Heredity **71** (1993) 177–184

Received 9 December 1992

Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis

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Three hundred random sequence 10-mer primers were used to screen a pair of near-isogenic lines of barley and their donor parent for markers linked to genes conferring resistance to *Rhynchosporium secalis*. One primer was identified which reproducibly generated a product, SC10-65-H400, from the donor parent and the *Rhynchosporium*-resistant near-isogenic line but not from the recurrent parent. Segregation analysis on a barley doubled haploid population and examination of a further three near-isogenic lines, their donor and recurrent parents confirmed that this marker was linked to the *Rhynchosporium* resistance locus (*Rh*) on chromosome 3L. The presence or absence of SC10-65-H400 was subsequently used along with the resistance phenotype to identify two groups of individuals in the doubled haploid population which possessed alternative alleles at both loci and defined a genetic interval between these two markers. Based on that information two bulked DNA samples were constructed by combining equal amounts of DNA from five individuals from each group. The two bulks and doubled haploid parental lines were screened with 700 10-mer primers. Seven products were identified which were present in the 'resistant' bulk and parent and were absent in the susceptible samples. Segregation analysis established their association with *Rh*. In addition co-segregation of the linked markers with a set of chromosome arm specific RFLPs confirmed the location of the *Rh* locus on the long arm of barley chromosome 3.

Keywords: barley, bulked segregant analysis, disease resistance, linkage, near-isogenic lines, RAPDs, *Rhynchosporium secalis*.

Introduction

The identification of suitable sources of disease resistance genes and their incorporation into adapted germplasm is a major challenge for plant breeders. However, assays for disease resistance are often based upon qualitative responses in disease nurseries and fail to identify specific resistance genes without the use of race testing. An easily scorable marker linked to the gene conferring a particular resistance phenotype would therefore represent an important tool for plant breeders. Furthermore, information on the chromosomal and genetic map location of a gene provides an alternative route to gene isolation and cloning (Rommens *et al.*, 1989).

The development of detailed genetic linkage maps based on molecular polymorphic assay procedures will facilitate the identification of markers linked to important disease resistance genes (Tanksley *et al.*, 1989). Methods for targeting markers to important plant genes have largely been based on the use of near-isogenic lines. The rationale of the approach is based on 'linkage drag' (Hanson, 1959; Brinkman & Frey, 1977; Stam & Zeven, 1981), a feature of chromosome behaviour whereby flanking DNA surrounding the target gene diminishes at a much slower rate than unlinked genes. A comparison of near-isogenic lines with the recurrent and donor parents provides a means of identifying DNA sequences arising from the introgressed region of the genome. Feuerstein *et al.* (1990) have used *Hordeum spontaneum* backcross derived lines to identify isozyme loci (*Est2, Acp3* and *Dip2*) which are linked to rust resistance genes (*Puccinia*

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hordei). In addition, a number of studies has screened near-isogenic lines with cDNA and genomic clones and identified markers linked to disease resistance genes (Young *et al.*, 1988; Sarfatti *et al.*, 1991; Hinze *et al.*, 1991).

The analysis of nucleotide sequence variability has been revolutionized by the development of the polymerase chain reaction (PCR) (Saiki *et al.*, 1988). Based on PCR, a new procedure termed 'Randomly Amplified Polymorphic DNAs (RAPDs)' has been developed for detecting polymorphism in plants (Williams *et al.*, 1990; Welsh & McClelland, 1990; Waugh & Powell, 1992). RAPD markers are generated by the use of short (10-mer) synthetic oligonucleotides in single primer PCR reactions. In contrast to RFLPs, genetic analysis with RAPDs is fast and does not involve the use of radioisotopes. RAPDs are therefore compatible with the high throughput requirements of breeding programmes (Rafalski *et al.*, 1991). Near-isogenic lines have been used in conjunction with RAPD markers to locate resistance genes (*Pseudomonas syringae*) in tomato (Martin *et al.*, 1991) and downy mildew resistance in lettuce (Paran *et al.*, 1991).

Scald or leaf blotch caused by the facultative fungus, *Rhynchosporium secalis*, is an important disease of barley, particularly winter barley. Resistance genes have been identified (Dyck & Schaller, 1961a; Habgood & Hayes, 1971) but the selection of resistant lines in breeding programmes can be difficult due to the influence of the environment on the development of symptoms in the host plant (Boyd *et al.*, 1987). The identification of markers linked to *Rhynchosporium* resistance genes would therefore be very useful to barley breeders. Recently, Michelmore *et al.* (1991) have described a 'bulked segregant' method which is not dependent on the availability of near-isogenic lines to identify RAPD markers linked to downy mildew resistance genes in lettuce. We have used a series of near-isogenic lines together with bulked segregant analysis to identify RAPD markers linked to genes determining *Rhynchosporium* resistance in barley.

Materials and methods

Plant material

The donor and recurrent parents used to create the near-isogenic lines are given in Table 1. Doubled haploid material was regenerated from a cross between the spring barley cultivar Blenheim (B) (*Rhynchosporium*-susceptible) and the SCRI breeding line E224/3 (E) (*Rhynchosporium*-resistant). Methods used to regenerate plants from microspores are given by Finnie *et al.* (1989). A total of 59 replicated doubled haploid families, together with their parents, were scored on a percentage leaf area infected basis for *Rhynchosporium* resistance at GS71 in disease nurseries that carried Octal race 14 which possesses *Rhynchosporium* virulence factors 3 (Athene) and 4 (Igri) but not virulence factor 5 (La Mesita) (Newton, personal communication). The scores ranged from 0 to 75 per cent and comparison of parental and control scores showed that ≤ 15 per cent represented the presence of major *Rhynchosporium* resistance gene(s).

RAPD analysis

Total genomic DNA was isolated from fresh leaf material of single plants by a modification of the method of Saghai-Maroof *et al.* (1984). Aliquots of DNA (5 μg) from five doubled haploid lines representing the two extremes of the distribution of *Rhynchosporium* resistance and alternate alleles at the SC10-65-H400 marker locus were combined to

Table 1 Parents used to create near-isogenic lines together with the number of backcrosses (BC)

Donor parent	Recurrent parent	Gene	Reference
Modoc	Ingrid (BC 7)	*Rh2Rh2rh6rh6*	Habgood & Hayes (1971)
CI 8162	Ingrid (BC 8)	*Rh3Rh3*	Habgood & Hayes (1971)
La Mesita	Ingrid (BC 7)	*Rh4Rh4Rh10Rh10*	Habgood & Hayes (1971)
Abyssinia	Ingrid (BC 6)	*Rh9*	Bockelman *et al.* (1977)
E224/3		*Rh4Rh4Rh10Rh10*	B. Clifford (pers. comm.)
Blenheim		Susceptible	

produce two sets of bulked DNAs (see Results). PCR reaction mixtures (50 μl) contained approximately 100 ng genomic DNA, dATP, dCTP, dGTP and dTTP each at 100 μM final concentration, 200 nM primer, 1 × *Taq* polymerase buffer (10 mM Tris HCl, *p*H 8.8, 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.1 per cent non-ionic detergent) and 0.5 unit *Taq* XL polymerase (Northumbria Biologicals Ltd., Cramlington, U.K.). Each reaction was overlaid with 50 μl of mineral oil to prevent evaporation. The random sequence 10-mer primers used in this study were synthesized on an applied Biosystems 391 PCR mate oligonucleotide synthesizer or were obtained from Operon Technologies Inc., Alameda, CA. The nucleotide sequences of those generating products linked to *Rh* resistance are given in Table 2. Samples for enzymatic amplification were subjected to 45 repeats of the following thermal cycle: 1 min at 92°C, 3 min at 35°C and 2 min at 72°C. Fragments generated by amplification were separated according to size on 2 per cent agarose gels, stained with ethidium bromide and visualized by illumination with ultraviolet light (312 mm). The notation used for primer products is the same as in Chalmers *et al.* (1992) with the prefix H (for barley) before the molecular size of the amplification product.

RFLPs

Total genomic DNA from the doubled haploid population was digested with a range of restriction enzymes, and the fragments separated by electrophoresis through 1 per cent agarose gels. Southern blots of these gels were sequentially hybridized to a set of barley chromosome specific RFLP probes as described previously (van de Ven *et al.*, 1990).

Genetic analysis

Segregating markers were scored for each of the doubled haploid lines as either B (Blenheim homozygotes) or E (E224/3 homozygotes) and linkage analysis performed using MAPMAKER software (Lander *et al.*, 1987). A logarithm of the odds ratio (LOD) score of 3.0 was established for linkage and Haldane's mapping function was used to convert recombination frequencies to map distances in centi-Morgans.

Table 2 Nucleotide sequence of primers used to amplify markers linked to a *Rhynchosporium* resistance locus

Primer designation	Sequence
OPR3	5′ ACACAGAGGG 3′
OPJ7	5′ CCTCTCGACA 3′
OPH16	5′ TCTCAGCTGG 3′
OPD8	5′ GTGTGCCCCA 3′
OPO6	5′ CCACGGGAAG 3′
OPO16	5′ TCGGCGGTTC 3′
OPH7	5′ CTGCATCGTG 3′
SC10-65	5′ CAGGGGTGAT 3′

Results

Near-isogenic lines and the putative association of RAPD markers with Rhynchosporium *resistance*

DNA from a pair of near-isogenic lines (CI 8162X and cv. Ingrid) and the *Rhynchosporium*-resistant donor accession (CI 8162), was used as the template for the amplification of RAPD markers with a total of 300 random 10-mer oligonucleotide primers. On average, each primer generated five amplification products which were considered to represent distinct genetic loci. Sixty primers detected polymorphism (approximately one polymorphic band per primer) between the donor and the recurrent parent. One product, SC10-65-H400 was reproducibly generated from both CI 8162 and CI 8162X but not from Ingrid. SC10-65-H400 was therefore considered to be putatively linked to the *Rhynchosporium* resistance gene *Rh3* derived from CI 8162.

Two approaches were used to confirm this association. First, a further three pairs of *Rhynchosporium*-resistant near-isogenic lines and their donor parents were examined for the presence of SC10-65-H400. Figure 1 shows that in addition to CI 8162X, the Modoc and La Mesita derived near-isogenic lines supported the generation of SC10-65-H400 while the

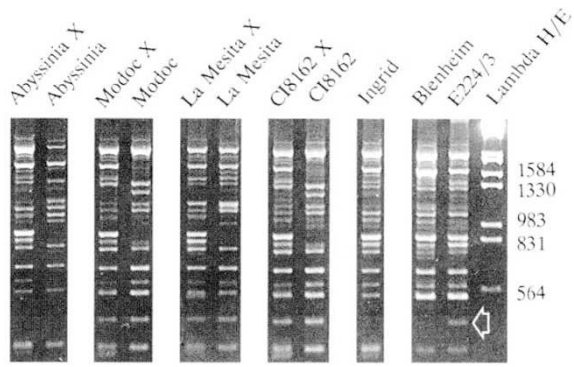

Fig. 1 RAPD products generated from four sets of near-isogenic lines, their recurrent parent Ingrid and the donor parents CI 8162, Modoc, La Mesita and Abyssinia with the primer SC10-65. The doubled haploid parents Blenheim and E224/3 are also shown. The position of SC10-65-H400 is indicated (arrow).

Abyssinian near-isogenic line did not. CI 8162, Modoc and La Mesita all possess *Rhynchosporium* resistance genes located on barley chromosome 3 (Dyck & Schaller, 1961b; Habgood & Hayes, 1971) and the presence of SC10-65-H400 in their near-isogenic lines suggests that this marker is physically linked to the loci on chromosome 3. The Abyssinia parent is known to possess a different *Rhynchosporium* resistance gene (*Rh9*) which is located on barley chromosome 4 (Bockelman *et al.*, 1977) and this supports the conclusion that SC10-65-H400 is linked to the *Rhynchosporium* resistance locus on chromosome 3. The second approach was based on the use of doubled haploid populations which had been previously scored for *Rhynchosporium* resistance (see Materials and Methods). SC10-65 detected polymorphism between Blenheim and E224/3 and SC10-65-H400 was transmitted from the *Rhynchosporium*-resistant parent (E224/3) to the doubled haploid progeny (Fig. 2). Furthermore, SC10-65-H400 exhibited significant linkage to the *Rhynchosporium* resistance locus in the doubled haploid populations ($\chi_{(1)}^2 = 7.51$, $P < 0.01$). The recombination distance between SC10-65-H400 and the *Rhynchosporium* resistance locus was estimated to be 34.7 cM.

Bulked segregant analysis

The loose linkage identified between SC10-65-H400 and *Rh* prompted us to use the presence or absence of this marker and the *Rhynchosporium* resistance phenotype to create two pools of DNA, each containing equal amounts of DNA from five individual doubled haploid genotypes. The composition of the pools consisted of either *Rhynchosporium*-resistant lines (E224/3 allele) together with SC10-65-H400 or *Rhynchosporium*-susceptible lines (Blenheim allele) lacking SC10-65-H400. A total of 700 primers were used to screen the two bulks and parents. Seven primers reproducibly generated products which were polymorphic between the bulked DNA samples and were derived from the *Rhynchosporium*-resistant parent E224/3. An example of the polymorphism detected between the bulks and the parents with one primer (OPD8) is shown in Fig. 3(a).

Linkage between the RAPD markers and the *Rhynchosporium* resistance locus was confirmed by monitoring the segregation of each informative primer product in the doubled haploid population. An example of the segregation of OPD8-H400 is shown in Fig. 3(b). Significant linkage between the *Rhynchosporium* resistance phenotype and the RAPD markers was detected.

Chromosome arm location of the Rhynchosporium *locus*

Several *Rhynchosporium* resistance genes have been described in barley and five alleles or pseudo alleles have been previously located to chromosome 3H (Habgood & Hayes, 1971) with *Rh, Rh3* and *Rh4* being clustered on the short arm (Takahashi, 1983). The linkage group representing the chromosomal location of the *Rhynchosporium* resistance locus described here was determined in the B × E cross by monitoring the co-segregation of the resistance phenotype and identified RAPD markers with a set of chromosome arm specific RFLPs. CDO1174, an RFLP probe previously mapped to the short arm of chromosome 3 (Heun *et al.*, 1991) co-segregated with *Rhynchosporium* resistance and the linked RAPD markers. However, the polymorphism detected with CDO1174 was linked to a number of other RFLP probes previously mapped to the long arm of chromosome 3 (WG940, WG110, BCD131, BD131) and an RFLP generated by CDO64 previously mapped to chromosome 2H. Wheat/barley ditelosomic addition lines were therefore used to confirm the location of the CDO1174 and CDO64 alleles segregating in the Blenheim × E224/3 cross. In both cases the segregating bands were located on chromosome 3HL (P. Jack & V. Lea, unpublished data). CDO1174 therefore probably identifies two distinct genetic loci, one on each arm of barley chromosome 3H and CDO64 a locus on both 2H and 3H. The order of the loci around *Rh* was investigated using MAPMAKER (Lander *et al.*, 1987).

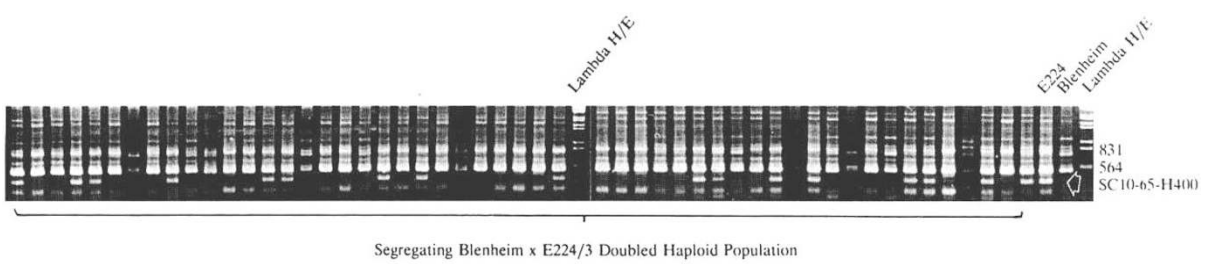

Fig. 2 Segregation of SC10-65-H400 in a Blenheim × E224/3 derived doubled haploid population.

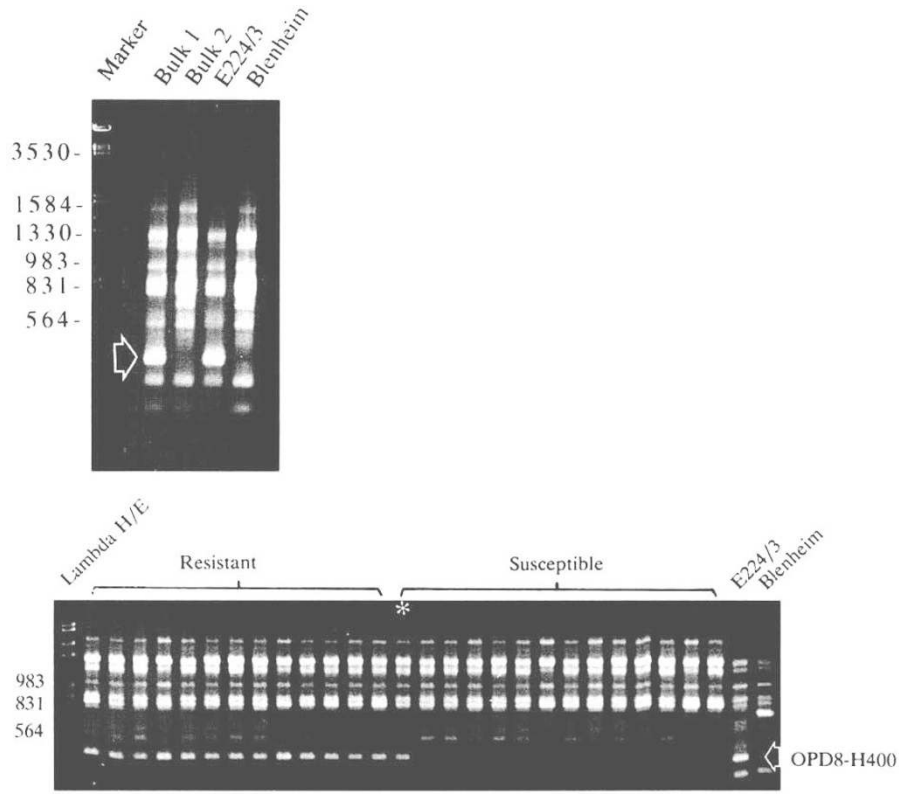

Fig. 3 (a) RAPDs from resistant (R) and susceptible (S) pooled DNA samples and from the doubled haploid parental lines Blenheim and E224/3 by primer OPD8. OPD8-H400 is indicated with an arrow. (b) Segregation of OPD8-H400 in the B × E derived doubled haploid population. Lane* is a susceptible recombinant line containing OPD8-H400.

This produced maps with similar LOD scores but different ordering of a subset of the loci (CDO1174, OPR3-H550, OPJ7-H1000, OPH16-H500 and OPD8-H500). The most likely map is shown in Figure 4 and covers a region of barley chromosome 3HL totalling 33.6 cm; approximately the same as the pairwise distance between *Rh* and the most distal marker SC10-65-H400.

Discussion

The identification of markers linked to a target genetic locus has routinely involved the construction of genetic linkage maps from appropriate segregating populations (Barone *et al.*, 1990; Kleinlankhorst *et al.*, 1991; Messeguer *et al.*, 1991) or identifying differences between near-isogenic lines (Young *et al.*, 1988; Sarfatti *et al.*, 1991; Hinze *et al.*, 1991). In the majority of cases the experimental procedure usually involves RFLP analysis. RAPDs (Williams *et al.*, 1990; Welsh & McClelland, 1990; Waugh & Powell, 1992) provide an alternative which can substantially reduce the time and effort required to identify markers linked to a target gene. The keys to the success of RAPDs are the ability of individual primers to detect multiple loci and the ease with which a large number of primers can be screened in a single run on a thermal cycler. In this manuscript we have used RAPDs in conjunction with

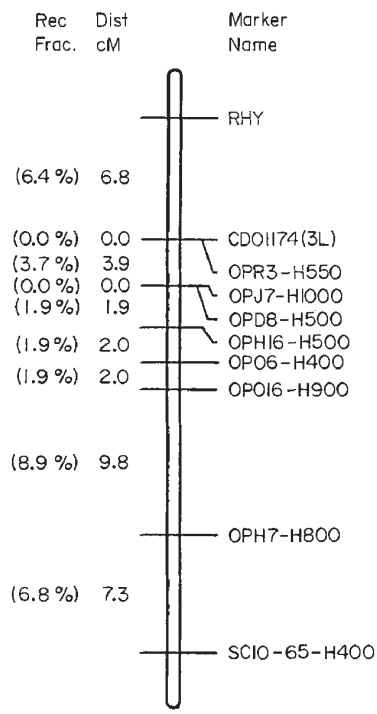

Fig. 4 Linear order of RAPD markers along the long arm of barley chromosome 3HL as generated by MAPMAKER. CDO1174 is an RFLP marker specific to chromosome 3HL.

near-isogenic lines and bulked segregant analysis to identify markers linked to a gene(s) determining resistance to *Rhynchosporium secalis*. In addition, previously mapped RFLP probes were used to assign the location of this resistance gene complex to the long arm of chromosome 3H. The significance of these findings is first, linked markers may be used to increase the efficiency of manipulating *Rhynchosporium* resistance genes particularly when considering gene introgression from *H. spontaneum* into adapted *H. vulgare* germplasm. Secondly, the concept of 'gene pyramiding' could be pursued when more than one resistance gene could be assembled in a single genotype.

The barley land races used to construct the near-isogenic lines used in this study have been classified as having different *Rhynchosporium* resistance genes on the basis of a complex series of crosses, and resistance testing with several *R. secalis* isolates (Habgood & Hayes, 1971). However, although their reaction to the different isolates was discriminatory, the major dominant resistance genes (with the exception of Abyssinia) were considered either allelic or tightly linked on the short arm of barley chromosome 3H (Takahashi, 1983). The breeding line E224/3 is the product of a cross between the resistant barley cv. Sergeant and the susceptible cv. Cytris. The origin of the resistance in Sergeant is derived from the USDA barley accession CI8256 which was classified as having the same resistance phenotype as La Mesita (*Rh*4*Rh*4*Rh*10*Rh*10) by Habgood & Hayes (1971). Generation of the same RAPD markers in both the near-isogenic lines and Blenheim × E224/3 double haploid populations is therefore consistent with available pedigree information but not with the previous map information. Takahashi (1983) presented a map of chromosome 3 with the *Rh4* locus on the short arm of chromosome 3H. Tsuchiya (1981) had previously reversed the arms based on new information from telotrisomic lines. This resulted in genes which had previously been assigned to the long arm of chromosome 3H (including *Rh4*) being assigned to the short arm. Our results suggest that the pre-1983 location of *Rh4* was correct. These results have implications for the other genes located on chromosome 3. However, as noted, the exact location of barley centromeres is unknown and the assignment of genes to chromosome arms is difficult (Søgaard & Wettstein-Knowles, 1987).

Recently, Hinze *et al.* (1991) screened a series of six near-isogenic lines (which had undergone seven backcrosses) selected for resistance to powdery mildew (*ml-o* locus) with 1100 RFLP probes and identified five markers which spanned an 8.6 cM interval on barley chromosome 4. The unexpectedly small size of the introgressed fragment defined by these markers was attributed to 'linkage drag' being lower than predicted from the theoretical estimates of Stam & Zeven (1981). In contrast to these findings, Young & Tanksley (1989) found that repeated cycles of backcrossing and selection were ineffective in reducing the size of the DNA segment flanking the *Tm-2* resistance gene introgressed from *L. peruvianum* into *L. esculentum*. These results concur with the hypothesis of Rick (1969) that there may be reduced opportunities for recombination in genotypes heterozygous for a segment originating from distantly related species. The intervarietal nature of the *ml-o* donor and recurrent barley parents may therefore be an explanation for the small size of the introgressed fragment reported by Hinze *et al.* (1991). The estimated minimum distance between the most distal marker, SC10-65-H400, and the *Rh* locus based on pairwise distances (34.7 cM in the B × E cross) is consistent with the theoretical predictions of Stam & Zeven (1981) assuming a total length of 200 cM for barley chromosome 3 (Heun *et al.*, 1991; Graner *et al.*, 1991). Maintenance of a DNA segment in the order of the predicted size may reflect the intermediate level of divergence between the land race donor and cultivated recurrent parents used to construct the near-isogenic lines.

Martin *et al.* (1991) used RAPDs and near-isogenic lines to identify markers linked to the *pto* locus (giving resistance to *Pseudomonas syringae*) in tomato. Less than 150 primers were required to isolate seven markers, three of which were confirmed by segregation analysis to be linked to the target locus. The substantially larger number of primers required in this study to isolate a single marker presumably reflects the low level of useable polymorphism detected by RAPDs in barley. Only 60 polymorphic products were detected between the near-isogenic line donor CI 8162 and the recurrent parent Ingrid after screening approximately 1500 genetic (RAPD) loci (300 primers, 5 loci per primer), reflecting a 4 per cent level of polymorphism. Assuming the barley genome is 1500 cM (Graner *et al.*, 1991; Heun *et al.*, 1991), by screening 60 randomly distributed markers the expected minimum distance between a marker and a target locus would be 12.3 cM increasing to 36.6 cM at the 95 per cent confidence limit (Martin *et al.*, 1991). The 34.7 cM map distance between SC10-65-H4O0 and the *Rh* locus in E224/3 is therefore consistent with those predictions. Thus, even when the level of polymorphism is low (4 per cent in the case of barley) the combination of RAPDs and near-isogenic lines was an effective means of identifying markers linked to a target gene and should also be an attractive strategy in other crops such as wheat where the inherent level of molecular polymorphism is also low.

Michelmore *et al.* (1991), using bulked segregant analysis, required only 300 PCR reactions to identify

seven markers linked to a downy mildew resistance locus (*Dm5/8*) in lettuce. Giovannoni *et al.* (1991) extended this approach in an attempt to saturate regions of the tomato genome containing genes responsible for pedicle abscission and fruit ripening which had previously been defined by flanking RFLP markers. By screening two genetic intervals of 6.5 cM and 15 cM with up to two hundred 10-mer primers, three linked markers were identified. However, only one marker mapped within the borders of the regions defined by RFLPs, one was 3 cM outside and the other was 45 cM away. The distance of the latter marker from the defined interval was attributed to unintentional skewing of a second genetic interval in conjunction with the competition for primer binding sites which occur in different ratios within the two DNA pools. In the present study, the genetic interval was defined firstly by the phenotype of the character (reaction to *Rhynchosporium*) and secondly by the presence or absence of SC10-65-H400 which was identified in the near-isogenic lines. Seven products were detected which differed between the bulks and were subsequently shown by segregation analysis to be linked to the resistance locus from E224/3. All of the markers identified mapped within the borders defined by SC10-65-H400 and the *Rh* locus.

The mapping of resistance genes based on well-characterized homozygous, genetically fixed doubled haploid barley populations (Powell *et al.*, 1986, 1990; Thompson *et al.*, 1991) is attractive. Although doubled haploids and bulked segregant analysis were used for the identification of markers linked to a major gene, a more significant impact of this approach will probably be in dissecting polygenically inherited characters. We have used doubled haploids, bulked segregant analysis and RAPDs to isolate markers linked to genetic factors contributing to the milling energy (ME) of the barley grain (Chalmers *et al.*, submitted). The availability of other doubled haploid populations and genetic linkage maps (Heun *et al.*, 1991; Graner *et al.*, 1991; Kleinhofs, 1992) should expedite the mapping and dissection of a wide range of major genes, and quantitative traits in cultivated barley. In conclusion, RAPDs currently provide the most efficient and cost effective means of isolating molecular markers linked to genes located on introgressed DNA segments. Two major future objectives will be the exploitation of RAPDs in marker-assisted breeding programmes and the isolation of target genes via map-based cloning strategies.

Acknowledgements

We thank J. I. Young and A. Wilson for assisting in the *Rhynchosporium* scores, and P. Jack and Plant Breeding International, Cambridge for the RFLP data. The work was funded by the Scottish Office Agriculture and Fisheries Department.

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