method is the higher background in comparison with the radioactive method. Application of the following parameters enable us to obtain successful results in wheat with a lower cost:

(1) Use of 0.5% SDS in the hybridization solution.

(2) Use of 100 µg/ml denatured herring sperm DNA in the hybridization solution.

(3) Use of 1% blocking reagent in the hybridization solution.

(4) Use of 37.5 mU/ml (1:20 000, v/v) anti-digoxigenin-AP in the buffer 2.

(5) Use of 50 μg/ml (1:200, v/v) Lumigen-PPD in the buffer 3.

(6) Despite the suggestion of Neuhaus-Url and Neuhaus (1993), we do not recommed the use of Tween 20 in buffer 1, because it increases the background in our conditions (data not shown).

In the protocol described here, all the steps of prehybridization, hybridization, washing and detection were performed in sealed bottles rotated at a constant speed in a hybridization oven using a minimum volume of reaction solution. If there was significant background, it could be eliminated by increasing the number and length of the washes, especially for the washes following the anti-digoxigenin-AP incubation. When large Southern blots are to be hybridized, as is usually the case for genome mapping programmes, this technique can be made less laborious and less expensive if all the washing and incubation steps are performed in a plastic box. For example, more than 10 blots of 20×20 cm can be washed together in a single box in 1 l of washing buffer and incubated in 400 ml of incubation buffer. The Lumigen-PPD incubation should be performed in a sealed plastic bag (40 ml for 2 blots back-to-back of 20×20 cm size in each bag) for a reduced cost.

The various advantages of this nonradioactive method over radioactive techniques are obvious and have been described elsewhere (Neuhaus-Url and Neuhaus, 1993). The ability to label, at the same time, a large number of DNA probes which are stable for a long time gives obvious logical advantages. It permits experiments to be planned more easily since we are not dependent on the availability and timeliness of ^{32}P shipments. The possibility of reusing the recovered labelled probe (5 times), Lumigen-PPD (10 times), and the Southern blot (8 times and sometimes more) increases the cost effectiveness of this chemiluminescent detection system over the traditionally used radioactive method for RFLP analysis. Nevertheless, the degree and characteristics of toxicity of the digoxigenin-11-dUTP is as yet unclear.

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REFERENCES

Beckmann JS, Soller M (1988) Detection of linkage between marker loci and loci affecting quantitative traits in crosses between segregating populations. *Theor Appl Genet* 76, 228-236

Beck S, Köster H (1990) Applications of dioxetane chemiluminescent probes to molecular biology. *Anal Chem* 62, 2258-2270

Burr B, Evola SV, Burr FA, Beckmann JS (1983) The application of restriction fragment length polymorphism to plant breeding. In: *Genetic Engineering: Principles and Methods* (JK Setlow and A Hollaender, eds) Plenum Press, NY, 5, 45-59

Devos KM, Gale MD (1993) The genetic maps of wheat and their potential in plant breeding. *Outlook Agric* 22, 93-99

Hart GE, Gale MD, McIntosh RA (1993) Linkage maps of *Triticum aestivum* (hexaploid wheat, $2n=42$, genomes A, B & D) and *T tauschii* ($2n=14$, genome D). *In: Progress in Genome Mapping of Wheat and Related Species* (D Hoisington and A McNab, eds) Proceedings of the 3rd public workshop of the International Triticeae Mapping Initiative. Mexico, DF, CIMMYT, 32-46

Höltke HI, Sagner G, Kessler Ch, Schmitz G (1992) Sensitive chemiluminescent detection of digoxigenin-labelled nucleic acids: a fast and simple protocol and its application. *Biotechniques* 12, 104-113

Ishii T, Panaud O, Brar DS, Klush GS (1990) Use of non-radioactive digoxigenin-labeled DNA probes for RFLP analysis in rice. *Plant Mol Biol Reporter* 8, 167-171

Kreike CM, Koning JRA, Krens FA (1990) Non-radioactive detection of single-copy DNA-DNA hybrids. *Plant Mol Biol Reporter* 8, 172-179

Lanzillo II (1991) Chemiluminescent nucleic acid detection with digoxigenin-labelled probes: a model system with probes for angiotensin converting enzyme which detect less than one attomole of target DNA. *Anal Biochem* 194, 45-53

Liu YG, Mori N, Tsunewaki K (1990) Restriction fragment length polymorphism (RFLP) analysis in wheat. I. Genomic DNA library construction and RFLP analysis in common wheat. *Jpn J Genet* 65, 367-380

Liu YG, Tsunewaki K (1991) Restriction fragment length polymorphism (RFLP) analysis in wheat. II. Linkage maps of the RFLP sites in common wheat. *Jpn J Genet* 66, 617-633

Lu YH, Nègre S (1993) Use of glycerol for enhanced efficiency and specificity of PCR amplification. *Trends Genet* 9, 297

Lu YH, Nègre S, Leroy P, Bernard M (1993) PCR-mediated screening and labelling of DNA from clones. *Plant Mol Biol Reporter* (in press)

Neuhaus-Url G, Neuhaus G (1993) The use of the nonradioactive digoxigenin chemiluminescent technology for plant genomic Southern blot hybridization: a comparison with radioactivity. *Transgenic Res* 2, 115-120

Panaud O, Magpantay G, McCouch S (1993) A protocol for non-radioactive DNA labelling and detection in the RFLP analysis of rice and tomato using single-copy probes. *Plant Mol Biol Reporter* 11, 54-59

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of beta-globin genomic sequences and restriction analysis of sickle-cell anemia. *Science* 230, 1350-1354

Sharp PJ, Chao S, Desai S, Gale MD (1989) The isolation, characterization and application in the Triticeae of a set of wheat RFLP probes identifying each homoeologous chromosome arm. *Theor Appl Genet* 78, 342-348

Tai TH, Tanksley SD (1991) A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. *Plant Mol Biol Reporter* 8, 297-303

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding : new tools for an old science. *BioTechonology* 7, 257-264