4 Comparative mapping of a barley QTL influencing black point formation

4.1 Introduction

Black point of barley and wheat grain is a trait that has been shown to have a genetic basis. Amongst cultivars of wheat and barley a range of susceptibility to black point has been observed (Conner and Davidson, 1988; de la Pena et al., 1999; Hadaway et al., 2003). Efforts to identify quantitative trait loci (QTL) controlling black point have identified several genomic regions. However, all studies commonly report QTL on chromosome 2H of barley and 2B of wheat associated with black point (refer to Chapter 1.2.4.2).

Barley and wheat are from the tribe Triticeae and share a large degree of macro-synteny with one another. Synteny refers to the conservation of gene order along chromosomes between two different chromosomes from two different species. The aforementioned chromosomes (2H and 2B) associated with black point have been reported to share high levels of sequence similarity and synteny (Devos et al., 1993). It is therefore plausible to speculate that if the black point QTL identified within the group 2 chromosomes of wheat and barley are in similar regions, then the same genes might control this trait. However, this hypothesis is currently untested.

Given the lack of complete sequence information for both the barley and wheat genomes, it is not surprising that little information on the genes that reside within the identified black point QTL is known. This is largely because these crops have considerably large genomes (5500 Mb for barley, and 17000 Mb for wheat)

(Bennett and Smith, 1976) and contain a high level of sequence repetition (Smith and Flavell, 1975). Rice on the other hand 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 has enabled the use of the rice genome (in some instances) as a tool to predict gene content in genomic regions of wheat and barley. The predicted genes are identified using bioinformatics and then subsequently mapped within the barley or wheat genome to validate the syntenic relationship *in vitro*.

The major objective of the research presented in this chapter was to identify candidate genes that resided within the barley 2H QTL, which is believed to be associated with black point formation. In achieving this goal, comparisons between the QTL identified on chromosomes 2H of barley and 2B of wheat were conducted. Using rice as a monocot model, *in silico* experimentation enabled the identification of genes that resided within the same area of the rice genome. A selection of those genes identified was then mapped to the barley genome to confirm the results *in vitro*. Knowledge of the genes underlying the black point QTL will aid in understanding the genetic mechanism of tolerance. Additional genetic markers mapped to the region will also assist in better defining the existing QTL.

4.2 Material and methods

4.2.1 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 4-2). Using a barley consensus map (Wenzl et al., 2006) and the program CMap (www.genica.net.au), the position of all the QTL on chromosome 2H were compared to determine if 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.

4.2.2 Identifying a syntenic relationship 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) and NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview) 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 (http://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/) to identify wheat ESTs within the region. The wheat ESTs where 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 the order of the barley genetic markers were compared to the order of the rice PAC and BAC clones along the rice chromosomes.

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

The rice genome sequence corresponding to the barley black point QTL that was identified in section 4.2.2 was downloaded from the rice genome sequence database (http://rye.pw.usda.gov/cgi-bin/gbrowse/japonica/). This sequence was then manually broken up into 126 contigs of 100 Kb in length. These fragments were then used in BLASTn searches against the GenBank barley EST database (release date, May 2005). To identify similar sequences, a cut off *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. The 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.

4.2.4 Selection of candidate genes

To confirm candidate genes identified from the *in silico* approach (section 4.2.3) mapped within the barley black point QTL on 2H, twenty ESTs were selected from the list of ESTs using two criteria. Firstly, 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 secondly, candidates were selected to ensure that there was an 'even' spread across what was the existing QTL at that time.

4.2.5 Designing RFLP probes

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 if the region was non-conserved. If this was the case, PCR primers were designed using Vector NTI Advance™10 to amplify the region of interest.

4.2.6 Amplification and sequencing of RFLP probes

Whole barley seeds (10 to 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)(as per section 3.2.2). 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)(as per section 3.2.2). PCR conditions were 2 min at 94°C; then 32 cycles of 30 sec at 94°C, 30 sec 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 4-1).

To determine if the correct sequences were amplified, the amplified fragments were cloned using the QIAGEN PCR Cloning Kit (Qiagen, Hilden, Germany). Cloned fragments were sequenced by AgGenomics Pty Ltd (Melbourne, Australia) using BigDye™ chemistry (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the program Vector NTI Advance™ 10 (Invitrogen).

Table 4-1. PCR primers used to amplify the RFLP markers. The optimum primer annealing temperature (Tm ^{o}C) and the predicted amplicon size for each primer pair is shown.

Marker ID	Forward Primer	Reverse Primer	Tm ^{o}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.t49	GCTGGCAGGCAGCCGCAACCCCAG	CACCGTGGCCTCGCTCTCGGCCT	66	520
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

4.2.7 Chromosome localisation

Southern membranes prepared from barley-wheat addition lines (kindly provided by Margaret Pallota, Australian Centre for Plant Functional Genomics, Adelaide) were probed to determine if each EST resided on barley chromosome 2H. Amplified ESTs were radioactively labelled with P^{32} (GE Healthcare, Uppsala, Sweden) using the Ready-To-Go DNA Labelling Beads (GE Healthcare). Labelled probes were denatured for 2 min at 95^{o}C and added to 20 mL of hybridisation buffer (0.5M 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^{o}C and then washed consecutively with 2x, 1x and 0.5x SSC containing 0.1% SDS for 15 min per wash at 65^{o}C. Washed membranes were subsequently exposed to Hyperfilm MP (GE Healthcare) for five days at -80^{o}C and then developed using a CP100 developer (AGFA-Geveart Group, Mortsel, Belgium).

4.2.8 RFLP analysis

To position the ESTs located on chromosome 2H (as per section 4.2.7), their genetic map location within the Alexis x Sloop double haploid mapping population (Barr et al., 2003) was determined using the following method. Aliquots of genomic DNA (20 µg) from the cultivars Alexis and Sloop (kindly provided by Kerrie Willsmore, South Australian Research and Development Institute, Adelaide) was separately digested with 20 units of each of the restriction enzymes *Eco*RI, *Dra*I, *Bam*HI, *Eco*RV, *Hind*III and *Xba*I (New England Biolabs, Ipswich, MA, USA) for 5 h at 37^{o}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 0.4M 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 x Sloop double haploid mapping population. Digested DNA was separated and transferred to a nylon membrane and probed as described above.

4.2.9 Mapping identified polymorphisms

To map the chromosome location of polymorphic ESTs (identified as per section 4.2.8) the banding pattern was scored across the Alexis x 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 the Alexis x Sloop genetic map (kindly provided by Kerrie Willsmore) using the 'find best location' function to determine the map location.

4.2.10 Re-mapping the black point QTL with new markers

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 x Sloop double haploid mapping population grown in Millicent, South Australia, during spring 2004 (Zerk et al., *unpublished*). Lines were phenotypically scored

for black point, by counting the number of grains with black point in six replicates of 100 grains using a seed tray, which has indents to hold 100 grains. The average of these counts was used as the black point score for each barley line. The Map Manager QTX program (version b13) was used to localise the QTLs responsible for the variability of barley black point phenotypes.

4.3 Results

4.3.1 Comparison of black point QTL between barley and wheat

Using previous reports (Table 4-2), genetic markers associated with 13 QTL for black point and kernel discolouration from various wheat and barley mapping populations were identified. These QTL were present on chromosomes 2H of barley and 2B of wheat. Comparison of the position of all the barley QTL on chromosome 2H using a previously published barley consensus map (Wenzl et al., 2006) grouped the QTL into three distinct regions (Figure 4-1). 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 x Sloop double haploid mapping population (Zerk et al., *unpublished*) was located within the 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 if the wheat black point QTL on chromosome 2B was in a similar position to the barley black point QTL on chromosome 2H, the genetic maps were aligned using the program CMap. No common markers were identified between the barley population Alexis x Sloop and the wheat population of Sunco x Tasman in

which the QTL were identified. To overcome this problem, two other genetic maps that shared common markers were used to bridge together the Alexis x Sloop and the Sunco x Tasman genetic maps (Figure 4-2). The wheat and barley black point QTL appear to be in similar positions near the centromere of their respective chromosomes.

Table 4-2 Summary of previously identified QTL for black point and kernel discolouration on chromosome 2H of barley and 2B of wheat.

QTL #	QTL group	Trait	Associated marker(s)	Species	Population	Reference
1	2	Black point	PSR131 - ABG14	Barley	Alexis x Sloop	Zerk et al., *unpublished*
2	2	Kernel discolouration	EBmac684	Barley	Alexis x Sloop	(Li et al., 2003)
3	2	Kernel discolouration	EBmac684	Barley	Sloop x Alexis	(Li et al., 2003)
4	2	Kernel discolouration	P13/M62-134	Barley	Arapiles x Franklin	(Li et al., 2003)
5	2	Kernel discolouration	Bmag125	Barley	Chebec x Harrington	(Li et al., 2003)
6	3	Kernel discolouration	HVM54	Barley	VB9104 x Dash	(Li et al., 2003)
7	2	Kernel discolouration	EBmac684	Barley	Galleon x Haruna Nijo	(Li et al., 2003)
8	2	Kernel discolouration	CDO474	Barley	Sloop x Halcyon	(Li et al., 2003)
9	1	Kernel discolouration	ABC311 - MWG858	Barley	Chevron x M69	(de la Pena et al., 1999)
10	3	Kernel discolouration	ABG497a - ABC157	Barley	Chevron x M69	(de la Pena et al., 1999)
11	1	Kernel discolouration	ABG008 - MWG858	Barley	Chevron x M69	(Canci et al., 2003)
12	1	Kernel discolouration	EBmac558 - HVM36	Barley	Chevron x M69	(Canci et al., 2003)
13	n/a	Black point	WMC154 - WMC149	Wheat	Sunco x Tasman	(Lehmensiek et al., 2004)

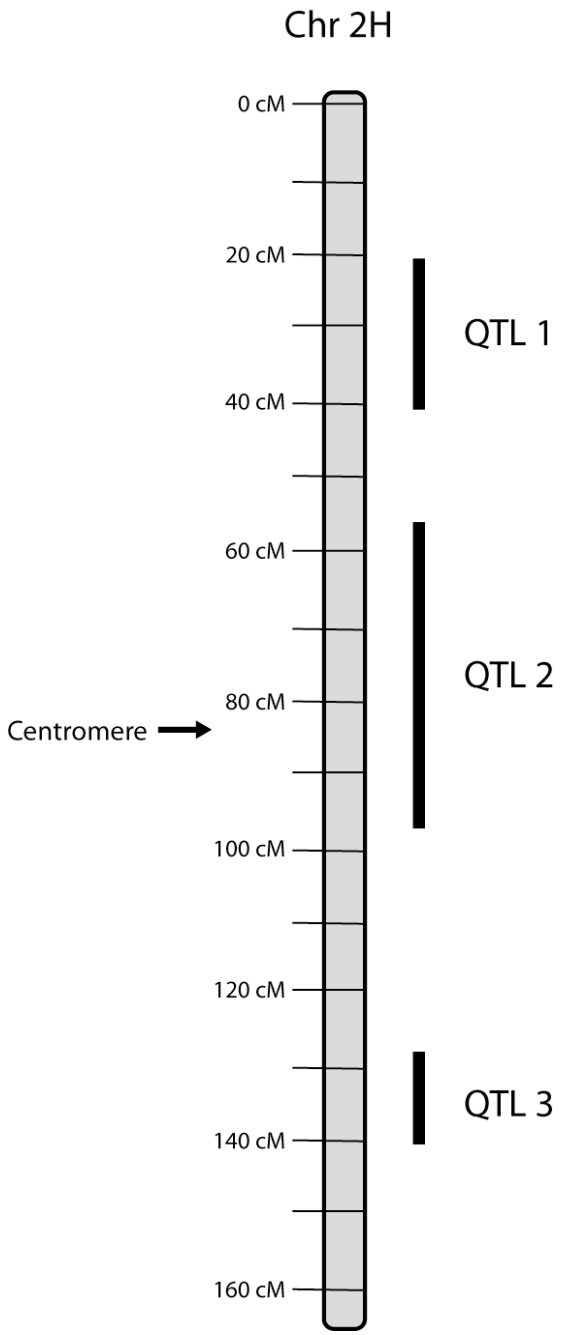

Figure 4-1 Relative positions of QTL associated with black point and kernel discolouration of barley grain. Using a previously published barley consensus map (Wenzl et al., 2006), thirteen previously identified QTL from various mapping populations (Table 4-2) were positioned on chromosome 2H. The QTL fell into three distinct locations along the chromosome.

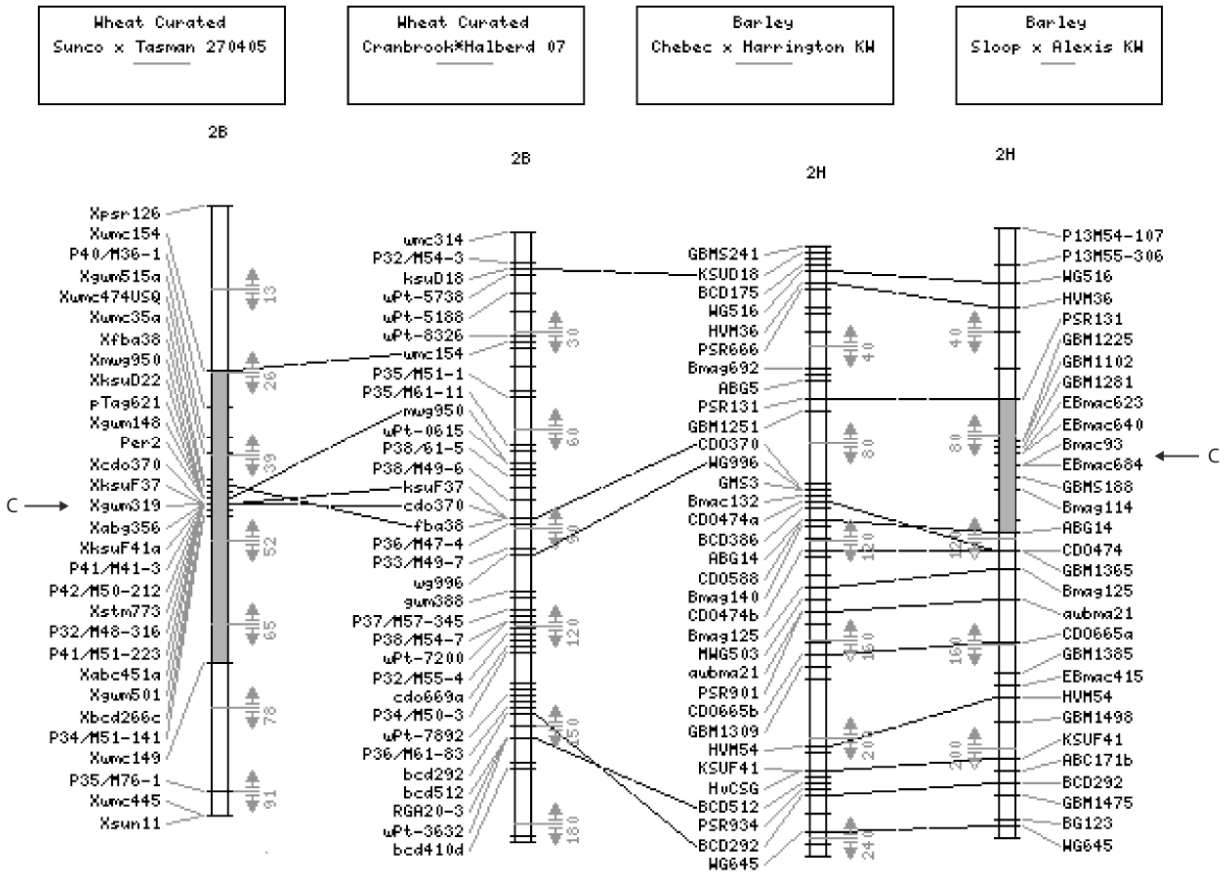

Figure 4-2 Alignment of black point QTL identified in wheat and barley mapping populations using the program CMap. Boxed grey regions show the area defined at the QTL on wheat chromosome 2B (far left map) and barley chromosome 2H (far right map). Lines connect common markers between maps while the centromere is indicated as C.

4.3.2 *The in silico* approach shows a syntenic relationship 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 approximately 13 cM (Figure 4-3).

For the top region of the QTL there was a lack of barley genetic markers that had sequence information available and 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. Unfortunately, the top of the wheat deletion region could not be aligned with the previous wheat map as no common markers were identified.

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 (Figure 4-3).

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 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 (Figure 4-3).

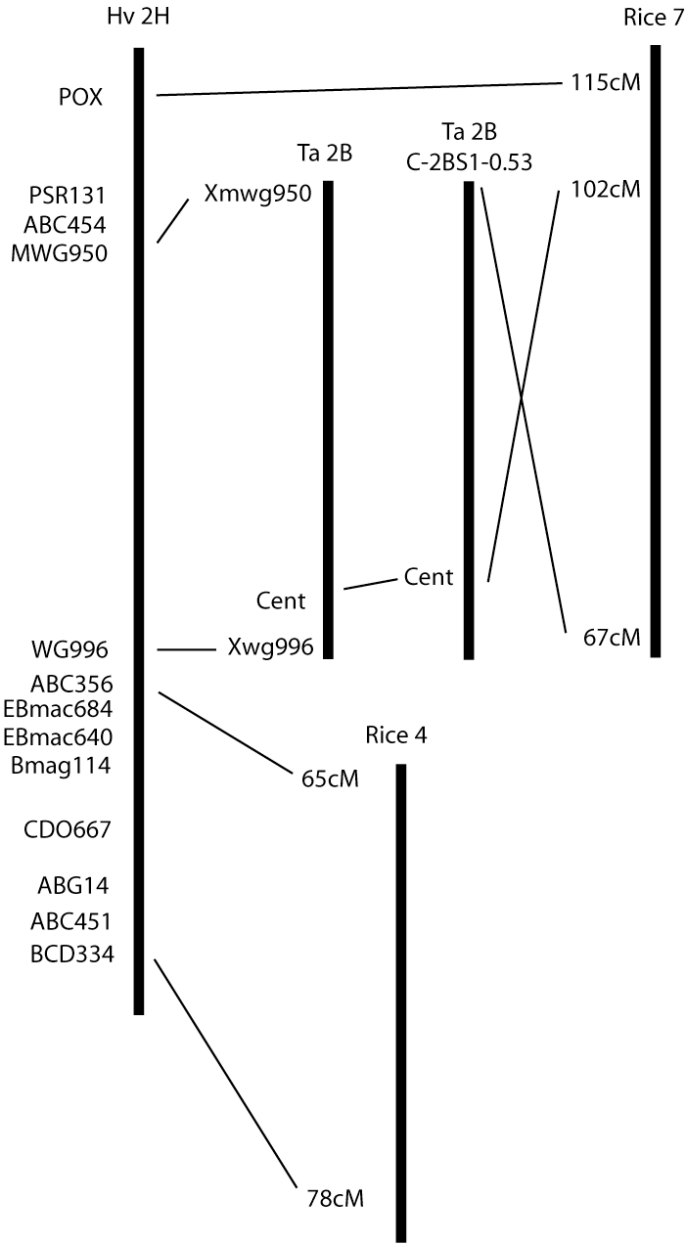

Figure 4-3 Comparison of the black point QTL region on chromosome 2H with wheat and rice. Barley genetic markers were identified using a BLASTn search against the rice genome to find regions of synteny. The wheat genetic map and wheat EST deletion map was used to bridge the barley and rice genomes where there were inadequate barley genetic markers.

4.3.3 Identification and mapping of selected barley EST sequences

From the identified rice sequence of chromosomes 4 and 7 that was syntenous with the black point QTL, barley ESTs with sequence similarity were identified. Annotation of these EST sequence using BLASTx searches returned 1928 unique annotations (see Appendix A) which were grouped into 11 functional categories (Figure 4-4). 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 (refer to Chapter 1.2.3), 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 4-3). ESTs showing high sequence similarity to peroxidase enzymes, 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 4-3). 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 grain cDNA except for Hv.t22 and Hv.t31, with these producing multiple or no PCR products that could be cloned. 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 (Figure 4-5; Table 4-4). Of these 15 RFLP probes, four also detected another copy of the gene located on separate chromosomes. From the 15 markers that were present on chromosome 2H, polymorphisms were detected with 10 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 (four markers). Polymorphisms were detected for the marker BCD334 with the restriction enzymes *Dra*I, *Eco*RI and *Hind*III. The marker BMY2 was polymorphic with all six restriction enzymes used (Figure 4-6; Table 4-4).

Mapping of the polymorphic markers within the Alexis x Sloop double haploid mapping population was successful for all markers except for Hv.t84.6, Hv.t 81 and Hv.t 67_1. This was due to the signal from the Southern membranes being too weak to score accurately across the entire Alexis x Sloop population.

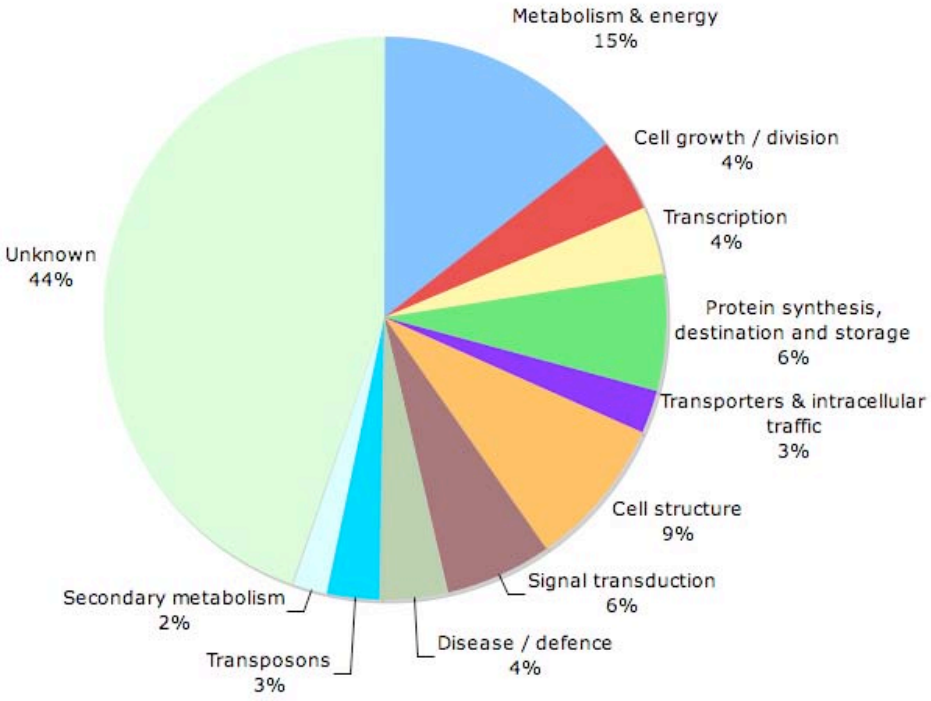

Figure 4-4 Functional classification of the ESTs predicted to reside within the barley black point QTL on chromosome 2H. ESTs identified from barley-wheat-rice comparison were grouped into functional groups based on the annotation obtained from the BLASTn searches. Functional groups were adapted from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

Table 4-3. Barley ESTs (20) predicted to reside within the black point QTL were selected. 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 Chr	Barley EST	Protein accession	Annotation	*E*-value
Hv.t102	7	BE591326	EAZ04931	Hypothetical protein OsI_026163 [*Oryza sativa* (indica cultivar-group)]	7.00E-46
Hv.t101	7	BE398439	AAM65274	NADH dehydrogenase [*Arabidopsis thaliana*]	1.00E-50
Hv.tPox1	7	CD056766	AAG46133	Putative peroxidase [*Oryza sativa*]	6.00E-54
Hv.t81.6	7	CB864764	CAA77237	Reversibly glycosylated polypeptide [*Triticum aestivum*]	1.00E-29
Hv.t81	7	BG314068	EAZ40456	Hypothetical protein OsJ_023939 [*Oryza sativa* (japonica cultivar-group)].	5.00E-16
Hv.tCsl	7	CB869766	AAL25133	Cellulose synthase-like protein OsCslF3 [*Oryza sativa*]	1.00E-95
BMY2	7	BE193999	AAG25638	Beta-amylase [*Hordeum vulgare*]	2.00E-98
Hv.tCHS	7	BQ466496	XP_479275	Putative chalcone synthase [*Oryza sativa* (japonica cultivar-group)]	7.00E-62
Hv.t71	7	BE636802	BAC57673	Putative glucose-6-phosphate/phosphate translocator precursor	3.00E-46
Hv.t70	7	DN156338	AAP80640	Succinate dehydrogenase subunit 3 [*Triticum aestivum*]	5.00E-60
Hv.t67_1	7	CA019486	BAD68985	Putative tRNA-(N1G37) methyltransferase [*Oryza sativa* (japonica cultivar-group)].	2.00E-47
Hv.t67	7	BJ464836	EAZ04028	Hypothetical protein OsI_025260 [*Oryza sativa* (indica cultivar-group)].	1.00E-68
Hv.t2	4	AJ473633	AAQ01197	COP9 [*Oryza sativa* (japonica cultivar-group)]	4.00E-46
Hv.t11	4	BI959763	BAD18962	Ferric reductase [*Oryza sativa* (japonica cultivar-group)]	9.00E-63
Hv.t13	4	BF264799	CAI84707	Lipoxygenase-like protein [*Hordeum vulgare* subsp. *vulgare*]	1.00E-68
Hv.t18	4	AV918365	BAD37679	Putative dehydration stress-induced protein [*Oryza sativa* (japonica cultivar-group)]	3.00E-23
Hv.t22	4	CB869939	BAD37858	Putative peroxidase [*Oryza sativa* (japonica cultivar-group)]	2.00E-45
Hv.t31	4	BQ767022	CAE92372	Peptide methionine sulfoxide reductase [*Secale cereale*]	2.00E-93
Hv.t38	4	AJ474981	AAL38796	Putative quinone oxidoreductase [*Arabidopsis thaliana*]	8.00E-60
bcd334	4	BE438762	CAA89005	Phenylalanine ammonia lyase [*Hordeum vulgare* subsp. *vulgare*]	2.00E-78

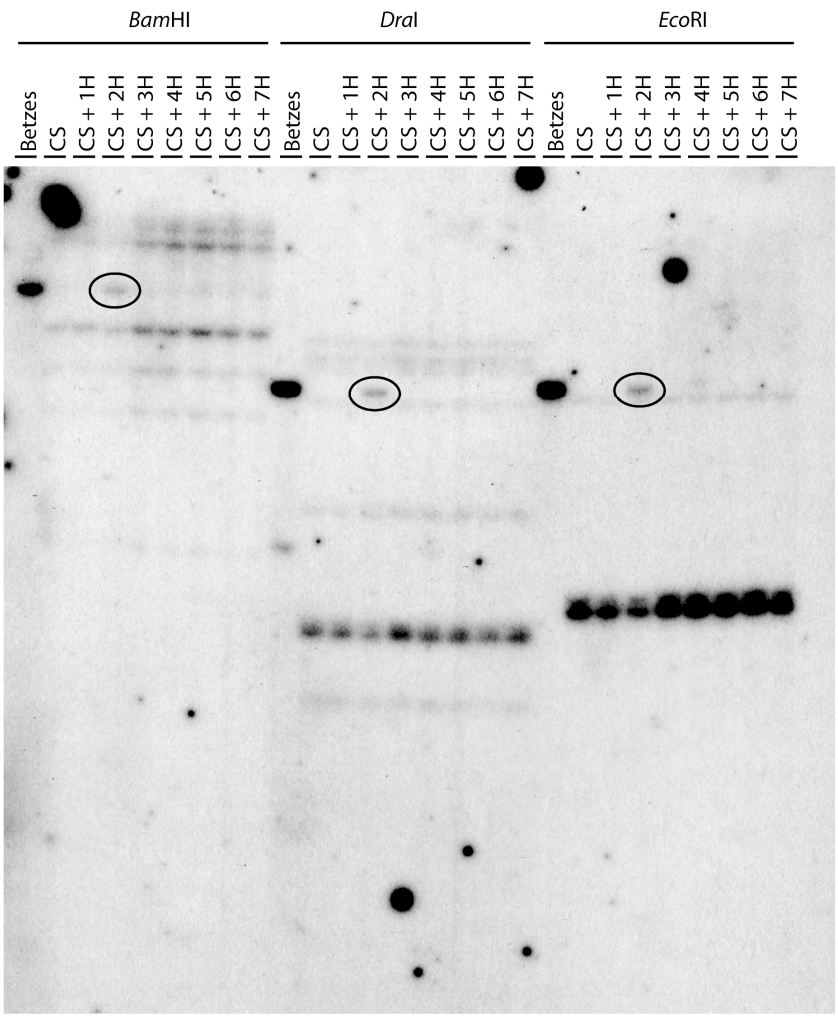

Figure 4-5 A representative Southern blot membrane used to determine the barley chromosome to which the ESTs localise. The barley cultivar Betzes, wheat cultivar Chinese Spring (CS), and 7 lines of Chinese Spring containing an additional barley chromosome (1H-7H), were digested with six different restriction enzymes (only *Bam*HI, *Dra*I and *Eco*RI are shown). Bands identified in Betzes were searched for in one of the seven barley wheat addition lines to indicate that the gene is located on that chromosome. This blot represents the marker BMY2, showing it is located on chromosome 2H (as indicated by the open black circles).

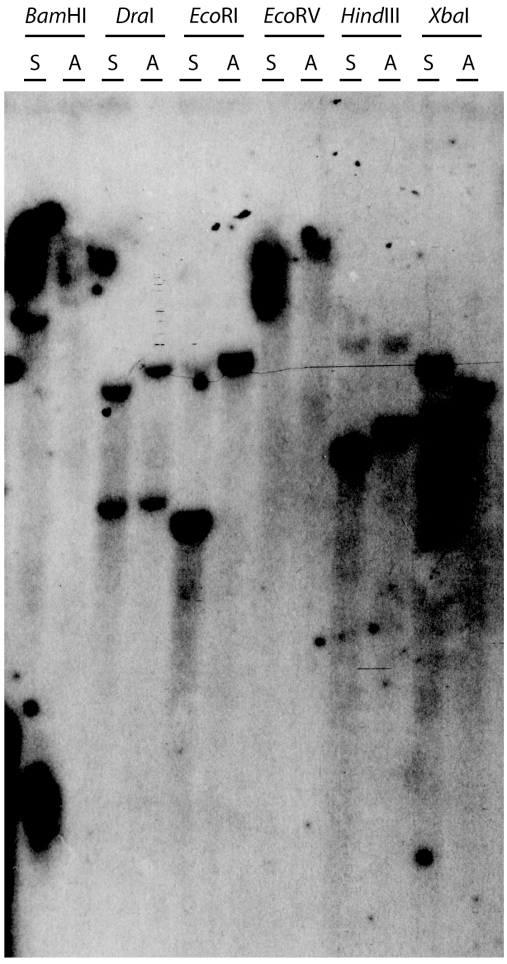

Figure 4-6 RFLP blot illustrating whether an EST is polymorphic between Alexis and Sloop. DNA from Alexis (A) and Sloop (S) was digested with six different restriction enzymes. This RFLP blot was hybridised with a BMY2 probe and indicates that there are polymorphisms in all six restriction enzymes used.

Table 4-4 Summary of each marker examined. For each marker, the functional group it belongs to, whether it was successfully PCR amplified and where it is positioned 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	PCR amplified	Chromosome location	Polymorphic between Alexis and Sloop	Mapped within Alexis x Sloop population	Located in QTL group 2
Hv.t102	Unknown	Yes	2H	*Dra*I	Yes	In
Hv.t101	Metabolism & energy	Yes	2H, 3H	*Dra*I	Yes	Out
Hv.tPox1	Disease / defence	Yes	3H	No	No	n/a
Hv.t81.6	Unknown	Yes	2H, 4H	*Eco*RI	No	n/a
Hv.t81	Unknown	Yes	n/a	*Eco*RI	No	n/a
Hv.tCsl	Cell structure	Yes	2H	No	No	n/a
BMY2	Metabolism & energy	Yes	2H	*Bam*HI, *Dra*I, *Eco*RI, *Eco*Rv, *Hind*III, *Xba*I	Yes	In
Hv.tCHS	Secondary metabolism	Yes	5H	No	No	n/a
Hv.t71	Metabolism & energy	Yes	2H	No	No	n/a
Hv.t70	Metabolism & energy	Yes	2H, 3H	*Dra*I	Yes	Out
Hv.t67_1	Metabolism & energy	Yes	2H	*Eco*RI	No	n/a
Hv.t67	Unknown	Yes	2H	No	No	n/a
Hv.t2	Signal transduction	Yes	2H	No	No	n/a
Hv.t11	Transporters & intracellular traffic	Yes	2H	No	No	n/a
Hv.t13	Metabolism	No	n/a	n/a	No	n/a
Hv.t18	Disease / defence	Yes	2H	No	No	n/a
Hv.t22	Disease / defence	No	n/a	n/a	No	n/a
Hv.t31	Protein synthesis destination and storage	No	n/a	n/a	No	n/a
Hv.t38	Secondary metabolism	Yes	2H, 5H	*Dra*I	Yes	Out
bcd334	Secondary metabolism	Yes	2H	*Dra*I, *Eco*RI, *Hind*III	Yes	In

4.3.4 Genetic location of mapped ESTs and effect on the black point QTL

From the original 20 ESTs identified using the *in silico* approach, six ESTs were mapped within the Alexis x Sloop double haploid population. The marker Hv.t38 produced three different polymorphisms with the restriction enzyme *Dra*I. These were designated Hv.t38a, Hvt38b (Figure 4-7) and Hv.t38c. Hvt.38c mapped 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 (Figure 4-7; Table 4-4).

The original black point QTL spanned a genetic map distance of 30 cM, and the peak height of the QTL had a logarithmic odds (LOD) score of 3.4 (Figure 4-8A). After adding the additional markers identified in this study to the genetic map, the QTL region has been reduced to 20 cM and the LOD score increased to a more significant value of 4.0 (Figure 4-8B). The marker Hv.t102 had the greatest effect in reducing the QTL region as it was able to better define the top region of the QTL by filling in a previously large gap that existed between markers PSR131 and EBmac607.

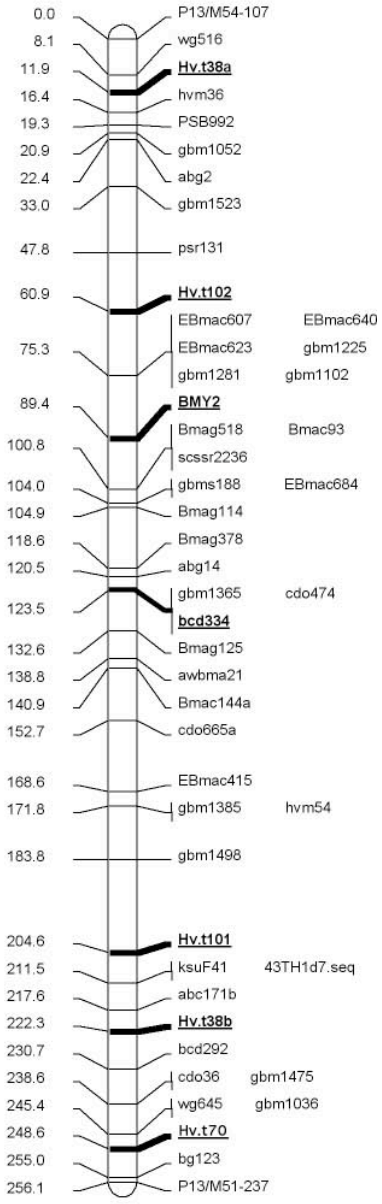

Figure 4-7 Genetic map of chromosome 2H. Polymorphic markers developed in this study were placed on the Alexis x Sloop double haploid genetic map. Markers added to the map are shown underlined with bold lines.

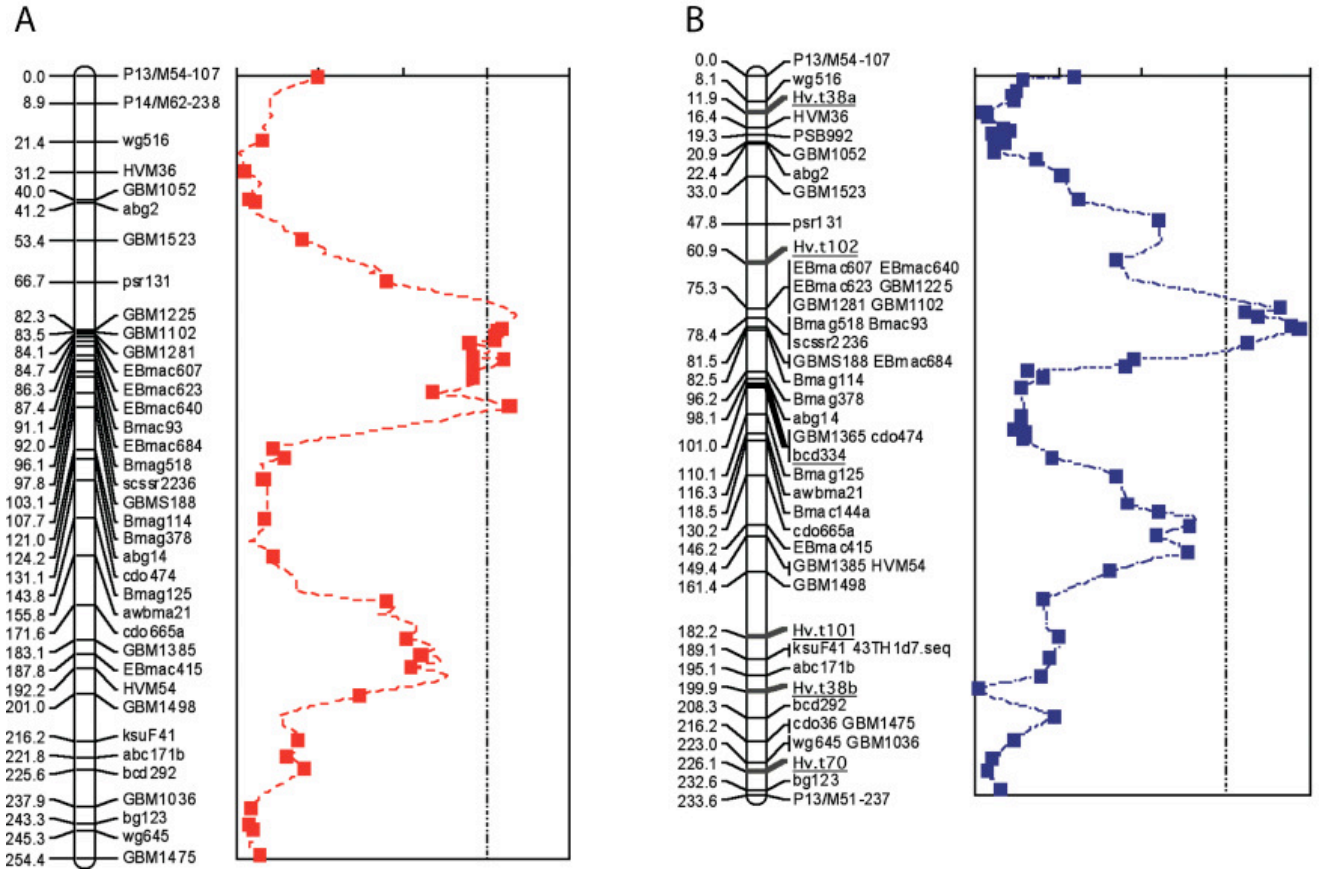

Figure 4-8 Effect of additional markers on the black point QTL. (A) Black point QTL prior to this study. (B) Effect of additional markers on the black point QTL. The vertical dashed line in (A) and (B) indicates a significant LOD score of 3.

4.4 Discussion

Several QTL have been identified throughout the barley genome that control black point and kernel discolouration. This present study set out to further characterise the QTL identified on chromosome 2H using a barley-wheat-rice comparison. The goal was to understand what genes are influencing the QTL and to further define the QTL size.

This aim was achieved by firstly comparing previously identified QTL for black point and kernel discolouration on chromosome 2H of barley. From this it was determined that there are three QTL regions on chromosome 2H that influence black point and kernel discoloration. QTL groups 1 and 3 were predominately identified from studies conducted in North America, where the symptoms are reported to be due to inoculation either naturally or artificially with *F. graminearum* (de la Pena et al., 1999; Canci et al., 2003). Within Australia, *F. graminearum* is not responsible for symptom development (Williamson, 1997). The only QTL in group 3 that was not reported to be *F. graminearum* related was identified from an Australian study in the population VB9104 x Dash and was associated with kernel discolouration.

The QTL group 2 was of most interest in this study as it is associated with both black point and kernel discolouration. Interestingly the same QTL was identified for black point and kernel discolouration within the Alexis x Sloop population (Li et al., 2003; Zerk et al., *unpublished*). This indicates that the same gene(s) within this QTL may influence the two traits. In further support of the importance of this region, it was also found that the wheat black point QTL on chromosome 2B

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.

To determine the genes underlying the black point QTL in barley, a barley-wheat-rice genome comparison was made. It was found *in silico* that the QTL on barley chromosome 2H shared synteny with rice chromosome 4 and 7, and the change between rice chromosomes coincided with the centromere of barley centromere 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 (Rota and Sorrells, 2004).

From the identified 12.2 Mb rice genome sequence, a significantly large set of barley EST sequences showing sequence similarity were identified. ESTs of interest were selected based on their possible role in black point development. As previously highlighted in this thesis, 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 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. It was

previously found in this study that barley grain peroxidase 1 (BP1) was more abundant in black pointed grains (refer to Chapter 2). However, BP1 is located on chromosome 3H of barley and thus not directly associated with this QTL.

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). 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). From the *in silico* approach an EST with sequence similarity to a quinone reductase was identified. Within Chapter 2 it was discussed that 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 was therefore of 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 phenylpropanoid metabolism pathway which produces phenolic compounds (Whitaker and Chang, 1996). PAL is also wound inducible and

it has been shown that an increase in the levels 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 beta amylase 2 gene (BMY2). Beta 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 classed as an ubiquitous beta amylase as it is expressed in all tissues of barley (Shewry et al., 1988) as opposed to 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 to 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, this would serve as a useful marker to select for this trait. 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.

Scattered throughout the QTL region were several MADS box genes (Appendix A). MADS box genes are involved in regulation and timing of flower development in plants (Trevaskis et al., 2003). This was of interest as this black point / kernel discolouration QTL is closely linked to a loci controlling heading date (Boyd et al., 2003). Sloop and Alexis also segregate for heading date, which raised the possibility that heading date is controlling black point susceptibility in this population by influencing when the grains are subjected to weather conditions that

induce black point. However it has been observed that kernel discolouration and heading date segregate independently of each other in this genomic region (Li et al., 2003) and it is therefore likely that there are two tightly linked loci for black point and heading date also.

From the *in silico* identification of candidate genes RFLP probes were made to determine whether the genes were located on chromosome 2H and also if they mapped within the black point QTL. From the 17 successfully amplified ESTs, 14 were located on chromosome 2H. Of these, 10 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 144 cM apart on barley chromosome 2H. Likewise the position 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. Such loss of syntenic relationship at the micro level is most likely due to chromosome restructuring events, such as gene duplication and deletion and chromosomal inversions and translocations, since barley and rice evolved from a common ancestor approximately 60 million years ago (Wolfe et al., 1989). In support of this several transposable elements were identified with the QTL region, which could facilitate such 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 did map to the predicted position on barley chromosome 2H that coincided 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 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 the black point susceptibility.

5 General Discussion

5.1 Introduction

Black point is a serious problem that faces the Australian barley industry, as affected grains are deemed unacceptable to the malting industry. Each year black point results in significant losses to the barley industry due to downgrading from malt to feed grade.

Despite causing significant economic losses, modern barley breeding varieties continue to be susceptible to black point. In part, this is because breeding for black point resistance relies on visual phenotypic assessment to identify tolerant lines. This process is time intensive and consistent phenotypic assessments are difficult due to black point levels varying considerably from year to year. To assist in breeding tolerant varieties there is a need to identify the genes controlling black point so that molecular breeding techniques can be implemented for screening.

This study has identified genes associated with black point formation using a two-pronged experimental approach involving proteomic and comparative mapping techniques. This comprised of identifying a potential role for barley grain peroxidase 1 (BP1) in black point along with the identification of a novel late embryogenesis abundant (LEA) protein that has given insight into the physiological events involved in black point formation. Furthermore this study has identified several candidate genes underlying a QTL for black point susceptibility on chromosome 2H and in doing so reduced the QTL size by 10 cM.

The identification of BP1 and the LEA protein has contributed significantly to

our understanding of how black point develops. With further validation, these genes can potentially be used as diagnostic genetic markers in future breeding programs, which will enable the constraints of phenotypic selection for black point tolerance to be overcome.

5.2 Evidence to propose a model for black point formation and future directions

There are two current models for black point formation. The first proposes that black point is the result of fungal colonisation and is widely accepted by North American researchers (refer to Chapter 1). The second more recently developed model, which was first proposed by an Australian study (Williamson, 1997), indicates black point is the result of an enzymatic browning reaction. The advantage of using a proteomics and comparative mapping approach in this study was that results gained were not influenced by a starting hypothesis assuming black point was due to either of these proposed models.

If black point were caused by a fungal infection it would be expected that fungal proteins would be isolated from black pointed grains. However the proteomic study did not identify any proteins of fungal origin. This was also the finding from a separate proteomics study comparing black pointed and healthy wheat grains (Mak et al., 2006). This knowledge, combined with the fact that black point can be reproduced *in vitro* in the absence of fungi (Williamson, 1997) suggests that fungal colonisation, at least under Australian conditions, is not the causative agent of black point.

This study has however provided further evidence to support the hypothesised

enzymatic model proposed in Chapter 1.2.3, whereby germination causes wounding which in turn induces enzymatic oxidation of phenolic compounds by peroxidase enzymes to result in discoloured end products.

5.2.1 The proteomics approach suggested physiological and biochemical changes in the grain are associated with black point

The proteomics approach used in this thesis revealed that the germination state in conjunction with enzymatic browning is likely to play a role in black point formation. Black pointed grains appear to have entered into a germination state based on the reduced levels of the LEA protein present. This germination may then lead to the physiological stress of tissues around the embryo resulting in wounding required to induce enzymatic browning. Whether the LEA protein is involved directly in causing black point by initiating the germination or is merely associated with the event was not determined. However, the LEA protein shares similarity to proteins identified in pea and soybean (Dehaye et al., 1997; Hsing et al., 1998) which are also biotinylated *in vivo* and proposed to act as biotin sinks providing biotin as a co-factor for enzymes involved in germination. Furthermore the identification of higher levels of BP1 in black pointed grains suggests that this enzyme could be directly involved in the oxidation of phenolic compounds needed to produce the discolouration. Immunolocalisation of BP1 showed it to be present in the embryo region where discolouration occurs further, thus supporting its involvement in black point.

Interestingly *LEA* and *BP1* mapped to locations on chromosome 6H and 3H respectively where QTL for black point have not previously been identified,

suggesting that neither gene is directly associated with susceptibility to black point. What may be occurring is that genes located within the black point QTL are controlling the expression of *LEA* and *BP1*. This phenomenon has recently been demonstrated with malt quality QTL that are associated with controlling the expression of genes that lie outside of the QTL region (Potokina et al., 2007). To determine if this is the case, gene expression for *LEA* and *BP1* could be measured across a mapping population to identify expression QTL and then compared to see if they coincide with known black point QTL.

To further confirm if *LEA* and *BP1* have a key role in black point, transformation could be conducted, generating either over-expression or knock-out transgenic plants to then functionally characterise. The phenotype of these *LEA* and *BP1* transgenic plants could be screened for increased or decreased incidence of black point. This approach may provide an enhanced phenotype for *BP1* over-expression lines, as increased *BP1* levels would be hypothesised to increase the rate of enzymatic browning. *LEA* transgenic lines on the other hand may not produce an enhanced phenotype. The reason for this is that in both healthy and black pointed grains *LEA* gene expression was at comparable levels. This indicates that the reduced LEA levels in healthy grain was due to protein degradation, therefore over- or reduced expression of *LEA* would not be expected to have an effect on black point.

The proteomics approach used in this study enabled the detection and characterisation of two proteins associated with black point, further research can now progress to help clarify their role in black point formation and grain development in general. Furthermore, the hypothesis of germination inducing black

point development can now be studied further. This can involve the identification and characterisation of other key genes, proteins and metabolites involved in the initiation of germination and determining their influence on black point development.

5.2.2 A black point QTL has been redefined and underlying genes identified

By utilising the established barley-wheat-rice syntenic relationship several candidate genes underlying the QTL for black point on chromosome 2H were identified. These included genes associated with phenolic acid biosynthesis including a phenylalanine ammonia-lyase, a putative quinone reductase and a putative chalcone synthase gene. This indicated that the QTL is possibly associated with controlling the supply of phenolic acid substrates needed for enzymatic browning to occur. Interestingly, a study in wheat concluded that the supply of phenolic substrates could be a tolerance mechanism for black point (Williamson, 1997).

This study has been successful in reducing the black point QTL on chromosome 2H to a size of 20 cM and identified genetic markers flanking this region that could potentially be used to select for this QTL in breeding programs. One constraint in doing so is that this QTL is still tightly linked to other agronomical important loci such as heading date (Boyd et al., 2003). For this QTL to be more easily utilised in breeding programs, further fine mapping to reduce the size of the QTL would be desirable to break the linkage with other loci. This study has helped to initiate this process by identifying *in silico* ESTs that are likely to reside within the QTL that can subsequently be used to generate more genetic markers.

In order to further reduce the QTL size and pinpoint specifically which gene is controlling the QTL, a map-based cloning approach would be beneficial. Map-based cloning involves the development of near isogenic lines that only differ genetically within the region of the QTL of interest, these lines are then crossed and offspring are selected using genetic markers where recombination events have occurred within the QTL (Salvi and Tuberosa, 2005). Typically in excess of 1000 offspring are developed to ensure adequate recombination events occur (Pourkheirandish et al., 2007). Thereafter, accurate phenotyping of recombinant lines is performed to determine the specific chromosome region controlling the trait.

Constructing and phenotyping such a population required for map-based cloning is not feasible for black point research for several reasons. Firstly, current methods of scoring black point require the manual counting of black pointed grains in 100 grain samples. This method is subjective due to variations in human scoring and difference in the severity of black point observed on individual grains. Secondly, there are no reliable glasshouse conditions that have been established to date in order to reproduce black point symptoms, thus field conditions are often relied upon to produce symptoms but these can vary dramatically from season to season. Both of these factors impact on the necessity to be able to accurately score recombinant lines as either susceptible or tolerant in map-based cloning studies (Varshney et al., 2006).

5.2.3 Reproduction of black point symptoms and germination

The development of glasshouse conditions that can routinely reproduce black point symptoms would greatly assist map-based cloning studies and black point

research in general. The knowledge gained from this study combined with that from previous studies could then assist in designing what environmental conditions are needed to reproduce black point reliably.

The results from this current study suggest that conditions are required that will cause the embryo to enter into a germination phase before the grain begins to desiccate. Previous studies have shown that by increasing the temperature during grain development the likelihood of precocious germination occurring was increased (Syankwilimba et al., 1999). Precocious germination is where the embryo continues to develop during grain development rather than entering into desiccation. Furthermore, correlations between field meteorological data and black point levels in wheat show that higher temperatures along with precipitation between the stages of milk and dough increase the incidence of black point (Moschini et al., 2006). Precipitation throughout grain development has been associated with black point development in several studies (Rees et al., 1984; Petr and Capouchova, 2001).

Based on the above studies, glasshouse conditions consisting of increased temperatures and humidity during the middle stages of grain development may lead to increased symptom development. Future studies trialling different growth conditions are needed so that a reliable system can be developed to produce black point. Being able to obtain consistent levels of black pointed grain would assist greatly in speeding up all facets of research conducted in understanding the trait.

5.3 Conclusion

This study has highlighted that there are likely to be several biochemical pathways involved in the enzymatic browning process that is proposed to cause

black point. The techniques utilised in this study have significantly narrowed down and identified individual genes associated within these biochemical pathways. The fact that there appears to be several pathways involved in producing symptoms suggests that pyramiding tolerance mechanisms to give enhanced tolerance will be feasible. However, further characterisation is necessary before tolerance mechanisms can be fully understood.

Black point research has now progressed to a stage where the research community have a much better understanding of the genetic and biochemical mechanisms involved. This knowledge can now be used to advise industry on the impact that black point has on the end-use of barley. More importantly there are now several research approaches as described above that can be utilised to further our understanding of black point. Ultimately this will enable breeding programs to introduce increased tolerance into new cultivars.