Theor Appl Genet (2004) 110: 116–125
DOI 10.1007/s00122-004-1805-7

ORIGINAL PAPER

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Use of new EST markers to elucidate the genetic differences in grain protein content between European and North American two-rowed malting barleys

Received: 13 May 2004 / Accepted: 24 August 2004 / Published online: 12 November 2004

Abstract A population comprising 102 doubled haploid lines were produced from a cross between Beka, a barley cultivar widely grown in Spain, and Logan, a north American cultivar with inherently low protein content, a character considered to derive from the cultivar Karl. The intentions were to determine whether low-nitrogen malting barleys could be developed in Spain, and if genetic factors that influenced protein content were similarly expressed in widely diverse environments, i.e. northeastern Spain and eastern Scotland. An extensive map comprising 187 molecular markers was developed. Expressed sequence-tagged-derived markers were used in addition to anonymous simple sequence repeats to determine the potential for identifying candidate genes for quantitative trait loci (QTLs), and 22 such markers were mapped for the first time. There was transgressive segregation for both yield and protein content, and the gene for low protein from Logan was not expressed in the Scottish environment. In 2002, high yield was associated with earlier heading date in Spain, while late heading at the Scottish site was associated with greater lodging and lower thousand-kernel weight. These appeared to be possible pleiotropic effects of a factor detected on chromosome 2H. Using information from a consensus map, it was shown that this locus on 2H was in the region of the photoperiod response gene *Eam6*. A QTL explaining 18% of the variation in grain protein content was detected on chromosome 5H in a region in which a gene for nitrate reductase was previously observed. No effect on grain protein was associated with chromosome 6H, which has been suggested as the location of the low protein gene from Karl. However, it is likely that Karl contained more than one genetic factor reducing protein, and we postulate that the gene on 6H may have been lost during the breeding of Logan.

Communicated by P. Shewry

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Introduction

The adverse effects of high barley grain protein content on malting quality are well documented, as the protein matrix surrounding the starch granules acts as a barrier to modification of the grain during malting. In addition, high levels of nitrogenous materials in a hot-water extract will reduce fermentability (Bathgate et al. 1978). Protein content is regarded as under environmental as well as genetic control and of low heritability (Sparrow 1970). Piper and Rasmusson (1984) suggested, however, that heritability varied between populations so that, for some crosses, it was possible to select for low protein content in the early generations of breeding programmes. Genes apparently associated with low protein content were detected in the six-row genotype Karl (Wesenberg et al. 1976) and some of its two-row derivatives (Sasaki et al. 1992) and have facilitated breeding for low grain protein content (Goblirsch et al. 1996).

Sasaki et al. (1992) suggested that low grain protein in Karl resulted from slow accumulation during the phase of grain filling in which the most rapid increase in dry matter occurred. This effect should be independent of the environment in which the plants are grown. By contrast, many of the genetic factors associated with grain protein content are only detected under certain environmental conditions. For example, Thomas et al. (1996) noted a quantitative trait locus (QTL) on chromosome 1H that was only detected in seasons where grain protein content was comparatively low. Of seven QTLs, affecting grain protein detected by Emebiri et al. (2003), only one, on chromosome 5H, occurred in all environments. This was in the area of the genome in which a gene influencing the activity of nitrate reductase, a key enzyme in the regulation of nitrogen incorporation into the grain, was located previously (Metzer et al. 1988).

Differences in the patterns of grain filling between sites in northern (Scotland) and southern (Spain) Europe have been demonstrated (Swanston et al. 1997). In Scotland, both dry matter and storage protein (hordein) accumulation peak at around 700 degree days after anthesis (a scale based on the product of number of days and average daily temperature), then level off during maturation. By contrast, in Spain, accumulation continues until harvest ripeness, so final protein levels exceed those observed at the Scottish site (Swanston et al. 1997). Consequently the desired phenotypic expression of grain protein content and, thus, its underlying genetic control, may differ between these two contrasting environments. The inclusion of factors that limit the uptake of protein into the grain would be beneficial in Spanish barley, enabling crops to receive sufficient fertiliser to achieve good yield, while producing grain protein contents suitable for malting. In Scotland, while low protein content is required for barleys used in malt whisky distilling and for brewing traditional ales, protein contents that are too low have been considered to reduce fermentability (Swanston et al. 2000), as they adversely affect yeast nutrition during fermentation.

To determine whether the genetic control of grain nitrogen content differed under Spanish and Scottish conditions and also to assess whether it was possible to breed inherently low protein malting barley in Spain, doubled haploid lines (DHLs) were produced for investigation from a cross between the malting barley cultivars Beka and Logan. Beka, derived from a Bohemian × Moravian cross (Bethge XIII × Kneifel), has been extensively cultivated in Spain as a malting variety, while Logan is a North American two-row cultivar, derived from Karl (J. Franckowiak, personal communication). In the work described here, DHLs from the cross were grown over two seasons at Lleida in northeast Spain and also in a single season at Dundee, Scotland. The intention was to determine the location of genetic factors affecting grain protein content and to observe whether they were detected across seasons and between highly contrasting environments. Previous work has also suggested an association between grain protein and agronomic characters including yield and ear emergence (Thomas et al. 1996; See et al. 2002) that may result from pleiotropic effects or close linkage. These characteristics were also scored in this experiment to determine whether they were associated with genetic factors that were also altering protein content in the population being assessed and whether any such effects were consistent across environments.

Thomas (2002) noted, however, that marker-assisted selection has not been taken up by barley breeders on a significant scale and offered several reasons for this situation. Although simple sequence repeats (SSRs) are multiallelic and highly polymorphic can therefore overcome the problem associated with restriction fragment length polymorphisms (RFLPs) and amplified fragment length polymorphisms (AFLPs) being largely monomorphic, they are still anonymous markers. Consequently, they are highly suited to genotyping, but not to assigning functionality. QTLs will therefore not be transferable between populations if the control of a particular trait differs, e.g. if malting performance is largely a structural trait in one population, but more strongly affected by enzyme activities in another. The use of the rapidly expanding database of expressed DNA sequences in particular means that these can be used to mine for robust sequence-based markers that are derived from genic regions and exhibit polymorphism that is due to the variation at these genes. With the large amount of sequence data being generated for barley, it is possible to identify SSRs from these expressed sequence tag (EST) libraries (Scott et al. 2000; Eujayl et al. 2001; Kantety et al. 2002; Woodhead et al. 2003; Russell et al. 2004). Furthermore, EST libraries are becoming a valuable source of single nucleotide polymorphic (SNP) markers, which can be used in mapping complex phenotypes. The development of gene-based markers offers the potential of identifying candidate genes for QTLs where sequence variation can be identified and markers developed. In this paper the use of a series of EST-derived markers mapped in Oregon Wolfe barley mapping populations (http://barleyworld.org/) is investigated, as well as genomic anonymous SSRs and AFLPs.

Material and methods

Plant material

To generate genetic variation for grain protein content in high-quality malting barley backgrounds of separate geographical origins, i.e. Europe and North America, a DHL population consisting of 102 lines was developed through anther culture from the cross Beka × Logan. Beka is prone to high and Logan to low protein content under Spanish conditions (J.L. Molina-Cano, unpublished data). Beka is a top-quality malting variety, bred in France in the 1960s and since then, widely grown in Spain because of its adaptation to Mediterranean environments and its interest to the Spanish brewing

industry. Its pedigree, Betghe XIII × Kneifel, is a Bohemian × Moravian cross, i.e. the highest traditional quality source within European germplasm. In turn, Logan (ND 11231-11), is a malting variety developed in North Dakota (USA) from the cross (ND7085 × ND4994) × ND7556, derived from the low-protein source Karl (J. Franckowiak, personal communication).

Field trials and phenotypic data assessment

Three field trials were carried out at two sites, Lleida (northeast Spain) and Dundee (eastern Scotland) as follows: Lleida 2001 and 2002, and Dundee 2002. By using these contrasting sites, we aimed to maximise the environmental factors influencing barley growth, particularly photoperiod, since in Spain barley is grown as an autumn crop (sowing in November, harvesting in June), whereas in Scotland it is a spring crop (sowing in March, harvesting in late August). In Spain, therefore, barley grows mostly under short days, while in Scotland it does so under long days. We have demonstrated over many years the strong influence of these environmental differences for malting quality of barley, particularly for protein content (e.g. Molina-Cano et al. 2001a, b; Swanston et al. 1995, 1997; Ellis et al. 1997).). In addition we have noted that differences between seasons are frequently greater in Lleida than Dundee, so that site × year interactions have been noted for some characters (Swanston et al. 1995). Thus, while our number of sites and seasons is limited, the contrasts are likely to be such that QTLs and QTL × site interactions should be readily identified. In fact, extensive research on environmental effects (reviews in Molina-Cano et al. 2000, 2004) indicated that the most effective environmental contrasts were between short-day sites (such as Lleida) and long-day ones (such as Dundee).

The trial at Lleida 2001 consisted of a single plot of each DHL plus repeated plots of the parents, laid out in a systematic design. Plot size was 1.2×5 m with eight rows 0.15 m apart. The trials at Lleida and Dundee in 2002 were organised by dividing the DHLs into six series of 17 plus three controls (Beka, Logan and Scarlett) in each. Each trial series was laid out in an alpha-lattice design with three replications, the constituent plots being 1.2×2 m with eight rows 0.15 m apart. At all trials both sowing rate and fertiliser application followed the standard practice for malting barley in the particular region. Phenotypic observations at both sites included days from sowing to heading (DSH) and grain yield [(YLD) kg m^{-2}]. At Dundee, differences in straw strength were clearly observed towards the end of the main grain-filling period, when adverse weather conditions caused lodging in the vulnerable DHLs. On grain from each replication thousand-kernel weight [(TKW) g] and total grain protein [(PRT) %] were recorded after harvest, the latter as Kjeldahl N × 5.5, the best estimate of barley grain protein content (P. Shewry, personal communication). For the trial of Lleida 2001, two samples of 50 spikes each were taken at random from each plot prior to harvest and considered as replications for the determination of grain parameters. YLD measurement was based on a single-plot measurement corrected in accordance with values obtained within blocks for the parental varieties. A precise estimation of the grain PRT requires a minimum plot area, and as the amount of available seed for the first year was limited, only a single plot could be seeded per each doubled haploid plant. Thus, we used an augmented design in which unreplicated DHLs were surrounded by a number of replicated plots of the parent varieties Beka and Logan for spatial adjustment. Two independent samples were individually threshed and processed per plot in order to estimate intra-plot error. Both the analysis of the replicated check plots and the intra-plot variance revealed that the degree of field heterogeneity for grain PRT was quite low and no further adjustments were needed.

Genotyping

For molecular marker analyses, genomic DNA was extracted from the leaves of greenhouse-grown seedlings of the entries, following a CTAB extraction method (Doyle and Doyle 1990) with minor modifications. Genotyping was carried with 187 markers, as follows: 86 AFLP, 78 SSRs, 22 EST-derived SSRs (EST-SSRs) and one random amplified polymorphism DNA (RAPD). AFLP analyses were conducted following the procedure of Vos et al. (1995). Seventeen *Eco*RI + 3/*Mse*I + 3 AFLP primers combinations were analyzed, and the polymorphic markers obtained were designed with the nomenclature proposed by Qi and Lindhout (1997). SSR primer sequences were obtained and analyses were performed according to the protocols of Becker and Heun (1995) and Ramsay et al. (2000). EST-SSRs (Table 1) were analysed according to the protocol of Woodhead et al. (2003).

Linkage map construction and statistical analysis

Linkage analysis of the molecular and morphological trait data was performed with MAPMAKER, version 3.0 (Lander et al. 1987), using the Kosambi mapping function (Kosambi 1944). Morphological sequence-tagged sites and SSR markers with common chromosomal locations in previously published maps (Kleinhofs et al. 1993; Ramsay et al. 2000) were used as anchor markers to assign linkage groups to chromosomes. Markers showing severe distortion were dropped for linkage analysis. Two- and three-point analyses were conducted at LOD 3.0, and markers were assigned to linkage groups defined by anchor markers using the *assign* command. The *order* command (LOD 3.0) was used to order markers within linkage groups, and those markers without unique placement were integrated by the *build* command. After these procedures, all markers uniquely placed were included in the genetic map.

Table 1 Description of the EST-derived microsatellites (simple sequence repeats) used in the genotyping of the plant material studied

Locus	New name	Affy contig	BLASTX hit	Acc. no.	Forward primer	Reverse primer
Ctig7981	scssr07759	abc07759	Hypothetical protein	AAK92570.1	GCAACTCCTCATCATCTCAGG	CAACAGCCAGAAGGTCTACG
Ctig12007	scssr00334	abc00334	OSJNBa0033G16.9	CAD40930.1	CAAACAGCCACTGTCCTAGC	AGGGCGAGGTAGATGACG
Ctig1158	scssr12203	abc12203	Early nodulin 75 precursor-like protein	BAC15501.1	AAGCCATGATCGGACTAGG	TACACGTAAAGGGAAGAAGG
Ctig4370	scssr02236	abc02236	Profilin 1	P52184	TTCCTGCTAGTTTGCTAATCG	TGGCGAGGAAGTAGAAGAGG
Ctig1624	scssr03381	abc03381	Subtilisin-chymotrypsin inhibitor 2	T06181	CAGGGACGTACAATATTACCG	AATAAGCAGGGTACCTTTTGG
Ctig6034	scssr07402	abc07402	P0698A10.29 [*Oryza sativa* (*japonica* cultivar-group)]	BAB92459.1	AGTTCCTGCCCTAGAAATGG	TCTTCCCCAATGTCATTACC
Ctig5436	scssr08623	abc08623	P0518C01.3 [*Oryza sativa* (*japonica* cultivar-group)]	BAB63667.1	AACATTTACACCCAATCTAATTCC	ACAGTAGAAGCTAGCCTTGG
Ctig5110	scssr10559	abc10559	Putative myb-related transcription activator	BAB16456.1	CATTTCCTCTCCCCTTGC	CTCACCTCCTGCCGATCC
Ctig5562	scssr01846	abc01846	Catalase 1 pir||T06478 catalase (EC 1.11.1.6) - wheat	Q43206	GGCTCGGTAAAATGAAGTAGC	AGCCGAGCATGTAATCACC
Ctig5280	scssr10477	abc10477	Glucan endo-1,3-beta-D-glucosidase	T06215	AGAGCAATGAGCTCCTACCC	GCTTACTCGCTCGTTTAGTCG
Ctig709	scssr08238	abc08238	Putative protein	NP_196721.1	CAGCAGCAGATCAAATCAGG	TACTCTTCTCTTGGCCTTGG
Ctig8484	scssr04163	abc04163	UDP-glucose 4-epimerase	BAC02925.1	GAAGAAACAACCCAACTTCC	AGGATCGTACGAAGAACAGC
Ctig4047	scssr04163	abc04163	UDP-glucose 4-epimerase [*Oryza sativa* (*japonica* cultivar-group)]	BAC02925.1	CAGAGCCAGTAGCAGTAGAGC	GGATCATCCGACTCACTCC
Ctig7136	scssr18076	abc18076	ead-box ATP-dependent RNA helicase	NP_768087.1	CAGCTAGTCGCGCATTTG	GAGTCCACTGCTGCCTTG
Ctig7358	scssr15334	abc15334	Putative ripening-related bZIP protein	CAB85632.1	GGGAGCCGTAAGTAAGAACC	CGACCTCTGAATCTCAAATCC
Ctig7646	scssr09041	abc09041	Sigma factor SIG6	AAD17856.1	CATGTCAGTGGGGTTCTAGC	TCTACTTGGACCTGCTGACC
Ctig7260t	scssr03906	abc03906	Ubiquitin-conjugating enzyme E2-23 kDa	P16577	ACCATGTCTTCCCCAAGC	GGAAGTGGACGAAGAACTCC
Ctig9331	scssr03686	abc03686	ABA-induced plasma membrane protein PM 19 – wheat	T06978	CCCACACCCACTACACTAGG	GTCACGTACGGTGTCGATG
Ctig3942	scssr03907	abc03907	Ubiquitin-conjugating enzyme E2-23 kDa	P16577	CTCCCATCACACCATCTGTC	GACATGGTTCCCTTCTTCTTC

QTL analyses were performed with the software package MQTL (Tinker and Mather 1995) adapted for the evaluation of progeny in multiple environments to identify possible QTL × environment (E) interactions. Genome-wide QTL searches were performed by simple interval mapping with a test statistic for QTL main effect and for QTL × E interaction across sites. Significance thresholds for the test statistic to maintain the genome-wise type I error rate below 5% were established by using 3,000 random permutations of the data. QTL position corresponded to the peaks of the test statistics scans. Individual effects of QTLs were used to estimate the percentage of phenotypic variation accounted for by significant QTLs and confirmed by stepwise multi-locus linear models, using SAS procedures (SAS Institute 2001). Box plots and correlation analyses were carried out with Statgraphics Plus, version 5 (Statistical Graphics Corporation 2002).

Results

Character variation

The box plots in Fig. 1 represent the recorded variation for DSH, TKW and PRT. For DSH from the Dundee trial, the DHLs show a normal distribution, with the mean, median and mid-parent values virtually identical. There is some transgressive segregation in both directions, suggesting genetic factors contributing to high and low values in both parents. In both years at Lleida, Logan is earlier to heading than Beka, as in Dundee, but the DHLs show slightly skewed distributions, with a greater proportion of lines earlier than the mean, but more extensive transgressive segregation towards later heading dates. These differences may reflect the later heading of a small proportion of lines being expressed more strongly when autumn rather than spring sown.

For TKW, Beka gave lower values than Logan in all three trials, but transgressive segregation is expressed more strongly than for DSH, especially in Lleida 2001, where it occurs almost exclusively towards higher TKW. This indicates that Beka is also likely to be contributing alleles that contribute to higher TKW. Transgressive segregation is also evident for PRT, with Beka contained within the upper quartile in both years at Lleida and both parents within the lower quartile at Dundee, where they gave similar values. At Lleida, Logan demonstrated much lower PRT content than Beka. These results suggest that both parents contain genetic factors contributing to low as well as high PRT content, while a low PRT factor observed in Logan in Spain may not be expressed in a Scottish environment.

YLD varied quite widely at Lleida in both years (Table 2), although 2002 was higher yielding, overall than 2001. In both years, YLD distribution was skewed, but in opposite directions. In 2001, the mean value exceeded those of both parents, with Beka yielding 3.07 and Logan 3.97 t ha^{-1}. In the higher yielding year, 2002,

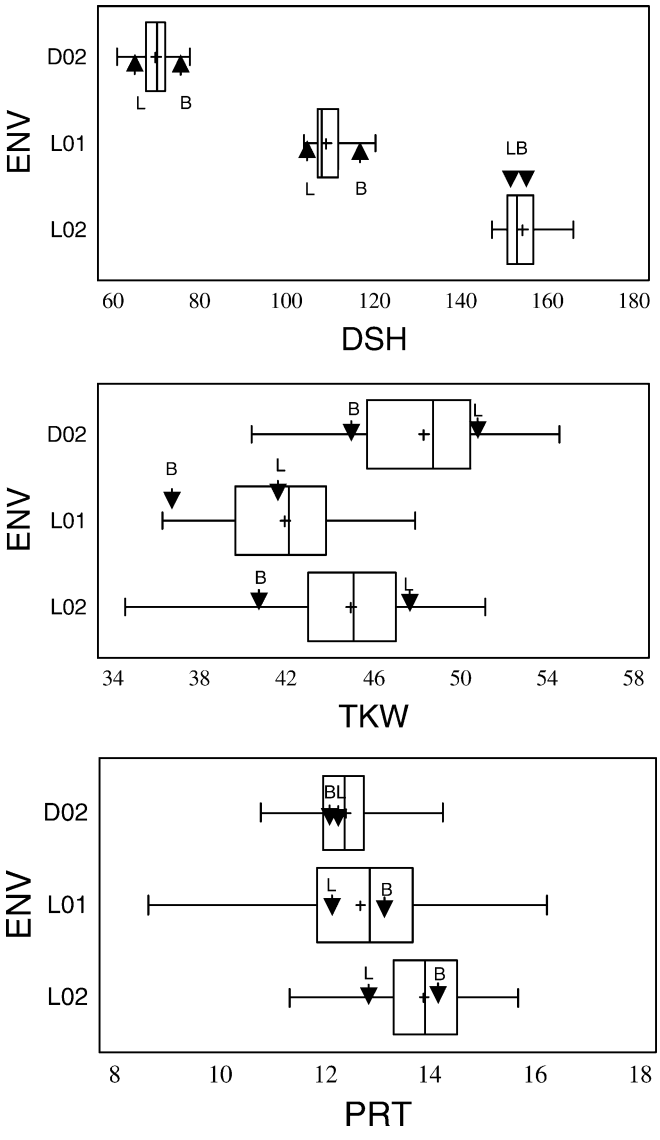

Fig. 1 Box-and-whisker plots showing the variation of days from sowing to heading (*DSH*), thousand-kernel weight (*TKW*) and Kjeldahl grain protein (*PRT*) at the three environments explored: *D02* Dundee 2002, *L01* Lleida 2001, *L02* Lleida 2002. The *central box* covers the middle 50% of the data; the *sides* of the box are the lower and upper quartiles, and the *vertical line drawn through the box* is the median. The whiskers extend out to the lower and upper values of the data (the range). The data are shown in four areas of equal frequency, which allows us to visually examine the shape of the distribution to detect skewness. *B* and *L* stand, respectively, for the phenotypic values of the different characters in the parent varieties Beka and Logan, marked approximately by the *arrows*

Logan (6.45 t ha^{-1}) again out-yielded Beka (6.17 t ha^{-1}), but the population mean was below the mid-parent value. In Dundee, both parents gave similar values, with Beka (4.86 t ha^{-1}) slightly, but not significantly, out-yielding Logan (4.77 t ha^{-1}). The mean and mid-parent values were very similar, but there was transgressive segregation in both directions, indicating that there were genetic factors for higher (and lower) YLD in both parents. The distribution for lodging was skewed

Table 2 Yield and lodging means and mid-parent values

Character	Yield (t ha^{-1})			Lodging (%)
Location/year	Lleida 2001	Lleida 2002	Dundee 2002	Dundee 2002
Mid-parent	3.52	6.31	4.82	33.8
Minimum	0.67	2.06	3.38	5.0
Maximum	6.00	8.63	5.55	67.5
Mean (SE)	4.38 (0.108)	6.01 (0.120)	4.87 (0.035)	23.9 (1.48)

Table 4 Correlations between environments for the characters DSH, TKW and PRT

Parameter	L01 vs L02	L01 vs D02	L02 vs D02
DSH	0.72***	0.87***	0.78***
TKW	0.58***	0.65***	0.69***
PRT	0.30**	0.45***	0.31**

Significance levels: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

towards lower values. With only one line slightly exceeding Beka (61.5%) and none significantly better than Logan (6%), there was no evidence of genetic factors in Logan contributing to weaker straw.

The matrix of correlations calculated on the means within environments is presented in Table 3. At all sites there was a significant negative correlation between PRT content and YLD. This may indicate a genetic association, but could also be due to the dilution effect of a higher plot YLD upon the PRT content. In 2002 at Lleida, YLD was also negatively associated with DSH, indicating a better performance from early-heading lines. This was not observed in Dundee. However, previous studies have shown that grain filling and ripening in Lleida generally occur during a period of increasing temperature and drought stress (Swanston et al. 1997). Late-heading types are thus likely to be at a disadvantage that will not occur at Dundee, where temperatures are cooler and more even across the grain-filling period. In 2001, there was also a correlation between YLD and DSH at Lleida, but it was a positive one. In that season, YLDs were much lower than in 2002 and, in particular, a number of early-heading lines performed very badly. Values for DSH were lower in 2001 compared to 2002, but that resulted from differences in time of sowing (18 December compared to 7 November), so ear emergence began in early April in both seasons. Examination of meteorological data showed heavy rainfall at the end of April 2001, following a prolonged dry spell, so it is likely that the earliest lines experienced drought stress during the early-to-middle grain filling period. MacNicol et al (1993) noted this to have a considerable deleterious effect on grain filling and, consequently, YLD. Although TKW was still negatively associated with DSH at Lleida in 2001, due to the combined effects of the Logan phenotype, the correlation was lower than at the other sites. This reflected the poor grain filling of some of the earlier genotypes.

In Dundee, later heading was associated with lower TKW and greater lodging (Table 3), indicating that several characteristics from Beka could be inherited together. However, although DSH was significantly correlated with PRT, the association was negative, which was unexpected given that Logan was considered to be the source of low PRT. It is possible that the low PRT factor is located elsewhere on the genome. Correlations between environments for a range of characters (Table 4) show a significant association in each instance. However, the highest correlations are for DSH, despite the Spanish-grown material being autumn-sown and the lowest for PRT. This suggests that DSH is under strong genetic control, while the other characters are affected to a greater extent by environment or genotype × environment interaction. For YLD, the correlation between Dundee and Lleida in 2002 was not significant ($r = 0.15$), indicating large differences in response to highly contrasting environments.

QTL analysis

Figure 2 shows the linkage map based on 187 markers, as follows: 86 AFLPs, 78 SSRs, 22 EST-SSRs and one RAPD. The 22 ESTs are mapped for the first time in the

Table 3 Correlation matrices over means of environments and within environments. In all cases $n = 104$

	Environment[a]															
	D02				L01				L02				Over environments			
Parameter	DSH	TKW	YLD	PRT	DSH	TKW	YLD	PRT	DSH	TKW	YLD	PRT	DSH	TKW	YLD	PRT
TKW	−0.57***	–	–	–	−0.24**	–	–	–	−0.38***	–	–	–	−0.51***	–	–	–
YLD	0.08	0.01	–	–	0.42***	0.22*	–	–	−0.50***	0.01	–	–	0.12	0.05	–	–
PRT	−0.34***	0.12	−0.40***	–	−0.43***	0.01	−0.23**	–	0.13	0.18	−0.49***	–	−0.42***	0.21*	−0.40***	–
LDG	0.49***	−0.49***	0.00	0.00												

Significance levels: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

[a] *DSH* Days sowing-heading, *TKW* thousand-kernel weight (g), *PRT* Kjeldahl protein (% dry matter), *LDG* lodging (observed at Dundee only), *D02* Dundee 2002, *L01* Lleida 2001, *L02* Lleida 2002

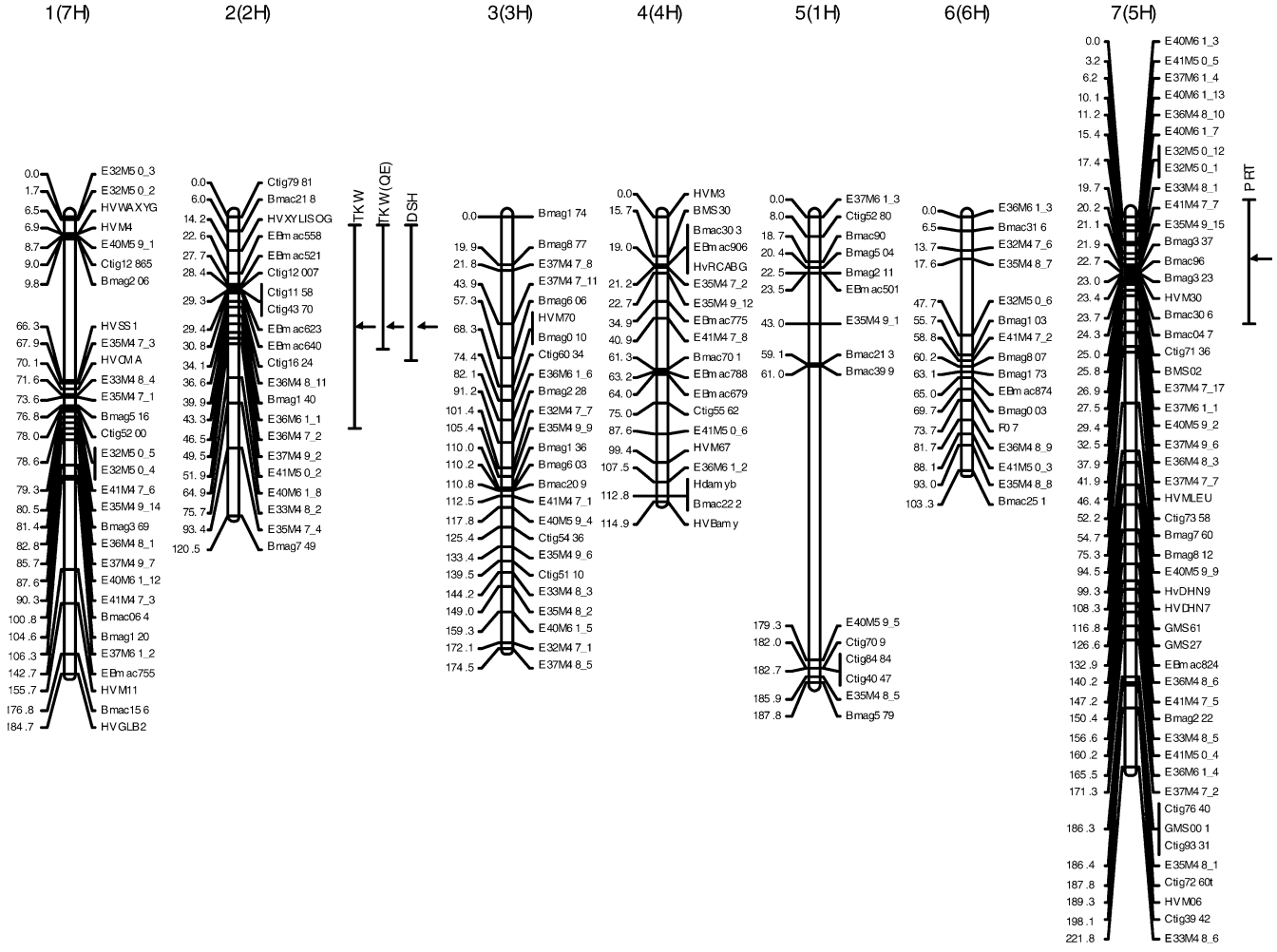

Fig. 2 Linkage map based on a mapping population of 102 doubled-haploid lines derived from the Beka × Logan barley cross. Chromosomes are oriented with short arms at the *top* of the figure. Map distances, on the *left side* of the bars, are in centiMorgans. *Arrows* on the *right side* of the bars represent the quantitative trait locus (QTL) position for each trait determined by the test statistic peak. Traits are coded as follows: *PRT* protein content (QTL main effect); *DSH* days sowing-heading (QTL main effect); *TKW* thousand-kernel weight (QTL main effect); *TKW(QE)* TKW (QTL × E Interaction). *Vertical bars* represent the confidence intervals derived from the tests for simple interval mapping for QTL main effects and QTL × environment (*E*) interaction for the traits. Barley chromosomes are oriented with the short arm at the *top*. *Horizontal lines* show significant test statistic (thresholds estimated from 3,000 permutations) *Arrows* represent the test statistic peak

barley chromosomes. This figure, together with Table 5, shows also the results of the QTL analysis carried out for DSH, TKW and PRT, because for YLD no QTLs were detected.

For DSH there was one QTL main effect on the centromeric region of chromosome 2H (Fig. 2), located at SSR marker Ebmac623, but with the ESTs scssr12203, scssr02236 and scssr03381 in its vicinity

Table 5 Quantitative trait locus (QTL) main effects in the Beka × Logan doubled haploid progeny across three environments

Trait	Chromosome	Closest marker	Test statistic[a]	Position[b] (cM)	LOD[c]	Allelic effect[d]	Variance explained (%)[e]
PRT	7(5H)	Bmac96	30.7	17	4.5	0.67(B)	18
Days sowing-anthesis	2(2H)	EBmac623	124.9	29	11.6	4.6(B)	40
TKW	2(2H)	EBmac623	92.2	29	9.4	3.1(L)	34

[a]Test statistic with MQTL (Tinker and Mather 1995). Threshold significance at $P \leq 0.05$

[b]QTL position within each chromosome determined by the test statistic peak

[c]According to Tinker and Mather (1995), LOD score is approximately equivalent to the test statistic averaged over environments divided by 2 ln(10)

[d]Positive effect attributed to: *B* Beka, *L* Logan

[e]Percentage of variance explained by each QTL

(Table 1). This QTL was at 29 cM (Table 5) with a LOD of 11.6 and explained 40% of variation. The positive allele comes from Beka and increases time to heading by 4.6 days. In the same region of chromosome 2H, a QTL main effect was observed for TKW (Fig 2), with a LOD of 9.4, explaining 34% of variation (Table 5). The effect of this QTL was to increase TKW by 3.1 g, and the positive allele is derived from Logan. There was a QTL main effect for PRT on the centromeric region of chromosome 5H, marked by the SSR Bmac96 at 17 cM, and with the EST scssr18076 in its vicinity (Figs. 2, 3; Table 1). This QTL has a LOD of 4.5 and explains 18% of variation, its positive allele, increasing PRT content by 0.67%, coming from Beka (Table 3). No association with YLD was noted for this QTL. Although there were significant negative correlations between YLD and PRT at all sites, the actual variation in YLD explained by PRT content (R^2) varied between 5% at Lleida in 2001 and 24%, also at Lleida in the following year. Given the very diverse nature of the growing environments, we are therefore confident that this QTL represents a genetic effect on PRT content and not a YLD dilution effect. The only character for which more than one QTL main effect was observed was lodging at Dundee 2002. Two factors explaining 35.3% and 15.2% of the variation, respectively, were associated with the regions of chromosomes 2H and 5H already identified for TKW and PRT, above. The factor on 2H accounted for the significant correlation between lodging and both TKW and DSH noted previously, but the factor on 5H was unexpected given the absence of any correlation between lodging and PRT.

There is one case of QTL × E interaction: that of TKW, located at the same region of SSR marker Ebmac623 in the centromeric region of 2H (Fig. 2), where the main effects for DSH and TKW were located. The allele from Logan increased TKW by 4.43 g at D02, 2.18 g at L01 and 2.86 g at L02, so the interaction was of magnitude and is thus likely to be the same allele as that identified as a main effect. In this same region there was a putative QTL × E for PRT that was slightly below the level of statistical significance.

Discussion

PRT variation

We have found a QTL for grain PRT content in the centromeric region of chromosome 5H, whose nearest marker, Bmac96, is in bin 6 in the Oregon Wolfe Barley map (http://www.barleyworld.org). On the other hand, nitrate reductase genes *nar2* and *nar5* are in bin 6 (Kleinhofs map, http://www.barleygenomics.wsu.edu); therefore, this QTL is near these nitrate reductase genes.

This region of chromosome 5H has been shown by other workers to harbour QTLs for grain PRT content, i.e. Marquez-Cedillo et al. (2000) with the cross Harrington × Morex, two-row × six-row North-American genotypes; Emebiri et al. (2003) with the cross VB9254 × ND11231-12, two-row Australian × two-row North-American sister line of Logan and, thus, considered it to be Karl-derived. See et al. (2002) found a QTL for PRT on the short arm of 6H marked by the SSR Hvm74, carrying the Karl allele that decreases PRT by 1.3% and hypothesised that this could be the main Karl low-protein gene. These authors also noted, however, that Karl appeared to derived its low PRT by transgressive segregation, so more than one factor would be present. Although Karl has been used as a parent to produce low-PRT progeny (Goblirsch et al. 1996), there has been limited success within North American six-row germplasm (See et al. 2002). See et al. (2002) also indicated that the low-protein gene on chromosome 6H in Karl had no pleiotropic effects, but noted other loci that permitted low PRT to be selected in conjunction with higher YLD, earlier heading and shorter straw. As the latter two factors are readily selected early in breeding programmes, it seems likely that these loci would be actively selected, while inheritance of the low PRT factor on chromosome 6H may have been more random. The absence of an effect on PRT on chromosome 6H here (we did not find Hvm74 to be polymorphic in our mapping population) or in a sister line of Logan (Emebiri et al. 2003) suggests that this particular genetic factor has been lost at some stage in the breeding process.

Emebiri et al. (2003) suggested that selection of this character could be difficult at sites where PRT was naturally low and correlation between PRT content and YLD or other agronomic factors could influence phenotypic expression of PRT content. This highlights the problem with selection for quantitative characters whose phenotype is influenced by environment and genotype × environment interaction as well as genetic factors. Growing genotypes under conditions to which they are well adapted, and under which, for example, they will give a strong phenotypic expression of YLD, can thus lead to over-estimation of the genetic component. Ideally this could be avoided by increasing the number of sites and seasons over which observations are made, but using a smaller number of environments, but ensuring adequate variation between them, can also be effective. The varied effects of early heading upon YLD at the three environments used here is indicative that environmental and genotype × environment effects differed markedly between the sites and that we were thus able to dissect out genotypic differences.

Interestingly, in none of these cases, nor in the present paper, was any QTL found on chromosome 1H, in the region of the structural hordein genes. It seems as if we may be dealing with regulatory genes, such as *nar2* and *nar5*. Nitrate reductase catalyses the reduction of NO_3 to NH_4; therefore, it is a key enzyme in the nitrogen assimilation pathway. It is considered to be the rate-limiting step in the assimilation of NO_3, because it initiates the reaction and is the logical point of control when NO_3 is available (Below 1995). Alternatively, we could state that the QTL on 5H is not the

main low-protein gene from Karl, which is actually located at 6H, and the two parents here did not segregate for this locus.

The effect of our QTL, explaining 18% of the variance, is similar to the estimate of 21% by Emebiri et al. (2003). It cannot be compared to the one detected by Marquez-Cedillo et al. (2000), because they gave only the total effect of the three QTLs they detected (38% explained variance). The QTL for PRT located in the short arm of 2H by Marquez-Cedillo et al. (2000), flanked by *vrs1* (bin 10) and MWG503 (bin 11), was related to the two-row/six-row character for which the population was segregating, therefore does not seem to be coincident with our QTL on this chromosome.

Grain size and earliness

We have found one QTL for DSH on the centromeric region of chromosome 2H with its peak at the SSR marker Ebmac623 and explaining 40% of variance. EBmac623 is close to HVBKASI in the Australian consensus map (Karakousis et al. 2003). HVBKASI signals the QTLs for heading date and PRT by See et al. (2002), which they claimed to be close to the photoperiod response gene *Ppd-H1*. However, HVBKASI is in bin 8 (Kleinhofs map, http://www.barleygenomics.wsu.edu), and the photoperiod response gene *Eam6* is in bin 8 (Franckowiak and Lundqvist 2002), while *Ppd-H1* is flanked by RFLPs MWG858 and BCD221B (Laurie et al. 1995), but these markers are in bin 4 (Kleinhofs map, http://www.barleygenomics.wsu.edu). We can thus conclude that our QTL is near *Eam6*, not *Ppd-H1*.

We have reported above that the same region of chromosome 2H, around the SSR marker Ebmac623, is related to grain weight, harbouring a QTL and a QTL × E for TKW. The main effect is high, with 34% of variance explained. In both cases, the Logan allele is increasing grain weight, in association with its earliness, induced by *Eam6*, a gene that appears to operate under autumn-sowing conditions in Lleida, but also under long-day conditions, as at Dundee. In the latter case, its effect is smaller than that of *Ppd-H1* (Tohno-oka at al. 2000). What is intriguing is the lack of direct effect of *Eam6* on PRT in our case, as no QTL was identified there except a QTL × E below the LOD threshold level. Moreover, looking at the correlation matrix (Table 3) there was no correlation whatsoever between PRT and TKW, despite negative correlations between PRT and DSH and between DSH and TKW. A possible explanation of this apparent contradiction is that Logan's allele at chromosome 2H is increasing earliness and grain weight and, at the same time, its allele at 5H is decreasing grain PRT content. The unexpected slightly higher PRT in Logan compared to Beka at D02 could also be interpreted by the joint action of both QTLs, those of 2H and 5H, because as Tohno-oka at al. (2000) reported, the effect of *Eam6* is smaller under long day than under autumn-sowing conditions. However, it may also be that, if the low PRT expression in Logan relates to the rate of assimilation into the ear, it may have little effect during the relatively slow grain filling that occurs in Scotland compared to Spain, unless the period is truncated by later ear emergence. The absence of any association between DSH and PRT in Lleida in 2002 suggests that the nature of association between the two factors is unlikely to be genetic.

The detection of two loci with very large effects suggests that, rather than QTLs, major genes may have been detected in this study. One, the Karl-derived low-protein gene present in Logan, appears to be strongly affected in its expression by environmental conditions. The fact that a number of Logan derived characters were inherited together may reflect the different seasonal adaptation in the two varieties—the Spanish genotype better adapted to autumn sowing and the North American one to short season (spring sowing). This was reflected in different alleles at the *Eam6* locus (J. Franckowiak, personal communication). Assessment of the influence of such genes on malting quality itself is underway at our laboratory.

Acknowledgements We want to thank Jerry Franckowiak (North Dakota, USA) for his valuable comments and information. The Spanish Group is partially funded by CICYT and INIA (Ministry of Science and Technology). The Scottish Crop Research Institute receives grant-in-aid from the Scottish Executive Environment and Rural Affairs Department (SEERAD).

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