CSIRO PUBLISHING
www.publish.csiro.au/journals/fpb *Functional Plant Biology*, 2008, **35**, 427–437

Comparative mapping of a QTL controlling black point formation in barley

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Abstract. The dark discoloration of the embryo end of barley grain (known as black point) is a physiological disorder and the discovery of a quantitative trait locus (QTL) on 2H confirms this trait is controlled genetically. The mechanisms underlying black point tolerance can now be dissected through identification of candidate genes. Comparisons between the QTL identified on chromosomes 2H of barley and 2B of wheat suggest that they are in similar positions near the centromere. *In silico* analysis, using rice, identified genes residing on two comparative chromosomes (4 and 7) of the rice genome. Analysis of the 12.6 Mb region revealed 1928 unique annotations classified into 11 functional categories. Expressed sequence tags (ESTs) with high sequence similarity to enzymes proposed to be involved in black point formation were used to develop restriction fragment length polymorphisms (RFLPs). To ensure an even coverage of markers across the QTL, RFLP markers were also developed from other ESTs. Mapping of these markers has reduced the QTL region from 28 to 18 cM. This study has identified candidate genes for the control of black point formation and paves the way for future research to develop black point resistant barley cultivars.

Additional keywords: barley black point, grain development, *Hordeum vulgare*, synteny.

Introduction

Black point is a dark discolouration of the embryo end of otherwise healthy, mature barley (*Hordeum vulgare* L.) grain. The discolouration occurs in the husk tissue (palea and lemma) and to a lesser extent in the germ aleurone tissue covering the embryo. Black point also affects wheat (*Triticum aestivum* L.) grains in a similar way, with the main difference being that mature wheat grains do not contain outer husk tissue. This results in the discolouration being concentrated in the germ aleurone tissue of wheat grains (Williamson 1997). Although black point has quite distinct symptoms, it is often categorised as kernel discolouration. However, kernel discolouration can also refer to a darkening or caramelisation of the whole grain (Li *et al*. 2003).

Black point of barley and wheat grain is a trait that appears to have a genetic basis with a range of susceptibility observed amongst cultivars when environmental conditions are suitable (Conner and Davidson 1988; de la Pena *et al*. 1999; Hadaway *et al*. 2003). Barley varieties range from the extremely susceptible including Schooner, Keel and Sloop to more tolerant varieties including Alexis, Mundah and Harrington. Black point tolerance has been found to segregate within the North American derived population Chevron × M69 (de la Pena *et al*. 1999) as well as the Australian doubled haploid (DH) mapping population Alexis × Sloop (A. J. Able, T. Zerk, K. R. Walker, unpubl. data). Using Alexis × Sloop, a single putative QTL associated with tolerance to discolouration of the embryo end of the grain has been identified on chromosome 2H over several years (A. J. Able, T. Zerk, K. R. Walker, unpubl. data). Mapping studies in wheat using DH populations derived from Sunco × Tasman and Cascades × AUS1408 identified QTL on chromosomes 2B and 2D, respectively (Lehmensiek *et al*. 2004).

The identification of these QTL provides evidence that black point formation is genetically controlled. Currently, phenotypic assessment is the only method available to screen for black point susceptibility in breeding programs. However, this is costly and often not reliable because of the exact environmental conditions that seem necessary. Understanding the genetic basis of black point formation would therefore be valuable to breeders.

Barley and wheat are from the tribe Triticeae and share a large degree of macro-synteny with one another, notwithstanding 2H and 2B, which are associated with black point in these cereals. It is, therefore, plausible to speculate that if the black point QTL identified within the group 2 chromosomes of barley and wheat are in similar regions, then the same genes might control this trait. However, this hypothesis is currently untested and therefore validation and alignment of these identified QTL is required to

 10.1071/FP08089 1445-4408/08/050427

determine whether the same genome regions are detected in all studies. Further research is then required to determine what genes underlie and control the identified QTL.

Given the lack of fully sequenced genomes for both barley and wheat, it is not surprising that there is limited information about the genes that reside within the identified black point QTL. This is largely because these crops have considerably large genomes (5500 Mb for barley and 17 000 Mb for wheat) (Bennett and Smith 1976) and contain a high level of sequence repetition (Smith and Flavell 1975). Rice (*Oryza sativa* L.), however, has a much smaller and less complex genome comprising 420 Mb that has been fully sequenced (Goff *et al*. 2002). Comparisons between rice, barley and wheat have found high levels of macro-synteny between specific regions of the genome (Sutton *et al*. 2003; Li *et al*. 2004). This allows the rice genome to be used as a tool to predict gene content in wheat and barley.

The major objective of this research was to identify candidate genes within the barley 2H QTL that we have previously associated with black point formation (A. J. Able, T. Zerk, K. R. Walker, unpubl. data). Comparisons between the QTL identified on chromosomes 2H of barley and 2B of wheat were conducted and *in silico* analysis, using rice, enabled the identification of genes that resided within the same area of the rice genome. A selection of genes identified was mapped to the barley genome to confirm the results *in vitro*.

Materials and methods

Comparison of black point and kernel discolouration QTL on barley chromosome 2H

Flanking markers from previously published black point and kernel discolouration QTL on chromosome 2H of barley and 2B of wheat were obtained from the literature (Table 1). Using a barley consensus map (Wenzl *et al*. 2006) and the program CMap (www.genica.net.au, accessed April 2007), the position of all the QTL on chromosome 2H were compared to determine whether they were in corresponding regions. The program CMap was also used to compare the position of a black point QTL on chromosomes 2H of barley and 2B of wheat.

Identifying the level of synteny between the barley black point QTL and the rice genome

Markers were selected that flanked the barley black point QTL on chromosome 2H (PSR131 and ABG14). This region was aligned with other publicly available barley genetic maps using the programs CMap (www.genica.net.au, October 2006 release) and NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview, October 2006 release) to identify additional genetic markers residing within the QTL. Restriction fragment length polymorphism (RFLP) markers that resided within the QTL were selected and the DNA sequence data was obtained from publicly available databases (www.ncbi.nlm.nih.gov and www.graingenes.com).

The sequences of the barley RFLP markers were then used in BLASTn searches (www.ncbi.nlm.nih.gov/BLAST/) against the GenBank rice genome sequence to identify P1-derived artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones with sequence similarity. If this approach yielded no sequence match, the wheat genetic map was used to bridge the barley and rice genomes. This was achieved by aligning the barley and wheat genetic maps using common markers. The region in wheat was subsequently aligned with the wheat deletion expressed sequence tag (EST) bin maps (www.wheat.pw.usda.gov/wEST/binmaps/, August 2007 release) to identify wheat ESTs which were then used in BLASTn searches against the rice genome to identify PAC and BAC clones with sequence similarity. For all BLASTn searches against the GenBank rice genome sequence an *E*-value cut-off significance level of $\leq 1e^{-10}$ was used. To identify syntenous regions between rice and barley

Table 1. PCR primers used to amplify RFLP markers
The optimum primer annealing temperature (Tm °C) and the predicted amplicon size for each primer pair is shown

Marker ID	Forward primer	Reverse primer	Tm (°C)	Size (bp)
Hv.t102	CTGCCTCCGGACATGCTGAAGGAT	GAGAGATTGCCCGCCATTAAGCA	60	527
Hv.t101	CGCAAGGCTGCATTTGAGTACTT	GGACGTCCATCAGAGACTTTGGG	57	404
Hv.tPox1	CAGGATCAACATCCAGATGCCGG	CTCTCCAGCATACCTTCCTGATCTCTGC	59	526
Hv.t81.6	TGAAGGTGTGATCCCATTCCGACA	TCCAGTCTGTGACCCTCTCAAAGGAG	58	420
Hv.t81	TGGTTGAGACCATCGAGGACGGT	CCGCCATCTAACCGTCCATCACT	61	501
Hv.tCsl	CAGAGTTACTTCGAAGCAAACAACGGC	TACACATAGCATATTTGGCTCCCAGC	58	447
BMY2	TGAGGGACTCTGAGCAAAGCGAAG	TACACAGCCACAGGGGCAAGGAG	55	484
Hv.tCHS	ACATCACCCACCTCATCGTCAGCA	GGGTGCACTACCCAGAAGAGGTCGT	59	549
Hv.t71	GATGATCTCCAACCTGGCTTTCG	CATTACTGCTTTGCCTGGGAGTA	60	438
Hv.t70	CCAAATCTTCACCGAGCTTCCGC	TACCCCTCATCATTGAAGGCCGC	61	479
Hv.t67_1	AGAGAAGCTCTGCCCCTGAGCTCCT	CAACAAATTGCCAGGTCCAGTACTTCAG	57	528
Hv.t67	TGCAGAGATGGACAAGATGATCTAGG	GCTCAGCATGAAGTCCAACACTT	56	430
Bcd334	ATGCTGTCAAGAGTTGTGTGAAGACGG	TGTTGTGTGGCAGTACCGTTCTCCA	59	330
Hv.t38	TTTCAAGATCAACCATCCTGAATTGCC	CATACAGACACCCGCCCGTGCAG	60	405
Hv.t31	CAACGCCATGTCGTCGAGCACCG	GTGGTTTCAGCCGTAGCAGCGGA	65	562
Hv.t22	CGTCCGCATGGGCACGCTTCTCC	ATGGCCCGCGCCGACATGTTGGT	69	536
Hv.t18	ATTTGACTCCCACCAGATCCTTCCG	GACTATTACGAGCGCGGCAACCT	62	500
Hv.t13	GATGCTGTCCGAGGGCGCCATCA	TTTAGGGGGGCCCCCGCCTGTAA	65	570
Hv.t11	TCGAGCAATGTATGCCATGCGCG	TTTAGCTCGCGCGATCAGGGCCC	63	537
Hv.t2	CCCTCGCCTCCAACACCCCACCG	GCCCCACTCGAACCCCTGTGCGG	65	360

the order of the barley genetic markers were compared with the order of the rice PAC and BAC clones along the rice chromosomes.

In silico *approach to identify barley ESTs within the QTL*

The rice genome sequence corresponding to the barley black point QTL on 2H was downloaded from the rice genome sequence database (http://rye.pw.usda.gov/cgi-bin/gbrowse/japonica/, August 2007 release). This sequence was manually fragmented into 126 contigs of 100 Kb. BLASTn searches against the GenBank barley EST database (release date, August 2007) were then conducted. To identify similar sequences, an *E*-value significance $\leq 1e^{-25}$ was used. The identified barley EST sequences were then used in BLASTx searches against the GenBank non-redundant protein database. Search results were used to view annotations and subsequently predict a putative function for the barley ESTs where possible. Based on the annotation, ESTs were grouped into 11 functional categories, adapted from those previously used to categorise genes identified from the *Arabidopsis* genome-sequencing project (Bevan *et al*. 1998). These categories included metabolism and energy; cell growth and division; transcription; protein synthesis, destination and storage; transporters and intracellular traffic; cell structure; signal transduction; disease and defence response; secondary metabolites; transposable elements; and unknowns.

Candidate gene selection

To confirm candidate genes identified from the *in silico* approach mapped within the barley black point QTL on 2H, 20 ESTs were selected from the list of ESTs using two criteria. First, the predicted function of the top hits was examined to determine whether an association with known physiological events during black point could be made; and second, candidates were selected to ensure that there was an even spread across what was the existing QTL.

Probe design and confirmation

Selected ESTs were used in BLASTn searches against the GenBank barley EST database to retrieve all ESTs with sequence similarity. These EST sequences were aligned into contiguous sequences using the program Vector NTI Advance 10 (Invitrogen, Carlsbad, CA, USA). Regions within the contiguous sequences were selected and used again in BLASTn searches against the GenBank barley EST database to determine whether the region was conserved. If this was the case, PCR primers were designed using Vector NTI Advance 10 to amplify the region of interest.

To amplify the probe of interest, whole barley seeds (10–15) were ground in liquid nitrogen using a mortar and pestle. RNA was extracted from ground tissue using the TRIzol reagent as per manufacturer's recommendations (Invitrogen). Probes for Southern analysis were PCR amplified from cDNA that was prepared from grain RNA using SuperScript II reverse transcriptase as per the manufacturer's instructions (Invitrogen). PCR conditions were 2 min at 94°C; then 32 cycles of 30 s at 94°C, 30 s at x°C and 1 min at 72°C; followed by 10 min at 72°C, where 'x' was determined based on the melting temperature of each primer pair (Table 1).

Amplified fragments were cloned using the QIAGEN PCR Cloning Kit (Qiagen, Hilden, Germany) and subsequently sequenced by AgGenomics Pty Ltd (Melbourne, Vic., Australia) using BigDye chemistry (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the program Vector NTI Advance 10.

Chromosome localisation

Southern membranes prepared from barley-wheat addition lines (kindly provided by Margaret Pallota, Australian Centre for Plant Functional Genomics, Adelaide, SA, Australia) were probed to determine whether each EST resided on barley chromosome 2H. Amplified ESTs were radioactively labelled with P^{32} (GE Healthcare, Uppsala, Sweden) using Ready-To-Go DNA Labelling Beads (GE Healthcare). Labelled probes were denatured for 2 min at 95°C and added to 20 mL of hybridisation buffer (0.5 M Na_2HPO_4 pH 7.2, 7% SDS and 1 mM EDTA). Membranes were hybridised overnight within bottles, in a rotating hybridisation oven at 65°C and then washed consecutively with 2×, 1× and 0.5× SSC containing 0.1% SDS for 15 min per wash at 65°C. Washed membranes were subsequently exposed to Hyperfilm MP (GE Healthcare) for 5 days at −80°C and then developed using a CP1000 developer (AGFA-Geveart Group, Mortsel, Belgium).

RFLP analysis

To position the ESTs located on chromosome 2H, their genetic map location within the Alexis × Sloop DH mapping population (Barr *et al*. 2003) was determined using the following method. Aliquots of genomic DNA (20 µg) from the cultivars Alexis and Sloop were separately digested with 20 units of each of the restriction enzymes *Eco*RI, *Dra*I, *Bam*HI, *Eco*RV, *Hin*dIII and *Xba*I (New England Biolabs, Ipswich, MA, USA) for 5 h at 37°C. Digested samples were separated in a 1% agarose gel in TAE buffer (40 mM Tris, 1 mM EDTA and 20 mM acetic acid) at 33V for 15 h then blotted to N+ nylon membrane (GE Healthcare) using 0.4 M NaOH as a transfer buffer and following the manufacturer's recommendations. Membranes were probed with the same radioactively labelled EST-derived probes as used with the barley-wheat addition line membranes.

If an RFLP was detected between Alexis and Sloop digested with one of the restriction enzymes, that enzyme was used to digest the DNA from 107 lines of the Alexis × Sloop DH mapping population. Digested DNA was separated and transferred to a nylon membrane and probed as described above.

Mapping identified polymorphisms

To map the chromosome location of polymorphic ESTs, the banding pattern was scored across the Alexis × Sloop double haploid mapping population as either Alexis or Sloop type. To map the genetic location of the polymorphism, the scores were entered into the program MapManager QTX (Manly *et al*. 2001) using the Kosambi mapping function. Linkage analysis was performed with existing markers on a curated Alexis × Sloop genetic map (Willsmore *et al*. 2006) using the 'find best location' function to determine the map location.

Re-mapping the black point QTL

Upon adding additional EST-derived genetic markers the black point QTL was re-mapped to determine the effect of the new markers. The original black point QTL was determined using black point symptom scores derived from the Alexis × Sloop DH mapping population grown in Port Wakefield South Australia, 2000 (A. J. Able, T. Zerk, K. R. Walker, unpubl. data). The Map Manager QTX program (version b13) was used to localise the QTL responsible for the variability of barley black point phenotypes.

Results

Comparing black point QTL in barley and wheat

Using previous reports (Table 2), genetic markers associated with 13 QTL for black point and kernel discolouration from various wheat and barley mapping populations were identified. Kernel discoloration is distinct from black point because the entire grain is discoloured rather than just the embryo end of the grain. These QTL were present on chromosomes 2H of barley and 2B of wheat. Using a previously published barley consensus map (Wenzl *et al*. 2006), comparison of the position of barley QTL on chromosome 2H grouped the QTL into three distinct regions (Fig. 1; Table 2). Group 1 QTL were located near the top of the short arm, group 2 QTL were located near the centromere and group 3 QTL were located near the end of the long arm. The barley QTL for black point in the Alexis × Sloop DH mapping population (A. J. Able, T. Zerk, K. R. Walker, unpubl. data) was located within QTL group 2. This QTL group also comprised several QTL associated with kernel discolouration. QTL groups 1 and 3 only comprised QTL identified for kernel discolouration.

To determine whether the wheat black point QTL previously identified on chromosome 2B was in a similar position to the barley black point QTL on chromosome 2H, the wheat and barley genetic maps were aligned using the program CMap. No common markers were identified between the barley population Alexis × Sloop and the wheat population of Sunco × Tasman in which the QTL were identified. To overcome this, two other genetic maps that shared common markers were used to bridge the Alexis × Sloop and the Sunco × Tasman genetic maps (Fig. 2). This highlights that wheat and barley black point QTL are in similar positions near the centromere of their respective chromosomes.

In silico *analysis reveals synteny between the barley black point QTL on chromosome 2H and rice chromosomes 4 and 7*

To determine what genes were present within the barley black point QTL the barley genome was aligned with the sequenced rice genome. Previously identified flanking and internal RFLP sequences for the QTL were used in BLASTn searches against the rice genome. This approach was successful for the lower half of the QTL region and identified a syntenous region on rice chromosome 4. This region in rice spanned 5 Mb and had a genetic map distance of ~13 cM (Fig. 3).

For the top region of the QTL there was a lack of barley genetic markers with sequence information available such that the same approach could not be used. The barley genetic map was therefore aligned with a wheat genetic map of chromosome 2B using the common markers wg996 and mwg950. From this, the wheat map was aligned with the wheat deletion EST bin map (http://wheat.pw.usda.gov/wEST/binmaps/). Using the centromere as a reference point, the wheat deletion region C-2BS1–0.53 was identified as being in alignment.

From the wheat deletion EST bin, several ESTs were selected and used in BLASTn searches against the sequenced rice genome. This enabled the identification of a syntenous region with rice chromosome 7 which spanned 7.6 Mb and a genetic map distance of 35 cM. When visualising this region against barley chromosome 2H, it became evident that the syntenic region on rice chromosome 7 was inverted in orientation (Fig. 3).

Within rice chromosome 7, at 115 cM, a peroxidase gene sequence was identified. The sequence was aligned with the barley POX RFLP marker (peroxidase gene) which showed

Table 2. Summary of previously identified QTL for black point (BP) and kernel discolouration (KD) on chromosome 2H of barley (B) and 2B of wheat (W)

Each QTL was assigned to a location as per Fig. 1

QTL #	QTL location	Trait	Associated marker(s)	Species	Population	Reference
1	2	BP	PSR131–ABG14	B	Alexis × Sloop	A. J. Able, T. Zerk, K. R. Walker, unpubl. data
2	2	KD	EBmac684	B	Alexis × Sloop	Li *et al.* (2003)
3	2	KD	EBmac684	B	Sloop × Alexis	Li *et al.* (2003)
4	2	KD	P13/M62–134	B	Arapiles × Franklin	Li *et al.* (2003)
5	2	KD	Bmag125	B	Chebec × Harrington	Li *et al.* (2003)
6	3	KD	HVM54	B	VB9104 × Dash	Li *et al.* (2003)
7	2	KD	EBmac684	B	Galleon × Haruna Nijo	Li *et al.* (2003)
8	2	KD	CDO474	B	Sloop × Halcyon	Li *et al.* (2003)
9	1	KD	ABC311–MWG858	B	Chevron × M69	de la Pena *et al.* (1999)
10	3	KD	ABG497a–ABC157	B	Chevron × M69	de la Pena *et al.* (1999)
11	1	KD	ABG008–MWG858	B	Chevron × M69	Canci *et al.* (2003)
12	1	KD	EBmac558–HVM36	B	Chevron × M69	Canci *et al.* (2003)
13	n/a	BP	WMC154–WMC149	W	Sunco × Tasman	Lehmensiek *et al.* (2004)

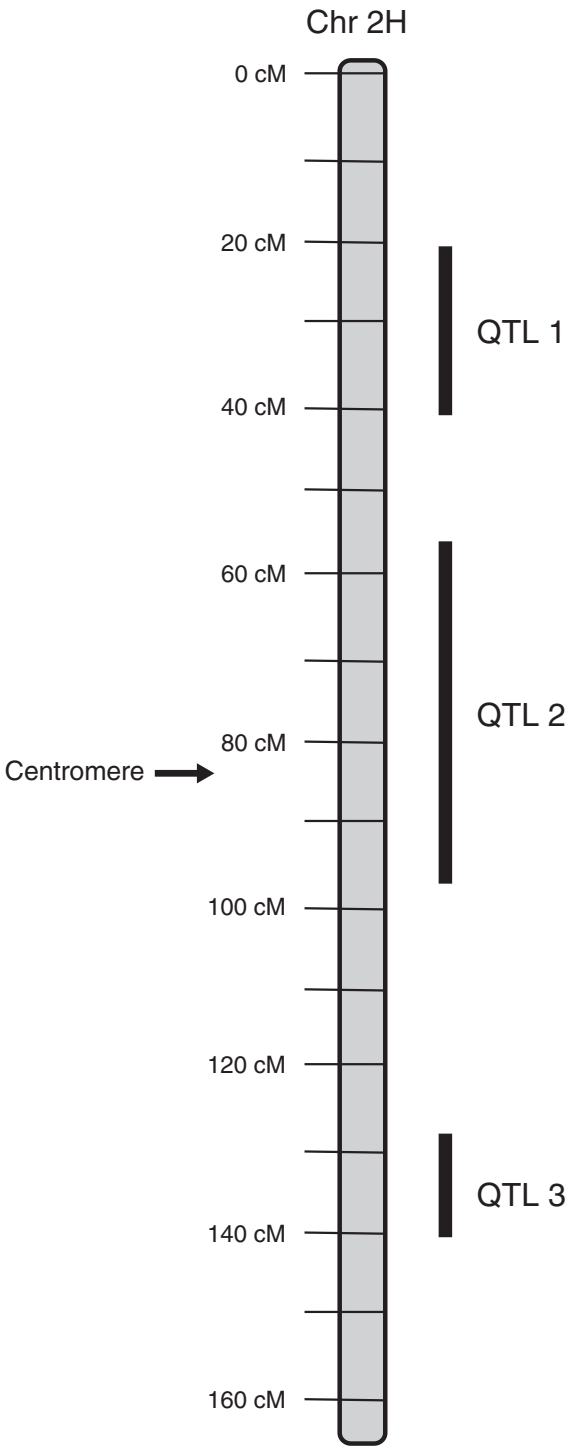

Fig. 1. Relative positions of QTL associated with black point and kernel discolouration of barley grain. Using a previously published barley consensus map (Wenzl *et al*. 2006), 12 previously identified QTL from various mapping populations (Table 1) were positioned on chromosome 2H. The QTL fell into three distinct locations along the chromosome.

that the two sequences were highly similar. This provided a genetic link from the identified rice chromosome 7 region back to the barley genetic map. It also confirmed that the wheat deletion bin C-2BS1–0.53 was likely to cover the entire top region of the barley black point QTL (Fig. 3).

Identification and mapping of selected barley EST sequences

The rice sequence from chromosomes 4 and 7 that was syntenous with the black point QTL was used to identify barley ESTs with sequence similarity. Annotation of these EST sequences using BLASTx searches returned 1928 unique annotations (see Table S1 in the accessory publication available from the online version of *Functional Plant Biology*) which were grouped into 11 functional categories (Fig. 4). From this, 864 ESTs could not be assigned a function. The largest known functional groups were involved in metabolism and energy (280 ESTs) and cell structure (167 ESTs). Given that secondary metabolism of phenolic compounds may play a role in black point (Williamson 1997), the 38 identified ESTs predicted to be involved in secondary metabolism were of interest.

Twenty barley ESTs were used to develop RFLPs with ESTs chosen based on their predicted function and possible role in black point (Table 3). ESTs showing high sequence similarity to peroxidases, phenylalanine ammonia lyase, quinone reductase and chalcone synthase were selected due to their published roles in phenolic compound synthesis or oxidation (Spitsberg and Coscia 1982; Rohde *et al*. 1991; Rasmussen *et al*. 1995; Regnier and Macheix 1996).

To ensure an even spread of markers across the QTL other ESTs showing high sequence similarity to identified regions on rice chromosomes 4 and 7 were selected (Table 3). Non-conserved regions were identified in all the ESTs based on BLASTn searches against the GenBank barley EST database. All of the EST sequences were successfully amplified as single products from mature barley grain cDNA except for Hv.t22 and Hv.t31, with these producing multiple or no PCR products, respectively. The marker Hv.t13 produced a single PCR product but upon sequencing was not the correct product. The remaining 17 EST markers amplified were the correct product.

Probing the barley wheat addition lines with the remaining 17 RFLP probes showed that 14 were located on barley chromosome 2H (Table 4). Of the 14 RFLP probes, four also detected another copy of the gene on separate chromosomes. From the 14 markers that were present on chromosome 2H, polymorphisms were detected with nine of the RFLP probes between Alexis and Sloop. The majority of the polymorphisms were detected with the restriction enzyme *Dra*I (six markers), followed by *Eco*RI (five markers). Polymorphisms were detected for the marker bcd334 with the restriction enzymes *Dra*I, *Eco*RI and *Hin*dIII. The marker BMY2 was polymorphic with all six restriction enzymes used (Table 4). Mapping the nine polymorphic markers specific for 2H within the Alexis × Sloop DH mapping population was successful for six of the markers. The markers Hv.t81.6, Hv.t81 and Hv.t67_1 were unsuccessful because the signal from the Southern membranes was too weak to score accurately across the entire Alexis × Sloop population.

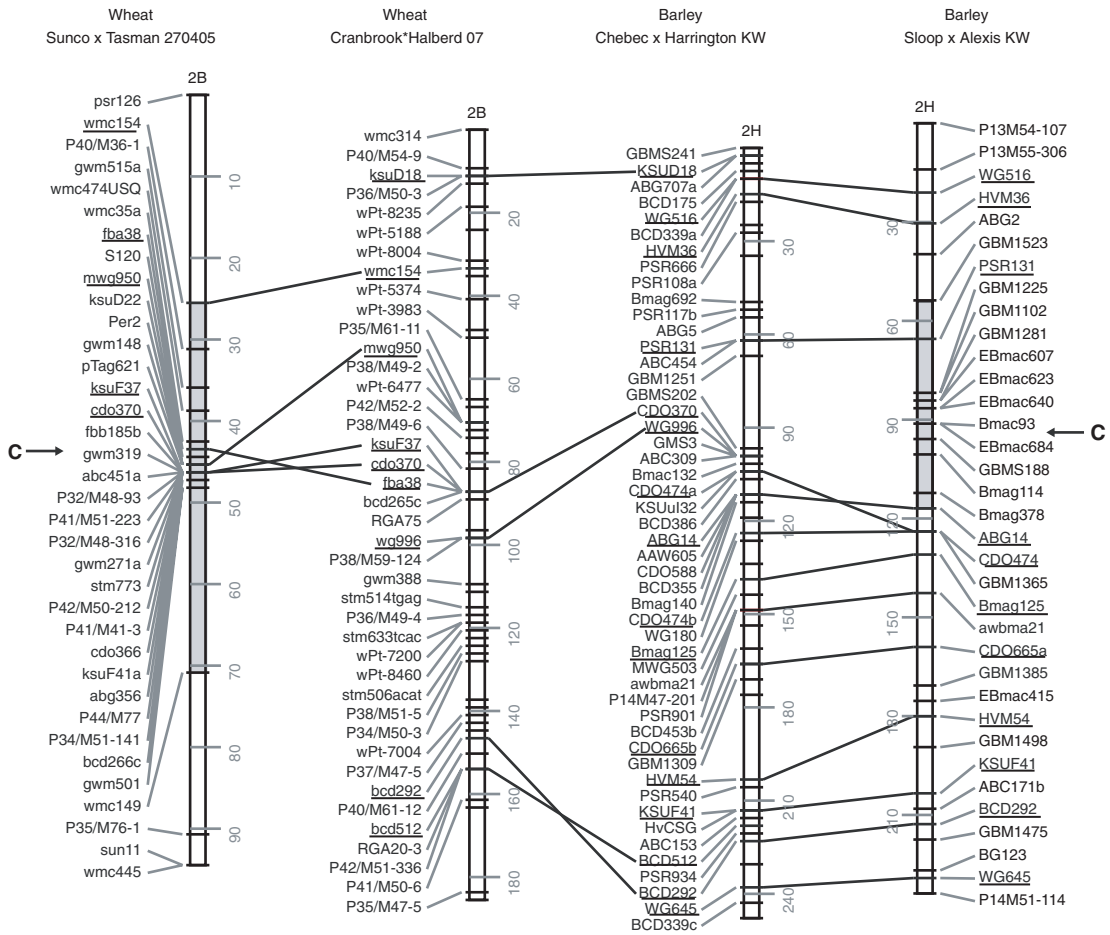

Fig. 2. Alignment of black point QTL identified in wheat and barley mapping populations using the program CMap. Boxed grey regions show the area defined at the QTL on wheat chromosome 2B (far left map) and barley chromosome 2H (far right map). Lines connect common markers between maps and the centromere is indicated as C.

Genetic location of mapped ESTs refines the black point QTL

From the original 20 ESTs identified *in silico*, six ESTs were mapped within the Alexis × Sloop DH population (Table 4). The marker Hv.t38 produced three different polymorphisms with the restriction enzyme *Dra*I. These were designated Hv.t38a, Hv.t38b (Fig. 5*B*) and Hv.t38c, with this marker mapping to chromosome 5H (data not shown).

The remaining mapped ESTs were located on chromosome 2H. Three of the markers (Hv.t102, BMY2 and bcd334) mapped to the original QTL group 2 region as expected from the *in silico* results. Markers Hv.t38a, Hv.t38b, Hv.t70 and Hv.t101 mapped outside of the original QTL region (Table 4; Fig. 5). The original black point QTL spanned a genetic map distance of 28 cM, and the peak height of the QTL had a logarithmic odds (LOD) score of 3.99 (Fig. 5*A*). After adding the additional markers identified in this study to the genetic map, the QTL region has been reduced to 18 cM with the LOD score remaining relatively unaffected at 3.88 (Fig. 5*B*). The markers Hv.t102 and BMY2 had the greatest effect in reducing the QTL region as it more accurately defined the top region of the QTL by filling in a previously large gap that existed between markers PSR131 and EBmac607.

Discussion

The two major outcomes of this study were the identification of candidate genes for barley black point and the fine mapping of the barley black point QTL on 2H. Several QTL have been identified throughout the barley genome that control black point and kernel discolouration (Table 2). By utilising the established barley-wheat-rice syntenic relationship, the first part of this study identified several candidate genes underlying the QTL for black point on chromosome 2H. The candidate genes were from QTL group 2 (Fig. 1), which is associated with both black point and kernel discolouration. Interestingly the same QTL was identified for black point and kernel discolouration within the Alexis × Sloop population (Li *et al*. 2003; A. J. Able, T. Zerk, K. R. Walker, unpubl. data). This indicates that the same gene(s) within this QTL may influence the two traits. Further support of the importance of this region is highlighted by the fact that the wheat black point QTL on chromosome 2B also aligned with the barley black point QTL on chromosome 2H. This suggests that the same genes may be influencing black point within the Triticeae tribe.

In silico analysis of the barley 2H region identified areas of synteny with rice chromosomes 4 and 7, and that the change

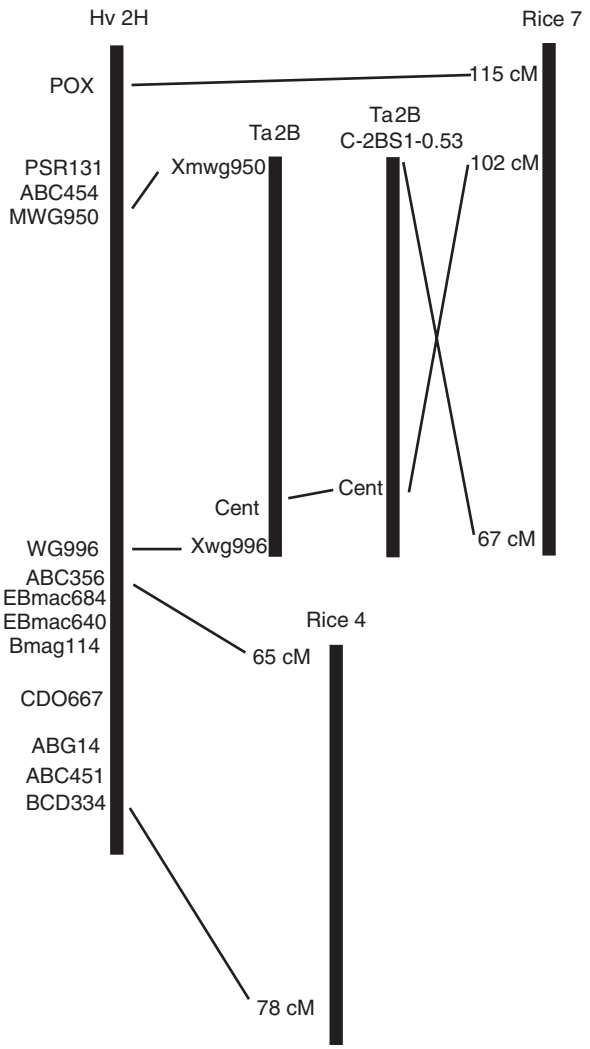

Fig. 3. Comparison of the black point QTL region on barley chromosome 2H with wheat and rice. Barley genetic markers were identified using a BLASTn search against the rice genome to find regions of synteny. The wheat genetic map and wheat EST deletion map was used to bridge the barley and rice genomes where there were inadequate barley genetic markers.

between rice chromosomes coincided with the centromere of barley 2H. In wheat a similar relationship has been observed, where there is a shift in synteny that occurs at the centromere of wheat chromosome 2B from rice chromosome 4 to rice chromosome 7 (La Rota and Sorrells 2004).

The 12.6-Mb region of the rice genome sequence that is syntenic with barley chromosome 2H identified a large set of barley EST sequences. ESTs for developing markers were selected based on their possible role in black point development. Black point has been proposed to be due to an enzymatic browning reaction where phenolic compounds within the grain are oxidised by peroxidase enzymes to form discoloured end products (Williamson 1997). Based on this, ESTs that were associated with the synthesis or oxidation of phenolic compounds were selected from the identified barley ESTs.

In direct support of the enzymatic browning model, two peroxidase genes were identified within the rice genome

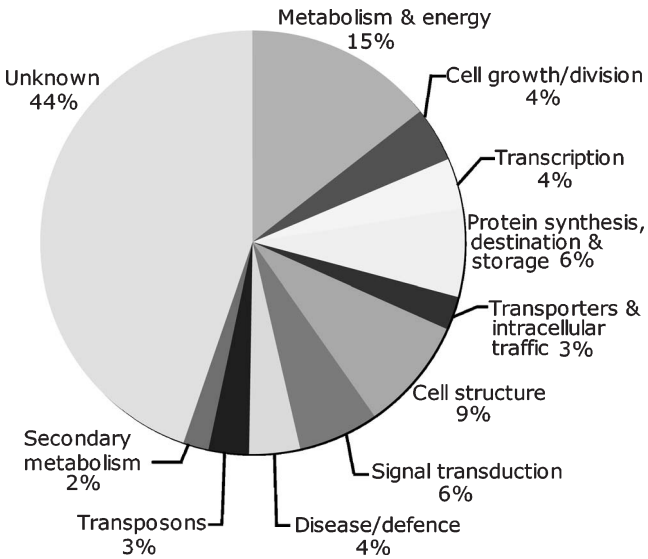

Fig. 4. Functional classification of the ESTs predicted to reside within the barley black point QTL on chromosome 2H. ESTs identified from the barley–wheat–rice comparison were grouped into functional groups based on the annotation obtained from the BLASTx searches. Functional groups were adapted from those described previously (Bevan *et al*. 1998).

sequence. Peroxidase enzymes are able to oxidise a range of phenolic substrates (Rasmussen *et al*. 1995). The oxidised phenols form quinones that can undergo auto-polymerisation or covalent hetero-condensation with proteins and carbohydrates to produce coloured compounds (Bittner 2006). Peroxidases could therefore play a direct role in black point development. Indeed, the peroxidase BP1 is found in black pointed grains but not healthy grains based on a proteomics study of barley grain (March *et al*. 2007).

Wounding of plant tissue results in the oxidation of phenolic compounds to quinones by enzymes such as peroxidases and polyphenol oxidases (Whitaker and Chang 1996). From the *in silico* approach an EST with sequence similarity to a quinone reductase was identified. Quinones are highly reactive compounds that serve several purposes in the cell. Upon wounding they can cross link cell walls to provide a physical barrier to protect the cell (Lynn and Chang 1990). Quinones can also oxidise the thiol groups of proteins and glutathione. This reaction results in semi-quinones that undergo rapid oxidation and produce superoxide that is damaging to the cell (Sparla *et al*. 1999). To regulate the levels of quinones, plants produce quinone reductase enzymes to reduce quinones into hydroquinones that can be removed from the quinone redox cycle by conjugation (Harborne 1979). March *et al*. (2007) suggested that the wounding of the grain may lead to black point. As a result of this wounding, quinones would likely be produced as a defence mechanism. The presence of a quinone reductase gene within the black point QTL is therefore of further interest. Quinone reductase may act as a tolerance mechanism to black point by removing their reactivity thus preventing their participation in enzymatic browning reactions.

Towards the distal end of the black point QTL an EST showing sequence similarity to phenylalanine ammonia lyase (PAL) was identified. PAL is a key enzyme involved in the

Table 3. Selected barley ESTs (20) predicted to reside within the black point QTL

ESTs were subjected to BLASTx searches to predict the putative function of the EST (gene transcript) and their possible role in black point

Marker ID	Rice chromosome	Barley EST	Protein accession	Annotation
Hv.t102	7	BE591326	EAZ04931	Hypothetical protein OsI_026163 [*Oryza sativa* (indica cultivar-group)]
Hv.t101	7	BE398439	AAM65274	NADH dehydrogenase [*Arabidopsis thaliana*]
Hv.tPox1	7	CD056766	AAG46133	Putative peroxidase [*Oryza sativa*]
Hv.t81.6	7	CB864764	CAA77237	Reversibly glycosylated polypeptide [*Triticum aestivum*]
Hv.t81	7	BG314068	EAZ40456	Hypothetical protein OsJ_023939 [*Oryza sativa* (japonica cultivar-group)].
Hv.tCsl	7	CB869766	AAL25133	Cellulose synthase-like protein OsCslF3 [*Oryza sativa*]
BMY2	7	BE193999	AAG25638	Beta-amylase [*Hordeum vulgare*]
Hv.tCHS	7	BQ466496	XP_479275	Putative chalcone synthase [*Oryza sativa* (japonica cultivar-group)]
Hv.t71	7	BE636802	BAC57673	Putative glucose-6-phosphate/phosphate translocator precursor [*Oryza sativa* (japonica cultivar-group)]
Hv.t70	7	DN156338	AAP80640	Succinate dehydrogenase subunit 3 [*Triticum aestivum*]
Hv.t67_1	7	CA019486	BAD68985	Putative tRNA-(N1G37) methyltransferase [*Oryza sativa* (japonica cultivar-group)].
Hv.t67	7	BJ464836	EAZ04028	Hypothetical protein OsI_025260 [*Oryza sativa* (indica cultivar-group)].
Hv.t2	4	AJ473633	AAQ01197	COP9 [*Oryza sativa* (japonica cultivar-group)]
Hv.t11	4	BI959763	BAD18962	Ferric reductase [*Oryza sativa* (japonica cultivar-group)]
Hv.t13	4	BF264799	CAI84707	Lipoxygenase-like protein [*Hordeum vulgare* subsp. *vulgare*]
Hv.t18	4	AV918365	BAD37679	Putative dehydration stress-induced protein [*Oryza sativa* (japonica cultivar-group)]
Hv.t22	4	CB869939	BAD37858	Putative peroxidase [*Oryza sativa* (japonica cultivar-group)]
Hv.t31	4	BQ767022	CAE92372	Peptide methionine sulfoxide reductase [*Secale cereale*]
Hv.t38	4	AJ474981	AAL38796	Putative quinone oxidoreductase [*Arabidopsis thaliana*]
bcd334	4	BE438762	CAA89005	Phenylalanine ammonia lyase [*Hordeum vulgare* subsp. *vulgare*]

Table 4. Summary of new barley RFLP markers

For each marker, the functional group it belongs to and its position within the barley genome is listed. Where markers were polymorphic between Alexis and Sloop, the restriction enzyme/s used are listed

Marker ID	Functional group	Chromosome location	Polymorphic between Alexis and Sloop	Mapped within Alexis × Sloop population	Located in QTL group 2
Hv.t102	Unknown	2H	*Dra*I	Yes	In
Hv.t101	Metabolism and energy	2H, 3H	*Dra*I	Yes	Out
Hv.tPox1	Disease/defence	3H	No	No	n/a
Hv.t81.6	Unknown	2H, 4H	*Eco*RI	—	n/a
Hv.t81	Unknown	n/a	*Eco*RI	—	n/a
Hv.tCsl	Cell structure	2H	No	No	n/a
BMY2	Metabolism and energy	2H	*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Xba*I	Yes	In
Hv.tCHS	Secondary metabolism	5H	No	No	n/a
Hv.t71	Metabolism and energy	2H	No	No	n/a
Hv.t70	Metabolism and energy	2H, 3H	*Dra*I	Yes	Out
Hv.t67_1	Metabolism and energy	2H	*Eco*RI	—	n/a
Hv.t67	Unknown	2H	No	No	n/a
Hv.t2	Signal transduction	2H	No	No	n/a
Hv.t11	Transporters and intracellular traffic	2H	No	No	n/a
Hv.t18	Disease/defence	2H	No	No	n/a
Hv.t38	Secondary metabolism	2H, 5H	*Dra*I	Yes	Out
Bcd334	Secondary metabolism	2H	*Dra*I, *Eco*RI, *Hind*III	Yes	In

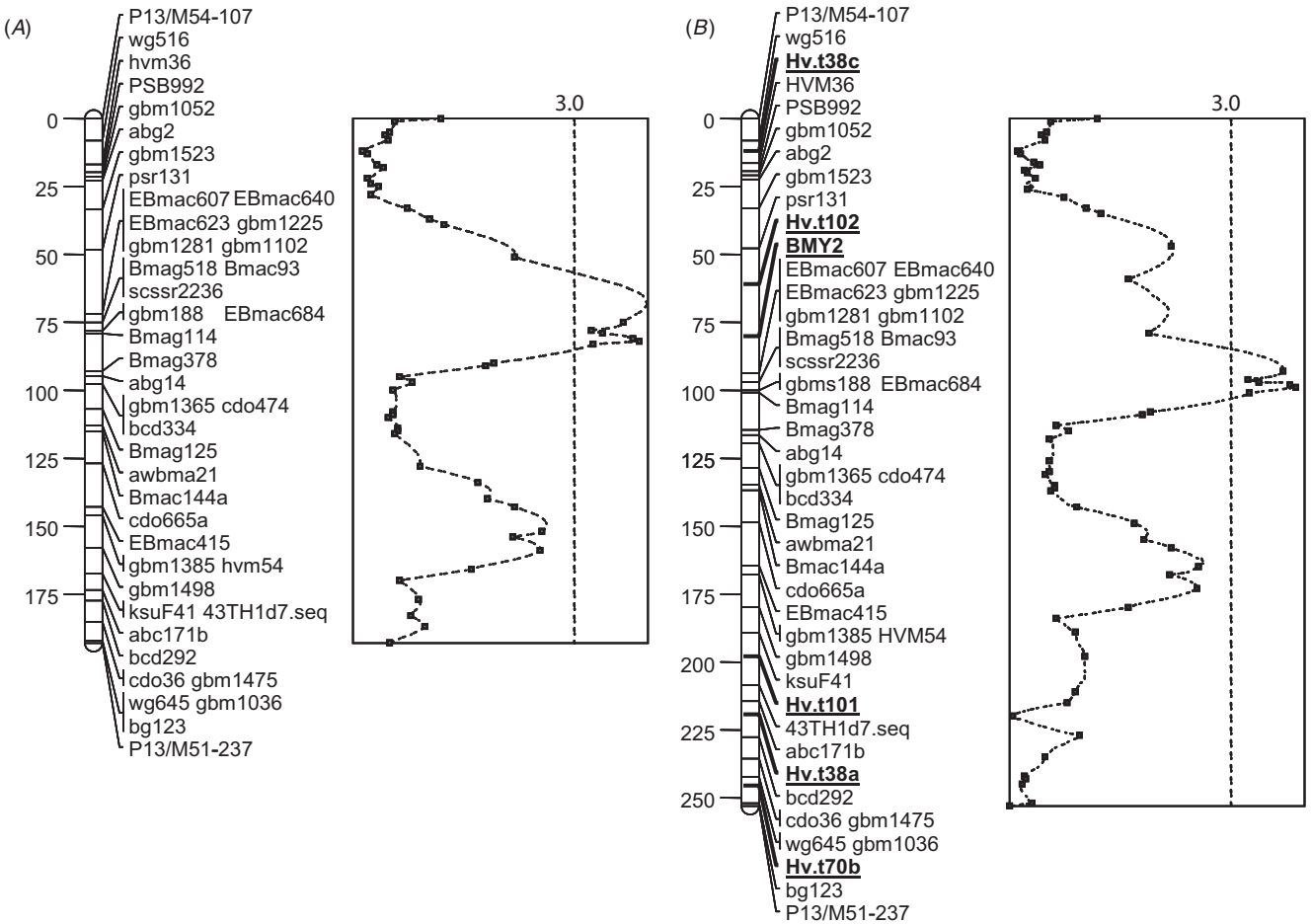

Fig. 5. Effect of additional markers on the black point QTL in the Alexis × Sloop doubled haploid mapping population. (*A*) Black point QTL before this study. (*B*) Effect of additional markers on the black point QTL. The vertical dashed line in (*A*) and (*B*) indicates a significant LOD score of 3. For clarity, AFLP markers have been removed.

phenylpropanoid metabolism pathway which produces phenolic compounds (Whitaker and Chang 1996). PAL is wound inducible and an increase in the level of PAL is directly related to increased production of phenolic compounds within plants (Chaman *et al*. 2003; Gaudet *et al*. 2003). In the context of black point formation, PAL could therefore be induced during the wounding associated with black point, thus increasing the phenolic substrates required to cause the discolouration.

Centrally located in the black point QTL was a β-amylase 2 gene (BMY2). β-amylases are exohydrolases that cleave α-1, 4-D-glucosidic bonds, releasing maltose from the non-reducing end of a range of polyglucans. BMY2 is an ubiquitous β-amylase and is expressed in all tissues of barley (Shewry *et al*. 1988) unlike BMY1, which is located on chromosome 4H and specifically expressed in the endosperm (Li *et al*. 2002). BMY1 has a large effect on diastatic power and hence, the malting quality of barley compared with BMY2 (Coventry *et al*. 2003). The function of BMY2 within the grain is still unknown. Given the central location of the BMY2 gene in the QTL, it would serve as a useful marker to select for barley black point. Furthermore, the BMY2 marker was polymorphic with all six restriction enzymes tested in this study, showing that it could potentially be a useful marker to select for the black point QTL across a range of mapping populations.

The second important outcome from this study was the reduction of the black point QTL on chromosome 2H from 28 to 18 cM, with the identification of additional markers flanking this region that could potentially be used to select for this QTL in breeding programs. *In silico* analysis enabled identification of candidate gene RFLP probes to initially determine whether they were located on chromosome 2H and if they mapped within the black point QTL. From the 17 successfully amplified ESTs, 14 were located on chromosome 2H. Of these, nine were found to be polymorphic between Alexis and Sloop using one of the six restriction enzymes. This level of polymorphism is consistent with a previous barley-rice comparative mapping study that found 31 of 54 RFLP probes tested were polymorphic (Perovic *et al*. 2004).

The location of ESTs along chromosome 2H was not always consistent with what was predicted *in silico*. For example, markers Hv.t101 and Hv.t102 reside 1 cM apart on rice chromosome 7 but were found to be 137 cM apart on barley chromosome 2H. Likewise, the position of markers Hv.t38a, Hv.t38b and Hv.t70 on barley chromosome 2H did not correspond to the predicted position from rice chromosome 4 and 7. This loss of synteny at the

micro-level is most likely due to chromosome restructuring events, gene duplication and/or deletion, since barley and rice evolved from a common ancestor ~60 million years ago (Wolfe *et al*. 1989). In support of this, several transposable elements were identified within the QTL region which could facilitate genome restructuring (Moffat 2000).

Despite rice and barley not always sharing micro-synteny, this study was able to identify new markers linked to the black point QTL. The markers Hv.t102, BMY2 and bcd334 mapped to the predicted position on barley chromosome 2H coinciding with the black point QTL. The marker Hv.t102 mapped to a region on barley chromosome 2H that previously had limited genetic markers. Remapping of the QTL including Hv.t102 and BMY2 redefined the distal end of the QTL and effectively reduced the QTL by 10 cM. Research can now progress to further fine map the black point QTL. This would involve generating larger mapping populations that segregate for black point susceptibility within this QTL region. This will increase the likelihood of finding polymorphic markers needed to further reduce the QTL size and ultimately pin point the gene(s) controlling black point susceptibility. In conclusion, the identification of candidate genes and their subsequent use to fine map the barley black point QTL on 2H have provided a complementary approach to help define the genetic basis of black point formation in barley.

Acknowledgements

The authors thank Margie Pallotta for supplying the barley-wheat addition line membranes. This research was supported by the Molecular Plant Breeding Cooperative Research Centre (MPB CRC) and the University of Adelaide.

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Manuscript received 19 March 2008, accepted 22 May 2008