Identification of genetic regions associated with black point in barley

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Abstract: Black point (BP) can cause severe losses to the barley industry through downgrading and discounting of malting barley. The genetic improvement in BP resistance of barley is complex, requiring reliable screening tools, an understanding of genotype by environment interactions and an understanding of the biochemical mechanisms of melanisation involved in BP development. Thus the application of molecular markers for resistance to BP may be a useful tool for plant breeders. We have investigated the genetic regions associated with BP resistance in the barley F_2 population, Valier/Binalong. Quantitative trait loci (QTLs) contributed by the resistant parent Valier, were detected on chromosomes 2HS, 2HC, 3HL, 4HL and a QTL contributed by the susceptible parent, Binalong was detected on 5HL. Three of the 4 QTLs were detected in two distinctly different environments. The differences observed in BP resistance between these two environments and the implications for accelerated screening are discussed. Identified SSR markers in these regions may be useful for selecting black point resistance in related breeding materials.

Keywords Black point · F_2 · Quantitative trait loci · DArT

Introduction

In barley, kernel colour is an important selection criteria for the malting industry. Kernel discolouration can result in thousands of tonnes of malting barley being downgraded each year both in Australia and other parts of the world (De la Pena et al., 1999; Fox et al., 2004). Three types of barley kernel discolouration have been observed in Australia, namely caramelizing or yellow pigmentation of the whole grain, black

point and distinctive spots or staining caused by fungal proliferation (Fox et al., 2004; Li et al., 2003).

Black point (BP) is a brown/black discolouration of the embryo end of the grain. Environmental conditions such as warm, humid, rainy periods are important factors in BP development and they are of greatest importance during the milk to dough kernel development or grain-filling stages (Fox et al., 2004; Li et al., 2003; Moschini et al., 2006).

Black point has been associated with fungal infection in the United States, Slovakia and the Czech Republic (Canci et al., 2003; Canci et al., 2004; De la Pena et al., 1999; Hudec, 2007; Prokinova, 1999), but in Australia the dark colour is thought to be caused largely by biochemical responses (Fox et al., 2004; Williamson, 1997). A number of proteins have been associated with BP, including a novel 75-kDa late embryogenesis protein, which was more abundant in healthy grain and a barley grain peroxidase 1 protein, which was more abundant in black pointed grain. Higher levels of protein associated with stress, disease and defence have also been observed in BP-free grain in wheat, suggesting that protection from BP may be due to increased levels of these proteins (Mak et al., 2006; March et al., 2007).

To date, two studies have indicated potential genomic regions involved in kernel discoloration in barley (Canci et al., 2003; Li et al., 2003). A cross between Chevron and M69 was used to identify nine quantitative trait loci (QTL) associated with kernel discolouration, one each on chromosomes 4H and 5H, two each on chromosomes 7H and 2H, and three on chromosome 6H (Canci et al., 2003). QTLs for kernel discolouration measured by grain brightness, redness and yellowness have also been identified in seven Australian barley populations, one each on chromosomes 4H and 7H

and two each on chromosomes 2H, 3H and 5H (Li et al., 2003). To date, only one study has identified a QTL for BP resistance and this QTL was mapped on 2H in the barley population Alexis/Sloop (March et al., 2008).

The genetic improvement in BP resistance of barley is thus complex requiring an understanding of genotype by environmental interactions and biochemical mechanisms of melanisation, as well as reliable screening tools. Given the difficulties associated with conventional phenotypic screening for BP, the use of more extreme environments (to maximise phenotypic discrimination) and the generation of reliable molecular markers for screening BP resistance would be useful tools for plant breeders.

The aim of this study was to identify QTLs linked to BP resistance in barley by using an F_2 segregating population developed between the BP resistant variety Valier and the susceptible variety Binalong. The implication of QTL results, obtained using phenotypic data from two distinct environments, is discussed.

Materials and Methods

Plant material

An F_2 population of one hundred and eighty four and 188 lines from an F_2 population derived from the cross Valier/Binalong, were planted in the field at the Hermitage Research Station (HRS), Warwick in 2004 and 2005, respectively. One hundred-and-ninety-two F_2 lines of the same cross were also planted at Bundaberg Research Station (BRS), Bundaberg in 2005. Valier is a USA feed variety with a good level of BP resistance, whilst Binalong is an Australian feed variety susceptible to BP. The F_2 lines were grown in hill plots with 0.5 m spacing between the plants and a distance of 0.5 m between rows. Trials were drip irrigated within the field during the season. The

irrigation was provided once a week with a water supply of 10-50 mm. At HRS, clear plastic sheeting of 2 mm thickness was used to cover the trial post anthesis to produce a more controlled environment. To increase humidity during grain fill at HRS, overhead high pressure misters were used on a daily basis for 15 min early in the morning. The BRS trial was left open with ground irrigation. Leaf samples were taken from individual F_2 plants at an early growth stage. DNA is extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation).

Black point screening

Lines were scored for BP by counting the number of black pointed seeds in three 100-grain samples using the Australian Wheat Board counting tray. A mean was taken of the three sets of samples and the percent BP is calculated.

Diversity Arrays Technology (DArT)

DArT markers were produced from 184 F_2 lines of the 2004 HRS trial and 5 resistant and 5 susceptible F_2 lines of the 2005 BRS trial by Diversity Arrays Technology Pty Ltd (DArT P/L). As DArT markers are dominant, the heterozygous genotypes could not be distinguished from the homozygous genotypes in the F_2 population. Therefore, lines that had the DArT marker (produced a signal) were scored as C or D depending on whether the paternal or maternal parent respectively was positive for that DArT marker. Homozygous lines in which a DArT marker was absent (did not produce a signal) were scored as A (maternal) or B (paternal).

Single Sequence Repeat (SSR) analysis

Primer sequences of SSR markers were obtained from GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml) and a standard protocol was used to amplify the markers. The reaction mixture consisted of 20 ng DNA, 5 µM of each primer, 100 µM of each dNTP, 1.5 mM $MgCl_2$, 1x buffer (Bioline Pty Ltd., Australia) and 0.5 U BIOTAQ™ DNA Polymerase (Bioline Pty Ltd., Australia) in a total volume of 10 µl. The following polymerase chain reaction (PCR) cycle profile was used: 5 min at 94°C, followed by 35 cycles 94°C for 1 min, 50-60°C (depending on annealing temperature) for 1 min and 72°C for 1 min and one cycle at 72°C for 10 min. The amplified products were visualized using a Gel-Scan 2000™ (Corbett Life Sciences, Sydney, Australia).

Linkage map construction and QTL analysis

MapManager QTXb20 (Manly et al., 2001) was used to partition DArT and SSR markers into linkage groups and RECORD (Van Os et al., 2005) was used to order markers within linkage groups. The Kosambi function was used to calculate map distances. The single marker regression analysis function in MapManager QTXb20 was used to identify significant marker trait associations. Composite interval mapping was conducted with Windows QTL Cartographer version 2.5 (Wang et al., 2007). A permutation test was performed using 1000 iterations (P=0.05) to determine the significance of linkage to the trait. Map figures were produced with MapChart version 2.1 (Voorrips, 2002).

Results

Phenotyping

As the BRS is situated along the coast at a significantly lower elevation and latitude than the HRS, the environmental conditions at these two sites can vary considerably. The grain-fill period from about October to December is critical for BP development. During this period in 2004 and 2005, the mean relative humidity at the HRS was 61.4 and 61.6%, respectively whilst it was 64.0% at the BRS in 2005. The total rainfall recorded from October to December for the HRS was 222 and 301 mm for 2004 and 2005, respectively and 375 mm for BRS in 2005. Only minor differences were observed in the average maximum temperature for both sites and years but the average minimum temperature was higher for BRS (20.7°C) than HRS (13.3 and 14.5°C, respectively for 2004 and 2005; (Table.4).

BP percent was variable across the two years and sites (Table 1; Fig. 1) with percent BP ranging from 1.0 to 40.7 at HRS in 2004 (HRS04), 0.0 to 29.7 at HRS in 2005 (HRS05) and 0.0 to 73.3 at BRS in 2005 (BRS05). The average percent BP was 7.2 (standard deviation 6.9), 5.7 (4.5) and 35.1 (16.6) for HRS04, HRS05 and BRS05, respectively. No significant correlations were observed between percent BP among years or environments ($P>0.05$; data not shown). Normal distributions of the percent BP were observed for the HRS trials, whereas the data from the BRS trial displayed a bi-modal distribution (Fig. 1). A large number of lines had lower percent BP scores those of the parents suggesting transgressive segregation.

Valier/Binalong genetic map

DNA was extracted from 184 individual F_2 lines of the Valier/Binalong population of the HRS04 trial and sent for DArT analysis. The 208 DArT markers obtained for this population were grouped into different linkage groups and assigned to chromosomes

based on a published barley consensus map (Wenzl et al., 2006). Forty-seven SSR markers were added to the map and the final map consisted of 255 markers (Fig. 2). Clustering, mainly of DArT markers, was observed on all chromosomes and 70 markers were redundant. Chromosomes 1H and 4H had the least markers, 11 and 26, respectively. The total map distance was 1291 cM with an average map density of 1 marker every 5 cM.

QTL analysis

Linkage groups 1H, 6H and 7H were split up into two groups each prior to composite interval mapping (CIM), as gaps greater than 50 cM were present on these chromosomes. Redundant loci were excluded from the CIM analysis. The Permutation test indicated that a log-likelihood (LOD) score of 2.5 was significant. With the HRS04 trial data significant QTL were identified on chromosomes 2HC (QBP.VaBi-2HC), 3HL (QBP.VaBi-3HL) and 5HS (QBP.VaBi-5HS) with LOD scores 3.7, 6.5 and 3.4, respectively (Table 2; Fig. 2). The phenotypic variances explained by the QTL on 2HC, 3HL and 5HS were 8, 28 and 12%, respectively. These QTLs were contributed by the resistant parent Valier.

Validation and bulked segregant analysis

SSR markers located in the region of the 2HC (Bmag692 and EBmac640), 3HL (Bmag13 and EBmac708) and 5HS (Bmac163 and Bmac113) QTLs were amplified on DNA of the HRS05 population and single marker regression analysis was performed. No association between these SSR markers and black point resistance was observed in this trial.

As the environment in Bundaberg is distinct from the HRS environment, it was thought that different QTL might be detected with the BRS05 trial. Therefore, a bulked segregant analysis (BSA) approach was undertaken with the BRS05 trial to rapidly identify markers linked to BP resistance. DNA of the five most resistant and five most susceptible lines, selected from the 192 lines of the BRS05 trial, was sent for DArT analysis. The DNA samples were not pooled into two bulks but kept separate so that they could be analysed individually. Four regions (on chromosomes 2HS, 3HL, 4HL and 5HL) were identified in which most of the resistant lines possessed the alleles of one parent and most of the susceptible lines the allele of the other parent (Table 3); i.e. for the 2HS, 3HL and 4HL regions three or more lines of the resistant bulk had the allele of the resistant parent, Valier, whereas four or more lines of the susceptible bulk had the allele of the susceptible parent, Binalong. For the 5H region, four lines of the resistant bulk possessed the Binalong allele whereas three lines of the susceptible bulk had the allele of the Valier parent. Thus in the latter, the resistance is contributed by the susceptible parent. The positions of the DArT markers identified by BSA are indicated in Fig. 2 (underlined). Marker bpb-9220 on chromosome 2HS underlined in the map is closely linked to marker bpb-8292 (Table 3) and underlined marker bpb-2501 is closely linked to both bpb-4261 and bpb-9682. Marker bpb-1999 on chromosome 4H is not present in the map but is closely linked to underlined marker bpb-0610. The region associated with BP resistance on 3H was in a similar region as QBP.VaBi-3HL. The putative QTL identified on 2H was on the short arm and was not in the same region as QBP.VaBi-2HC.

SSR markers in the regions of the putative QTL on 2HS (HVM36 and Bmag692), 4HL (EBmac635 and EBmac788) and 5HL (GMS01 and HVM6) identified by BSA were

amplified on DNA of the HRS05 trial to determine whether these QTLs can be detected with this trial data. Single marker regression analysis results indicated a significant association of black point resistance with the 5H region (LOD score of 2.7; phenotypic variance explained of 6%) and a suggestive association with the 4H region (LOD score of 1.4; phenotypic variance explained of 3%; Table 2). No association with the 2HS region and BP resistance was detected in this trial.

Discussion

We have identified QTL for BP resistance using an F_2 cross with Valier as the BP resistant parent and Binalong as the susceptible parent. Different lines of this population were planted in two different sites over two consecutive years. The coastal Bundaberg site (BRS) has a higher average minimum temperature, higher rainfall and slightly higher relative humidity than the Warwick site (HRS), where barley is typically grown. Even though the Bundaberg site does not reflect the typical barley growing environment, but it was chosen for black point screening due to the environmental conditions favourable for BP development. The phenotypic data manifests these environmental differences with higher percent BP observed at the BRS site (up to 73%) compared to the HRS site (up to 41%).

A genetic linkage map was produced for the Valier/Binalong F_2 population using both dominant (DArT) and co-dominant (SSR) markers. The marker order was consistent with the marker order of the DArT-based consensus map (Wenzl et al., 2006) and the total map distance was similar to that observed in other studies (Stein et al., 2007; Varshney et al., 2007; Wenzl et al., 2006; Willsmore et al., 2006). A redundancy level of 34% was observed, which was mainly due to clustering of the DArT markers. This

level is similar to the level (38%) previously observed with DArT-based maps (Wenzl et al., 2006). The 1H and 4HS chromosomes have the least markers suggesting that the parents are not polymorphic in these regions. Other studies have also indicated that chromosomes 1H and 4H are less polymorphic (Karakousis et al., 2003; Wenzl et al., 2006).

QTL analysis of the HRS04 trial detected QTL on 2HC, 3HL and 5HS. These QTL regions were investigated in the HRS05 trial but no associations with BP resistance could be detected at these loci. As the SSR markers, which were used to validate the QTL on chromosome 5H, were more than 40 cM distal to the QTL region, no association with the trait was expected. Attempts to identify polymorphic SSR markers closer to the QTL were unsuccessful.

A BSA approach (Michelmore et al., 1991) was used to identify genomic regions associated with BP resistance in the BRS05 trial. This approach has been applied successfully in other studies to identify QTL (Cheong et al., 2006; Lehmensiek et al., 2001; Shen et al., 2003; Wenzl et al., 2007). Four putative QTL on chromosomes 2HS, 3HL, 4HL and 5HL were identified by BSA of which one (3HL) had previously been identified in the HRS04 trial (Table 3). The other QTLs were examined in the HRS05 trial and marker regression analysis confirmed the presence of QTL on chromosomes 4HL and 5HL. The QTL on 3HL, 4HL and 5HL were therefore detected at two sites and this suggests that similar genomic regions are associated with BP resistance in these two distinctive environments. Thus, even though the environment at the BRS does not reflect the typical environment where barley is grown, it is a reliable environment in which to score for BP resistance and provides a BP screening site suitable for genetic studies.

Although the QTL on 2HS was only detected at the BRS05 site, it was in the same region where a QTL associated with kernel discolouration severity had previously been identified in the Chevron/M69 population by Canci et al., (2003). The QTL, QBP.VaBi-2HC and QBP.VaBi-3HL, are in the same genomic region as QTL identified by Li et al., (2003) for kernel discolouration using seven different Australian mapping populations. Both these QTLs were also associated with heading date and it was suggested that a more detailed mapping study needs to be undertaken to determine whether these are two different QTLs or whether the concurrent QTLs were due to pleiotropy (Li et al., 2003). QBP.VaBi-4HL is in the same region as the kernel discolouration QTL identified by Canci et al., (2003) in the Chevron/M69 population.

In wheat, BP QTLs have been identified on the short arms of the group 2 chromosomes (Lehmensiek et al., 2004). The 2B chromosome region in wheat has been compared to the 2H QTL identified in barley to identify candidate genes involved in BP formation (March et al., 2008).

In the light of these several studies, robust PCR markers closely linked to the identified QTL regions of chromosomes 2HS, 2HC, 3HL, 4HL and 5HL should be of use in selecting germplasm with improved resistance to BP in barley. As the nucleotide sequences for DArT markers become increasingly available, their conversion to PCR-based markers suitable for routine screening will assist in this endeavour.

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Table 1. Descriptive statistics of trait data of BP of barley trials of HRS 2004, BRS 2005 and HRS 2005 are shown (SE = Standard Error; st.dev = standard deviation; Min: Minimum; Max: Maximum)

Parameter	**Black point (%)**		
	BRS05	**HRS05**	**HRS04**
Mean	35.1	5.7	7.2
Min	0.0	0.0	1.0
Max	73.3	29.7	40.7
SE	1.3	0.3	0.6
St.dev	16.6	4.5	6.9

Table 2. QTL for BP resistance identified in the HRS04, BRS05 and HRS05 trials are listed. QTLs were given the same name if they are in the same location in the different sites. Flanking markers are given together with the LOD scores and the percentage phenotypic variance (R^2) explained. The parent contributing the resistance is indicated. References for QTLs previously identified in the same location in other studies are given.

QTL name	**Marker interval**	**LOD**	**R^2**	**Parent**	**Previously identified by**
HRS04					
QBP.V/B-2HC	bPb-1664-Ebmac640	3.7	8	Valier	Li *et al.* 2003
QBP.V/B-3HL	bPb-4830-Ebmac708	6.5	28	Valier	Li *et al.* 2003
QBP.V/B-5HS	bPb1084-bPb2460	3.4	12	Valier	
BRS05 (DArT results)					
QBP.V/B-2HS	bpb-6847-bPb-2501			Valier	Canci *et al*. 2003
QBP.V/B-3HL	bPb-3630-bpb-4830			Valier	Li *et al.* 2003
QBP.V/B-4HL	bPb-0610-bpb-8164			Valier	Canci *et al*. 2003
QBP.V/B-5HL	bPb-4809-bpb-9244			Binalong	
HRS05					
QBP.V/B-4HL	EBmac635-EBmac788	1.4	3	Valier	Canci *et al*. 2003
QBP.V/B-5HL	GMS01-HVM6	2.7	6	Binalong	

Table 3. Selected DArT markers amplified on the parents Valier (Va) and Binalong (Bi) and the 5 most resistant (R) and 5 most susceptible (S) lines of the BRS05 F_2 population. The chromosome (chr) and marker name are indicated on the left, "1" indicates that the DArT marker is present whereas "0" indicates that it is absent and "-" indicates that the data is missing

Chr	Marker	Va	Bi	R	R	R	R	R	S	S	S	S	S
2H	bPb-6847	1	0	0	0	1	1	1	0	0	0	1	0
2H	bPb-8292	1	0	0	0	1	1	1	0	0	0	1	0
2H	bPb-4261	1	0	0	0	1	1	1	0	0	0	1	0
2H	bPb-9682	1	0	0	0	1	1	1	0	0	0	1	0
3H	bPb-3630	1	0	0	0	1	1	1	0	0	1	0	-
3H	bPb-4830	1	0	0	0	1	1	0	0	0	0	0	0
4H	bPb-1999	1	0	1	0	1	1	-	0	0	0	0	-
4H	bPb-0610	1	0	-	0	1	1	1	0	0	0	0	1
4H	bPb-8164	1	0	0	0	1	1	1	0	0	0	0	1
5H	bPb-9660	0	1	1	1	0	1	1	0	1	1	0	0
5H	bPb-4809	0	1	1	1	0	1	1	0	1	1	0	0
5H	bPb-5766	0	1	1	1	0	1	1	0	1	1	0	0
5H	bPb-4725	0	1	1	1	0	1	1	0	1	1	0	0
5H	bPb-7360	0	1	1	1	0	1	1	0	1	1	0	0
5H	bPb-6124	0	1	1	1	0	1	1	0	1	1	0	0
5H	bPb-9244	0	1	1	1	0	1	1	0	1	1	0	0

Table 4. Environmental data (total monthly rainfall [mm], mean minimum and maximum monthly temperature [ºC] and mean relative humidity [%]) during the grain-fill months (October-December) for HRS and BRS in 2004 and 2005 (source: Bureau of Meteorology).

	Mean minimum temperature (°C)			Mean maximum temperature (°C)			Mean relative humidity (%)			Total monthly rainfall (mm)		
Site	HRS 2004	HRS 2005	BRS 2005	HRS 2004	HRS 2005	BRS 2005	HRS 2004	HRS 2005	BRS 2005	HRS 2004	HRS 2005	BRS 2005
May	4.8	7.0	14.0	21.5	21.5	25.1	77.0	73.4	68.0	5.4	45.4	81.6
June	1.4	6.9	13.6	19.1	18.7	22.7	76.8	86.9	82.0	3.0	94.1	170.4
July	1.9	3.9	12.6	18.8	18.4	22.8	73.3	86.9	72.0	10.2	6.2	14.6
Aug	2.1	2.4	12.0	20.0	19.2	22.8	66.7	71.5	67.0	30.0	8.4	43.0
Sep	5.6	8.4	14.2	23.4	23.7	25.7	64.1	64.7	62.0	26.8	48.1	3.2
Oct	11.3	12.4	19.7	27.1	27.5	28.3	62.1	63.6	66.0	68.8	153.8	99.6
Nov	13.0	14.5	20.0	27.4	27.0	28.7	60.5	65.0	65.0	88.6	63.0	122.2
Dec	15.7	16.6	22.5	29.1	32.3	32.0	61.7	56.1	61.0	64.2	84.0	152.8
Mean (Oct-Dec)	13.3	14.5	20.7	27.8	28.9	29.7	61.4	61.6	64.0	73.8	100.2	124.8
Total (Oct-Dec)										221.6	300.8	374.6

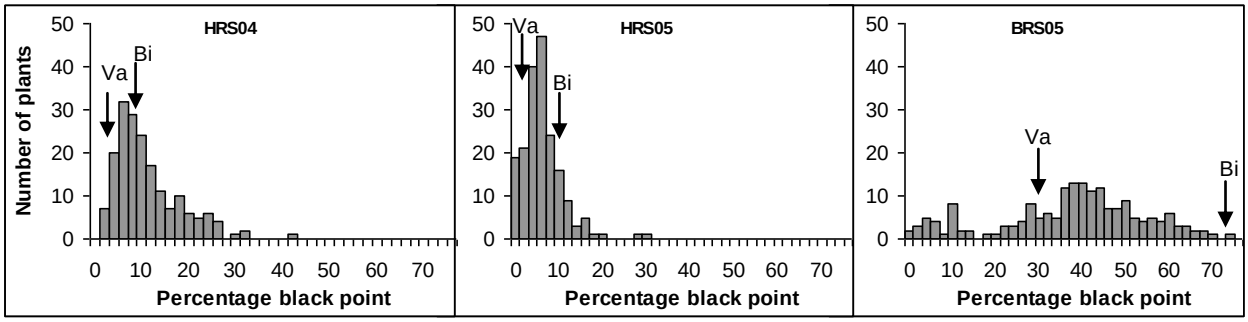

Fig 1. Distribution of percent BP for a Valier/Binalong F_2 population planted in 2004 and 2005 at HRS (HRS04 and HRS05, respectively) and in 2005 at BRS (BRS05). The parental BP mean scores of each trial are indicated by arrows (Va,Valier; Bi, Binalong)

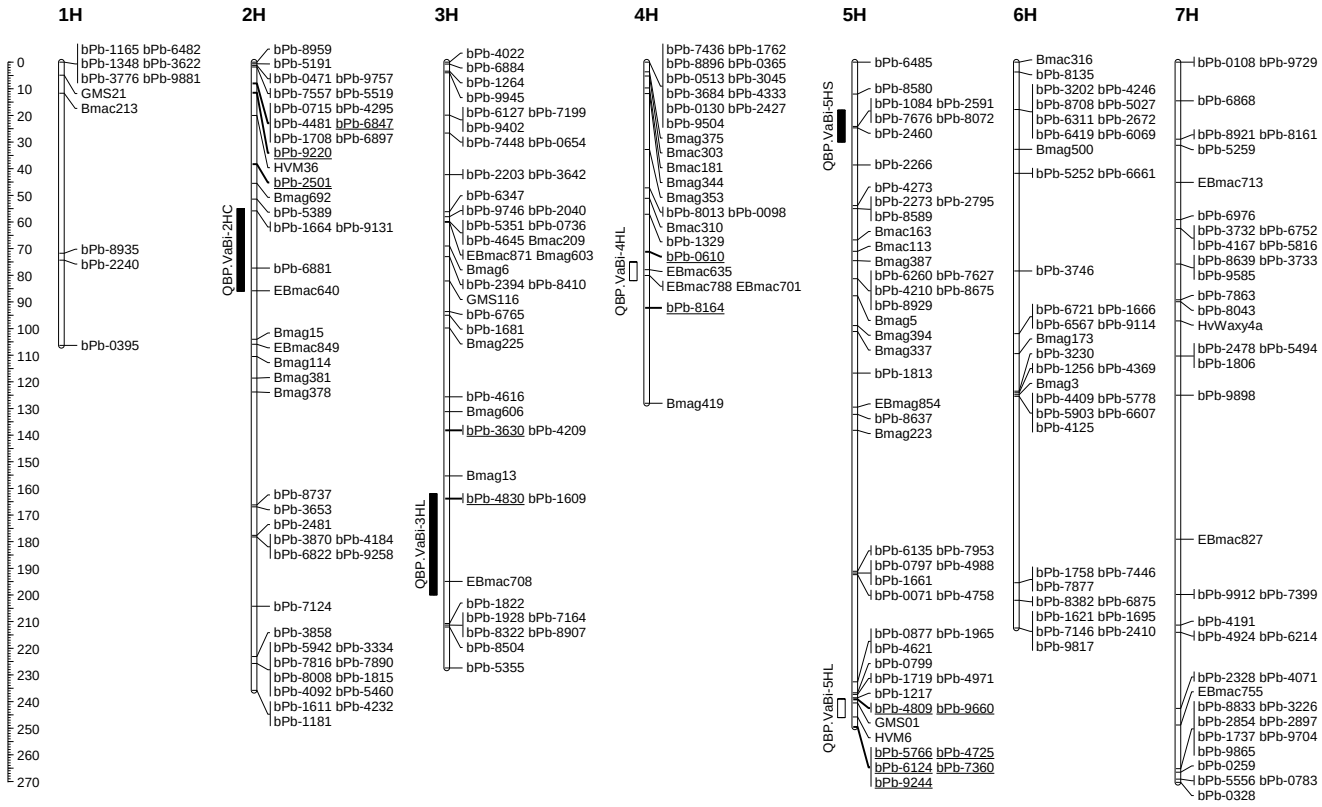

Fig 2. Genetic map of the HRS04 Valier/Binalong F_2 population indicating positions of identified QTLs (black bar). The location of the QTLs identified with the HRS05 (white bar) and the BRS05 data (underlined) are also indicated. Map distance in cM is indicated on the left.

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