November 2019

Project Report No. 614

Maximising the potential of *Aegilops ventricosa* introgression for *Pch1* eyespot resistance and increased grain protein in wheat

Paul Nicholson[1] and Marianna Pasquariello[1]

[1]John Innes Centre, Norwich Research Park, Norwich NR4 7UH

This is the final report of a 42-month project (21130018) that started in July 2014. The work was funded by BBSRC and a contract for £62,000 from AHDB.

CONTENTS

1. Abstract

Eyespot is a damaging disease that affects the stem base of cereals, particularly in second-wheat situations. The most potent source of resistance to eyespot was introduced into wheat from the relative *Aegilops ventricosa*. This resistance is believed to be due to a single gene (but this is unproven) on chromosome 7D. Other factors were also believed to have been transferred to wheat along with the eyespot resistance. These include the positive trait of increased grain protein content and the negative trait of reduced yield under some conditions. The relationship between the three traits is unknown.

This project aimed to produce plant materials, knowledge on the location of the three traits (*Pch1*, protein content and yield) and DNA markers to track the three traits in breeding programmes. This will allow breeders to select the desirable traits conferred by this segment while removing the deleterious ones from their varieties. The project achieved its three objectives:

1. To establish whether *Pch1* eyespot resistance gene on chromosome 7D is in an identical position to a second weaker eyespot resistance (*Pch2*) are located on chromosome 7A.

   It was determined that the two resistances are not in equivalent positions on their respective chromosomes. DNA markers were produced to both resistances.

2. To establish the location of the positive grain protein and the negative yield penalty traits and to produce diagnostic DNA markers for these to allow selection for the former and against the latter.

   It was demonstrated that both traits were distant from *Pch1* and from each other. Selected lines carrying a very short segment of $7D^{V}$ containing *Pch1* and/or the grain protein trait were provided to plant breeders for incorporation into new varieties.

3. To sequence the region of chromosome containing *Pch1* and attempt to identify the *Pch1* gene. The region about *Pch1* on the *Ae. ventricosa* chromosome was sequenced and several candidate genes identified.

It was shown that the introgressed segment contains several resistance genes not found in wheat. It was demonstrated that a gene previously suggested by several other researchers as a candidate for *Pch1* is not, in fact, involved in resistance to eyespot. The remaining candidates for *Pch1* await further work to establish whether any of them can confer increased resistance to eyespot.

2. Introduction

Eyespot is a disease of the stem base of cereals that poses a frequent, but often underestimated, threat to the UK wheat crop being particularly serious in second wheat situations. It is caused by two closely related fungal species *Oculimacula yallundae* and *O. acuformis*. Eyespot yield losses of up to 30% can occur, even in the absence of lodging. Eyespot is the most important disease of the stem base of cereals in the UK causing £12-20 million per annum in lost yield, in addition to significant expenditure on fungicides (Hardwick et al., 2001). A previous HGCA/AHDB-funded project (Diagnosis, forecasting, risk assessment and control of stem-base diseases of wheat using new molecular technologies: Project Report no. 216) demonstrated that the application of fungicides provided a yield benefit in second wheat situations for varieties highly and moderately susceptible to eyespot. Most significantly, no fungicide application produced a yield benefit in varieties carrying the *Pch1* eyespot resistance gene. This demonstrates the potency of this resistance whereby its presence removes the need for fungicide application to control eyespot.

Significantly, it has been shown that eyespot facilitates entry of secondary invaders responsible for brown foot rot (Bateman and Munnery, 1995) This indicates that the deployment of *Pch1* would also contribute to control of brown foot rot.

The two eyespot species have exhibited differential fungicide sensitivity in recent years leading to changes in species abundance that has implications for disease forecasting, disease levels and yield losses (Parnell et al., 2008). The changes in pathogen population have cast into question the validity of previous risk assessments undertaken to establish whether fungicide application is merited.

Only three sources of resistance to eyespot are known to be present in modern wheat cultivars. The most potent of these is termed *Pch1*, and it originates from the wheat relative *Aegilops ventricosa.* This resistance was introduced into wheat by replacing a large segment chromosome 7D of wheat with the equivalent portion from *Ae. ventricosa* (so-called chromosome $7D^V$) (Maia, 1967).

The *Pch1* resistance is referred to as being a single gene but the effect on resistance may be due to the action of a cluster of genes carried on the chromosome $7D^V$ segment of *Aegilops ventricosa.* Wheat varieties carrying this segment are highly resistant to eyespot but, unfortunately, they also suffer from 'linkage-drag' due to deleterious genes carried on the $7D^V$ segment and a significant yield deficit may be observed in the absence of the disease in some environments (Koen et al., 2002). It is only relatively recently that *Pch1* carrying varieties have been developed that also possess high yield potential and have succeeded in being placed on the AHDB Recommended List. We have shown that many of these varieties carry the full-size original segment and so it appears that the negative yield effect of the $7D^V$ segment is compensated by other factors in these new varieties

rather than being removed (Burt and Nicholson, 2011). A reduction of the *Ae. ventricosa* segment in varieties should further increase yield potential.

In contrast to the negative effect on yield, there is evidence that the *Ae. ventricosa* introgression carries other beneficial traits along with eyespot resistance. The *Pch1* $7D^V$ segment also confers a 2-3% higher grain protein content and 100-150 greater Alveograph dough strength (W) units, resulting in enhanced bread making potential (Groos et al., 2004). The effect on grain protein has been observed repeatedly in other *Pch1* breeding materials (E. Flatman, RAGT, personal communication).

Efforts to separate the desirable eyespot resistance and protein content traits from the deleterious yield effect and locate their relative positions on the $7D^V$ segment have been seriously hindered by two factors: an apparent reduction in recombination between the native wheat 7D and *Ae. ventricosa* $7D^V$ regions and a lack of suitable co-dominant DNA markers. An HGCA/AHDB-funded PhD studentship (Burt, 2013) at the JIC, however, showed that this segment can be recombined and also identified co-dominant gene-based markers to the $7D^V$ region (Burt and Nicholson, 2011). This raises the potential for breeders to select the desirable traits conferred by this segment while removing the deleterious ones from their varieties.

The other two eyespot resistances, '*Pch2*' and 'QTL5A' present in UK wheat varieties are only of moderate effect and both derive from the French variety Cappelle Desprez. It has been found that *Pch2* is less effective against *O. yallundae* than against *O. acuformis* in resisting leaf sheath penetration towards the stem (Burt et al., 2010) while QTL5A is equally effective against both species (Burt et al., 2011). Evidence to date suggests that *Pch1* lies in a region of $7D^V$ that is similar to that of the eyespot resistance, *Pch2*, located on chromosome 7A of wheat. This observation makes it possible that the two resistances are due to similar genes (homoeologues), one on $7D^V$ (*Pch1*) and the other on 7A (*Pch2*).

The corresponding region to that for *Pch1* in wheat was identified in the fully sequenced genome of the grass models *Brachypodium*, rice and sorghum enabling the development of further genetic markers in the region and the potential to identify candidate genes for the resistance (Burt and Nicholson, 2011). The observation that *Pch1* and *Pch2* are in similar locations, combined with the identification of the equivalent region in model species should also enable the identification of similar closely linked DNA markers for the *Pch2* gene as well. This would be beneficial as *Pch2,* although of moderate effect, is useful when combined with other resistances in varieties.

Aim: This project aims to produce plant materials, knowledge on the location of the three traits (*Pch1*, protein content and yield) and DNA markers to track the three traits for plant breeders to use in their

breeding programmes. This will allow breeders to select the desirable traits conferred by this segment while removing the deleterious ones from their varieties.

Objective 1

1) To establish whether *Pch1* and *Pch2* eyespot resistance genes are located at identical positions on chromosomes 7D and 7A, respectively and to develop co-dominant closely-linked/perfect DNA markers for use by plant breeders.

Resources for Objective 1:

A population of 244 F_7 lines segregating for *Pch2* has been developed. These lines will be genotyped and screened for eyespot resistance to precisely define the location of *Pch2* and establish whether *Pch1* and *Pch2* eyespot resistance genes are located at identical positions on chromosomes 7D and 7A, respectively. This information will be used to develop co-dominant closely-linked/perfect DNA markers for *Pch2* use by plant breeders.

Objective 2:

1) To produce a series of wheat lines in which the $7D^V$ segment is recombined and to establish the location of the positive grain protein and the negative yield penalty traits, and to produce diagnostic DNA markers for these to allow breeders to select for the former and against the latter. Selected lines that carry a very short segment of $7D^V$ containing *Pch1* and/or the grain protein trait will be provided to plant breeders where they can be incorporated into breeding programmes and followed using the associated DNA markers.

Resources available for Objective 2:

A population of lines carrying different portions of the original $7D^V$ segment has been produced to allow the regions for the three target traits (eyespot resistance, protein content and yield penalty) to be identified. Newly developed co-dominant DNA markers to produce additional lines in which the $7D^V$ segment is recombined and to establish the location of the positive eyespot resistance, grain protein content and the negative yield penalty traits.

Objective 3:

To fine map *Pch1* and use candidate genes to screen a DNA library of a *Pch1*-containing variety (Renan) to isolate *Pch1*.

Resources available for Objective 3:

A very large population (1000's) of progeny has been developed from a cross between lines that differ only for a small $7D^V$ segment containing *Pch1*. These lines will be used to precisely define the location of *Pch1,* and DNA sequences very closely linked to *Pch1* will be used to screen a large

insert size Bacterial Artificial Chromosome (BAC) DNA library of the *Pch1*-containing variety Renan. BACs will be sequenced to identify the gene content of the *Pch1* region and to identify the genes in this region that are potential candidates for *Pch1*. The most promising candidate genes will be introduced into an eyespot susceptible variety to identify which of the candidates is actually *Pch1*. If the gene responsible for the *Pch1* eyespot resistance is identified, perfect markers to *Pch1* will be designed for breeders to use in their programmes.

3. Comparison of the locations of *Pch1* and *Pch2*

3.1. Introduction

Pch1 has been mapped to a 0.26 cM region between five co-segregating proximal markers (*Xcos7-6, X4CD7A8, Xorw5, Xtr383* and *Xwg7S*) and the distal COS (Conserved Orthologous Sequence) marker *Xcos7-9* (Burt and Nicholson, 2011). Until recently, marker-assisted selection for *Pch1* commonly relied upon the presence of allele *Ep-D1b* of a co-segregating endopeptidase (McMillin, 1986). This assay has been replaced by the PCR-based STS (Sequence Tagged Site) marker *Xorw1* developed by Leonard et al. (2008).

It has been speculated that *Pch1* resistance may be conferred by the *Ep-D1b* protein (Worland et al., 1988) because of the apparent lack of recombination between *Xorw1* and *Pch1*. However, Mena et al. (1992) identified a recombination event between *Ep-D1b* and *Pch1* in a line that carried the endopeptidase allele *Ep-D1b*, but was susceptible to eyespot, suggesting that the resistance was not a product of the Ep-D locus. Eyespot resistance was also transferred to durum wheat from a second *Ae. ventricosa* accession (Vent 11) (Huguet-Robert et al., 2001). This accession lacks a functional Ep-D1 endopeptidase allele, further indicating that *Pch1* is not a product of *Ep-D1b*.

The eyespot resistance gene *Pch2* derives from the French cultivar Cappelle Desprez (CD) (Vincent et al., 1952). Since the 1950s, this has proved to be a moderate but highly durable resistance, dominating European wheat markets for over two decades. *Pch2* is located on the distal end of chromosome 7AL (de la Peña et al., 1996) and has been mapped to a 7 cM interval between SSR markers *Xwmc346* and *Xcfa2040* and it is closely associated with SSR *Xwmc525* (Chapman et al., 2008).

The relative positions of *Pch1* and *Pch2* on the long arm of chromosomes 7D and 7A, respectively, suggest that they are homoeoloci. A number of previous studies support this hypothesis. For example, in the work of Chapman et al. (2008), the SSR marker *Xcfa2040*, which has homoeoloci on 7A and 7D, was found to be close to *Pch2* on 7A, and also to the *Pch1*-linked SSR marker *Xwmc14* on 7D. Unfortunately, due to the homoeologue-specific nature of most SSR markers, it was impossible to make other direct comparisons between the two regions. The potential homoeology is

further supported by the study of de la Peña et al. (1997) where they mapped *Ep-D1* and *Ep-A1*, respectively, on the distal portion of the long arms of the 7D and 7A chromosomes. However, Leonard et al. (2008), who identified a candidate gene for Ep-D1 and developed a DNA-based marker on it, reported a large discrepancy between the genetic distances of the two endopeptidase loci (*Ep-D1* and *Ep-A1*) and the *Pch* loci on their respective chromosomes and could not provide any clear evidence to support them being homoeologous based upon their genetic positions. In the more recent paper of Burt and Nicholson (2011), the authors found that the region containing *Pch1* (flanked by the markers Xcos7-6 and Xcos7-9) on chromosome 7D overlapped with the *Pch2* QTL between markers *Xcos7-6* and *Xorw1* (Figure 3.1). The *Pch2* QTL region originally spanned a 7 cM region (Chapman et al. (2008), but the addition of COS and sequence tagged site (STS) markers extended both the map and the QTL interval (Burt and Nicholson, 2011). The large size of the QTL interval was believed to be, largely, a result of inaccuracies in phenotyping F3 families for eyespot resistance conferred by *Pch2*, as this gene is only of moderate effect (Burt et al., 2010; Chapman et al., 2008).

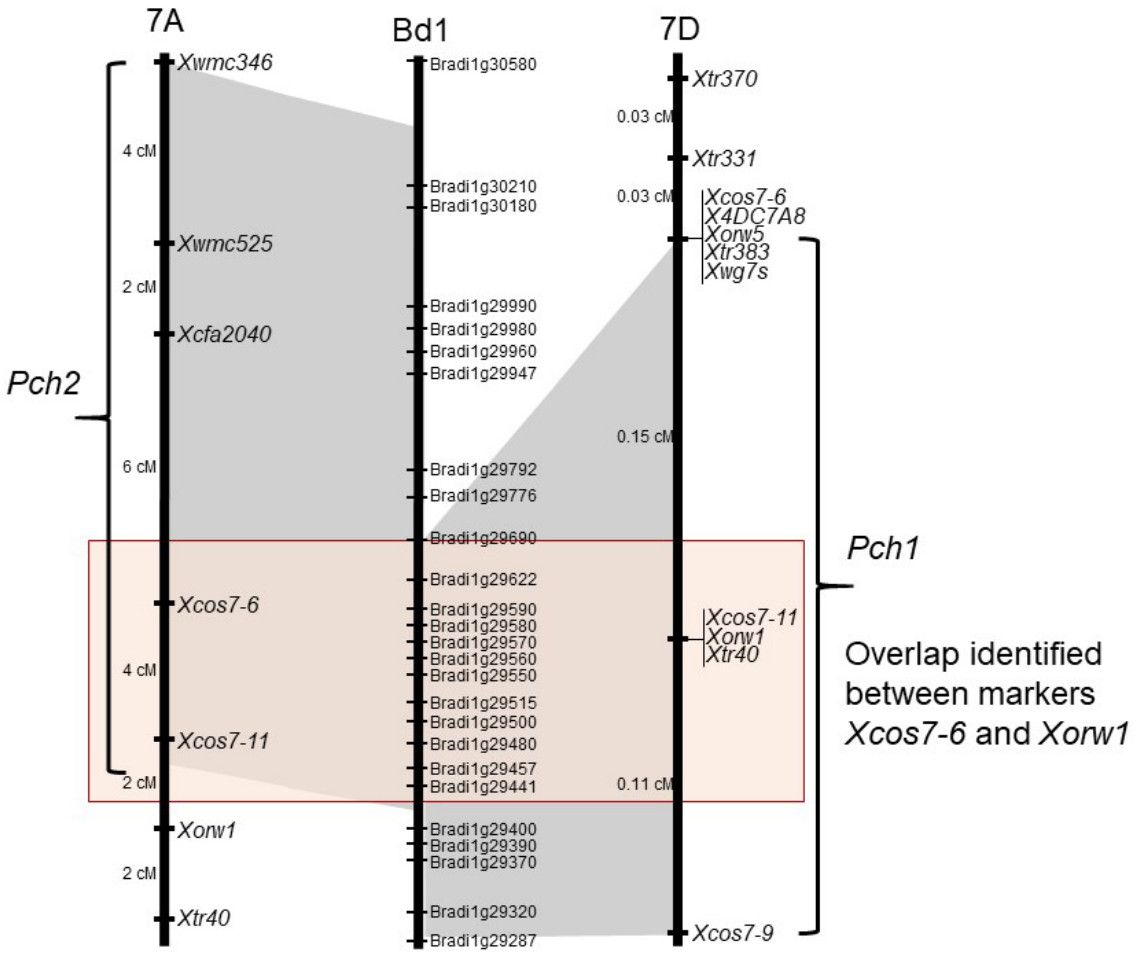

Figure 3.1. Knowledge on the relative locations of *Pch1* and *Pch2* at the outset of the project (Burt and Nicholson, 2011). Comparison of the location of *Pch1* on the HSxRVPM25 genetic map of

chromosome 7D (Ta7D) with the CSxCS/CD7A chromosome 7A genetic of *Pch2* using physical marker locations on *Brachypodium distachyon* chromosome 1 (Bd1) as a reference.

No wheat genome sequence was available at the beginning of this project, so marker development relied upon the relationship to closely related species. A high level of genome-synteny between wheat and model species, such as rice sorghum and *Brachypodium distachyon* (Bd) has been widely reported (Vogel et al., 2010), with Bd providing the highest level of co-linearity with wheat due to its closer evolutionary relationship. For example, the panel of co-dominant COS markers developed by Burt and Nicholson (2011) were obtained by exploiting the syntenic relationship between mainly Bd (but also rice and sorghum) and wheat. The availability of complete genomic sequence of these species is a great advantage for the development of molecular markers, for the identification of candidate genes for traits of interest and for predicting biological gene functions. From the eyespot point of view, this co-linear relationship could be further exploited for developing homoeologue (7A/7D) transferable markers by which to finally determine the potential homoeology between *Pch1* and *Pch2*.

The three goals of this objective were: 1) develop new gene-based molecular markers closely linked to *Pch1* and *Pch2* by exploiting synteny between wheat and Bd, 2) refine the genetic position of *Pch1* and *Pch2,* respectively on the 7D and 7A chromosomes, 3) clarify the potential homoeologous relationship between *Pch1* and *Pch2.*

3.2. Materials and methods

3.2.1. Plant material

2256 BC_6 F_2 plants from the cross between the recombinant substitution line RVPM25 and Hobbit-Sib (HS) produced by Burt and Nicholson (2011) were genotyped to identify additional recombinants in the *Pch1* region. The 25 BC_6 F_2 plants reported by Burt and Nicholson (2011) to be recombinant between the SSR markers *Xbarc76* and *Xcfd175* were re-analysed with additional markers to refine the recombination break-points.

A total of 92 F_7 lines obtained by single seed decent (SSD) and deriving from a cross between Chinese Spring (CS) and the substitution line Chinese Spring/Cappelle Desprez 7A (CS/CD7A) produced by Chapman et al. (2008) were used to define the genetic position of *Pch2*.

3.2.2. Seedling bioassays

The 92 CS x CS/CD7A F_7 lines were tested for eyespot resistance against *O. acuformis* at the seedling stage. Five plants from each of the F_7 lines were grown in 7 x 7 cm square pots containing peat and sand compost. Three replicate pots of each, with five plants per pot, were arranged in a

complete randomized block design. The parental lines, CS and CS/CD7A, were included in each block as a control. The plants were grown for 2-3 weeks in a controlled-environment-room (CER) at 8°C and 8/16hrs light/dark condition, then inoculated using inoculum slurry constrained within PVC cylinders and incubated as described by Chapman et al. (2008). Plants were harvested 8 weeks after inoculation and scored for disease using the method described by Scott (1971). This experiment was subsequently repeated using identical methods to confirm the findings.

For the eyespot inoculation, a homogenized mixture of twenty-five isolates of *O. acuformis* was selected from the JIC culture collection. A mixture of different isolates was used to ensure that a successful infection was achieved. Each isolate was grown on V8 agar (9 g of bacto-agar, 50 ml of V8 vegetable juice in 450 ml of deionised water) at 15°C for 21 days prior to preparation of inoculum as described previously (Chapman et al. (2008).

3.2.3. Statistical Analysis

For the two CER experiments, analysis of variance was performed on visual disease scores to assess the variation attributable to line, blocks and interactions between line and blocks, using a general linear model (GLM) in Genstat v.16 (Copyright 2009 Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Predicted mean disease scores were calculated for each line using the GLM for use in the QTL analysis.

3.2.4. Map construction and QTL analysis

Genetic maps were generated for *Pch1* and *Pch2* populations in JoinMap© (version 3.0) (Stam, 1993) using default parameters. The *Pch2* linkage map data were combined with phenotypic data from the two seedling bioassays for QTL analysis. The QTL analysis was carried out using data from each phenotype trial individually as well as using a data set in which the data from the two trials was combined.

The search of QTLs was done using Single Trait Linkage Analysis in three different steps: 1) initial genome-wide scan by simple interval mapping (SIM) to obtain candidate QTL positions; 2) one or more rounds of composite interval mapping (CIM), in the presence of cofactors, which are potential QTL positions detected at previous step; 3) fit the final QTL model.

3.2.5. Molecular Markers

The mapping reported by Burt and Nicholson (2011) located *Pch1* to the region between the markers *Xcos7-6* (corresponding to *Bradi1g29690*) and *Xcos7-9* (corresponding to *Bradi1g29287*). To refine the position of *Pch1* on the chromosome 7D, markers were developed taking advantage of the syntenic relationship between *Brachypodium distachyon* (Bd) chromosome 1 and wheat Group 7

chromosomes. The *Pch1* homologous location on Bd chromosome 1 was identified in EnsemblPlants (http://plants.ensembl.org/Brachypodium_distachyon/Info/Index) and found to cover approximately a region of 424 Kb (Bd1:24810968-25235129) containing 43 genes from *Bradi1g29690* to *Bradi1g29287*.

Wheat genes on the group 7 chromosomes corresponding to each of these Bd genes were identified and PCR primers pairs were designed at locations with high levels of sequence conservation between Bd and wheat to maximise the probability of obtaining successful amplification of the *Ae.ventricosa* introgressed DNA. These primers were tested on genomic DNA from HS and RVPM25 and PCR products were used in two different strategies for producing either Single-Strand Conformation Polymorphism (SSCP) or Kompetitive Allele Specific PCR (KASP) markers. In the first case, PCR products were examined by SSCP assay (Martins-Lopes et al., 2001) using Sequa Gel® MD (National Diagnostics, UK Ltd.) and visualised by silver staining (Bassam et al. 1991). For KASPs, instead, PCR products were sequenced and aligned to identify SNPs between *Ae. ventricosa* and wheat 7D. KASP marker PCR assays were manually designed on the basis of the identified SNPs.

A similar procedure was followed for identifying new markers to define the *Pch2* locus and to allow the genetic maps for 7A and 7D to be related to one another. *Pch2* was originally located on the chromosome 7A to a position between the markers *Xwmc346* (SSR) and *Xorw1* (corresponding to *Bradi1g29400*) (Burt and Nicholson, 2011). Due to the non-gene based nature of SSR markers, it was not possible to identify a syntenic position for *Xwmc346* on Bd1. To ensure coverage of the potential homologous region of *Pch2* on Brachypodium, a large Bd1 region containing about 100 genes was identified. As one of the aims of this work is to determine the homoeologous relationship between *Pch1* and *Pch2*, most attention was focused on developing markers to the overlapping segment of the two loci. Eight Bd genes were selected at equal intervals to span the region from *Bradi1g29480* at the proximal end of *Pch1* to *Bradi1g29960* at the distal end of *Pch2*.

Initially, primer pairs were designed in the most conserved region and tested on CS and CS/CD7A. Sequencing of PCR products then allowed the identification of 7A homoeologue-specific SNPs on the basis of which KASP assays were designed.

A second set of KASP markers specific for the *Pch2* map was developed using the high-density single nucleotide polymorphism (SNP) genotyping iSelect array of 81,587 SNPs published by Wang et al. (2014) and available for 400 wheat lines on the CerealDB website (http://www.cerealsdb.uk.net/cerealgenomics/CerealsDB/iselect_search.php). Both Chinese Spring (CS) and Cappelle Desprez were included among this panel. After analysing the complete set of SNPs mapping to 7A and polymorphic between the two parental lines CS and Cappelle Desprez, a

set of SNPs located across the *Pch2* region interval were selected. KASP primers were designed using PolyMarker, an automated bioinformatics pipeline for SNP assay development which is designed to increase the probability of generating homoeologue-specific assays for polyploid wheat (http://polymarker.tgac.ac.uk/).

Thermodynamic properties of designed primers were verified after adding the standard FAM or HEX compatible tails (FAM tail: 5′ GAAGGTGACCAAGTTCATGCT 3′; HEX tail: 5′ GAAGGTCGGAGTCAACGGATT 3′). 7A and 7D specific KASP markers were initially tested against the parental lines (CS and CS/CD7A, HS and RVPM25, respectively) and the ones which were polymorphic between the parental line pairs were then applied to the F_7 CSxCS/CD7A and BC6 F_2 HSxRVPM25 populations, respectively.

Genomic DNA extraction from the parental lines was performed using the CTAB method (Nicholson et al., 1996) to obtain large quantities of high quality nucleic acid, whereas genomic DNA of the two population lines was extracted using the extraction protocol for 96-well plates, adapted from Pallotta (2003). PCR reactions were prepared in a 6.25 µl final volume containing 2.5 µl DNA (10 ng/µl), 3.125 µl Taq mastermix (Qiagen) and 0.625 µl of the relevant primer pair (2 µM). A common PCR programme was used throughout consisting of a denaturing step of 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min, with a final elongation step of 72°C for 7 min. Where required, PCR products were then purified using QIAquick Gel Extraction Kit (Qiagen), sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (following the manufacturer's instructions) and aligned in VectorNTI® (ThermoScientific).

For the KASP assay, 2 µl (5 ng/µl) of the extracted DNA were added to 0.056 µl of primer mix (12 µl each of specific primer, 30 µl of the common primer and 46 µl deionized water) and 2 µl of KASP master mix (LGC). The PCR included an initial denaturation step of 94°C for 15 minutes followed by10 cycles of touchdown PCR (annealing 62°C to 56.6°C, decreasing 0.6°C per cycle), then 25 cycles of 94°C for 10s and 60°C for 1 minute. After amplification, plates were read into the Tecan Safire plate reader and genotyped using the Klustercaller™ software (version 2.22.0.5, LGC).

3.3. Results

3.3.1. Refining the position of *Pch1* on chromosome 7D

To refine the position of *Pch1* on chromosome 7D, the available set of 6 COS and SSR markers previously produced by Burt and Nicholson (2011) was supplemented by a set of eleven new markers, three SSCPs (*X29550, X29570 and X29577*) and eight KASPs (*Xorw1_D, Xcos7-11, Xcos7-9, Xtr40, X29457, X29500, X29515* and *X29560*) developed on the basis of the syntenic relationship between wheat and Bd (Table 3.1). The number relates to the syntenic Bd gene on Bd1.

Different names were used for *Xorw1_D, Xcos7-11, Xcos7-9, Xtr40* KASPs to maintain the link between new KASP markers and those developed on the same genes by Burt and Nicholson (2011). These markers were applied to 2256 lines of the HS x RVPM25 BC6 F_2 population.

Table 3.1 Summary of markers used to identify recombinants in the region of *Pch1*

Marker Name	Brachypodium Gene	Wheat 7D homolog	Marker Type	Primer sequences[1]
X29577	*Bradi1g29577*	Traes_7DL_C255A109C	SSCP	tccacttgaggttgtcaaga
				attgcaagctctaggaaaca
X29570	*Bradi1g29570*	Traes_7DL_471B4134B	SSCP	caccgttgttttcactgctg
				gtccctagaataggggagca
X29560	*Bradi1g29560*	Traes_7DL_6F30AD134	KASP	ggatgacacatgatctCctacgA
				ggatgacacatgatctCctacgC
				atctgttatcagGaagcgggcA
X29550	*Bradi1g29550*	-	SSCP	ccaactcgcacctcatcac
				cgttgatctcgtccacgtc
X29515	*Bradi1g29515*	Traes_7DL_0B4336C25	KASP	ctttcttctctcgcatgtgaacatA
				ctttcttctctcgcatgtgaacatG
				atttggtggaccttctgtcactG
X29500	*Bradi1g29500*	Traes_7DL_35DFF1FCF	KASP	cttattggattggatcagtttggcA
				cttattggattggatcagtttagcT
				ctcaaagtcctcaactatctggaaa
X29457	*Bradi1g29457*	Traes_7DL_B2EB69EC9	KASP	agaccttgtatttggtgcagcG
				agaccttgtatttggtgcagcA
				ttaggtgtgggctctaggtttct
Xorw1_D	*Bradi1g29400*	Traes_7DL_DBBA5FD6C	KASP	ctcatatccattgtgcttggttGcC
				ctcatatccattgtgcttggttAcT
				ccctctatctttgactatgacatgg
Xcos7-11	*Bradi1g29441*	Traes_7DL_973A33763	KASP	tattgatgcagaaacaccctTcgcT
				tattgatgcagaaacaccctAcgcA
				aattcctcgtcgtcgtcaccg
Xtr40	*Bradi1g29320*	Traes_7DL_434E0F3E1	KASP	tgtcatatactctccagcagaagaAA
				tgtcatatactctccagcagaagaGG
				gacataagtgcataggtgcttgtg
Xcos7-9	*Bradi1g29287*	Traes_7DL_CA193C813	KASP	ccctagctgaccgCcC
				ccctagctgaccgGcT
				caccgccgtggcatt

[1] Uppercase letter denotes the selective nucleotide in the KASP assay

Six BC6 F_2 plants from the 2256 tested (0,26%) were identified with recombination events occurring between the KASP markers *X29577* (corresponding to *Bradi1g29577*) and *Xcos7-9* (corresponding

to *Bradi1g29287*). Two groups of co-segregating markers, *X29560-X29550-X29515* and *X29500-X29457-Xcos7-11-Xorw1_D-Xtr40*, were identified within the six plants. These were used to determine the marker order in the *Pch1* region, and a new genetic map was constructed as shown in Figure 3.2. In the new map, *Pch1* spans a genetic region of 0.07 cM from the distal marker *Xcos7-9* (*Bradi1g29287*) to the proximal group of co-segregating markers *X29500-X29457-Xcos7-11-Xorw1_D-Xtr40*. The refined position of *Pch1* on the chromosome 7D eliminates 19 genes from the list of candidates in comparison to the previous version of the map published by Burt and Nicholson (2011). The *Pch1* syntenic region of Bd1 is reduced from 424 Kb to 250 Kb long, in which 24 genes have been identified in wheat (Figure 3.2).

3.3.2. *Pch2* mapping and QTL analysis

A set of 21 KASP markers was developed and used to refine the position of *Pch2* on chromosome 7A. Eleven of them (*X29960, X29947, X29792, X29776, X29560, X29500, X29480, Xcos711, Xorw1_A, Xtr40* and *X29390*) are gene-based markers and were based on the syntenic relation between Bd and wheat. In particular, *X29960, X29947, X29792, X29776, X29560, X29500* and *X29480* were designed on 7A wheat genes which, based on the synteny with Bd1, were determined to be located in the overlapping region of the intervals in which *Pch1* and *Pch2* were estimated to be located.

The other ten markers (*XBS1_30210, XBS2, XBS3_29990, XBS4_29980, XBS5, XBS6, XBS7, XBS8_29590, XBS9_29370* and *XBS10*) were developed from the high-density single nucleotide polymorphism (SNP) genotyping iSelect array (Wang et al., 2014) and are based on 7A specific SNPs able to distinguish between CS and CD. Only five of them could be related to wheat genes and their corresponding Bd gene code is indicated in their nomenclature (*XBS1_30210, XBS3_29990, XBS4_29980, XBS8_29590* and *XBS9_29370*).

Table 3.2 Summary of KASP markers used to identify recombinants in the *Pch2* region

Marker name	Brachypodium gene	Wheat 7A homolog	Primer sequences[1]
X29390	*Bradi1g29390*	Traes_7AL_ACE7F3AC3	gtttgtgtagaacagaggaagcaT
			gtttgtgtagaacagaggaagcaG
			acacatcaatgtttgacttccg
X29480	*Bradi1g29480*	Traes_7AL_1522CB55B	gtcgtctagtgtttcttacttgC
			gtcgtctagtgtttcttacttgG
			gacgactgctgttgcctagacttat
X29500	*Bradi1g29500*	Traes_7AL_AFA2C29C9	ggatgttgatgagagctgagaggA
			ggatgttgatgagagctgagaggC
			atgggcaaatccagcataatgc
X29560	*Bradi1g29560*	Traes_7AL_3F2C16688	tcctctggctatcaaggtcatT
			tcctctggctatcaaggtcatC
			catcgctattcaggaccatctg
X29776	*Bradi1g29776*	Traes_7AL_13AE7EEC0	attctaggtgcagaagggaatcA
			attctaggtgcagaagggaatcG
			ggtctgctttgtcatcctgaaatc
X29792	*Bradi1g29792*	Traes_7AL_DA8343C6B	cctcttccatcggaaacctcA
			cctcttccatcggaaacctcG
			agtgctgaaagttttgtcaaattc
X29947	*Bradi1g29947*	Traes_7AL_842775A88	ctttgacagtgacattctgttcC
			ctttgacagtgacattctgttcA
			cctcatcagccatcaaatattagcT
X29960	*Bradi1g29960*	Traes_7AL_DF750E6B1	catgcacttgttgttctccatccG
			catgcacttgttgttctccatccA
			cagtttgataaccatgtgctcctcaa
XBS1_30210	*Bradi1g30210*	Traes_7AL_09A36AAE41	gctccgcttctaggccaT
			gctccgcttctaggccaG
			gtagtagtaacggcagagacaa
XBS2	-	-	gccttaccaaactacgcacG
			gccttaccaaactacgcacA
			attgtttcttcgtgcgatccg
XBS3_29990	*Bradi1g29990*	Traes_7AL_6ACB3177B	cgacgatgactcggacatctcA
			cgacgatgactcggacatctcG
			ggggcgcaaagataaaatcgtccat
XBS4_29980	*Bradi1g29980*	Traes_7AL_BB11D77B5	gaggcccatcatgcgcagcA
			gaggcccatcatgcgcagcA
			agaggcccgatgtcaagttggataa
XBS5	-	-	gaaggtcgccagtatatatgtgT
			gaaggtcgccagtatatatgtgC
			atttggcataaggaagcgcg
XBS6	-	-	ccaagcaatttcggtgcagttA
			ccaagcaatttcggtgcagttG
			gtagctggggaaactaatacaagtc
XBS7	-	-	cccaaaggacaacttagtgtcG
			cccaaaggacaacttagtgtcA
			caagacatacatgacgtgaggta
XBS8_29590	*Bradi1g29590*	Traes_7AL_5262BD5AE	taggctagatagaatgaaacatggcA
			taggctagatagaatgaaacatggcG
			ggatgcagagctccaaggcagat
XBS9_29370	*Bradi1g29370*	-	agagaactgatatgtcggcgaT
			agagaactgatatgtcggcgaC
			gaagaaactcgagctgctcaaggtt
XBS10	-	-	cacagatcacgcgggcgcaG
			cacagatcacgcgggcgcaA
			ggcgcaggtcgacccgcat
Xcos7-11	*Bradi1g29441*	Traes_7AL_04C7DF2DE	tcctgagagttctttgcattgaaG
			tcctgagagttctttgcattgaaA
			cctaccaattacactggagaagtcttt
Xorw1_A	*Bradi1g29400*	Traes_7AL_D1DB1B9EE	cgaattggttgcttgcccA
			cgaattggttgcttgcccG
			cgacaagactgaccttccag
Xtr40	*Bradi1g29320*	Traes_7AL_33AA9A079	aaatggccaaataacagcaacA
			aaatggccaaataacagcaacG
			tgtttccttccatgtagtctcc

[1] Uppercase letter denotes the selective nucleotide in the KASP assay

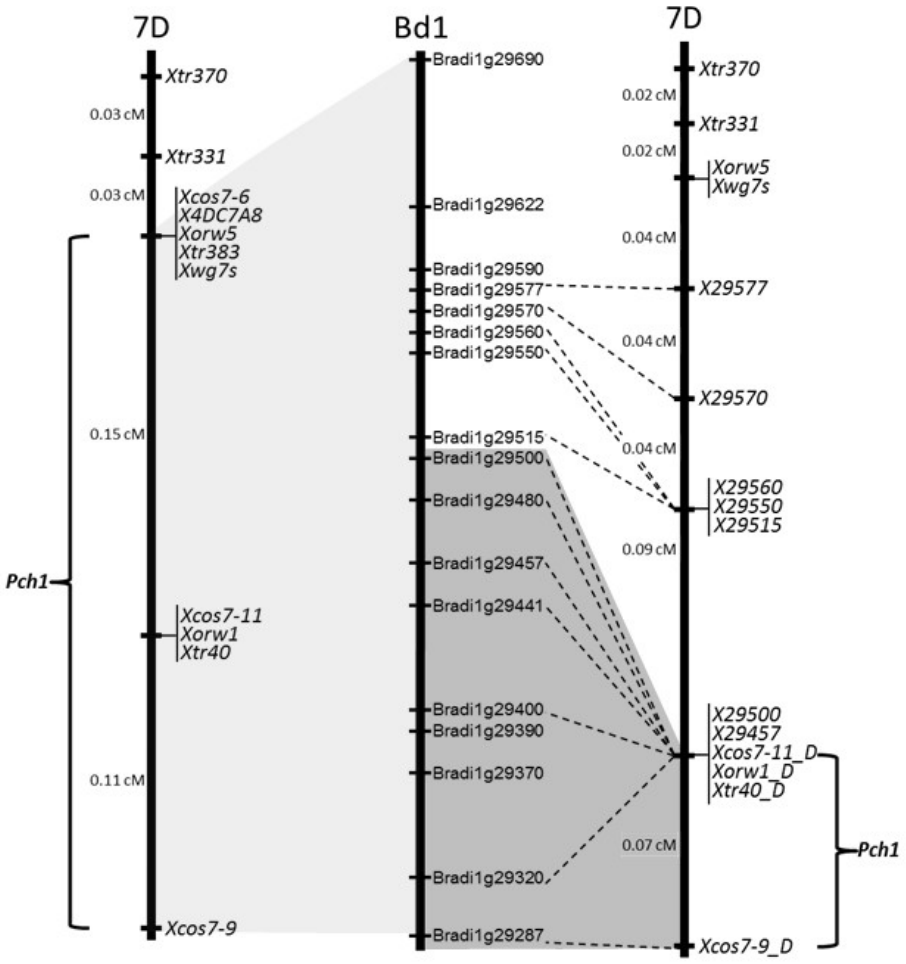

Figure 3.2. Comparison of the newly refined location of *Pch1* on the HSxRVPM25 genetic map of chromosome 7D (Ta7D) with the CSxCS/CD7A chromosome 7A genetic of *Pch2* using physical marker locations on *Brachypodium distachyon* chromosome 1 (Bd1) as a reference.

These two new sets of markers, the Bd-wheat based one (X) and the iSelect one (XBS), are listed in Table 2 along with primer sequences, corresponding Bd gene code (where available) and 7A wheat ESTs gene names where known. All these markers, along with those described by Burt and Nicholson (2011) (*Xwmc346, Xwmc525 and Xcfa2040*) were used to genotype 92 lines of the CS x CS/CD7A SSD F7 population and a new genetic map of the region, spanning a total of 43 cM, was produced (Figure 3.2).

Moreover, the same set of lines was phenotyped for eyespot resistance in two replicated and independent seedling disease bioassays inoculated with *O. acuformis*. It has been reported that *Pch2* is more effective against *O. acuformis* than against *O. yallundae* (Burt et al., 2010). Earlier attempts to localise *Pch2* were hindered because of the use of F_3 families (still segregating for *Pch2*)

rather than more advanced fixed lines for assessing disease resistance (Chapman et al., 2008; Chapman et al., 2009). For these reasons, in the current work disease assessment was carried out using an SSD F7 population challenged with isolates of *O. acuformis*. An interval mapping QTL analysis was carried out combining marker data with phenotypic results and it revealed that the *Pch2* QTL peaks at *Xcfa2040* marker in each of the two bioassays as well as using combined data from the two trials (LOD = 10.41) and explained 42.4% of the phenotypic variance observed (Figure 3.3). Overall, the analysis determined that the *Pch2* QTL region spans 15.6 cM in the interval between XBS1_30210 and the co-segregating group of markers XBS3_29990-XBS4_29980-X29960-XBS5 (Figure 3.3).

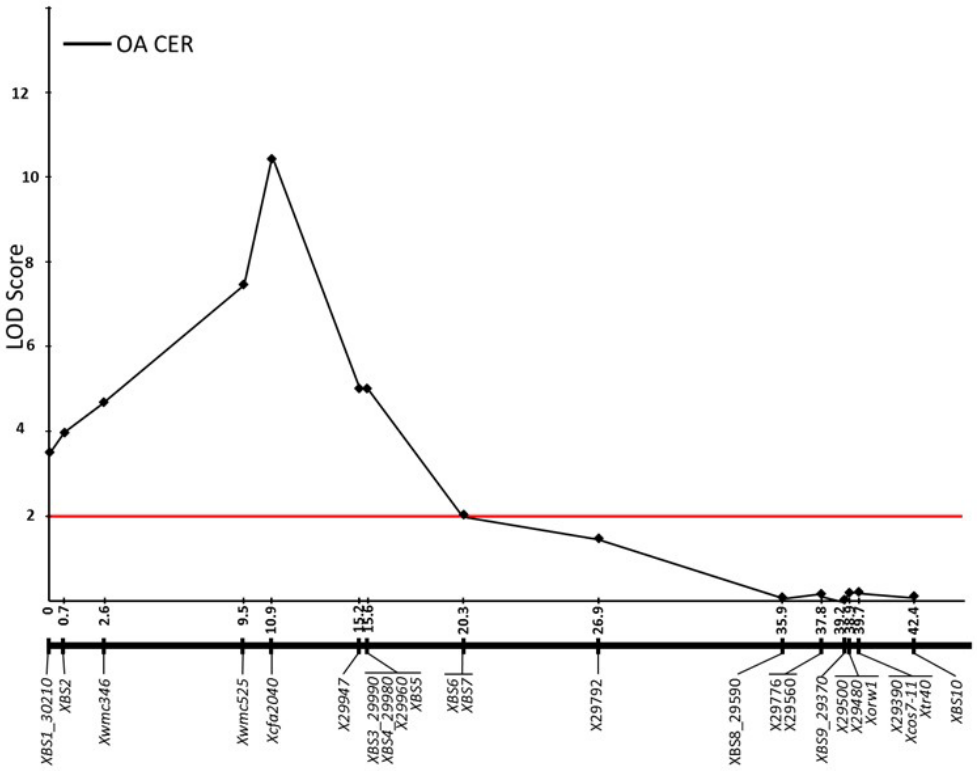

Figure 3.3: QTL interval mapping analyses of *Pch2* resistance to *Oculimacula acuformis* of the 92 wheat CSxCS/CD7A 7A F_7 lines. This LOD profile derives from combined data of two CER trials. Genetic map data are aligned to the LOD profile. The red line indicates the threshold for significance.

3.3.3. *Pch1 – Pch2* homoeology

The new genetic maps of 7D and 7A with the respective positions of *Pch1* and *Pch2* were used to examine the potential homoeology between the two loci. The two maps were aligned to the relevant region of chromosome Bd1. Markers present on both 7A and 7D chromosomes were anchored to the Bd gene-spaced map of the region (Figure 3.4) and co-linearity was checked for both

chromosomes against Bd1. The overall co-linearity was conserved across the entire length of the *Pch1* map. It should be noted that this map is that for the wheat-*Ae. ventricosa* population. Due to the low level of recombination between wheat and *Ae. ventricosa*, it was not possible to dissect and confirm the gene order in the regions with the two co-segregating groups of markers. Similarly, co-linearity was largely conserved between wheat 7A and Bd1 across the region analysed. However, a breakdown of the micro-synteny was identified in the *Pch2* map where X29447, X29776, X29370 and Xcos7-11 seem to be located in a different position in respect to the physical order in Bd1 (Figure 3.4). Using Bd1 as reference, the homoeologous region to *Pch1* on 7D was identified on chromosome 7A. The equivalent locus was located in a genetic region of 0.5 cM between the two co-segregating groups of markers *X29500-X29480-Xorw1_A and X29390-Xcos7-11-Xtr40*. The genetic distance between the most proximal of these groups (*X29500-X29480-Xorw1_A*) and the most distal group of markers (*XBS3_29990-XBS4_29980-X29960-XBS5*) for the *Pch2* QTL was 23.6 cM. On the basis of these new findings, it is clear that the *Pch1* and *Pch2* respective regions on chromosomes 7D and 7A do not overlap and hence there is no evidence that they represent homoeoloci.

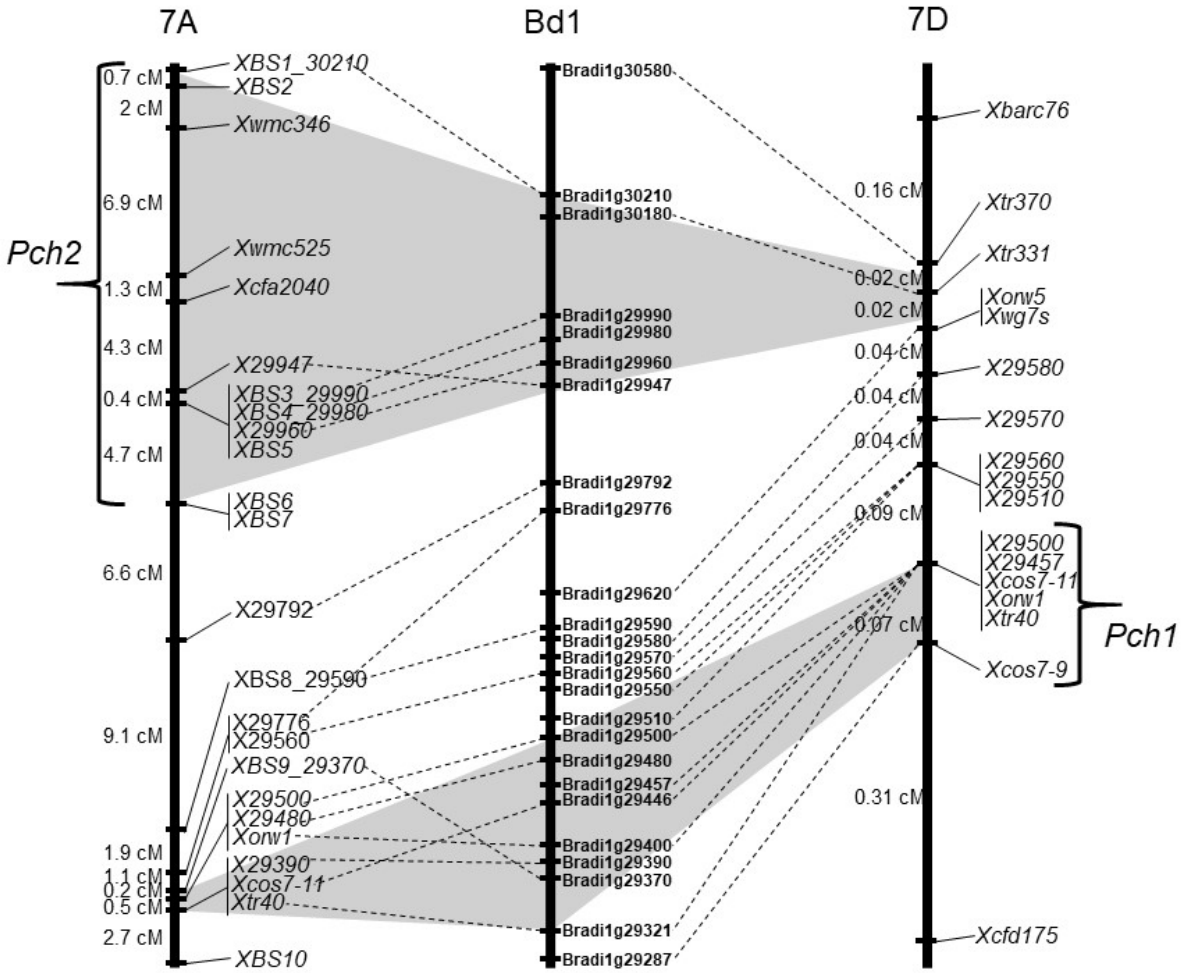

Figure 3.4: Comparison of the location of *Pch1* on the HSxRVPM25 genetic map of chromosome 7D (Ta7D) with the CSxCS/CD7A chromosome 7A genetic of *Pch2* using physical marker locations on *Brachypodium distachyon* chromosome 1 (Bd1) as a reference.

4. Establishing the location of the positive grain protein and the negative yield penalty traits relative to that of *Pch1*

4.1. Introduction

Eyespot poses a frequent threat to the UK wheat crop being particularly serious in current farming systems with a high proportion of cereal crops and a reduced crop rotation. Disease is caused by two closely related fungal species *Oculimacula yallundae* and *O. acuformis* and cases of severe infection, the disease results in lodging and premature ripening of grain, leading to yield losses of up to 40% (Meyer et al., 2011).

The growing of genetically resistant cultivars is one of the most effective, economic, environmental-friendly and sustainable strategy to control plant diseases. In wheat, wild relatives represent an important source of genetic variation for disease resistance. Over 52 wheat related species from 13 genera have been exploited as donors thanks to the plasticity of the wheat genome (Wulff and Moscou, 2014). Although several barriers restricting interspecies hybridization and introgression have been overcome, the simultaneous introduction of genetically linked deleterious traits still hinders the widespread deployment of introgressed resistance genes for crop improvement. The leaf rust resistance gene *Lr19*, transferred to wheat from *Thinopyrum ponticum*, is accompanied by a yellow colouration of the flour (Knott,1980) and is an example of lower quality caused by resistance breeding. In addition, the use of the 1BL–1RS wheat–rye (*Secale cereale*) chromosome translocation to improve resistance to stem rust compromises bread making quality (Dhaliwal et al., 1987).

The most potent source of resistance against eyespot is conferred by the gene *Pch1* which derives from chromosome $7D^V$ of the wheat relative *Aegilops ventricosa* and was introduced into hexaploid wheat by introgression of a large segment of $7D^V$ into chromosome 7D of wheat (Maia, 1967). Although *Pch1* is highly effective against both eyespot species, a significant reduction of yield and thousand-kernel mass has been sometimes observed in the absence of the disease. For example, Koen et al. (2002) found that the presence of the $7D^V$ segment carrying *Pch1* gene had a detrimental effect on thousand-kernel mass or yield in three wheat lines. Kwiatek et al. (2016) also demonstrated that the *Pch1* gene, together with the *Ae. ventricosa* segment, caused significant yield losses, both when used as a single eyespot resistance source or in a combination with *Q.Pch.jic-5A*. Previously, Worland et al. (1990) observed a yield reduction associated with the *A. ventricosa* introgression in recombinant lines developed from crosses between VPM1 (carrying *Pch1*) and Hobbit Sib and mapped it on the chromosome 7D between the *Pch1*/Ep-D1 and *LrVPM* loci.

Although recombination does occur between the *Ae. ventricosa* $7D^V$ and the wheat 7D chromosome segments, it does so at a much lower frequency than normal (Worland et al. 1988), and it is only relatively recently that *Pch1*-carrying varieties have been developed that also possess high yield potential. Burt and Nicholson (2011), showed that many of these varieties still retain a large portion of the original segment, and so they speculated that the negative yield effect of the $7D^V$ segment is compensated by other factors rather than being removed. Reducing the size of the *Ae. ventricosa* segment to remove the deleterious portion should further increase yield potential in new varieties.

In contrast to the negative effect on yield, there is evidence that the *Ae. ventricosa* introgression carries other beneficial traits along with eyespot resistance. The *Pch1* $7D^V$ segment also confers a 2-3% higher grain protein content and 100-150 greater Alveograph dough strength (W) units resulting in enhanced bread making potential (Bogard et al., 2010; Groos et al., 2004).

The nutritional quality of wheat grains is significantly impacting human health and well-being. Grain protein content (GPC) is an important trait for both nutritional value and end-use quality of wheat (Veraverbeke and Delcour, 2002). Protein level in modern wheat cultivars is naturally low and improvements in the nutritional quality of wheat, such as increased protein and grain micronutrient levels are important traits to be considered in breeding programs. Producing wheat varieties high in both GPC and grain yield is a major task in wheat breeding programs (Zhao et al., 2009). The simultaneous improvement in the conventional wheat breeding of GPC and grain yield has been hampered by the strong negative correlation between these two traits (Simmonds, 1995). The transfer of alien genes from wheat related species has become an effective approach in the development of new wheat varieties for high GPC or yield.

The main objective of this part of the project was to locate the relative positions of the yield penalty and increased GPC loci on the $7D^V$ segment in relation to the *Pch1* locus. Knowledge on the relative positions of the three loci would reveal whether the desirable eyespot resistance and GPC traits could be separated from those conferring the deleterious yield effect.

4.2. Materials and Methods

4.2.1. Plant Material

A BC5 recombinant population (RVPM7D) of 90 lines, originally produced by Worland et al. (1988), between the eyespot susceptible line Hobbit ‘sib’ (HS) and the eyespot resistance substitution lines Hobbit 'sib'-VPM7D (HS/VPM7D), was used to define the genetic position of the potential Yield Penalty (YP) and increased GPC historically associated to the *Pch1* mediated eyespot resistance. HS/VPM7D is an intergenotypic single chromosome substitution line where chromosome 7D has

been replaced by chromosome $7D^v$ of *Aegilops ventricosa* Tausch (2n = 4x = 28, genomes $D^vD^vM^vM^v$) (Doussinault et al., 1983; Maia, 1967). This population was previously used by Chapman et al. (2008) to map the position of *Pch1* at the distal end of chromosome $7D^V$.

4.2.2. Field Trials

Eight field trials were carried out over four years (one trial in 2014, three in 2015, three in 2016 and one in 2017) in the following locations of United Kingdom:

- John Innes Centre - Church Farm, Bawburgh, Norfolk in 2014, coded as 2014_JIC;

- R.A.G.T. Seeds Ltd, at Stapleford and Great Shelford, Cambridgeshire, in the 2015 (coded as 2015_RAGT_SF and 2015_RAGT_WH respectively); at Elmdon, Essex (coded as 2016_RAGT_BT) and Ickleton, Cambridgeshire (coded as 2016_RAGT_SD);

- Limagrain UK Ltd, at Burnt Fen, Littleport, Ely in 2015 (codes as 2015_Limagrain) and at Lower Barn, Gedding, Bury Saint Edmunds in 2016 (coded as 2016_Limagrain);

- Morley St. Botolph, Wymondham, Norfolk in 2017, coded as 2017_JIC.

For 2014_JIC and 2017_JIC, all entries were planted in three replications at each location in randomized complete block designs. The plots were 4 x 1.5 m corresponding to $6m^2$.

2015_Limagrain and 2016_Limagrain trials were randomized using an Alpha design with six replications and six sub-blocks of seven varieties/replicate. Plot size was 5.4 m^2 with plot length of 6 m and plot width of 1.55 m.

Finally, R.A.G.T. trials were conducted in a randomized complete block design with 2 blocks, each block containing 1 replicate. A plot size of 7.2 m^2 (6m x 1.2m) was used.

All trials were run using standard agronomic packages of fertilisers, pesticides and growth regulators.

4.2.3. Thousand Grain Weight

TGW, grain length and grain width were performed using the grain analyser Marvin (GTA Sensorik GmbH, Neubrandenburg, Germany) using pooled seed material from the field grown BC5 plants.

4.2.4. Yield

Field plots were harvested with combine harvesters possessing onboard weighing systems. The yield weight was adjusted to 14% moisture content for use in down-stream analysis.

4.2.5. Grain Protein Content

GPC was assessed using Near-infrared (NIR) spectroscopy with the method previously published by Chia et al. (2017). A FOSS 6500 wavelength scanning near-infrared microscope incorporating ISIscan™ Routine Analysis Software was used to measure protein content, moisture content, and grain hardness for each sample according to the manufacturer's instructions. Each sample of ~5 g of grain was run in duplicate using a ring cup. The sample spectra were compared with calibration set spectra taken from samples with known protein content, moisture, and hardness compositions.

4.2.6. Molecular markers

Both HS and HS/VPM7D were genotyped using the Axiom® wheat HD Genotyping Array (Winfield et al., 2016). After analysing all the SNPs mapping to the 7D chromosome, a set of SNPs polymorphic between the two parental lines located across the full chromosome were selected. KASP primers were designed on these SNPs using PolyMarker (http://polymarker.tgac.ac.uk/ (Ramirez-Gonzalez et al., 2015). Thermodynamic properties of designed primers were verified after adding the standard FAM or HEX compatible tails (LGC Ltd). 7D specific KASP markers were initially tested against HS and HS/VPM7D and the ones which were polymorphic between them were then applied to the RVPM7D population (Table 4.1).

Genomic DNA extraction from the parental lines was performed using the CTAB method (Nicholson et al., 1996) to obtain large quantities of high quality nucleic acid, whereas genomic DNA of the population lines was obtained using the extraction protocol for 96-well plates, adapted from Pallotta (2003).

For KASP assay, 2 µl (5 ng/µl) of the extracted DNA was added to 0.056 µl of primer mix (12 µl each of specific primer, 30 µl of the common primer and 46 µl deionized water) and 2 µl of KASP master mix (LGC). The PCR amplification included an initial denaturation step of 94°C for 15 minutes followed by 10 cycles of touchdown PCR (annealing 62°C to 56.6°C, decreasing 0.6°C per cycle), then 25 cycles of 94°C for 10 s and 60°C for 1 minute. After amplification, plates were read using a Tecan Safire plate reader and genotyped using the Klustercaller™ software (version 2.22.0.5, LGC).

PCR reactions were prepared in a 6.25 µl final volume containing 2.5 µl DNA (10 ng/µl), 3.125 µl Taq mastermix (Qiagen) and 0.625 µl of the relevant primer pair (2 µM). A common PCR programme

was used throughout consisting of a denaturing step of 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min, with a final elongation step of 72°C for 7 min. Where required, PCR products were then purified using QIAquick Gel Extraction Kit (Qiagen), sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (following the manufacturer's instructions) and aligned in VectorNTI® (ThermoScientific).

4.2.7. Map construction and QTL analysis

The genetic map of chromosome 7D was generated in JoinMap© (version 3.0) (Stam, 1993) using default parameters. Mapping data were combined with phenotypic data from the field for QTL analysis. The QTL analysis was carried out using data from each phenotype trial individually as well as using a data set in which the data from all trials were combined.

The search of QTLs was done using Single Trait Linkage Analysis of Genstat v.19 (Copyright 2009 Lawes Agricultural Trust, Rothamsted Experimental Station, UK) in three different steps: 1) initial genome-wide scan by simple interval mapping (SIM) to obtain candidate QTL positions; 2) one or more rounds of composite interval mapping (CIM), in the presence of cofactors, which are potential QTL positions detected at previous step; 3) fit the final QTL model. Default threshold based on the estimation of the effective number of tests (Li and Ji, 2005) has been chosen for the QTL analysis.

4.2.8. Statistical Analysis

Analysis of variance was performed on yield, GPC and TGW scores to assess the variation attributable to line, blocks and interactions between line and blocks, using a general linear model (GLM) in Genstat v.19 (Copyright 2009 Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Predicted mean scores were calculated for each line using the GLM for use in the QTL analysis.

4.3. RESULTS

4.3.1. Genetic mapping

The genetic map of chromosome 7D in the Hobbit-sib (HS) x Hobbit-sib/VPM7D (HS/VPM7D) recombinant population (RVPM lines) published by Chapman et al. (2008) was updated in this work. A new set of 20 7D specific KASP markers (7D_KASP1, 7D_KASP2, 7D_KASP3, 7D_KASP4, 7D_KASP5, 7D_KASP6, 7D_KASP7, 7D_KASP8, 7D_KASP9, 7D_KASP10, 7D_KASP11, 7D_KASP12, 7D_KASP13, 7D_KASP14, 7D_KASP15, 7D_KASP16, 7D_KASP17, 7D_KASP18, 7D_KASP19 and 7D_KASP20) were developed from the Axiom® wheat HD Genotyping Array (Winfield et al., 2016). They are based on 7D genome specific SNPs able to distinguish between the HS and HS/VPM7D parents. The markers are listed in Table 4.1 along with primer sequences, corresponding BA/BS SNP code (where available) and SNP genomic location where known. The

positions of the DNA sequence relating to each of the markers was identified and used to produce a physical map showing their positions on chromosome 7D (Figure 1a). The SNPs relating to the *Xbarc97* and *Ep-D1* markers found previously to co-segregate with *Pch1* locate at 631,323,507 nucleotide position on chromosome 7D (Figure 4.1a).

The KASP and other markers were used to genotype the 90 RVPM lines of the HS x VPM7D population and a new genetic map of the 7D chromosome, spanning a total of 38.8cM and divided in three linkage groups, was produced (Figure 4.1b). As anticipated, based upon previous work, the *Xbarc97* and *Ep-D1* markers associated with *Pch1* located on the linkage group relating to the distal end of the long arm of 7D (Figure 4.1b).

Table 4.1: Summary of markers used for mapping yield and GPC traits in the HS x HS/VPM7D population

Marker name	BA/BS SNP code	Marker type	Primer sequence[1]	Genomic location
7D_KASP1	BA00229805	KASP	cgccaaaccgatcattccC cgccaaaccgatcattccT cgctaggttacttccctgtg	7D: 1,260,492-1,254,548
7D_KASP2	BA00123654	KASP	ggccgctgttattgctacaG ggccgctgttattgctacaT gtgtttgcagaatctctatcgg	7D: 2,410,287-2,410,341
7D_KASP3	BA00820637	KASP	gagtgcagggttcagctC gagtgcagggttcagctG atgatccgccgccccaac	7D: 5,205,975-5,206,075
Xgdm86	-	SSR	ggtcaccctctcccatcc ggcgctccattcaatctg	7D: 19,033,865-19,033,983
7D_KASP4	BA00159833	KASP	cagaagagtcagtgacagaagcaG cagaagagtcagtgacagaagcaT agatataagaacgacaccaaactga	7D: 167,362,311-167,362,411
7D_KASP5	BA00870707	KASP	gctaactacagagagcaccacaG gctaactacagagagcaccacaA cttagcctgcgattacattgctgc	7D: 165,947,999-165,948,099
7D_KASP6	BA00509217	KASP	tgcacgaaatcgaccatgtA tgcacgaaatcgaccatgtC cgaaggcgctctcggtaat	7D: 186,404,679-186,404,779
Xwmc221	-	SSR	acgataatgcagcggggaat gctgggatcaagggatcaat	7D: 364,632,884-364,633,165
7D_KASP7	BA00181566	KASP	tgaaccgtggatctattgtgcG tgaaccgtggatctattgtgcA ccgttaaatcagcagcttaatcc	7D: 412,758,968-412,759,068
7D_KASP8	BS00180865	KASP	agaaggcaaatatgttgtagatcttgA agaaggcaaatatgttgtagatcttgC caactcttgttgaaggggttatctttgta	7D: 451,050,671-451,050,701
7D_KASP9	BA00236459	KASP	ctggggcagcgacatggA ctggggcagcgacatggG ctgacgctcggcttcgga	7D: 476,754,123-476,754,223
7D_KASP10	BA00386263	KASP	cagtgtgttcgccttagatgtaC cagtgtgttcgccttagatgtaT accggaattagacaaactgagac	7D: 497,949,876-497,949,976
Xgdm67	-	SSR	aagcaaggcacgtaaagagc ctcgaagcgaacacaaaaca	7D: 563,309,984-563,310,113
Xgdm150	-	SSR	actagcctggcagttgatgc ccgaccggttcacttcc	7D: 605,875,095-605,875,208
7D_KASP11	BA00121106	KASP	tgtactgccaaaatacgcctG tgtactgccaaaatacgcctC cggcgaacctcatccact	7D: 622,547,411-622,547,511

Table 1 continued: Summary of markers used for mapping yield and GPC traits in the HS x HS/VPM7D population

Marker name	BA/BS SNP code	Marker type	Primer sequence[1]	Genomic location
7D_KASP12	BA00386209	KASP	gctcacaacacccaccaA gctcacaacacccaccaT catgaactgaatctgttctgtgg	-
7D_KASP13	BS00011507	KASP	tgcctttggtcgaagagttcT tgcctttggtcgaagagttcG cagccttattcttcttgcttcaagatcaa	7D: 623,528,591-623,528,754
Xwmc273	-	SSR	agttatgtaattctctcgagcctg ggtaaccactagagtatgtcct	-
Xgwm37	-	SSR	acttcattgttgatcttgcatg cgacgaattcccagttaaac	-
7D_KASP14	BA00084318	KASP	tttcgctgcagaacccaaG tttcgctgcagaacccaaC tcaacaaggaggttcagaatgtt	7D: 620,985,854-620,985,954
7D_KASP15	BA00110518	KASP	cctcgagattgtgctttagattcG cctcgagattgtgctttagattcA cagttcccaaacaggacca	7D: 625,523,827-625,523,927
7D_KASP16	BA00894181	KASP	atgaccgaggagcatgcT atgaccgaggagcatgcC aggttcttcatcagcacacG	-
7D_KASP17	BA00558682	KASP	gtgttgctattagcattcctccT gtgttgctattagcattcctccC taacatacacatcaatgctgcttgaggtt	7D: 629,433,570-629,434,116
Xbarc97	-	SSR	gcgccaactacggagctcggagaa gcaggatcaaacgtagccatggtg	7D:631,323,507-631,323,748
7D_KASP18	BA00747082	KASP	agtcaaacctcgcaaacgC agtcaaacctcgcaaacgT catgagcagcaatgccgac	7D: 629,830,916-629,831,016
7D_KASP19	BA00578168	KASP	atcctcgccttcatgccA atcctcgccttcatgccG tgGagagcaagatatgtatgttcG	7D: 638,463,092-638,463,163
Xwmc14	-	SSR	acccgtcaccggtttatggatg tccacttcaagatggagggcag	7D: 635,511,763-635,512,503
Xgwm428	-	SSR	cgaggcagcgaggattt ttctccactagccccgc	
7D_KASP20	BA00244883	KASP	accgcacatcaaactgagC accgcacatcaaactgagT ctgcgagtgtggtggggt	-

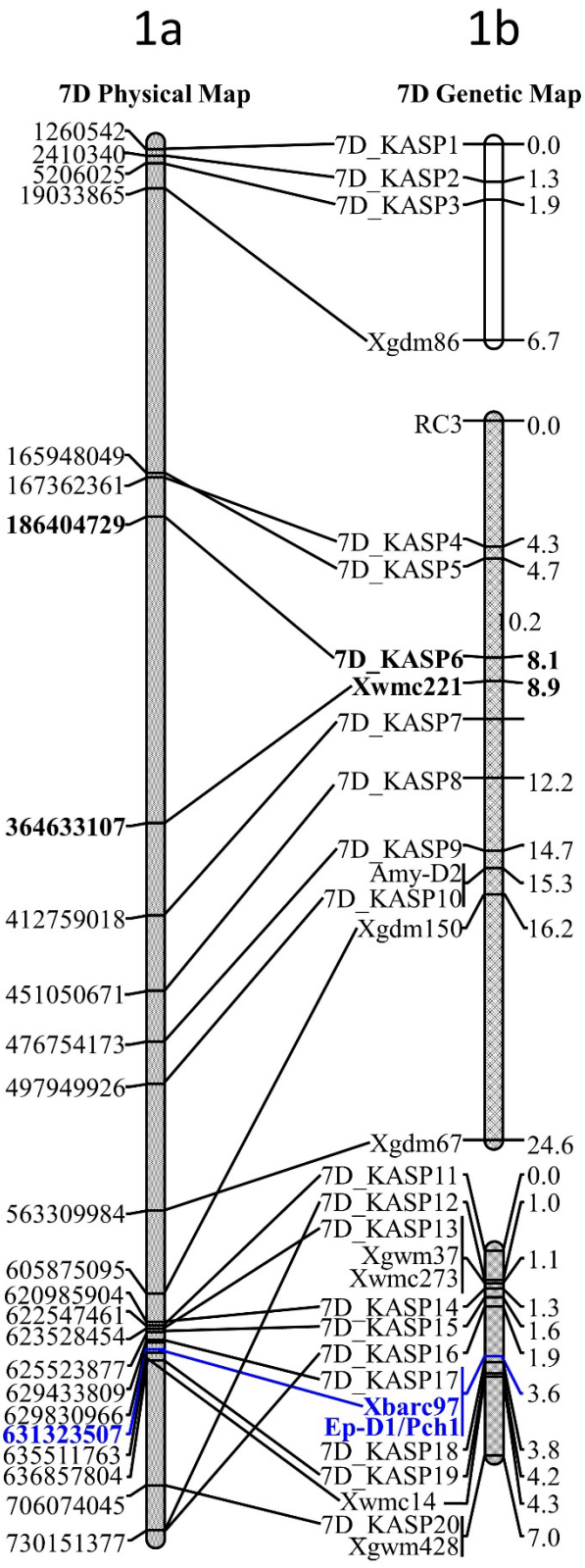

Figure 4.1. Physical and genetic maps produced for chromosome 7D. Physical map of chromosome 7D (1a) showing the positions of the markers used for genetic mapping. Genetic map (1b) of chromosome 7D for the wheat x *Ae. ventricosa* cross showing recombination distances in centiMorgans. The position of *Pch1* and associated markers is indicated in blue.

4.3.2. Field trials

A subset of 34 lines representing all the recombination groups identified in the population was used for Yield and GPC assessment in four consecutive years of field trials as described above. Thousand Grain Weight (TGW) was also assessed for two (2014_JIC and 2017_JIC) out of the four years trials. Predicted means of these traits from individual field trials were calculated. An interval mapping QTL analysis was carried out combining marker data with field trials results for yield and GPC.

In the 2014-JIC trial, QTL for GPC and TGW were obtained but no effect on yield was observed (Figure 4.2). The QTL peak for GPC was at the end of linkage group2 at marker RC3 and explained 40.695% of the phenotypic variance). The QTL peak marker for TGW was positioned 15.3 cM away at *Amy-D2 7D_KASP10*.

In the 2015_Limagrain field trial, a large QTL for yield penalty was observed on linkage group 2 with the peak at marker *Xwmc221* (57.345% explained variance) and a QTL of smaller effect for GPC was identified on the same linkage group at marker *7D_KASP4* (Figure 4.3).

Only a single QTL was detected in the two RAGT trials in 2015 (2015_RAGT_WH and RAGT_SF) and this was for GPC with the peak at marker *7D_KASP6* on linkage group 2 (Figure 4.4 and Figure 4.5).

In the 2016_Limagrain trial, a highly significant QTL for GPC was detected but no QTL effect was observed for yield. The GPC QTL peak was at marker *Xgdm86* on the distal part of linkage group 1 and the QTL explained 26.86% of the phenotypic variance (Figure 4.6).

Very large QTL effects for GPC were observed in both the 2016_RAGT_BT and 2016_RAGT_SD trials. The peak marker for GPC was at marker *Xwmc221* for both trials and the QTL explained 67.337% of variance at 2016_RAGT_BT and 55.66% at 2016_RAGT_SD) (Figure 4.7 and Figure 4.8). A minor QTL effect was detected for yield penalty in the 2016_RAGT_BT trial with the peak at *Amy-2D 7D_KASP10* but no effect was observed for yield in the 2016_RAGT_SD trial.

In the 2017_JIC trial, highly significant QTL were obtained for yield penalty GPC and TGW (Figure 4.9). The QTL peak marker for GPC was *7D_KASP4* which explained 38.433% of the variance. The peak marker for yield was 3.4 cM away at marker *7D_KASP6* which explained 47.02% variance. The peak marker (*Xwmc221*), for TGW explaining 45.5% of the variance was positioned only 0.8 cM from that for yield (Figure 4.9)

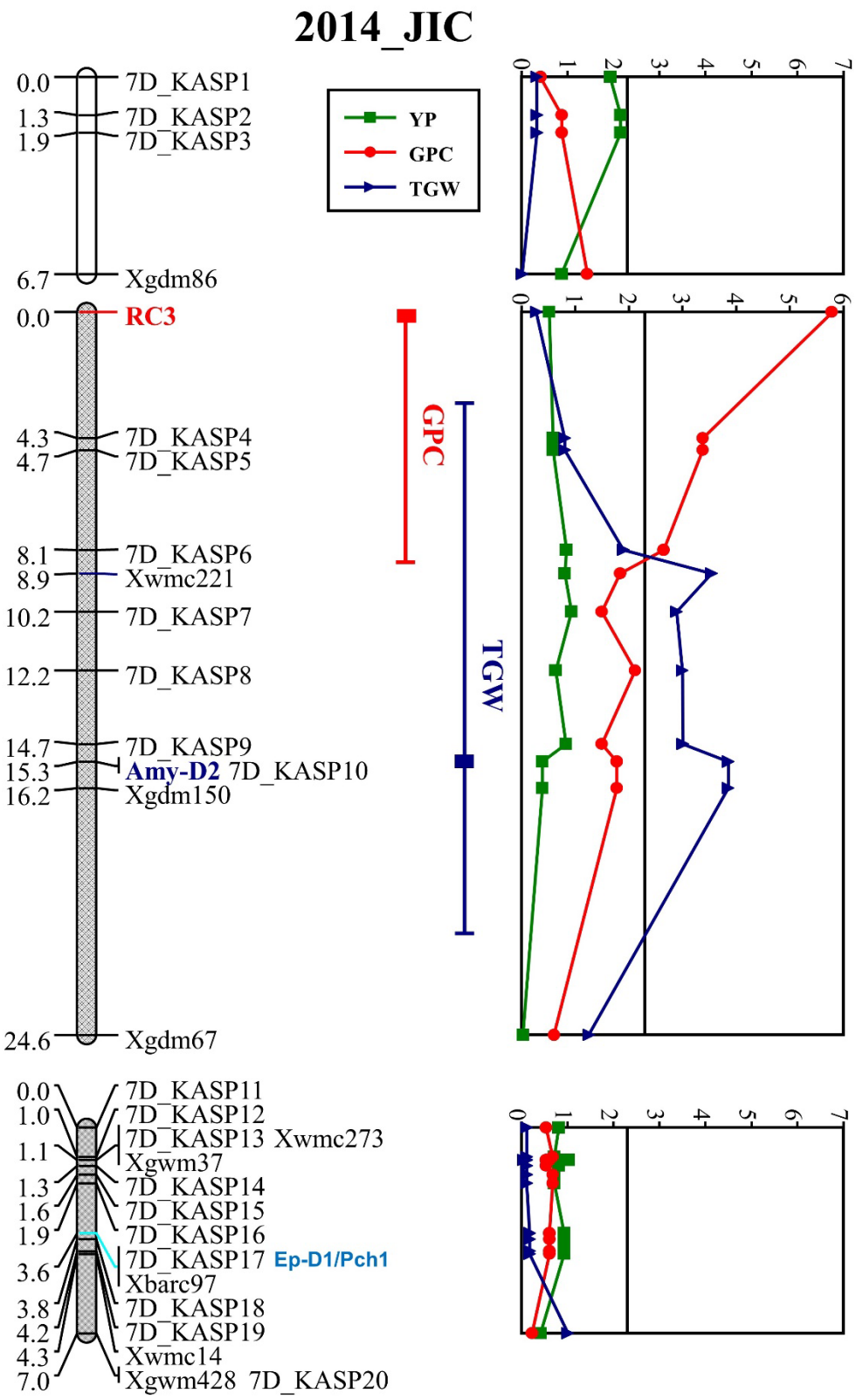

Figure 4.2. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2014_JIC field trial for Yield Penalty, GPC and TGW.

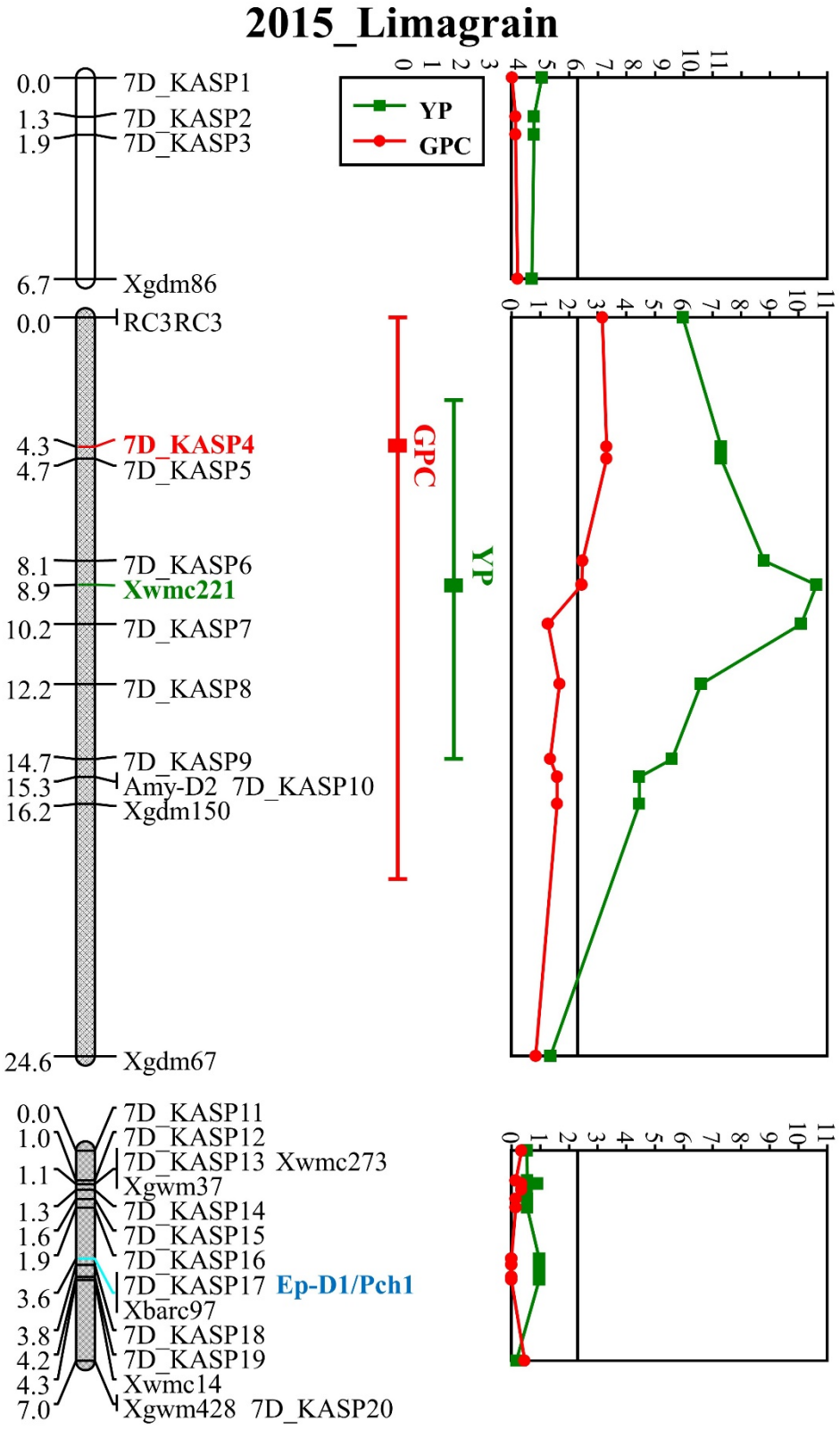

Figure 4.3. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2015_Limagrain field trial for Yield Penalty and GPC

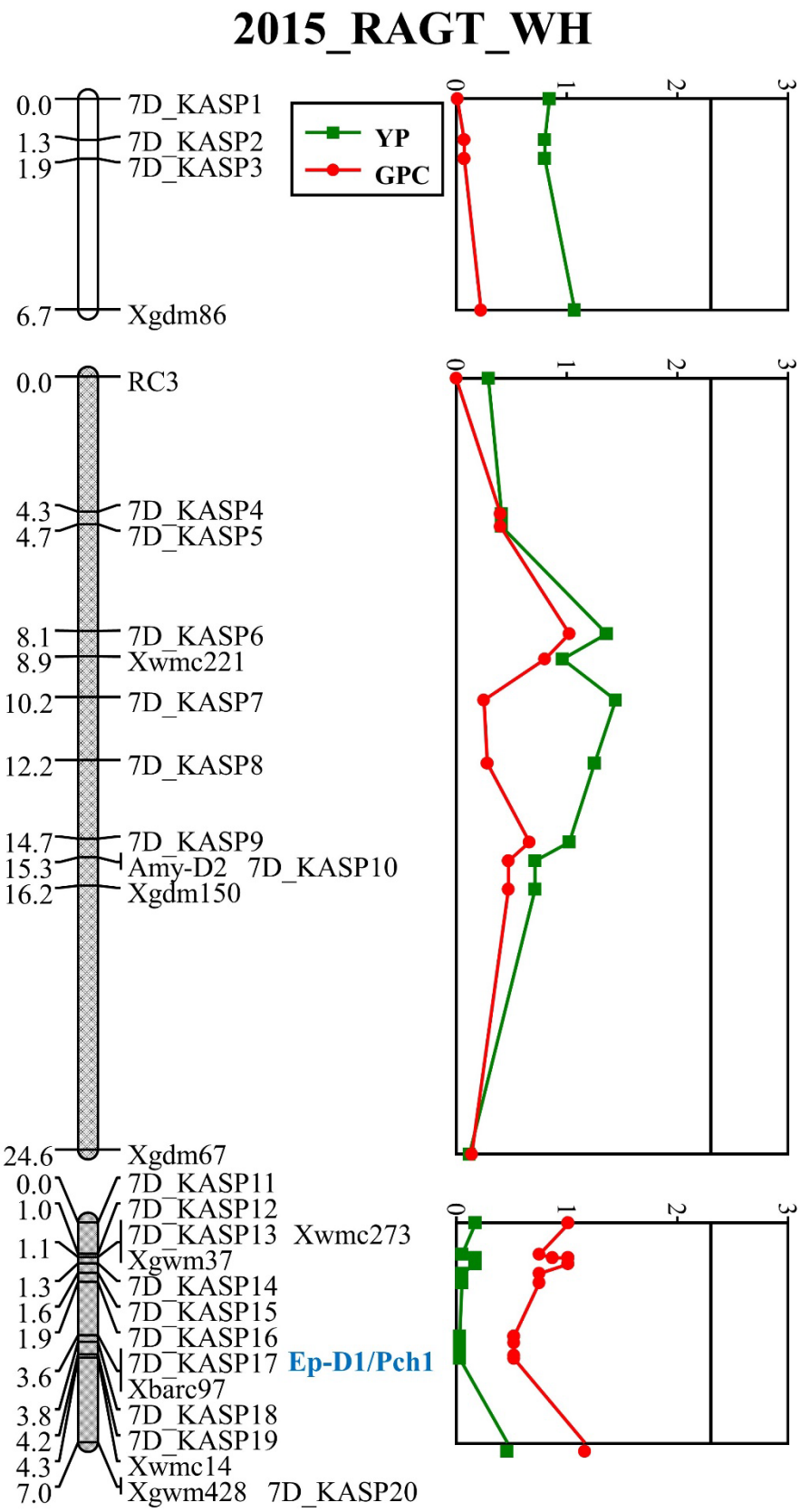

Figure 4.4. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2015_RAGT_WH field trial for Yield penalty and GPC.

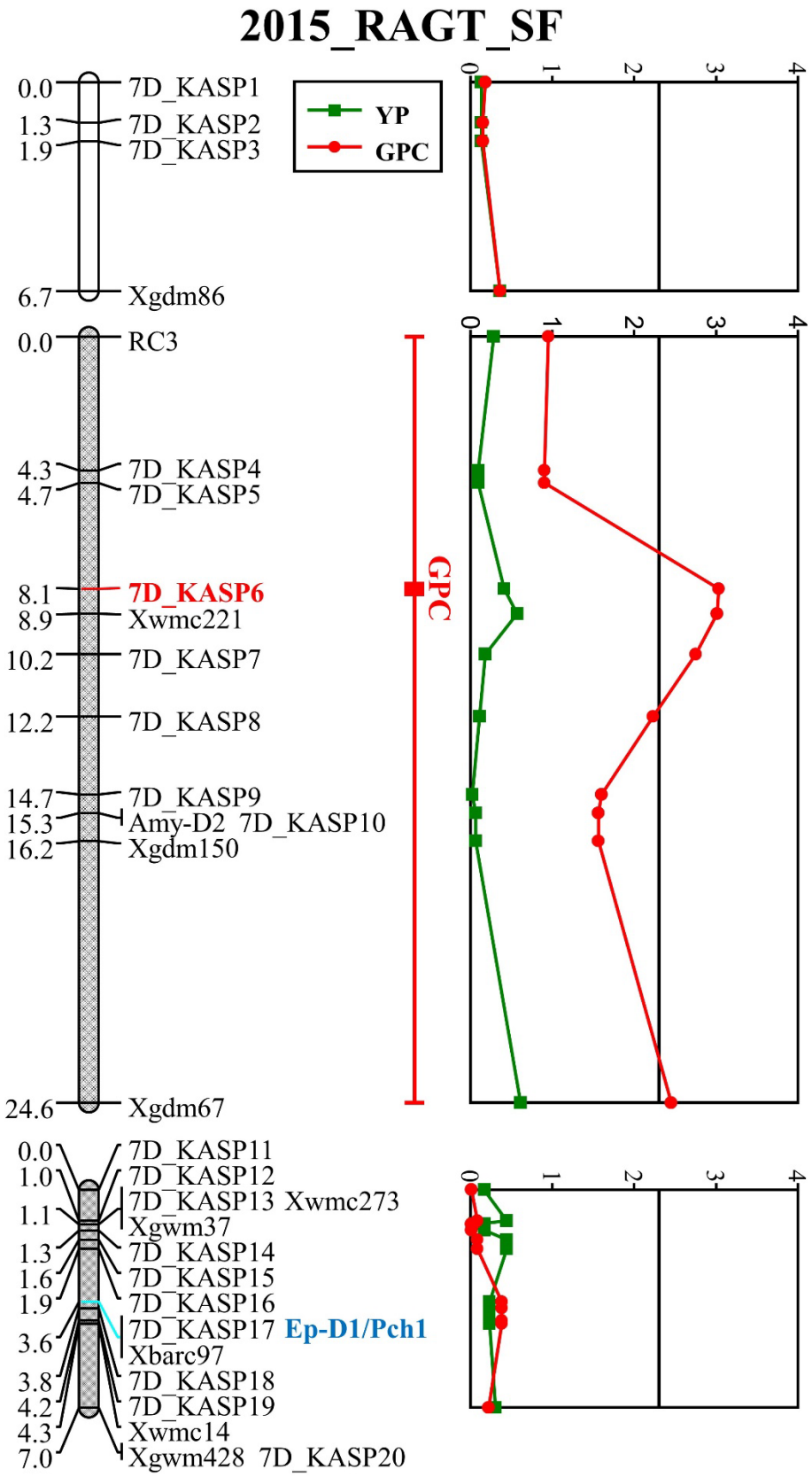

Figure 4.5. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2015_RAGT_SF field trial for Yield penalty and GPC.

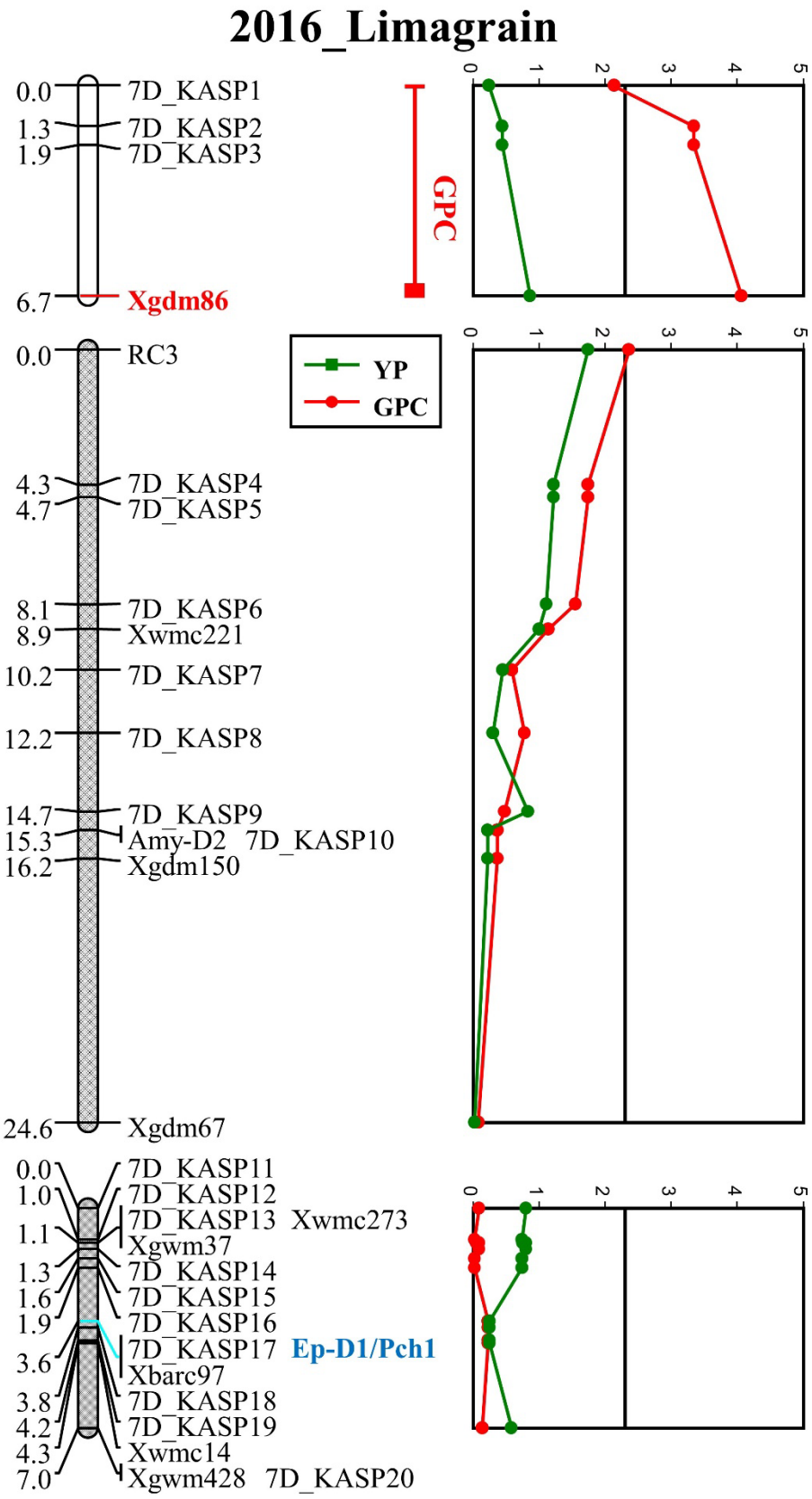

Figure 4.6. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2016_Limagrain_ field trial for Yield penalty and, GPC.

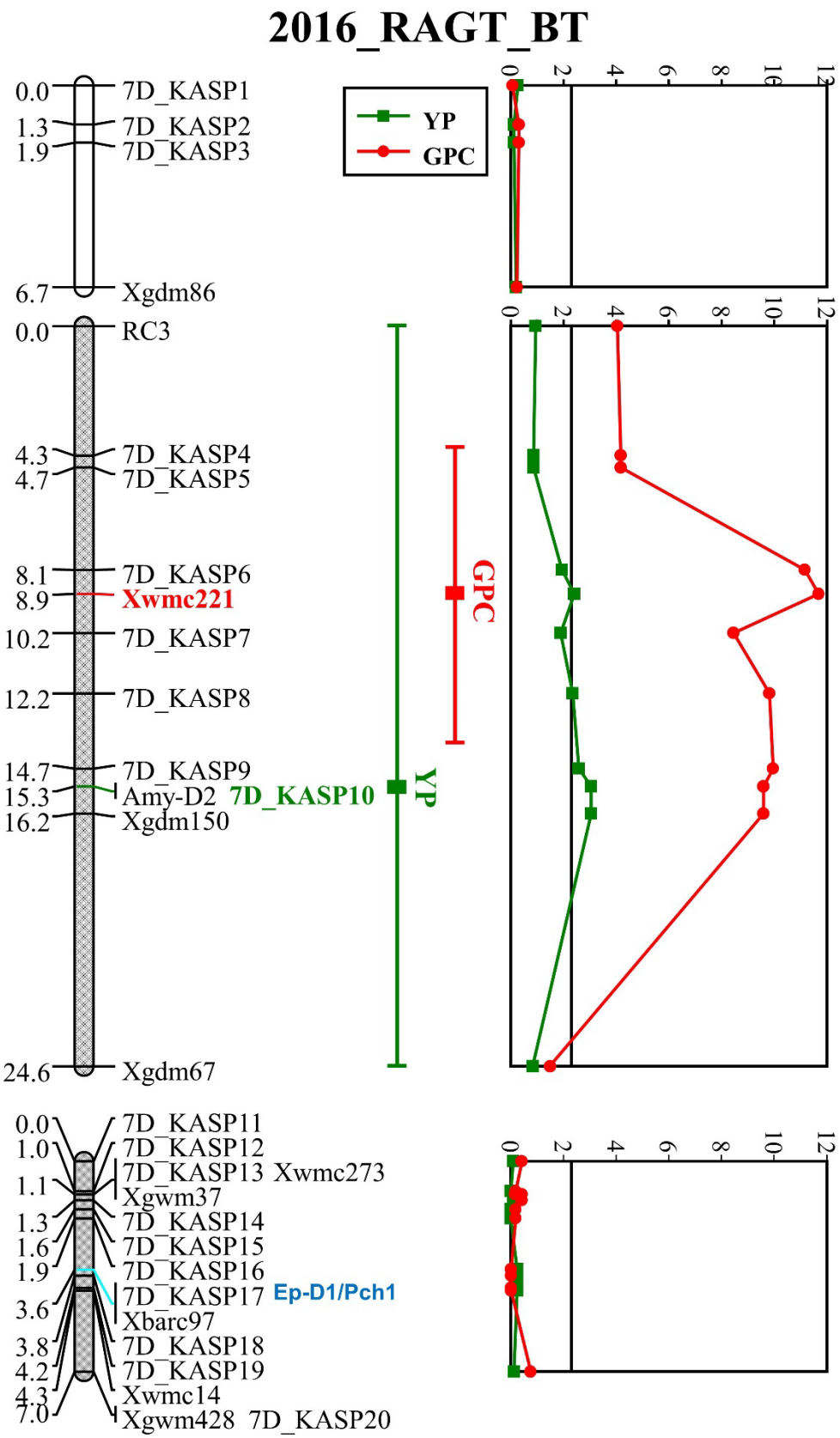

Figure 4.7. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2016_RAGT_BT field trial for Yield penalty and GPC.

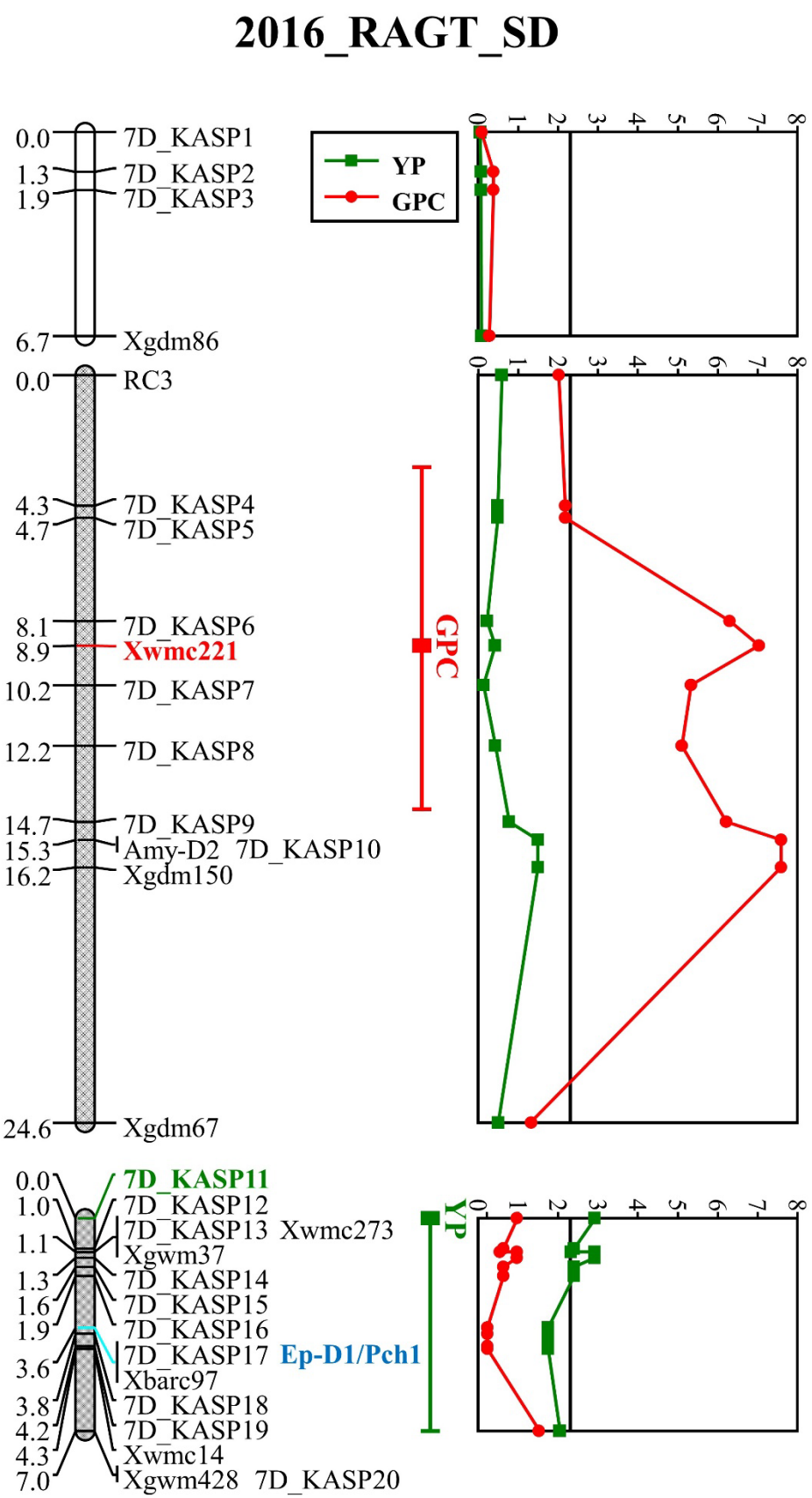

Figure 4.8. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2016_RAGT_SD field trial for Yield penalty and GPC.

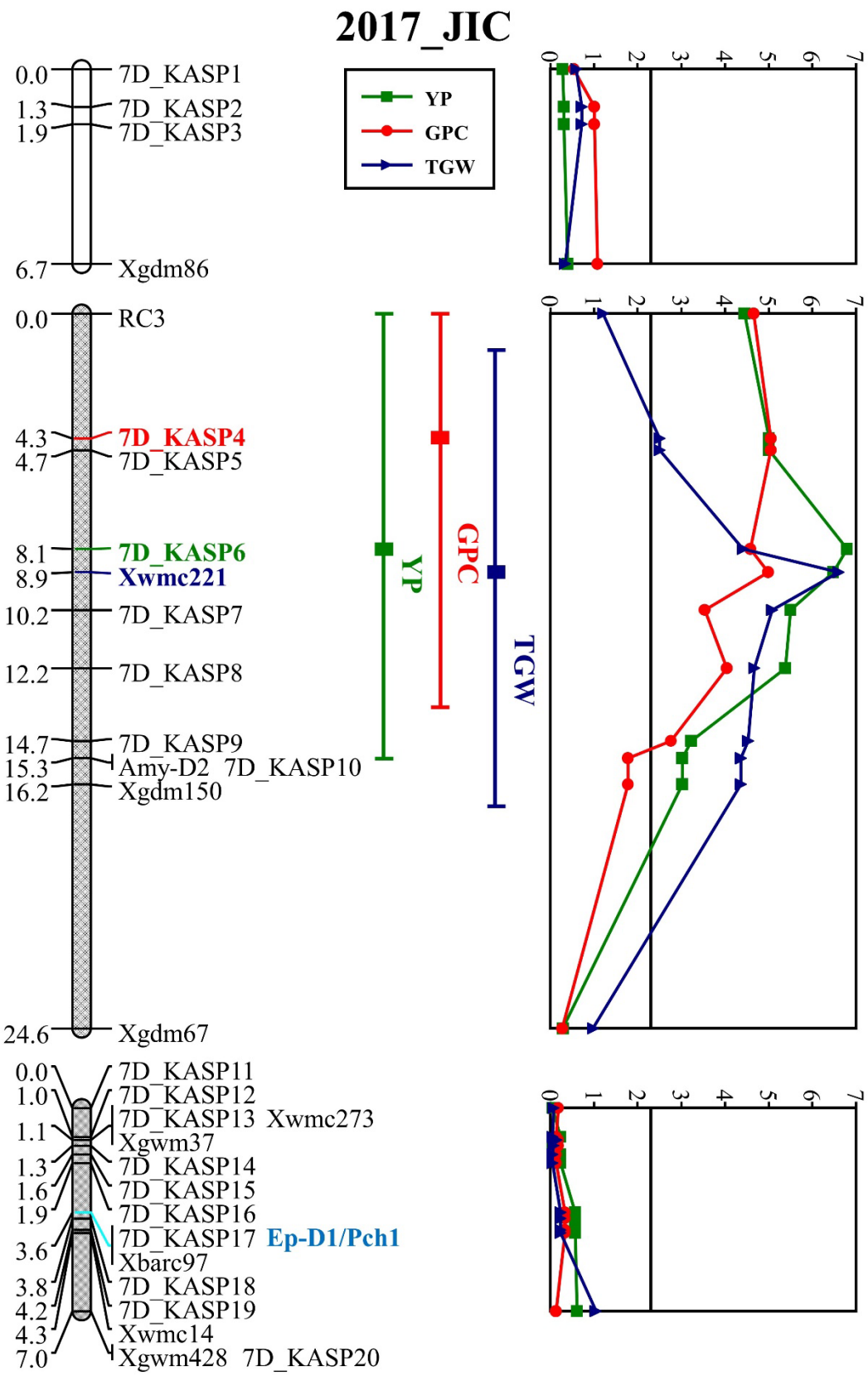

Figure 4.9. Genetic map of chromosome 7D in the HS x HS/VPMD7D population aligned to the LOD profile of the QTL interval mapping analysis of 2017_JIC field trial for Yield penalty, GPC and TGW.

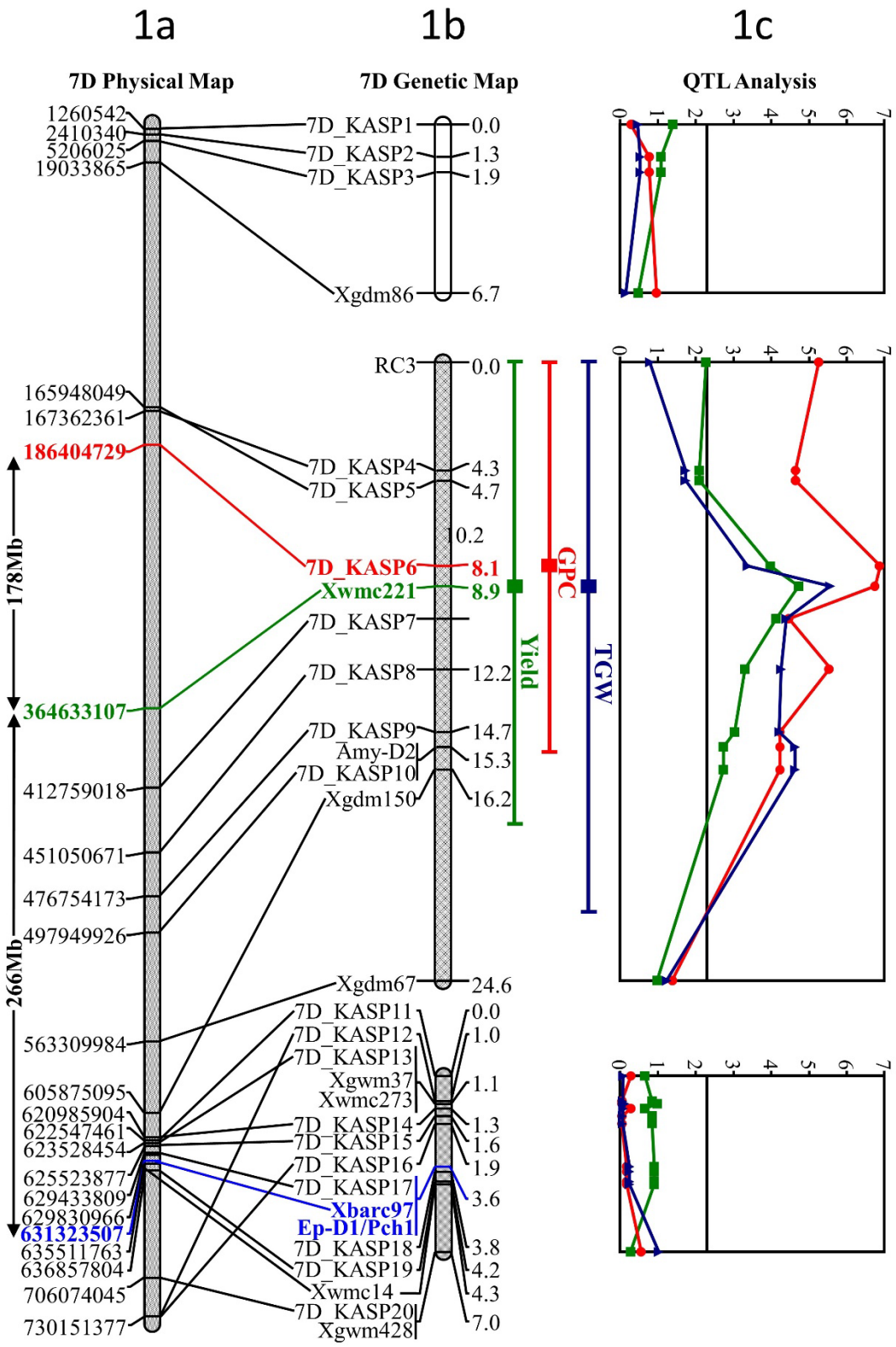

Figure 4.10. Relationship between the physical map (1a) and the genetic map (1b) of chromosome 7D in the HS x HS/VPMD7D population. Summary of QTL effects across all trials for yield penalty (Yield), GPC and thousand grain weight (TGW) (1c)

In the present study, we confirmed that the *Ae. ventricosa* introgression provides a new source of alleles to increase GPC in wheat and it corresponds to an increase of 2.6% in respect to the wheat GPC (Hobbit-sib). This increase is consistent with the data published by Bogard et al. (2010) and Groos et al. (2004). Moreover, combining mapping data and GPC content collected in our field trials, this effect has been mapped for the first time on the chromosome $7D^V$. In contrast to the Yield Penalty effect that was not observed in most trials, the QTL for GPC was robust, and a QTL was identified in every trial except for the 2015_RAGT_WH. This indicates that the effect of the introgressed *Ae. ventricosa* segment on GPC is stable. The GPC increase QTL is on the 'linkage group 2' of the HS x HS/VPM7D map being located only 0.8 cM apart from the Yield Penalty effect in the analysis of the combined data. However, in individual trials the effect on GPC was always associated with a marker that was proximal to that for Yield Penalty, suggesting that the loci controlling the two effects are not coincident. The overall genetic distance may be very small (0.8 cM) but this corresponds to a very large physical distance (178Mb). Despite the small size of the HS x HS/VPM7D population, one recombinant line containing *Pch1* and the GPC QTL peak marker, but not that for the Yield Penalty was identified indicating that an improvement of GPC may be obtained without sacrificing yield. Further study of this line is required to confirm that the GPC and Yield Penalty traits have been separated. A large population recombinant for chromosome $7D^V$ has been produced and is available for future study to generate additional resources for plant breeders to produce varieties carrying the positive GPC and eyespot resistance traits but without the detrimental effect on yield.

5. Fine mapping of *Pch1* and Bacterial Artificial Chromosome (BAC) library screening to isolate *Pch1*

5.1. Introduction

Introgressions into wheat (*Triticum aestivum*) from cultivated relatives such as rye (*Secale cereale*) (Schlegel and Korzun, 1997) and wild species such as *Aegilops* (Schneider et al., 2008) provide a valuable source of genetic diversity for plant breeders. However, use of this germplasm in wheat breeding is often limited because of linkage on the introgressed segments between the trait of interest and deleterious traits (Feuillet et al., 2008). In addition, the presence of a large introgressed segment may prevent beneficial alleles from other sources being introduced into these regions. For these reasons, it is highly desirable to reduce the size of the introgression to minimise these effects. However, reducing the size of such introgressions and breaking adverse linkages is often very difficult due to suppressed recombination between wheat chromosomes and wild relative homeologues (Riley et al., 1959).

Comparative mapping in grass species has provided evidence for conservation of markers and gene order, (co-linearity), between genomes (Devos and Gale, 2000). The release of the wheat and barley genome sequences has revolutionised cereal research (IWGSC, 2018; IBGSC, 2017). Similarly, the availability of several rice genome sequences the annotated *Brachypodium distachyon* (Brachypodium) genome (International Brachypodium, 2010) and the development of large EST collections from other cereals have greatly enhanced the resolution of comparative mapping and has enabled co-linearity based gene cloning (Han et al., 1998). Wheat is more closely related to Brachypodium than to rice (Bossolini et al., 2007) and it is anticipated that Brachypodium may provide higher levels of sequence conservation and co-linearity for marker development, fine-mapping and positional cloning studies.

However, small rearrangements of gene content and order are often found in studies of micro-co-linearity between rice and wheat (Bennetzen and Ramakrishna, 2002; Feuillet and Keller, 2002). It is anticipated that this will further enable the exploitation of co-linearity in the economically important Pooideae sub-family of cool season cereals and grasses, which includes wheat.

The introgression of *Pch1* from the wild grass *Aegilops ventricosa* into hexaploid wheat (Maia, 1967; Doussinault et al., 1983) has provided a potent source of eyespot resistance in wheat. Unfortunately, the negative association between the presence of *Pch1* and yield observed in early varieties has limited the full exploitation of this important source of disease resistance.

A large segment of chromosome $7D^V$ of *Ae. ventricosa* was introgressed into chromosome 7D of wheat as a source of resistance to eyespot. The *Ae. ventricosa* eyespot resistance appears to be

conferred by a single dominant gene, termed *Pch1*, on the chromosome 7D^{V} introgression (Worland et al., 1988) and has been shown to provide a potent resistance that is effective against both pathogen species (*Oculimacula yallundae* and *Oculimacula acuformis*), (Burt et al., 2010) at seedling and adult plant stages (Jahier et al., 1989). However, varieties carrying *Pch1* appear to achieve a lower yield in the absence of the disease than varieties lacking this gene (Koen et al., 2002). Although the *Ae. ventricosa* 7D^{V} and the wheat 7D chromosomes do recombine, they do so at a much lower frequency than normally observed in crosses between conventional hexaploid varieties.

Despite the perceived linkage-drag associated with the *Ae. ventricosa* introgression, significant interest remains in *Pch1* due to its potency and also to the paucity of alternative eyespot resistances. A co-dominant endopeptidase marker *Ep-D1b* was identified by McMillin et al. (1986) with an apparent tight linkage to *Pch1* resistance (Santra et al., 2006). Although this isozyme marker has been used widely for indirect selection of the gene, there have been efforts to replace it with a more user-friendly PCR alternative. Leonard et al. (2008) developed a *Pch1*-linked STS marker, termed *Orw1*, as a potential PCR replacement for the endopeptidase isozyme marker. This was achieved by identifying an oligopeptidase B gene on the wheat 7DL syntenous region of rice chromosome 6. In the same study, *Pch1*-linked STS markers *Orw5* and *Orw6* were developed from a callose synthase gene and a putative photosystem II assembly factor gene, respectively that resided on the same rice bacterial artificial chromosome (BAC).

It has been speculated that *Pch1* resistance may be conferred by the *Ep*-D1b protein (Worland *et al.,* 1988). However, recombination has previously been identified between *Ep-D1* and *Pch1*, suggesting that the *Pch1* eyespot resistance is not a product of this locus (Mena et al., 1992). In addition, a second *Ae ventricosa* source of *Pch1* has been identified but in which the *Ep-D1* allele is non-functional. Huguet-Robert et al. (2001) obtained durum wheat lines, using a second *Ae. ventricosa* accession (Vent 11), which are resistant to eyespot but lack a functional version of the Ep-D1 endopeptidase. These findings discount the *Ep-D1* endopeptidase gene as a candidate for *Pch1*.

It is possible to identify candidate gene regions for *Pch1* in wheat, rice and in Brachypodium on the basis of orthologous locations of the flanking markers. At this stage, it is not possible to infer whether wheat, rice or Brachypodium provides a more suitable reference sequence for further dissection of this region in *Ae. ventricosa*. However, these resources should enable the development of high density of markers and targeting of candidate genes to facilitate positional cloning of *Pch1*.

From the putative gene functions identified in wheat, rice and Brachypodium, there are no clear candidates for *Pch1*. Currently, we have no prior expectations as to the likely function of *Pch1*. To our knowledge no resistance genes of quantitative effect against a necrotrophic fungal pathogen

such as eyespot have been cloned and characterised in wheat. It is important to appreciate that *Pch1* may not be a single gene. The lack of recombination in the region of *Pch1* means that the region has always been inherited as a single unit. It is possible that the potent effect on eyespot resistance is due to the action of closely linked genes that have never been separated through means of recombination.

Identification of novel recombinants in the *Ae. ventricosa* chromosome 7D introgression and development of co-dominant PCR markers in the region would enable the fine-mapping and potential map-based cloning of *Pch1*. This would, in turn, assist the development of high yielding and eyespot resistant wheat varieties. In this study we aimed to identify additional recombination events in the region of *Pch1* to refine further the interval. Following this, the intention was to use a bacterial artificial chromosome (BAC) library of the wheat cultivar Renan (contains *Pch1*) to determine the gene content of the *Pch1* locus and identify gene candidates for *Pch1*. As part of this work, we utilised co-linear regions in the sequenced wheat, Brachypodium and rice genomes to generate a physical map of the region and to identify candidate genes.

5.2. Materials and methods

5.2.1. Plant material

Two new populations were produced within this project and genotyped to identify additional recombinants in the *Pch1* region.

1) An F_2 population of 2400 plants from the cross between the recombinant substitution line F9-8, containing the smallest segment of $7D^V$ including the *Pch1* locus, and Hobbit-Sib (HS)

2) An F_2 population of 2700 plants from the cross between the recombinant substitution line 9-4-H3 (*Pch1*+) and Paragon *Ph1*-. Paragon *Ph1*- was chosen to increase the probability of pairing between the chromosome 7D of wheat and the $7D^V$ chromosome of *Ae. ventricosa* and increasing the recombination rate about the *Pch1* locus.

5.2.2. BAC library screening

The Renan BAC library, Tae-B-Renan, held by CNRGV was screened to identify gene-bearing clones residing at the *Pch1* locus. The 2D pooling strategy used for organising the BAC library allowed the screening to be performed in two steps. Firstly, PCR screening of the 2D pools allows the identification of the plates containing a specific clone of interest. Then, for each positive plate, secondary row and column pools are then produced and screened to identify the positive clone. (https://cnrgv.toulouse.inra.fr/Services/Screening-services/DNA-Pool-production). Real-Time PCR

amplification was performed in a total volume of 20 µl consisting of 5 µl of Sigma SYBR Green JumpStart Taq ReadyMix, 0.05 µl of both the forward and the reverse primer (50 µM), and 2 µl of 2D pool DNA. PCRs were performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using the following cycling protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 20 s and 60 °C for 1 min. A dissociation curve with light measurement every 0.1°C was included to confirm amplification of single gene products.

5.2.3. Seedling bioassay

Four replicates of five plants each of line R25 (contains *Pch1*) were grown in 7 x 7 cm square pots containing peat and sand compost. Pots were arranged in a complete randomized block design and plants were grown for 2-3 weeks in a controlled-environment-room (CER) at 8°C and 8/16hrs light/dark condition. Then they were inoculated using inoculum slurry of *O.yallundae* constrained within 3 cm long transparent PVC cylinders (5 mm internal diameter) and incubated as described by Chapman et al. (2008). Control plants were inoculated with V8 media not containing the fungus. For the expression analysis test, a section of 3 cm of the stem base was collected 8 hrs, 8 and 16 days after inoculation. For the phenotyping of the transgenic lines, plants were harvested 9 weeks after inoculation and scored for disease using the method described by Scott (1971).

5.2.4. Molecular markers

For designing new markers to distinguish the *Ae. ventricosa* 7D introgression from wheat, the new BAC gene sequences were 'BLASTed' in the EnsemblPlants database and the orthologous wheat genes were identified. A set of SNPs polymorphic between *Ae. ventricosa* and wheat were selected and *Ae. ventricosa* specific primers were then designed (Table 5.2). These new markers were initially tested against HS and R25 DNA. The ones giving amplification only for R25 and not for HS were then used for the BAC library screening.

Genomic DNA extraction from the parental lines was performed using the CTAB method (Nicholson et al., 1996) to obtain large quantities of high quality nucleic acid, whereas BAC DNA was obtained using the QIAGEN® Plasmid Midi Kit according to the manufacturer's protocol.

For fine mapping of *Pch1*, two markers *X29550* and *XCos7-9* present at the edges of the *Pch1* genetic map and previously described in this report in chapter 3, were used (Table 3.1).

5.2.5. Expression analysis

RNA extraction was performed from the stem base samples collected from the seedling bioassays described above. Total RNA was prepared using Qiagen RNeasy Plant Mini Kit according to the manufacturer's protocol and subsequently treated with TURBO DNA-free™ Kit (Thermo Fisher

Scientific, US) to remove residual genomic DNA contamination. First-strand cDNA was synthesized using Superscript III 1st strand RT-PCR system (Life Technologies Ltd) according to manufacturer instructions. RT-qPCR was carried out in a total of 20 µl consisting of 10 µl of Sigma SYBR Green JumpStart Taq ReadyMix, 1 µl of both the forward and the reverse primer (10 µM), and 4 µl of 1 to 5 diluted template cDNA solution. Three independent amplifications were performed from each cDNA sample, and reactions were done in triplicates. PCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using the following cycling protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation curve was included to confirm amplification of single gene products. Primer pairs used are shown in Table 5.1. Primers were chosen to amplify a fragment between 50 and 150 bp. Relative quantitation of gene expression was carried out using the 2ΔCT method (Livak and Schmittgen, 2001). The ubiquitin gene was used as an endogenous reference gene to normalize expression levels of target genes.

5.3. Results

5.3.1. Fine mapping of *Pch1*

As shown previously in this report, the genetic position of *Pch1* has been successfully refined to a very small region spanning 0.7cM at the distal end of the chromosome 7D (Figure 3.2). In order to obtain more recombination events with the aim of further refining the locus position, two new F_2 population were produced. The first population consists of 2400 F_2 plants from the cross between the recombinant substitution line F9-8, containing the smallest segment of $7D^V$ including the *Pch1* locus, and Hobbit-Sib (HS). The aim in this case was to try to identify some new natural recombination events. The second population, instead, contains 2700 F_2 plants from the cross between the recombinant substitution line 9-4-H3 (*Pch1*+), a line with a small introgressed segment of the chromosome $7D^V$, and Paragon *Ph1*-. In hexaploid wheat, *Ph1* is the major locus controlling chromosome pairing and ensuring that recombination at meiosis is restricted to true homologues (Griffiths et al., 2006). Mutation at this gene increases the probability of pairing between non homologous chromosomes (homoeologous chromosomes). In this study, the aim was to increase the pairing of the wheat chromosome 7D and the $7D^V$ chromosome of *Ae. Ventricosa,* thereby, increasing the recombination rate at the *Pch1* locus.

These two populations were screened with *X29550* and *XCos7-9*, the two markers present at the edges of the *Pch1* genetic map (Table 3.1 and Figure 3.2). Surprisingly, despite the very large number of plants screened (representing 10,200 gametes), no new recombination events were identified in either population. The lack of recombination may be for two reasons: the physical size of the *Pch1* locus is very small or recombination at the *Pch1* locus is prevented due to factors such as deletion, inversion or reorganization of the locus in comparison with the wheat version on chromosome 7D.

5.3.2. Physical Mapping of *Pch1* and identification of candidate genes.

The lack of recombination within the *Pch1* locus prevented a conventional fine mapping approach to identify candidates for the causal gene(s). For this reason, a physical mapping approach was employed using the BAC library of Renan, a variety that contains *Pch1*. The Tae-B-Renan BAC library, (https://cnrgv.toulouse.inra.fr/en/library/genomic_resource/Tae-B-Renan) was screened to identify gene-bearing clones residing at the locus. Four markers were initially chosen for the PCR-based screening: *X29500*, *XCos7-11_D*, *Xorw1_D* and *Xtr40_D* (Table 3.1), previously designed and used for the genetic mapping. Although these markers co-segregate in the genetic map of *Pch1*, the positions of their orthologous genes on the Brachypodium genome (*Bradi1g29500*, *Bradi1g29400*, *Bradi1g29441* and *Bradi1g29321* respectively) were found to cover a region of ~237Mb (Figure 5.1 c and d).

The BAC library screening identified four positive BACs: 509K05, 397C12, 1935M10 and 1461B10. Complete sequencing and assembly of the BAC clones was done by the Earlham Institute (Norwich Research Park, Norwich, NR4 7UZ, UK) custom sequencing service using the PacBio platform.

Sequence analysis revealed that the BACs did not overlap. Three gaps remained in the physical map (denoted by '???????' in Figure 5.1a).

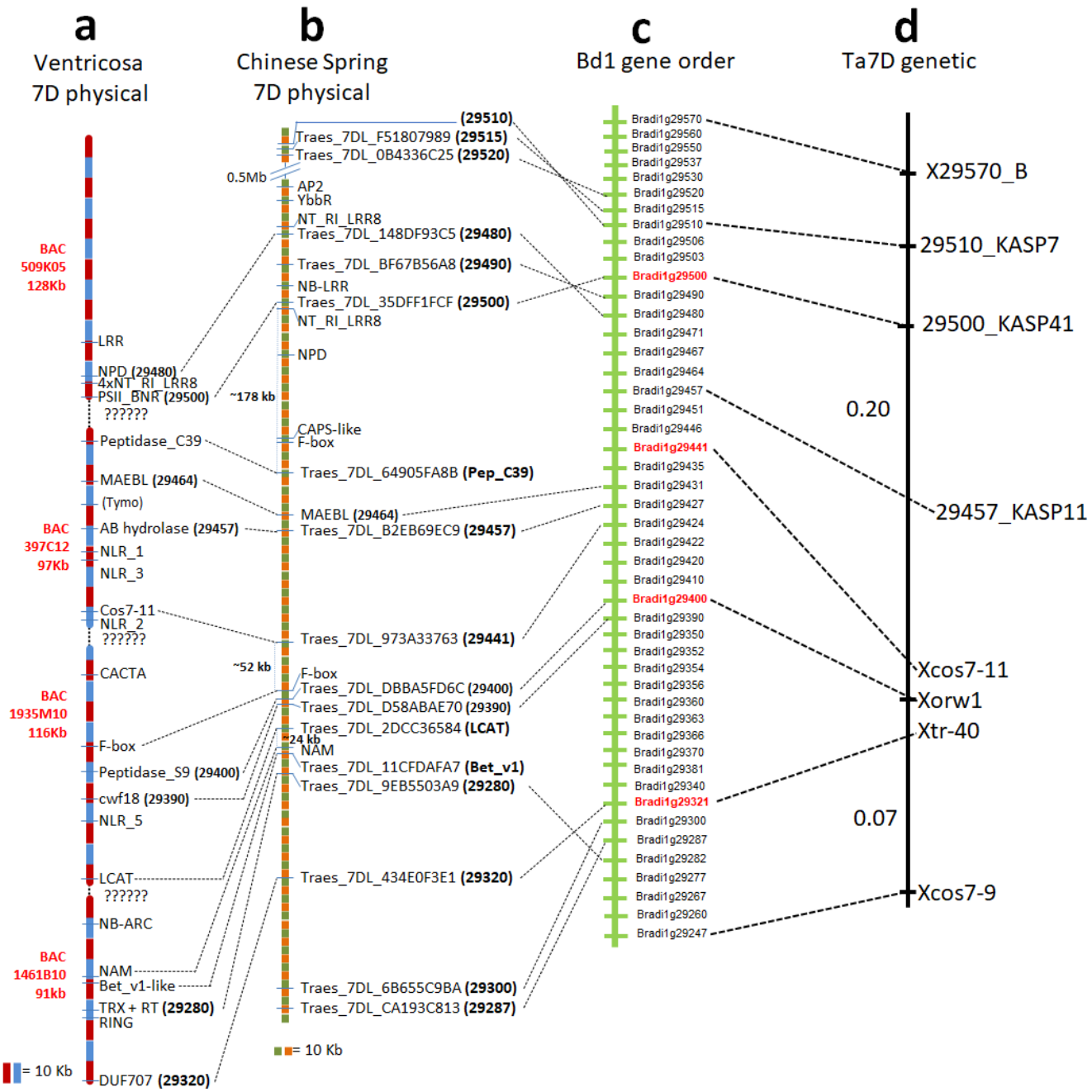

Figure 5.1: Comparison of the genetic map of *Pch1* with the physical map of chromosome 1 (Bd1) of *Brachypodium distachyon*. Highlighted in red are the Brachypodium genes (*Bradi1g29500*, *Bradi1g29400*, *Bradi1g29441* and *Bradi1g29321*) representing the orthologous positions of the four markers chosen for the PCR based screening: *X29500*, *XCos7-11_D*, *Xorw1_D* and *Xtr40_D,* respectively.

The software Fgenesh (Solovyev et al., 2006) was successfully used for annotating a total of 25 genes including 6 NBS-LRRs, 2 peptidases, 15 other protein coding genes and 2 unknown protein domain genes (Figure 5.1a and Table 5.1). The expression of the 15 protein coding genes was tested in R25 stem base tissues inoculated with *O.yallundae* as described in the methods section at three time points (8 hrs, 8 and 16 days after infection). As shown in Table 5.2, only 6 genes were expressed in at least one timepoint: the two peptidases (Peptidase_C39 and Peptidase_S9), MAEBL, AB Hydrolase, Cos7-11 (NBS-LRR) and TRX, but no differential expression was observed in any of these genes when plants inoculated with the fungus were compared to the non-inoculated ones. Among these genes, *Cos7-11* was particularly interesting because it encodes a nucleotide-binding site leucine-rich repeat (NBS-LRR) protein. Most of the disease resistance genes (R genes) in plants cloned to date encode NBS-LRR proteins characterized by a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain as well as variable amino- and carboxy-terminal domains (McHale et al., 2006). For this reason, *Cos7-11* was considered a very good candidate for *Pch1* and was transformed into the wheat eyespot susceptible variety Fielder under the control of the *Zea Mays* Ubiquitin promoter to provide constitutive expression. The transformation was undertaken by the BRACT crop transformation service (Biotechnology Resources for Arable Crop Transformation) of the John Innes Centre (Norwich Research Park, Norwich, NR4 7UZ, UK). T2 plants were tested for eyespot resistance after being inoculated with *O.yallundae* strains as described above. Unfortunately, as shown in Figure 5.2, no statistical difference was observed in disease levels between the transgenic lines containing one or multiple copies of the *Cos7-11* gene and Fielder. This result indicates that *Cos7-11* is highly unlikely to be the *Pch1* resistance gene.

Table 5.1: Annotated genes in the BACs. The software FGenesh was used for the *ab initio* annotation of new genes in the BACs sequencing.

BAC	Protein Domain	Brachypodium orthologous gene	Wheat gene
509K05	PSII_BNR	BRADI1G29500	TRIAE_CS42_7DL_TGACv1_602769_AA1968060
509K05	LRRNT_2 + 5x LRR_RI + 9x LRR_8	No orthologs on Brachy	No annotated wheat gene
509K05	No protein Domain	Bradi1g29480	No annotated wheat gene
509K05	LRRNT_2 + LRR_8	No orthologs on Brachy	No annotated wheat gene
397C12	RX-CC_like/NB-ARC/LRR	Bradi1g29427/Bradi1g29434/Bradi1g29441	TRIAE_CS42_7DL_TGACv1_603214_AA1978490
397C12	RX-CC_like/NB-ARC/LRR	Bradi1g29427/Bradi1g29434/Bradi1g29441	TRIAE_CS42_7DL_TGACv1_603214_AA1978490
397C12	No protein Domain	No orthologs on Brachy	TRIAE_CS42_7DL_TGACv1_603214_AA1978500
397C12	RX-CC_like/NB-ARC/LRR	Bradi1g29427/Bradi1g29434/Bradi1g29441	TRIAE_CS42_7DL_TGACv1_603214_AA1978490
397C12	RX-CC_like/NB-ARC/LRR	Bradi1g29427/Bradi1g29434/Bradi1g29441	TRIAE_CS42_7DL_TGACv1_603214_AA1978490
397C12	AB hydrolase / DUF829	Bradi29457	TRIAE_CS42_7DL_TGACv1_605035_AA2004040
397C12	MAEBL	Bradi1g29464	TRIAE_CS42_7DL_TGACv1_605035_AA2004020
397C12	Peptidase_C39_like super family	No orthologs on Brachy	TRIAE_CS42_7DL_TGACv1_606879_AA2010680
1935M10	F-box (pfam00646)/DUF295 super family (cl04101)	No orthologs on Brachy	No annotated wheat gene
1935M10	Peptidase_S9 (pfam00326)/Peptidase_S9_N (pfam02897)	Bradi1g29400	TRIAE_CS42_7DL_TGACv1_604548_AA1999320
1935M10	cwf18 pre-mRNA splicing factor super family (cl07070)	Bradi1g29390	TRIAE_CS42_7DL_TGACv1_604548_AA1999300
1935M10	RX-CC_like/NB-ARC/LRR	Bradi1g29370/Bradi1g29360	TRIAE_CS42_7DL_TGACv1_604548_AA1999300
1935M10	Protein of unknown function (DUF679 pfam05078)	No orthologs on Brachy	No annotated wheat gene
1935M10	phosphatidylcholine-sterol O-acyltransferase (PLN02733 super family)	No orthologs on Brachy	No annotated wheat gene
1461B10	DUF707	BRADI1G29320	TRIAE_CS42_7DL_TGACv1_602635_AA1963390
1461B10	FAP	No orthologs on Brachy	No annotated wheat gene
1461B10	RING	No orthologs on Brachy	TRIAE_CS42_7DL_TGACv1_604284_AA1996200
1461B10	TRX + RT_like	Bradi1g29280	No annotated wheat gene
1461B10	Bet_v1-like	No orthologs on Brachy	TRIAE_CS42_7DL_TGACv1_604284_AA1996210
1461B10	NAM	No orthologs on Brachy	No annotated wheat gene
1461B10	RX-CC_like/NB-ARC/LRR	No orthologs on Brachy	No annotated wheat gene

Table 5.2: Expression levels of candidate genes identified in the *Pch1* physical map. Expression levels were calculated using the 2∆Ct method and control plants inoculated with V8 media only were used for normalization. Ubiquitin gene was used as reference gene.

Gene	**8 hours a.i.**	**8 days a.i.**	**16 days a.i.**	**Primer Forward**	**Primer Reverse**
Peptidase_C39	-	0,83	0,97	GCTTTCATACGCTCGTCGTCCTCT	TTTATACAGTAGCGGCAGCAGAGCG
MAEBL	-	0,81	0,58	CACTTGTTCAGGCTAATTTGTCGA	ATTCGCTTGCTTCCTTCTTCGCA
Tymo	-	Not expressed	-	TGAGCTAAGTTGACAACGATTGGG	CCCTACCTTATTCAAGCATTTTCTTT
AB Hydrolase	-	0,94	0,77	TATACCTGTTGCTGTCCCTGATTCC	AGATAGTAGAACAGCCTCGGTAGCA
Cos7-11 (NLR)	1.26	1,36	0,76	AAGTCTGCTTGGCTTTGAGG	TGAATGCACCCACCAATTACGA
NLR_2	Not expressed	Not expressed	-	CATCCAGAGTTAAGGAGCACTTTA	TCCCATGTATCTGTTTCCCAAACG
NLR_3	Not expressed	Not expressed	-	GCATCAACAGGAAGAACAGTGAGA	CTCTCCCACTCTGGCAACTTGTA
NLR_4	Not expressed	Not expressed	Not expressed	TCATGGGTGCTATTTCAAGGTTTGCC	GCTTCCTTGTTACACTTTGAATTTGG
F-Box	-	Not expressed	-	ACCTACCAACAACAGAAACTGGGTA	TCTTTCATCGTTCGGTAAGGGAGGC
Peptidase_S9 (Xorw1)	0.76	0,8	0,89	GATGGGTGCTGTCCTAAATATGAGA	AGTTGTGAGAACATCGACGAAAGGG
NLR_5	-	Not expressed	Not expressed	CGTTAAGTTGAGACACTGTAAAGGG	AGTCTGCTAGTAAAGGAGTTGTCCC
LCAT	-	Not expressed	-	AAATACCCTTGTGCGTCGTGTTGGC	TGGTGTTCTGGGACGGTAACTTG
NAM	-	Not expressed	-	AAGAGGCTACAAGTGCTTCCCTTCG	GGTAGATGTCGAGTAGCTCCTCGTG
BET	-	Not expressed	-	AACACCACCATCTCCAACCACAACG	GAGCCTCTGCCATGAGTTCAAGAC
TRX	-	0,83	1	TTTACACTTGCAGTGGGCGATGTA	CCCTCAGACATCTCAGCTTGCCGA

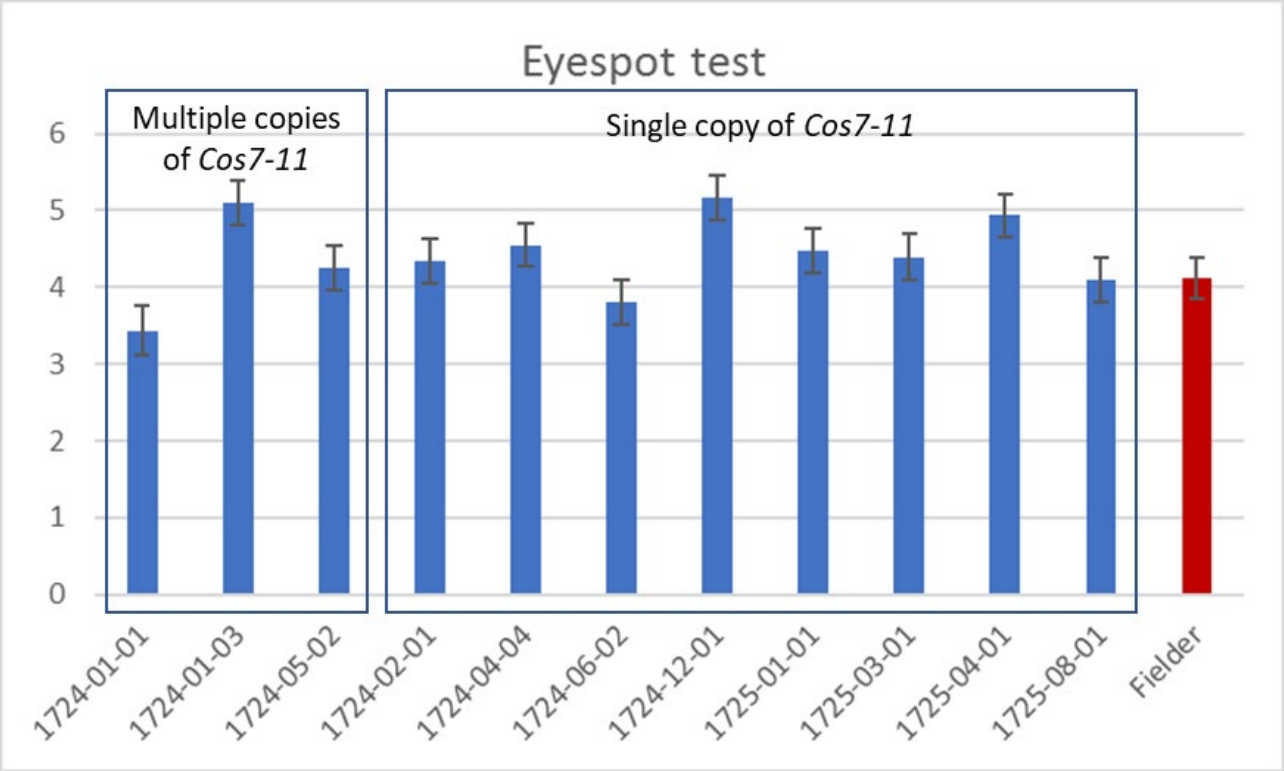

Figure 5.2: Eyespot seedling test of the transgenic lines constitutively over-expressing the candidate gene *Cos7-11*. Non-statistical difference was identified in the resistance levels in comparison to Fielder.

A further round of BAC library screening was necessary to try to close the three gaps left in the physical map and identify potential new candidate genes. Data from a different project (data not shown) suggested that the most probable region involved in the eyespot resistance conveyed by *Pch1* was located between the *Peptidase_C39* and the *F-Box* genes. These findings led to a focus on closing the gap in this region.

Two PCR-based molecular markers were designed to the ends of the physical map close to the target gap. They were designed to amplify only *Ae. ventricosa* DNA but not wheat DNA and were used for the BAC library screening. Two new positive BACs, 2026K18 and 154M06, were identified, one for each marker, and again they were sequenced by the Earlham Institute custom sequencing service. Sequence analysis showed that each BAC was partially overlapping to one end of the physical map, providing some new sequence information to be investigated for new genes. BAC 2026K18 that overlaps to the proximal end of the map, was found to contain a new sequence segment 35 Kb long. This new sequence revealed itself to be a duplication of the last part of the proximal region of the map with a similarity of 99.8%. Importantly, this BAC was found to contain three new genes (Figure 5.3).

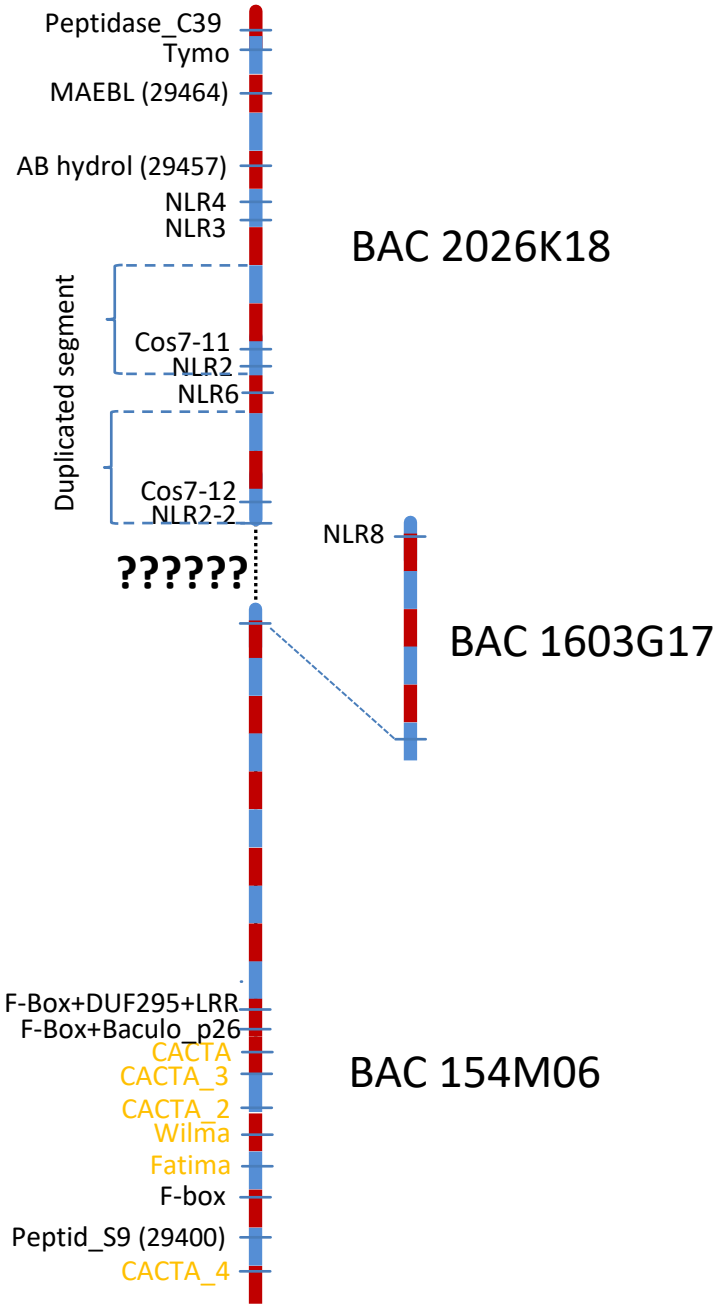

Figure 5.3: Detail of the physical map of *Pch1* showing the results of the second BAC library screening. On the proximal end of the physical map (BAC 2026K18) there is a duplicated segment 35 kb long containing a duplication of two genes: Cos7-11 and NLR2. On the distal end, (BAC 154M06), a new NBS-LRR was identified (NLR8).

Two of the genes (termed *Cos7-12* and *NLR2-2*) were duplicates of *Cos7-11* and *NLR2,* respectively previously identified on BAC 397C12 (see Figure 5.1). Notably, *NLR2-2* was 100% identical to *NLR2*. The second gene was very similar to *Cos7-11* (99.85%) and for this reason it was named *Cos7-12*. Only 5 SNPs were identified between *Cos7-11* and *Cos7-12* leading to two amino acid changes at the protein level. The third gene was an NBS-LRR gene named *NLR6*. Expression analysis of these

new genes showed that *NLR2_2* and *NLR6* were not expressed. Whereas *Cos7-12* was expressed. Data from a different project, however, have allowed us to exclude *Cos7-12* from the list of candidates.

No new genes were identified on 154M06 and unfortunately, 2026K18 and 154M06 sequences were also not found to overlap. So, another BAC library screening became necessary to try to close the gap in the physical map. Due to the presence of the duplication segment on 2026K18, it was not possible to design new *Ae. ventricosa* specific markers on the proximal end of the map, but a new marker was designed at the end of 154M06. This marker was used to re-screen the BAC library. A single new positive BAC (1603G17) was identified. Sequencing of this BAC revealed that it overlapped the distal end of the map and gene annotation revealed the presence of a new NBS-LRR, named *NLR8*. Significantly, *NLR8* was expressed and its expression levels doubled in the stems 2 days after eyespot infection (Figure 5.3), indicating that it is responsive to infection and so making *NLR8* a very good candidate for *Pch1*.

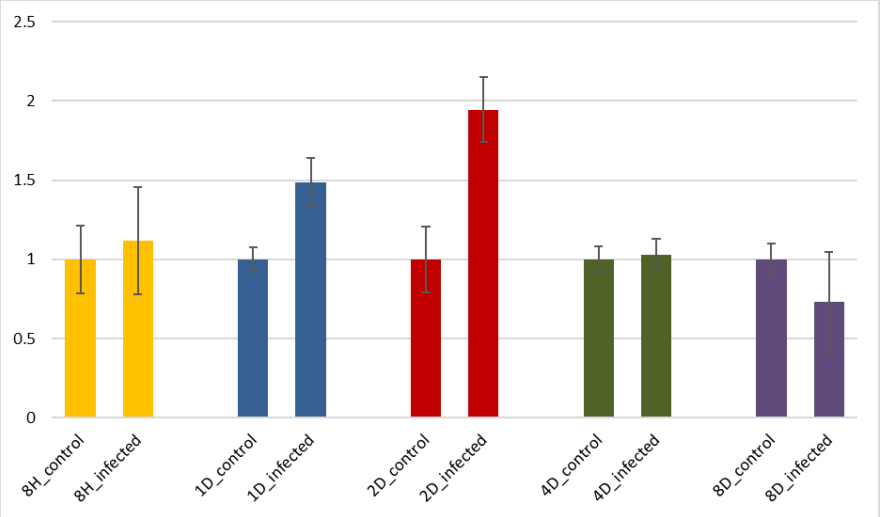

Figure 5.3: Expression levels of *NLR8* in R25 lines (Pch1+) infected with *O. yallundae*. Stem samples were collected 8 hours, 1 day, 2 days, 4 days and 8 days after infection.

The wheat eyespot susceptible variety Fielder was transformed with the *NRL8* gene under the control of the *Zea Mays* Ubiquitin promoter for constitutive expression. At the time of writing this report, it is not known whether this gene confers resistance to eyespot. This will be the subject of future investigations. Additional screening of the BAC library has been undertaken in an attempt to close the gap in the physical map. Preliminary data indicate that the region contains a large number of very closely related repeated segments making it extremely challenging to create a reliable consensus sequence for the interval.

6. Discussion

6.1. Establishing the relationship between *Pch1* and *Pch2*

The three goals of this study were to refine the genetic positions of *Pch1* and *Pch2*, to develop new gene-based molecular markers closely linked to *Pch1* and *Pch2,* and clarify the potential homoeologous relationship between *Pch1* and *Pch2*

The *Pch1* resistance gene was introgressed to wheat from *Ae. ventricosa* but characterisation of the gene responsible has been hindered by the lack of marker availability and very low recombination rate. To deal with these issues, we exploited the syntenic relationship between *Brachypodium distachyon* and wheat to develop high-throughput KASP markers and applied these to a large population in the search for new recombinants. *Pch1* was located to a very small genetic region spanning only 0.07 cM between *Xcos7-9* and the group of co-segregating markers *X29500-X29457-Xcos7-11-Xorw1-Xtr40* (Figure 3.1). Despite the larger number of lines screened (4512 gametes) a total of only 36 recombination events were identified between the two most external markers *Xbarc76* and *Xcdf175*, corresponding to recombination rate of 0.8%. Considering that the rate of recombination between homoeologous chromosomes of wheat and wild grass relatives is estimated at around 3% (Niu et al. 2011), our results suggest that the actual *Pch1* physical region is very small and that a recombination event within the introgressed segment is very unlikely to be observed. Confirmation of this hypothesis is that the *Pch1*-corresponding equivalent genetic region on chromosome 7A in a conventional wheat x wheat cross (CS x CS/CD7A) spans only 0.5 cM (Figure 3.4). Compared with the previous version of the map (Burt and Nicholson, 2011), the new map eliminates 19 ‘candidate’ genes and now physically spans only 250 Kb in which only 24 genes are reported in wheat.

The syntenic relationship between wheat and Bd, together with a high-density SNP genotyping 9K array iSelect (Wang et al. 2014), were used to refine the position of *Pch2*. The QTL analysis performed combining marker and phenotypic data resulted in positioning *Pch2* in a genetic region of 15.6 cM between *XBS1_30210* and the co-segregating group of markers *XBS3_29990-XBS4_29980-X29960-XBS5* (Figure 3). Although the genetic size of the interval containing *Pch2* remains unchanged in comparison to the earlier version (Burt and Nicholson, 2011) the distal edge of the locus moved from the *Xcos7-11* marker (Burt and Nicholson 2011) to the group of co-segregating markers *XBS3_29990-XBS4_29980-X29960-XBS5*, which sits 24.1 cM away from *Xcos7-11* (Figure 2). Moreover, using Brachypodium as a syntenic reference, this distance (from *Xcos7-11* (*Bradi1g29441*) to *X29960* (*Bradi1g29960*) corresponds to a region containing 56 genes on Bd1 (Figure 3.2).

Two KASP markers, *Xorw1_A* and *Xorw1_D*, were designed to the 7A (*Traes_7AL_D1DB1B9EE*) and 7D (*Traes_7DL_DBBA5FD6C*) orthologous genes of the endopeptidase encoding gene *Bradi1g29400*. *Xorw1_D* is the KASP replacement of the STS marker (Xorw1) developed by (Leonard et al. 2008) towards the wheat *EST AB246917*. *AB246917* is 96% and 91% similar to *Traes_7DL_DBBA5FD6C* and *Bradi29400* respectively and encodes the endopeptidase protein Ep-D1b. A number of previous studies detected a complete linkage between *Pch1* and either the isozyme marker (McMillin 1986; Santra et al. 2006) or the STS marker (Burt and Nicholson 2011; Leonard et al. 2008; Meyer et al. 2011). In accordance with these studies, our data also did not identify any recombination event between *Xorw1_D* and *Pch1*, making this new KASP marker highly diagnostic for the presence of *Pch1*.

Koebner and Martin (1990) reported the eyespot resistance on the chromosome 7A of Cappelle Desprez to be associated to the endopeptidase allele *Ep-A1b* and the isozyme marker of Ep-A1b was located at the end of chromosome 7A by de la Peña et al. (1997). In this work we developed a KASP marker (*Xorw1_A*) that can be considered the first DNA-based replacement of the isozyme marker for *Ep-A1b*. Our data revealed that recombination between *Pch2* and *Xorw1_A* was extensive with *Xorw1_A* being 28.1 cM from the *Pch2* peak marker (Xcfa2040) (Figure 2). These results confirm the findings of de la Peña et al. (1997) who mapped the isozyme marker for *Ep-A1b* 32.8 cM distal to *Pch2*.

Several papers have postulated about the possible homoelogy of *Pch1* and *Pch2.* Burt and Nicholson (2011) found that the region containing *Pch1* on chromosome 7D overlapped with the *Pch2* QTL on the chromosome 7A. Moreover, Chapman et al. (2008) identified an SSR marker *Xcfa2040* that is close to *Pch2* on the 7A, and also to the *Pch1*-linked SSR marker *Xwmc14* on the 7D.

The data from the present study does not support the view that *Pch1* and *Pch2* are homoeologous. Using new DNA markers and large populations, we found that the homoeologous region to *Pch1* on chromosome 7A is located 23.6 cM far away from the group of co-segregating markers (*XBS3_29990-XBS4_29980-X29960-XBS5*) that define the distal end of the *Pch2* QTL (Figure 3.4). The high level of resolution of genotyping and phenotyping allowed us to refine the positions of *Pch1* and *Pch2* resulting in a clear a clear separation between them.

In conclusion, our results reveal that *Pch1* and *Pch2* do not overlap and that they do not appear to be homoeoloci. This result has implications for the cloning of *Pch1* because it prevents *Pch2* being used as a proxy target in which the higher level of recombination in wheat x wheat cross could be exploited to fine map and identify the causal gene. On the other hand, the high level of synteny between wheat and *Brachypodium distachyon* has been proven to be extremely helpful for guiding

the development of new molecular markers and the identification of candidate genes, as a means to towards the *Pch1* cloning.

6.2. Establishing the location of the positive grain protein and the negative yield penalty traits relative to that of *Pch1*

In the present study, KASP markers data were integrated to the SSR-based map of the 7D chromosome from RVPM lines published by Chapman et al. (2008). The new version of the map remains divided into three linkage groups due to the small size of the RVPM population. Nevertheless, the inclusion of new markers distributed along the chromosome, has proved effective for the purpose of this study. For the first time, the Yield Penalty effect deriving from the *Ae. ventricosa* introgression that has been reported by different authors (Koen et al. 2002; Kwiatek et al. 2016; Worland et al. 1990) has been mapped onto the chromosome 7D. The effect on yield was markedly affected by environment as indicated by the absence of effect in most years and trials. When each trial was analysed independently, YP was identified only in one of the 2015 field trials (2015_Limagrain), in both 2016 field trials (with only a small QTL detected on the 2016_RAGT_BT field trial) and in the 2017 field trial, indicating that the YP QTL is not stable and its effect is probably affected by environmental factors, such as high summer temperature. More importantly, our results clearly show that Yield penalty locus and the *Pch1* eyespot ressitance are located on different linkage groups, indicating that, if a YP effect is present, it is not linked to *Pch1* and it can easily be separated from the desirable disease resistance locus. This is confirmed by the huge physical distance of 443Mb found between the YP QTL peak marker *Xwmc221* and *Xbarc97*, the SSR marker co-segregating with *Pch1* (Chapman et al. 2008).

For having the overall picture of the 7D introgressed segment effect on yield and protein content, predicted means of YP, GPC and TGW from each field trial were combined and a new interval mapping QTL analysis was carried out. Overall, this analysis revealed that *7D_KASP6* is the marker most significantly associated to the increased GPC explaining 46.625% of the observed phenotypic variance with the QTL interval sitting between *RC3* and *Xgdm150* markers and spanning a region of 16.2cM (Figure 4.10). *Xwmc221*, instead, is the marker most significantly associated with Yield Penalty explaining 40.089% of the observed phenotypic variance with the QTL interval located between *7D_KASP6* and *Xgdm150* markers and spanning 8.1cM (Figure 4.10). Finally, combining TGW data of the two years tested, a QTL was identified with *Xwmc221* as the most significantly associated marker, explaining 35.85% of the observed variance and with the QTL region spanning 8.1cM.

Exploiting the wheat genome sequence, the physical position on the chromosome 7D of all the markers used for the genetic map was identified and, although the two peak markers for YP and GPC appear genetically very close (0.8 cM), they are physically distant being 178Mb apart (Figure

4.10). More importantly, YP and *Pch1* are separated by a very large distance of 443Mb (physical distance calculated from *7D_KASP6*, YP peak marker to *Xbarc97*, SSR marker co-segregating with *Pch1*(Chapman et al. 2008)), showing again that YP and *Pch1* are not physically linked (Table 4.10).

To quantify the 7D introgressed segment effect on YP and GPC, a regression analysis has been performed dividing individuals of the RVPM population in two groups: group A containing all genotypes the wheat haplotype of the yield penalty peak marker *Xwmc221* and group B with all genotypes having the ventricosa haplotype of the same marker. Same process has been performed using the GPC peak marker *7D_KASP6* respectively. Results showed that the presence of $7D^V$ segment causes an overall increase of 2.6% in GPC and a 3.1% reduction in yield.

The QTL interval for TGW assessed in this study is, in fact, coincident to YP QTL (Figure 4.10). We postulate that the locus acts primarily through an effect on grain size that, under some circumstances, leads to an overall loss of yield.

Considering all these results, *Xwmc221*, the YP QTL peak marker, can be negatively selected by breeders preventing this deleterious trait of reduced yield to be included in the genetic background of new wheat breeding lines but still retaining Pch1 eyespot resistance. Moreover, Burt and Nicholson (2011), showed that most of the wheat varieties listed on the UK Recommended List for cereals possess a large portion of the *Ae. ventricosa* original segment and they speculated that the negative yield effect must have been compensated by other factors. The findings of the present study reveal that, most of these varieties, based upon the evidence for marker *Xwmc221,* possess the wheat version of the locus and should not suffer any yield penalty. The deleterious portion of *Ae ventricosa* $7D^V$ affecting yield had already been removed by natural recombination.

6.3. Fine mapping of *Pch1* and Bacterial Artificial Chromosome (BAC) library screening to isolate *Pch1*.

The objective of this work was to use a combination of resources to develop markers as close to *Pch1* as possible and then use these markers to screen a bacterial artificial chromosome library of the *Pch1*-containing wheat variety Renan to produce a physical map of the region containing *Pch1* and identify potential candidate genes for *Pch1* itself.

Despite screening 10,200 gametes of recombinants between wheat chromosome 7D and the equivalent region from *Ae. ventricosa*, no new recombinants were identified. This finding was unexpected as half of the recombination took place in the absence of the *Ph1* gene that controls pairing between homoeologues. The absence of this gene should promote pairing between the wheat 7D chromosome and the *Ae. ventricosa* chromosome (Griffiths et al 2006). This finding

indicated that, either the introgressed segment of *Ae. ventricosa* DNA is very small or that recombination between the wheat 7D and *Ae. ventricosa* chromosome equivalent is highly suppressed.

At the start of this project no genome sequence was available for wheat and so all progress relied upon exploiting the syntenous relationship between wheat chromosome 7D, *Brachypodium distachyon* chromosome 1, Sorghum chromosome 10 and rice chromosome 6. The closest relation to wheat among these grasses is *Brachypodium distachyon* and, for this reason this species was used as the anchor for generating a physical map of the *Ae ventricosa* introgressed segment.

Using sequences to genes at the *Pch1* locus it was possible to identify the equivalent regions in *Ae ventricosa* within the BAC library of the wheat *Pch1* containing variety Renan. Selected BACs were sequenced to determine their gene content and identify potential candidate genes for *Pch1*.

Burt and Nicholson (2011) showed that the marker COS7-11 segregated with Pch1. This marker was designed to a wheat CC-NBS-LRR gene that corresponded to a complete wheat gene sequence termed DQ205351 (Wang et al 2006). This gene has undergone several name changes and was also identified as a potential candidate by Zanke et al (2017) when it was termed Traes_7DL_973A33763. The *Ae. ventricosa* allele of this gene was identified on BAC 397C12. The *Ae. ventricosa* allele was transformed into the eyespot susceptible wheat variety Fielder. Unfortunately, none of the transformants carrying this gene exhibited any increase in eyespot resistance. Thus, despite the repeated identification of this gene as a candidate for *Pch1* our data indicates that this is not the gene responsible.

Sequencing of additional BACs revealed numerous full-length CC-NBS-LRR type resistance genes. Some of these appeared to be represented multiple times indicating that parts of *the Ae. ventricosa* chromosome had undergone duplication. Analysis of RNA indicated, however, that many of these were not expressed in seedling tissues making it highly unlikely that they are candidates for *Pch1*. One gene, termed LRR-8 exhibited an increase in expression following exposure to eyespot. The *Ae. ventricosa* allele of LRR-8 was transformed into the eyespot susceptible wheat variety Fielder. At the time of writing this report it is not known whether LRR8 has any influence on eyespot resistance.

7. References:

Bassam BJ, Caetanoanolles G, Gresshoff PM (1991) Fast and Sensitive Silver Staining of DNA in Polyacrylamide Gels. Ann Biochem 196:80-83

Bateman GL, Munnery IC (1995) Development of Eyespot and Brown Foot Rot in Wheat Plants Inoculated with Mixtures of *Pseudocercosporella herpotrichoides* Types and Fusarium Species. J Phytopathol 143:147-151

Bennetzen JL, Ramakrishna W (2002) Numerous small rearrangements of gene content, order and orientation differentiate grass genomes. Plant Mol Biol 48: 821-827

Bogard M, Allard V, Brancourt-Hulmel M, Heumez E, Machet JM, Jeuffroy MH, Gate P, Martre P, Le Gouis J (2010) Deviation from the grain protein concentration-grain yield negative relationship is highly correlated to post-anthesis N uptake in winter wheat. J Exp Botany 61:4303-4312

Bossolini E, Wicker T, Knobel PA, Keller B (2007) Comparison of orthologous loci from small grass genomes Brachypodium and rice: implications for wheat genomics and grass genome annotation. Plant Journal 49:704–717

Burt C (2013) Identification and characterisation of eyespot resistance in wheat. HGCA Student Report SR 22

Burt C, Nicholson P (2011) Exploiting co-linearity among grass species to map the *Aegilops ventricosa*-derived Pch1 eyespot resistance in wheat and establish its relationship to Pch2. Theor Appl Genet 123 1387-1400

Burt C, Hollins TW, Nicholson P (2011) Identification of a QTL conferring seedling and adult plant resistance to eyespot on chromosome 5A of Cappelle Desprez. Theor Appl Genet 122 119-128

Burt C, Hollins TW, Powell N, Nicholson P (2010) Differential seedling resistance to the eyespot pathogens, *Oculimacula yallundae* and *Oculimacula acuformis*, conferred by Pch2 in wheat and among accessions of *Triticum monococcum*. Plant Pathol 59: 819-828

Chapman NH, Burt C, Dong H, Nicholson P (2008) The development of PCR-based markers for the selection of eyespot resistance genes Pch1 and Pch2. Theor Appl Genet 117:425-433

Chapman NH, Burt C, Nicholson P (2009) The identification of candidate genes associated with Pch2 eyespot resistance in wheat using cDNA-AFLP. Theor Appl Genet 118:1045-1057

Chia T, Adamski NM, Saccomanno B, Greenland A, Nash A, Uauy C, Trafford K (2017) Transfer of a starch phenotype from wild wheat to bread wheat by deletion of a locus controlling B-type starch granule content. J Exp Bot 68:5497-5509

de la Peña RC, Murray TD, Jones SS (1996) Linkage relations among eyespot resistance gene Pch2, endopeptidase Ep-A1b, and RFLP marker Xpsr121 on chromosome 7A of wheat. Plant Breeding 115:273-275

de la Peña RC, Murray TD, Jones SS (1997) Identification of an RFLP interval containing Pch2 on chromosome 7AL in wheat. Genome 40:249-252

Devos KM, Gale MD (2000) Genome relationships: The grass model in current research. Plant Cell 12: 637-646

Dhaliwal A, Mares D, Marshall D (1987) Effect of 1B/1R chromosome translocation on milling and quality characteristics of bread wheats. Cereal Chem 64:72-76

Doussinault G, Delibes A, Sanchezmonge R, Garciaolmedo F (1983) Transfer of a Dominant Gene for Resistance to Eyespot Disease from a Wild Grass to Hexaploid Wheat. Nature 303:698-700

Feuillet C, Keller B (2002) Comparative Genomics in the grass family: Molecular characterization of grass genome structure and evolution. Ann Botany 89: 3-10

Feuillet C, Langridge P, Waugh R (2008) Cereal breeding takes a walk on the wild side. Trends in Genetics 24:24–32

Griffiths S, Sharp R, Foote TN, Bertin I, Wanous M, Reader S, Colas I, Moore G (2006) Molecular characterization of *Ph1* as a major chromosome pairing locus in polyploid wheat. Nature 439: 749–752

Groos C, Bervas E, Charmet G (2004) Genetic analysis of grain protein content, grain hardness and dough rheology in a hard X hard bread wheat progeny. J Cer Sci 40:93-100.

Han F, Kleinhofs A, Ullrich SE, Kilian A, Yano M, Sasaki T (1998) Synteny with rice: analysis of barley malting quality QTLs and rpg4 chromosome regions. Genome 41: 373-380

Hardwick NV, Jones DR, Slough JE (2001) Factors affecting disease of winter wheat in England and Wales 1989-98. Plant Pathol 34 272-283.

Hollins TW, Lockley KD, Blackman JA, Scott PR, Bingham J (1988) Field Performance of Rendezvous, a Wheat Cultivar with Resistance to Eyespot (Pseudocercosporella-Herpotrichoides) Derived from Aegilops-Ventricosa. Plant Pathol 37:251-260

Huguet-Robert V, Dedryver F, Roder MS, Korzun V, Abelard P, Tanguy AM, Jaudeau B, Jahier J (2001) Isolation of a chromosomally engineered durum wheat line carrying the Aegilops ventricosa Pch1 gene for resistance to eyespot. Genome 44:345-349

Knott D (1980) Mutation of a gene for yellow pigment linked to Lr19 in wheat. Can J Genet Cytol 22:651-654

Koebner RMD, Martin PK (1990) Association of Eyespot Resistance in Wheat Cv Cappelle-Desprez with Endopeptidase Profile. Plant Breeding 104:312-317

Koen E, Labuschagne MT, Viljoen CD (2002) The influence of eyespot resistance genes on baking quality and yield in wheat. J Sci Food and Agr 82:1537-1540.

Kwiatek M, Wiśniewska H, Korbas M, Gawłowska M, Belter J, Majka M, Danielewicz J (2016) How do eyespot resistance genes transferred into winter wheat breeding lines affect their yield? J Plant Protect Res 56:319-322

Leonard JM, Watson CJW, Carter AH, Hansen JL, Zemetra RS, Santra DK, Campbell KG, Riera-Lizarazu O (2008) Identification of a candidate gene for the wheat endopeptidase Ep-D1 locus and two other STS markers linked to the eyespot resistance gene Pch1. Theor Appl Genet 116:261-270

Li J, Ji L (2005) Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. Heredity 95:221-227

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods25: 402-8.

Maia N (1967) Obtention de blés tendres résistants au piétin-verse (Cercosporella herpotrichoides) par crôisements interspécifiques blés x Aegilops. Comptes Rendus de l'Academie d'Agriculture de France 53:149–154

Martins-Lopes P, Zhang H, Koebner R (2001) Detection of single nucleotide mutations in wheat using single strand conformation polymorphism gels. Plant Mol Biol Rep 19:159-162

McMillin D, Allan, RE, Roberts, DE (1986) Association of an isozyme locus and strawbreaker foot rot resistance derived from Aegilops ventricosa in wheat. Theor Appl Genet 72:743–747

Mena M, Doussinault, G, Lopez-Brana, I, Aguaded, S, Garcia-Olmedo, E, Delibes, A (1992) Eyespot resistance gene Pch1 in H-93 wheat lines. Evidence of linkage to markers of chromosome group 7 and resolution from the endopeptidase locus Ep-D1b. Theor Appl Genet 83:1044–1047

Meyer N, Lind V, Heindorf M, Korzun V, Friedt W, Ordon F (2011) Diagnostic value of molecular markers linked to the eyespot resistance gene Pch1 in wheat. Euphytica 177:267-275

McHale L, Tan X, Koehl P, Michelmore R.W. (2006) Plant NBS-LRR proteins: adaptable guards. Genome Biology 7: Article number 212

Nicholson P, Lees AK, Maurin N, Parry DW, Rezanoor HN (1996) Development of a PCR assay to identify and quantify Microdochium nivale var nivale and Microdochium nivale var majus in wheat. Physiol Mol Plant P 48:257-271

Niu ZX, Klindworth DL, Friesen TL, Chao SM, Jin Y, Cai XW, Xu SS (2011) Targeted Introgression of a Wheat Stem Rust Resistance Gene by DNA Marker-Assisted Chromosome Engineering. Genetics 187:1011-1021

Pallotta M, Warner, P, Fox, RL, Kuchel, H, Jefferies SJ, Langridge, P (2003) Marker assisted wheat breeding in the southern region of Australia. In: Pogna N, McIntosh, RA (ed) Proceedings of

the Tenth International Wheat Genetics Symposium Istituto Sperimentale per la Cerealicoltura. Paestum, Italy, pp 1-6

Parnell S, Gilligan CA, Lucas JA, Bock CH, van den Bosch F (2008) Changes in fungicide sensitivity and relative species abundance in *Oculimacula yallundae* and *O. acuformis* populations (eyespot disease of cereals) in Western Europe. Plant Pathol 57: 509-517

Ramirez-Gonzalez RH, Uauy C, Caccamo M (2015) PolyMarker: A fast polyploid primer design pipeline. Bioinformatics 31:2038-2039

Riley R, Chapman V, Kimber G (1959) Genetic control of chromosome pairing in intergeneric hybrids with wheat. Nature 183:1244–1246

Santra DK, Watt C, Little L, Kidwell KK, Campbell KG (2006) Comparison of a modified assay method for the endopeptidase marker Ep-D1b with the Sequence Tag Site marker XustSSR2001-7DL for strawbreaker foot rot resistance in wheat. Plant Breeding 125:13-18

Schlegel R, Korzun V (1997) About the origin of 1RS.1BL wheat–rye chromosome translocations from Germany. Plant Breeding 116:537–540

Schneider A, Molnar I, Molnar-Lang M (2008) Utilisation of Aegilops (goatgrass) species to widen the genetic diversity of cultivated wheat. Euphytica 163:1–19

Scott P (1971) The effect of temperature on eyespot (Cercosporella herpotrichoides) in wheat seedlings.. Ann Appl Biol 68:169–175

Simmonds NW (1995) The relation between yield and protein in cereal grain. J Sci Food Agr 67:309-315

Stam P (1993) Construction of Integrated Genetic-Linkage Maps by Means of a New Computer Package - Joinmap. Plant J 3:739-744

Veraverbeke WS, Delcour JA (2002) Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. Crit Rev Food Sci Nutr 42:179-208

Vincent A, Ponchet, J, Koller, J (1952) Recherche de variétés de blés tendres peu sensibles au piétin-verse: résultats préliminaires. Annales de l'Amélioration de Plantes 2:459–472

Vogel JP, Garvin DF, Mockler TC, Schmutz J, Rokhsar D, Bevan MW, Barry K, Lucas S, Harmon-Smith M, Lail K, Tice H, Grimwood J, McKenzie N, Huo NX, Gu YQ, Lazo GR, Anderson OD, You FM, Luo MC, Dvorak J, Wright J, Febrer M, Idziak D, Hasterok R, Lindquist E, Wang M, Fox SE, Priest HD, Filichkin SA, Givan SA, Bryant DW, Chang JH, Wu HY, Wu W, Hsia AP, Schnable PS, Kalyanaraman A, Barbazuk B, Michael TP, Hazen SP, Bragg JN, Laudencia-Chingcuanco D, Weng YQ, Haberer G, Spannagl M, Mayer K, Rattei T, Mitros T, Lee SJ, Rose JKC, Mueller LA, York TL, Wicker T, Buchmann JP, Tanskanen J, Schulman AH, Gundlach H, de Oliveira AC, Maia LD, Belknap W, Jiang N, Lai JS, Zhu LC, Ma JX, Sun C, Pritham E, Salse J, Murat F, Abrouk M,

Bruggmann R, Messing J, Fahlgren N, Sullivan CM, Carrington JC, Chapman EJ, May GD, Zhai JX, Ganssmann M, Gurazada SGR, German M, Meyers BC, Green PJ, Tyler L, Wu JJ, Thomson J, Chen S, Scheller HV, Harholt J, Ulvskov P, Kimbrel JA, Bartley LE, Cao PJ, Jung KH, Sharma MK, Vega-Sanchez M, Ronald P, Dardick CD, De Bodt S, Verelst W, Inze D, Heese M, Schnittger A, Yang XH, Kalluri UC, Tuskan GA, Hua ZH, Vierstra RD, Cui Y, Ouyang SH, Sun QX, Liu ZY, Yilmaz A, Grotewold E, Sibout R, Hematy K, Mouille G, Hofte H, Pelloux J, O'Connor D, Schnable J, Rowe S, Harmon F, Cass CL, Sedbrook JC, Byrne ME, Walsh S, Higgins J, Li PH, Brutnell T, Unver T, Budak H, Belcram H, Charles M, Chalhoub B, Baxter I, Initiative IB (2010) Genome sequencing and analysis of the model grass Brachypodium distachyon. Nature 463:763-768

Wang HY, Yang WX, Zhang LR, Meng QF, Liu DQ, Zhang T (2006) Isolation and Characterization of Resistance Gene Homology Sequence from Wheat. J. Phytopathol 154: 670–675

Wang SC, Wong DB, Forrest K, Allen A, Chao SM, Huang BE, Maccaferri M, Salvi S, Milner SG, Cattivelli L, Mastrangelo AM, Whan A, Stephen S, Barker G, Wieseke R, Plieske J, Lillemo M, Mather D, Appels R, Dolferus R, Brown-Guedira G, Korol A, Akhunova AR, Feuillet C, Salse J, Morgante M, Pozniak C, Luo MC, Dvorak J, Morell M, Dubcovsky J, Ganal M, Tuberosa R, Lawley C, Mikoulitch I, Cavanagh C, Edwards KJ, Hayden M, Akhunov E, Sequencing IWG (2014) Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. Plant Biotechnol J 12:787-796

Winfield MO, Allen AM, Burridge AJ, Barker GL, Benbow HR, Wilkinson PA, Coghill J, Waterfall C, Davassi A, Scopes G (2016) High-density SNP genotyping array for hexaploid wheat and its secondary and tertiary gene pool. Plant Biotechnol J 14:1195-1206

Worland A, Law C, Hollins T, Koebner R, Giura A (1988) Location of a Gene for Resistance to Eyespot (Pseudocercosparella herpotrichoides) on Chromosome 7D of Bread Wheat. Plant Breeding 101:43-51

Worland A, Law C, Li W (1990) The location of genes depressing yield associated with the transfer of eyespot resistance from Aegilops ventricosa. Annual report, AFRC Institute of Plant Science Research, John Innes Institute and Sainsbury Laboratory, 1989 7-8

Wulff BB, Moscou MJ (2014) Strategies for transferring resistance into wheat: from wide crosses to GM cassettes. Front Plant Sci 5:692

Zanke CD, Rodemann B, Ling J, Muqaddasi QH, Plieske J Polle A, Kollers S,·Ebmeyer E Korzun V Argillier O, Stiewe G, Zschäckel T, Ganal MW, Röder MS (2017) Genome-wide association mapping of resistance to eyespot disease (Pseudocercosporella herpotrichoides) in European winter wheat (Triticum aestivum L.) and fine-mapping of Pch1. Theor Appl Genet 130: 505–514

Zhao F, Su Y, Dunham S, Rakszegi M, Bedo Z, McGrath S, Shewry P (2009) Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. J Cereal Sci 49:290-295