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Analysis of quantitative traits in barley by the use of Amplified Fragment Length Polymorphisms

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Amplified fragment length polymorphisms (AFLPs) produced with *Eco*RI and *Pst*I both in combination with *Mse*I restriction enzymes have been studied in the parents of four barley mapping populations. Averages of 15.9 and 18.7 polymorphic products per assay were produced for the *Eco*RI/*Mse*I and *Pst*I/*Mse*I combinations, respectively. There was some evidence of interaction between cross combinations and restriction enzyme combinations, with *Pst*I/*Mse*I generating relatively more polymorphic products than *Eco*RI/*Mse*I in the Blenheim × E224/3 cross combination, the least polymorphic of the four. Three hundred and ninety-eight AFLP products, using both restriction enzyme combinations, were generated in a doubled haploid population of 68 lines produced from the Blenheim × E224/3 cross. These were added to existing marker data for the cross to study the effects of incorporation of AFLPs produced by different restriction enzyme combinations upon genetic maps. Addition of the AFLP data resulted in greater genome coverage, both through linking previously separate groups and extensions to other groups. This increase in coverage appeared to result from AFLPs sampling some different regions of the genome compared to RAPDs and RFLPs, as the map distances spanned by the RAPD and RFLP linkage groups were similar with and without incorporation of AFLPs. There was also evidence that the *Eco*RI and *Pst*I restriction enzymes sampled different regions of the genome. The revised maps were used in scanning for QTLs controlling a subset of 12 economically important traits measured in the cross. Overall, the QTLs accounted for an average of 53 per cent of the phenotypic variation for the characters. Positive and negative alleles were present in each parent for each character, apart from hot water extract corrected to 1.5 per cent nitrogen (HWEc). Several regions of the genome appeared to be involved in the control of several characters, notably chromosome 2, the *denso* locus on chromosome 3, the short arm of chromosome 5 and chromosome 7. Although there was considerable similarity to previous results of QTL mapping for the subset of characters, the greater genome coverage afforded by the inclusion of the AFLPs revealed some new QTL locations.

Keywords: AFLPs, barley, genes, mapping, markers, QTL.

Introduction

The repertoire of genetic marker systems available for genome analysis has increased considerably and falls into two main technological categories: assays based on hybridization and on amplification (Rafalski *et al.*, 1996). Hybridization-based methods have been dominated by Restriction Fragment Length Polymorphism (RFLPs; Botstein *et al.*, 1980) and this technology has been widely deployed in plants (Helentjaris & Burr, 1989). However, the RFLP assay is time consuming and labour intensive. The development of PCR (Mullis *et al.*, 1986) has expanded the range and efficiency of amplification marker systems available. These include: Randomly Amplified Polymorphic DNA (RAPDs; Williams *et al.*, 1990; Welsh & McClelland, 1990), Simple Sequence Repeat Polymorphisms or microsatellites (SSRPs; Tautz, 1989; Weber & May, 1989) and Amplified Fragment Length Polymorphisms (AFLPs; Zabeau

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& Vos, 1993). Two important aspects of a marker system's utility are: information content and multiplex ratio (Powell *et al.*, 1996). Standard measures of diversity may be used to evaluate information content and the multiplex ratio is the number of loci simultaneously analysed per experiment. These two metrics have been used to compare RFLPs, AFLPs, SSRPs and RAPDs in common soybean and barley genotypes (Powell *et al.*, 1994, 1995, 1996). Multiplex ratio and the diversity index were combined to provide an overall measure of marker utility defined as the Marker Index. To date, all comparative studies concur in identifying AFLP as an unique technology with high marker utility arising mainly from its high multiplex ratio.

The AFLP approach has recently been used to identify markers tightly linked to disease resistance loci (Meksem *et al.*, 1995; Thomas *et al.*, 1995a), to fingerprint plant and bacterial genomes (Lin & Kuo, 1995) and to examine genepool variation in potato cyst nematode populations (Folkertsma *et al.*, 1996). The power of AFLP technology to create rapidly linkage maps in a doubled haploid population of barley has also been recently demonstrated (Becker *et al.*, 1995).

The AFLP approach relies on the selective amplification of small genomic restriction fragments, produced by double digestion with a rare and a frequent cutting restriction enzyme, into which known sequence adaptors have been ligated. As with RFLP, it is reasonable to assume that different restriction enzymes may show different abilities to detect/reveal polymorphism and/or that the polymorphism detected may show a biased distribution throughout the genome. Despite this, the majority of studies published to date have used a combination of *Eco*RI and *Mse*I restriction enzymes and a relatively small number of primers. The objectives of the present study were therefore to: (i) identify informative protocols (enzymes/primers) for identifying polymorphic AFLP products for barley; (ii) compare the genome distribution of *Eco*RI/*Mse*I and *Pst*I/*Mse*I generated AFLP products in comparison with RFLP and RAPD loci; and (iii) examine the utility of AFLP mapping procedures to characterize polygenic, quantitative traits in barley.

Materials and methods

DNA isolation

Genomic DNA was isolated from fresh leaf material of Blenheim, E224/3 and 68 DH produced from the F_1 of the cross between them by a modification of the CTAB method of Saghai-Maroof *et al.* (1984). The DNA was assessed, quantified and used for AFLP analysis without further purification. DNA from Igri and Franka was supplied by Andreas Graner, Grünbach, Germany. Dicktoo and Morex DNA was supplied by Pat Hayes, Oregon State University, USA.

AFLP analysis

AFLP methodology was essentially as described by Zabeau & Vos (1993) with minor modifications. Template DNA was prepared using two combinations of restriction enzymes. Each combination consisted of a pair of enzymes, one of which cut DNA rarely (*Pst*I or *Eco*RI) and the other cutting frequently (*Mse*I). Genomic DNA (1.25 μg) was digested as outlined by Vos *et al.* (1995) and specific double-stranded adaptors were ligated to the fragment ends. The digested and ligated DNA was then preamplified using either an *Eco*RI or *Pst*I directed primer and an *Mse*I directed primer. The primers did not have additional selective nucleotides at the 3′ end (Vos *et al.*, 1995). Adaptor and preamplification primer sequences are given in Table 1. All adaptors and primers used were synthesized by Genset, France.

Preamplification was performed in a total volume of 25 μL containing 75 ng each of primers M00 and either E00 or P00, 0.2 mM of all four dNTPs (Pharmacia), 1 × PCR buffer (Perkin Elmer Cetus), 1 U Amplitaq DNA polymerase LD (Perkin Elmer Cetus) and 30 ng of the digested and ligated DNA.

The cycle profile used for preamplification was as follows; denaturation for 30 s at 94°C, annealing for 30 s at 60°C, extension for 60 s at 72°C, for 30 cycles. After preamplification the product was diluted by the addition of 55 μL of buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA). Once diluted the preamplification product was used as a template for selective amplification. Selective amplification was carried out using adaptor directed primers with additional selective nucleotides. The primer combinations and their sequences are listed in Appendix I. In each case the *Eco*RI or *Mse*I primer was end labelled using [γ^{33}P] ATP and T4 polynucleotide kinase (Gibco) as described by Vos *et al.* (1995). The selective amplification reactions were carried out in a total volume of 20 μL, comprising 6.7 ng labelled *Eco*RI or *Pst*I primer, 25 ng unlabelled *Eco*RI or *Pst*I primer, 30 ng *Mse*I primer, 0.2 mM of all four dNTPs, 1 × PCR buffer (Perkin Elmer Cetus), 0.5 U Amplitaq DNA Polymerase (Perkin Elmer Cetus) and 2 μL of template DNA. Reactions were carried

Table 1 Adaptor and preamplification primer sequences

Primer		Sequence
*Eco*RI	Forward adaptor	5′-CTCGTAGACTGCGTACC
*Eco*RI	Reverse adaptor	5′-AATTGGTACGCAGTC
*Pst*I	Forward adaptor	5′-CTCGTAGACTGCGTACATGCA
*Pst*I	Reverse adaptor	5′-TGTACGCAGTCTAC
*Mse*I	Forward adaptor	5′-GACGATGAGTCCTGAG
*Mse*I	Reverse adaptor	5′-TACTCAGGACTCAT
E00	Preamplification	5′-GACTGCGTACCAATTC
M00	Preamplification	5′-CATGAGTCCTGAGTAA
P00	Preamplification	5′-GACTGCGTACATGCAG

out using the cycle profile described by Vos *et al.* (1995), i.e. one cycle of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s, followed by 11 cycles over which the annealing temperature is decreased by 0.7°C per cycle, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

All PCR reactions were performed using a Perkin Elmer 9600 thermocycler. Reactions were stopped by the addition of an equal volume of formamide loading buffer (98 per cent formamide, 10 mM EDTA pH 8, 0.5 mg/mL Bromophenol Blue, 0.5 mg/mL Xylene Cyanol FF). The samples were denatured at 90°C for 5 min, 3.5 μL of each sample was loaded on to a 40 cm, 6 per cent denaturing polyacrylamide gel (Easigel, Scotlab) which had been preheated by running at 80 W for 30 min. The samples were then electrophoresed at a constant power of 80 W for 1 h 45 min. Gels were transferred to Whatman 3 MM paper and dried for 2 h at 80°C on a gel drier (Biorad). They were then exposed to autoradiographic film (X-OMAT S, Kodak) to visualize the results.

Results were scored twice, independently, as presence/absence of a given band.

Linkage map construction

Linkage groups for the Blenheim × E224/3 DH population were formed using JOINMAP 2.0 (Stam & Van Ooijen, 1995) with a LOD of 6.0 to form groups and ignoring previous information about linkage groups and orders (Thomas *et al.*, 1995b). Groups were merged where they were known to be separate segments of a chromosome and mapping was carried out with a LOD of 0.05 and a JUMP (Stam & Van Ooijen, 1995) which varied between 2.8 and 5.0 according to the group. A ripple was performed after the addition of every three markers and the robustness of the ordering was tested by raising the mapping LOD to 0.5. Map distances were calculated using the Kosambi function. JOINMAP 2.0 constructs maps in three cycles (Stam & Van Ooijen, 1995). In the first cycle, markers which cause the JUMP threshold to be exceeded are excluded until the program has attempted to include all the markers in the group. In the second cycle the program attempts to insert these markers but with the same restriction that if the JUMP threshold is exceeded, they are excluded. In the third cycle markers excluded after the second cycle are inserted in the map but with no restrictions on the JUMP threshold. As the positioning of markers inserted in the third cycle generally gave large rises in the JUMP value, the ordering produced by the second cycle was taken.

Plant material

Fifty-nine of the Blenheim × E224/3 DH lines were grown in replicated trials at the Scottish Crop Research Institute (SCRI) in the years 1989–92. In addition, replicated trials were grown at Plant Breeding International, Cambridge (PBIC) in 1992 and 1993. A number of characters were measured either on the plots or on samples of seeds harvested from the plots (Thomas *et al.*, 1995b, 1996). In this paper, we will consider a subset of those characters (Table 2).

Data analysis

QTL analysis was carried out using MQTL 0.95 with significance thresholds being established for an experimental error rate of 0.05 by 1000 random permutations of the data for each character (Tinker & Mather, 1995). MQTL searches for QTLs using both simple interval mapping (SIM) and simplified compound interval mapping (sCIM). In the latter,

Table 2 Quantitative traits studied

Symbol	Character	Units	Description
HD	Heading date	Days	From 31 May
Ht	Height	cm	Ground to collar
PY	Plot yield	t/ha	Weight of harvested plot @ 13 per cent moisture
TGW	1000 grain weight	g	(Weight of 100 seed) × 10
SPW	Specific weight	kg/hL	(Weight (g) of 1 L seed)/10
>25	Sievings > 2.5 mm	per cent	(Sample weight (g) passing over sieve × 100)/total sample weight (g)
GE4	Germinative energy	per cent	Numbers of seeds from a sample of 100 germinating in 4 mL after 72 h
1GE8	Water sensitivity	per cent	Numbers of seeds from a sample of 100 germinating in 8 mL after 72 h
ME	Milling energy	J	Energy required to mill 5 g grain sample
GN	Grain nitrogen	per cent	Near-infrared reflectance or combustion analysis of nitrogen in the grain
DP	Diastatic power	per cent	Percentage β-amylase activity in milled sample
HWEc	Hot water extract	L°/kg	Hot water extract of malted grain corrected to 1.5 per cent GN

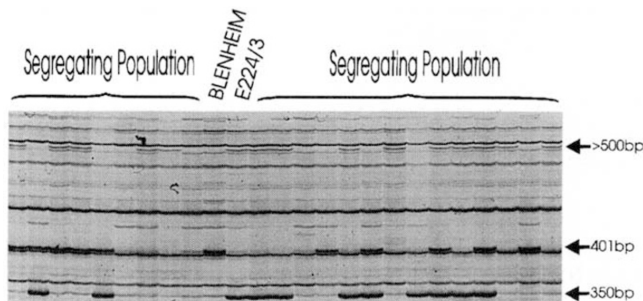

Fig. 1 Segregation of AFLP loci in a doubled haploid population of barley. Template DNA was prepared from *Eco*RI/*Mse*I digests.

background markers are used to account for possible variation in regions of the genome other than that under test which can refine the location of QTLs revealed by SIM or reveal additional QTLs (Tinker & Mather, 1995). Some of the mapped markers from Fig. 1 were dropped from the analysis if they were within 1 cM of each other. A total of 222 markers was included in the analysis, 25 of which were background markers. All the background markers were located on the larger chromosome segments and at an approximate spacing of 35 cM. The *denso* locus was deliberately chosen as one of the background markers as previous studies had highlighted its importance in the control of many quantitative traits (Thomas *et al.*, 1991, 1995b, 1996). 'Primary' QTL locations were established where peaks for SIM and sCIM coincided (Tinker & Mather, 1995). In addition, 'secondary' QTL locations (Tinker *et al.*, 1996) were also established by including effects where the SIM or the sCIM peak was at least equivalent to an increase of LOD 1.0 in the Test Statistic and, in analyses of single environments, the peak exceeded the sCIM threshold at least once.

Results

Comparison of restriction enzymes and primer combinations

Data for the total number of amplification products, number of polymorphic products and the proportion of polymorphic products are given for each primer combination in Appendix I. A total of 39 *Eco*RI/*Mse*I and 18 *Pst*I/*Mse*I combinations were evaluated. Within each restriction enzyme category there are significant differences ($P<0.001$) between primer pairs for the total number of amplification products generated. The number of polymorphic products differs significantly ($P<0.05$) for the *Eco*RI/*Mse*I primer pairs. For each of the three parameters measured there are differences between crosses (Tables 3a,b). There are significant differences between restriction enzymes (*Eco*RI/*Mse*I vs. *Pst*I/*Mse*I) for the number and proportion of polymorphic products. Furthermore there is a statistically significant interaction ($P<0.05$; Table 3b) between restriction enzyme and crosses for the proportion of polymorphic products detected. This is manifested in the different response of Blenheim × E224/3 and Igri × Franka template DNA compared to that of the other crosses to *Pst*I/*Mse*I primers (Table 3a).

Map construction

An example of segregating AFLP products in the Blenheim × E224/3 DH population obtained from *Eco*RI/*Mse*I template DNA is given in Fig. 1. A total of 398 polymorphic AFLP markers were obtained,

Table 3a A comparison of different restriction enzyme combinations in the parents of four barley mapping populations

	Total number of amplified products		Number of polymorphic products		Proportion of polymorphic products	
	*Eco*RI/*Mse*I	*Pst*I/*Mse*I	*Eco*RI/*Mse*I	*Pst*I/*Mse*I	EcoRI/*Mse*I	*Pst*I/*Mse*I
Blenheim × E224/3	92.3 ± 3.90	88.8 ± 6.01	8.5 ± 1.03	13.7 ± 1.59	0.092 ± 0.0049	0.154 ± 0.0096
Dicktoo × Morex	102.4 ± 3.95	104.4 ± 6.42	21.9 ± 1.05	23.6 ± 1.70	0.213 ± 0.0067	0.226 ± 0.0109
Igri × Franka	90.8 ± 3.90	82.3 ± 6.42	10.8 ± 1.03	11.5 ± 1.70	0.119 ± 0.0055	0.138 ± 0.0102
Lina × *H. spontaneum*	90.0 ± 4.00	91.0 ± 6.66	22.4 ± 1.06	25.8 ± 1.76	0.248 ± 0.0076	0.283 ± 0.0131

Table 3b Analysis of variance for various parameters influencing the efficiency of AFLP assays in barley

	d.f.	Total number of amplified products MS	Number of polymorphic products MS	Proportion of polymorphic products M. deviance
Between crosses	3	2201.4 ***	2614.3***	167.5***
Between restriction enzymes	1	233.8	326.8***	28.2***
Crosses by restriction enzymes	3	237.0	42.0	6.5*
Error	198	577.1	40.4	2.6

*** $P<0.001$; ** $P<0.01$; *$P<0.05$.

236 using *Eco*RI and 162 using *Pst*I. The Blenheim allele was dominant for 105 of the *Eco*RI and 82 of the *Pst*I markers and a chi-squared test for the distribution of dominant alleles between the two enzymes showed no significant association ($\chi^2_1 = 1.45$; $P = 0.23$). Together with the 120 markers previously used for map construction (Thomas *et al.*, 1995b) in this population, 518 markers were now available for mapping. Thirty-eight of the markers were discarded, mainly because they either showed no significant linkage with any other marker at LOD 6.0 or formed a small linkage group of four or fewer markers. Nine of these were putative chromosome-specific RFLPs (PBI30x, PBI30y and PBI30z; PSR100 and PBI24; PSR1077, WG178 and ABG377; and ABG390 on chromosomes 1, 2, 3 and 7, respectively), 21 were AFLPs, six were RAPDs and two were isozyme markers. Fifteen individual linkage groups remained and it was possible to merge some of these by reducing the LOD to 3.0 resulting in 12 groups. These groups were assigned to chromosomes on the basis of chromosome-specific RFLPs. Two groups were ambiguous in that they contained two markers that had been previously assigned to different chromosomes. Thomas *et al.* (1995b) assigned a small segment consisting of two RAPDs and one RFLP (WG282) to chromosome 6b as WG282 was a chromosome 6 marker (Heun *et al.*, 1991). However, with the additional data, this group was found to contain a microsatellite within the Rubisco activase GenBank sequence HVRCABG which had previously been reported on chromosome 4 (Becker & Heun, 1995). As several of the AFLP products located in this group were also found to be present on chromosome 4 of the Dicktoo × Morex linkage map (unpublished data), this group was re-assigned to chromosome segment 4b. The other ambiguous group contained CDO36 which had been mapped to barley chromosome 1 (Heun *et al.*, 1991) and MWG546 which had been mapped to chromosome 3 (Graner *et al.*, 1991). Neither of these RFLPs showed any significant linkage with any other chromosome-specific RFLPs and none of the AFLPs in this group was segregating in other mapping populations. However, some of the AFLPs showed significant linkage at LOD 3.0 with markers in the region of PSR687 on chromosome 2, so this group has been tentatively assigned as chromosome segment 2b. In constructing the maps from these linkage groups, 129 markers were excluded after the second cycle. These comprised 109 AFLPs, 11 RAPDs, five RFLPs and four other markers, resulting in maps constructed from 268 AFLPs, 34 RAPDs, 38 RFLPs and 11 other markers.

Sixty-two markers covered 176.9 cM on chromosome 1 (Fig. 2). This arose from the joining of segments 1a, 1c and 1d of a previous study (Thomas *et al.*, 1995b) through the inclusion of AFLP

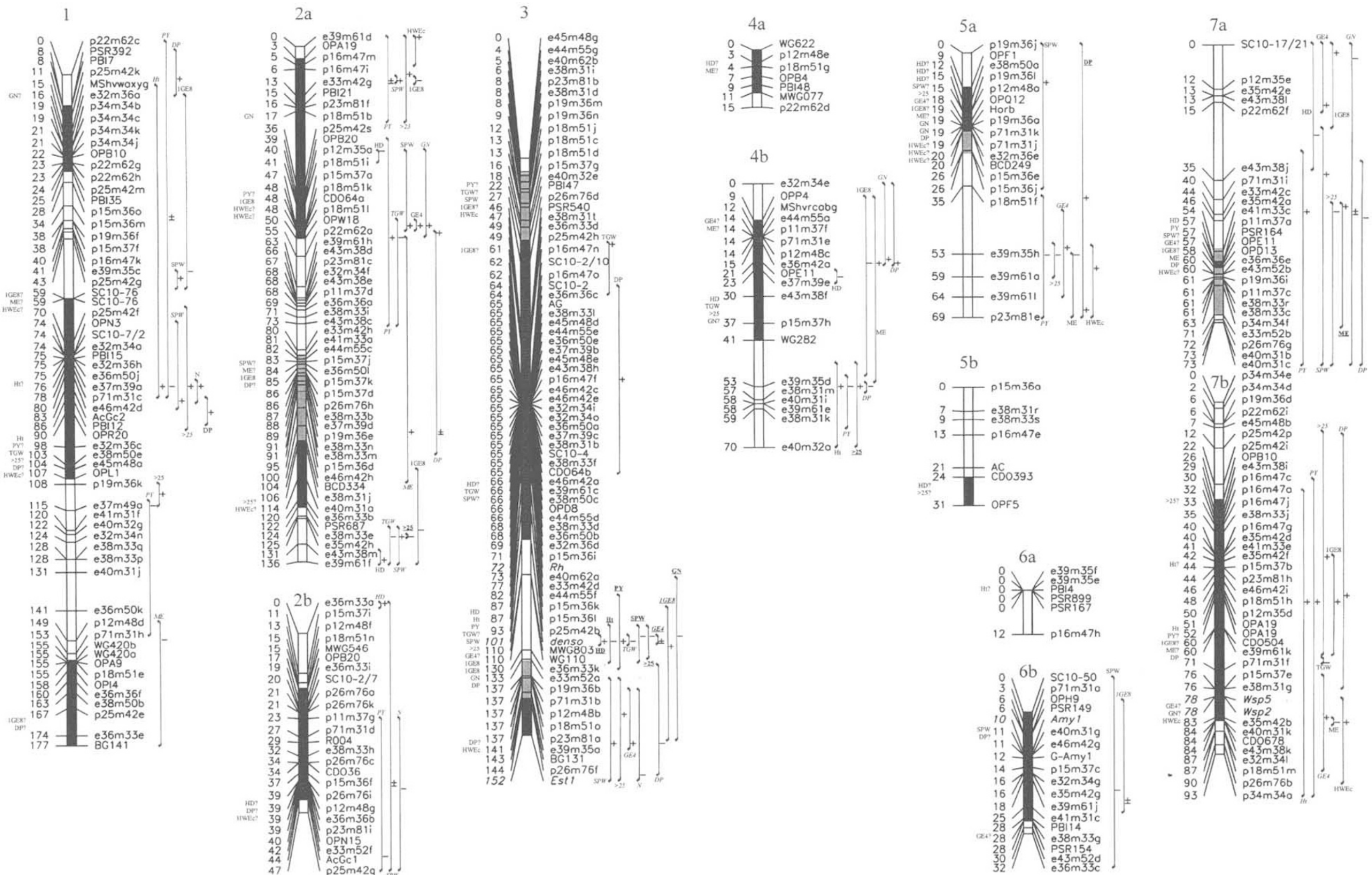

Fig. 2 Linkage map of barley, showing the distribution of different markers. Heavily and lightly shaded regions denote portions of the genome covered by maps produced largely from RAPDs and RFLPs (Thomas *et al.*, 1995b). The location of quantitative traits is also indicated. Arrowed lines indicate the marker regions over which the test statistic exceeded the threshold with a '+', '−' or '±' at the location of the test statistic peak and indicating the effect of the Blenheim allele upon the character. Interaction QTLs are denoted by italic type and primary QTLs are in bold type and underlined. Variate names to the left of chromosomes indicate the locations of QTLs from previous studies with a '?' denoting those significant in just one environment (Thomas *et al.*, 1995b, 1996).

markers. Similarly, chromosome segments 2a and 2b and 3b and 3c of Thomas *et al.* (1995b) were also joined to form segments 136.1 and 152.3 cM long, respectively, (Table 4; Fig. 2). A marker (SC10–17/21) from a previously unassigned segment (Ua) (Thomas *et al.*, 1995b) was linked to chromosome segment 7a with the AFLPs to form a segment 72.7 cM long (Fig. 2). The other marker in segment Ua was not linked to any other marker at LOD 6.0 and only linked with SC10–17/21 above LOD 4.0 and so was excluded from the linkage group. At LOD 3.0, there was no other bridging of the other groups previously reported by Thomas *et al.* (1995b) although some were extended (Table 4; Fig. 2).

Overall, the map covers 906.2 cM compared to under 700 cM constructed mainly with RAPDs and RFLPs (Thomas *et al.*, 1995b). However, the Haldane function was used to produce the latter map and, if the Kosambi function is used, the RAPD and RFLP map covers 502 cM (Table 4). The inclusion of AFLPs in the data set has produced bridges and extensions of 420 cM when compared to the previous map. Some of the markers used to construct the RAPD and RFLP maps were excluded after the second cycle of mapping by JOINMAP but if we include the results from the third cycle of mapping, we can directly compare the lengths spanned by the regions reported by Thomas *et al.* (1995b) and the present study (Table 4; Fig. 2). The map distances are very similar with or without inclusion of AFLPs (Table 4) so the greater genome coverage is unlikely to be caused by map extension in these regions. Fifty-two *Eco*RI and 43 *Pst*I AFLP markers were found in the bridges and extensions with 91 *Eco*RI and 82 *Pst*I markers in the previously mapped areas. These values are significantly different from those expected from a random distribution ($\chi_1^2 = 12.79$; $P = 0.005$) with fewer markers than expected in the bridges and extensions. However, there was no significant difference between the distributions of *Eco*RI and *Pst*I markers across the two regions ($\chi_1^2 = 0.11$; $P = 0.74$).

Microsatellites, isozymes, proteins, a *Rhynchosporium* resistance locus and a morphological marker were amalgamated into one group and compared to AFLP, RFLP and RAPD marker groups. The numbers for linkage groups smaller than 30 cM were summed and a contingency chi-squared calculated which showed no significant interaction between linkage groups and marker classes ($\chi_{30}^2 = 38.0$; $P = 0.15$). The distribution of AFLPs is therefore no different from the other markers. We can further classify the AFLPs into those produced with either *Eco*RI or *Pst*I and into those where the dominant product came from either Blenheim or E224/3. There was a 'borderline' significant contingency chi-squared for the distribution of the AFLPs produced by the two different enzymes across the 11 groups ($\chi_{10}^2 = 19.5$; $P = 0.04$). This was largely caused by discrepancies in the distribution of products in linkage groups 2b and 3, suggesting that the two enzymes can generate polymorphism in different regions of the genome. However, there was no difference in the origin of the dominant AFLP products over the 11 groups ($\chi_{10}^2 = 7.0$; $P = 0.73$).

Table 4 Distances (cM) spanned by markers mapped by Thomas *et al.* (1995b) ('old') and after addition of AFLPs in these ('previous') and new regions ('bridges and extensions')

		New		
Chr.[1]	Old	Previous	Bridges & extensions	Total
1	80.8	87.3	89.6	176.9
2a	84.4	87.4	48.7	136.1
2b	26.4	25.3	21.6	46.9
3b	117.5	116.1	36.2	152.3
4a	14.6	11.2	3.8	15.0
4b	34.4	31.7	37.8	69.5
5a	22.0	16.7	52.6	69.3
5b	12.8	7.5	23.9	31.4
6a	0	0	11.6	11.6
6b	28.8	28.3	3.3	31.6
7a	23.7	16.9	55.8	72.7
7b	56.2	58.3	34.6	92.9
Total	501.6	486.7	419.5	906.2

[1]Chr., chromosome.

Location of quantitative traits

Results from the MQTL analyses based on the complete mapping information are summarized in Table 5. Thirteen primary QTL inferences, 12 of which were main effects, and 77 secondary QTL inferences, 29 of which were main effects, were detected for the 12 characters. The main effects accounted for over 30 per cent of the phenotypic variation for nine of the characters. The average phenotypic variation accounted for when interactions were included was 53 per cent and the range was from 87 per cent for Ht to 22 per cent for HWEc. The percentages of phenotypic variation accounted for by the main effects and interactions were less than those detected in the previous studies for HD, Ht, TGW, 1GE8, GN and HWEc (Thomas

Table 5 Numbers of QTLs detected by MOTL using primary and secondary inferences for each character and the percentages of phenotypic variation accounted for by QTL main effects and QTL × E interactions compared to the average amount detected by single environment regression analysis (Thomas *et al.*, 1995b, 1996)

	Main effect QTLs				Interaction QTLs					Per cent phenotypic variation		
	Primary		Secondary		Primary		Secondary					
Character	+	−	+	−	+	±	+	−	±	Main effects	Main effects and interactions	Previous analysis
HD	1		1	3				1		37	53	72
Ht		1	1				2			83	87	90
PY	1						3	5	2	52	66	64
TGW			1	1				3		32	36	44
SPW		1	3	1			4		2	44	57	53
>25	1	2	1				4	2		43	57	57
GE4			2			1	3			16	42	30
1GE8			2	4	1		1			24	45	59
ME	1		1	2			1	1		47	50	48
GN		1	1				2	3	1	50	58	69
DP	2		1	1			4	3	1	35	63	58
HWEc			3							11	22	58
Total	6	5	17	12	1	1	24	18	6			

+ and − indicate the effect of alleles from Blenheim upon a trait. ± indicates a cross-over interaction where significant effects from Blenheim were positive in one environment and negative in another.

et al., 1995b, 1996). For the remainder, the percentages were the same or slightly greater, apart from GE4, where considerably more variation was accounted for by sCIM analysis (Table 5). The average Test Statistic for main effects was permuted to be equivalent to LOD 7.7, ranging from values equivalent to LOD 4.1 for GE4 to LOD 10.9 for Ht. For QTL × E interactions, the average Test Statistic was lower, equivalent to LOD 2.7 with a range equivalent to 0.7 for Ht to 5.2 for HWEc.

Positive and negative QTLs were identified in each parent for each character apart from HWEc, where all the positive QTLs were derived from Blenheim and possibly GE4, where the primary QTL was a cross-over interaction and all the other Blenheim alleles gave an increase in the character (Table 5). With these possible exceptions, this confirms the dispersion of QTLs between parents leading to the transgressive segregation reported in previous studies of this cross (Thomas *et al.*, 1995b, 1996). More than 67 per cent of the phenotypic variation accounted for by the sCIM analysis was attributable to main effects for most of the characters, the exceptions being GE4, 1GE8, DP and HWEc (Table 5).

There appear to be a number of QTL 'hot-spots' where the region around a particular marker was found to be involved in the control of a number of characters. This is particularly noticeable on chromosome 2 where three such regions were found, each involved in the control of four or more characters (Fig. 2). The region around *denso* on chromosome 3 was also important, being involved in the control of all the characters in the current study apart from ME and HWEc. Both arms of chromosome 7 were also important with a 'hot-spot' around e41m33c on the short arm being involved in the control of six characters. The long arm of chromosome 7 was also involved in the control of nine characters but, apart from some clustering around p18m51h, these QTLs were spread more evenly over the chromosome. In contrast, chromosome 1 had the longest map length of any segment but, apart from a region around e37m39a, showed no great clustering of QTLs. Eight of the primary QTLs were located in the region around *denso*, probably reflecting the action of a major developmental gene (Fig. 2).

Discussion

Several reports have now demonstrated that AFLPs are a reliable and reproducible molecular marker assay. Furthermore, large numbers of AFLP loci can be detected in a single experiment providing an experimental system capable of generating vast numbers of informative markers. Most experiments reported to date have focused on *Eco*RI/*Mse*I

digested DNA and associated adaptors but *Hin*dIII/*Mse*I and *Pst*I/*Mse*I have also been used in potato (Meksem *et al.*, 1995) and tomato (Thomas *et al.*, 1995a). Our results indicate that *Pst*I/*Mse*I primers are more efficient in detecting polymorphism than *Eco*RI/*Mse*I primers in barley. This is an important observation because it will improve the cost-effectiveness of deploying AFLP technology in barley genetics. The Blenheim × E224/3 population is the least polymorphic intraspecific cross examined but nearly 400 AFLP markers were added to the data set used to produce the linkage map based on RFLP and RAPD markers (Thomas *et al.*, 1995b).

Contingency chi-squared tests revealed that the distributions of *Eco*RI/*Mse*I- and *Pst*I/*Mse*I-generated polymorphic products between linkage groups were different. An examination of the distribution of the AFLPs detected by the different enzyme combinations within the genome also revealed a nonrandom distribution of data points.

*Pst*I is a methylation-sensitive enzyme and there is a possibility that some of the observed polymorphisms were influenced by methylation changes. In plants, 5-methyl cytosine is the most common methylated base occurring in up to 30 per cent of all C (Gruenbaum *et al.*, 1981) in plants and an estimated 25.7 per cent in barley (Amasino *et al.*, 1990b). Changes in methylation status have been reported previously in barley when *Hpa*II- and *Msp*I-digested DNA from anther-culture derived doubled haploids of cv. Igri was screened with seven RFLP probes (Devaux *et al.*, 1993). In that case 11.7 per cent of the assays performed (49 out of 420) revealed polymorphism based on a methylation change. These polymorphisms appeared to be distributed randomly amongst the DHs examined but not amongst the probes.

Overall, the AFLP markers filled gaps in the previously constructed map (Thomas *et al.*, 1995b), providing good genome coverage. A previous study (Becker *et al.*, 1995) with barley AFLPs reported evidence of map extension. In our study increase in map length by the inclusion of AFLP markers was caused by map extension in specific regions of the genome that were poorly represented by RFLP and RAPD markers.

A major consideration is the usefulness of the AFLP data generated in localizing quantitative traits. Good overall agreement in the location of QTL to chromosomal segments is observed between previous (Thomas *et al.*, 1995b, 1996) studies based on RAPD and RFLP maps, and the present study which included AFLPs (Fig. 2). With some exceptions, the QTL in the current study are either in the same segment or in an adjacent segment which has been newly mapped. Thirty-three QTL peaks are located outside regions covered by the RAPD and RFLP map which demonstrated that these regions were also important in the control of traits.

In conclusion, AFLPs provide a fast and reproducible method for producing linkage maps in breeding populations which have a relatively narrow genetic base. The technology will allow more effort to be devoted to examining larger segregating populations which are chosen for their biological or breeding relevance rather than the ease with which polymorphism can be detected. Finally, because the polymorphism detected with AFLPs is analogous to that revealed by RFLPs (restriction site variation) greater emphasis can be placed on comparative mapping between different barley populations.

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Appendix I Total number of products, number of polymorphic products and percentage polymorphic for AFLP primers evaluated on four barley crosses (a) Blenheim × E224/3, (b) Dicktoo × Morex, (c) Igri × Franka, (d) Lina × *H. spontaneum*

	3′ selective nucleotides		Total number of products				Polymorphic products				% polymorphic products			
	G/p	m	a	b	c	d	a	b	c	d	a	b	c	d
*Eco*RI/*Mse*I primers														
e11m37	AA	ACG	118	111	115	108	16	24	18	29	13.6	21.6	15.7	26.9
e16m42	CC	AGT	67	63	70	70	8	19	10	18	11.9	30.2	14.3	25.7
e19m36	GA	ACC	80	78	92	82	6	15	7	19	7.5	19.2	7.6	23.2
e31m34	AAA	AAT	120	120	70	140	5	12	3	20	4.2	10.0	4.3	14.3
e32m34	AAC	AAT	128	148	115	128	15	36	22	32	11.7	24.3	19.1	25.0
e32m36	AAC	ACC	71	96	70	78	8	27	9	18	11.3	28.1	12.9	23.1
e33m42	AAG	AGT	107	*	100	34	9	*	22	8	8.4	*	22.0	23.5
e33m52	AAG	CCC	64	86	76	84	6	16	10	31	9.4	18.6	13.2	36.9
e34m39	AAT	AGA	*	130	109	86	*	15	5	15	*	11.5	4.6	17.4
e35m31	ACA	AAA	136	150	130	150	3	36	9	37	2.2	24.0	6.9	24.7
e35m42	ACA	AGT	98	72	*	*	9	21	*	*	9.2	29.2	*	*
e36m33	ACC	AAG	82	112	110	100	11	28	24	21	13.4	25.0	21.8	21.0
e36m36	ACC	ACC	68	94	82	96	6	27	8	30	8.8	28.7	9.8	31.3
e36m42	ACC	AGT	71	*	72	*	5	*	11	*	7.0	*	15.3	*
e36m50	ACC	CAT	98	97	66	74	12	21	3	20	12.2	21.6	4.5	27.0
e37m39	ACG	AGA	75	82	76	*	6	13	2	*	8.0	15.9	2.6	*
e37m49	ACG	CAG	53	85	64	89	2	25	6	20	3.8	29.4	9.4	22.5
e38m31	ACT	AAA	127	137	126	108	20	37	10	15	15.7	27.0	7.9	13.9
e38m33	ACT	AAG	99	115	74	78	19	23	3	10	19.2	20.0	4.1	12.8
e38m50	ACT	CAT	65	90	95	89	6	32	13	20	9.2	35.6	13.7	22.5
e39m35	AGA	ACA	100	117	97	96	8	15	18	28	8.0	12.8	18.6	29.2
e39m61	AGA	CTG	67	78	67	72	12	24	11	25	17.9	30.8	16.4	34.7
e40m31	AGC	AAA	88	110	125	108	11	12	7	22	12.5	10.9	5.6	20.4
e40m32	AGC	AAC	122	99	128	98	8	11	11	33	6.6	11.1	8.6	33.7
e40m62	AGC	CTT	118	74	62	57	3	7	15	12	2.5	9.5	24.2	21.1
e41m31	AGG	AAA	82	109	115	100	6	28	12	32	7.3	25.7	10.4	32.0
e41m32	AGG	AAC	86	120	79	81	9	20	9	20	10.5	16.7	11.4	24.7
e41m33	AGG	AAG	103	90	85	110	6	16	9	27	5.8	17.8	10.6	24.5
e41m35	AGG	ACA	119	108	115	72	5	17	24	25	4.2	15.7	20.9	34.7
e42m47	AGT	CAA	80	92	69	75	9	11	5	31	11.3	12.0	7.2	41.3
e43m38	ATA	ACT	101	110	105	105	13	28	13	28	12.9	25.5	12.4	26.7
e43m52	ATA	CCC	53	56	54	62	6	13	8	18	11.3	23.2	14.8	29.0
e44m55	ATC	CGA	89	99	80	91	7	22	8	20	7.9	22.2	10.0	22.0
e45m48	ATG	CAC	110	101	80	98	7	22	5	25	6.4	21.8	6.2	25.5
e46m42	ATT	AGT	89	93	90	55	9	18	4	7	10.1	19.4	4.4	12.7

Appendix I *Continued*

	3′ selective nucleotides		Total number of products				Polymorphic products				% polymorphic products			
	G/p	m	a	b	c	d	a	b	c	d	a	b	c	d
e95m307	AAAA	TCAG	72	103	113	68	3	36	14	14	4.2	35.0	12.4	20.6
e121m238	ACGG	GATC	134	132	105	142	15	31	12	14	11.2	23.5	11.4	9.9
e181m181	CCCC	CCCC	38	99	70	64	1	19	16	24	2.6	19.2	22.9	37.5
e181m213	CCCC	CTGA	131	133	101	93	12	33	15	37	9.2	24.8	14.9	39.8
*Pst*I/*Mse*I primers														
p11m14	AA	AT	103	167	*	115	9	25	*	25	8.7	15.0	*	21.7
p11m37	AA	ACG	43	68	55	44	7	24	11	12	16.3	35.3	20.0	27.3
p12m16	AC	CC	110	110	124	105	20	38	12	29	18.2	34.5	9.7	27.6
p16m18	CC	CT	129	143	110	120	9	18	7	27	7.0	12.6	6.4	22.5
p18m12	CT	AC	137	118	96	108	16	17	13	24	11.7	14.4	13.5	22.2
p18m51	CT	CCA	112	108	94	91	15	25	12	30	13.4	23.1	12.8	33.0
p19m36	GA	ACC	62	73	58	60	14	21	10	18	22.6	28.8	17.2	30.0
p22m62	GT	CTT	65	89	86	88	10	27	14	24	15.4	30.3	16.3	27.3
p23m81	TA	TAG	53	70	62	*	9	22	13	*	17.0	31.4	21.0	*
p25m42	TG	AGT	76	*	*	*	19	*	*	*	25.0	*	*	*
p26m76	TT	GTC	80	*	*	*	18	*	*	*	22.5	*	*	*
p31m31	AAA	AAA	81	113	*	100	9	25	*	29	11.1	22.1	*	29.0
p40m31	AGC	AAA	112	*	59	79	23	*	6	37	20.5	*	10.2	46.8
p40m32	AGC	AAC	65	100	76	*	13	24	14	*	20.0	24.0	18.4	*
p213m121	CTGA	ACGG	*	73	102	78	*	17	22	22	*	23.3	21.6	28.2
p238m140	GATC	AGTC	92	127	83	95	15	23	14	33	16.3	18.1	16.9	34.7
p238m238	GATC	GATC	*	*	70	100	*	*	10	25	*	*	14.3	25.0
p307m181	TCAG	CCCC	100	103	77	*	13	25	3	*	13.0	24.3	3.9	*

*Not measured.