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Identification and characterisation of eyespot resistance in wheat

by

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1. ABSTRACT

Eyespot is an economically important disease of the stem base of wheat caused by two species of fungi, *Oculimacula yallundae* and *Oculimacula acuformis*. The aim of this work was to develop genetic tools for plant breeders so that resistances against these pathogen species can be introduced into wheat varieties to provide reliable control of the disease.

This study provided further understanding of the genetic location, function and efficacy of resistances in the variety Cappelle Desprez. The genetic location of *Pch2* resistance from this variety was confirmed on the distal portion of chromosome 7AL and, using information from a previous gene expression study, two candidate genes for involvement in the *Pch2* resistance response were identified. However, this study also revealed that *Pch2* confers a significantly lower resistance against penetration by *O. yallundae* than against *O. acuformis*. As both pathogen species are present in the UK, this suggests that *Pch2* should not be used as a 'stand-alone' resistance in wheat varieties.

In addition, an adult plant resistance on chromosome 5A of Cappelle Desprez was shown to also be effective in seedlings and was shown to provide protection against both *O. yallundae* and *O. acuformis*, suggesting that it could provide reliable protection against the disease in the field. This resistance was mapped on chromosome 5AL in both field trials and seedling bioassays, and the molecular marker *Xgwm639* was identified to be closely associated with the resistance to aid selection by plant breeders.

Finally, this study fine-mapped the potent resistance gene *Pch1,* previously introduced into wheat from the relative *Aegilops ventricosa*. Conserved Orthologous Sequence (COS) markers were targeted to the *Pch1* region of chromosome $7D^{V}$. These were used to identify recombinants in the *Ae. ventricosa* segment and to fine map *Pch1* using Brachypodium and rice as reference sequences. Molecular markers were identified for the efficient selection of the resistance and, furthermore, candidate gene regions in Brachypodium and rice were identified to enable map-based cloning of the resistance gene.

2. INTRODUCTION

2.1. Eyespot disease

Eyespot is an important fungal stem-base disease of winter wheat, barley and rye in temperate regions including north-west Europe, Russia, north-west America, South Africa, southern Australia and New Zealand (Fitt 1992). Eyespot is the most prevalent stem base disease of winter wheat in England and Wales and surveys suggest that its prevalence has increased (Hardwick *et al.* 2001). Chemical control of the disease can be problematic because it requires precise application timings (Burnett and Hughes 2004) and it is not always cost effective (Nicholson and Turner 2000). In addition, resistance has arisen to many of the widely-used chemicals (Parnell *et al.* 2008).

UK national crop surveys (http://www.cropmonitor.co.uk) in the period from 1999–2009 recorded an average of 10.5% of stems with moderate or severe lesions. Although eyespot can often be found on winter cereals in these areas, it is usually only deleterious to yield when severe epidemics occur (Clarkson 1981). It is estimated that this amount of infection would result in an average yield loss in the UK of 130,000 tonnes per annum.

2.2. Symptoms

Eyespot causes characteristic oval brown-bordered lesions at the base of cereal stems. Severe eyespot lesions can weaken stem bases and cause them to bend or break. If severe eyespot lesions are widespread in a crop, lodging is likely to occur, resulting in yield losses of up to 50%. Less severe lesions may affect water and nutrient uptake resulting in shrivelled ears, or 'whiteheads,' again resulting in yield loss (Scott and Hollins 1974).

2.3. Causal organisms

The two species of fungus that cause eyespot disease in cereals are *Oculimacula yallundae* and *Oculimacula acuformis.* These were previously classified as one species, *Pseudocercosporella herpotrichoides* (Deighton 1973). However, the fungus was discovered to have two pathotypes, W-type and R-type, on the basis of differential pathogenicity to wheat and rye (Lange-de la Camp 1966). Subsequently, the W-type and R-type forms were found to form two separate breeding groups, leading to their re-classification as two distinct species, *Tapesia yallundae* and *Tapesia acuformis*. More recently the species were further re-classified as *O. yallundae* (Figure 1a) and *O. acuformis* (Figure 1b), by Crous *et al.* (2003).

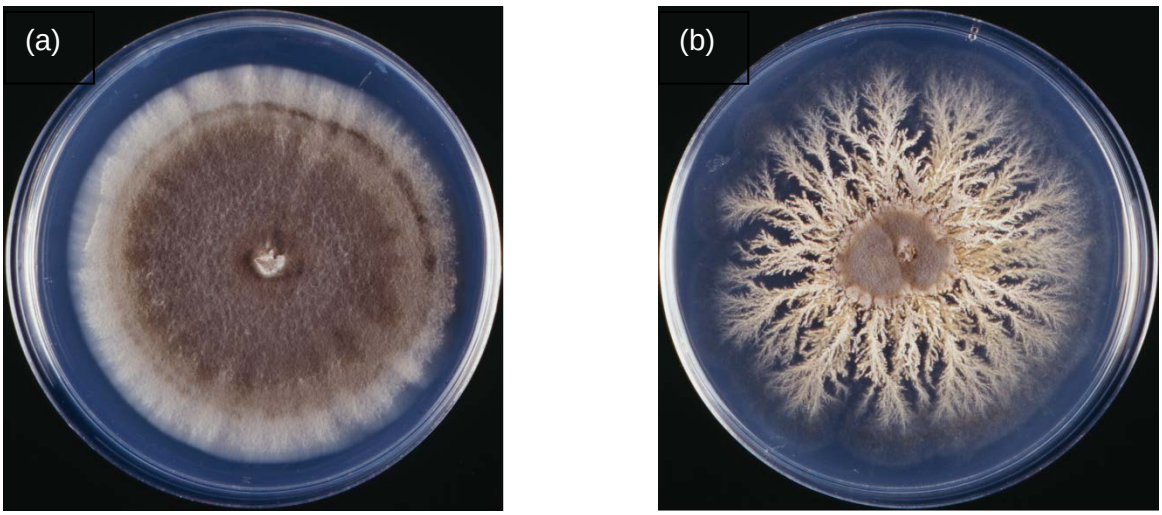

Figure 1. Isolates of (a) *Oculimacula yallundae* and (b) *Oculimacula acuformis* growing on potato dextrose agar.

2.4. Genetic resistance to eyespot

The use of genetic resistance in crops provides a method of disease control that is agriculturally, economically and environmentally desirable. Using resistant cultivars enables the grower to pay a fixed cost for disease control, imposes no great constraints on methods for growing the crop and decreases the need for chemical applications that may affect non-target organisms.

2.4.1. *Pch1* resistance

A potent seedling resistance was found in the wild goat grass, *Aegilops ventricosa* (Sprague 1936), and subsequently transferred to a hexaploid wheat line (Maia 1967) as part of a chromosome segment on chromosome 7D. The resistance gene *Pch1* was identified as a major dominant gene on this segment on the long arm of chromosome 7D (Worland *et al.* 1988) and has been widely used as a source of resistance in attempts to produce eyespot resistant cultivars. Although *Pch1* is highly effective against both eyespot species, loss in grain yield can still occur when eyespot disease pressure is severe (Hollins *et al.* 1988).

The use of *Pch1* in commercial cultivars in the UK has been limited as the resistance gene was transferred to wheat from *Ae. ventricosa* as part of a segment of chromosome along with agronomically undesirable genes from the wild grass. Hence a significant yield deficit may sometimes be observed in the absence of the disease (Koen *et al.* 2002). This association has proven difficult to break because of limited recombination of the translocated *Ae. ventricosa* segment (Johnson 1992). Early *Pch1* varieties, such as Rendezvous, were not widely used in the UK as a result of their relatively low yield in the absence of the disease. However, a number of *Pch1* carrying varieties have been released more recently, such as Battalion, that do not appear to suffer from this reduced yield potential (HGCA 2010). It is not clear if this is due to a reduced

Ae. ventricosa segment in which the linkage has been broken, or to background yield promoting effects from elsewhere in the genome. In contrast to the situation in the UK, *Pch1* has been widely used in cultivars in the US Pacific northwest where there is a high disease pressure (Leonard *et al.* 2008). Furthermore, there is evidence that the *Ae. ventricosa* introgression may also confer a higher grain protein content resulting in enhanced bread making ability (Groos *et al.* 2004).

2.4.2. Cappelle Desprez resistance

The first source of genetic resistance to be widely used against eyespot was from the French variety Cappelle Desprez (Vincent *et al.* 1952). Cappelle Desprez was widely grown in the UK between 1953 and 1976 partly due to its durable resistance. This resistance was attributed mainly to a gene located on chromosome 7A (Law *et al.* 1976) and termed *Pch2* (de la Peña *et al.* 1996). *Pch2* confers a moderate durable resistance and is adequate where disease pressures are not too high (Scott *et al.* 1989). However, the use of *Pch2* in current commercial cultivars is limited as the level of resistance conferred is not sufficient to protect the crop under high disease pressure and to obviate the need for fungicidal control (Johnson 1992).

The resistance observed in Cappelle Desprez appears to be quite complex and may involve additional genes on chromosomes other than 7A. In particular, a major gene for eyespot resistance, expressed in adult plants, has been identified on chromosome of 5A of Cappelle Desprez (Muranty *et al.* 2002). A more precise understanding of the resistance factor(s) on 5A including its chromosomal location, mode of action, time of effectiveness and comparative efficacy against *O. yallundae* and *O. acuformis* is required. The development of tightly linked PCR-based markers would enable its use in breeding programmes to provide adult-plant stage resistance to eyespot.

2.5. Molecular markers for eyespot resistances

Phenotyping breeding lines for eyespot resistance in cultivar development programmes involves replicated seedling bioassays and field trials. This involves significant costs in terms of controlled environment room, glasshouse and field trial facilities. Conducting large-scale eyespot phenotyping trials is also highly labour intensive. Furthermore, there is a high level environmental variance often associated with seedling bioassays and field trials of eyespot (de la Peña *et al.* 1996; Lucas *et al.* 2000) and this can lead to inaccuracies when determing the level of eyespot resistance within wheat breeding lines or varieties. Therefore, the development of molecular markers for eyespot resistance genes should provide greater efficiency and accuracy when selecting for eyespot resistance within plant breeding programmes and result in higher levels of resistance within wheat varieties in the UK.

2.5.1. Molecular markers for *Pch1* resistance

Developing recombinants and markers to determine the genetic locations of factors influencing yield and protein content relative to *Pch1* would be beneficial in the development of high yielding, high quality and eyespot resistant wheat varieties. Molecular markers have been developed for the mapping and marker assisted selection of *Pch1.* A tight linkage has been detected between an endopeptidase isozyme marker *Ep-D1b* and the *Pch1* gene, positioning the gene at the distal end of the long arm of chromosome 7D (McMillin *et al.* 1986; Worland *et al.* 1988). Isozyme methods can be technically challenging and time-consuming, whilst in comparison PCR markers are technically straightforward and provide rapid results. Consequently there have been attempts to develop PCR markers that are closely linked to *Pch1*. For example, the tight linkage observed between *Ep-D1b* led to its conversion to a PCR-based marker *Orw1* (Leonard *et al.* 2008). However, it has proven difficult to identify co-dominant markers that function in both wheat and *Ae. ventricosa* due to differences in their non-genic regions (Chapman 2005).

Further markers to the distal end of long arm of chromosome 7D are required for the fine mapping of *Pch1.* Markers for alien introgressions in wheat and other cereals could be generated through the generation of cross-species transferable Conserved Orthologous Sequence (COS) markers. This involves designing primers to genes that are highly conserved between the fully sequenced reference genomes of rice, *Brachypodium* and available sequence from wheat or other cereals (Bertin *et al.* 2005).

2.5.2. Molecular markers for *Pch2* resistance

There have also been attempts to find suitable molecular markers for *Pch2* to facilitate marker-assisted selection. Recently, *Pch*2 resistance to *O. acuformis* has been mapped as a QTL in a 7.5 cM region in between SSR markers *Xwmc346* and *Xcfa2040* at the distal end of the long arm of chromosome 7A (Chapman 2005). There is a requirement for further mapping studies in order to refine the position of *Pch2* and to determine whether there are any differences in the genetics of resistance to *O. yallundae* and *O. acuformis* on chromosome 7A. This would enable the development of closely linked PCR markers suitable for marker-assisted selection of eyespot resistance in breeding programmes.

2.6. Overall objectives

The ability to combine multiple eyespot resistance genes through marker assisted selection could enable the production of cultivars with durable resistance to both species of eyespot at all stages of plant growth and hence reduce the requirement for fungicide inputs.

The objectives of this study are:

i) To confirm the genetic location of *Pch2* and to develop gene-based markers for its selection.

ii) To further characterise the resistance conferred by *Pch2* and to determine its level of efficacy against *O. yallundae* and *O. acuformis* separately.

iii) To determine the efficacy and genetic location of the Cappelle Desprez chromosome 5A resistance.

iv) To identify recombinants in the *Ae. ventricosa* segment containing *Pch1* and to fine map the resistance by exploiting synteny between wheat and Brachypodium.

3. DEVELOPMENT OF PCR-BASED MARKERS ASSOCIATED WITH *PCH2* EYESPOT RESISTANCE

To identify further markers on chromosome 7A and to identify candidate genes for involvement in the *Pch2* resistance, a previous study used a cDNA-AFLP gene analysis to identify differences in gene expression between the eyespot susceptible wheat cultivar Chinese Spring (CS) and the resistant chromosome substitution line Chinese Spring / Cappelle Desprez 7A (CS/CD7A) containing *Pch2*. 29 differentially expressed fragments were cloned and sequenced. The function of these sequences was then determined by comparison to databases of plant genes (Chapman 2005). This study aimed to develop genetic markers on chromosome 7A using these sequences to provide additional markers for the mapping of *Pch2* to confirm its location on chromosome 7A and to identify if any of the cDNA-AFLP sequences map to the *Pch2* region and can be considered candidate genes for the resistance.

3.1. Locating SSR markers and cDNA-AFLP fragments

Previously the CS x CS/CD7A F_2 population has been used to generate an SSR map of chromosome 7A and to locate *Pch2* resistance to the distal end of the long arm of the chromosome (Chapman 2005). In this study fourteen of the cDNA-AFLP fragments were found to originate from chromosome 7A. The genetic location of these fourteen 7A fragments was more precisely determined using CS deletion bin lines that are missing sections of chromosome 7A, or where polymorphisms existed by genetic mapping using the CS x CS/CD7A F_2 population alongside the existing SSR map of chromosome 7A.

One sequence (*X19CD7A4*) was located at the distal end of chromosome 7A in the region of *Pch2* by genetic mapping using the population and two sequences (*X4CD7A8* and *X33CD7A8*) were located in the distal deletion bin of chromosome 7AL, in approximately the same region as *Pch2* (Figure 2). Therefore, the genes relating to these sequences can be considered as candidates for *Pch2*.

3.2. Locating SSR genetic markers

Existing SSR genetic markers that have previously been associated with *Pch2* were physically mapped to integrate the physical map developed using deletion bins and the genetic map developed using the population. *Xwmc525* was found to be located in the 7AL deletion bin (0.897AL20-0.997AL15), whilst *Xcfa2040* was located in the immediately distal, and terminal, deletion bin (0.99 7AL15) (Figure 2). Therefore it appears likely that *Pch2* is positioned around the 0.99 bin breakpoint, possibly in the region within the terminal bin.

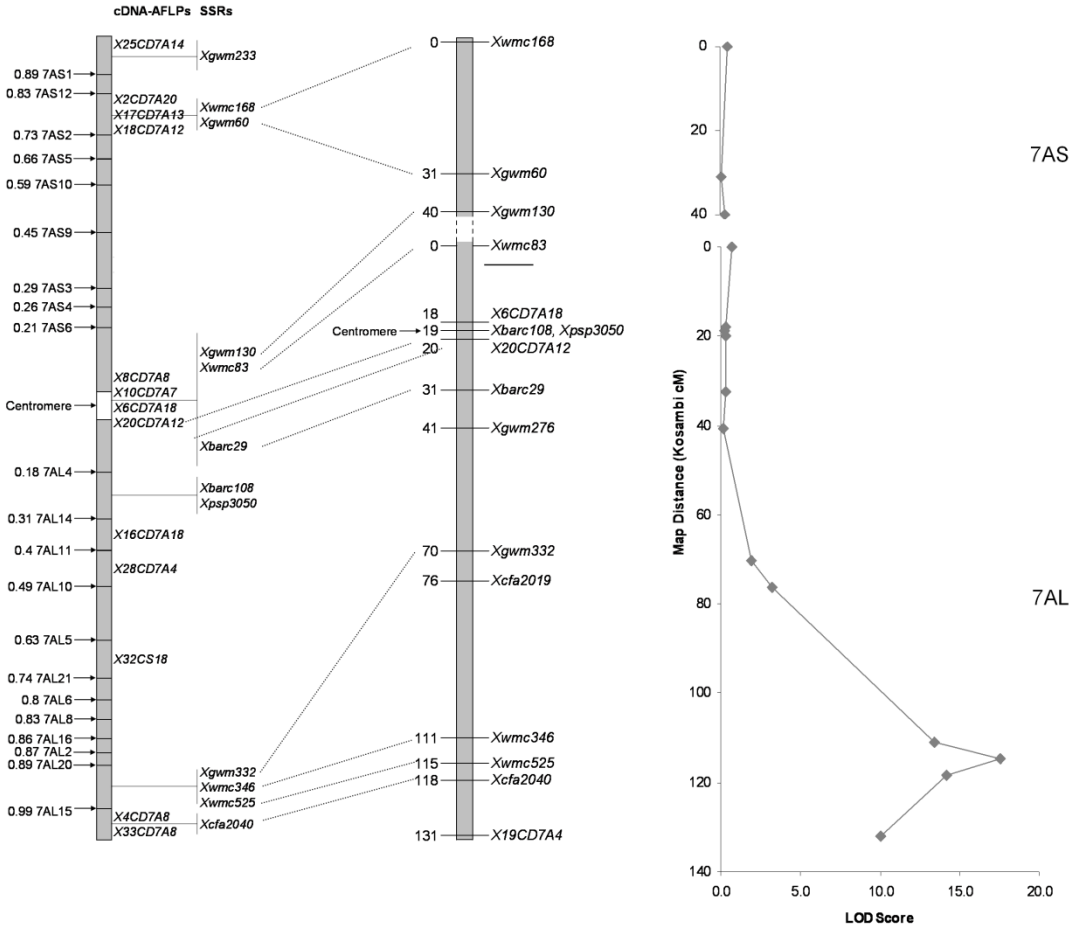

Figure 2. A comparison of integrated SSR and cDNA-AFLP physical and genetic maps of chromosome 7A. A LOD profile from the QTL analysis of *Pch2* on chromosome 7A is also shown aligned to the genetic map to demonstrate to position of *Pch2.*

3.3. Locating and characterising *Pch2* resistance using QTL analysis

186 F_3 families developed from the CS x CS/CD7A population were phenotyped for resistance to *O. acuformis* in a controlled environment room (CER) bioassay by Chapman (2005); and in a glasshouse seedling bioassay in the present study. The results of both experiments were in agreement (individual experiment data not shown) and were combined for a quantitative trait loci (QTL) analysis in which phenotypic data was compared to the genetic mapping data to determine the likelihood that each marker is associated with the *Pch2* resistance. This experiment positioned *Pch2* to a 7 cM region between the SSR markers *Xgwm346* and *Xcfa2040* and has identified the SSR marker *Xwmc525* to be tightly linked to the resistance (Figure 2).

3.4. Candidate genes for *Pch2*

Sequence 4CD7A8 is particularly interesting as a candidate for *Pch2* as in addition to its chromosomal location, it showed homology to an *Oryza sativa* putative callose synthase protein. Callose synthase is produced in response to wounding and as a defence response to pathogen attack (Østergaard *et al.* 2002). The second marker that physically mapped into the terminal deletion bin in the region of *Pch2* (*X33CD7A8*) showed homology with a gene encoding a protein involved in photosynthesis, suggesting it is unlikely to be involved directly with resistance.

Genetic mapping revealed that *X19CD7A4* is located 16cM distal of the SSR marker *Xwmc525* and is therefore relatively close to the proposed location of *Pch2*. The sequence for 19CD7A4 has been shown to be similar to a gene encoding a plant disease resistance protein. Therefore, this gene may contribute towards *Pch2* resistance, potentially by pathogen recognition. This could be investigated in future studies by identifying additional markers to enhance the genetic map along with the development, identification and disease testing of more recombinant lines.

To summarise, this study has confirmed the location of *Pch2* and confirmed the SSR marker *Xwmc525* to be tightly linked to the resistance. It is anticipated that this marker will facilitate marker assisted selection of *Pch2* in plant breeding programmes. Further insight into the *Pch2* resistance response has been obtained by determining the genetic location of cDNA-AFLP fragments. Further study of these will aid future attempts to fine-map the *Pch2* resistance so that it can be used effectively by plant breeders.

4. DIFFERENTIAL SEEDLING RESISTANCE TO THE EYESPOT PATHOGENS CONFERRED BY *PCH2*

In order to provide effective eyespot control it is important that any genetic sources of resistance are effective against both forms of the pathogen, *Oculimacula yallundae* and *Oculimacula acuformis*. Therefore, this study aimed to determine the level of efficacy of *Pch1* and *Pch2* to *O. yallundae* and *O. acuformis* separately.

4.1. Controlled environment seedling bioassays of wheat varieties

Wheat varieties known to have different susceptibilities to eyespot were tested for resistance to *O. yallundae* and *O. acuformis* independently using seedling bioassays in controlled environment rooms. Andante, Lynx and Rendezvous are highly resistant and were found to be likely to contain the resistance gene *Pch1* after testing with linked SSR markers. Cappelle Desprez, Riband and Hobbit 'sib' are moderately resistant and were found to contain the *Pch2* resistance. Holdfast, Talon and Chinese Spring are susceptible and were found to have either *Pch1* or *Pch2* after marker testing (Table 1).

Table 1. Mean disease scores for wheat lines when inoculated with *Oculimacula acuformis* (OA) and with *Oculimacula yallundae* (OY).

Wheat Variety	Resistance Genes	OA	OY	t-prob*
Andante	*Pch1*	3.0	2.9	0.473
HS/VPM7D	*Pch1* and *Pch2*	3.1	3.2	0.246
Lynx	*Pch1* and *Pch2*	3.4	3.2	0.185
Rendezvous	*Pch1* and *Pch2*	3.2	3.1	0.568
Cappelle Desprez	*Pch2*	3.9	4.6	<0.001
Riband	*Pch2*	4.0	4.9	<0.001
Holdfast	None	5.3	6.0	<0.001
Talon	None	5.1	4.7	0.002
Chinese Spring	None	5.8	5.5[c]	0.104

* The statistical significance of the difference between OA and OY disease scores for each line are shown by t-probabilities.

These results demonstrate that those wheat lines containing *Pch2* had significantly higher levels of disease when inoculated with *O. yallundae* than with *O. acuformis*. This suggests that *Pch2* confers a lower level of resistance to *O. yallundae* than to *O. acuformis.* However, it is also possible that other as yet uncharacterised genes may be influencing resistance. An additional resistance to eyespot has previously been identified on chromosome 5A of Cappelle Desprez (Muranty *et al.* 2002) and it is possible that this may account for a higher level of resistance

towards *O. yallundae* in this variety than in Riband. In contrast, lines containing *Pch1*, or *Pch1* in combination with *Pch2*, demonstrate similarly high levels of resistance to both pathogen species.

4.2. Controlled environment and glasshouse seedling bioassays of wheat varieties of wheat single chromosome substitution lines

The precise wheat single chromosome substitution lines Hobbit 'sib'-VPM7D (HS/VPM7D) containing the resistance genes *Pch1* and *Pch2* (Worland *et al.* 1988) and Chinese Spring-Cappelle Desprez 7A (CS/CD7A) containing *Pch2* (Law *et al.* 1976) were tested for resistance to *O. yallundae* and *O. acuformis* in controlled environments and in glasshouses, alongside their recurrent parents Hobbit 'sib' and Chinese Spring (Table 2). Two CER experiments were conducted at JIC, Norwich and three glasshouse trials; two at the JIC, and one at RAGT Seeds Ltd., Cambridge.

These experiments on precise genetic stocks, rather than cultivars with unknown genetic backgrounds, demonstrated that lines containing *Pch2* as a sole eyespot resistance had significantly lower levels of resistance against *O. yallundae* compared to *O. acuformis*, in agreement with the previous results on wheat varieties. In comparison, no reduction in resistance to *O. yallundae* was observed in HS/VPM7D combining *Pch1* and *Pch2*, suggesting that the potent effect of *Pch1* is sufficient to mask the differential resistance conferred by *Pch2*.

Table 2. Mean disease scores for the inter-varietal substitution lines when inoculated with *Oculimacula acuformis* (OA) and with *Oculimacula yallundae* (OY).

Line	Resistance Genes	CER			Glasshouse			Overall		
		OA	OY	t-prob*	OA	OY	t-prob*	OA	OY	t-prob*
HS/VPM7D	*Pch1* and *Pch2*	2.9	2.7	0.433	4.4	5.0	0.030	3.6	3.9	0.117
Hobbit-Sib	*Pch2*	5.3	5.8	<0.001	6.5	7.6	<0.001	5.8	6.7	<0.001
CS/CD7A	*Pch2*	5.2	6.4	<0.001	6.4	7.6	<0.001	5.8	7.0	<0.001
Chinese Spring	None	7.3	6.9	0.163	8.3	8.3	0.889	7.8	7.7	0.3

* The statistical significance of the differences between OA and OY disease scores are shown by t-probabilities.

4.3. QTL analysis of CSxCS/CD7A resistance to *O. yallundae* and *O. acuformis*

186 F_3 families developed from the CS x CS/CD7A population, were phenotyped for resistance to *O. yallundae* in controlled environment room (CER) and glasshouse seedling bioassays.

Predicted mean disease scores for *O. yallundae* inoculations of each F_3 family from CER and glasshouse trials were used alongside the map of chromosome 7A generated from SSR and cDNA-AFLP marker data (section 2) for QTL mapping of *O. yallundae* resistance. Existing datasets of predicted mean disease scores for *O. acuformis* inoculations of each F_3 family from CER (Chapman 2005) and glasshouse trials from the present study, were used to finalize the location of the previously identified *Pch2* QTL for *O. acuformis* resistance.

The location of the *O. acuformis* resistance QTL was confirmed to be centred on SSR marker Wmc525 (Figure 3). However, it was not possible to detect any significant QTL for resistance to *O. yallundae* on chromosome 7A in either the CER or glasshouse trials.

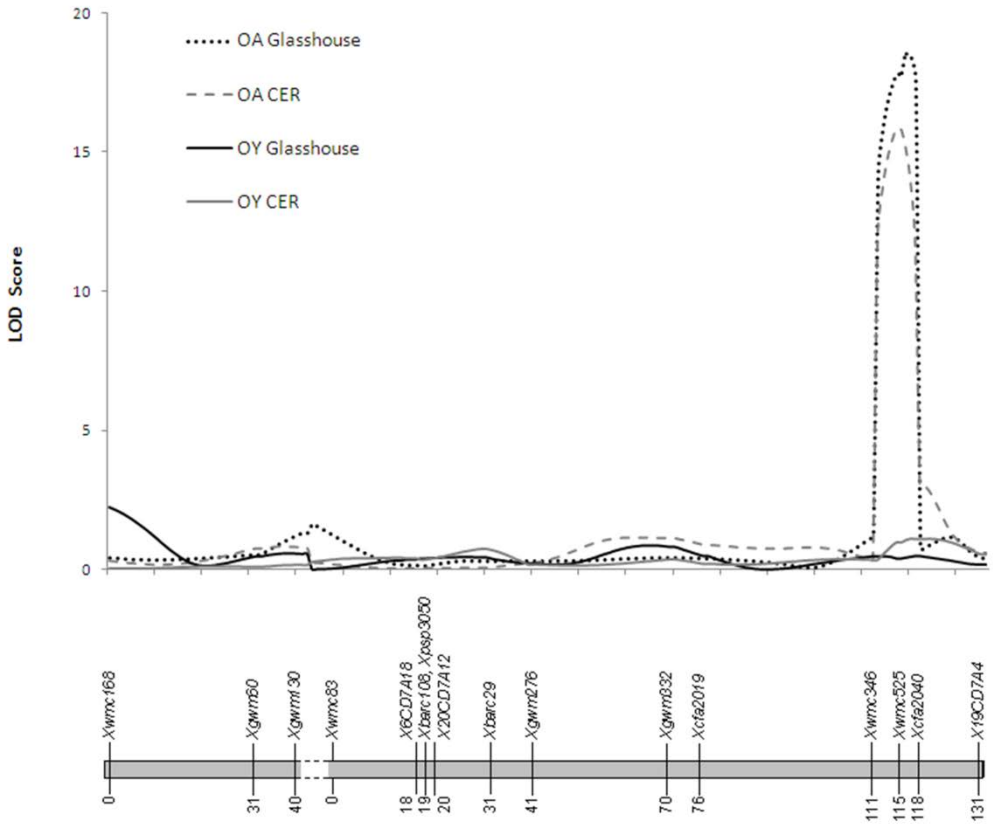

Figure 3. A comparison of QTL mapping analyses of *Pch2* resistance to *Oculimcula acuformis* and *Oculimacula yallundae*, in CER and glasshouse trials of the wheat Chinese Spring x Chinese Spring-Cappelle Desprez 7A F_3 families.

The results from the QTL analysis provide evidence, alongside the results of seedling bioassays of wheat cultivars and single chromosome substitution lines, that *Pch2* confers relatively little resistance to *O*. *yallundae* at the seedling stage. This has important implications for the use of *Pch2* in commercial varieties as it is necessary to have genes that confer resistance to both pathogens for effective eyespot control, as both species are present in the UK. Although, it is not recommended that *Pch2* is deployed as a stand-alone resistance in wheat varieties, it may still be beneficial when combined with other resistances.

5. 'ALL-ROUND' EYESPOT RESISTANCE ON CHROMOSOME 5A OF CAPPELLE DESPREZ

There are only two sources of resistance that have been characterised and are known to be widely used in commercial wheat varieties: *Pch1* and *Pch2*. However, there are drawbacks associated with both resistances; *Pch1* is linked to deleterious traits carried on the *Ae. ventricosa* introgression and *Pch2* has been shown to have limited effectiveness (section 3). An additional resistance has been reported on chromosome 5A of Cappelle Desprez (CD) that confers resistance to eyespot in adult plants (Muranty *et al.*, 2002).

The aim of the present study was to characterise the adult plant eyespot resistance previously identified on chromosome 5A of Cappelle Desprez, to determine whether its presence could be detected at the seedling stage, and to identify whether it confers resistance towards both *O. yallundae* and *O. acuformis*. This study also sought to identify the genetic location of the resistance at both the seedling and adult plant stages and, furthermore, to identify SSR markers suitable for marker-assisted selection of the resistance.

5.1. Inter-varietal single chromosome substitution line experiments

Seedling bioassays were conducted to determine the relative effectiveness of *Pch2* and 5A resistances at the seedling stage using wheat lines Chinese Spring (CS), CS/CD7A, CS/CD5A, CD, CD/Bez 5A, and Bezostaya (Figure 4).

The substitution line CS/CD5A exhibited a significantly lower mean disease score than CS ($P<0.001$) when inoculated either with *O. yallundae* or *O. acuformis* (Figure 4a), demonstrating that chromosome 5A of CD confers resistance at the seedling stage when operating in a susceptible background.

The substitution line CD/Bez5A exhibited a mean disease score that was significantly higher than CD ($P<0.001$) when inoculated with either *O. yallundae* or *O. acuformis* (Figure 4b). This demonstrates that chromosome 5A provides an important component of the eyespot seedling

resistance observed in CD, because when chromosome 5A from CD was replaced by chromosome 5A from the susceptible line Bezostaya, the level of resistance conferred to both pathogens was significantly reduced.

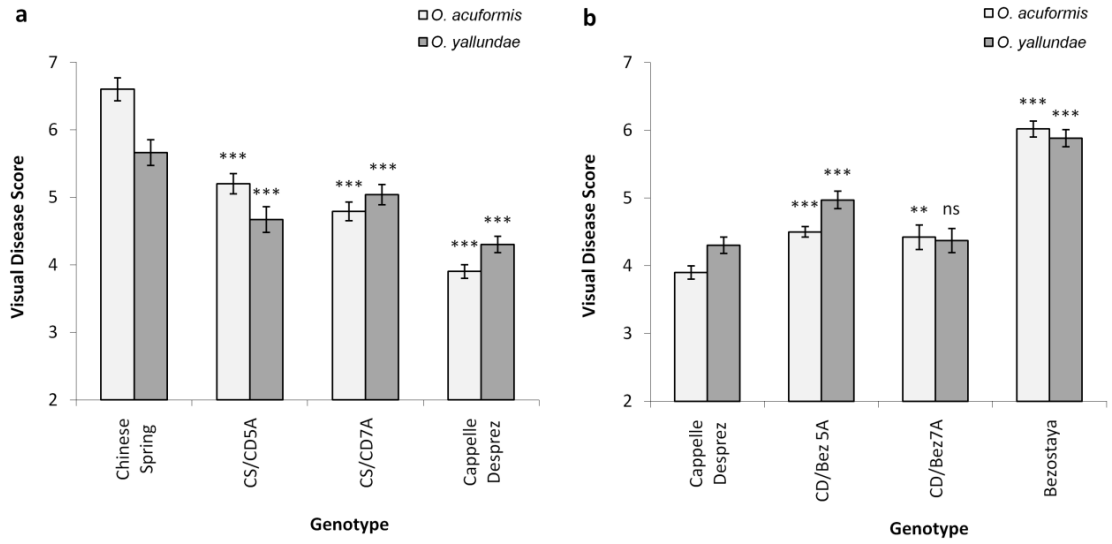

Figure 4. Predicted mean disease scores for (a) Chinese Spring – Cappelle Desprez substitution lines and (b) Cappelle Desprez – Bezostaya substitution lines when inoculated with *Oculimacula yallundae* and *Oculimacula acuformis* in seedling bioassays. Error bars are all ± standard error of the mean. Mean disease scores are compared to Chinese Spring in 1a, and to Cappelle Desprez in 1b, using t-probabilities: ns = non-significant, * P<0.05, ** P<0.01, ***P<0.001.

5.2. Mapping chromosome 5A using SSR markers

Two chromosome 5A recombinant populations were used to determine the genetic location of the eyespot resistance on chromosome 5A of CD. These were a population of 88 recombinant inbred lines (RILs) previously generated from the cross CD x CD/Bez5A by Tony Worland at the Plant Breeding Institute, Cambridge, and a population of 147 RILs generated from the cross CS x CS/CD5A.

The parent lines of the two populations, CS, CS/CD5A, CD and CD/Bez5A, were screened with 47 publically available SSR markers, reported to be located on chromosome 5A, to identify markers which were polymorphic in either or both populations. These markers were applied to the populations and linkage maps were calculated. The markers were resolved into identical orders in both populations and it was possible to compare QTL locations in the two populations directly (Figure 5).

5.3. Locating adult plant resistance on chromosome 5A

To locate the 5A resistance at the adult plant stage, 88 RILs from the population CD x CD/Bez5A were grown in two independent field trials at RAGT Seeds, Cambridge, UK and at JIC, Norwich, UK. Genetic and phenotypic data were combined in a QTL analysis to detect a QTL for eyespot resistance at the adult plant stage on the long arm of chromosome 5A, centred on the SSR marker *Xgwm639* (Figure 5), in both the JIC and RAGT field trials. It was not possible to conduct a field trial to map adult plant resistance in the CS x CS/CD5A population because of the very poor agronomic performance and growth habit of Chinese Spring-based materials in the field.

5.4. Locating seedling resistance on chromosome 5A

To identify the genetic location of the resistance at the seedling stage, 88 RILs from the CD x CD/Bez5A population were phenotyped for resistance to *O. yallundae* and 147 RILs from the CS x CS/CD5A population were phenotyped for seedling resistance to *O. acuformis* in seedling bioassays.

The seedling bioassay of CD x CD/Bez5A, identified a QTL for seedling resistance to *O. yallundae*, which was most significantly associated with the SSR marker *Xbarc197* (Table 3). Although this QTL is centred on a different SSR marker to that identified in the field trials, *Xbarc197* is only 1 cM proximal to *Xgwm639*, and the QTL regions overlap (Figure 5). The seedling bioassay of the CS x CS/CD5A population also identified a single major QTL for resistance to *O. acuformis* in the same location (Table 3), again centred on marker *Xgwm639*.

The QTL location of resistance to both pathogen species at the seedling stage was confirmed in a verification experiment. Seedling bioassays were conducted on sub-sets of 13 lines from the CD x CD/Bez5A population and 28 lines from the CS x CS/CD5A population. These lines were selected on the basis of recombination around the detected QTL. This experiment confirmed *Xgwm639* to be highly associated with seedling resistance to both pathogen species in both populations.

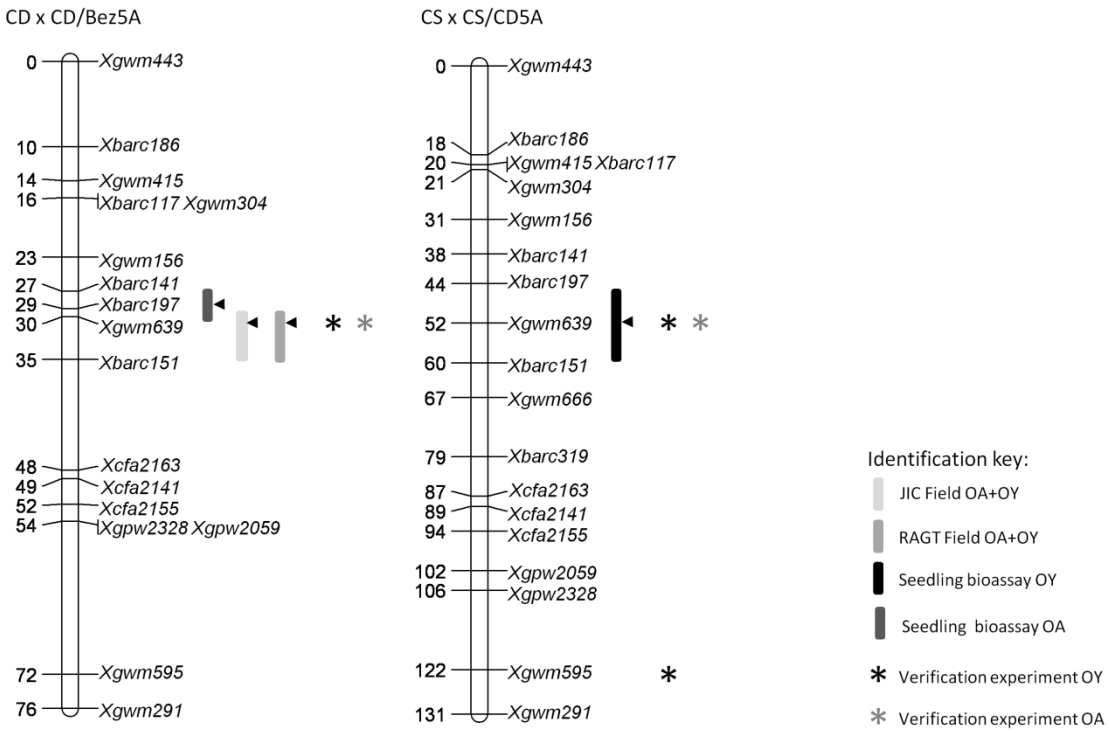

Figure 5. Genetic maps of chromosome 5A in CD x CD/Bez5A and CS x CS x CS/CD5A populations. QTL positions for resistance to eyespot are shown to the right of the genetic maps by bars and arrows indicate the location of the QTL peak. Asterisks indicate markers with significant associations (P<0.05) with resistance to *Oculimacula* in the verification experiments.

Table 3. QTL identified in CS x CS/CD5A and CD x CD/Bez5A populations.

Population	Test	Pathogen	Closest Marker	Map Position	LOD Threshold	LOD	R^2
CS x CS/CD5A	Seedling	OA	*Xgwm639*	52	2.0	10.62	33.9
CD x CD/Bez5A	Seedling	OY	*Xbarc197*	29	1.9	4.62	23.9
CD x CD/Bez5A	Field JIC	OA+OY	*Xgwm639*	30	1.8	4.72	23.2
CD x CD/Bez5A	Field RAGT	OA+OY	*Xgwm639*	30	1.8	4.83	23.5

OA, *Oculimacula acuformis;* OY, *Oculimacula yallundae*; LOD, Logarithm of the odds ratio; R^2, % phenotypic variance explained.

In conclusion, we identified a single major QTL, termed *QPch.jic-5A*, on the long arm of chromosome 5A conferring resistance to both *O. yallundae* and *O. acuformis* at both the seedling and adult plant stages. We have also identified *Xgwm639* as a closely linked SSR marker that can be used for the marker assisted selection of the resistance. The effect of this resistance requires validation in commercial varieties, but could provide an additional useful resistance gene that can be utilised by plant breeders.

6. FINE MAPPING *PCH1*

Introgressions into wheat from related species have been widely used as a source of agronomically beneficial traits. An example is the introduction of the potent eyespot resistance gene *Pch1* from the wild relative *Aegilops ventricosa* onto chromosome 7DL of wheat. The potency of *Pch1* to both eyespot species in a range of genetic backgrounds combined with its widespread use in areas of high eyespot pressure such as the pacific north-west USA clearly demonstrates the agricultural value of the gene. However, in common with genes carried on many other such introgressions, the use of *Pch1* in commercial wheat varieties has been limited by linkage drag with yield limiting traits.

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 aim to apply species transferable conserved orthologous sequence (COS) markers to the *Ae. ventricosa* alien introgression to identify recombinants, to utilise co-linear regions in the sequenced Brachypodium and rice genomes identified through gene-based COS markers, to fine-map the gene and to identify candidate gene regions.

6.1. Development and identification of genetic markers

Using Brachypodium sequence as a genetic model for the *Pch1* region in wheat five COS markers previously developed by Dr. Simon Griffiths at the John Innes Centre were found to be polymorphic between Hobbit ‘sib’ (HS) and the VPM recombinant line RVPM25, containing a small *Ae. ventricosa* segment with the *Pch1* resistance on the distal portion of chromosome 7D. Using the Brachypodium sequence as a reference two further COS markers were developed in the region and the cDNA-AFLP derived marker *4CD7A8* (section 2) was also found to function on chromosome 7D and included in the COS marker panel.

This marker panel was supplemented by the markers *Orw1* and *Orw5*, developed in the *Pch1* region by Leonard *et al.* (2008) which were also found to be polymorphic between HS and RVPM25. In addition to the COS markers, a panel of 10 publically available chromosome 7D SSR markers were identified that were polymorphic between HS and the single chromosome substitution line HS/VPM7D, which has an *Ae. ventricosa* introgression along the length of chromosome 7D.

6.2. Screening wheat cultivars to determine *Ae. ventricosa* introgression sizes

A panel of 23 European wheat varieties, consisting of 21 commercial varieties and 2 breeding lines all believed to carry *Pch1*, were obtained from RAGT Seeds Ltd and the John Innes Centre (JIC) wheat collection. These were screened with the 10 SSR markers to identify recombination in their *Ae. ventricosa* introgressions. A range of recombination events were detected in the varietal panel, including recombination at the distal end of 7DL in the lines Hermann, Striker and RAGT 2 (Table 4). These distal recombinant varieties were included in further analysis using the full set of polymorphic COS and STS markers and were included in a seedling bioassay to determine the presence or absence of *Pch1*. This suggested that all three varieties contain *Pch1* with a recombination event immediately proximal to the gene. Such wheat lines with small *Ae. ventricosa* introgressions in commercial backgrounds would provide suitable breeding material to provide *Pch1* with less linkage drag with deleterious traits.

Table 4. SSR haplotypes of wheat varieties and breeding lines along chromosome 7D.

Line	*Xwmc221*	*Xbarc121*	*Xbarc111*	*Xbarc53*	*Xgwm428*	*XustSSR2001*	*Xbarc76*	*Xwg7s*	*Xbarc97*	*Xwmc14*	*Xtr40*	*Xcfd175*
HS	A	A	A	A	A	A	A	A	A	A	A	A
HS/VPM7D	B	B	B	B	B	B	B	B	B	B	B	B
RVPM25	A	A	A	B	B	B	B	B	B	B	B	B
Grafton	A	A	B	B	B	B	B	B	B	B	B	B
Hyperion	A	A	B	B	B	B	B	B	B	B	B	B
Phare	A	A	B	B	B	B	B	B	B	B	B	B
Rendezvous	A	A	B	B	B	B	B	B	B	B	B	B
Amundsen	A	A	A	B	B	B	B	B	B	B	B	B
Boregar	A	A	A	B	B	B	B	B	B	B	B	B
Buenno	A	A	A	B	B	B	B	B	B	B	B	B
Galactic	A	A	A	B	B	B	B	B	B	B	B	B
Renan	A	A	A	B	B	B	B	B	B	B	B	B
Sorrial	A	A	A	B	B	B	B	B	B	B	B	B
Azimut	A	A	A	A	B	B	B	B	B	B	B	B
Aardvark	A	A	A	A	A	B	B	B	B	B	B	B
Battalion	A	A	A	A	A	B	B	B	B	B	B	B
Iridium	A	A	A	A	A	B	B	B	B	B	B	B
Marksman	A	A	A	A	A	B	B	B	B	B	B	B
Sankara	A	A	A	A	A	B	B	B	B	B	B	B
RAGT 1	A	A	A	A	A	A	B	B	B	B	B	B

Cardos	A	A	A	A	A	A	A	B	B	B	B	B
Ochre	A	A	A	A	A	A	A	B	B	B	B	B
Tuerkis	A	A	A	A	A	A	A	B	B	B	B	B
Hermann	A	A	A	A	A	A	A	A	A	A	B	B
RAGT 2	A	A	A	A	A	A	A	A	A	A	B	B
Striker	A	A	A	A	A	A	A	A	A	A	B	B

A = wheat

B = *Aegilops ventricosa*

6.3. Mapping *Pch1*

The recombinant substitution line RVPM25 was further backcrossed to HS and a recombinant population of 376 F_2 plants was generated. This population segregates for HS and *Ae. ventricosa* over the distal portion of chromosome 7D in a susceptible HS background, and was used to identify novel recombinants in the *Pch1* region.

The seven polymorphic COS markers, two STS markers, and four SSR markers were all applied to the HS x RVPM25 F_2 population. Eight F_2 plants from the 376 tested were identified with recombination events occurring between the SSR markers *Xbarc76* and *Xcfd175*. This data was combined with marker data from the three recombinant varieties; Hermann, Striker and RAGT 2 to create a recombinant set to determine the marker order in the *Pch1* region (Figure 6).

Recombinant lines from the F_2 population were selfed to produce F_3 families for phenotypic evaluation of eyespot resistance using seedling bioassays with *O. yallundae*. Lines were classified as *Pch1*+, *Pch1*- or as heterozygotes. The phenotypic data was combined with the marker data to determine the position of *Pch1* relative to the markers (Figure 6). The resistance gene was found to be flanked proximally by a group of five co-segregating markers; *Xcos7-6*, *X4CD7A8*, *Xorw5*, *Xtr383* and *Xwg7S* and flanked distally by the COS marker *Xcos7-9*. In addition, *Pch1* was found to co-segregate with the STS marker *Xorw1* and the COS marker *Xtr40*, suggesting that these markers would be suitable for selection of the resistance.

The F_2 recombinants identified could be developed into stable near isogenic lines (NILs) to be used in replicated yield and quality trait trials to determine the genetic locations of these traits relative to *Pch1*. This would facilitate the breeding of high yielding varieties with good quality traits and a high level of resistance to eyespot.

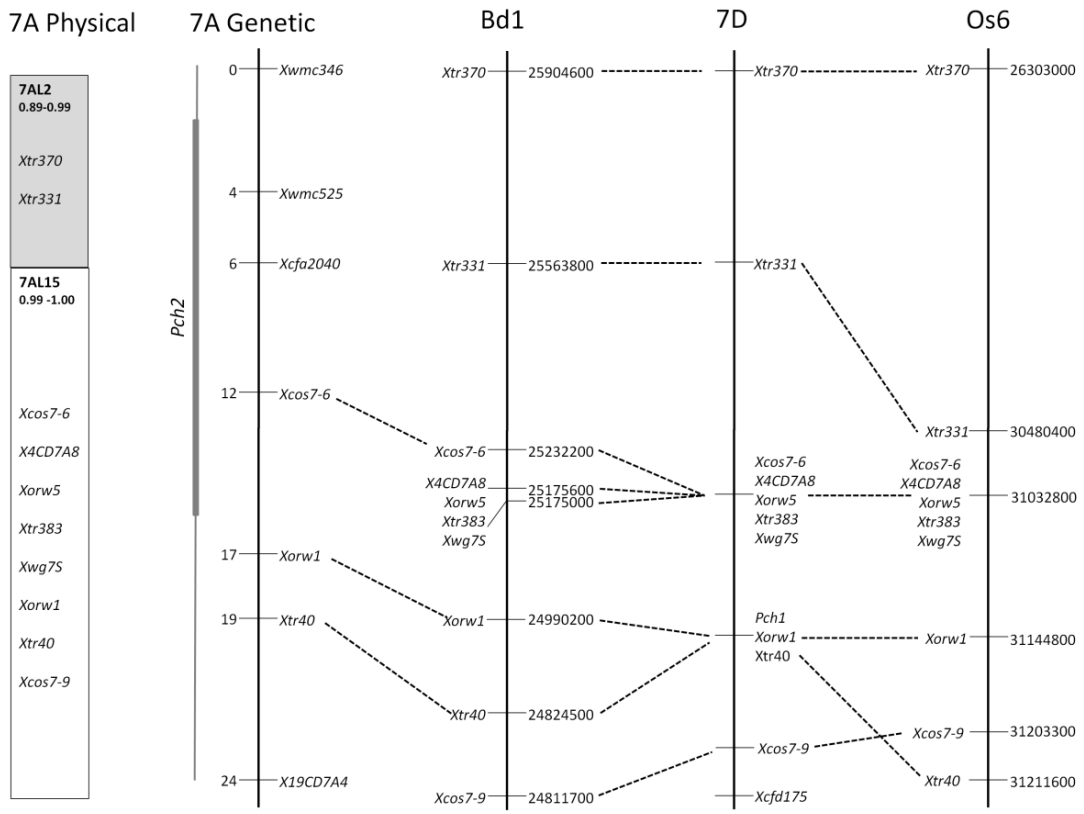

Figure 6. Comparison of the HS x RVPM25 wheat chromosome 7D genetic map including *Pch1* with physical marker locations on Brachypodium chromosome 1 (Bd1) and rice chromosome 6 (Os6), and also with the CS x CS/CD7A chromosome 7A genetic and CS deletion bin maps.

6.4. Co-linearity between wheat chromosome 7D, Brachypodium chromosome 1 (Bd1) and rice chromosome 6 (Os6)

The physical location of orthologues of COS and STS markers could be identified over an approximately 1.1 Mb region on Brachypodium chromosome 1 and a 4.9 Mb region on rice chromosome 6. On the basis of the polymorphic markers tested there was perfect co-linearity between wheat, Brachypodium and rice in this region, with the exception of an apparent inversion between rice and Brachypodium at loci *Xtr40* and *Xcos7-9* (Figure 6). This suggests that both rice and Brachypodium provide a useful resource for further marker development and fine-mapping of *Pch1*.

From the physical locations of the *Pch1* flanking markers on Bd1, the *Pch1* region in Brachypodium can be defined as an approximately 364 Kb region (Figure 6). This region contains thirty-four predicted genes, which includes four genes with homology to plant disease resistance proteins. These could be investigated further as potential candidate genes for *Pch1.*

6.5. Evidence for homeology between *Pch1* and *Pch2*

The direct transferability of gene-based COS and STS markers across chromosomes 7A and 7D enabled integration of the maps of the two chromosomes. It was therefore possible to identify an overlap in the region of *Pch1* and the region of the *Pch2* QTL between markers *Xcos7-6* and *Xorw1*. If the two genes are indeed homeoloci then it is anticipated that the higher level of recombination on chromosome 7A in *Pch2* populations may assist the orientation of homeologue transferable COS markers on chromosome 7D and directly inform mapping of the more potent *Pch1* resistance.

In conclusion, this study has demonstrated that COS markers could be used to identify recombinants in a previously recalcitrant *Ae. ventricosa* alien introgression in wheat. Using COS markers and previously developed STS markers we fine-mapped the potent eyespot resistance *Pch1* and identified candidate genes within the *Pch1* region on the basis of co-linearity between wheat and the reference genomes of rice and Brachypodium. This research provides a springboard for the map-based cloning of the *Pch1* eyespot resistance gene. This would allow the development of perfect markers for accurate selection of the resistance by plant breeders. Application of the same techniques to other introgressions from relatives of wheat may assist the exploitation of the numerous beneficial alleles that they contain in commercial varieties.

7. CONCLUSION

Eyespot is an economically important fungal disease of wheat that attacks the stem base and causes significant yield losses in northern Europe. There are only two genetic sources of eyespot resistances in wheat for which molecular markers have been identified; *Pch1* and *Pch2*. This thesis has provided further information about the genetic location of these resistances to improve their selection in plant breeding programmes, and characterised a third resistance *QPch.jic-5A* so that it can be utilised in wheat varieties. The study has provided important tools to plant breeders to enable the pyramiding of the eyespot resistances *Pch1*, *Pch2* and *QPch.jic-5A* to develop commercial varieties of wheat with a high level of resistance to eyespot. In addition, it has provided a foundation for the map-based cloning of both *Pch1* and *Pch2* to facilitate more accurate marker-assisted selection.

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