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Fast resolution of identification problems in seed production and plant breeding using molecular markers

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Summary. Polymerase chain reaction (PCR) analysis with specific and arbitrary primers was used to identify germplasm from a barley breeding program. Through practical application of the technology, an F_1 plant's identity was confirmed, the correct pedigrees of 2 incorrectly labelled F_2 populations were determined, confusion in the identity of seed lots of an advanced breeding line was resolved and off-type barley plants were identified in a seed increase block. This illustrated the value of PCR-based genotype analysis in plant breeding programs.

Introduction

DNA marker technology has become well established as a research tool. Techniques such as restriction fragment length polymorphism (RFLP) (Beckman and Soller 1983), amplification fragment length polymorphism (AFLP) (Zabeau *et al.* 1994), polymerase chain reaction (PCR) (Saiki *et al.* 1988) and random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990) have gained widespread use in plant genetic research. These technologies are often being used for developing genomic maps and the identification of markers to assist in selection of specific traits. Other uses have included cultivar identification (barley, Ko and Henry 1994; sweet potato, Connolly *et al.* 1994), studies to observe evolutionary relationships (Shields 1993; Campos *et al.* 1994) and observation of genetic diversity (M'Ribu and Hilu 1994).

Suggested uses for marker-based identification of plant cultivars have mostly been concerned with commercial aspects of plant breeding; e.g. plant breeders rights applications or the identification of plant products for industrial use. The most common use of this 'DNA fingerprinting' in plant breeding research has been the assessment of gene pool diversity (Lawson *et al.* 1994). However, there are several other ways in which cultivar identification by DNA markers, particularly with PCR, could be used in practical plant breeding programs.

For reasons including relative cost, space and waste disposal, PCR is superior to other available technologies such as RFLP for establishing facilities at field research centres. PCR requires only small amounts of template DNA and is superior to protein electrophoresis because of its ability to detect greater variation between lines. PCR can produce reliable results within days; a vast improvement on the conventional field-based identification techniques which may not produce useful data for several months. Quick answers to problems concerning the correct identity of breeding material would help to maintain continuity of research in field crop and horticultural breeding programs, and would have even greater benefits in longer-term research programs such as fruit tree or forestry breeding. PCR was used by Keil and Griffen (1994) to solve misslabelling in a set of clones from *Eucalyptus grandis* and *E. camaldulensis*.

In this paper, we report how PCR techniques were used to solve 4 germplasm identification issues encountered during the routine operations of a barley breeding program: (i) an F_1 hybrid's identity was confirmed before harvesting pollen for anther culture; (ii) the pedigrees of 2 incorrectly labelled F_2 breeding populations were determined; (iii) correct seed lots of an advanced breeding line were identified following a labelling error; and (iv) off-type barley plants were identified in a precommercial seed increase block.

Materials and methods

DNA extraction

All DNA samples were extracted by the cetyltridimethylethylammoniumbromide (CTAB)

method described by Graham *et al.* (1994). Samples were frozen in liquid nitrogen, ground and incubated in 2% CTAB buffer at 55°C for 15 min in 1.5 mL Eppendorf microfuge tubes. After centrifugation at 13000 rpm for 5 min, the supernatant was removed and about 50% volume of 24:1 chloroform:iso-amyl alcohol (v/v) added to the tubes and mixed gently. Centrifugation at 13000 rpm for 1 min followed and the upper aqueous phase was transferred to clean Eppendorf tubes to which about 10% volume of 7.5 mol/L ammonium acetate (CH_3COONH_4) was added. The DNA was precipitated in ice-cold absolute ethanol, washed in 70% (v/v) ethanol, vacuum-dried and resuspended in TE buffer (1 mol Tris-HCl/L, 0.5 mol EDTA/L, pH 8.0) containing 10 µg/mL RNAase. DNA concentration in the final solutions was determined spectrophotometrically.

Confirming an F_1 hybrid's identity

Glasshouse-grown F_1 barley plants from the cross between the cultivars Tallon and Kaputar were to be used as pollen donors for anther culture. Self-pollination of the female parent, Tallon, may have taken place and confirmation of the identity of the F_1 plants was necessary before any pollen was harvested.

Leaf tissue samples from parental bulks (5 plants each) and 5 individual F_1 plants were collected for DNA extraction. PCR was performed using primers based on the barley α-amylase genes (Weining *et al.* 1994), of which the combinations BSW3/BAS2 and BAS1/BAS2 (Table 1) showed polymorphism. The PCR mixture consisted of: 1 x PCR buffer [4 mmol $MgCl_2$/L, 0.01 mol Tris-HCl/L, 0.05 mol KCl/L, 0.1 mg gelatin/mL, pH 8.3], 200 µmol of each of dATP, dCTP, dGTP and dTTP, 25 pmol of each primer and 0.8 units of *Taq* DNA polymerase (Boehringer Mannheim). Template DNA (1 µL, 100–600 ng) was added to each mixture and the total volume made up to 25 µL with sterile water. The mixture was overlaid with about 0.05 mL of paraffin oil.

Table 1. Selected primer sequences used for PCR analysis

Primer	Sequence (5' to 3')
BSW3	CAG CTT GGC CTC CGG GCA AGT C
BAS1	CCG CCG TCG CAC TCC GTC
BAS2	CAC CTT GCC GTC GAT CTC
OPA-08	GTG ACG TAG G
OPA-11	CAA TCG CCG T
OPA-19	CAA ACG TCG G
OPA-20	GTT GCG ATC C
OPU-03	CTA TGC CGA C
OPU-06	ACC TTT GCG G
OPU-17	ACC TGG GGA G
OPV-06	ACG CCC AGG T
OPV-20	CAG CAT GGT C

PCR was carried out in a Perkin Elmer 480 Thermal Cycler with an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min, and a final 5 min cycle at 72°C.

PCR products were subjected to electrophoresis on agarose gels with concentrations chosen for optimum resolution of products <2 kb. Gels were stained for about 45 min with 50 µg/L ethidium bromide ($C_{21}H_2ON_3Br$) and photographed under UV light.

Determining the identity of two F_2 seed lots

Labels on 2 F_1 plants, Cameo/Harrington and Manley/Ellice, were accidentally removed before harvesting the F_2 seed. Because both crosses were potentially useful, it was necessary to identify them correctly. In an adaptation of the bulk segregant analysis method (Michelmore *et al.* 1992), bulk DNA samples were extracted from 5 seedlings of each of the 2 barley F_2 populations and also from the parent cultivars. Eighteen 10-mer oligonucleotide primers (Operon Technologies) were used to screen the samples by PCR. The primers were known to produce amplification products from barley as the result of earlier work (D. M. E. Poulsen unpublished data). Each reaction mix contained 50 ng of template DNA, 0.05 µmol of primer, 1.0 unit of *Taq* DNA polymerase, 200 µmol of each of dATP, dCTP, dGTP and dTTP, and 1 x PCR buffer and was made up to a total volume of 25 µL with sterile water.

PCR was performed on a Perkin Elmer 9600 Gene Amp PCR system and was initiated by a denaturation step of 1 min at 94°C, followed by 10 cycles of 30 s at 36°C, 1 min at 72°C and 20 s at 94°C. Subsequently, a cycle of 30 s at 37°C, 1 min at 72°C and 15 s at 94°C was repeated 30 times.

Amplication products were subjected to electrophoresis on 1.5% (w/v) agarose gels and stained with ethidium bromide as in the previous experiment.

Identifying correct seed lots of an advanced breeding line

Morphological differences were detected between plants from different seed sources of an advanced breeding line. To trace the error and determine the correct seed sources without resorting to out-of-season planting, it was decided to use the RAPD technique to test the identity of 8 different seed lots, including the original (1988) F_7 selection material. Of the remaining 7 seed sources, 1 each were from plots grown in 1991 and 1992, 2 from 1993 and 3 from 1994. Seedlings from each seed source were grown in 5 cm peat 'pots'. Bulk tissue samples from between 3 and 5 seedlings per seed source were harvested for DNA extraction. PCR was conducted on the samples, using the same 18 primers, PCR reaction mixtures, amplification cycle and gel electrophoresis procedure as in the previous experiment.

Identifying 'off-types' in a seed production block

Several off-type plants with an increased level of anthocyanin (red) pigmentation of the glumes and awns were found in a precommercial increase of a potential new barley cultivar with the pedigree Triumph/Grimmett. Other morphological differences between these plants and the new line were not obvious, so DNA samples were collected from individual off-type and true-to-type plants to determine if there were significant genetic differences between them. As well as including the parents of the new line in the PCR analysis, another barley cultivar, Gilbert, was tested as it had been grown in the same field the previous year.

DNA was extracted from bulked leaf samples of 3 barley cultivars (Grimmett, Triumph and Gilbert), 5 individual samples from the potential new cultivar and suspected off-type plants found in a seed production block of the new line. Eighteen arbitrarily selected 10-mer oligonucleotide primers were used for performing PCR on the DNA samples. The PCR reaction mixtures, amplification cycle and gel electrophoresis procedure were the same as in the previous 2 experiments.

Results and discussion

Confirming an F_1 hybrid's identity

The resulting gels showed that when amplified with the BSW3/BAS2 primer combination, Tallon expressed an extra band at about 600 bp which was missing in the Kaputar and F_1 samples (Fig. 1*a*). Between the 947 and 1375 bp markers, Kaputar and the F_1 showed an identical pattern of 3 bands. Tallon possessed 2 bands in this area and only the smaller band matched with the Kaputar and the F_1 samples.

With the BAS1/BAS2 primer combination, a band at about 800 bp was amplified from Tallon and the F_1 but not from Kaputar (Fig. 1*b*). Additionally, Kaputar has a marker at about 990 bp, which was missing for Tallon and the F_1 sample.

These results showed that for the BAS1/BAS2 primer combination, all 5 F_1 plants resembled Tallon (the female parent), while for the other pair of primers they resembled Kaputar. While it is possible for bands to be generated from mitochondrial or ribosomal DNA and be maternally inherited, that explanation is unsuitable here because of the similarities shown to both maternal and paternal parents. The most likely explanation comes from the nature of the PCR reaction itself. Because of the very low concentration of template DNA and the reliance on many amplification cycles, generation of reaction products becomes competitive, and the observed banding pattern probably represents the most successfully amplified products (Heun and Helentjaris 1993). These competition effects have been observed in amplifications with relatively few product bands, and it could be expected that this effect would be commonplace in PCR where many products are produced, such as in the current experiment. Because resemblances to both parents were shown from the 2 primer combinations, it was considered unlikely that the putative F_1s were selfs, since only similarities to the maternal parent, Tallon, would have been observed in that case.

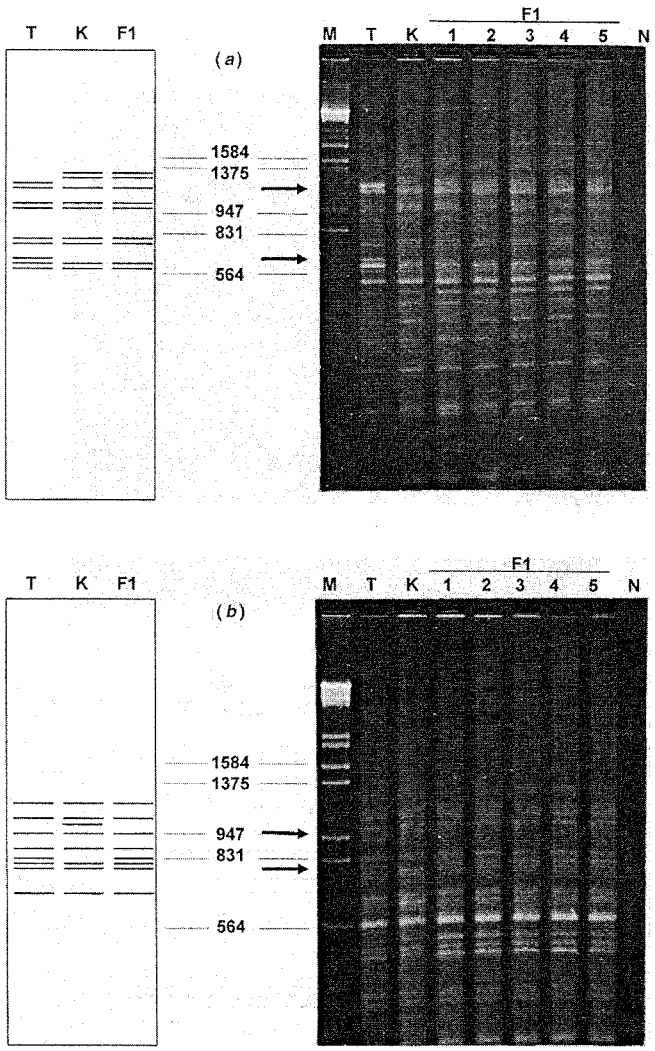

Figure 1. (*a*) Agarose gel (2.5%) showing BSW3/BAS2 primer combination PCR amplification products from: T, Tallon DNA bulk; K, Kaputar DNA bulk; and 1–5 individual F_1 plants. M, λ digested with *Eco*R1 and *Hin*dIII; N, negative control. Arrows indicate polymorphisms. (*b*) Agarose gel (2.2%) showing BAS1/BAS2 primer combination PCR amplification products from: T, Tallon DNA bulk; K, Kaputar DNA bulk; and 1–5, individual F_1 plants. M, λ digested with *Eco*R1 and *Hin*dIII; N, negative control (no DNA). Arrows indicate polymorphisms.

Anther culture is timely and resource consuming. Therefore, pollen donors must be the correct genotype. We have shown that PCR markers can be used to

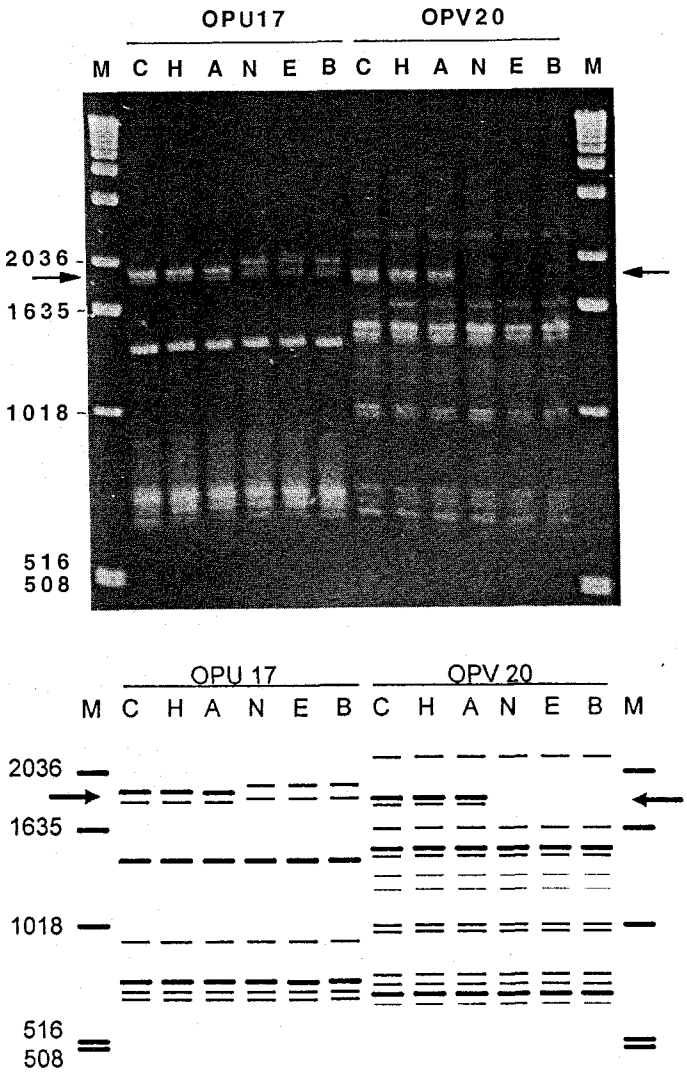

Figure 2. Agarose gel (1.5%) showing OPU-17 and OPV-20 primer PCR amplification products from: C, Cameo; H, Harrington; A, F_2 population A bulk DNA; N, Manley; E, Ellice; and B, F_2 population B bulk DNA. M, λ digested with *Hin*dIII. Arrows indicate polymorphisms.

identify F_1 and ensure that the correct double haploid populations can be generated for plant breeding and molecular mapping.

Determining the identity of two F_2 seed lots

Following PCR of the F_2 and parent samples, polymorphisms were observed from primers OPA-19, OPU-03, OPU-06, OPU-17 and OPV-20. Several of the primers did not produce amplification products; but this was suspected to have been caused by problems with the reagents rather than failure of the primers themselves.

The detected polymorphisms were used to match the F_1 and parent DNA samples. For example, amplification with the primer OPU-17 resulted in the production of 4 polymorphic bands in the region of 2000 bp. There were 2 matching bands in the Cameo, Harrington and F_2 A lanes and 2 different matching bands for Manley, Ellice and F_2 B. OPV-20 produced 2 bands in the Cameo, Harrington and F_2 A samples at about 2000 bp (Fig. 2). The banding patterns from these primers grouped Ellice and Manley with the F_2 B sample and Cameo and Harrington with the F_2 A sample. This result reflected the parentage of the 2 F_2 populations. Banding profiles with other primers confirmed the data.

The experiment clearly showed that the PCR technique offers a means of fast resolution of identification problems in early generation breeding material. Whereas the conventional method of using known morphological traits to distinguish the populations in the field would have taken considerable time and may have given dubious results, the use of RAPDs gave a quick, reliable answer. Additional time was saved by using DNA bulks. This significantly reduced the number of PCRs.

Identifying correct seed lots of an advanced breeding line

As with the preceding experiment, problems with reagents in the PCR mixtures appear to have caused several of the primers to fail to produce products. However, useful results were obtained, including a very clear result from primer OPA-19 (Fig. 3) which showed 2 distinct banding patterns within the set of samples. Intense twin bands of about 1500 bp were only produced from the 1988, 1991, 1994*b* and 1994*c* samples while a single band of about 500 bp was only produced by the 1992, 1993*a*, 1993*b* and 1994*a* samples. Identical classification of the seed sources was obtained with other primers, including OPA-20 which produced an approximately 1700 bp band in the 1988, 1991, 1994*b* and 1994*c* samples. This classification was further

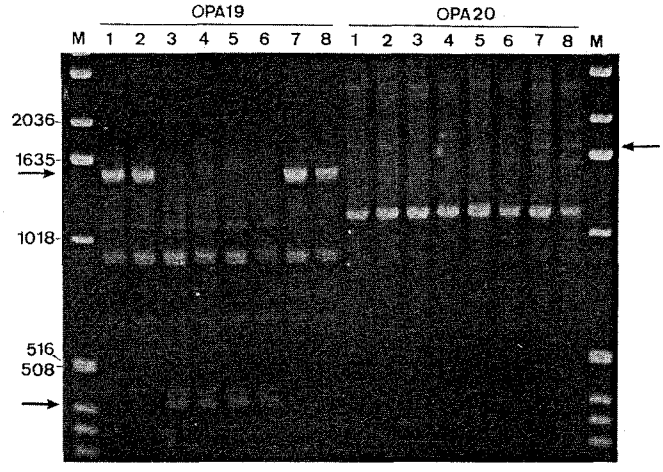

Figure 3. Agarose gel (1.5%) showing OPA-19 and OPA-20 primer PCR amplification products from eight separate seed lots: 1, 1988; 2, 1991; 3, 1992; 4, 1993*a*, 5, 1993*b*; 6, 1994*a*; 7, 1994*b*; 8, 1994*c*. M, λ digested with *Hin*dIII. Arrows indicate polymorphisms.

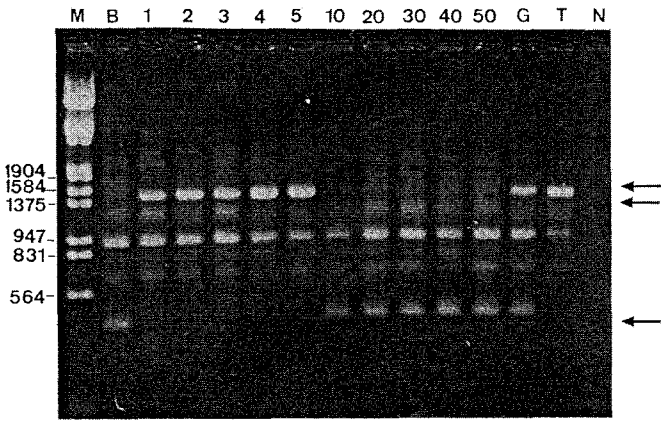

Figure 4. Agarose gel (1.5%) showing OPA-19 PCR amplification products from: B, Gilbert; G, Grimmett; T, Triumph; 1, 2, 3, 4, and 5, individual plants of Triumph/Grimmett advanced breeding line; and 10, 20, 30, 40, and 50, individual off-type plants. M, λ digested with *Eco*R1 and *Hin*dIII; N, negative control. Arrows indicate polymorphisms.

supported by net blotch and spot blotch susceptibility data from a separate experiment (G. J. Platz and R. G. Rees pers. comm.). It appeared, therefore, that the 1991, 1994*b* and 1994*c* samples were from seed sources of the correct line. When this information was combined with available records, the problem was traced to the apparent occurrence of a labelling error between the 1991 and 1992 growing seasons.

The use of molecular techniques negated the need for field-based 'grow outs' and resulted in the identification of correct seed sources before trial preparation began.

Identifying off-types in a seed production block

Seventeen out of 18 oligonucleotide primers tested on the material yielded amplification products. Each primer yielded specific banding profiles and there were several amplification products per sample. Four of the primers, OPA-08, OPA-11, OPA-19 and OPA-20, showed polymorphism between 2 distinct sample groups. The remaining 13 primers produced monomorphic banding profiles across all templates. Cultivars Triumph, Grimmett and the true-to-type plants showed a similarity in their banding patterns that was distinct from the patterns shown similarly by the off-types and Gilbert. For example, primer OPA-19 produced a product at about 500 bp and a fainter band at about 1700 bp from cv. Gilbert and the off-type DNA samples. A strongly amplified band at about 1500 bp was produced from cvv. Triumph, Grimmett and the 5 true-to-type plants (Fig. 4).

The results showed convincingly that the off-type plants were the same as Gilbert and different from either Triumph, Grimmett or the Triumph/Grimmett line. From this we concluded that the seed increase crop had probably been contaminated by self-sown Gilbert.

As in the previous experiments, PCR techniques were able to produce clear results in a relatively short time. This enabled action to be swiftly taken in the seed production block and resolved a problem, which if undetected could have had serious consequences in future commercial seed production.

Conclusions

The 4 experiments reported in this paper are representative of the type of problems which occur occasionally in breeding programs. Each situation could have resulted in significant difficulties if left unresolved. Although conventional techniques could have been used in each case, the use of PCR-based methods provided useful results more quickly. This demonstrated the value of PCR technology as a diagnostic tool in plant breeding programs.

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