Theor Appl Genet (1993) 87:314–320

THEORETICAL AND APPLIED GENETICS

Identification of RAPD markers linked to genetic factors controlling the milling energy requirement of barley

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Received: 30 December 1992 / Accepted: 29 March 1993

Abstract. Doubled haploid (DH) populations of barley have been used in combination with PCR-based polymorphic-assay procedures to identify molecular markers linked to genes controlling the milling energy requirement of the grain. Milling energy (ME) is a quantitative trait and locating individual quantitative trait loci (QTLs) involved the construction of bulks by combining DNA from DH families representing the extreme members of the distribution for ME. In addition, the individuals had alternative alleles at the *Rrn2* locus that has previously been shown to be linked to an ME QTL. The DNA bulks were screened with Randomly Amplified Polymorphic DNA (RAPD) markers and polymorphic amplification products tested for linkage to genes influencing the expression of ME in a DH population. Several markers were identified which are linked to a QTL controlling ME and the recombination fraction determined by maximum likelihood procedures. The results indicate that DHs in combination with RAPDs and bulked segregant analysis provide an efficient method for locating QTLs in barely. Furthermore, this approach is applicable to mapping other QTLs in a range of organisms from which DH or recombinant inbred lines can be extracted.

Key words: RAPDs – DHs – Bulked segregant analysis – QTLs – Barley

Communicated by J. W. Snape
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Introduction

The identification of genetic factors influencing quantitative traits in plants has been greatly facilitated by the development of detailed genetic linkage maps (reviewed by Tanksley et al. 1989). Two types of molecular markers have been used to develop these maps. The majority of studies have used restriction fragment length polymorphisms (RFLPs) but more recently randomly amplified polymorphic DNA markers (RAPDs) have been shown to be useful in a range of organisms (Welsh and McClelland 1990; Williams et al. 1990). RAPDs provide an alternative polymorphic assay procedure for use in plant genetics and breeding (Rafalski et al. 1991; Waugh and Powell 1992).

Many opportunities now exist for the exploitation of genetic markers in crop improvement programmes. Central to this challenge is the requirement to quickly locate markers to specific regions of the genome which are important in controlling the expression of traits of economic or biological importance. The availability of near-isogenic lines (NILs) for various disease resistances has identified both RFLP (Hinze et al. 1991; Yu et al. 1991) and RAPD markers (Martin et al. 1991; Paran et al. 1991) that are linked to important plant disease resistance genes. Michelmore et al. (1991) have recently described a bulked segregation analysis method, that is not dependent on the availability of NILs, to identify RAPD markers linked to major genes conferring resistance to downy mildew in lettuce. This approach was based on progeny testing of F_2 individuals to identify and eliminate heterozygous individuals at a downy mildew resistance locus. In this manuscript we report the use of bulked segregation analysis in conjunction with doubled haploids (DHs) to efficiently identify RAPD markers linked to a quantitative trait in

barley. We illustrate this mapping strategy with the trait known as the milling energy requirement of barley but stress that the approach has general applicability to a range of quantitative traits.

The mechanical energy required to mill a given sample of barley grain can be quantified. This value, termed milling energy (Allison et al. 1976), is important in predicting malting quality, since the ease with which the structure of the endosperm can be disrupted mechanically appears to relate to the ease with which it is broken down by enzymes during malting, a major industrial use of the barley crop. Consequently, low milling energy of barley grain is generally associated with high levels of hot-water extract in the malt made from it (Allison et al. 1976). Although some barley cultivars with low milling energy do not give high malt extracts, presumably due to low enzyme levels (Allison et al. 1976), no genotypes with high milling energy have been found to produce high levels of extract. Milling energy is a rapid, small-scale test which is an important screening procedure for the barley breeder and serves here as a typical quantitative trait. In a previous study, we had identified a strong association between the ribosomal DNA RFLP marker (*Rrn2*) located on the short arm of barley chromosome 7 (Powell et al. 1992) and milling energy. This information was used in conjunction with bulked segregant analysis to make a more detailed study of the region around the *Rrn2* locus and its association with genes controlling milling energy.

Materials and methods

Plant material

Fifty-nine doubled haploids (DHs) were regenerated from a cross (B86-13) between the spring barley cultivar Blenheim and the SCRI breeding line E224/3. Methods used to regenerate plants from microspores are given by Finnie, Powell and Dyer (1989). DH families together with their parents were evaluated in replicated field trials in 1989, 1990 and 1991 (for details of the 1989 trials see Powell et al. 1992). The 1990 and 1991 trials were repeats of the 1989 trial but with different randomisations and were grown at different sites on the SCRI experimental farm. After each harvest a weighed sample of grain that passed over a 2.5-mm sieve was used to determine milling energy (joules) by the modified comparamill (Cowe, Cuthbertson and Swanston 1989) adjusted to joules per 5 g sample.

DNA procedures and bulk analysis

Total genomic DNA was isolated from fresh leaf material of single plants grown under disease-free conditions by a modification of the method of Saghai-Maroof et al. (1984). Aliquots of DNA (5 µg) from five DH lines, representing the two extremes of the distribution for milling energy requirement and having alternative alleles at the *Rrn2* locus, were combined to produce two sets of bulked DNAs. The bulks were screened with a range of arbitrary RAPD primers. The genotypic composition of the bulks is given in Table 1.

Randomly amplified polymorphic DNA markers (RAPDs)

PCR reaction mixtures (50 µl) contained approximately 100 ng of genomic DNA, dATP, dCTP, dGTP and dTTP each at 100 mM final concentration, 200 nM of primer, 1 × *Taq* polymerase buffer [10 mM Tris HCl pH 8.8 (25 °C), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.1% non-ionic detergent] and one unit of *Taq* polymerase (Northumbria Biologicals Limited, Cramlington, UK). Each reaction was overlaid with 50 µl of mineral oil to prevent evaporation. The random sequence 10-mer primers used in this study were obtained from Operon Technologies Inc., Alameda, Calif., or were synthesised on an Applied Biosystems PCR-mate oligonucleotide synthesiser at SCRI.The notation used for primer products is the same as proposed in Chalmers et al. (1992) with the prefix H (for barley) before the molecular size of the amplification product.

Genetical analyses

Segregating RAPD markers were scored for each of the DH lines as either B (Blenheim homozygotes) or E (E224/3 homozygotes) and linkage analysis was 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 centimorgans. Linkage between ME and each RAPD marker was estimated using both interval mapping by

Table 1. Allelic composition at six RAPD loci of five individuals used to construct two ME bulks representing high (H) and low (L) values. The mean and standard deviation of the H and L bulks are given

RAPD locus	Parents		Bulk H 742.8 (6.35)		Bulk L 612.38 (7.97)	
	Blenheim	E224/3	Blenheim	E224/3	Blenheim	E224/3
OPM14-H2000	−	+	1	4*	5	0*
OPD13-H980	−	+	0	5*	5	0*
OPE11-H1500	−	+	0	5*	5	0*
OPJ5-H850	+	−	0*	5	4*	1
OPE11-H400	−	+	0	5*	5	0*
OPB4-H300	−	+	4	1*	1	4*

* Allele generating dominant amplification product
+, Product present as dominant amplification product
−, Null allele

MAPMAKER/QTL (Lander and Botstein 1989) and normal mixture models. The latter were proposed for F_2 data by Weller (1986) and adapted for doubled haploid data by Luo and Kearsey (1991). Such models have previously been used successfully to model linkage in barley by Jensen (1989) and Hackett et al. (1992). In the present case the distribution of milling energy associated with each RAPD marker genotype is represented as a mixture of two normal distributions, one associated with the BB genotype at the QTL and the other associated with the EE genotype, in proportions depending on the recombination fraction between the marker and the QTL. The model parameters are thus the mean and standard deviation of the two normal distributions and the recombination fraction. All five parameters were estimated simultaneously using the NAG implementation of the Nelder-Mead algorithm (Nelder and Mead 1965).

Results

The two bulked DNA samples (Table 1) were screened with a total of 440 random 10-mer oligonucleotide primers to generate RAPD markers. In barley, under the conditions outlined in Materials and methods, each primer generates on average 5–6 discrete amplification products. Five primers generated clear polymorphisms in the DNA bulks. One primer, OPE11, generated two polymorphic products. To confirm that the polymorphisms detected were not artefacts, both the parents and the individuals used to construct the bulks were examined with all five primers. The allelic composition of all six genetic loci for both the parents and the individuals is shown in Table 1 and an example of the polymorphisms detected using OPD13 is given in Fig. 1a. The polymorphic band, OPD13-H980, is clearly present in bulk 1 and E224/3. To confirm potential associations between the RAPD markers and ME, segregation of the polymorphic amplification products was monitored in 52 of the DH families. Segregation of OPD13-H980 in a selection of these families is shown in Fig. 1b. With the exception of OPE11-H400 the polymorphic loci did not deviate significantly from the expected 1:1 ratio. Multipoint linkage analysis indicated that four of the loci revealed with RAPDs are tightly linked to the *Rrn2* locus and the most likely genetic map of this region of the barley genome is outlined in Fig. 2. The *Rrn2* locus has been previously mapped to the short arm of chromosome 5H (barley chromosome 7) (Saghai-Maroof et al. 1984) and the linkage group shown in Fig. 2 corresponds to the short arm of chromosome 5H. The locus OPB4-H300 did not co-segregate with markers on chromosome 5HS but is linked to a 1H long-arm-chromosome-specific RFLP marker (MPIII/46; Fig. 2b).

The means of the Blenheim (B) and E224/3 allele groups for milling energy are given in Table 2 for the 1989 growing season. In each case there are significant differences between marker classes for the trait. Normal mixture models were used to obtain estimates of recombination fractions between QTLs and the molecular markers on chromosomes 5H and 1H (Table 3). Estimates of the recombination fractions indicate that there is a QTL for ME on 5HS, probably between the *Rrn2* and OPD13-H980 loci. There also appears to be a second QTL for ME on the long arm of chromosome 1H in the vicinity of the OPB4-H300 locus. This locus contrasts with the other markers in that the Blenheim allele is associated with decreasing ME but for the other markers it is associated with increasing ME.

Estimates of the additive genetic variation (D) associated with allelic variation at each marker locus were calculated for the three growing seasons (Fig. 3). The

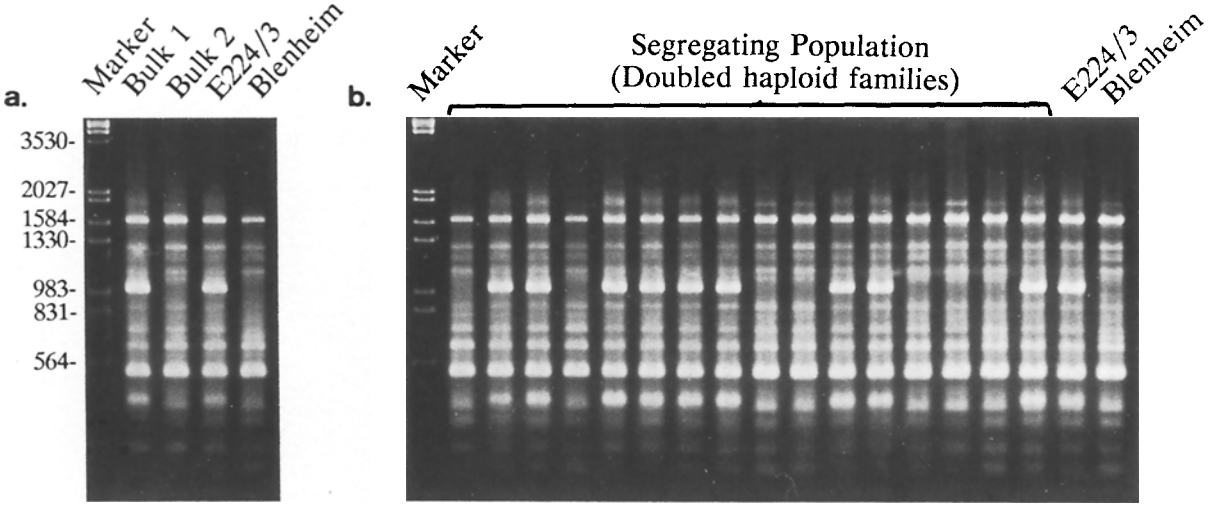

Fig. 1. **a** Polymorphism detected between bulked DNA samples with OPD13-H980. **b** Segregation of a polymorphic amplification product detected with OPD13-H980 in a DH population of barley

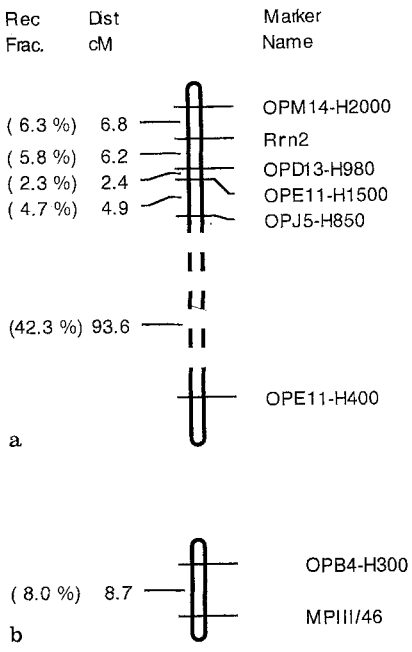

Fig. 2. **a** Genetic linkage map of the short arm of chromosome 5H of barley. **b** Linkage of OPB4-H300 to a 1HL-chromosome-specific RFLP marker (MP111/46)

Table 2. Means of the allelic classes for milling energy (1989 data) with six RAPD loci and *Rrn2*

Molecular marker	Chromosomal location	Mean and standard deviation	
		Blenheim allele	E224/3 allele
OPM14-H2000	5HS	694.5 (39.7)	643.3 (22.0)
Rrn2	5HS	700.6 (29.7)	653.2 (37.9)
OPD13-H980	5HS	698.7 (35.1)	645.3 (24.9)
OPE11-H1500	5HS	686.0 (38.4)	647.5 (32.5)
OPJ5-H850	5HS	692.5 (36.7)	650.0 (32.3)
OPE11-H400	5HS	686.0 (38.4)	647.5 (32.5)
OPB4-H300	1HL	656.5 (37.1)	687.5 (39.5)

magnitude of the genetic variation associated with the molecular markers varies over seasons with the associations being most pronounced in 1989. Four markers, *Rrn2*, OPE11-H150, OPD13-H980 and OPB4-H300, exhibit significant association with ME over the three growing seasons. The remaining three markers exhibit significant associations for two seasons but are not significantly associated with ME in the 1991 growing season. Based on data for the three growing seasons the highest percentage of genetic variation is associated with OPM14-H2000 and OPD13-H980 which flank the *Rrn2* locus (Fig. 2).

Table 3. Estimates of the recombination fractions and the means and standard deviations associated with the two genotypes at the milling energy QTL

Item	Molecular markers						
Parameter estimates	OPM14-H2000	*Rrn2*	OPD13-H980	OPE11-H1500	OPJ5-H850	OPE11-H400	OPB4-H300
Recombination fraction	0.108 ± 0.079	0.055 ± 0.052	0.053 ± 0.067	0.157 ± 0.123	0.101 ± 0.085	0.111 ± 0.233	0.232 ± 0.162
Mean of Blenheim (B) group	705.6 ± 7.9	705 ± 7.7	718 ± 10.2	704 ± 8.1	698 ± 8.6	690.2 ± 11.3	646 ± 11.0
Mean of E224/3 (E) group	643.0 ± 5.5	647 ± 5.4	638 ± 7.2	641 ± 7.0	645 ± 6.1	646.0 ± 9.2	697 ± 11.4
Standard deviation of (B) group	29.4 ± 6.1	33.3 ± 5.3	33.3 ± 8.2	31.1 ± 5.7	33.6 ± 6.1	37.5 ± 6.7	28.6 ± 9.1
Standard deviation of (E) group	22.9 ± 3.8	25.1 ± 3.8	30.2 ± 6.9	29.9 ± 6.8	25.2 ± 4.5	29.9 ± 7.5	33.7 ± 8.2

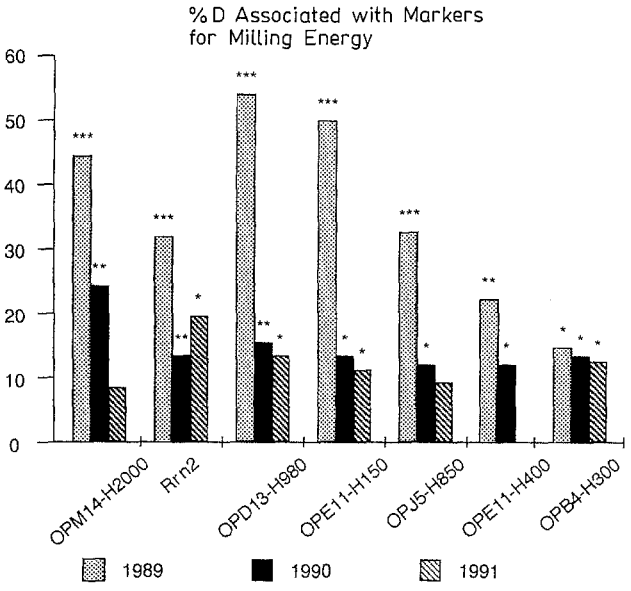

Fig. 3. Estimates of the percentage genetic variation (*D*) associated with markers for ME in three growing seasons (1989, 1990 and 1991)

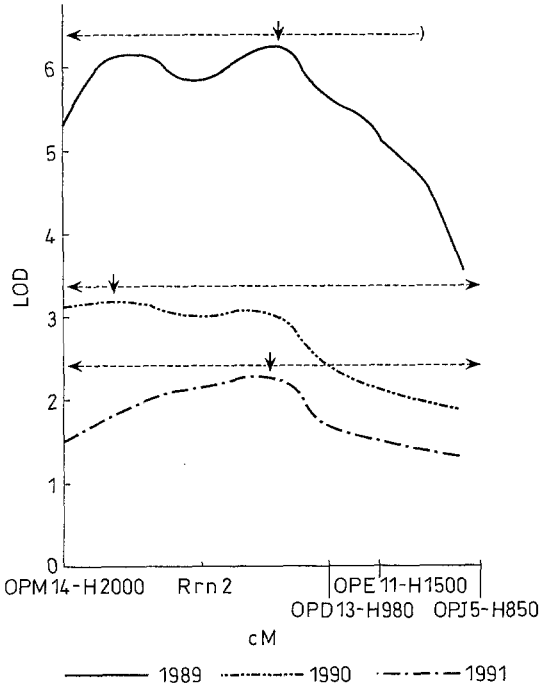

Fig. 4. Likelihood plots of a QTL for milling energy relative to the *Rrn2* locus and RAPD loci on chromosome 5HS for three growing seasons (1989, 1990 and 1991).

The method of interval mapping (Lander and Botstein 1989) implemented in the MAPMAKER/QTL package was also used to analyse the linkage of ME to the tightly-linked markers on chromosome 5HS. Plots of the three likelihood surfaces are shown in Fig. 4 and the most likely positions for the ME QTL are indicated. These are in similar locations between *Rrn2* and OPD-13-H980 for the 1989 and 1991 data but is located between *Rrn2* and OPM14-H2000 based on the 1990 data. In each case the likelihood surface is rather flat and the confidence interval for the QTL position is wider than the interval OPM14-H2000 to OPJ5-H850. The QTL accounted for 45%, 20% and 15% of the trait variances in the 1989, 1990 and 1991 growing seasons respectively.

Discussion and conclusions

The phenotype of a quantitative character is the consequence of both genetical and environmental factors. It is important, therefore, to partition the phenotypic variation into heritable and non-heritable components. the concept of using bulk segregant analysis to map a QTL hinges on the precision with which individual genotypes representing the tails of the distribution for a metrical trait can be identified. These genotypes are expected to differ at most of the loci controlling the character so that the alleles (increasing and decreasing) are highly associated. The choice of the segregating generation in which to evaluate the quantitative trait is of crucial importance. Dominance-related effects are likely to confound the assessment of quantitative traits, particularly in the early generations of a segregating cross. Recombinant inbred lines, particularly doubled haploids, can overcome many of these problems and have been used to identify associations between marker genes and quantitative traits (Powell et al. 1990; Kjaer et al. 1991; Thomas et al. 1991). Furthermore, linkage data on the segregation of RAPD markers can be obtained directly from DHs without the need for progeny testing to reveal heterozygotes. DHs in conjunction with RAPD analysis of bulked samples provide a highly efficient strategy to map QTLs. Reiter et al. (1992) working with *Arabidopsis thaliana* used recombinant inbred lines (RIL) generated by single-seed descent (SSD) in combination with pooled DNA samples to localise markers to a selected region of the genome. Although quantitative traits were not mapped in the *Arabidopsis* RILs, the approach outlined in this manuscript could also be used to map QTLs in RILs derived by SSD.

We have previously reported an association between *Rrn2* and a QTL for ME in barley (Powell et al. 1992). Using this information, one phenotype at the *Rrn2* locus was superimposed on the ME requirement data to select the individual DH families for bulking. The advantage of this approach is that by differentiating the bulks on the basis of two linked markers (*Rrn2* and the QTL for ME) differences in a chromo-

somal interval have been defined rather than in a single genetic locus. This approach is essentially similar to that described by Giovannoni et al. (1991) who used previously-mapped RFLP markers to target specific chromosomal intervals in tomato. For QTL analysis, a potential disadvantage is that such bulks are enriched for differences at only one of the genomic regions contributing to an overall quantitative trait. However, two primers, OPE11 and OPB4, generated amplification products which were linked to genetic factors controlling ME but which were unlinked to each other. Thus, the approach of defining a chromosomal interval influencing the expression of a QTL does not exclude the possibility of detecting markers at discrete unlinked genetic loci that also influence the expression of the trait. In this respect, it should be stressed that the quality of the data for the QTL is of primary importance.

The milling energy of barley grain was originally devised by Allison et al. (1976) as a practical method for predicting malting quality. The technique is now used in breeding and research programmes (Swanston 1987; Swanston and Taylor 1990) and is considered to be an important measure of grain texture though it is known to be influenced by environmental factors. Parallel studies with aneuploid stocks of wheat and barley disomic addition lines (Forster and Ellis 1990) located a QTL affecting milling energy to the short arm of homoeologous group 5 chromosomes. RFLP studies (Powell et al. 1992) also established associations between the *Rrn2* locus and milling energy in barley. Although the parents are not significanlty different (Table 1), the means of the parental groups of loci on the short arm of chromosome 5 linked to ME are in the opposite direction to the parental means. This is just the situation one would find with dispersion of alleles amongst the parents of the original cross giving no significant differences between them. This is confirmed by the identification of a primer (OPB4-H300) which accounts for 13% of the genetic variation for ME over all 3 years for which the Blenheim allele is linked to low ME. Thus the parental genotypes must differ for genetic factors at two genomic regions which are dispersed between the parents but which exhibit an excess of repulsion linkages between the markers and the ME QTL. Blenheim is derived from a cross between the cultivars Triumph and Egmont, and Egmont has the cultivar Vada in its ancestry. Many cultivars with Vada, and therefore *H. laevigatum*, in their pedigree are characterised by adverse quality characters (Swanston 1987), one of which is an increased level of ME. The fact that Blenheim should possess both deleterious and advantageous ME factors is therefore not surprising. However, the identification of molecular markers linked to the QTL for ME on chromosomes 1H and 5H provides the potential to manipulate the ME requirement, and therefore the malting quality, of a genotype such as Blenheim which is already high in extract.

Acknowledgements. The authors acknowledge the comments of J. S. Swanston and R. P. Ellis. The research was supported by the Scottish Office Agriculture and Fisheries Department.

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