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Chromosomal loci associated with endosperm hardness in a malting barley cross

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Abstract A breeding objective for the malting barley industry is to produce lines with softer, plumper grain containing moderate protein content (9–12%) as they are more likely to imbibe water readily and contain more starch per grain, which in turn produces higher levels of malt extract. In a malting barley mapping population, 'Arapiles' × 'Franklin', the most significant and robust quantitative trait locus (QTL) for endosperm hardness was observed on the short arm of chromosome 1H, across three environments over two growing seasons. This accounted for 22.6% (Horsham 2000), 26.8% (Esperance 2001), and 12.0% (Tarranyurk 2001) of the genetic variance and significantly increased endosperm hardness by 2.06–3.03 SKCS hardness units. Interestingly, Arapiles and Franklin do not vary in *Ha* locus alleles. Therefore, this region, near the centromere on chromosome 1H, may be of great importance when aiming to manipulate endosperm hardness and malting quality. Interestingly, this region, close to the centromere on chromosome 1H, in our study, aligns with the region of the genome that includes the *HvCslF9* and the *HvGlb1* genes. Potentially, one or both of these genes could be considered to be candidate genes that influence endosperm hardness in the barley grain. Additional QTLs for endosperm hardness were detected on chromosomes 2H, 3H, 6H and 7H, confirming that the hardness trait in barley is complex and multigenic, similar to many malting quality traits of interest.

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Introduction

The effect of endosperm hardness in barley (*Hordeum vulgare*) on processing grain for human or animal consumption is well studied (Beecher et al. 2002; Fox et al. 2007a, b; Takahashi et al. 2009; Turuspekov et al. 2008a, b). Soft, plump kernels are generally lower in protein and therefore have more starch per grain. The higher starch content per grain is associated with improved feed barley quality because the starch provides a high metabolizable energy source (Fairbairn et al. 1999; Sola-Oriol et al. 2009). The malting industry also prefers barley to be plump, uniform and moderately soft so that water imbibes readily and evenly into the grain during germination, resulting in malt with a high portion of fermentable sugars (Coventry et al. 2003; Psota et al. 2007). In order to breed for softer grain, the genetic inheritance of barley endosperm hardness and how this relates to barley quality needs to be elucidated.

For classifying and processing bread wheat, much importance is also placed on endosperm hardness for which

a large portion of the genetic variance has been attributed to the *Hardness* (*Ha*) locus on chromosome 5D (Morris 2002; Turner et al. 2004). *Ha locus component* genes include *Puroindoline-a* (*Pina-D1*) and *Puroindoline-b* (*Pinb-D1*) and *Grain Softness Protein* (*GSP-D1*). These genes encode the grain softness proteins, Puroindoline A (PinA), Puroindoline B (PinB) and Grain Softness Protein-1 (GSP-1), respectively (Bhave and Morris 2008; Gautier et al. 1994). Mutations in the tightly linked *Pina-D1* and *Pinb-D1* sequences have been shown to directly influence hardness as shown in a survey of soft and hard bread wheats from near isogenic lines, commercial varieties, breeding lines and landraces (Giroux and Morris 1998; Lillemo and Morris 2000). Also, a quantitative trait locus (QTL) in the *Ha* region of the short arm of chromosome 5D contributed 63 and 71% of the phenotypic variation in endosperm hardness over 2 years within a segregating hard/soft hexaploid wheat population of 155 recombinant inbred lines (Igrejas et al. 2002).

Grain softness proteins, similar to the puroindolines, were isolated and identified in barley, and are known as the hordoindolines (Gautier et al. 2000). Genes for the wheat puroindolines on chromosome 5D and the barley hordoindolines on chromosome 5H reside at syntenic chromosomal locations. The *hordoindoline-a* (*Hina*), paralogous *hordoindoline-b1* (*Hinb-1*) and *hordoindoline-b2* (*Hinb-2*) and *Grain Softness Protein* (*GSP*) genes have been isolated from the *Ha* region and sequenced in a range of barley accessions. These accessions include commercial varieties from North America, Europe and Australia, and landraces of spring barley (Beecher et al. 2001; Caldwell et al. 2006; Darlington et al. 2001; Turuspekov et al. 2008a). To date, 5 *Hina*, 6 *Hinb-1*, 18 *Hinb-2* and 18 *GSP* alleles have been reported in varietal surveys (Beecher et al. 2001; Darlington et al. 2001; Fox et al. 2007a; Turuspekov et al. 2008a). Recently, allelic variation at the *Ha* locus was reported to significantly influence endosperm hardness in 32 two-rowed barley accessions, attributed to the pleiotropic effect of this locus on seed size (Turuspekov et al. 2008a).

Genome linkage mapping of a North American doubled haploid (DH) barley population, 'Steptoe' × 'Morex', revealed a QTL on the short arm of chromosome 5H, explaining up to 22% of endosperm hardness (Beecher et al. 2002). The chromosomal location also coincided with a small barley malt-extract QTL, indicating allelic variation of the hordoindoline genes may also affect malting quality (Beecher et al. 2001). Also, genomic regions on chromosome 5H were associated with NIR-predicted endosperm hardness in an Australian barley mapping population ('Patty' × 'Tallon') (Fox et al. 2007b). The hordoindoline region on the 5HS explained a similar amount of the genetic variation in endosperm hardness (20%). In addition to a QTL on chromosome 5HS, another QTL was reported on the long arm of chromosome 5H associated with endosperm hardness within mapping population studies (Beecher et al. 2002; Fox et al. 2007b).

Endosperm hardness in wheat has been reported to be a complex quantitative trait, where many chromosomal regions contribute to the overall hardness phenotype as found in a genetic by environment (G × E) study (Gazza et al. 2008). Similarly, in barley, the genetic control of endosperm hardness is polygenic and influenced by many determinants such as grain weight, grain size (diameter) and grain protein percent (Fox et al. 2007b; Psota et al. 2007; Takahashi et al. 2009; Turuspekov et al. 2008a). Therefore, gene effects other than those associated with the *Ha* locus on chromosome 5HS are expected. Three genomic regions on chromosome 7H found in isogenic lines were associated with grain size, texture and modification during malting by Swanston (1995). Endosperm hardness has been negatively correlated with kernel diameter and kernel weight, with a possible epistatic interaction between the *Ha* locus on chromosome 5HS and the *Vrs1* on chromosome 2H when surveying barley accessions (Turuspekov et al. 2008a). The *Vrs1* gene controls the head type characteristic in barley, with two-row types generally larger seeded and having high starch and lower protein compared to six-row types (Turuspekov et al. 2008b). These differences affect endosperm hardness, grain size, malt extract and friability. In mapping studies, other significant QTLs have been reported on chromosomes 1H, 4H, and 7H to be associated with endosperm hardness (Beecher et al. 2002) and QTLs were also reported on chromosomes 2H, 3H, 6H and 7H to be associated with NIR-predicted endosperm hardness (Fox et al. 2007b).

The co-location of QTL conditioning different traits thought to be related to hardness may result in pleiotropic gene effects (Groos et al. 2003). Previously, significant correlations have been detected between endosperm hardness and grain size in barley varietal surveys (Nielsen 2003; Turuspekov et al. 2008b) and in a barley mapping population (Thomas et al. 1996). Significant correlations have also been found between endosperm hardness and protein content in wheat mapping studies (Breseghello et al. 2005; Giroux et al. 2000).

The Australian malting varieties Arapiles and Franklin have contrasting endosperm hardness attributes. Therefore, the aims of this study were to (1) determine if the *Ha* genomic region in the 'Arapiles' × 'Franklin' mapping population is associated with the endosperm hardness trait; (2) identify any other major QTLs associated with hardness; and (3) determine if QTLs governing grain size, grain weight and grain protein percent are co-located with barley endosperm hardness QTL.

Materials and methods

The 'Arapiles' × 'Franklin' DH population

The 'Arapiles' × 'Franklin' DH mapping population was developed as a part of the Australian National Barley Molecular Marker (ANBMM) program with the aim to map quality and disease traits (Panozzo et al. 2007; Reinheimer et al. 2004). *Cv*. Arapiles was released in 1994 and has the pedigree Noyep/Proctor/2/CI3576/Union/4/Kenia/3/Research/2/Noyep/Proctor/5/Domen, with moderate grain softness and moderate grain size (unpublished data). *Cv*. Franklin was released in 1989, derived from a cross between *Cv*. Triumph and *Cv*. Shannon with harder grain and a smaller size (unpublished data).

Trials

The parents, Arapiles and Franklin, and up to 180 of the DH lines were grown at three field locations over 2 years (Horsham, 2000; Esperance, 2001; and Tarranyurk, 2001; Tables 1, 2).

Phenotypic data collection

Grain traits consisting of endosperm hardness, size, 1,000 grain weight and grain protein percent were collected on parental and DH lines in all three trials. The endosperm hardness for all lines grown at Horsham (2000), Esperance (2001), and Tarranyurk (2001) was measured by an SKCS 4100 (Single Kernel Characterisation System, Perten Instruments, USA). The SKCS measurements were performed on 150 kernels of grain cleaned over a 2.2-mm screen.

Grain weight was defined as the mass of 1,000 individual grains (g) (EBC 1998a). The grain size of barley as defined by industry standards was determined by size fractionation through three fixed-width slotted sieves, 2.2, 2.5 and 2.8 mm (EBC 1998b). Plump grain was determined by weighing the percentage of the sample above a 2.5-mm slotted sieve (w/w). Thin grain, or screenings, was determined by weighing the percentage of sample below a 2.2-mm slotted sieve (w/w). Grain protein percent was measured on whole grain using a NIR Systems 6500 machine (Foss Pacific Pty Ltd, USA) as previously described (Panozzo et al. 2007), and expressed as % protein on a dry basis.

Table 1 Mean values for endosperm hardness, percent grain plumpness, percent screenings, 1,000 grain weight and grain protein percent in the 'Arapiles' × 'Franklin' population

Year and environment	Endosperm hardness (SK units)	Plump grain (>2.5 mm, %w/w)	Screenings (<2.2 mm, %w/w)	1,000 grain weight (g)	Grain protein (dry basis, %)
2000 Horsham, 117 doubled haploid lines grown					
Arapiles	58.8	93.79*	2.26*	48.16*	10.02*
Franklin	59.6	56.90*	4.87*	39.84*	9.31*
Minimum value, doubled haploid	37.4	51.93	0.48	36.50	8.47
Maximum value, doubled haploid	68.3	98.50	5.63	51.15	13.85
Mean value, doubled haploid	57.5	87.98	1.67	44.69	10.08
Standard error of the mean	2.1	2.50	0.53	0.73	0.41
2001 Esperance, 173 doubled haploid lines grown					
Arapiles	57.9*	98.81*	0.10*	51.16*	10.46
Franklin	64.3*	79.77*	2.31*	43.69*	10.23
Minimum value, doubled haploid	41.7	73.46	0.00	38.83	9.53
Maximum value, doubled haploid	77.5	99.50	3.50	53.34	12.11
Mean value, doubled haploid	59.1	94.70	0.43	45.97	10.46
Standard error of the mean	2.5	2.09	0.24	1.33	0.33
2001 Tarranyurk, 180 doubled haploid lines grown					
Arapiles	74.1*	97.47*	0.38	49.28*	10.62
Franklin	82.8*	90.80*	0.57	44.91*	10.66
Minimum value, doubled haploid	59.8	69.08	0.16	39.43	9.72
Maximum value, doubled haploid	94.2	98.51	2.75	54.50	12.82
Mean value, doubled haploid	76.4	93.02	0.57	46.30	10.80
Standard error of the mean	2.1	1.79	0.16	0.99	0.41

* Arapiles and Franklin were significantly different at the 0.01 probability level

Table 2 Reported significant quantitative trait loci (QTLs) for endosperm hardness (SKCS units), percent plump grain (>2.5 mm, %w/w), percent screenings (<2.2 mm, %w/w), 1,000 grain weight (g), and grain protein (%) analyzed in the 'Arapiles' × 'Franklin' population grown at three environments: Horsham (2000), Esperance (2001), and Tarranyurk (2001)

Environment	Chr	Closest marker	Contributing parent	LOD	Size	Gen. var. (%)
Endosperm hardness (SK units)						
Esperance	1H	P12/M50-87	Arapiles	>15.0	3.03	26.8
Tarranyurk	1H	Bmag0345	Arapiles	11.23	2.28	12.0
Horsham	1H	0211F	Arapiles	5.44	2.06	22.6
Tarranyurk	2H	GBM1214	Franklin	>15.0	−4.32	42.9
Esperance	3H	P11/CGT109	Franklin	4.02	−1.46	6.2
Tarranyurk	3H	P14/M47-166	Franklin	3.70	−1.30	3.9
Esperance	6H	P11/M47-253	Franklin	4.29	−1.52	6.7
Esperance	7H	P14/M59-297	Franklin	7.81	−2.06	12.4
Tarranyurk	7H	P13/M60-134	Franklin	8.90	−2.12	10.3
Screenings (<2.2 mm, %w/w)						
Esperance	1H	P12/M50-87	Arapiles	5.49	0.14	16.3
Tarranyurk	2H	Bmac218	Franklin	4.44	−0.11	13.3
Esperance	2H	Bmag518	Franklin	4.65	−0.13	12.6
Tarranyurk	5H	GBMS141	Arapiles	7.68	0.15	22.1
Plump grain (>2.5 mm, %w/w)						
Esperance	1H	Bmag105	Franklin	5.80	−1.20	12.1
Horsham	2H	GBM1459	Arapiles	9.28	6.08	47.5
Tarranyurk	2H	Bmag518	Arapiles	11.66	2.12	27.7
Esperance	2H	Bmag518	Arapiles	9.00	1.52	19.4
Esperance	6H	P14/M61-62	Arapiles	3.75	0.96	7.8
Esperance	7H	P13/M60-134	Arapiles	3.30	0.95	7.6
1,000 grain weight (g)						
Horsham	2H	GBM1459	Arapiles	14.93	2.28	51.0
Esperance	2H	Bmag518	Arapiles	>15.0	1.45	30.3
Tarranyurk	2H	P14/M48-337	Arapiles	>15.0	1.79	42.3
Esperance	5H	P11/M62-248	Arapiles	5.13	0.74	7.9
Tarranyurk	5H	P14/M48-259	Arapiles	4.01	0.71	6.7
Esperance	6H	P14/M61-62	Arapiles	6.03	0.81	9.3
Esperance	7H	P13/M53-61	Franklin	4.01	−0.71	7.3
Grain protein (%, dry basis)						
Esperance	2H	Bmag745	Arapiles	7.31	0.16	15.4
Tarranyurk	3H	P13/M62-299	Franklin	5.55	−0.16	14.1
Esperance	3H	P14/M49-210	Arapiles	12.05	0.19	22.3
Esperance	4H	GBM1027	Franklin	3.52	−0.11	6.7
Tarranyurk	4H	GBM1027	Franklin	7.35	−0.18	17.5
Esperance	7H	P13/M60-134	Arapiles	5.78	0.14	12.3
Horsham	7H	P14/M49-447	Arapiles	4.44	0.31	18.7
Tarranyurk	7H	P14/M51-228	Arapiles	3.86	0.13	9.3

Chr chromosome number, *nearest marker* marker nearest the QTL, *contributing parent* parent contributing the alleles, *LOD* logarithm of the odds, *size* the difference between the two parental allele classes, measured in the units of the trait, *Gen. var. (%)* the percentage of genetic variation for that trait attributed to that QTL at that environment

The 'Arapiles' × 'Franklin' linkage map

A genetic linkage map, constructed with 323 AFLP, RFLP and microsatellite markers scored across 225 DH lines, was used (Panozzo et al. 2007). This map was drawn using MapChart (Voorrips 2002).

Before QTL analysis, redundant markers were removed whilst ensuring that there was approximately one marker every 10 cM. Microsatellite markers were retained where possible, over AFLP and RFLP markers. In total 277 markers over the 7 chromosomes were retained for QTL analysis.

QTL analysis

QTL analysis was performed for each trait/site combination using the ASREML-R package in the R statistical environ-

ment (R-Development-Core-Team 2009) method as described in Panozzo et al. (2007). An advantage of this method is that non-genetic components such as spatial variation are estimated simultaneously with QTL effects. From these models, QTL significances were calculated using LOD scores, and QTL peaks with LOD scores greater than 3.0 were identified along with flanking markers.

DNA isolation and sequencing of the *Ha locus component genes*

Genomic DNA was extracted from Arapiles and Franklin plants and PCR amplifications were performed according to the method described by Cane et al. (2004). *Hina* sequences were amplified using primers described by Beecher et al. (2001), the *Hinb-1* and *Hinb-2* sequences were amplified using primers described by Turuspekov et al. (2008a) and the *GSP* sequences were amplified using primers described by Caldwell et al. (2004). *Hina* primers were annealed at 60°C, *Hinb-1* and *Hinb-2* specific primers at 66.6°C, and *GSP* primers at 66.6°C, with at least three independent PCR reactions performed to characterize the two cultivars. Sequencing using an AB 3730xl-96 capillary sequencer was carried out by the Australian Genome Research Facility (AGRF, Brisbane, Australia).

Map alignment chromosome 1H

For chromosome 1H, the 'Arapiles' × 'Franklin' map was aligned with two high-density SNP maps (Voorrips 2002). These were from an integrated SNP and SSR maps which contained 80 loci and the 'Derkado' × 'B83-12/21/5' SNP and SSR maps, which contained 70 loci (Ramsay 2010; Stein 2010; Thomas 2010).

Results

Grain phenotypes and variations

Franklin had significantly harder, had smaller grain size with a lower grain weight than Arapiles when grown at Esperance (2001) and Tarranyurk (2001) (Table 1). The DH progeny was more diverse than Arapiles and Franklin in grain size, softness, percent plump grain, and percent screenings. Mean endosperm hardness for samples grown at Tarranyurk (2001) was greater than samples from the trials grown at Horsham (2000) and Esperance (2001).

A wider variation was observed in endosperm hardness within the DH population and parental lines grown at Esperance (2001) and Tarranyurk (2000) than those grown in Horsham (2000). The endosperm hardness distribution of the DH progeny compared to the parents grown at all three sites indicated quantitative inheritance, and was influenced by environmental factors (Fig. 1). Lines grown at Tarranyurk (2001) and Esperance (2001) tended to be plumper and have lower percent screening values (Table 1) than lines grown at Horsham (2000). In addition, lines grown at Horsham (2000) had more variable grain protein percent than those grown at Esperance (2001) and Tarranyurk (2001).

QTL mapping

Multiple QTLs were observed across the seven chromosomes for all of the physical grain traits and from all of the trials (environments) assessed (Fig. 2; Table 2).

Endosperm hardness QTL

A major QTL was detected for endosperm hardness on chromosome 1H in all three environments. This accounted

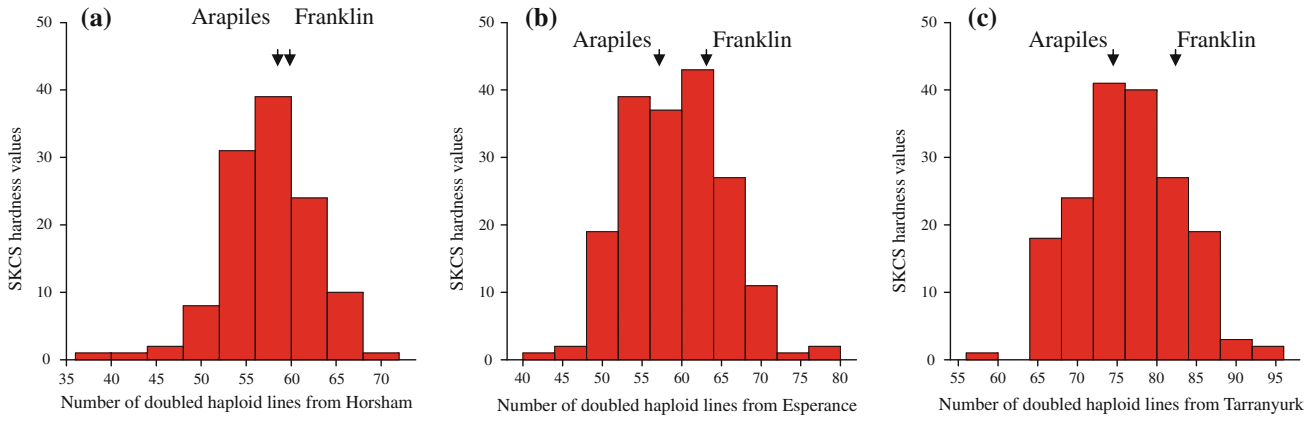

Fig. 1 Frequency and distribution of endosperm hardness for the 'Arapiles' × 'Franklin' mapping population grown at three environments: **a** Horsham 2000, **b** Esperance 2001, and **c** Tarranyurk 2001. The *arrows* indicate the average endosperm hardness values for Arapiles (the soft parent) and Franklin (the hard parent)

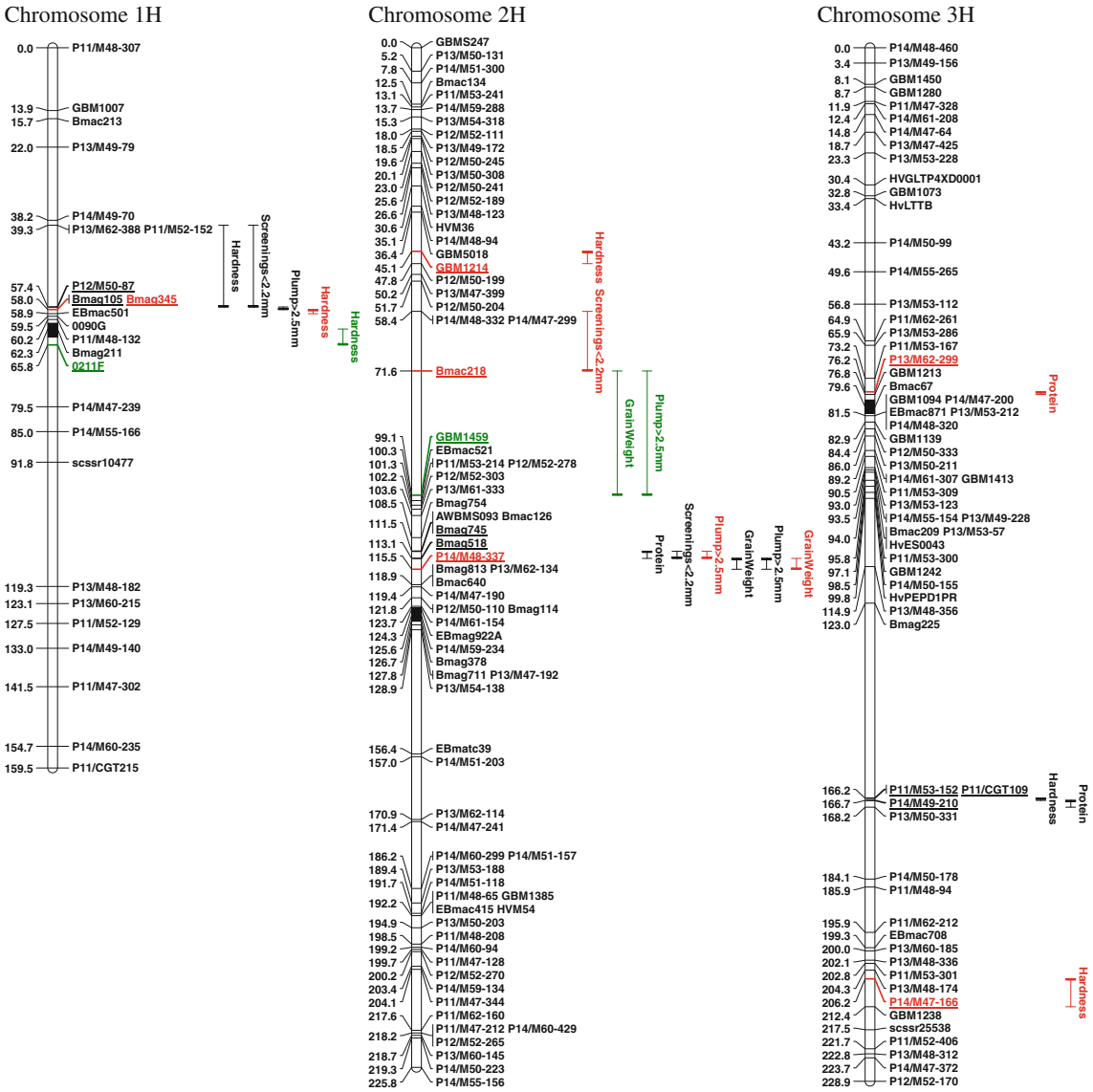

Fig. 2 The genetic map of the 'Arapiles' × 'Franklin' mapping population and associated significant quantitative trait loci (QTLs) observed for endosperm hardness (hardness), percent plump grain (plump > 2.5 mm), percent screenings (screenings < 2.2 mm), 1,000 grain weight (grain weight), and grain protein percent (protein) analyzed at three environments, Horsham 2000 (*green*), Esperance 2001 (*black*), and Tarranyurk 2001 (*red*). Markers are presented to a scale of genetic distance (centiMorgans)

for 22.6% (Horsham 2000), 26.8% (Esperance 2001), and 12.0% (Tarranyurk 2001) of the genetic variance (Table 2). This QTL was the largest and most consistent for endosperm hardness to be identified at all three environments. This QTL was contributed by Arapiles and significantly increased endosperm hardness by 2.06–3.03 SKCS hardness units. The endosperm hardness QTL detected on chromosome 1H at Esperance (2001) was coincident with QTL detected at Esperance for percent screenings (nearest marker P12/M50-87), also contributed by Arapiles alleles, and for plump grain (nearest marker Bmag105, 0.6 cM apart) contributed by Franklin alleles.

A major QTL was detected on chromosome 2H (nearest marker GBM1214), which explained 42.9% of the genetic variance in endosperm hardness for lines grown at one site, Tarranyurk (2001). Franklin alleles at this locus contributed to an increase in SKCS hardness by 4.3 units.

Two additional QTLs for endosperm hardness were detected on chromosome 7H, which explained 12.4 and 10.3% of the genetic variation in lines grown at two sites, Esperance (2001) and Tarranyurk (2001), respectively. Franklin alleles contributed an increasing effect at both loci, increasing hardness by 2.1 and 2.1 SKCS units, respectively. These QTLs were in different regions of the chromosome,

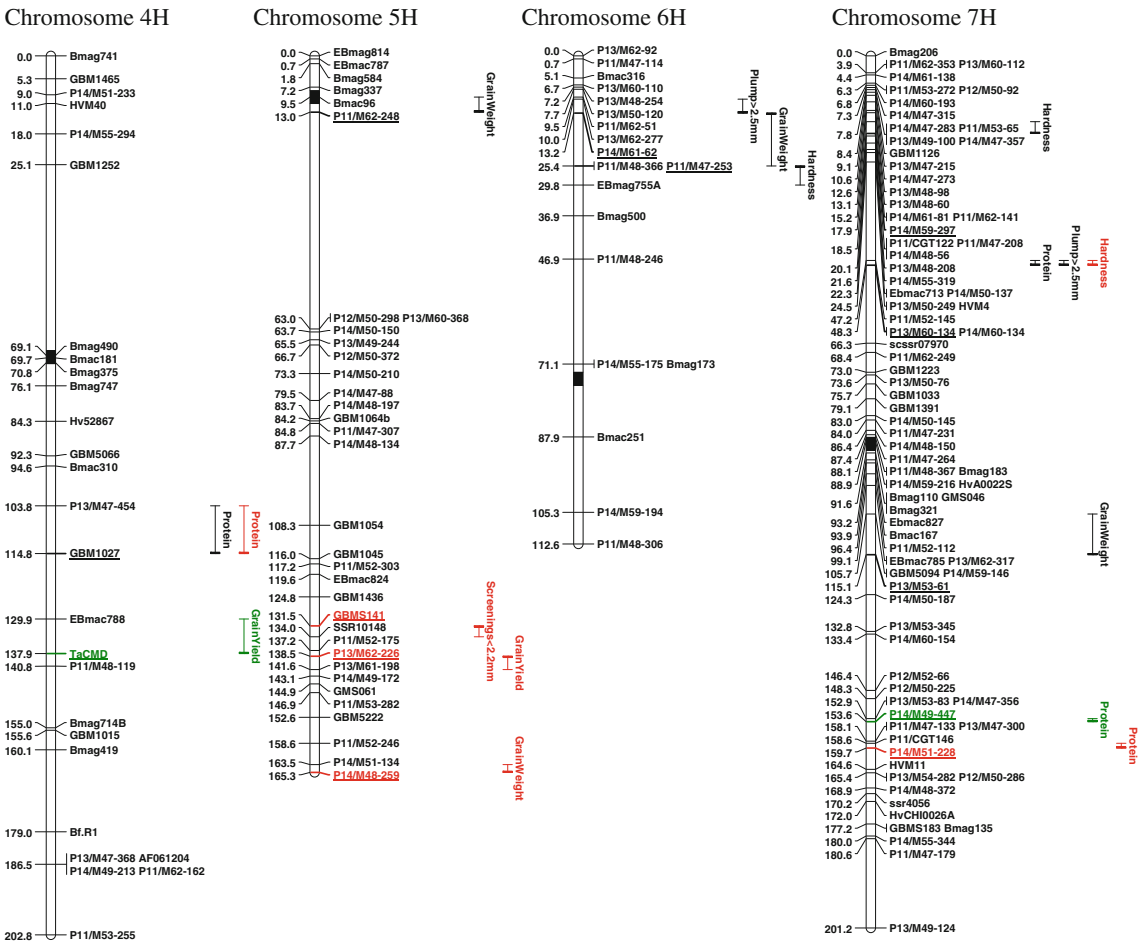

Fig. 2 continued

30.4 cM apart, with the nearest marker to the Esperance (2001) QTL being P14/M59-297, whilst the Tarranyurk (2001) QTL was associated with the P13/M60-134 allele. The endosperm hardness QTL (Tarranyurk 2001) was at the same location as QTL for plump grain and grain protein percent detected at Esperance (2001) with Arapiles contributing the alleles for both traits.

Franklin alleles were associated with an increase in endosperm hardness at a QTL on chromosome 6H detected when the population was grown at Esperance (2001). This explained 6.7% of the genetic variation and the nearest marker was P11/M47-253. QTLs for percent plump grain and 1,000 grain weight were also detected in the same region, with Arapiles contributing the alleles associated with increased plump grain and grain weight.

Two QTLs for endosperm hardness were detected on the long arm of chromosome 3H for lines grown at Esperance (2001) nearest marker P11/CGT109 and Tarranyurk (2001) nearest marker P14/M47-166, 40.0 cM apart. The QTL explained 6.2 and 3.9% of the genetic variation, respectively. Franklin alleles, also, contributed an increasing effect at both QTLs and the hardness QTL was in the same region as a QTL, contributed from Arapiles alleles, for grain protein percent detected in the Esperance (2001) environment.

Ha locus component genes on chromosome 5HS

No significant endosperm hardness QTL was detected at the *Ha* locus on chromosome 5HS in the 'Arapiles' × 'Franklin' population. To confirm if the allele state of the *Ha* locus between the parents of the DH population varies and possibly influences endosperm hardness, full length sequences of the *Ha locus component* genes were obtained. The two cultivars, Arapiles and Franklin, did not differ from each other in the nucleotide sequences for the *Ha locus component* genes, *Hina*, *Hinb-1*, *Hinb-2* and *GSP*. *Hina-b*, *Hinb-1a* and *Hinb-2a* alleles were present in both cultivars, and aligned with those derived from Morex, GenBank accession numbers AY644179 for *Hina-b* and AY644056 for *Hinb-1a* and *Hinb-2a* (Caldwell et al. 2006). The *GSP-i* allele was present in the parents and aligned

with the gene derived from the cultivar Harrington, GenBank accession number AY644276 (Caldwell et al. 2006). Therefore, QTL found in this study on chromosome 1H, 2H, 3H, 6H and 7H had a significant effect on endosperm hardness in the absence of *Ha* locus variation.

Map alignment chromosome 1H

To aid in identifying possible genes that influence endosperm hardness from chromosome 1H, the loci within the significant QTL identified for endosperm hardness, Bmag0105, EBmac0501 and Bmag0211, were aligned with SNP- and SSR-based maps (Fig. 3). The significant interval included an SSR marker, HvBDG associated with (1-3, 1-4)-beta-D-glucanase (Becker and Heun 1995).

Grain size, grain weight and grain protein percent QTL

A major QTL was identified on chromosome 2H between the markers GBM1459 and P14/M48-337, for 1,000 grain weight trait and percent plump grain at all three sites, and for percent screenings at two sites (Esperance and Tarranyurk). The contributing allele, from Arapiles, increased grain weight and percent plump grain for all significant QTLs observed in this region. Franklin alleles were also associated with an increase in percent screenings at this QTL. The QTL on chromosome 2H influenced most of the observed genetic variation for grain weight (>30.3%) and percent plump grain (>19.4%).

For percent plump grain, QTLs were detected on chromosomes 1H and 6H when the population was grown at Esperance (2001), where the overall grain weight was lower than for the trials grown at Horsham (2000) and Tarranyurk (2001). The QTL on chromosome 1H for percent screenings and plump grain explained between 16.3 and 12.1%, of the variation, respectively. At this QTL, Arapiles contributed an allele for higher screenings (0.1%, w/w), whilst Franklin contributed the allele for increased grain plumpness (1.2%, w/w).

Significant QTLs for increased grain protein percent were observed on chromosome 7H in all three environments, contributed by alleles from Arapiles. In contrast, the QTLs for grain protein percent detected at Horsham (explaining 18.7% of the genetic variation) and at Tarranyurk (explaining 9.3% of the genetic variation) were on the long arm of chromosome 7H, whereas the QTL detected at Esperance (2001) (explaining 12.3% of the genetic variation) was on the short arm of 7H. As previously reported by Panozzo et al. (2007), major QTLs were confirmed for grain protein percent on chromosomes 3H, 4H, and 2H, for lines grown at Esperance. In this study, the major QTLs for grain protein content on chromosomes 3H and 4H were verified when the lines were grown at Tarranyurk. Also, the QTLs on chromosome 2H were close to QTL for 1,000 grain weight, percent plump grain and percent screenings for lines grown at Esperance.

Discussion

The differences in endosperm hardness between the parents of the DH population, Arapiles and Franklin, were 6.4 and 8.7 SKCS units at Esperance (2001) and Tarranyurk (2001), respectively. In a previous QTL study using a barley DH population, the parents of the 'Steptoe' × 'Morex' population did not significantly vary from each other in endosperm hardness (Beecher et al. 2002). So, even though Steptoe and Morex were not different in endosperm hardness, their progeny varied by 39.5 SKCS units, where significant QTLs were reported in a number of genomic regions. Small but significant negative correlations (−0.37, $P < 0.01$) were reported between SKCS hardness and dry matter digestibility for 2 years data (Beecher et al. 2002). On average, this translated to an increase of 1% in dry matter digestibility for every decrease in endosperm hardness of 5 SKCS units within the DH progeny. In a G × E study, different advanced barley lines were surveyed and significant negative genetic correlations were also reported between SKCS hardness of barley and malt friability, −0.60 to −0.23 (Osborne et al. 2007). Therefore, even though barley varieties have not been reported as having extreme endosperm hardness values compared to that typical of hard and soft wheats, a small decrease in barley endosperm hardness through conventional breeding may translate to improved quality for the both the feed and malting industries.

Even though the Arapiles and Franklin parents were significantly different in endosperm hardness at Esperance (2001) and Tarranyurk (2001), no significant association between endosperm hardness and the *Ha* region on chromosome 5HS was detected in the progeny DH population. In addition, no sequence variation for the *Ha locus component* genes between the two cultivars, Arapiles and Franklin, was observed. Instead, several other genomic regions contained major QTL governing endosperm hardness, which in total accounted for 52.1% of the variance at Esperance (2001) and 69.1% at Tarranyurk (2001), and were detected across various chromosomal linkage groups. Many of the hardness QTL identified in this study were repeatedly present in different environments, indicating their robust expression. Information on their genomic locations and closely associated molecular markers may be useful for reducing hardness in future breeding efforts for improving malting quality.

Although the *Ha*-like locus on chromosome 5HS (Beecher et al. 2002; Fox et al. 2007b) was not associated

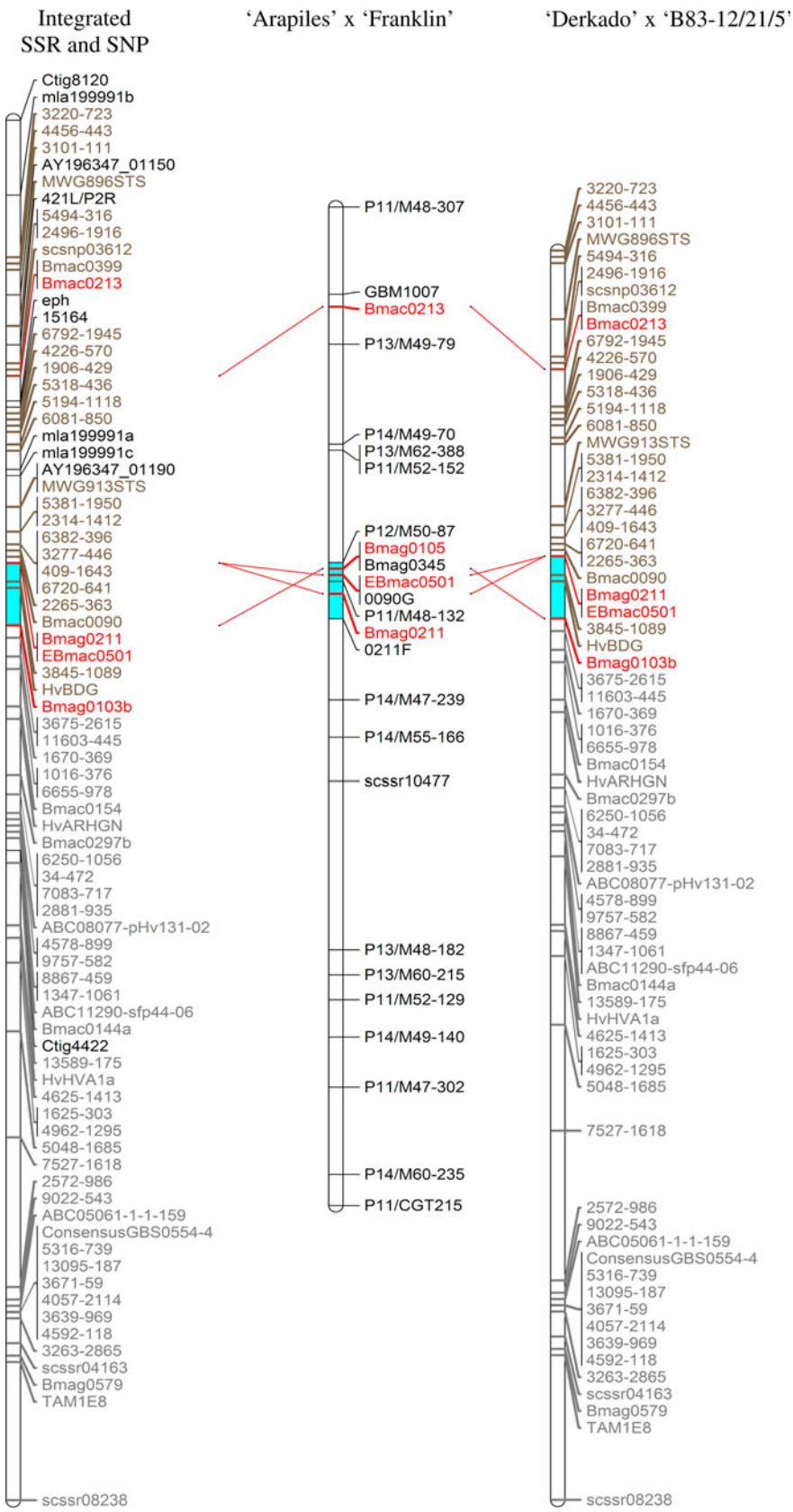

Fig. 3 Alignment for chromosome 1H of the 'Arapiles' × 'Franklin' map (*middle panel*) to an integrated barley SNP and SSR maps (*left panel*) and the 'Derkado' × 'B83-12/21/5' map (*right panel*). The aqua segment is the significant quantitative trait loci (QTLs) observed for endosperm hardness (hardness) at all three environments, loci linking all three maps are in *red*, the loci labeled in *black* are not in the other two maps, and the loci labeled in *gray* found in both the integrated barley SNP and SSR maps and the 'Derkado' × 'B83-12/21/5' map. The Bmag0103b marker is the distal to Bmag0105; therefore, Bmag0105 from the 'Arapiles' × 'Franklin' map was aligned to Bmag103b in the SSR maps

with endosperm hardness variation in this population, the involvement of the functionality of the hordoindolines cannot be discounted in the barley hardness trait. Arapiles and Franklin are both highly selected commercial malting varieties, and this is most likely why there is a lack of variation in the nucleotide sequence for the three genes at the *Ha* locus. Indeed, only two different haplotypes in the *Hina* and *Hinb* (*Hinb-1* and *Hinb-2*) genes were detected in a survey of 40 Australian commercial malting varieties (Fox et al. 2007a). Also, there may be variant forms of the *hordoindoline* genes at remote loci; e.g., in wheat, the existence of expressed *Pin*-related genes has been found on chromosome 7A, where small grain hardness QTLs have been reported in two mapping populations (Wilkinson et al. 2008). In addition, the variation in hardness values, as measured by the SKCS, could be a result of a combination of other traits in barley, such as husk thickness and husk adherence to the endosperm, beta-glucan content, arabinoxylan content, protein composition and content, or grain diameter. Any of these grain components may have a greater influence on the SKCS hardness value than the functionality of the hordoindolines in the 'Arapiles' × 'Franklin' population.

The largest and most consistent QTL associated with barley endosperm hardness in this study, detected in the DH population when grown in all three environments, was on chromosome 1HS near the centromere. This coincides with a previously reported QTL for SKCS hardness in a 'Steptoe' × 'Morex' barley population (Beecher et al. 2002) and a minor QTL reported in a 'Patty' × 'Tallon' population (Fox et al. 2007b), indicating that an understanding of the alleles present at this locus could allow effective marker-based selection for endosperm hardness. The same genomic region on chromosome 1HS co-locates with major malting quality QTL detected in the 'Arapiles' × 'Franklin' DH population at which the Franklin allele had beneficial effects on all malting quality traits (Panozzo et al. 2007). In addition, this coincides with QTL reported on chromosome 1H associated with variation in diastatic power reported in the 'Steptoe' × 'Morex' DH population (Beecher et al. 2002).

Interestingly, whilst Arapiles produces a softer, plumper grain than Franklin, at the QTL on chromosome 1H with the major effect on endosperm hardness, Arapiles contributed the alleles for increased hardness and reduced grain plumpness. Hence, due to the degree of transgressive segregation within this population, progeny has been identified with softer and plumper grain than the superior parent. Therefore, lines with the Franklin alleles, at this locus, tended to have softer plumper grain, which is likely to increase hot water extract. This study and other mapping studies (Beecher et al. 2002; Edney and Mather 2004; Emebiri et al. 2004; Laidò et al. 2009; Panozzo et al. 2007; Rajasekaran et al. 2004) have indicated that the region near the centromere on chromosome 1H is of great importance in the determination of malting quality. In this study, we have shown that this region is also associated with grain size and endosperm hardness with a pleiotropic relationship between the traits.

In this study, the focus was on chromosome 1H, as the QTLs for endosperm hardness were found to be consistent at all three environments, whereas the QTL associated with endosperm hardness on chromosome 2H, even though it accounted for 42% of the genetic variation, was only significant at the Tarranyurk location. As the whole-genome sequence of barley is not yet available, potential candidate genes on chromosome 1H that influence endosperm hardness may be predicted by aligning our target mapping population with other SSR and SNP barley maps (Szücs et al. 2009). The HvBDG locus flanked the endosperm hardness QTL in the aligned SSR and SNP barley maps when compared to the 'Arapiles' × 'Franklin' map. Interestingly, the HvBDG locus was previously associated with QTL for milling energy, grain splitting, grain weight, size and shape in the 'Tankard' × 'Livet' mapping population (Rajasekaran et al. 2004). Also, this region is associated with beta-glucan content in barley grain (Han et al. 1995). All of these barley grain traits have been reported to influence endosperm hardness in varietal studies (Gamlath et al. 2008; Turuspekov et al. 2008a). Interestingly, this region, close to the centromere on chromosome 1H, the map in our study, aligns with the *HvCslF9* (close to EBmac 501) and *HvGlb1* genes (close to Bmag345), which encodes barley (1,3;1,4)-beta-D-glucan endohydrolase isoenzyme EI (Burton et al. 2008; Emebiri 2009). The *HvCslF9* gene is involved in cell wall deposition during the early stages of grain development, and the *HvGlb1* gene is also expressed in the developing endosperm, where it is involved in (1,3;1,4)-beta-glucan synthesis (Burton et al. 2006, 2008). Therefore, one or both of these genes, *HvCslF9* and *HvGlb1*, may be implicated in influencing endosperm hardness in the barley grain.

Grain size and grain protein percent have been reported in varietal molecular studies to influence endosperm hardness in both barley and wheat (Gazza et al. 2008; Turuspekov et al. 2008b) and several of the endosperm hardness QTL detected in the present study co-located or were very close to QTL for percent plumpness, grain protein content, 1,000 grain weight or percent screenings. This may potentially be due to the presence of the *esp2* locus which also maps to this region (Coventry et al. 2003; Walker et al. 2008). The exceptions were the QTL detected on chromosome 2HS for lines grown at Tarranyurk (2002) and on chromosome 7HS for lines grown at Esperance (2002). Both of these coincided with QTL reported in the 'Patty' × 'Tallon' population for endosperm hardness predicted by NIR (Fox et al. 2007b).

Understanding the key physical and biochemical factors that influence endosperm hardness and their impacts on malting and feed quality in barley is of importance to the barley industry. This QTL study highlights the complexity of the genes that influence endosperm hardness. Continued progress in the sequencing of alleles underpinning key traits will improve our ability to predict phenotypic effects. In the case of endosperm hardness, it is clear that, even when all the allelic variations at the *Ha* locus has been fully described, there will remain a need to understand the pleiotropic effects on endosperm hardness of a range of other gene loci that are currently considered to be important only in terms of physical grain size or phenology. Other factors, such as the synthesis of (1,3;1,4)-beta-glucan during grain development, along with the use of gene-based makers, will help define the biochemistry of endosperm hardness and the relationship with key quality traits in barley.

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References

Becker J, Heun M (1995) Barley microsatellites: allele variation and mapping. Plant Mol Biol 27:835–845

Beecher B, Smidansky ED, See D, Blake TK, Giroux MJ (2001) Mapping and sequence analysis of barley hordoindolines. Theor Appl Genet 102:833–840

Beecher B, Bowman J, Martin JM, Bettge AD, Morris CF, Blake TK, Giroux MJ (2002) Hordoindolines are associated with a major endosperm-texture QTL in barley (*Hordeum vulgare*). Genome 45:584–591

Bhave M, Morris CF (2008) Molecular genetics of puroindolines and related genes: allelic diversity in wheat and other grasses. Plant Mol Biol 66:205–219

Breseghello F, Finney PL, Gaines C, Andrews L, Tanaka J, Penner G, Sorrells ME (2005) Genetic loci related to kernel quality differences between a soft and a hard wheat cultivar. Crop Sci 45:1685–1695

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB (2006) Cellulose synthase-like *CslF* genes mediate the synthesis of cell wall (1,3;1,4)-β-D-glucans. Science 311:1940–1942

Burton RA, Jobling SA, Harvey AJ, Shirley NJ, Mather DE, Bacic A, Fincher GB (2008) The genetics and transcriptional profiles of the cellulose synthase-like *HvCslF* gene family in barley. Plant Physiol 146:1821–1833

Caldwell KS, Langridge P, Powell W (2004) Comparative sequence analysis of the region harboring the hardness locus in barley and its colinear region in rice. Plant Physiol 136:3177–3190

Caldwell KS, Russell J, Langridge P, Powell W (2006) Extreme population-dependent linkage disequilibrium detected in an inbreeding plant species, *Hordeum vulgare*. Genetics 172:557–567

Cane K, Spackman M, Eagles HA (2004) Puroindoline genes and their effects on grain quality traits in southern Australian wheat cultivars. Aust J Agric Res 55:89–95

Coventry SJ, Barr AR, Eglinton JK, McDonald GK (2003) The determinants and genome locations influencing grain weight and size in barley (*Hordeum vulgare* L.). Aust J Agric Res 54:1103–1115

Darlington HF, Rouster J, Hoffmann L, Halford NG, Shewry PR, Simpson DJ (2001) Identification and molecular characterisation of hordoindolines from barley grain. Plant Mol Biol 47:785–794

EBC (1998a) Thousand corn weight of barley. European Brewery Convention Analytica. Verlag Hans Carl Geranke-Fachverlag, Nurnberg, Germany

EBC (1998b) Sieving test for barley. European Brewery Convention Analytica. Verlag Hans Carl Geranke-Fachverlag, Nurnberg, Germany

Edney MJ, Mather DE (2004) Quantitative trait loci affecting germination traits and malt friability in a two-rowed by six-rowed barley cross. J Cereal Sci 39:283–290

Emebiri LC (2009) EST-SSR markers derived from an elite barley cultivar (*Hordeum vulgare* L. 'Morex'): polymorphism and genetic marker potential. Genome 52:665–676

Emebiri LC, Moody DB, Panozzo JF, Read BJ (2004) Mapping of QTL for malting quality attributes in barley based on a cross of parents with low grain protein concentration. Field Crops Res 87:195–205

Fairbairn SL, Patience JF, Classen HL, Zijlstra RT (1999) The energy content of barley fed to growing pigs: characterizing the nature of its variability and developing prediction equations for its estimation. J Anim Sci 77:1502–1512

Fox GP, Nguyen L, Bowman J, Poulsen D, Inkerman A, Henry RJ (2007a) Relationship between hardness genes and quality in barley (*Hordeum vulgare*). J Inst Brew 113:87–95

Fox GP, Osborne B, Bowman J, Kelly A, Cakir M, Poulsen D, Inkerman A, Henry R (2007b) Measurement of genetic and environmental variation in barley (*Hordeum vulgare*) grain hardness. J Cereal Sci 46:82–92

Gamlath J, Aldred GP, Panozzo JF (2008) Barley (1-3;1-4)-b-glucan and arabinoxylan content are related to kernel hardness and water uptake. J Cereal Sci 47:365–371

Gautier M-F, Aleman M-E, Guirao A, Marion D, Joudrier P (1994) *Triticum aestivum* puroindolines, two basic cystine-rich seed proteins: cDNA sequence analysis and developmental gene expression. Plant Mol Biol 25:43–57

Gautier M-F, Cosson P, Guirao A, Alary R, Joudrier P (2000) Puroindoline genes are highly conserved in diploid ancestor wheats and related species but absent in tetraploid *Triticum* species. Plant Sci 153:81–91

Gazza L, Taddei F, Corbellini M, Cacciatori P, Pogna NE (2008) Genetic and environmental factors affecting grain texture in common wheat. J Cereal Sci 47:52–58

Giroux MJ, Morris CF (1998) Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. Proc Natl Acad Sci USA 95:6262–6266

Giroux MJ, Talbert L, Habernicht DK, Lanning S, Hemphill A, Martin JM (2000) Association of puroindoline sequence type and grain hardness in hard red spring wheat. Crop Sci 40:370–374

Groos C, Robert N, Bervas E, Charmet G (2003) Genetic analysis of grain protein-content, grain yield and thousand-kernel weight in bread wheat. Theor Appl Genet 106:1032–1040

Han F, Ullrich SE, Chirat S, Menteur S, Jestin L, Sarrah A, Hayes PM, Jones BL, Blake TK, Wesenberg DM (1995) Mapping of β-glucan content and β-glucanase activity loci in barley grain and malt. Theor Appl Genet 91:921–927

Igrejas G, Leroy P, Charmet G, Gaborit T, Marion D, Branlard G (2002) Mapping QTLs for grain hardness and puroindoline content in wheat (*Triticum aestivum* L.). Theor Appl Genet 106:19–27

Laidò G, Barabaschi D, Tondelli A, Gianinetti A, Stanca AM, Nicosia OLD, Fonzo ND, Francia E, Pecchioni N (2009) QTL alleles from

a winter feed type can improve malting quality in barley. Plant Breed 128:598–605
Lillemo M, Morris CF (2000) A leucine to proline mutation in puroindoline b is frequently present in hard wheats from Northern Europe. TAG 100:1100–1107
Morris CF (2002) Puroindolines: the molecular genetic basis of wheat grain hardness. Plant Mol Biol 48:633–647
Nielsen JP (2003) Evaluation of malting barley quality using exploratory data analysis. II. The use of kernel hardness and image analysis as screening methods. J Cereal Sci 38:247–255
Osborne BG, Fox GP, Kelly AM, Henry RJ (2007) Measurement of barley grain rheology for the quality selection of breeding material. J Inst Brew 113:135–141
Panozzo JF, Eckermann PJ, Mather DE, Moody DB, Black CK, Collins HM, Barr AR, Lim P, Cullis BR (2007) QTL analysis of malting quality traits in two barley populations. Aust J Agric Res 58:858–866
Psota V, Vejrazka K, Famera O, Hrcka M (2007) Relationship between grain hardness and malting quality of barley (*Hordeum vulgare* L.). J Inst Brew 113:80–86
Rajasekaran P, Thomas WTB, Wilson A, Lawrence P, Young G, Ellis RP (2004) Genetic control over grain damage in a spring barley mapping population. Plant Breed 123:17–23
Ramsay L (2010) Genetics program. Scottish Crop Research Institute, Invergowrie, Dundee
R-Development-Core-Team (2009) R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. ISBN 3-900051-07-0. http://www.R-project.org
Reinheimer JL, Barr AR, Eglinton JK (2004) QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.). Theor Appl Genet 109:1267–1274
Sola-Oriol D, Roura E, Torrallardona D (2009) Feed preference in pigs: relationship with feed particle size and texture. J Anim Sci 87:571–582
Stein N (2010) Genome diversity. Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany
Swanston JS (1995) Effects on barley-grain size, texture and modification during malting associated with 3 genes on chromosome-1. J Cereal Sci 22:157–161
Szücs P, Blake VC, Bhat PR, Chao S, Close TJ, Cuesta-Marcos A, Muehlbauer GJ, Ramsay L, Waugh R, Hayes PM (2009) An integrated resource for barley linkage map and malting quality QTL alignment. Plant Genome 2:134–140
Takahashi A, Ikeda T, Takayama T, Yanagisawa T (2009) A barley hordoindoline mutation resulted in an increase in grain hardness. Theor Appl Genet 120:519–526
Thomas W (2010) Genetics program. Scottish Crop Research Institute, Invergowrie, Dundee
Thomas WTB, Powell W, Swanston JS, Ellis RP, Chamlers KJ, Barua UM, Jack P, Lea V, Forster BP, Waugh R (1996) Quantitative trait loci for germination and malting quality characters in a spring barley cross. Crop Sci 36:265–273
Turner AS, Bradburne RP, Fish L, Snape JW (2004) New quantitative trait loci influencing grain texture and protein content in bread wheat. J Cereal Sci 40:51–60
Turuspekov Y, Beecher B, Darlington Y, Bowman J, Blake TK, Giroux MJ (2008a) Hardness locus sequence variation and endosperm texture in spring barley. Crop Sci 48:1007–1019
Turuspekov Y, Martin JM, Bowman JGP, Beecher BS, Giroux MJ (2008b) Associations between *Vrs1* alleles and grain quality traits in spring barley *Hordeum vulgare* L. Cereal Chem 85:817–823
Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Heredity 93:77–78
Walker CK, Panozzo JF, Ford R (2008) Quantitative mapping of the chromosomal loci associated with physical grain traits in barley. In: Panozzo JF, Walker CK (eds) Proceedings of the 58th Australian cereal chemistry conference. Grains Research and Development Corporation and AACC International Downunder, Surfers Paradise, Queensland, pp 278–281
Wilkinson M, Wan Y, Tosi P, Leverington M, Snape J, Mitchell RAC, Shewry PR (2008) Identification and genetic mapping of variant forms of puroindoline b expressed in developing wheat grain. J Cereal Sci 48:722–728